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Biosynthesis of laccase by *Aspergillus flavus* NG85 Isolated from Saint Catherine protectorate

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THE MICROFLORAL pictures of Saint Catherine protectorate, Giza Zoo Garden and Cairo University soil were studied. The obtained 31 microbial isolates were qualitatively and quantitatively screened for laccase production. *Aspergillus flavus* from Saint Catherine protectorate achieved highest laccase production on both solid and liquid media. Identification of this fungal species was further confirmed at the molecular level based on nuclear ribosomal DNA internal transcribed spacer (ITS) identities and was found to be *A. flavus* strain NG85. The fungus produced statistically highest amounts of laccase after 10 days of growth at 36.7^{o}C and when growth medium was adjusted at pH 5. D-glucose at a concentration of 24 g/l was the best carbon source. The leading nitrogen source was peptone used at 2.51 g/l. Supplementation of copper sulfate at concentration 10 µM to the optimized growth medium caused an increase of 122% in enzyme yield. The crude laccase preparation of *A. flavus* NG85 from Saint Catherine protectorate showed antiproliferative activity against colon carcinoma cells (HCT-116) andbreast carcinoma cells (MCF-7) with IC_{50} values of 24.3 and 41.3 µg/ml, respectively, and a less inhibitory effect against hepatocellular carcinoma cells (HepG-2).

Keywords: Laccase, *Aspergillus flavus* NG85, Biosynthesis, Optimization, Cytotoxic activity.

Laccase (benzenediol oxygen oxidoreductase, EC 1.10.3.2) is a highly unspecific enzyme containing up to 4 copper atoms within their catalytic sites (Duran and Esposito, 2000).It catalyses the oxidation of various phenolic compounds and aromatic amines with molecular oxygen as an electron acceptor (Palmeri *et al.*, 1993).

Laccase production has been described in fungi, plants and bacteria (Mayer and Staples, 2002). Yoshida first described laccase in 1883 from the exudates of the Japanese lacquer tree, *Rhusvernicifera*. However in 1896, for the first time, both Bertrand and Laborde demonstrated laccase to be a fungal enzyme (Levine, 1965 and Thurston, 1994). Laccase production occurs in various fungi over a wide range of taxa. Fungi from the deuteromycetes, ascomycetes (Aisemberg *et al.*, 1989) as well as basidiomycetes (Sadhasivam *et al.*, 2008) are the known producers of laccase. Some soil bacteria were also reported to produce extracellular laccase (Martins *et al.*, 2002).

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In fungi, laccases play a variety of physiological roles including morphogenesis, fungal plant pathogen/host interaction, stress defense, and lignin degradation (Giardina *et al.*, 2010).

Due to its relatively broad substrate specificity, it has wide potential industrial applicability, including pulp bleaching in the paper industry, dye decolorization, oxygen cathode development for biofuel cells, biosensors, bioremediation, and detoxification of environmental pollutants.Heap *et al.* (2014) used laccase in an enzymatic pretreatment method to improve lignocellulosic saccharification. Laccases have also applications in the medical side to prepare certain drugs like anticancer drugs and they are added in cosmetics to minimize their toxic effects (Couto and Herrera, 2006).

In view of the importance of laccases, the present study aimed at investigating laccase production by some microorganisms isolated from different habitats. More consideration was given to optimize cultural conditions for maximum laccase production by the most potent microorganism. Furthermore, the antitumor activity of the crude laccase extract was tested against some cancer cell lines.

Material and Methods

Microfloral picture of collected soil samples

For isolation of fungi and bacteria, soil samples were collected from different habitats; Saint Catherine protectorate (South Sinai), Cairo Zoo Garden (Giza), and Cairo University (Giza).The microfloral picture of each soil sample was investigated. Fungal isolation was carried out according to Johnson *et al.* (1960) using soil dilution plate method. Czapek-Dox agar medium was used for isolation of fungi which contained (g/l): sucrose, 20; $NaNO_3$, 2; K_2HPO_4, 1; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.01 and agar, 15. Streptomycin (30µg/ml) was added to the above medium after sterilization and cooling.

Bacterial isolation was carried out using nutrient agar medium which contained (g/l): Peptone, 5; Beef extract, 3; Nacl, 5; Agar, 15. The final pH was adjusted to 6.8 at 25^o C.The medium was then sterilized by autoclaving at 121^oC and 1.5 bars for 15 min.

The developing colonies were counted (colonies/g dry soil). The relative density of each microorganism (R.D. %) was calculated as percentage of the total microorganisms count.

The isolated fungal colonies were identified up to the species level according to the morphological characters and microscopic examination. This was made through the help of Gilman (1957),Moubasher (1993), Raper and Fennell (1965) and Watanabe (2002). Species diversity (H) was determined according to Pielou (1966).

Qualitative screening for laccase production *by isolated microorganisms*

The basal medium (Olga *et al.,* 1998), containing chromogenic substrate as indicator, was used for screening microorganisms for laccase production. It contained (g/l): Peptone, 3.0; Glucose, 10.0; KH_2PO_4, 0.6; $ZnSO_4$, 0.001; K_2HPO_4, 0.4; $FeSO_4$,0.0005; $MnSO_4$, 0.05; $MgSO_4$,0.5; Agar ,20.0; Dist. H_2O up to 1000 ml. Guaiacol 0.1% (w/v) was added to the above medium (Kiiskinenand Saloheimo, 2004).

Each microorganism was inoculated onto these plates. Positive results were indicated bycolor production from chromogenic substrate metabolism after incubation at 30°C for 3 or 7 days in case of bacteria or fungi, respectively.

Quantitative estimation of laccaseproduction by isolated microorganisms

Triplicate flasks containing 50 ml of the previous sterilized medium were inoculated with 1 cm diameter fungal discs taken from the periphery of 7-day old cultures grown on the Czapek-Dox agar plates or bacterial discs taken from 2-day old cultures grown on nutrient agar plates. The flasks were incubated at 30^{o} C for 3 or 10 days for bacteria or fungi, respectively. The culture filtrate was used for measuring the extracellular laccase production.

Laccase activity was assayed with guaiacol as substrate. The reaction mixture contained 3.9 ml acetate buffer (10 mmol/l, pH 5.0), 1 ml guaiacol (2 mmol/L) and 0.1ml properly diluted enzyme solution and was incubated at 35 ^{o}C for 30 min. Absorbance was read at 470 nm. In the blank, guaiacol was replaced with acetate buffer (Das *et al.,* 2008).

Laccase activity unit is expressed in µmol of substrate oxidized per milliliter per minute, in the following equation: $U\ ml^{-1}\ min^{-1} = \Delta Abs\ (10^6)\ (\varepsilon RT)^{-1}$, where ΔAbs is the difference between final and initial absorbances, ε is the extinction coefficient of laccase product (εmax = 6,740 $Mol^{-1}\ cm^{-1}$), R is the volume in milliliters of supernatant, and T is the reaction time in minutes.

Molecular identification of Aspergillus flavus from Saint Catherine protectorate

Identification of *Aspergillus flavus* from Saint Catherine protectorate was further confirmed usingnuclear ribosomal DNA internal transcribed spacer (ITS) sequencing. Genomic DNA was isolated using Qiagen kit. Internal transcribed spacer (ITS) region of 5.8S rRNA was amplified using the primers ITS1 and ITS2. Sequencing of PCR amplified product was performed at Macrogen (South Korea). The resulting sequence was entered into the BLAST algorithm of National Centre of Biological Information (NCBI) database to obtain closely related phylogenetic sequences. A phylogenetic tree was constructed using MEGA 6 software. The obtained sequence was then submitted to the GenBank of NCBI database.

Effect of some cultural conditions on extracellular laccase production *by Aspergillus flavus*

Eight factors were investigated to study their effects on laccase production by *A. flavus*. Those factors were, incubation periods at 2, 4, 6, 8, 10, 12 or 14 days; incubation temperature at 5, 15, 25, 35, 40, 50, 60 or 70^{o}C; pHs at 2, 3, 4, 5, 6, 7 or 8; carbon source: glucose, sucrose, lactose, fructose, starch or maltose; glucose concentrations at 0.1, 5, 10, 15, 20, 25, 30 or 40 g/l; nitrogen source:$NaNO_3$ (2g/l), NH_4NO_3(1.9 g/l), KNO_3 (2.4 g/l), NH_4Cl (2 g/l), $(NH_4)_2SO_4$ (2.1 g/l), glycine (1.8 g/l), or peptone (2.64 g/l); peptone concentrations at 0.5, 1, 2, 3, 4 or 5 g/l and metal ions (10 µM) as$FeSO_4.7H_2O$, $CaCl_2$. $2H_2O$, $MgSO_4.7H_2O$, $MnSO_4.H_2O$, NaCl, KCl or $CuSO_4.5H_2O$. Triplicate flasks containing the optimized Czapek-Dox's medium were prepared. The medium was inoculated with fungal discs and incubated under the previous successive conditions.

Evaluation of Cytotoxic Effects of crude protein extract

The crude protein extract from *A.flavus* NG85filtrate was partially purified. Cold ethanol was slowly added with stirring to the fungal extract until reachedthe concentration of 80% (v/v). After standing for 1 hr, the protein precipitate was removed by centrifugation at 10,000 rpm for 15 min. at 4^{o}C.

The cytotoxic activity of protein extract was tested against colon carcinoma cells (HCT-116 cell line), breast carcinoma cells (MCF-7 cell line) or hepatocellular carcinoma cells (HepG-2 cell line). These cell lines were obtained from the American Type Culture Collection (ATCC). The cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, HEPES buffer and 50µg/ml gentamycin. All cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 and were subcultured two times a week.

For cytotoxicity assay (Wilson, 2000), eacht ested cell line was seeded in 96-well plate at a cell concentration of 1×10^4 cells per well in 100µl of growth medium. Fresh medium containing different concentrations of enzymic protein extract was added after 24 hr of seeding. Two-fold serial dilutions of protein extract were added to confluent cell monolayer dispensed into 96-well, flat-bottomed microtiter plates (Falcon, NJ, USA) using a multichannel pipette. The microtiter plates were incubated at 37°C in a humidified incubator with 5% CO_2 for a period of 48 hr. Three wells were used for each concentration of protein extract. Control cells were incubated without protein extract. After the end of incubation period, the viable cells yield was determined by a colorimetric method. In brief, the media were aspirated and the crystal violet solution (1%) was added to each well for at least 30 min. The plates were rinsed using tap water to remove excess stain. Glacial acetic acid (30%) was then added to all wells and mixed thoroughly, and then the absorbance of the plates were measured after gently shaken on microplate reader (TECAN, Inc.), using a test wavelength of 590 nm. The absorbance is proportional to the number of surviving cells in the culture plate. All the results were corrected for background absorbance detected

in wells without adding stain. Treated samples were compared with the control cells. All experiments were carried out in triplicate. The cell cytotoxic effect of each tested compound was calculated (Mosmann, 1983).

The percentage of cell viability in relation to control was calculated. The 50% cell cytotoxic concentration (IC_{50}), the concentration required to kill or cause visible changes in 50% of intact mammalian cells, was estimated from graphic plots. STATA statistical analysis package was used for the dose response curve drawing in order to calculate IC_{50}.

Statistical analysis

Data presented in each experiment were means of triplicate assays. The SPSS 16.0 software was used in determination of standard error (SE), the least significant difference (LSD) and for regression analysis.

Results

Microfloral picture

In case of soil of Saint Catherine protectorate (SCP), it appears that 5 fungal isolates were isolated using Dox's medium with species diversity (H) = 0.4 (Table 1). A total of 952.5 colonies/g dry soil from Saint Catherine protectorate were isolated. Genus *Aspergillus* was dominant and constituted 50.0 % of the total count. It was represented by *Aspergillus flavus* (R.D. 28.6%) followed by *A. fumigatus* (R.D. 17.1 %). *Alternaria alternata* was the third in rank of density (21.4 % R.D. of the total count). *Penicillium chrysogenum*and *Rhizopusstolonifer* accounted for 17.9 % and 10.7 % of the total fungal count, respectively.One bacterial isolate from the soil of SCP was encountered on nutrient agar medium (Table 2).

For the soil of Giza Zoo Garden (GZG), Genus *Aspergillus* was also dominant on Dox's medium and constituted 63.7 % of the total count (Table 1). It was represented by *A.fumigatus* (R.D. 28.0 %) followed by *A. niger* (R.D. 15.5%), *A.terreus* (R.D. 7.8%,) *A.versicolor* and *A. flavus* (R.D. 6.2%, each).Two bacterial isolates were isolated on nutrient agar medium from this soil (Table 2).

Similarly, Genus *Aspergillus* was dominant in Cairo University soil (CUS) constituting 67.2% of the total count on Dox's medium (Table 1). It was represented by 5 species. *A.niger* was the highest in count (R.D. 19.7 %), followed by *A.flavus* (R.D. 16.7%). *Aspergillus terreus*, *A.fumigatus* and *A.aculeatus* came next with relative densities of 13.6, 10.6 and 7.6%, respectively. Three bacterial isolates (no. 4, 5 and 6) were medium (Table 2).

TABLE 1. Total count (colony/g dry soil), relative density percentage (R.D %)and species diversity (H)of fungal isolates from Saint Catherine Protectorate (SCP), Giza Zoo Garden (GZG) and Cairo University Soil (CUS).

Fungal isolate	SCP		GZG		CUS	
	Total count	**R.D. %**	**Total count**	**R.D. %**	**Total count**	**R.D. %**
Aspergillus species						
A. aculeatus	N.D.	N.D.	N.D.	N.D.	175.4	7.6
A. flavus	272.1	28.6	143.4	6.2	386.0	16.7
A. fumigatus	204.1	21.4	645.2	28.0	245.6	10.6
A. niger	N.D.	N.D.	358.4	15.5	456.1	19.7
A. terreus	N.D.	N.D.	179.2	7.8	315.8	13.6
A. versicolor	N.D.	N.D.	143.4	6.2	N.D.	N.D.
Other species						
Alternaria alternate	204.1	21.4	251.0	10.8	140.4	6.1
Cladosporium sphaerospermum	N.D.	N.D.	215.1	9.3	N.D.	N.D.
Emericella nidulans	N.D.	N.D.	10.8	0.5	N.D.	N.D.
Fusarium oxisporum	N.D.	N.D.	71.7	3.1	105.3	4.5
Penicillium chrysogenum	170.1	17.9	215.1	9.3	210.5	9.1
Rhizopusstolonifer	102.1	10.7	71.7	3.1	280.7	12.1
Total	**952.5**		**2304.9**		**2315.8**	
Number of species	**5**		**11**		**9**	
Species diversity (H)	**0.4**		**0.6**		**0.6**	

R.D. % = Relative density as percentage of total species count.N.D. = not detected .

TABLE 2. Total count (colony/g dry soil) and relative density percentage (R.D %) of bacterialisolates from Saint Catherine Protectorate (SCP), Giza Zoo Garden (GZG) and Cairo University Soil (CUS).

Bacterial isolate	SCP		GZG		CUS	
	Total count	**R.D. %**	**Total count**	**R.D. %**	**Total count**	**R.D. %**
Isolate (1)	238.1	100	N.D.	N.D.	N.D.	N.D.
Isolate (2)	N.D.	N.D.	143.4	30.8	N.D.	N.D.
Isolate (3)	N.D.	N.D.	322.6	69.2	N.D.	N.D.
Isolate (4)	N.D.	N.D.	N.D.	N.D.	350.9	50.0
Isolate (5)	N.D.	N.D.	N.D.	N.D.	315.8	45.0
Isolate (6)	N.D.	N.D.	N.D.	N.D.	35.1	5.0
Total	**238.1**		**466**		**701.8**	

R.D. % = Relative density as percentage of total isolate count.N.D. = not detected .

Qualitative screening and quantitative assay of laccase production by 31 microbial isolates

Table 3. reveals qualitative screening (diameter of colored zone, mm) and quantitative assay (U/ml) for laccase production by microbial isolates from the three isolation habitats. It appears that *Aspergillus flavus*from Saint Catherine protectorate was highly distinctive in laccase production (15.2 mm). It was followed by *A. flavus* from Giza Zoo garden (12.0 mm). A bacterial isolate (no. 3) from Giza Zoo garden came third (6.00 mm) along with *A. fumigatus* from Cairo University soil (5.50 mm).Concerning quantitative estimation of the enzyme, *Aspergillus flavus* from SCP produced the highest significant amounts of laccase (59.50 U/ml). The *A. flavus* isolate from GZG came next (31.01 U/ml) then followed by *A. flavus* from CUS (18.99 U/ml).

By the end of the screening part of the work, *Aspergillus flavus* from SCP proved to be the most potent laccase producer on both solid and liquid media. Its identification was further confirmed at the molecular level.

Molecular identification of Aspergillus flavus

DNA was isolated from *Aspergillus flavus* from SCP and the ITS region of 5.8S rRNA was amplified using the specific primers ITS1 and ITS2. The sequence was determined using automated sequencers. Amplification and sequencing of fungal rRNA gene resulted in 545bp-long nucleotide sequence. NCBI Blast search sequence similarity was conducted against the existing non-redundant nucleotide sequence database. This showed maximum identity (100 %) with *A. flavus* species. The phylogenetic tree was constructed (Fig. 1) to show sequence alignment with available sequences from NCBI data bank (first 15 hits in Blast results). The 545bp-long nucleotide sequence was deposited in NCBI Gen Bank and was given a strain identifier, *A. flavus* NG85, with accession number: KJ855143.

Effect of some cultural conditions on laccase production by A. flavus NG85
Incubation period

The effect of incubation period on laccase production by *A. flavus* NG85 growth is shown in Fig. 2. The gradual increase in time led to concomitant increase in the amount of laccase. Optimum incubation period was found to be 10 days where 41 U/ml were assayed. More increase in incubation period above optimum led to decrease in enzyme activity.

TABLE 3. Qualitative and quantitative screening for laccase production by microorganisms isolated from Saint Catherine protectorate (SCP), Giza Zoo Garden (GZG) and Cairo University Soil (CUS) .

Microorganism	Colored zone (mm)	Laccase activity (U/ml)
SCP		
Alternaria alternata	2.00 g	10.43 j
Aspergillus flavus	15.2 a	59.50 a
Aspergillus fumigatus	2.50 f	3.41 o
Rhizopus stolonifer	0.00 i	0.15 w
Penicillium chrysogenum	0.00 i	0.46 uv
Bacteria (1)	0.00 i	3.12 p
GZG		
Alternaria alternata	1.00 h	5.05 n
Aspergillus aculeatus	0.00 i	31.01 b
Aspergillus flavus	12.0 b	17.95 d
Aspergillus fumigatus	3.45 d	6.82 l
Aspergillus niger	0.00 i	15.13 h
Aspergillus versicolor	3.20 e	16.62 e
Aspergillus terreus	0.00 i	6.08 m
Emericella nidulans	2.00 g	12.02i
Fusarium oxisporum	3.50 d	0.74 stu
Penicillium chrysogenum	0.00 i	1.93 r
Rhizopus stolonifer	0.00 i	12.17 i
Bacteria (2)	0.00 i	15.43 g
Bacteria (3)	6.00 c	7.33 k
CUS		
Aspergillus aculeatus	0.00 i	0.45 v
Aspergillus flavus	3.50 de	18.99 c
Aspergillus fumigatus	5.50 c	16.02 f
Aspergillus niger	0.00 i	0.89 s
Alternaria alternata	2.00 fg	12.02 i
Rhizopus stolonifer	0.00 i	0.74 st
Fusarium oxisporum	2.5 f	15.58 g
Penicillium chrysogenum	0.00 i	0.45 tuv
Aspergillus terreus	1.50 gh	14.99 h
Bacteria (4)	1.00 h	0.30 vw
Bacteria (5)	0.00 i	2.23 q
Bacteria (6)	0.00 i	3.12 p
LSD (at 0.01)	**0.34**	**0.24**

The least significant difference (LSD) was calculated at 99% confidence interval (probability of error p=0.01). Means followed by the same letters are statistically non-significant.

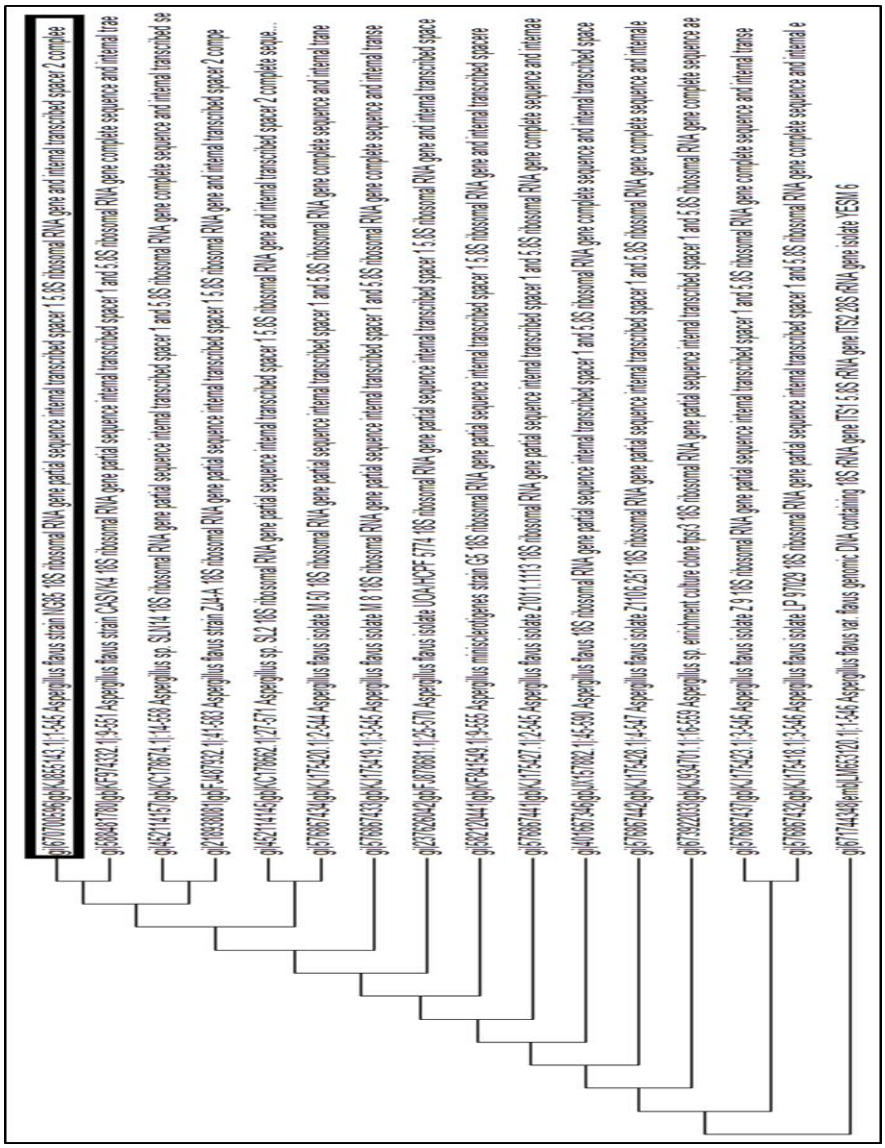
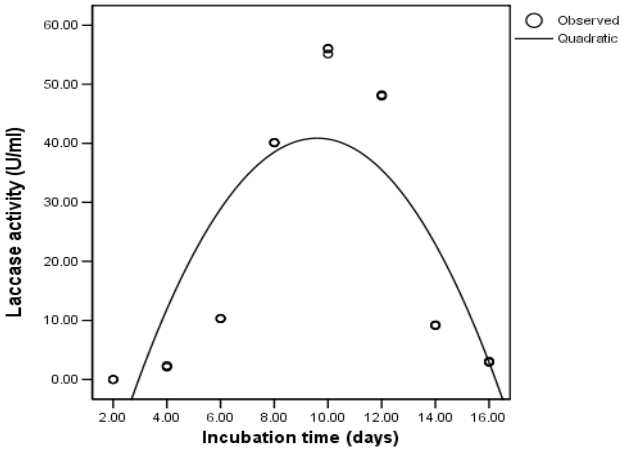


Fig. 1. Phylogenetic tree showing genetic relationship between the isolate *A. flavus* NG85 (first one from the top) and other closely related reference microorganisms.

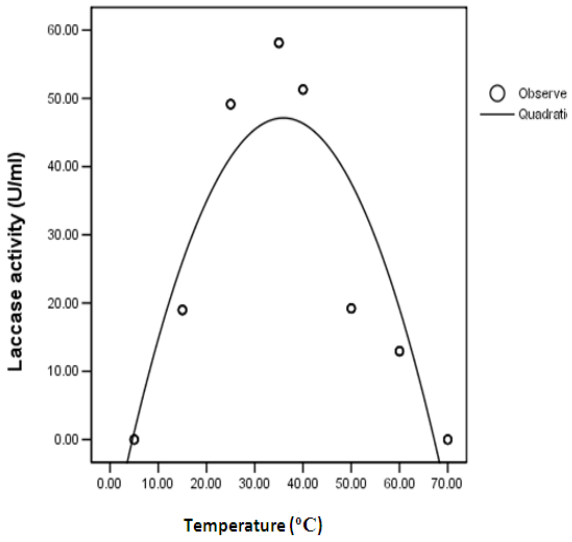


R^2= 0.682 y = - 44.359 + 17.7661 x - 0.9259 x^2

Fig. 2. Effect of incubation period on laccase production by *A. flavus* NG85 .

Incubation temperature

It appears from Fig. 3 that the gradual increase in temperature led to corresponding increase in the amount of laccase with optimum at 36.7^{o}C with activity of 42.2U/ml. The increase in temperature above optimum temperature caused a decrease in enzyme activity.

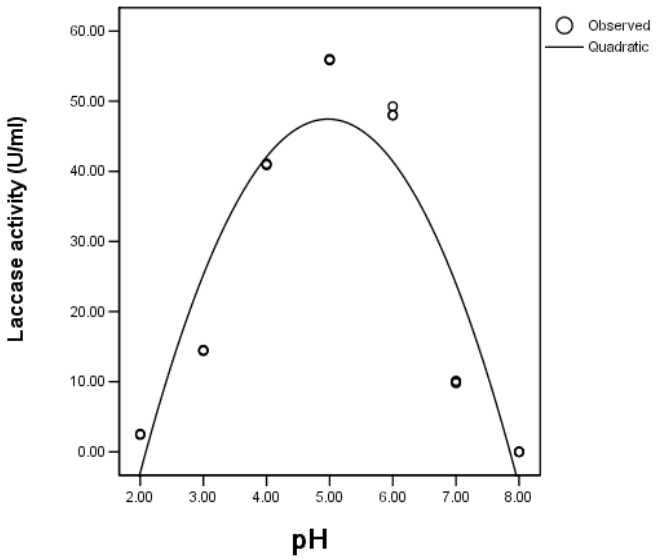


R^2= 0.81386 y=-15.056 + 3.4627 x -0.0482 x^2

Fig. 3. Effect of incubation temperature on laccase production by *A. flavus* NG85 .

pH

From Fig. 4, it was found that the statistical optimum pH for enzyme production was 5 with activity of 46.0U/ml. Any change in this pH value led to a decrease in enzyme production.



R^2= 0.849 y=-93.667 + 56.7648 x -5.7086 x^2

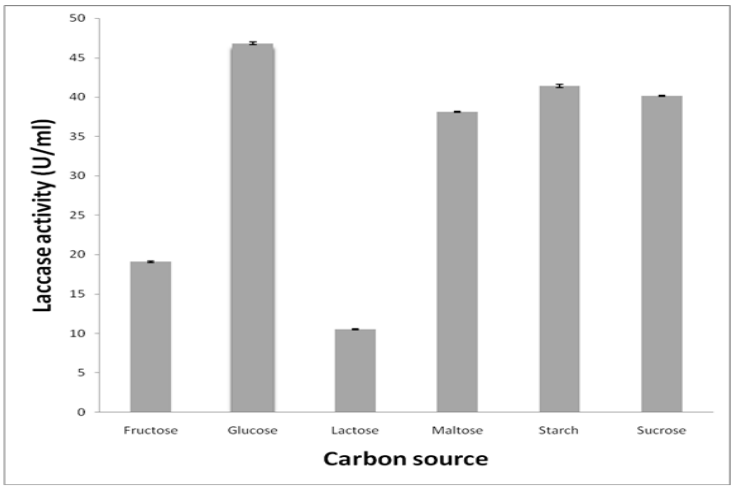
Fig. 4. Effect of different pH values on laccase production of *A. flavus* NG85 .

Carbon sources

Figure 5 reveals the effect of different carbon sources at concentration 2% on laccase production by *A. flavus* NG85after 10 days of growth at 36.7°C and pH 5. Sucrose was used as carbon source in control experiment, yielded 40.13 U/ml. Obviously, D-glucose induced highest laccase production (46.83 U/ml) as compared with control. Starch exhibited statistically significant increases in laccase production (41.41U/ml). On the other hand, maltose and fructose exhibited statistically significant decreases in laccase production (38.12, 19.06 U/ml, respectively), while lactose exhibited the lowest decrease in laccase production (10.51 U/ml) compared with control.

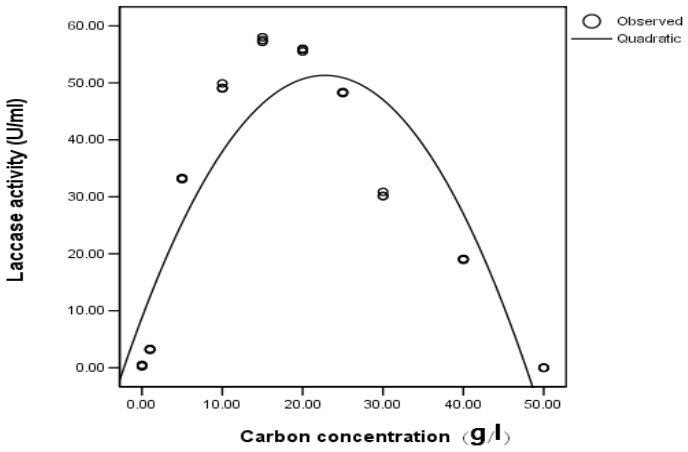
Glucose concentrations

The increase in glucose concentration up to 24 g/l led to a high increase in the amount of laccase enzyme (Fig. 6). Statistically, this is the optimum glucose concentration for enzyme production with 51.9U/ml activity. Decrease in enzyme production was achieved above the optimum concentration.



Bars show Means. Error Bars show Mean ± SE.

Fig. 5 . Effect of different carbon sources on laccase production of *A. flavus* NG85 .

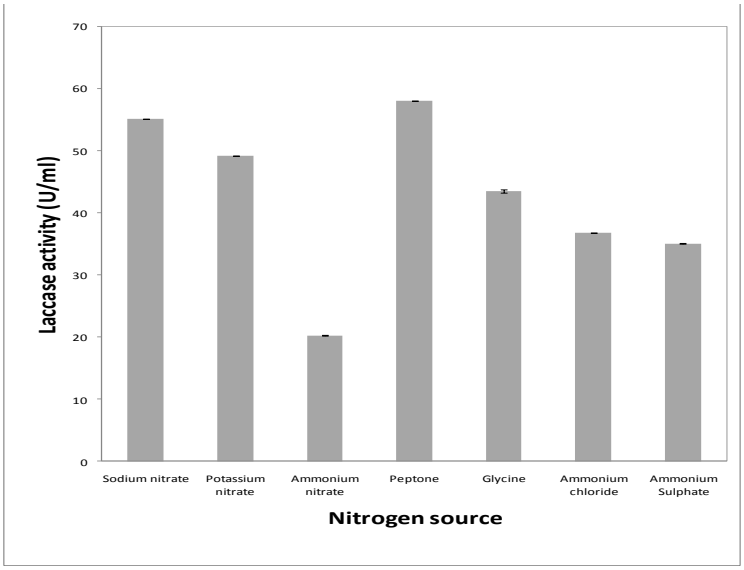


R^2= 0.803 $y=8.8107 + 3.7311x - 0.0819x^2$

Fig. 6. Effect of different glucose concentrations on laccase production by *A. flavus* NG85 .

Nitrogen sources

Figure 7 shows the effect of different nitrogen sources on laccase production by *A.flavus* NG85. Sodium nitrate was used as control. Obviously, peptone induced highest significant laccase production with activity of 53.10 U/ml. Glycine exhibited statistically significant decrease in enzyme activity (43.55U/ml). Also significant decrease in laccase production was evident when using the inorganic nitrogen sources.

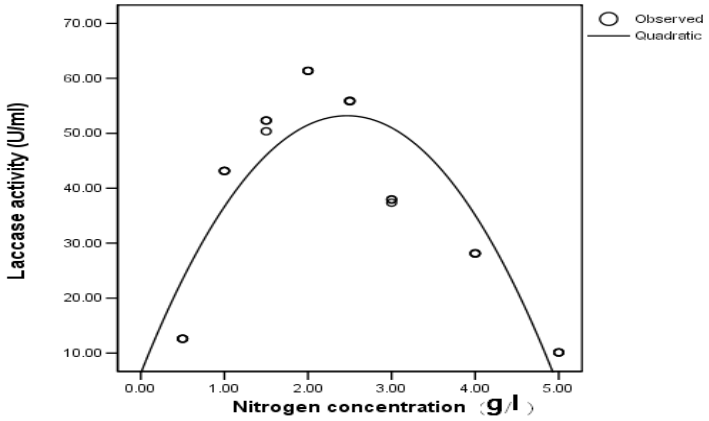


Bars show Means. Error Bars show Mean ± SE.

Fig. 7. Effect of different Nitrogen sources on laccase production of *A. flavus* NG85 .

Peptone concentrations

Figure 8 reveals the effect of different peptone concentrations on laccase production by *A. flavus* NG85. The gradual increase in peptone concentration led to a concomitant increase in the amount of laccase enzyme with maximum activity of 53.9U/ml at 2.51 g/l. A decrease in enzyme activity was noticed above this concentration.

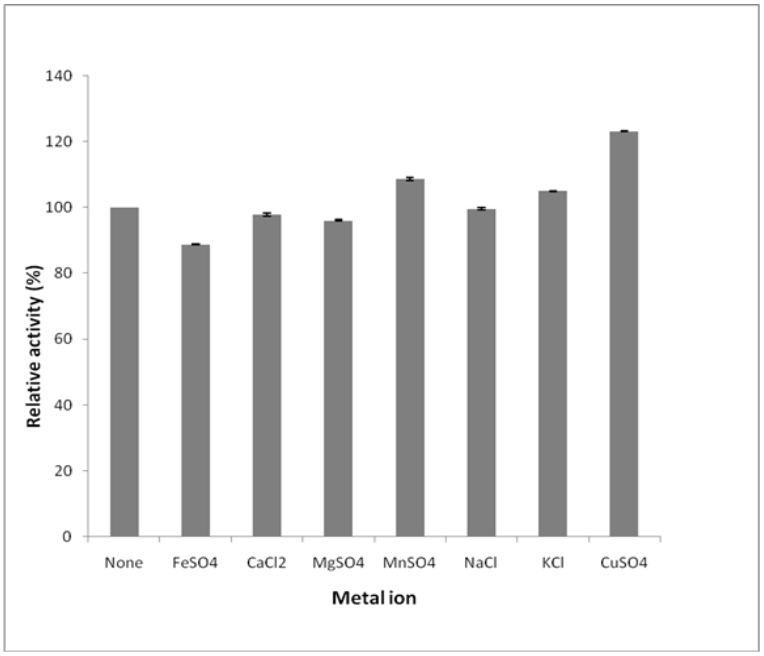


R^2= 0.784 y=6.37 + 37.96 x -7.69 x^2

Fig. 8. Effect of different peptone concentrations on laccase production by *A. flavus* NG85.

Metal ions

Figure 9 depicts the effect of different metal ions on laccase production. Among the tested compounds there was a significant inhibition in laccase induction exhibited by ferrous sulfate, magnesium sulfate, calcium chloride or sodium chloride. A statistically non-significant change was noticed for potassium chloride. An enhancement in laccase production was caused by manganese sulfate or potassium chloride. However, the highest significant promotive effect was due to copper sulfate addition with percentage increase over control (0.0 metal ion) of 122 %.



Bars show means. Error Bars show Mean ± SE.

Fig. 9. Effect of different metal ions on laccase production by *A. flavus* NG85 .

Evaluation of antitumor activity of crude laccase preparation ofA. flavus NG85

The crude protein extract of *A. flavus* NG85 was tested against colon carcinoma cells (HCT-116 cell line), breast carcinoma cells (MCF-7 cell line) and hepatocellular carcinoma cells (HepG-2 cell line). The crude protein extract showed strong inhibitory activities toward colon carcinoma cells (Fig. 10a) and breast carcinoma cells (Fig. 10b) with IC_{50} values of 24.3 and 41.3 µg/ml, respectively. However, a weaker depressive effect was shown against hepatocellular carcinoma cells (Fig. 10c).

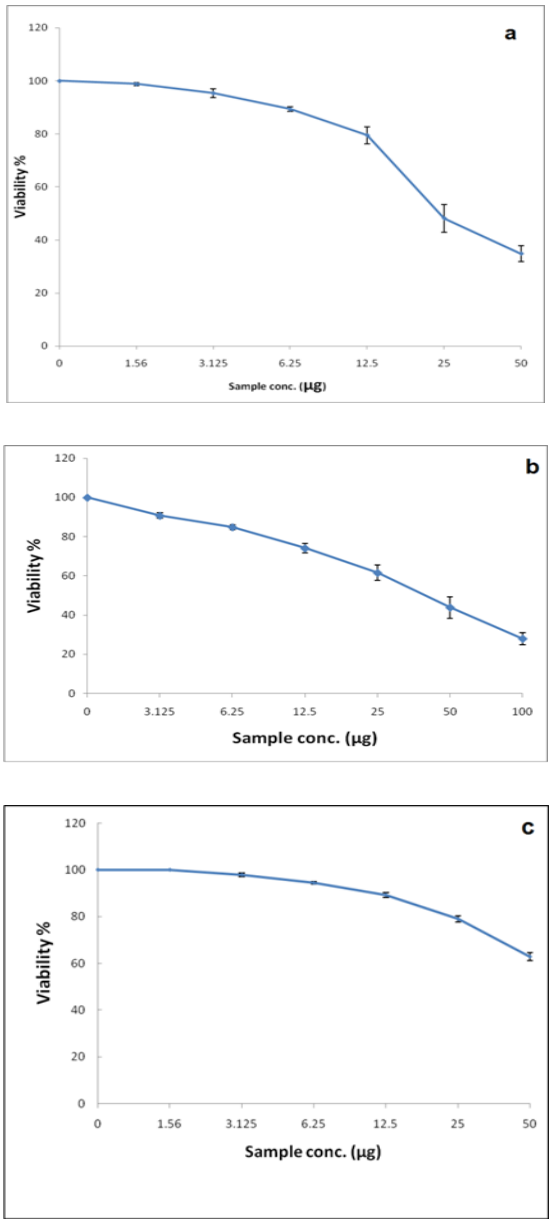


Fig. 10. Effect of crude laccase preparation of *A. flavus* on cell viability ratio of: a- Colon carcinoma cells (HCT-116 cell line), b- Breast carcinoma cells (MCF-7 cell line) and c-hepatocellular carcinoma cells (HepG-2 cell line).

Discussion

Laccase is most widely distributed in a wide range of higher plants and fungi (Benfield *et al.,* 1964) as well as in bacteria (Diamantidis *et al.,* 2000). Most of the laccases described in literature were isolated from higher fungi. Laccases have been isolated from ascomycetes, deuteromycetes and basidiomycetes (Assavanig *et al.,* 1992).The present study firstly aimed to isolate fungi and bacteria from different localities. The microfloral pictures in Saint Catherine protectorate, Cairo Zoo garden and Cairo University soil were studied. Despite the fact that the Saint Catherine protectorate soil showed the least species diversity (H= 0.4), compared with the other tested soils, *Aspergillus flavus* isolate obtained from this soil exhibited the highest laccase production on solid as well as on liquid media. Accordingly, this isolate was chosen to optimize its cultural conditions for maximum laccase production and activity. It was thought advisable to confirm its identification at the molecular level. The obtained 545bp-long nucleotide sequence was deposited in NCBI GenBank and was given a strain identifier, *A. flavus* NG85, with accession number: KJ855143.

It was found that maximum laccase production by *A. flavus* NG85 was reached after10 days of growth. Various incubation periods were achieved by different microorganisms. The white rot fungus *Pycnoporus cinnabarinus* produced maximum level of laccase on day 5 and accounted for about 70% of the total extracellular protein (Eggert *et al.,* 1996). Laccase production from *Cyathusbulleri* was detectable after 2 days and reached maximum on day 7 (Vasdev *et al.,* 1994). The onset oflaccase activity in *Trichoderma harzianum* WL1occurred on day 2 and reached its maximum on day 4 and then the rate of enzyme production declined gradually. The difference in time course of laccase production by the various fungal systems mainly dependson the source, media composition and type of inducers (Sadhasivam *et al.,* 2008).

Pointing *et al.* (2000) stated that the optimum temperature range for laccase production is between 25 and 30^{o}C. However, *A. flavus* NG85 in this work showed maximum production of laccase at 36. 7^{o}C.

Maximum production of laccase by *A. flavus* NG85 occurred in the acidic pH (5). Earlier reports suggested that pH between 4.5 and 6.0 is suitable for laccase production (Thurston, 1994).

Laccase production by fungi has been found to be largely affected by nutritional conditions, such as carbon and nitrogen source and related concentrations and microelements. Laccases are generally produced in low concentrations by fungi, but higher concentrations could be obtained by adding various supplements to liquid growth media (Lee *et al.,* 1999 and Vasconcelos *et al.,* 2000).

In this work, the monosaccharide D-glucose was the most potent carbon source inducing maximum laccase production at an optimum concentration of 24g/l.This is in accordance with Leifa *et al.* (2007) who reported that utilization

of monosaccharides for laccase production is better compared to sugar alcohol and complex sugar due to their simple nature.

A. flavus NG85 in this study achieved highest laccase production when using peptone as a nitrogen source with optimum concentration of 2.51g/l. It was proven that nitrogen sources and their concentrations were as important nutritional factors as carbon sources in regulating laccase production (Minussi *et al.,* 2002). Vahidi *et al.* (2004) reported that when yeast extract was used as nitrogen source it increased laccase production. A decrease in enzyme production was seen when inorganic nitrogen sources were used alone in the growthmedium. Oppositely, Elisashvili *et al.* (2001) reported high laccase production in *C. unicolor* IBB 62, grown in a medium with ammonium sulfate as the only nitrogen source.

The data reached in this study showed that supplementation of copper sulfate at a concentration of 10 μM caused significant enhancement in laccase production. These findings are in agreement with previous reports showing that the addition of 2 mM CuSO4 during the exponential growth phase of fungal growth led to a remarkably increased laccas eproduction (Galhaup *et al.,* 2002; Couto and Sanroman, 2005).Almost a similar effect was observed in the cultures of *Trametes multicolor* MB 49 and *Trametes trogii* BAFC 463 with copper concentrations ranging from 0.5 to 2.0 mM. Sadhasivam *et al.* (2008) found that copper sulfate supplementation at 1 mM concentration yielded high amounts of laccase. The formation of laccase by a *Bacillus* sp. was considerably increased by addition of 1 mM copper sulfate (Kaushikand Thakur, 2014). Addition of inducers enhanced the production of laccase at the level of gene transcription. The promoter regions of the genes encoding for laccase contain various recognition sites that are specific for xenobiotics and heavy metals. It has been demonstrated that the *Pleurotuso streatus* laccase genes poxc and poxa1b are transcriptionally induced by copper, and several putative metal responsive elements (MREs) were found in the promoter regions of thes egenes (Faraco *et al.,* 2003).

Urgent need for novel anticancer drugs has paved way for the usage of fungi and their products withanti-cancer properties. In this work, the crude enzyme preparation produced by *A. flavus* NG85 showed high cytotoxic activities against colon carcinoma cells or breast carcinoma cells and a less inhibitory effect against hepatocellular carcinoma cells was detected. This effect could be due to laccase and/or other proteins produced by *A. flavus* NG85, since the cultivation medium was oriented to high laccase production. However, it is recommended for future work to purify and characterize laccase produced by *A.flavus* NG85. Hu *et al.* (2011) purified alaccase from fresh fruiting bodies of the edible white common *Agrocybe cylindracea* mushroom. It caused HIV-1 reverse transcriptase inhibitory activity (IC_{50}=12.7 μM) and antiproliferative activity against HepG2 cells (IC_{50}=5.6 μM) and MCF7 cells (IC_{50}=6.5 μM).

In conclusion, *A. flavus* NG85 strain isolated from Saint Catherine protectorate achieved highest amounts of extracellular laccase. Its growth medium was directed toward maximum biosynthesis of the enzyme. The crude enzyme extract of the fungal filtrate proved high antitumor potency against colon carcinoma cells and breast carcinoma cells and a less cell toxicity against hepatocellular carcinoma cells.

References

Aisemberg, G.O., Grorewold E.,Taccioli, G.E. and Judewicz, N. (1989) A major transcript in the response of *Neurospora crassa* to protein synthesis inhibition by cycloheximide.*Exp. Mycol.,* **13**: 121 – 128.

Assavanig, A., Amornkitticharoen, B., Ekpaisal, N., Meevootisom,V. and Flegel, T. W. (1992) Isolation, characterization andfunction of laccase from *Trichoderma*. *Appl.Microbiol. Biotechnol*., **38**: 198–202.

Benfield, G., Bocks, S.M., Bromley, K. and Brown, B.R. (1964) Studies in fungal and plant laccases.*Phytochem.,* **3**: 79 – 88.

Couto, S.R. and Herrera, J.L.T. (2006) Industrial and biotechnological applications of laccases: A review. *Biotechnol. Advances*, **24**: 500 – 513.

Couto, S.R. and Sanroman, M.A. (2005) Coconut flesh: A novel raw material forlaccase production by *Trametes hirsuta* under solid state conditions. application to lissamine green B decolorization. *J. Food Eng*., **71**: 208–13.

Das, P., Mukherjee, S. and Sen, R. (2008) Improved bioavailability and biodegradation of a model polyaromatic hydrocarbon by a biosurfactant producing bacterium of marine origin.*Chemosphere*,**72**: 1229–1234.

Diamantidis, G., Effosse, A., Potier, P. and Bally, R. (2000) Purification and characterization of the first bacterial laccase in the rhizospheric bacterium *Azospirillum lipoferum. Soil Biol. Biochem.,* **32**: 919-927.

Duran, N. and Esposito, E. (2000) Potential applications of oxidative enzymes and phenoloxidase-like compounds in wastewater and soil treatment: A review: *Appl.Catal. B Environ*., **28**: 83–99.

Eggert, C., Temp, U. and Eriksson, K.E.L. (1996) The ligninolytic system of the white rot fungus *Pycnoporus cinnabarinus*: purification and characterization of the laccase. *Appl. Environ.Microbiol*., **62**: 1151–8.

Elisashvili, V., Parfar, H., Kachlishvili, E., Chichua, D., Bakradze, M. and Kokhreidze, N. (2001) Ligninolytic activity of basidiomycetes grown under submerged and solid-state fermentation on plant raw material (sawdust of grapevine cuttings). *Adv. in Food Sci.,***23**: 117-123.

Faraco, V., Giardina, P. and Sannia, G. (2003) Metal-responsive elements in *Pleurotus ostreatus* laccase gene promoters. *Microbiol*., **149**: 2155–62.

Galhaup, C., Wagner, H., Hinterstoisser, B. and Haltrich, D. (2002) Increased production of laccase by the wood-degrading basidiomycete *Trametes pubescens*. *Enzyme Microb. Technol.,* **30**: 529–36.

Giardina, P., Faraco, V., Pezzella, C., Piscitelli, A., Vanhulle, S. and Sannia, G. (2010) Laccases: A never-ending story.*Cell Mol. Life Sci*.,**67**: 369 – 385.

Gilman, J. C. (1957) *"A MAnual of Soil Fungi"*. The Iowa State College Press, Ames, Iowa, U.S.A., pp. 450.

Heap, L., Green, A., Brown, D., van Dongen, B. and Turner, N. (2014) Role of laccase as an enzymatic pretreatment method to improve lignocellulosic saccharification. *Catal. Sci. Technol.,* **4**: 2251-2259.

Hu, D.D., Zhang, R.Y., Zhang, G.Q., Wang, H.X. and Ng, T.B. (2011) A laccase with antiproliferative activity against tumor cells from an edible mushroom, white common *Agrocybe cylindracea*. *Phytomed*., **18**: 374-9.

Johnson, L. F.,Curi, E. A., Bond, J. H. and Fribourg, H. A. (1960) *"Methods for Studying Soil Microflora-Plant Disease Relationship"*. Burgess Publishing, Minneapolis, U.S.A., pp. 178.

Kaushik, G. and Thakur, I.S. (2014) Production of laccase and optimization of its production by *Bacillus* sp. using distillery spent wash as inducer. *Bioremed. J.,* **18**: 28-37.

Kiiskinen, L. and Saloheimo, M. (2004) Molecular cloning and expression in *Saccharomyces cerevisiae*of a laccase gene from the ascomycete *melanocarpus albomyces*. *Appl. Environ. Microbiol*.,**70**: 137-144.

Lee, I.Y., Jung, K.H., Lee, C.H. and Park, Y.H. (1999) Enhanced production of laccase in *Trametes versicolor* by the addition of ethanol. *Biotechnol. Lett.,* **21**: 965–8.

Leifa, F., Andra, T.S., Ashok, P. and Carlos, R.S. (2007) Effect of nutritional and environmental conditions on production of exopolysaccharide of *Agaricus brasiliensis* by sub-merged fermentation and its antitumor activity. *LWT J.,* **40**: 30 – 35.

Levine, W. **(1965)** *"The Biochemistry of Copper"*, Academic Press Inc., New York,p. 371-385.

Martins, L., Soares, C., Pereria, M., Teixeira, M., Costa, T., Jones, G. and Henriques, A. (2002) Molecular and biochemical characterization of a highly stable 28 bacterial laccase that occurs as a structural component of the *Bacillus subtilis* endospore coat. *J. Biol. Chem*., **277**: 18849-18859.

Mayer, A. and Staples, R. (2002) Laccase : New functions for an old enzyme: *Phytochem*., **60**: 551–65.

Minussi, R.C., Pastore, G.M. and Duran, N. (2002) Potential applications of laccase in the food industry. *Trends in Food Sci. Technol.,* **13**: 205–216.

Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assay. *J. Immunol. Methods*, **65:** 55-63.

Moubasher, A. H. (1993) *"Soil Fungi in Qatar and Other Arab Countries"*. Scientific and Applied Research Center, University of Qatar, pp. 566.

Olga, V.K.S., Elena, V.S., Valeria, P.G., Olga, V.M., Natalia, V.L., Aida, N.D., Alexander, J.J. and Alexander, M. (1998) Purification and characterization of the constitutive form of laccase from basidiomycete *Coriolushirsutus* and effect of inducers on laccase synthesis. *Biotechnol. Appl. Biochem.*, **28**: 47-54.

Palmeri, G., Bianco, C., Cennamo, G., Giardina, P., Marino, G., Monti, M. and Sannia, G. (2001) Purification, characterization, and functional role of a novel extracellular protease from *Pleurotus ostreatus*. *Appl. Environ.Microbiol*., **67**: 2754 – 2759.

Pielou, E.C. (1966) The measurement of diversity in different types of biological collection. *J. Theor. Biol.*, **13**: 131-144.

Pointing, S.B., Jones, E.B.G. and Vrijmoed, L.L.P. (2000) Optimization of laccase production by *Pycnoporussanguineus* in submerged liquid culture. *Mycologia*, **92**: 139–144.

Raper, K.B. and Fennell, D.L.(1965) *"The Genus Aspergillus"*. Williams, Baltimore, U.S.A., pp. 876.

Sadhasivam, S., Savitha, S., Swaminathan, K. and Lin, F.H, (2008) Production, purification and characterization of mid-redox potential laccase from a newly isolated *Trichoderma harzianum* WL1. *Process Biochem.,***43**: 736 – 742.

Thurston C. (1994) The structure and function of fungal laccases. *Microbiol*., **140**: 19-26.

Vahidi, H., Kobarfard, F. and Namjoyan, F. (2004) Effect of cultivation conditions on growth and antifungal activity of *Mycena leptocephala. Afr. J. Biotechnol*., **3**: 606-609.

Vasconcelos, A.F., Barbosa, A.M., Dekker, R.F.H., Scarminio, I.S. and Rezende, M.I. (2000) Optimization of laccase production by *Botryospaeria* sp. in the presence of veratryl alcohol by the response-surface method. *Process Biochem*., **35**: 1131–8.

Vasdev, K. and Kuhad, R.C. (1994) Induction of laccase production in *Cyathus bulleri* under shaking and static culture conditions. *Folia Microbiol*., **39**: 326–30.

Watanabe, T. (2002) Pictorial Atlas of Soil and Seed Fungi. *"Morphologies of Cultured and Key to Species"*, 2nd ed, CRC Press, Boca Raton London New York Washington D.C.

Wilson, A. P. (2000) *"Cytotoxicity and Viability Assays in Animal Cell Culture: A Practical Approach"*, 3rd ed. (Ed. Masters, J.R. W.), Oxford University Press.

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التخليق الحيوى لانزيم اللاكيز المنتج بواسطة اسبرجيللس فلافس NG85 المعزول من محمية سانت كاترين

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تم دراسة صورة الفلورة الميكروبية لتربة محمية سانت كاترين و حديقة الحيوان بالجيزة و جامعة القاهرة. تم عمل مسح كيفى و كمى لانتاج اللاكيز بواسطة ٣١ عزلة ميكروبية. حققت فطرة اسبرجلس فلافس من محمية سانت كاترين اعلى انتاجية لانزيم اللاكيز على الوسط الصلب و السائل. تم تأكيد تعريف هذه الفطرة على المستوى الجزيئى اعتمادا على هوية منطقة ال ITS الموجودة فى ال DNA، و وجد انه سلالة NG 85 *Aspergillus flavus*. انتج الفطر احصائيا اعلى كمية من اللاكيز بعد ١٠ ايام من النموعند درجة الحرارة المئوية ٣٦,٧. وجد ان pH 5 هى الافضل لاعلى انتاجية من الانزيم. كان الجلوكوز هو احسن مصدر كربونى عند تركيز g/l 24 و كان الببتون افضل مصدر نيتروجينى عند استخدامه بتركيز g/l 2.51 . و ثبت ان اضافة كبريتات النحاس الى الوسط الامثل للنمو ادى الى زيادة قدرها ١٢٢ % فى انتاج الانزيم. اظهر المستخلص اللاكيز الخام لفطرة اسبرجلس فلافس NG85 تأثيرا مضادا لنشاط التكاثر لخلايا القولون السرطانية (HCT-116) و خلايا الثدى السرطانية (MCF-7) بقيم IC_{50} هى 24.3 و 41.3µg /ml بالتوالى، واظهر نشاط تثبيطى اقل لخلايا الكبد السرطانية (HepG-2).