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Optimisation of Fungal Laccase Production from *Monodictys castaneae*

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Laccases are copper-containing oxidase enzymes that have broad substrate specificity and applicability in industrial processes. The purpose of this work was to optimise laccase production by a certain endophytic fungus. *Monodictys castaneae* (Wallr.) Hughes was cultured and incubated using different nutritional and physiological factors affecting laccase production. It was found that the optimum physiological conditions for laccase production were 4 disc inoculum size, a 9-day fermentation period, pH 5, and 28°C. The enzyme was purified using acetone precipitation, gel filtration, and ion-exchange chromatography. The enzyme was identified as a monomeric protein with a molecular mass of 63 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Different concentrations of $CuSO_4$ were used to stimulate laccase production and activity. A 0.15 g/l concentration of $CuSO_4$ was the optimum dosage for laccase activity enhancement. For enzyme-guaiacol interaction, the optimal pH and temperature were 5.4 and 35 °C, respectively. The laccase activity was highly stimulated using 3 mM and 7 mM concentrations of chloride salts of Cd^{+2}, Ca^{+2}, Cr^{+2} and Fe^{+3}, but the enzyme activity was slightly inhibited by 10 mM concentrations of these metal ions. According to all previous results, *Monodictys castaneae* showed promising potential as a producer of laccase.

Keywords: Endophyte; Fermentation; Enzyme; Monomeric Protein

I. INTRODUCTION

Laccases are multinuclear oxidase enzyme which contain copper and have ability to act on diverse aromatic and aliphatic compounds. Laccases have broad substrate specificity, so they are widely used in various industrial and biotechnological fields (Agrawal *et al.*, 2018). Laccases are isolated from different sources such as higher plants, bacteria, insects, fungi and lichens (Claus & Decker, 2006).

They have been represented as a "Green Tool", because they need molecular oxygen (O_2) as the only co-substrate for bio-catalysis and not hydrogen peroxide (Surwase *et al.,* 2016). They can reduce oxygen to water through one-electron oxidation of substrate which are at most substituted phenols.

Laccases have high catalytic efficiency and are used for various applications in different industrial and biotechnological fields such as environmental hazard removal, bio-detection, degradation of synthetic dyes, printing and dyeing manufacture, bio-pulping in paper manufacture, a different process for aromatic compounds, and removal of phenols which have different negative effects on living organisms health, especially when they found in polluted water (Pang *et. al.*, 2016; Xenakis *et. al.*, 2016; Zheng *et al.*, 2016). In general, enzymes are preferred in many processes rather than chemicals. Chemical treatments are costly and need complicated instruments. Enzymes not only are widely used for removal of pollutants, but also are an eco-friendly, cost-effective, and efficacious tool in bioremediation (Khan *et al.*, 2013). Laccases are currently used in bioremediation of different pollutants as phenols, aromatic hydrocarbons, lignin, phosphorus compounds and various dyes (Viswanath *et al.*, 2014). In prokaryotes, laccases play different important roles such as morphogenesis, pigment biosynthesis, and copper homeostasis (Strong & Claus, 2011). Furthermore, the fungal laccases have important roles during different physiological process such as sporulation, pigment production and plant

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pathogenesis (Sadhasivam *et al.*, 2008). Fungal laccases likely have additional functions in nature, including lignocellulose degradation, soil organic matter cycling, fungal plant–pathogen/host interaction and defence, and stress responses on diverse environmental challenges (Kues & Ruhl, 2011). Endophytic microorganisms colonise living, internal tissues of the plants without causing any negative effects (Wasser, 2002). Endophytes have proved to be the promising sources of biologically active products and extracellular enzymes (Strobel, 2002). Different articles reported that *Monodictys castaneae* was isolated from different sources such as dead stem, home dust, rotting wood, leaf surface, and soil. For the first time ever, *Monodictys castaneae* was isolated as endophytic fungi from Opuntia ficus-indica Mill. (Forage cactus) that is farmed in the semi-arid region of the Brazilian northeast and used as forage and food (Bezerra *et al.,* 2012). In addition to different extracellular enzymes that were produced by this endopyhtic fungus, there are some articles stated that *Monodictys castaneae* had the ability to produce significant yield of fungal pigments that have anti-microbial activity against human pathogens (Visalakchi S & Muthumary J, 2010). The purpose of this work was to detect the ability of a strain of endophytic *Monodictys castaneae* for laccase production. This fungus has indicated its promising potential for deployment in enzymology sector such as production of different enzymes as proteases, xylanases, pectinases and cellulases (Bezerra *et al.,* 2012). In our study, we optimise several conditions for increasing and enhancing extracellular laccase production and activity, then purify, characterise and determine of Electro-phoretical profile of laccase extracted from this fungus.

II. MATERIALS AND METHOD

A. Identification and Characterisation of The Fungus

The fungus was originally isolated from the soil collected from Western desert in Egypt, close to Crude oil well. The soil sample was serially diluted and appropriate dilution was plated using Czapek's agar medium (CZA) containing Rose bengal. The pure fungus obtained was maintained on Czapek's slants following sub-culturing and storage at 4°C. Colony morphology of the fungus was examined on 5 days old culture, grown on Czapek's agar (CZA) that contain (30.0 g sucrose, 2.0 g sodium nitrate, 1.0 g dipotassium phosphate, 0.5 g magnesium sulfate, 0.5 g potassium chloride, 0.01 g ferrous sulfate, 20 g agar, and 1000 ml distilled water) and potato dextrose agar (PDA) that contain (4 g from 200 g infused potato, 20 g dextrose, 20 g agar, and 1000 distilled water) at 28° C (Asmaa AH *et al.*, 2021). Macroscopic and Microscopic observations of the growth of fungus were recorded. The fungus identified at Assiut University Moubasher Mycological Center as AUMC 15308 *Monodictys castaneae* (Wallr.).

B. Laccase Detection

The fungal strain was cultivated in Czapek's agar medium containing 4 mM guaiacol for fungal laccase detection. Morphological observation and colour change on the fungal mycelia and solid medium were recorded every day for 10 days (Senthivelan *et al.*, 2019).

C. Optimisation of Nutritional, Physiological, and Environmental Factors Affecting Laccase Production

1. Effect of incubation period

Monodictys castaneae was cultured and incubated at 28°C and pH 5 for different time intervals (3, 5, 7, 9 and 11 days).

2. *Effect of inoculum size*

In order to test the effect of the inoculum size on cell mass and laccase production, the sterilised medium was dispended in 250 ml Erlenmeyer flasks (100 ml each), inoculated by different inoculum sizes (2, 3, 4, 5 & 6 discs), and incubated at the optimum incubation period (Aisha U & Shakil A, 2022; Yang X *et. al.*, 2020; Risdianto *et. al.*, 2012; Visalakchi S & Muthumary J, 2010).

3. *Effect of different pH values*

The effect of different starting pH values on the laccase production by the fungus was investigated using initially adjusted fermentation medium either with 1N HCl or NaOH to pH values 3, 4, 5, 6, and 7. All the pH adjustments were carried out by means of iSTEK pH meter.

4. *Effect of different temperatures*

The fungus was cultured and incubated at different temperatures (24, 28, 30, 32, 37, and 42 ±1°C) to find out the optimum temperature for the fungal growth and laccase production.

5. *Effect of shaking incubation*

The fungus was cultured and incubated in darkness for 9 days under shaken conditions using a reciprocal shaker with 150 rpm to test their effect on the laccase production.

6. *Effect of different types of nitrogen source*

Different types of nitrogen source used to determine the best one for laccase production. Sodium nitrate, yeast extract, peptone and ammonium sulfate at 2 gl^{-1} of each were added to other components of the Czapek's liquid medium without nitrogen source, and the discs of *Monodictys castaneae* were inoculated and incubated at other optimum physiological conditions.

D. Effect of Different Concentrations of Copper Sulfate for Increasing of Laccase Production

Various concentrations of copper sulfate were added to the media to enhance laccase production. Copper sulfate weight 0.20 g, 0.175 g, 0.15 g, 0.10 g were added to 1L Czapek's liquid medium and laccase activity was measured for each to determine the effect of copper sulfate as laccase production inducer.

E. Purification of Laccase

1. *Organic_solvent precipitation*

Refrigerated acetone was added to the crude laccase production liquid medium as 2:1 volume by volume at 0°C for 2 h, and then centrifuged (9,000 rpm) at 3 °C for 12 min. The precipitated material was dissolved in 2 ml sodium acetate (pH 5.4) as a buffer solution (Moubasher H *et al.*, 2017).

2. *Gel filtration chromatography*

A volume (3 ml) of the concentrated enzyme obtained after 70% organic solvent precipitation was transferred to Sephadex G-100 column buffered using Tris buffer (0.1 M, pH 7.1). Fractions of 4 ml were collected at a flow rate of 0.20 ml/min and then assayed for protein concentration which was performed according to both Layne E (1957) and Stoscheck CM (1990) methods then laccase activity which was colourimetric detected using guaiacol at 470 nm (Ayla S *et al.*, 2018).

3. *Ion-exchange chromatography (IEC)*

A volume (2 ml) of the purified enzyme using Sephadex column was applied to a Q-sepharose anion column previously equilibrated with Tris buffer (0.1 M, pH 7.1). The column was washed with 50 ml of the same buffer followed by gradual extraction of proteins using NaCl solution (0.1 – 1 M) prepared with the buffer solution (Moubasher H *et al.*, 2017). All fractions resulted from IEC were collected in 3 ml fractions at a flow rate of 0.20 ml/min and assayed for laccase activity. Fractions which contain high laccase concentration were collected and concentrated for performing Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Moubasher H *et al.*, 2017).

4. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)*

After IEC, pooled fractions which showed high laccase concentration were introduced to SDS-PAGE and Native-PAGE analysis to confirm the purity of the enzyme and to determine the sub-unit molecular weight of the purified laccase. SDS-PAGE was performed following the method by Laemmli (1970). Both Coomassie Brilliant Blue R-250 and silver stain were used for SDS-PAGE. Native-PAGE was performed using 40 mM guaiacol.

F. Characterisation of Laccase Enzyme

1. *Effect of temperature and determination of thermal stability*

Optimum temperature for the maximum activity of crude enzyme was evaluated by performing the enzymatic assays

at different temperatures. The activity was investigated by incubating the crude enzyme in sodium acetate buffer (100 mM, pH 5.4) with 4 mM guaiacol as a substrate at temperatures 25, 30, 35, 40, 50, 60, and 70 °C for a period of 30 minute.

2. Effect of pH

Optimum pH for the maximum activity of crude enzyme was evaluated by carrying out the enzymatic assays at pHs (2.6, 3.6, 4.6, 5.4, 6.4, and 7). The different levels of pH were adjusted using citrate-phosphate buffer solution.

3. Effect of different laccase inducers and inhibitors

The effect of metal ions ($CdCl_2$, $CaCl_2$, $CrCl_2$, $FeCl_3$) on laccase activity was studied with the concentrations of 3 mM, 7 mM and 10 mM. The effect of inhibitors, ethylene diamine tetra acetic acid (EDTA) and SDS (sodium dodecyl sulfate) on laccase activity was evaluated at 1 and 2 mM concentrations. The laccase-guaiacol mixture was incubated with each of these metal ions and inhibitors for ½ hour according to the method stated by Reda *et al.* (2019).

III. RESULTS AND DISCUSSION

A. Identification and Characterisation of The Fungus

The isolated fungus was morphologically identified at Assiut University Moubasher Mycological Center. It was identified as AUMC 15308 *Monodictys castaneae* (Wallr.) Hughes. Macroscopic identification was performed by determination the morphological characters of the fungal Colony on 5 days old culture, grown on Czapek's agar (CZA) and Potato dextrose agar (PDA) at 28° C and the fungal colony morphology was as shown in Figure1. Microscopic identification was performed by microscopic examination of a 5-day fungal colony. According to AUMC protocol and scheme the fungal spore, conidia, and conidiophores were identified.

B. Qualitative Detection of Laccase Activity

Qualitative analysis for laccase activity was carried out in both PDA and CZA plates supplemented with 4 mM guaiacol. This Screening of fungi for laccase production was performed by using plate assay method that was used at previous different studies such as (Senthivelan *et al.*, 2019). In this study *Monodictys castaneae* showed brown colour zone (Figure 1) when it was cultured in the media containing guaiacol which is the substrate of laccase indicating laccase production from the fungus that caused oxidation of guaiacol and convert its colour from pale yellow into red brown colour.

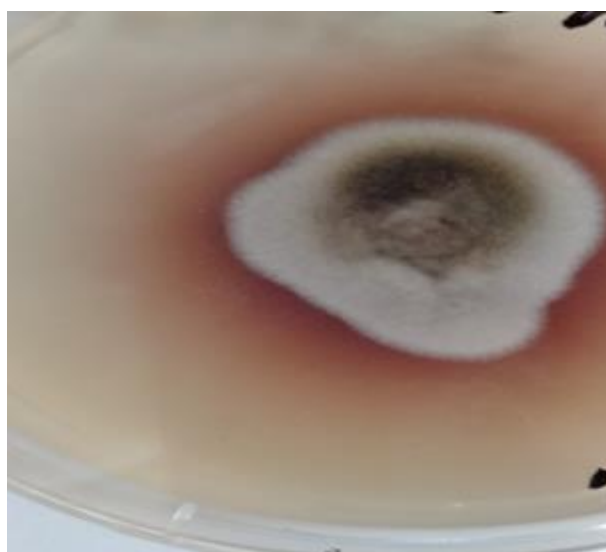

Figure 1. Fungal colony of A 5-day mycelia on CZA with guaiacol media

After the 3rd day of incubation, the fungus exhibited intense brown colour zone under and around the fungal colony especially in the case of CZA plates. The colour and diameter of brown zone became more intense and larger by increasing in the incubation period. The brown colour of the zone was light at the 3rd day of incubation, then it became darker and larger by the time of incubation indicating increasing in the laccase production.

C. Optimisation of Laccase Production

1. Fermentation period

Maximum laccase activity was found to be 2.103 ± 0.106 U/ml using Czapek's liquid medium after 9 days of fermentation period when incubation temperature was 28 °C, pH of the medium was 5 using 4 discs inoculum size (Figure 2). Fermentation period of 11 days showed a

decrease in the laccase activity which was found to be 1.781 ± 0.017 U/ml. Different optimum fermentation periods have been reported for different fungal species (Niladevi & Perma, 2008; Strong, 2011). In the present study *Monodictys castaneae* showed slow growth rate till the 1st five day of incubation periods, so its laccase activity was the minimum through this period. The fungus at day 9 showed the best growth which led to the maximum laccase production and activity. At a certain level, the incubation time cannot increase the activity of enzyme-produced laccase which is according to the fungal logarithmic/exponential phase. The decrease in enzyme activity is caused by the decrease of growth in the medium of the fungus. This results in the lower production of laccase enzyme.

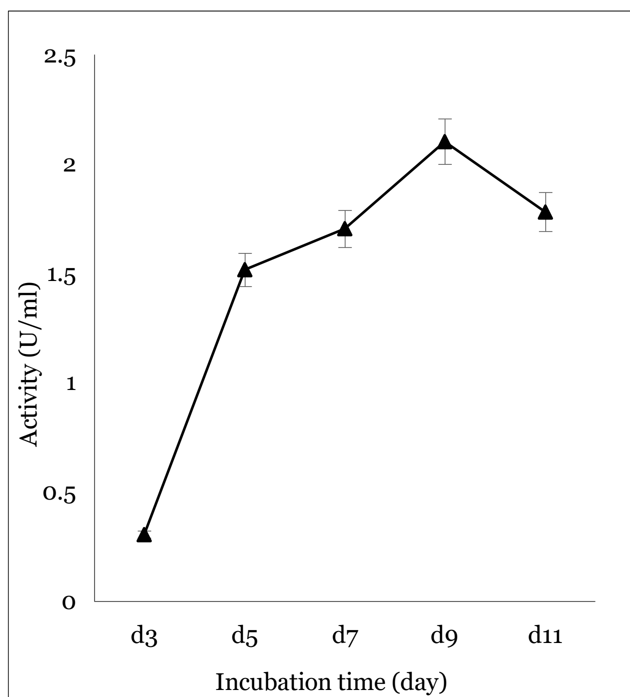

Figure 2. The relation between laccase activity and incubation periods (the data are the mean values of triplicate samples)

2. Size of inoculum

Results in Figure 3 showed that 4 discs of the inoculum was the optimum inoculum size for the production of laccase by *Monodictys castaneae* with laccase activities of 2.103 ± 0.106 U/ml. Different study using another fungus reported that the 5-disc inoculum size was the optimum inoculum for laccase production (Elshafie *et al.*, 2012). There was no significant difference in the laccase production between 4 discs and 5 discs inoculum. After that there was decreased in the production of laccase, showing laccase activities of 1.697 ± 0.016 U/ml using Czapek's liquid medium of pH 5 which incubated at 28 °C for a 9-day incubation time. This decrease in the laccase production after certain inoculum size is possibly due to the competition between the fungal spores for nutrition and decreased production of laccase (Revankar *et. al.*, 2007; Patel *et al.*, 2009).

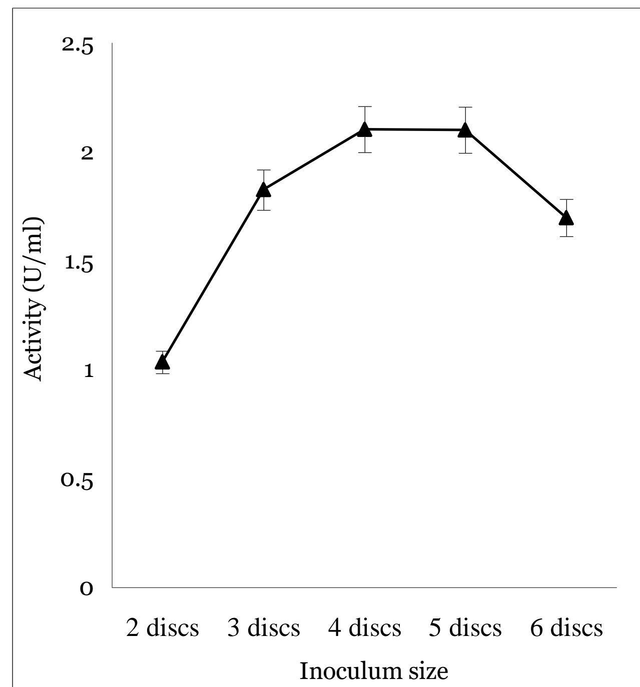

Figure 3. The relation between laccase activity and different inoculum size (the data are the mean values of triplicate samples)

3. Effect of different pH values

Fermentation media pH adjusted to different values (pH 3 - 7) before sterilisation and incubation of the fungus to determine the optimum pH for fungal growth and laccase production. Results showed that pH 5 was the optimum one for growth and production of laccase by *Monodictys castaneae* with laccase activities of 2.103 ± 0.106 U/ml. There are no significant difference in the laccase production between pH 5 and pH 6. After that there was decreased in the production of laccase, showing laccase activities of 1.901 ± 0.086 U/ml using Czapek's liquid medium with 4 discs inoculum and 9 days incubation period at 28 °C. These data

are shown in Figure 4. This results are similar with Thurston (1994) who found that when fungi are grown in the medium at pH 5.0, the laccase will be produced in excess while several studies proved that pH range between 4.5 and 6.0 is suitable for laccase production. These results are convenient with those obtained by Kalra *et al.* (2013) who found that the optimum pH value for laccase activity was 4.5 – 5.5.

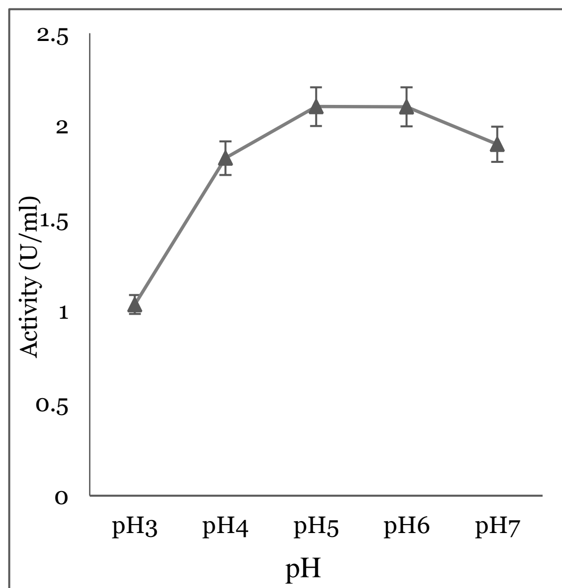

Figure 4. The relation between laccase activity and different liquid media pH (the data are the mean values of triplicate samples)

4. Effect of different temperatures

Temperature plays a very important role in both microorganism growth and enzyme production. Different studies reveal that an average temperature of 25-35°C is best for fungal biomass growth and laccase production (Sabiha *et al.*, 2022). The optimum temperature for fungal growth was determined by measurements of linear growth and macroscopic observations of fungal mycelia in the liquid media. Figure 5 shows that optimal temperature for *Monodictys castaneae* growth and laccase production was 28 °C with laccase activities of 2.103 ± 0.106 U/ml. The optimum temperature for growth and laccase production in the present work agrees with different other studies. In this study, there was no significant difference in the laccase production at temperatures ranging 28 to 32 °C. Following that, there was a slight decrease in laccase production with the lowest laccase activity of 1.578 ± 0.03 U/ml achieved at 42 °C using the optimum previous conditions.

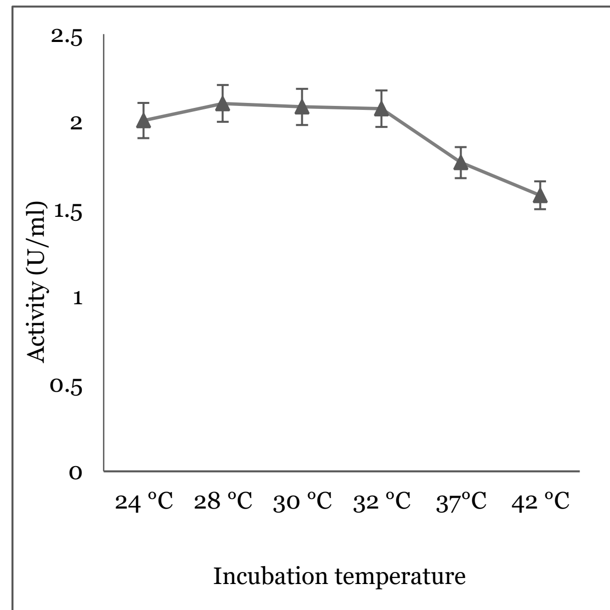

Figure 5. The relation between laccase activity and incubation temperature (the data are the mean values of triplicate samples)

5. Effect of shaking incubation

It is known that shaking of the fungal growth medium during incubation period enhance fungal growth because of good aeration. In aerobic fermentation, the presence of oxygen affect the enzyme production which may be attributed to the metabolic activities in the microorganism. Shaking process plays very important role during aerobic fermentation by homogenisation of the production medium and dissolution of oxygen (Enas MM *et al.*, 2021). It was reported by Enas MM *et al.* that the agitation speed affect the fungal growth and specific growth which affect directly the enzyme production and specific activity. In this study, it was found that shaking at 150 rpm with other optimum conditions resulted in the highest laccase production by *Monodictys castaneae*. Shaking condition achieved laccase production higher than stationary incubation, aeration system gave 2.711 ± 0.05 U/ml laccase activity, while stationary incubation system gave 2.103 ± 0.106 U/ml laccase activity.

6. Effect of different types of nitrogen source

Using peptone as nitrogen source for laccase production achieved the best laccase activity of 3.505 ± 0.007 U/ml, then yeast extract, sodium nitrate and finally ammonium sulphate which showed the lowest laccase activity of 2.4 ± 0.012 U/ml (Figure 6). In another article Nona *et al.* (2006) mentioned that peptone, followed by casein hydrolysate, appeared to be the best nitrogen sources for laccase accumulation by different fungi. They also mentioned that the positive effect of nitrogen source on enzyme production may be imputed to higher fungal biomass production.

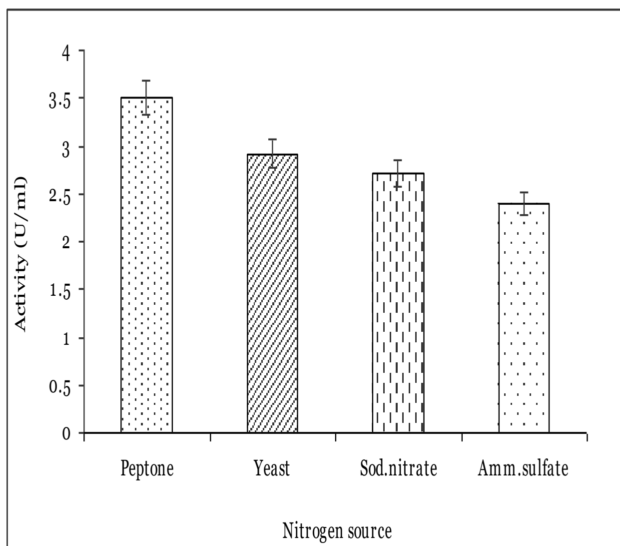

Figure 6. The relation between laccase activity and different nitrogen source (the data are the mean values of triplicate samples)

7. Effect of various concentrations of copper sulfate for laccase production enhancement

Copper sulfate weight 0.10 g, 0.15, 0.175, and 0.2 added to 1L Czapek's liquid medium and determine laccase activity for each. Copper sulfate with 0.15 g/l concentration showed the best positive result to induce laccase activity which achieved 4.058 ± 0.054 U/ml (Figure 7). Other concentrations showed laccase activity more than that of Czapek's liquid using peptone as nitrogen source which achieved 3.505 ± 0.007 U/ml, but lower than 0.15 g/l concentration (the optimum concentration). At another study Galhaup and Haltrich (2001) reported that certain fungal laccase production and activity were greatly stimulated by the addition of Cu^{2+} to the growth medium.

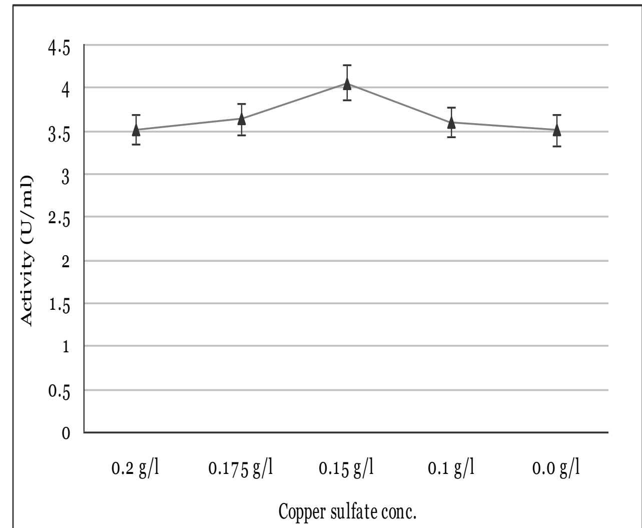

Figure 7. The relation between laccase activity and different copper sulfate concentrations (the data are the mean values of triplicate samples)

D. Purification of Laccase

Using spectrophotometer, absorbance of each fraction determined at 280 nm to detect protein content. Fractions which have the highest protein content were selected to detect Laccase activity in them. Fractions which showed high laccase activity were blended together and concentrated for Ion-exchange chromatography. Fractions corresponding to 600 mM which showed the best laccase activities and protein concentrations pooled and concentrated for SDS-PAGE and molecular weight determination. Table 1 shows the protein concentrations and corresponding laccase activity related to each purification step. SDS-PAGE and native-PAGE analysis was performed for molecular weight determination. Figure 8 shows the electro-phoretical profiles of the proteins molecular weight standards and the purified laccase after IEC. Electro-phoretical profile of laccase extracted from *Monodictys castaneae* showed distinct and clear protein band at 63 KDa. We can conclude, based on this profile, that *M.castaneae* laccase was a monomeric protein with a molecular mass of 63 kDa. The molecular mass of *M.castaneae* laccase was within the range of molecular masses for most of the fungal laccases reported 50-100 kDa (Giardina *et al.*, 2010).

Table 1. The protein concentration and laccase activity after each purification step

Purification Step	Enzyme activity (U/ml)	Protein concentration (mg/ml)
Crude enzyme (step 0)	4.058	22.319
Gel filteration chromatography	0.855	4.53
Ion-exchange chromatography	0.46	2.6

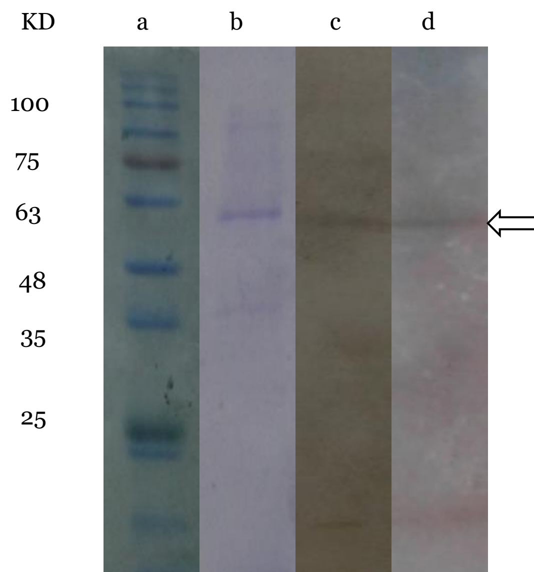

Figure 8. a. SDS-PAGE of Proteins molecular weight standard, b. SDS-PAGE of partially purified laccase after SEC using Coomassie Brilliant Blue R-250 stain, c. SDS-PAGE of purified laccase after IEC using silver stain and d. Native-PAGE of purified laccase after IEC using 40 mM Guaiacol.

E. Characterisation of Laccase Enzyme

1. Thermal stability

The results in Figure 9 show that the laccase enzyme is stable in a wide range of temperature. At 35 °C, the reaction rate of guaiacol and crude enzyme was the farthest which had laccase activity of 4.058 ± 0.054 U/ml. There was no significant difference between 35 °C and 40 °C reaction temperature. The enzyme activity has weak effect toward reaction temperature, it showed very high stability in wide range of temperature from 25 °C to 70 °C which achieved the lowest activity of 3.403 ± 0.039 U/ml. Similar results were obtained by Sadhasivam *et al.* (2008) who found that the maximum of laccase enzyme activity was found at 35 °C, also Palonen *et al.* (2003) indicated that in general, laccases are stable at 30 – 50 °C.

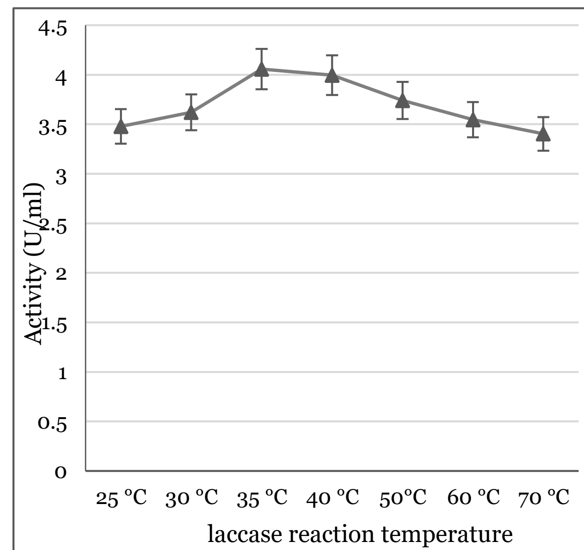

Figure 9. The relation between laccase activity and different laccase-guaiacol reaction temperature (the data are the mean values of triplicate samples)

2. pH stability

The different levels of pH were adjusted using citrate-phosphate buffer solution which wide pH range (2.6 - 7). The results in Figure 10 shows that the laccase enzyme is stable in the wide range of pH. The reaction rate of guaiacol and crude enzyme was the maximum at pH 5.4 which had laccase activity of 4.058 ± 0.054 U/ml, there are little difference of the enzyme activity at both pH 5.4 and pH 6.4, laccase activity became lower at other pH, whether more acidic or more basic, pH 2.6 achieved the lowest enzyme activity 2.157 ± 0.06 U/ml. Similar results mentioned by Kumar *et al.* (2016) who reported that the laccase enzyme activity of a different fungus was steady between pH 4 and 6.

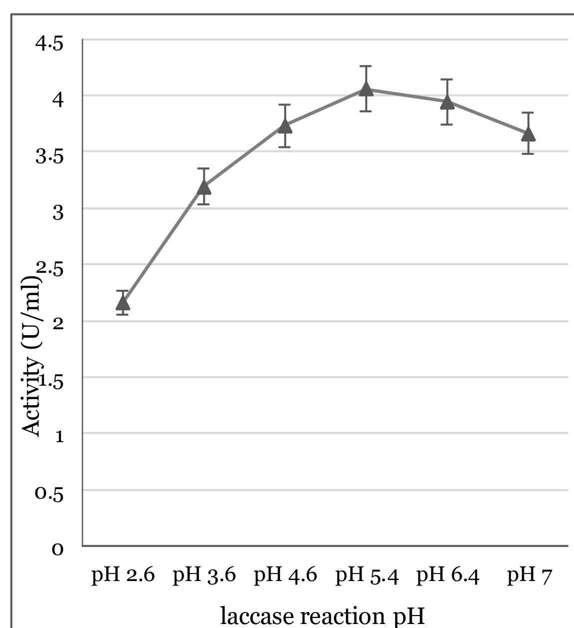

Figure 10. The relation between laccase activity and different laccase-guaiacol reaction pH, using phosphate-citrate buffer (the data are the mean values of triplicate samples)

F. Effect of Different Laccase Inducers and Inhibitors

Although laccases are efficient on a wide range of substrates without cofactors, in most cases, the addition of Cu^{2+}, Cd^{2+}, Ni^{2+}, Mo^{2+}, and Mn^{2+} ions increases the activity of laccases, whereas Ag^{2+}, Hg^{2+}, Pb^{2+}, Zn^{2+}, NaN_3, NaCl, and H_2O_2 inhibit their activity (Manavalan *et al.*, 2015). Metal ions have varying degrees of impact on activity of laccase and most of other enzymes. It has been reported that metal ions stimulated laccase formation when added to actively growing culture of a different fungus (Galhaup *et al.*, 2002). At present study concentrations of metal ions ($CdCl_2$, $CaCl_2$, $CrCl_2$, $FeCl_3$) played a vital role for stimulation or inhibition of laccase activity, the lowest concentration (3 mM) showed stimulation efficacy of laccase, while the greatest one (10 mM) exhibited the inhibition action for laccase activity (Table 2). The inhibition effect of Inhibitors (SDS and EDTA) increased with increasing of their concentrations (Table 2).

Table 2. Effect of some metal ions, some activators and inhibitors on the laccase activity

Sample	The relative activity % (Mean ± SD) inhibitor/inducer concentration		
	1 mM	2 mM	
Control	100.00 ± 0.00	100.00 ± 0.00	
EDTA	24.03 ± 0.26	10.06 ± 0.41	
SDS	32.02 ± 0.36	15.18 ± 1.3	
	3 mM	7 mM	10 mM
Control	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
Cd^{+2}	199.71 ± 4.76	128.39 ± 1.62	67.89 ± 3.08
Cr^{+2}	146.77 ± 4.42	103.51 ± 3.18	62.77 ± 2.38
Ca^{+2}	293.38 ± 7.45	205.69 ± 4.40	93.11 ± 7.85
Fe^{+3}	241.52 ± 9.02	165.84 ± 6.39	79.21 ± 5.42

IV. CONCLUSION

The endophytic fungus *Monodictys castaneae* which isolated from Egyptian Western Desert showed significant ability to be new microbial laccase supplier. The monomeric laccase was produced by this fungus and its activity was enhanced using different optimised conditions. In the present study, it was found that laccase production enhancement had been performed using Czapek's liquid media with pH5, incubated at 28 C for 9 days in the dark and shaking condition. Copper sulfate showed good laccase production stimulant when it was added to the fermentation medium, using 0.15 g/l concentration of $CuSO_4$ led to laccase activity increasing by 0.5 U/ml. For enzyme-guaiacol interaction, the optimal pH and temperature were 5.4 and 35 °C, respectively. The laccase activity was highly stimulated using 3 mM and 7 mM concentrations of chloride salts of Cd^{+2}, Ca^{+2}, Cr^{+2} and Fe^{+3}. 3 mM Ca Cl_2 showed the best stimulant for laccase activity which was increased by 293 % using this salt. This laccase was purified, characterised and its electro-phoretical profile was determined to be used at another further study as bioelectricity enhancer.

V. ACKNOWLEDGEMENT

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