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Production, Purification, Characterization and Immobilization of Laccase from *Phoma betae* and its Application in Synthetic Dyes Decolorization

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LACCASE (EC 1.10.3.2, p-diphenol: dioxygen oxidoreductases) is an extracellular enzyme found mainly in bacteria, fungi and plants. It has the ability to decolorize the hazardous synthetic dyes. Our study aimed to produce, purify, characterize and immobilize laccase from the fungus *Phoma betae* and its application in decolorization of synthetic dyes. Laccase was produced using both submerged and solid-state fermentations. Some inducers (ferulic acid, vertyl alcohol and $CuSO_4$) for laccase production were used singly and in mixture. Significant increase in laccase production with all tested compounds was detected. Submerged fermentation induced laccase production than solid-state fermentation. Fast protein liquid chromatography (FPLC) used for laccase purification and through Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) the molecular mass of the purified laccase was 37 kDa. Optimum temperature for lacasse activity was determined at 30°C using guaiacol assay. Enzyme was more stable at -4ºC than at -20 and -80ºC. Maximum laccase activity was found at pH 8. Effect of metal ions and inhibitors on laccase activity was studied. Ni^{2+} and K^{1+} induced laccase activity, while Na^{1+}, Ag^{1+}, Hg^{2+}, Zn^{2+}, Cu^{2+}, EDTA and SDS decreased laccase activity with different ratios. Laccase was immobilized in alginate beads. Both free and immobilized laccase were used to decolorize five synthetic dyes (Eriochrome Cyanine R, Merantine Brilliant Yellow 8G, Rhodamine 123, Solvent Orange 20 and Solvent Yellow 47) at concentration 0.2 g dye/L. Immobilized laccase decolorized dyes with higher yields than free one in all tested dyes. *Phoma betae* laccase is an efficient biocatalyst for synthetic dye decolorization.

Keywords: Characterization, Dye decolorization, Immobilization, Laccase, *Phoma betae*, Purification.

Introduction

Laccases are found widely in fungi, bacteria, algae, plants and insects. They are multicopper oxidases that act on a wide range of substrates. They induce the transformation of aromatic and non-aromatic compounds with reduction of molecular oxygen to water. Laccase has many applications such as pulp bleaching, detoxification, removal of phenolic compounds from wines, effluent decolouration and dye transfer blocking functions in detergents and washing powders (Desai et al., 2011; Rivera-Hoyos et al., 2013; Hoballah & Salem, 2015; Khalil et al., 2016).

Synthetic dyes are complex compounds applied in several industries such as textile, paper, printing, cosmetics and food (Khalid et al., 2010; Khalil et al., 2016). The main synthetic dyes comprise azo, anthraquinone, triphenylmethane

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and metal dyes. They are carcinogenic and their releases into the environment threaten the public health. About 10-15% of the unbound dyes from the industries are released into water bodies without any actual treatment which affects all biological systems. Hence, their elimination from the industrial effluents is a main environmental problem (Selvam et al., 2003; Carmen & Daniela, 2012; Ali et al., 2014). Physical and chemical methods are used for degradation or elimination of these dyes. The disadvantages of these methods are the high cost and the production of some toxic compounds (Kim et al., 2005).

The degradation of hazardous wastes such as synthetic dyes using microorganisms is gaining more attention. This is chiefly because of the low cost, ability to produce smaller amounts of sludge and environmental compatibility (Rauf & Ashraf, 2012). Several microorganisms such as *Aeromonashydrophila*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Penicillium* sp., *Pestalotiopsis* sp., *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Proteus mirabilis*, *Pseudomonas cepacia* and *Pseudomonas putida* were very efficient in degrading different dyes (Ashrafi et al., 2013; Maulin et al., 2013; Blyskal, 2014; Tuttolomondo et al., 2014; Mandic et al., 2019; Valliyaparambil et al., 2019; Wikee et al., 2019).

Phoma is a fungal genus belonging to Ascomycota that was less known to produce ligninolytic enzymes (Cragg et al., 2015) in contrast to the white-rot fungi that are well known to be the most effective microorganisms in degrading synthetic dyes because of their ability to produce extracellular ligninolytic enzymes such as manganese peroxidase, lignin peroxidase and laccase (Wesenberg et al., 2003; Daâssi et al., 2013; Lakshmi et al., 2017). The enzymatic operation has received great attention providing a rapid mechanism of decolorization rather than the eco-friendly treatment of textile dyes in wastewaters (Iqbal & Asgher, 2013). Dye decoloration by laccase has been suggested to handle textile waste water as a potential solution, because of their oxidation capacity and relative specificity, to oxidize various chemical structures (Husain, 2006).

The immobilization of enzymes on supportive materials have contributed mainly to the success of diagnosis and enzyme therapy approaches (Reda & El-Shanawany, 2020). Enzyme immobilization can offer various benefits over native enzymes involving fast processing, variable reaction recovering and repeating, better operational stability and higher efficiency (Ferreira et al., 2003). Several matrices have been used for cell immobilization by the entrapment technique. These are synthetic polymers such as polyvinyl, polyacrylamide and polyurethane or natural polymeric gels like agar, alginate, carrageenan, chitosan and cellulose derivatives (Katzbauer et al., 1995). Among all mentioned media, alginate is the most commonly used polymer for cell entrapment (Gerbsch & Buchholz, 1995). Alginate is nontoxic and the immobilization process is done under mild conditions (Kourkoutas et al., 2004).

This research studied the production of laccase from *Phoma betae* using submerged and solid-state cultures then purification of the produced enzyme. Some factors effect (temperature, pH-value, and chemical compounds) on laccase activity was displayed. The ability of both free and immobilized laccase to decolorize five types of synthetic dyes was also determined.

Materials and Methods

Laccase production

Submerged fermentation

Phoma betae A.B. Frank was isolated in a previous work (Abedin et al., 2013) from agriculture soil in Al-Nubaria, Egypt and was identified using molecular techniques (Accession number: JQ624871). Basal medium was used for cultivation of *Phoma betae*. It was composed of the following (g/L) in distilled water: Glucose, 20; KH_2PO_4, 1; Yeast extract, 0.01; $(NH_4)_2SO_4$, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $CuSO_4.5H_2O$, 0.001; $CaCl_2 \cdot 2H_2O$, 0.01; $MnSO_4.H_2O$, 0.001 and $Fe_2(SO_4)_3$, 0.001.

In 250ml Erlenmeyer conical flasks, 100ml of the previously stated medium were added. To each flask, one of the following activators (ferulic acid, vertyl alcohol and $CuSO_4$) was added with the concentration of 1.0mM. The final flask contained a mixture of all the tested activators. Ethylene glycol tetra acetic acid was added (0.5%, v/v) to suppress peroxidase production. All media were sterilized then inoculated with the test fungus. Incubation was at 30^oC for 5 days. The flasks contents were then prepared for further analysis of enzymatic activities.

Solid-state fermentation

Five grams of dry grinded rice straw was placed in 250ml Erlenmeyer conical flask and saturated with 20ml distilled water. A mixture of the previously stated activators was added to the flask. The flasks were sterilized and inoculated with the tested fungus. The flasks were incubated for 5 days at 37°C under static condition. The fermentation mass was extracted with 100ml of distilled water at room temperature for 1 hour on a rotator shaker (Infors Multitron HT AJ103S, England) at 200rpm , followed by centrifugation using cooling centrifuge (Heraeus Biofuge primo R, Germany) at 4°C and 5000rpm for 20min as described by Lakshmi et al. (2017) with few modifications. The supernatant was used for determination of laccase activities.

Laccase purification

All the steps were done at 4°C. Cultures were filtered through filter paper (Whatman No. 1) and centrifuged at 10,000×g for 10 min. To the supernatant, ammonium sulfate was added to give 80% saturation. Centrifugation was done at 10,000 ×g for 30min to precipitate proteins. The precipitate was dissolved and dialyzed with 10mM NH_4OAc buffer (pH 4.5) then was applied to Q-Sepharose column (10 mM Tris/HCl buffer, pH 7.4). The enzyme fraction was finally purified by fast protein liquid chromatography (FPLC) on a Superdex 75 HR 10/30 column (0.2M NH_4HCO_3 buffer, pH 8.5) (Park & Park, 2008; Tian et al., 2012).

Molecular mass determination

The molecular mass of laccase was determined by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) as described by Laemmli & Favre (1973) using 12% resolving gel and 5% stacking gel. Then, the gel was stained by Coomassie brilliant blue. The molecular weight of the purified laccase was calibrated with ladder-extra broad molecular weight (protein 6.5– 270kDa, abcam, USA).

Determination of laccase activity using guaiacol assay

For laccase assay, guaiacol has been reported as efficient substrate. Laccase activity was determined by formation of intense reddish brown color which formed due to oxidation of guaiacol by laccase. The reaction mixture was 1.0ml enzyme, 3.0ml sodium acetate buffer (10mM pH 5) and 1.0ml guaiacol (2mM). The blank contained 1.0ml distilled water instead of enzyme. The mixture was incubated for 15min at 30ºC, then absorbance was read using UV spectrophotometer (Shimadzu, Japan) at 450nm. The laccase activity was calculated in U/L using the guaiacol extinction coefficient (12,100$M^{-1}cm^{-1}$) at 450nm. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1µmol of guaiacol per min under optimal conditions (Desai et al., 2011; Kalra et al., 2013).

The calculation formula was:

$$E.A = (A * V) / (t * e * v)$$

Where, E.A= Enzyme Activity (U/ml), A= Absorbance, V= Total volume of reaction mixture (mL), t= Incubation time (min), e= Extinction Coefficient (M^{-1} cm^{-1}) and v= enzyme volume (ml).

Factors affecting laccase activity

The effect of temperature on the activity of laccase was determined by guaiacol assay at different incubation temperatures (25, 30, 35, 40, 45 and 50ºC) for 15min. Optimum temperature was the temperature at which enzyme showed maximum activity. The effect of freeze storage on laccase activity was studied. The enzyme was stored at temperatures -4, -20 and -80ºC for 6hrs in freezer (New Brunswick™ Premium, England), then laccase activity was determined.

The pH effect on laccase activity was determined by using buffers of different pH-values and incubated at 30ºC for 15min (Desai et al., 2011).

The effect of metal ions (NaCl, KCl, $AgNO_3$, $HgCl_2$, $ZnCl_2$, $Cu(NO_3)_2$ and $NiCl_2$) on laccase activity was studied with the concentration of 10 mM. Also, the effect of inhibitors on laccase activity was evaluated at the same concentration. Inhibitors were ethylene diamine tetraacetic acid (EDTA) and SDS. The reaction mixture of laccase was incubated with each compound for 30min before adding guaiacol according to the method described by Reda et al. (2019).

Immobilization of laccase in alginate beads

Laccase was mixed with Na-alginate solution (4%). The prepared mixture was then added drop by drop onto 2% $CaCl_2$ solution using a sterile

syringe. The solution was stirred gently to form the immobilized enzyme beads. The beads were kept for hardening in the same solution overnight at 4°C. The beads were then filtered and washed carefully several times with sterile distilled water until there was no detectable protein in the wash out solution then stored in dark at 4°C until further use (Wang et al., 2014).

Decolorization studies

The decolorization of five synthetic dyes was studied by the purified free and immobilized laccase. The tested dyes were Eriochrome Cyanine R (λ_{max}= 550nm), Merantine Brilliant Yellow 8G (λ_{max}= 570nm), Rhodamine123 (λ_{max}= 510nm), Solvent Orange 20 (λ_{max}= 449nm) and Solvent Yellow 47 (λ_{max}= 580nm). All dyes were used at concentration 0.2g/L. Decolorization was studied spectrophotometrically by determining the reduction in the absorbance at maximum wavelength for each dye during 6 hrs. Control samples were done with heat denatured laccase. Measurements were done in triplicates. The decolorization percentage was calculated as follows:

$$\text{Decolorization (\%)} = (A_c - A_t) / A_c * 100$$

Where, A_c is the absorbance of the control and A_t is the absorbance of the test sample as reported by Bagewadi et al. (2017).

Statistical analysis

All measurements were carried out in triplicate. The results were expressed as mean ± standard deviations (mean±SD). Data were analyzed by one-way analysis of variance (ANOVAs) using SPSS 20 statistical software. The differences between mean values were evaluated at $P \leq 0.05$ with Duncan's multiple range test (Duncan, 1955).

Results

Laccase production

Laccase was produced from the fungus *Phoma betae* cultivated using both submerged and solid-state fermentations. In submerged fermentation, some laccase activators were used singly and in combination to increase laccase production. As shown in Table 1, all used compounds had a positive effect on laccase production with maximum effect when used in mixture. Submerged fermentation was significantly more effective than solid-state fermentation (Table 2).

Molecular mass determination

The laccase molecular mass was estimated by using SDS/PAGE. Laccase purified from *Phoma betae* was shown to be homogeneous with a single band at 37kDa according to the size with SDS-PAGE (Fig. 1).

TABLE 1. Activation of laccase production using submerged fermentation

Days	Laccase activity (U/L)				
	Control	Ferulic acid	Vertryl alcohol	$CuSO_4$	Mixture of activators
Day 1	86 ± 1.5[a]	248 ± 2.3[a]	98 ± 0.7[a]	109 ± 3.1[a]	479 ± 2.8[a]
Day 2	103 ± 1.9[b]	532 ± 2.4[b]	257 ± 2.2[b]	217 ± 1.7[b]	720 ± 3.1[b]
Day 3	132 ± 1.3[c]	763 ± 3.5[c]	646 ± 3.1 [c]	525 ± 2.6[c]	915 ± 3.6[c]
Day 4	166 ± 2.4[d]	953 ± 2.9[d]	836 ± 4.2[d]	701 ± 3.4[d]	1228 ± 4.1[d]
Day 5	187 ± 2.1[e]	1097 ± 4.1[e]	964 ± 3.7[e]	751 ± 5.1[e]	1421 ± 4.5[e]

- Values are mean of triplicate readings (mean ± SD).
- Across the same column, mean values with different letters are significantly different at 5% level according to Duncan's multiple range test.

TABLE 2. Comparison between submerged fermentation and solid-state fermentation

Days	Laccase activity (U/L)	
	Submerged fermentation	Solid-state fermentation
Day 1	479 ± 2.8[a]	208 ± 1.7[a]
Day 2	720 ± 3.1[b]	532 ± 1.4[b]
Day 3	915 ± 3.6[c]	764 ± 4.1[c]
Day 4	1228 ± 4.1[d]	1155 ± 3.9[d]
Day 5	1421 ± 4.5[e]	1350 ± 2.8[e]

- Values are mean of triplicate readings (mean ± SD).
- Across the same column, mean values with different letters are significantly different at 5% level according to Duncan's multiple range test.

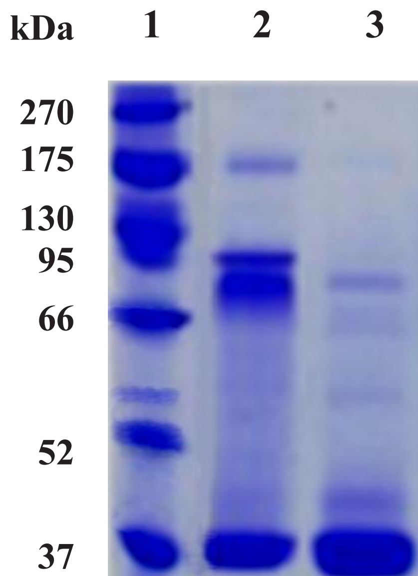

Fig. 1. SDS-PAGE of purified *Phoma betae* laccase [Lane 1: Molecular mass standards (kDa) [Xanthine oxidase, 270; Glycogen debranching enzyme, 175; Cluster of differentiation 163 (CD163), 130; Phosphorylase b, 95; Bovine serum albumin, 66; Streptavidin, 52; Glyceraldehyde-3-phophate dehydrogenase, 37], Lane 2: Purified laccase produced by solid-state fermentation, Lane 3: Purified laccase produced by submerged fermentation].

Factors affecting laccase activity

The temperature effect on laccase activity was studied using guaiacol assay at different incubation temperatures (25, 30, 35, 40, 45 and 50ºC). The optimum temperature for lacasse activity was found to be at 30°C followed by 35°C. Lowest activities were observed at 45 and 50ºC. Moderate activities were shown at 25 and 40ºC (Table 3).

TABLE 3. Effect of different temperatures on laccase activity.

Temperature (°C)	Laccase activity (U/L)
25	1570 ± 4.2^{d}
30	1963 ± 2.7^{f}
35	1825 ± 3.6^{e}
40	1112 ±1.3^{c}
45	131 ± 2.0^{b}
50	62 ± 0.8^{a}

- Values are mean of triplicate readings (mean ± SD).
- Mean values with different letters are significantly different at 5% level according to Duncan's multiple range test.

The effect of laccase storage at temperatures of -4, -20 and -80ºC for 6hrs on its activity was studied. The enzyme was more stable at -4ºC than at -20 and -80ºC (Table 4).

The data in Table 5 presented the effect of different pH-values on laccase activity. High activities were observed at pH range 6-10 with optimum pH at 8. Low activities were obtained at extreme acidic and alkaline media.

The effect of some chemical compounds on laccase activity was determined. As shown in Fig. 2, Ni^{2+} was the most effective metal ion followed by K^{+}. Other compounds decreased laccase activity.

Decolorization of synthetic dyes using free and immobilized laccase

Free and immobilized laccase were tested to decolorize five synthetic dyes (0.2g/L) at different incubation times. The used dyes were Eriochrome Cyanine R, Merantine Brilliant Yellow 8G, Rhodamine 123, Solvent Orange 20 and Solvent Yellow 47. The results displayed in Table 6 indicated that as the incubation time increased, the decolorization percent increased using free and immobilized laccase. Free and immobilized laccase were able to decolorize all tested dyes with higher decolorization percentages in case of immobilized laccase than in case of free one.

Discussion

Laccase is an extracellular enzyme secreted by several fungi (Agematu et al., 1993). It has been isolated from fungi of Ascomycete, Basidiomycete and Deuteromycte to which more than 60 fungal strains belong (Gianfreda et al., 1999).

Phoma betae was used for laccase production in the present work. Some laccase activators (ferulic acid, vertryl alcohol and $CuSO_4$) were added to the production media singly and in combination. All added compounds induced laccase activity with maximum results when added in combination.

The production of laccase was estimated to be greatly dependent on the cultivation of the fungus (Heinzkill et al., 1998). Laccase is commonly secreted in small concentrations by laccase producing fungi (Vasconcelos et

al., 2000). Addition of some supplements like xenobiotics to production media showed greater concentrations of laccase. Phenolic compounds like vanillic acid, and ferulic acid increased laccase production (Munoz et al., 1997). Aromatic compounds addition like lignin, veratryl alcohol and 2,5-xylidine induced laccase activity. Veratyl alcohol induced laccase activity than xylidine (Bollag & Leonowicz, 1984; Barbosa et al., 1996; Lee et al., 1999; Xavier et al., 2001). Copper is an important inducer for laccase production. Addition of Cu^{+2} in small concentrations to the cultivation media induced laccase production in white-rot fungi (Shutova et al., 2008; Neifar et al., 2009). Ali et al. (2015) reported that the addition of copper sulfate (10µM) to the growth medium of *Aspergillus flavus* NG85 isolated from Saint Catherine protectorate produced an increase of 122% in enzyme yield.

Although several studies assayed the inducers of laccase production, combined induction has not been widely explored. Only few researches evaluated the effect of mixed inducers on the production of laccase and activity changes. Tong et al. (2007) increased laccase production in *Trametes* sp. using a combination of Cu^{+2} and O-toluidine (0.5mM and 6mM, respectively). Values of laccase activity ranged between 68.1 x 10^2 and 78.8 x 10^2 UL^{-1}.

TABLE 4. Effect of freeze storage on laccase activity.

Temperature (°C)	Laccase activity (U/L)			
	1hr	2hrs	4hrs	6hrs
-4	27 ± 1.7^c	45 ± 2.4^c	64 ± 3.2^c	88 ± 3.5^c
-20	7 ± 0.6^b	18 ± 1.9^b	22 ± 1.6^b	30 ± 2.2^b
-80	0 ± 0.0^a	0.8 ± 0.1^a	11 ± 0.9^a	19 ± 1.3^a

- Values are mean of triplicate readings (mean ± SD).
- Across the same column, mean values with different letters are significantly different at 5% level according to Duncan's multiple range test.

TABLE 5. Effect of different pH-values on laccase activity.

pH	Laccase activity (U/L)
2	90 ± 1.2^a
4	433 ± 2.1^c
6	1461 ± 3.3^e
8	1757 ± 2.9^f
10	1343 ± 3.1^d
12	121 ± 1.7^b

- Values are mean of triplicate readings (mean ± SD).
- Mean values with different letters are significantly different at 5% level according to Duncan's multiple range test.

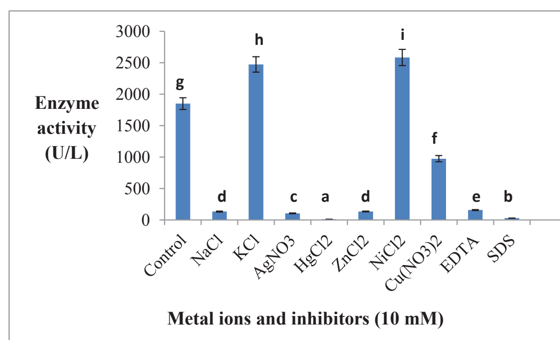

Fig. 2. Effect of metal ions and inhibitors on laccase activity [Values are mean of triplicate readings (mean ± SD), mean values with different letters are significantly different at 5% level according to Duncan's multiple range test].

TABLE 6. Decolorization (%) of different synthetic dyes (0.2g/L) using free and immobilized *Phoma betae* laccase.

Time (hrs)	Synthetic dye									
	Eriochrome Cyanine R		Merantine Brilliant Yellow 8G		Rhodamine 123		Solvent Orange 20		Solvent Yellow 47	
	Free	Immobilized	Free	Immobilized	Free	Immobilized	Free	Immobilized	Free	Immobilized
1	18± 1.7^a	43± 2.0^a	24± 2.7^a	55± 3.1^a	25± 1.1^a	32± 0.9^a	20± 2.6^a	62± 4.0^a	34± 2.1^a	56± 2.6^a
2	46± 2.3^b	63± 1.9^b	40± 3.1^b	61± 3.4^b	38± 1.8^b	58± 2.5^b	42± 2.0^b	85± 1.3^b	48± 3.3^b	70± 3.6^b
4	60± 1.9^c	79± 2.7^c	51± 2.2^c	74± 2.7^c	58± 3.2^c	71± 3.1^c	73± 2.2^c	96± 3.2^c	64± 3.1^c	89± 2.1^c
6	83± 3.3^d	93± 3.6^d	64± 1.8^d	87± 2.3^d	74± 2.4^d	89± 3.3^d	85± 3.1^d	98 ± 1.1^c	79± 1.4^d	98± 2.0^d

- Values are mean of triplicate readings (mean ± SD).
- Across the same column, mean values with different letters are significantly different at 5% level according to Duncan's multiple range test.

The laccase encoding promoter region contains many recognition sites which are specific for heavy metals and xenobiotics (Faraco et al., 2002). They combined to the recognition sites and increase laccase production.

Submerged and solid-state fermentations were used for laccase production from *Phoma betae* in the current study. Laccase production was significantly higher in case of submerged fermentation than in solid-state fermentation. Solid-state fermentation (using natural substrates such as agricultural residues) is suitable for enzymes production because they resemble the natural growth conditions of fungi (Moo-Young et al., 1983; Pandey et al., 1999; Couto & Sanroman, 2005; Brijwani et al., 2010). But in our results we found the production of laccase by submerged fermentations was significantly greater than the other treatment especially at the fifth day of cultivation. Submerged fermentation provides certain advantages such as an easier isolation of extracellular enzymes and an improved process control, as compared to solid-state fermentation (Junior et al., 2012).

Purification of laccase is an important step in deciding exact criteria with the occurrence of potential fungal compounds or analogous enzymes which may display distinctly different kinetic reactions (Rittstieg et al., 2003). The laccase was produced from the culture filtrate of *Phoma betae* as described previously according to optimum culture conditions. The obtained crude filtrate was then exposed to the purification process. Filtrates were centrifuged then; ammonium sulfate was added to the supernatants to give 80% saturation. The complete laccase precipitation from filtrate required at least 75% saturation of supernatants with ammonium sulphate (Patel et al., 2014).

According to the size with SDS-PAGE, laccase purified in this study was displayed to be homogeneous with a single band at 37kDa. Suganya & Soundhari (2019) showed that partially purified laccase from *Calocybe indica* had a molecular weight of 45kDa using SDS-PAGE analysis. Laccase purified from *Fomitella fraxinea* had a molecular mass of 47kDa on SDS-PAGE (Park & Park, 2008). Most of the fungal laccases are monomeric glycoproteins between 50-140kDa of molecular masses (Bollag & Leonowicz, 1984) with a major variety in size and glycosylation patterns (Claus, 2004). Thus, in our experiment the purified laccase was slightly smaller than the others.

It was indicated that high laccase activities were obtained at temperature ranges from 25-40ºC using guaiacol assay in the current study with optimum temperature at 30°C. Laccase B produced from *Trametes* sp. AH28-2 displayed optimum temperature for oxidizing guaiacol at 45ºC (Xiao et al., 2004). The purified laccase from *Streptomyces mutabilis* A17 displayed maximum activity at 40°C (Reda et al., 2019). Al-Hagar et al. (2019) reported that the optimum temperature was at 35ºC for laccase activity produced from irradiated *Pleurotus ostreatus*. Khalil et al. (2016) indicated that laccase enzyme retains 85% of its activity after 4 hours of incubation at 50°C and 88% of its activity after 4hrs of incubation at pH 5.

Storage of purified enzymes in optimal conditions is very important to keep their structure and activity. In the present study the effect of freeze storage on laccase activity was determined. Laccase was more stable at -4ºC than at -20 and -80ºC. Laccase from *Pleurotus ostreatus* HP-1 (Genbank Accession No. EU420068) was quite stable at -4ºC for months (Patel et al., 2014). Tamiya et al. (1985) displayed that some enzymes (ADH, G-6-PDH, LDH, MDH and PK) lost their activity during frozen storage at -20°C. Effects with different storage temperatures and times in details may be observed in future works.

The effect of different pH-values on laccase activity was estimated in the current study using guaiacol assay. At pH range 6-10, induced activities were detected with optimum pH at 8. At high acidic and high alkaline media, low activities were observed. Junghanns et al. (2009) reported that laccase produced from *Phoma* sp. UHH 5-1-03 displayed optimum pH at 5.0 by using guaiacol as a substrate. The optimum pH for laccase activity from *Pleurotus ostreatus* was found to be at 5.5-5.6 using guaiacol as a substrate (Palmieri et al., 1993; Patel et al., 2014). The optimum pH for laccase B produced from *Trametes* sp. AH28-2 for oxidizing guaiacol was at 4.7. The enzyme displayed a good stability in a pH-range of 3.5–7.5 (Xiao et al., 2004). Reda et al. (2019) found that optimum pH for laccase produced from *Streptomyces mutabilis* A17 was at 8.0 within stability pH-range of 7.0 to 9.0. Salony et al. (2006) observed that at higher pH, there was

a gradual decrease in the oxidation rate of many substrates. This may be due to ionization of critical amino acids (Asp and Glu), showing that the enzyme was inactive at higher pH-value. Junior et al. (2012) concluded that each fungal species has unique stability characteristics and physiological parameters, which demonstrate how effective it will be for applications with specific needs.

Oxidation of phenolic compounds by laccase activity has been caused by two effects. On one hand, ionization potential of phenolic compounds decreases with increase in the pH-value of the solution as a result of formation of phenolate anion that has to increase the enzymatic reaction. On the other hand, the rate of laccase catalyzed reactions decreases with increase in the pH of the solution at the cost of -OH binding with the T2/T3 site of the enzyme (Patel et al., 2014).

The results of this study evaluated some activators and inhibitors of laccase activity which showed that, $Ni^{2+}$and K^{1+}induced laccase activity, while Na^{1+}, Ag^{1+}, Cu^{2+}, Hg^{2+}, Zn^{2+}, EDTA and SDS decreased purified laccase activity. In relation to the present results, Manavalan et al. (2015) stated that Ni^{2+} ions promoted laccase activity, while Ag^{2+}, Hg^{2+}, NaCl and Zn^{2+} prevented their activity. D'Souza-Ticlo et al. (2009) informed that Lac-II activity was inhibited with 32–37% in the presence of Ag and Hg. Park & Park (2008) showed that activity of laccase from *Fomitella fraxinea* was slightly stimulated by K^{+} and unaffected by Cu^{2+}. Kumar et al. (2012) found that the effect of metal ions is concentration dependent. They displayed that laccase activity was slightly inhibited (32% inhibition) in the presence of 2mM Cu^{2+} and by increasing the concentration up to 10 mM, the enzyme activity decreased by 61%. Although laccase is containing copper, extra addition of Cu^{2+} ions may cause a change in laccase structure.

Patel et al. (2014) found that EDTA and cysteine inhibited laccase activities to a lesser extent as compared with sodium azide. Dube et al. (2008) displayed that 5Mm EDTA inhibits the total laccase activity. De-Souza & Peralta (2003) reported that the chelating agent EDTA inhibited the enzyme only at higher concentrations. EDTA was not an efficient laccase inhibitor except in high concentration (Heinzkill et al., 1998; Liu et al., 2010). Xiao et al. (2004) found that laccase B activity from *Trametes* sp. AH28-2 was inhibited mildly by SDS.

Abedin et al. (2013) reported that *Phoma betae* showed 99% decolorization rate with crystal violet and 90% with safranin at dye concentration of 0.2 g/L. Junghanns et al. (2008) reported that *Phoma* sp. professionally decolorize synthetic anthraquinone and azo dyes.

Decolorization studies were applied to five synthetic dyes in the present study using free and immobilized laccase. Both free and immobilized laccase decolorized the tested dyes with higher decolorization rate in case of immobilized enzyme than in free one. The results verified that the immobilization of laccase displayed significant enhancement in enzyme properties for dye decolorization.

Free and Ca-alginate immobilized fungi were used to decolorize the metal textile dye Lanaset Grey G (50mg/L). The tested fungi were *Coriolopsis gallica*, *Bjerkandera adusta*, *Trametes versicolor* and *Trametes trogii*. The percentages of decolorization were 2-fold greater in the case of immobilized cultures than those obtained by the free fungal cultures except for *T. versicolor* cultures which had similar decolorization percentages (Daâssi et al., 2013). Allam (2017) reported that immobilized mixture of three bacterial species (*B. subtilis, R. radiobacter* and *S. paucimobilis*) decolorized two azo dyes efficiently more than free bacterial cells of single or mixed cultures. This may be due to the nature of the matrices (Ca-alginate) which acts as protector for cells against surrounded environment by decreasing the number of attack sites.

Conclusion

This work highlighted *Phoma betae* as a good source of laccae. Maximization of enzyme production can be achieved under submerged fermentation. Immobilization enhanced laccase decolorization properties. *Phoma betae* is a promising strain for biotechnological interest in view of xenobiotics (like synthetic dye) degradation in future.

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