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Evaluation of Dye Decolourization ability of Laccase Produced *Curvularia lunata* SS17

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Abstract

The increasing discharge of dyes into the environment and their consequential ecological effects has necessitated the need for the ecofriendly decolourization methods. Laccases became enzymes of research interest due to their broad substrates specificities. The aim of the study was to purify, characterize and determine the decolourization ability of Curvularia lunata SS17 produced laccase. Laccase was produced using maize cob as substrate under optimized culture conditions and purified using Gel Filtration Chromatography. Biuret method was then used to estimate the protein contents of the crude and partially purified laccase. Specific activities, purification fold and yield (%) of the crude and purified laccase enzyme were also estimated. Decolourization potentials of the crude and partially purified enzyme were evaluated using four dyes namely: Congo red, Methylene blue, Bromocresol green and direct yellow. Elution profile of partially purified laccase revealed that fraction 5 had the highest laccase activity (3.654 U/mL). Optimum conditions for enzyme activity were estimated to be 35°C and pH 6. Enzyme activity of the partially purified laccase (3.654 U/mL) was observed to be higher than that of the crude laccase (1.635 U/mL). Also, the partially purified laccase had higher specific activity (1.87 U/mg) compared to that of the crude laccase (0.41 U/mg). Higher percentage dye decolourization potential was observed using the partially purified laccase compared to the crude laccase. Increase in percentage decolourization of the dyes by the partially purified laccase as well as crude laccase was observed as incubation time proceeds.

Keywords: Laccase, Curvularia lunata, purified, decolourization, dye

INTRODUCTION

Laccases are oxidoreductase enzymes that oxidizephenolics (diphenols, polyphenols, substituted phenols aromatic amines and benzenethiols (Vantamuri and Kaliwal, 2015). This reaction is accompanied by the production of water through oxygen reduction (Giardina *et al.*, 2010; Vantamuri and Kaliwal, 2015).

Laccases produced by fungi havethe ability of degrading different organic pollutants including polycyclic aromatic hydrocarbons (Faradi de Souza *et al.*, 2011; Mtui, 2012). They are enzymes of high interest for diverse application due to their substrate flexibility and ability toserve as environmentally friendly alternative (Buddolla *et al.*, 2008).

Increase in dye discharge into the environment has been recognized as a major source of water pollution. Their release into surface water system makes the water unfit for use and limits light passage thereby affecting aquatic life as well as their biodiversity (Gudelj *et al.*, 2011; Karim *et al.*, 2018). The presence of dyes in aquatic ecosystems is associated with health threats as a result oftoxicity and carcinogenic effects (Karim *et al.*, 2018).

Increased application of synthetic dyes is worrisome and their releaseinto the ecosystem can results in ecological effects (Abdulredha, 2013). Discharge of dyes has been reported as a major problem of water toxicity and threat to aquatic life (Gudelj *et al.*, 2011).

Laccases are relatively stable, which makes their purification easy (Toca-herrera *et al.*, 2007). Such characteristics make them suitable for application in biobleaching, and industrial wastewater treatment (Toca-herrera *et al.*, 2007). Laccases are able to degrade several dye structures and transform their toxic compounds into safer compounds making them useful in bioremediation (Karim*et al.*, 2018).

MATERIALS AND METHODS

Preparation of Stock Solutions of Dyes

To prepare 1000 ppm stock solutions of the test dyes: Congo red, Methylene blue, Bromocresol green and direct yellow, 1g each was dissolved in 1 L of distilled water.

Laccase Production and Extraction

Curvularia lunata SS17 previouslyisolated and characterized by Bello *et al.*(2021) was used in this study. Laccase production was conducted out under solid state fermentation using five grams of corn cob moistenedwith 15 mL of mineral medium (3.0 $gL^{-1}$of NH_4NO_3; 3.0 gL^{-1} of $(NH_4)_2HPO_4$; 0.5 gL^{-1} of $MgSO_4$. $7H_2O$; NaCl; 0.5 gL^{-1} of $CaCO_3$; 0.35 gL^{-1} of $FeSO_4.7H_2O$ and 0.6 gL^{-1} of $CuSO_4.5H_2O$) (Szabo *et al.*, 2015). Fermentation was set upunder the previously determined optimumincubation conditions for laccase production of inoculum size = 3*5mm fungal plug, pH = 5, temperature = 30°C and incubation for 6 days(Bello *et al.*, 2020).

After the fermentation, the fermented media was reconstituted with 50 mL of CH_3COONa buffer at pH 4.5, shaken for 2 hours at 100 rpm and centrifuged for 5 minutes at 8000 rpm (Risdianto *et al.*, 2012). Enzyme activity of crude laccase was evaluated in the filtered supernatant as described below. The culture filtrate from this set up was used for enzyme purification.

Laccase Purification

Filtered supernatant was loaded onto column of Sephadex G-100 previously equilibrated with 25 mMCH_3COONa buffer (pH 4.5) based on gel filtration chromatography technique (Wuyep *et al.*, 2012). Elution of the enzymes was set at 1 mL per minuteflow rate using the CH_3COONa buffer. A total of 25 fractions of 20 mL each were collected and assayed for laccase activity as well as concentration of protein (Wuyep *et al.*, 2012).

Characterization of Enzyme Activity

Optimum temperature for enzyme activity was determined by setting the enzyme mixture composed oflaccase enzyme, guaiacol and CH_3COONa buffer at 25°C, 30°C, 35°C, 40°C and 45°C for 15 min. Optimum pH for enzyme activity was estimated by adjusting the enzyme mixture to 3, 5, 7, 9 and 11. Following 15 min of incubation, absorbance of the reaction mixtures were read and used for calculation of enzyme activity (Kalra *et al.*, 2013).

Laccase Activity Determination

Formation of reddish brown coloration as a result of guaiacoloxidation by laccase is used to determine enzyme activity at 450 nm. Preparation of the reaction mixture was done as described by Abd El Monssef *et al.* (2016) and is composed of: 2mMguaiacol (1 mL), 10mMCH_3COONa buffer (3 mL) andfiltered supernatant (1 mL) serving as enzyme source. In place of filtered supernatant the blank contains distilled water (1 mL). After incubation for 15 min at 30°C, Ultraviolet spectrophotometer (Model No. 752N)was used to read the absorbance at 450 nm (Abd El Monssef *et al.*, 2016). Laccase activity in U/ml was estimated using the formula below:

$$\text{Enzyme activity} = \frac{\text{Absorbance x Total mixture volume}}{\text{incbation time x extinction coefficient x enzyme volume}}$$

Extinction coefficient for guaiacol = 0.6740 µM/cm.

Estimation of Protein concentration

Concentration of protein in the crude and purified enzyme sample was estimated using Biuret method as described by Saqib *et al.* (2015) (Table 1). Briefly, different concentrations of Bovine Serum Albumin (BSA) were prepared (Table 2) and used to plot a standard curve of absorbance against BSAconcentrations (Figure 1). Equation of simple linear regression was generated and used to estimate protein concentration in the crude and purified enzyme samples.

Table 1: Biuret Reagent Composition

S/N	Chemicals	Quantity/Liter
1	NaOH	8.0 g
2	$CuSO_4.5H_2O$	3.0 g
3	KI	5.0 g
4	$C_4H_4KNaO_6$	96.0 g
5	Distilled water	Make up to 1000 mL

Source: Saqib*et al.* (2015)

The specific activity, purification folds and % yield were estimated using the following formulae as described by Wuyep*et al.* (2012):

$$\text{Specific activity} = \frac{\text{Total Activity (U)}}{\text{Total protein (mg)}}$$

$$\text{Purification fold} = \frac{\text{Specific activity of enzyme after a purification step}}{\text{Specific activity of the crude enzyme}}$$

$$\% \text{ yield} = \frac{\text{Total amount of enzyme after purification}}{\text{Total amount of enzyme in the crude extract}} \times 100$$

Decolourization of Dye by Laccase

The decolourization of the four dyes by crude and partially purified laccase was determined as follows: The reaction mixture (1.6 mL) contained 1 mL citrate phosphate buffer (pH 6), 0.5 mL laccase (crude or partially purified) and 0.1 mL of the four dyes for each dye five concentrations (100 ppm). The reaction was initiated after the addition enzyme at 35˚C (Mechichi *et al.*, 2006). Absorbance of samples collected at 3 hours intervals were measured at 450 nm. The dye decolourization percentage was calculated as:

$$\text{Percentage dye decolourization} = \frac{\text{initial absorbance} - \text{final absorbance}}{\text{Initial absorbance}} \times 100$$

RESULTS

Table 2 shows the concentration of protein in the sample as estimated by Biuret Assay. Figure 1 represents the standard curve graph of absorbance against the different concentrations of BSA.

Table 2: Estimation of Protein concentration

S/N	Distilled water	Protein Standard (4mg/mL)conc. (mL)	Biuret Reagent conc. (mL)	Total Vol. (mL)	Protein conc. (mg/mL)	Absorbance at 540nm
1(Blank	0.5	-	1.00	1.50	0.00	0.000
)	0.40	0.10	1.00	1.50	0.40	0.054
2	0.30	0.20	1.00	1.50	0.80	0.095
3	0.20	0.30	1.00	1.50	1.20	0.148
4	0.10	0.40	1.00	1.50	1.60	0.188
5	-	0.50	1.00	1.50	2.00	0.243
6						

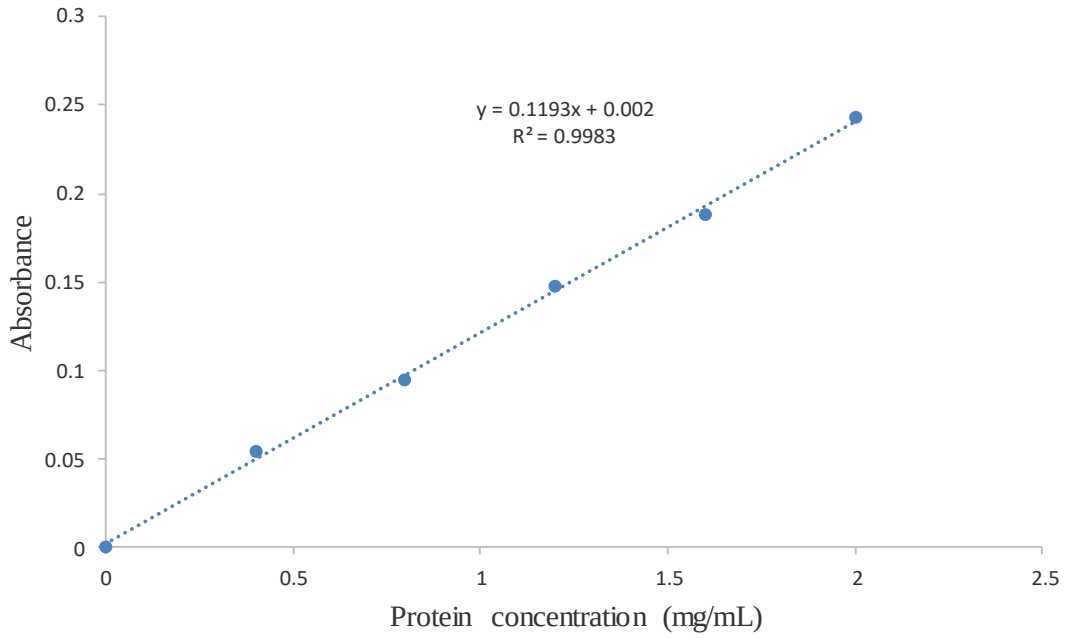

Figure 1: Standard curve plotted using concentrations of Bovine Serum Albumin against absorbance for the protein concentration determination

Of the 25 elution fractions collected during gel filtration chromatography, fraction 5 had the highest enzyme activity of 3.654 U/mL followed by fraction 10 with enzyme activity of 1.724 U/mL while fraction 24 had the least enzyme activity of 0.08 U/mL (Figure 2).

Effect of temperature on enzyme activity of laccase produced by *Curvularia lunata* is illustrated in Figure 3. The optimum temperature was revealed to be 35°C (1.584U/mL). The laccase activity reduces significantly with increase in temperature above the optimum temperature ($p \leq 0.05$).

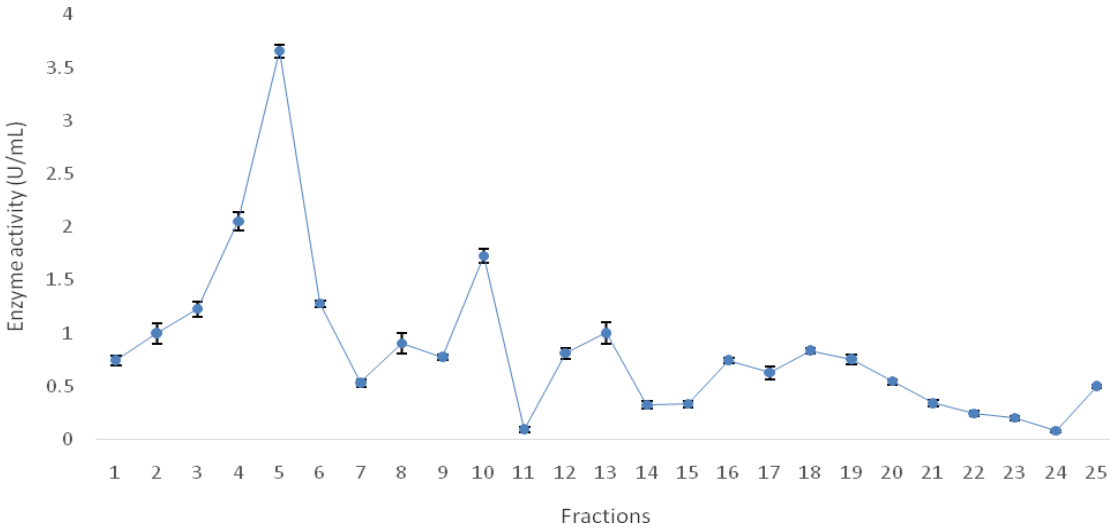

Figure 2: Elution profile of laccase produced using optimum culture conditions and purified by Sephadex G-100 gel filtration chromatography using Guaiacol oxidation assay.

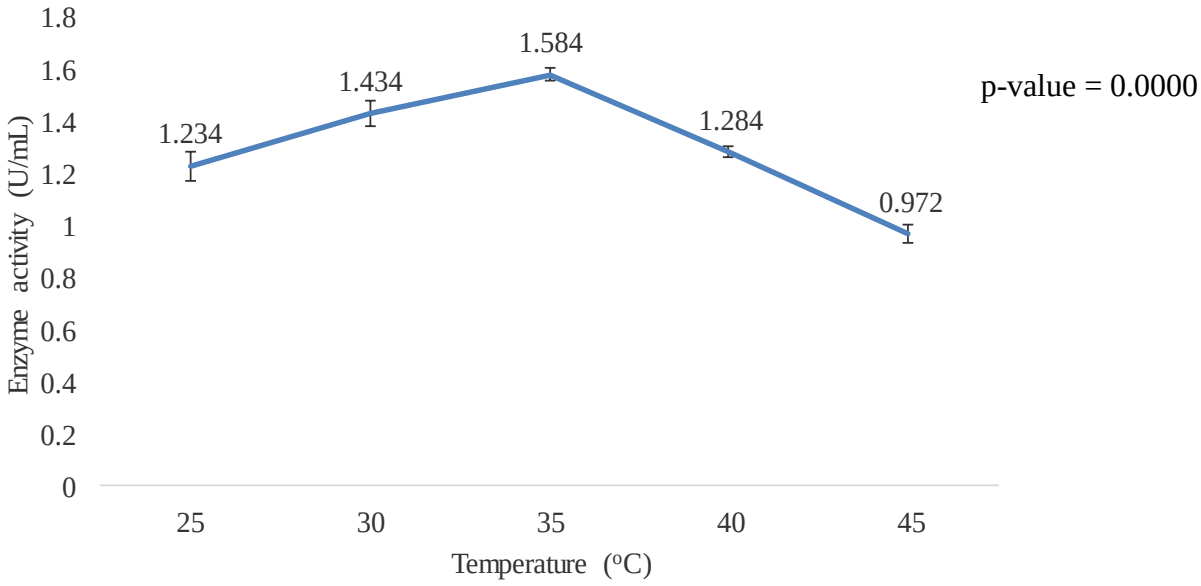

Figure 3: Effect of Temperature on Enzyme Activity of Laccase Produced by *C. lunata*

Optimum pH for laccase enzyme activity was estimated to be pH 6 (1.604 U/mL). Enzyme activity was significantly reduced at pH lower than the optimum and pH higher than the optimum pH ($p \leq 0.05$) (Figure 4).

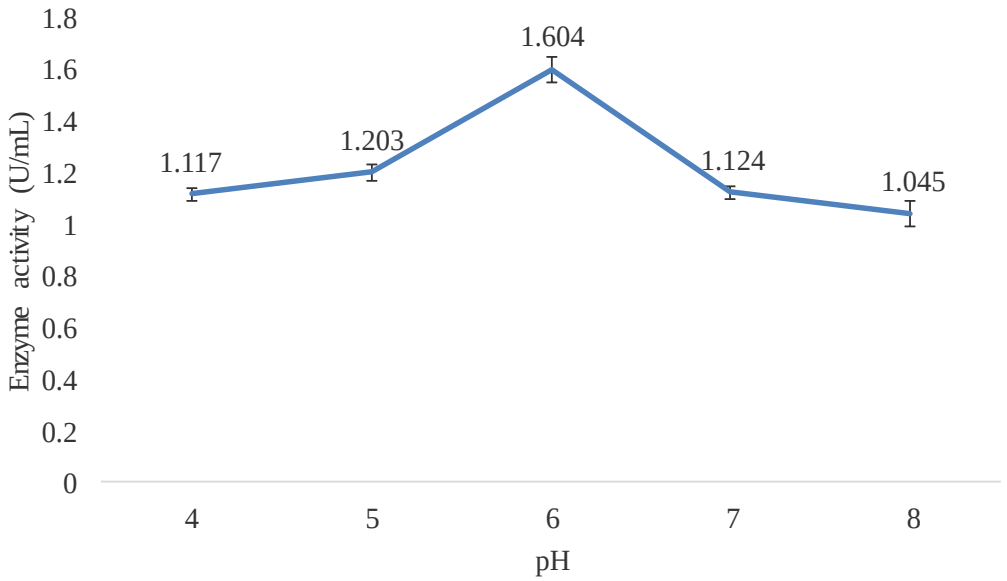

Figure 4: Effect of pH on Enzyme Activity of Laccase Produced by *C. lunata*

Purification chart of laccase from *Curvularia lunata* purified by gel filtration chromatography is illustrated in Table 3. The enzyme activity (1.635 U/mL) and specific activity (0.41 U/mg) of the crude laccase were increased to 3.654 U/mL and 1.87U/mg respectively after partial purification. However, decreases in total activity from 81.75 U to 73.08 U and protein concentration from 4.04mg/mL to 1.95mg/mL were observed after purification. The purification fold and percentage yield of gel filtration chromatography was found to be 4.56 and 89.40% respectively.

Table 3: Purification Chart of Laccase from *Curvularia lunata* Purified By Gel Filtration Chromatography

Enzyme	Vol. (mL)	Protein concentration (mg/mL)	Enzyme activity (U/mL)	Total protein (mg)	Total laccase activity (U)	Specific Activity (U/mg)	Purification Fold	% Yield
Crude enzyme	50± 0.00	4.04± 0.02	1.635± 0.01	202± 1.0	81.75± 0.30	0.41± 0.00	1.00± 0.00	100± 0.00
Partially purified enzyme	20± 0.00	1.95± 0.01	3.654± 0.01	39± 0.20	73.08± 0.14	1.87± 0.02	4.56± 0.01	89.40± 0.16

Values are mean± SD

Figure 5 shows the mean decolourization (%) of the dyes at 100 ppm concentration by crude and partially purified laccase enzymes. The result shows that the purified enzyme had higher decolourizing ability compared to the crude enzyme. The highest percentage decolourization was observed in Methylene blue (56.48%) followed by Direct yellow (55.04%) after 3 hours by the purified enzyme. The percentage decolourization was found to be higher after 6 hours and highest after 9 hours for all the dyes by both the purified and crude enzyme.

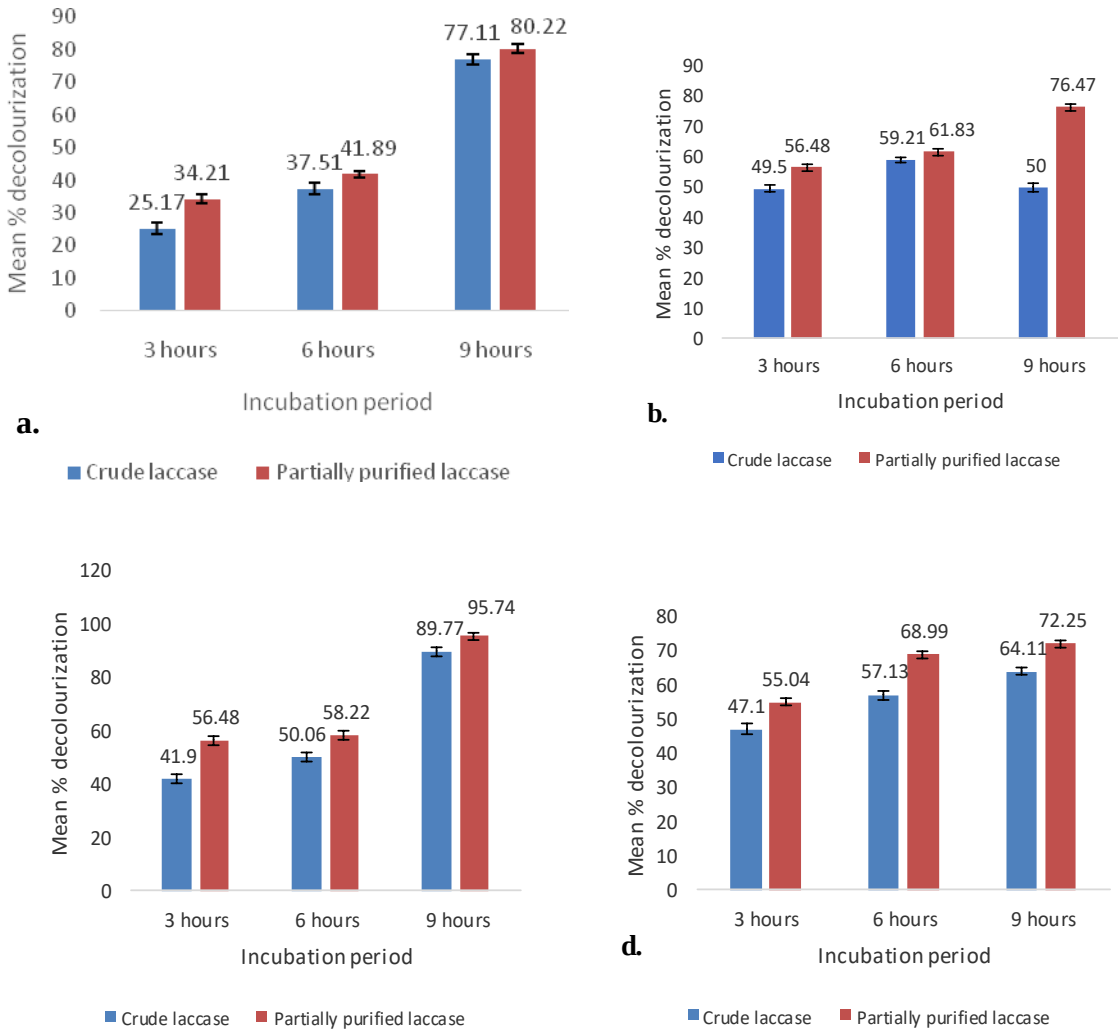

Figure 5: Percentage Decolourization of (a) Congo red(b) Methylene blue (c)Bromocresol greenand (d) Direct yellow at 100ppm Concentration by Crude and Partially Purified Laccase Enzyme Produced by *Curvularia lunata*. Bars indicate standard deviation (±SD)

DISCUSSION

Among the 25 fractions from the gel filtration chromatography, fraction 5 was observed to be the optimum with the highest enzyme activity of 3.654 U/mL. This is more than two times the laccase activity (1.635 U/mL) of the crude laccase produced using the optimum culture conditions. Increased enzyme activity of the partially purified laccase is likely due to its increased purity level and removal of interfering non-enzyme protein. Similar trend was observed by Saqib *et al.* (2015) where the partially purified laccase had an enzyme activity of 9.06 U/mL which ismore than twice that of the crude laccasewith enzyme activity of 3.995 U/mL.

The result of this study indicated that 35ºC was the optimum temperature for enzyme activity. However laccase remained stable between 25ºC and 40ºC. There was decrease in laccase activity at 45ºC likely due to destruction of laccase structure (Saqib *et al.*, 2015). Decrease in laccase activity at 45ºC might due to enzyme denaturation and subsequent loss of activities.

Different optimum temperatures ranging from 30ºC to 60ºC have been reported for laccase produced by different organisms. This is likely due to differences in the characteristics and properties of laccases produced by different organisms. Saqib *et al.* (2015) reported 30ºC, Kammoun*et al.* (2009) reported 55ºC while Sahay *et al.* (2009) reported 60ºC as the optimum temperatures for laccase activity. The differencessuggest that fungi species produce various types of laccases produced.

The result of this study revealed pH 6 as the optimum pH for laccase activity. Lower laccase activities were observed at pH above and below the optimum, this might be due to changes in the 3 dimensional structures of the enzyme active sites and substrates binding speed. Different pH values have been reported as optimum for enzyme activity of laccase. Saqib *et al*. (2015) reported pH 5 as optimum for laccase activity.

The enzyme activity of the crude laccase (1.635 U/mL) and partially purified laccase (3.654 U/mL) observed in this study is lower than the enzyme activity of crude laccase (3.995 U/mL) and partially purified laccase (9.06 U/mL) produced by *Neurospora sitophila* reported by Saqib *et al*. (2015) using corn cob as substrate. This difference in enzyme activity is likely due to the fact that laccase activity varies and depends on the microorganism producing it. However, the enzyme activity of the partially purified laccase observed is higher compared to the enzyme activity of laccase produced by *Curvularia kusanoi L7* strain (2800U/L equivalent to 2.8U/mL) after a three-phase purification system in a study carried out by Vázqueza *et al*. (2019).

The protein concentration decreases from 4.04 mg/mL to 1.95 mg/mL while the specific activity increases from 0.41 U/mg to 1.87 U/mg after the partial purification. Decrease in protein concentration observed after purification is as a result of the removal of other protein sources during gel filtration chromatography. Increased specific activity observed could also be linked to the purification procedure, since the purified sample contains mainly the desired enzyme. This trend is similar to the reports of Wuyep *et al*. (2012) who observed a decrease in protein concentration from 12.78mg/mL to 3.91mg/mL as well as an increase in specific activity from 0.31 U/mg to 2.32 U/mg and Saqib *et al*. (2015) who also observed a decrease in protein concentration from 2.5 mg/mL to 0.201 mg/mL as well as an increase in specific activity from 0.44 U/mg to 34.33 U/mg after partial purification. The percentage yield of partially purified laccase enzyme by gel filtration chromatography (89.40%) observed in this study is higher than the 27.0% and 17.0% reported by Wuyep *et al*. (2012) using gel filtration chromatography and anion exchange chromatography respectively. This might be due to difference in amount of total enzyme after purification relative to amount in crude sample.

The total protein concentration was estimated to be 202 mg for the crude enzyme and 39 mg for the partially purified enzyme. The reduction in protein concentration is an indication that there were other protein sources apart from the laccase enzyme in the crude enzyme which was removed during the purification by gel filtration chromatography. Similar trend in total protein reduction after purification was reported by Wuyep *et al*. (2012) using gel filtration chromatography and Minari and Agho (2018) using ammonium sulphate purification.

Percentage decolourization of the dyes varies with the enzyme type (crude or partially purified), type of dye and incubation period. Generally, the mean percentage dye decolourization by the partially purified laccase enzyme was higher than the percentage decolourization by the crude laccase enzyme. This is likely due to the higher enzyme activity and specific activity of the partially purified laccase enzyme. This result contrasts with the finding of Wakil *et al*. (2019) who reported that the partially purified laccase mostly had lower decolourization. Methylene blue had the highest percentage decolourization of 49.50% and 56.48% by crude and partially purified laccase after 3 hours of incubation respectively. The least decolourized dye after 3 hours was Congo red (25.17% and 34.21% for crude and partially purified laccase respectively). This implies that Congo red is more resistant to enzymatic treatment or requires longer time for decolourization. This is in linewith the finding of Forootanfar *et al*. (2012) who observed that Congo red was the least decolourized dye (18.5%) by laccase produced by *P. variabile* after 3 hrs of incubation alongside laccase mediator (5mM hydroxybenzotriazole). Variation in the rate of decolourization of dyes that are structurally different by laccase might be linked to redox potential differences and their suitability with the enzyme active site. This is similar to the result of Afreen *et al*. (2018) and Wakil *et al*. (2019).

Increase in mean percentage decolourization of the dyes by laccase was recorded with increase in incubation period. The mean percentage decolourization of Methylene blue by partially purified enzyme increases from 56.48% after 3 hours to 61.83% and 76.47% after 6 hours and 9 hours respectively. Increase in percentage decolourization of dyes by laccase with incubation period was also observed by Wakil *et al*. (2019)

CONCLUSION

The enzyme activity (3.654 U/mL) and specific activity (1.87 U/mg) of the partially purified laccase were higher than those of the crude laccase (1.635 U/mL and 0.41 U/mg respectively).The optimum conditions for

laccase activity wereobserved to be 35°C and pH 6. The dyes were decolourized by both the crude and partially purified laccase, with partially purified laccase having higher decolourization ability.

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