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Research Article

Purification and Characterization of Laccase Enzyme from Locally Isolated *Aspergillus flavus* Strain

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Authors' Contributions
SRZ and SAM conceived the idea and designed the research. SRZ performed the experiments. SAM pefromed statistical analysis and reviewed the manuscript.

Keywords
Laccase, Purification, *Aspergillus flavus,* Pakistan

Abstract | Laccases belong to family oxidoreductase are widely present in nature and possess broad range of substrates. Vast application of these enzymes demands their production in a large quantity. In this study production and optimization of laccase enzyme production from locally isolated and screened potential fungal strain of *Aspergillus flavus* was monitored. During this study culture medium and different parameters such as incubation period and inoculum size were optimized and inducers were employed for enhanced production of laccase. Partial purification was accomplished through Ammonium-Sulphate precipitation and for further purification gel filtration and anion exchange methods were used along with protein estimation. The purified fraction was then subjected to enzymatic characterization to find its thermal stability, effect of pH and selected chemical compounds. Other significant experimental findings were; optimum incubation period was 7 days, inoculum size was 3 discs of 0.5 cm, $CuSO_4$ as inducer improved production, laccase activity of crude extract *i.e.* 10.89U/ml increased to 15.07U/ml with purification of the enzyme, the purified samples of enzyme was significantly greater protein concentration than crude sample so, the optimum pH was 5.0 for laccase activity and the enzymatic extract was thermostable up to 60°C for 1 hour with guaiacol as substrate. The biological production of enzyme have an ecofriendly impact in current scenario.

Novelty Statement | The study is novel in suggesting ecofriendly impact of production of laccase enzyme from locally isolated *Aspergillus flavus* strain.

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Introduction

The most widely occurring group of phytochemicals as plant secondary metabolites is polyphenols (Aberoumand and Deokule, 2008). Laccases are polyphenol oxidases and also known as multicopper oxidases because they are members of the blue multi-copper protein family as their catalytic site contains four copper atoms (Baldrian, 2006). As laccases are oxido-reductive enzymes, oxidize substrates with the elimination of electrons and a free unstable radical is formed that undergoes non enzymatic processes like polymerization or hydration. Electrons are picked up by the oxygen molecule and reduced into water at the same time (Xiao *et al.*, 2004). These enzymes are advantageous because they do not require hydrogen per oxide for oxidation of substrate rather oxygen act as an unlimited electron acceptor (Michizoe *et al.*, 2005). It is an important industrial catalyst, helpful in fuel production (Giardina and Sannia, 2015; Abd El-Monssef *et al.*, 2016). Besides that, it has a variety of actions, such as azodye oxidation (Gomi *et al.*, 2011), pulp bleaching via biochemical means (Kalyani *et al.*, 2012), processes of polymerization or depolymerization, as well

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as biosensors (Bilal *et al*., 2019).

Laccases have been found in bacteria, plants, and numerous fungal species (Marques de Souza and Peralta, 2003). Fungal laccases are useful in food microbiology like ethnol production from pulp, clarification of wine and manufacturing of lightening cream etc. (Couto and Herrera, 2006). These enzymes are attracting attention because of their potential industrial application in textile dye bleaching, effluent detoxification, degradation of aromatic pollutants bio-pulping, bio-sensor and bio-remediation (Couto and Herrera, 2006). Both extracellular and intracellular types of laccases are present in fungi but most fungal laccases are extracellular (Octavio *et al*., 2006). Extra cellular types of laccases are produced in small amount but inducers can be used to enhance their production (Octavio *et al*., 2006). Fungal laccases are preferred over plant and bacterial laccases because of their high redox potential, they are involved in a variety of biotechnological processes (Brijwani *et al*., 2010).

In recent years major interest of scientists lies in production of active and stable laccases in enhanced quantity but at relatively low cost by using biotechnological techniques. Laccases obtained from filamentous fungi provides stable, reliable and cost effective source due to production of improved level and ease of extraction. (Desai and Hityanand, 2011). A serious issue regarding the low production rate of fungal laccases by native hosts makes them suitable for industrial utilization and high cost. This problem can be solved by using inducers to enhance production rate among laccase positive producers including *Trichoderma* and *Aspergillus* species and using cost-effective substrates.

Due to the presence of many isoforms of laccases, the most appropriate system of purification has not been established (Flukery, 2003). Purification of laccases obtained from white rot fungi, on the other hand, has been documented (Niladevi *et al*., 2008). It has been found that purified laccases remained stable if stored frozen (Flukery, 2003). Moreover, use of these enzymes in food industry require high level of purified preparations so the discovery of a simple and effective purification method is eagerly awaited (Freixo *et al*., 2011). The aim of present research was to produce and partially purify laccases from extracellular crude culture extract of locally isolated and screened *Aspergillus flavus* strain including enzymatic characterization for thermal stability, optimum level of pH and substrate optimization.

Materials and Methods

The current studies were conducted in PCSIR Laboratories, Ferozpur road, Lahore.

Production of laccase

Screening of culture medium

A locally isolated fungal strain of *A. flavus* was cultured on five different media for inducing enzyme production, using submerged fermentation technique at 30°C for ten days of incubation. Then filtered and centrifuged culture was used as enzyme source. Sampling was done after every 24 hours for determination of enzyme activity.

Inoculum size

The enzyme activity was measured by using various inoculum discs sizes. The flasks were incubated at 120 rpm on ±30°C for 7 days.

Incubation period

The impact of incubation time for maximum enzyme production was determined through sampling and measuring laccase activity at regular intervals such as 24, 48, 72, 96, 120, 144, 168, 196 hours.

Impact of copper sulphate laccase production

To investigate the effect of copper sulphate on enzymes, various Conc. of Copper sulphate was used and compare with control.

Impact of inducers on laccase production

Various inducers were introduced to enhance enzyme production and its activity 0.02 % of each inducer, crystal violet, methylene blue, congo red, trypan blue, ethanol were used.

Purification of laccases

Partial purification of enzyme

The partial purification of recovered concentrated crude, the ammonium sulphate precipitation technique was used to obtain the enzymatic extract at temperature of 4°C (Madani *et al*., 1997). The crude laccase suspension was fed by solid ammonium-sulphate and stirred continuously at saturation of 0 to 20%. The suspension was then allowed to settle down for approximately half an hour. We get precipitates after centrifugation for half an hour at 10,000 g. The supernatant obtained as a result was further subjected to saturation at different concentrations like 20-40%., 40-60%, 60-80% and 80-100%. Following that, the fractions were pooled separately and centrifuged 10,000 g, for half an hour and resulting precipitates were dissolved in a minimum amount of 0.1 M sodium acetate buffer having pH 5.0 and desalted by dialysis, utilizing a dialysis tubing membrane that has a cut-off 12-14 kDa and using a 0.01 M sodium acetate buffer with a pH of 5.0. The protein fraction obtained as a result of dialyzation was assayed for enzyme positive activity. The fraction containing laccase enzyme (60-80%) had been selected as the partially purified enzymatic extracts and lyophilized in the presence of 2.5% of mannitol. The purified fraction after lyophilization was kept at -80°C and then used for

the characterization in further studies.

Purification of enzyme

We got precipitates as a result of partial purification of laccases by ammonium sulphate method for Gel-permeation chromatography sephadex G 100 column of size 2.0 x 40 cm was used. The sample obtained after DEAE-cellulose anion-exchange chromatography was then loaded into this column for further purification. A 3 ml of enzyme fraction was pooled and active eluted fraction was dialysed. A graph was plotted between dependent and independent variables to get a slope of variables for the calculation of protein concentration.

Protein estimation

Protein was estimated using the Lowery *et al.* (1951). To prepare alkali solution of copper, 2g sodium carbonate (Na_2CO_3), 2ml copper sulphate, 0.5%, 2 ml of sodium potassium tartrate, 1% dissolved in 100ml of 0.1N sodium hydro-oxide. Protein solution 0.5ml and alkaline copper reagent 5ml was combined and left at room temperature for 10 to 15 minutes. Follins reagent 0.5ml, diluted with distilled water in 1:1 was added in order to complete the reaction. Absorbance was taken at 600nm and values were compared to that of bovine serum albumin.

Total protein= Absorbance value × Standard factor (BSA)

Laccase characterization

For characterization of purified laccases fraction, the comparative bio-assay was carried out against various treatments including temperature, pH, activators and inhibitors.

Impact of pH on enzyme stability and activity

Laccase activity was checked at different pH levels. Other cultural conditions such as temperature, time of incubation, concentration of substrate were remained constant. The purified laccase enzyme was incubated in a variety of buffers ranging in pH from 2 to 8. After incubation of 1 h laccase enzyme activity was calculated. For all enzyme assay, assay conditions were kept same.

Impact of temperature on enzyme activity and stability

Laccase activity was measured in percentage and investigated at different temperatures for 1 to 4 hours. All enzyme assays used the same conditions.

Effects of activators and inhibitors

Different solutions of inhibitors (strontium chloride, bismuth nitrate, lead chromate, sodium chloride, silver nitrate) were prepared to investigate the effect on activity of laccase enzyme. Enzyme extract mixed with inhibitor solution and incubated at 30°C for one hour and then the assay method was used to determine laccase activity.

Statistical analysis

From the initial data of this present investigation, mean and standard error were determined. Values regarding the coloured oxidizing zones under different parameters were also analysed statistically by using LSD (least significant difference) method to deliberate mean differences (Steel and Torrie, 1980).

Results

Production of laccases

Screening of different culture media

Aspergillus flavus strain SAf_5 was culture on five different media for inducing enzyme production using submerged fermentation technique at 30°C for ten days of incubation. Results showed that among different media used for cultivation of *A. flavus* strain and laccase enzyme production, medium 3 i.e. of Olga *et al.* (1993) proved best for maximum production under shake flask conditions with enzyme activity 0.246 U/ml followed by medium 4 (Coll *et al.*, 1993).

Fungal Strain and culture conditions

Following protocol of Harley *et al.* (1993), *A. flavus* locally isolated from industrial waste water and screened for laccase enzyme synthesis was sustained by transferring enzymes on a regular basis to malt extract agar medium plates and incubating them at 20°C. A basal liquid medium was chosen for laccase synthesis, screened with the previously optimized conditions in which 30°C temperature and 5pH with the addition of 3.5% sugarcane bagasse as carbon and peptone (2%) for nitrogen source.

Incubation time

Figure 1 shows the effect of range of incubation time duration on the production of laccase enzyme. Laccases enzyme production and fungal dry mycelial mass was maximum at seventh day.

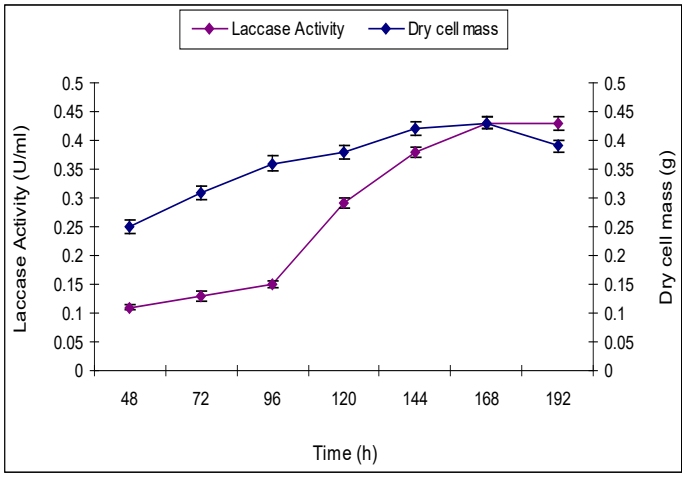

Figure 1: Effect of Incubation time on the production of laccase and mycelial mass of *Aspergillus flavus*.

Inoculum size

Inoculum size optimization was carried out for laccase activity by employing different number of sterilized inoculum discs (0.5 cm diameter) such as 1, 2, 3, 4 and 5 discs in medium. Optimum size of inoculum was 1.5 cm with highest production (0.390 U/ml). However, the optimum size of inoculum, activity of enzyme was low gradually. But the dry mycelial mass was found utmost in reaction mixture having inoculum amount 1.09g while smallest amount in reaction mixture 0.38g having 02 discs of inoculum (Figure 2).

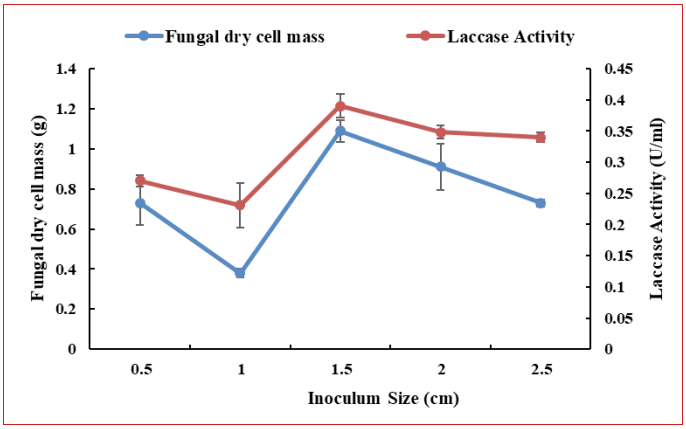

Figure 2: Optimization of size of Inoculum for enzyme production from *A. flavus*.

Effect of copper sulphate on enzyme production

Enzyme activity and dry cell mass of *A. flavus* was estimated at various copper sulphate conc. i.e. 0.2, 0.4, 0.6, 0.8 and 1.0 mM $CuSO_4$ conc., the maximum laccase activity was observed as 0.4mM $CuSO_4$ concentration. Dry cell mass was also estimated maximum with o.4 mM $CuSO_4$ concentration. This investigation indicated, laccase production from *A. flavus* was improved by Copper sulphate.

Inducers effect on Laccase Activity

Effect of various compound were investigated on enzyme production. Results indicated that some of the compounds encourage laccase production (Figure 4). The highest activity was noticed trypan blue (12.54 U/ml) while the smallest amount was noted in case of congo red (11.23 U/ml). But, maximum mycelial biomass was calculated in case of crystal violet (2.46 g).

Purification of enzyme

Recovery of crude extract

After seven days of incubation of culture in selected medium, the mycelia pellets were removed with cheese cloth and liquid culture medium was filtered by using Whatman filter paper no 1. The filterate was used as concentrated crude enzyme extract.

Partial purification of enzyme

Partial purification of active laccases was conducted from culture filtrate of fermentation medium by using the simple technique of ammonium-sulfate percentage saturation method. In this method, we employed different concentrations of ammonium sulphate from 20 to 100% for protein purification. The results indicated that complete precipitation of proteins was obtained at 80% saturation of ammonium sulfate at 0°C. Thus the enzyme activity of partially purified proteins started at 25% saturation and maximum activity was calculated at 80% ammonium sulfate saturation in pellet at 0°C (Kalra *et al.*, 2013).

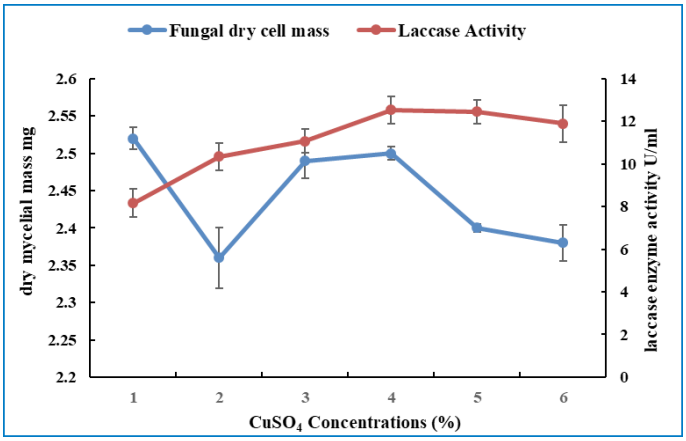

Figure 3: Optimization of Copper sulphate conc. for enzyme production from *A. flavus*.

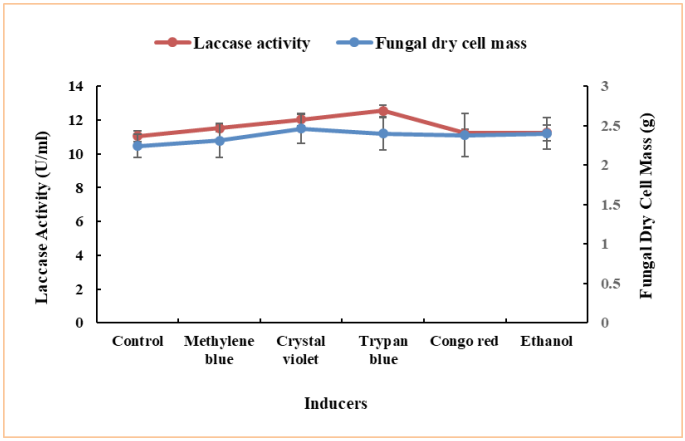

Figure 4: Effect of various Inducers on enzyme production from *A. flavus*.

Purification of extracellular laccase

After purification of crude enzyme by ammonium sulphate saturation method, the partially purified laccase enzyme was further subjected to different purification steps. By employing these purification steps, enzyme proteins were further purified 5.23 folds and 21.4% yield. Fungal laccases obtained from *A. flavus* adsorbed strongly on DEAE cellulose and then displaced creating a linear gradient of citrate buffer with 40-370 Mm concentration. Single band of extracellular laccase enzyme was obtained when we employed activity staining for purified protein. Gel chromatography using sephadex G-100 was performed for further verification of molecular wt. of laccase that was determined as 65±2 KDa (Chefetz *et al.*, 1998).

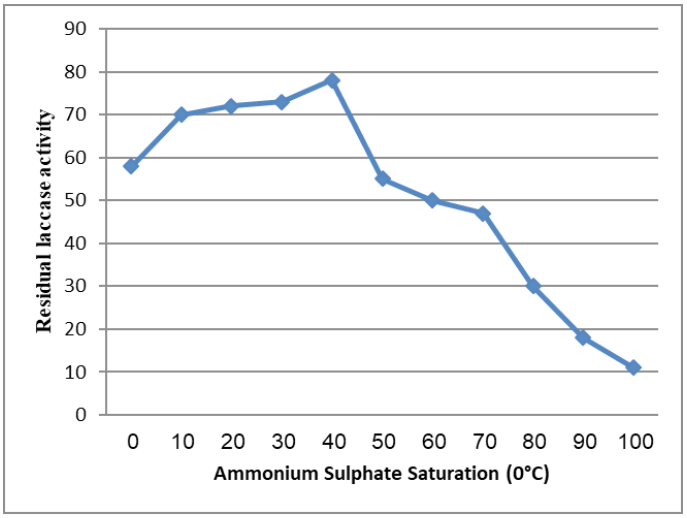

Figure 5: Ammonium sulphate precipitation of laccase enzyme by *A. flavus*.

Total proteins (mg) defined as multiplication of total volume of culture medium by protein concentration (mg / mL).

Total enzyme activity (U) defined as multiplication of total protein by specific activity and expressed as nmol of product/min.

Specific activity defined as nmol of product/ mg of protein/ min.

Purification factor of enzymes can be calculated by dividing specific activity of each sample by that of crude enzyme extract.

Evaluation of protein concentration

The data showed very low protein pellets activity was obtained underneath 30% of ammonium sulfate saturation however; protein pellets active conc. was noticed subsequent gradual boost from 30% to 80% dispersion of ammonium sulfate. This clearly showed, in crude sample protein conc. was considerably high as compared to purified protein samples. The specific activity was considerably improved by purification steps and overall purification was found to be 1.82 folds.

Characterization of purified laccase enzyme from A. flavus

To study the properties of laccase enzyme by *A. flavus* comparative bioassay was performed to differentiate the purified laccase beside different treatments including temperature, pH, activators and inhibitors.

Effect of different pH levels on enzyme activity and stability

Two buffer systems were used to determine the pH range. It was shown that the purified laccase enzyme was active in a broad range of pH i.e., 3.0 to 6.0, and the maximum activity was noticed at pH 5 (Figure 6).

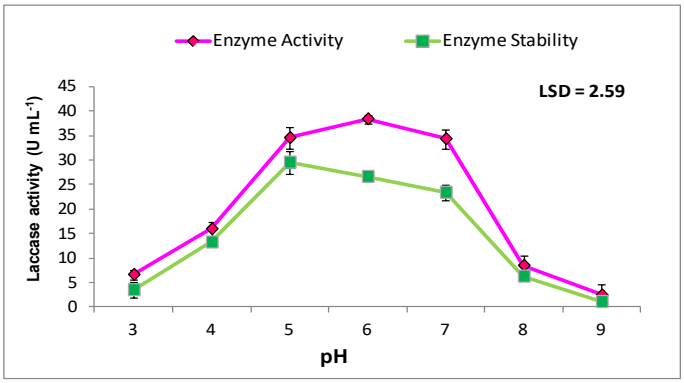

Figure 6: Impact of different pH on laccase activity by *A. flavus*.

Effect of temperature on laccase enzyme activity and stability

The purified sample of enzymes was incubated to various temperatures range between 5°C to 75°C. The results indicated that laccase enzyme activity was found to be weakening at 70°C. The laccase activity increased sharply at and above 50°C and became 100% between 60 and 65°C then gradual decline in activity started till 75°C. In stability of enzyme exposure time also played an important role towards different temperatures. In this investigation laccase was stable at 60°C for about 1 hour. Enzyme stability was up to 40 minutes at 75°C and completely lost after 120 minutes i.e inhibition in enzyme activity was 100% (Figure 7).

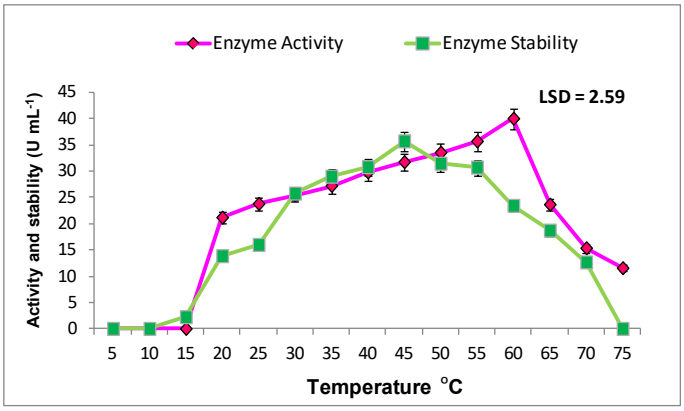

Figure 7: Impact of Temperature on laccase activity by *A. flavus*.

Effects of inducers

Effect of different ions and compounds were studied, it was shown that only 2 mM copper sulphate ($CuSO_4$) induce the activity of laccase enzyme among selected ions and compounds. Laccase activity showed no significant increase or decrease in case of Mn^{2+}. In the presence of mercury (Hg^{2+}) 3 mM, almost 70% of enzyme activity was lost and sodium azide completely inhibited enzyme activity while EDTA acted as moderate inhibitor of purified fraction of laccase. Another tested compound lead nitrate act as a denaturing agent and also effectively inhibited enzyme activity. Mercaptethanol lowers activity

of laccases and acted as repressor. TEMED did not show any significant effect on the enzyme activity (Figure 8).

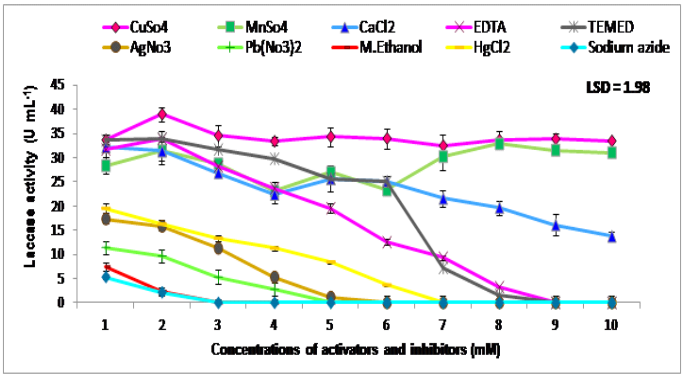

Figure 8: Impact of activators and inhibitors on laccase activity by *A. flavus*.

Discussion

The most widespread saprophytic organisms are fungi that degrade organic materials by the secretion of ligninolytic enzymes including laccase. In present work production, purification and characterization of laccase enzyme from isolated strain *A. flavus* was investigated. Previous studies showed that different parameters such as inoculum size, incubation period and addition of inducers were found vital for improved enzyme production. In this study, as fungal inoculum increased, the production was increased till optimum level. Similar results have been described regarding laccase production from *Penicillium martensii* by (Elshafei *et al*., 2012; Elsayed *et al*., 2012; Kumar *et al*., 2016).

In the growth of microorganisms, enzyme secretion, and enzyme activity, the incubation time is critical. In current investigation maximum enzyme production and activity was monitored at 168 h of culture growth due to a lack of macro and micronutrients in the production medium, enzyme synthesis began to decline. The incubation time in this study is shorter than studies carried out by Sivakumar *et al*. (2010) observed highest laccase activity at 240 h of incubation with *Ganoderma* sp.

Enzymes are sensitive to metal ions even at low concentrations and inhibit enzyme activity. This is because copper ions attach to type-2 copper binding sites and also because catalytic center of laccase enzymes contains copper ions (Bukh and Bjerrum, 2010). Figure 3 showed increased production of enzyme by the addition of $CuSO_4$, this can be described as the culture organism's defence response to the imposed metallic stress (Fernandez-Larrea and Stahl, 1996). As copper induces production of laccase isozymes that leads to improved enzyme production (Saparrat *et al*., 2002). Higher copper concentrations beyond the optimum level, according to Baldrian (2003), may be harmful to fungi and limit their growth and enzymatic activity. Similar to present study results *Ganoderma* sp. produced the most laccase when exposed to CuSO4 at a concentration of 30 µM. sp. (Sivakumar *et al*., 2010). Baldrin *et al*. (2003) came to similar conclusions. Inhibition of enzyme activity by other metals (like sodium, silver, hydrogyrum) as shown in our results may be due to formation of SH groups with enzymes that leads to inactivation of enzyme (Fortina *et al*., 1996). Figure 8 shows when testing the effect of some inhibitors the enzyme activity was inhibited by sodium azide at different tested concentrations. This inhibition may be due to binding of azide ions to the laccase copper ions causing disruption in laccase inhibition is caused by the electron transport system (Gianfreda *et al*., 1999). In this study, Figure 8. The enzyme interaction with different metals are essential for the understanding and improvement of a bioremediation process. Metal ions have a significant impact on enzyme catalytic activity, depending on the type of metals employed as metal ions. Compared to the previous literature (Sahay *et al*., 2009) the experimental findings (Table 1) showed that partial purification of enzymes leads to higher purification folds and higher enzymatic activity with lower yield. Table 1 also indicate partial purification of enzyme precipitated at 60-80% Ammonium sulphate contain higher laccase activity as compared to crude extract. In further purification by ion–exchange methods, experimental findings showed that although activity of enzyme increased as compared to crude extract but close to that of partially purified fraction. Purification yield with ammonium sulphate precipitation method is minimum as compared to gel filtration method, it may be because of denaturation of the enzyme by the salt ammonium sulphate. Using ion-exchange method, purification yield is also not considerable because of enzyme ability of absorption on cellulose matrix. Maximum purification yield obtained with gel filteration using sephadex G-100 resin column owing to improved enzyme adsorption on sephadex gel this resin has purified a lot of fungal laccases. Kumar *et al*. (2016) described purification of laccases, produced by *A. flavus* on sephadex G-100 resins. It also suggests purification of enzymes by size exclusion eliminates reasonable amount of proteins that had no effects on activity of laccases. Results also showed that procedures of purification of enzymes may not have inhibitory effects on laccase activity.

In many fungal species laccase productions were reported at mesophilic temperature and acidic pH (Yang *et al*., 2013). In its natural state, the laccase under investigation was mesophilic, and it was stable up to its optimal temperature (Figure 7). The enhance in laccase activity via pre incubation process was because of conformational changes that increases flexibility and so the catalytic activity. It is also investigated that conformational changes in metallo-proteins like laccases lead to efficient electron transfer (Saparrat *et al*., 2008). Koroleva *et al*. (2001) reported increase in temperature cause changes

Table 1: Purification of fungal laccase by *A. flavus*.

Purification	Total proteins (mg)	Total enzyme units (U)	Specific activity (U mg^{-1})	Purification factor
Laccases (crude)	144	9268	63	1
(NH4)2SO4 precipitation	87	7548	86	1.36
DEAE-cellulose	32	4149	129	2.04
Sephadex G-100	12	1856	154	2.57

in copper centers and protein conformation. They also indicated enzyme activity increase with the increase in temperature up to certain limits and then thermos inactivation starts. This cannot be explained by protein unfolding, as protein unfolding starts at temperature above 80°C. Inactivation of enzymes at 60°C and above may because Increases in temperature trigger the release of type 2-copper ions, and at temperatures more than 70°C, the types 1 and 3 copper sites are entirely dissolved. The activity and stability of laccase for longer duration at broad range of temperatures from 20°C to 60°C and at variable pH range from acidic to alkaline is because of presence of specific substrates for example guaiacol or ABTS and is made to enable for the most cost-effective production of it as well as its utilisation in bioremediation of effluents. Several researchers, including Kumar *et al.* (2016), have found excellent laccase stability and activity across a wide temperature range and pH in strains of *A. flavus.* Most fungi have indicated that the optimal pH for laccase activity is between 3-5. (Galhaup *et al.*, 2002; Wesenberg *et al.*, 2003; D'Souza *et al.*, 2006). The instability of the enzyme at enhance pH is mostly because of the fact that there is inhibition of the enzyme activity by the presence of hydroxide (OH) ions that generally stop catalysis of the substrate. The presence of numerous laccase isoforms with different properties may account for the differences in experimental results (Flurkey, 2003; Lisova *et al.*, 2010).

Conclusion

Laccases have a lot of biotechnological importance because they can help oxidize a lot of different chemical substances. Because fungus create enzymes and grow swiftly on cheap food, there has been a lot of investigation into how laccases are made by them. The successfully produced and purified laccase enzyme to homogenity from *A. flavus* strain isolated from industrial wastewater sample collected from industrial area near Lahore Pakistan showed thermal as well as pH stabilities. In the future, we'd like to investigate environmentally friendly laccase mediators for industrial use. Moreover, it is possible to obtain high levels of production by the exploration of genes using suitable hosts.

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Conflict of interest

The authors have declared no conflict of interest.

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