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GANODERMA LUCIDUM: A CASE STUDY FOR LACCASE BIOSYNTHESIS

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A B S T R A C T

Laccase production by white rot fungus *Ganoderma lucidum* (Curtis) P. Karst. was investigated through submerged fermentation using effluents of paper and pulp industry as substrate. The effect of variation in culture and nutritional conditions was optimized for industrial scale production of enzyme. Among the optimized parameters incubation period 16 days, inoculums size 13 discs (5 mm), initial pH 5, incubation temperature 30 ^{o}C, glucose 4.0 g 50 mL^{-1} and yeast extract 2 g 50 mL^{-1} were found the best for laccase synthesis (22.54 U mL^{-1}). Optimum pH and temperature for enzyme activity were 3.0 and 65 ^{o}C. The laccsae activity was strongly inhibited by cobalt at 1 mM and 5 mM concentrations.

Keywords: *Ganoderma lucidum,* laccase, submerged fermentation, white rot fungus.

INTRODUCTION

Laccases (E.C. 1.10.3.2) are multi copper containing enzymes that speed up oxidation of various aromatic compounds, purposely phenols and anilins, while concurrently reduce molecular oxygen to water, and are widely distributed among plants, fungi and insects (Shraddha *et al*., 2011). Unlike lignin peroxidases and manganese peroxidases, laccases have the potential to oxidize organic compounds even in the absence of H_2O_2 or Mn^{2+}. Laccases have extensive substrate range, serving for industrial purposes and/or bioremediation and bio reclamation processes (Justino *et al*., 2010). Polluted waters may be most suitable targets for lignin degrading enzyme systems including laccases for its reclamation at cheaper costs (Kallel *et al*., 2009).

White rot fungi have been proved as potent laccases producers leading towards the exposure of cellulose. The wood rotting fungi include *Pleurotus ostreatus* (Liu *et al*., 2009; Hou *et al*., 2004), *Pleurotus eryngii* (Wang and Nagai, 2006a), *Trametes versicolor* (Lorenzo *et al.,* 2002), *Agaricus blazei* (Ullrich *et al*., 2005), and *Clitocybe maxima* (Zhang *et al*., 2010). They have been given extensive attention as the most appropriate laccase producers for industrial applications. *Ganoderma lucidum* has been proved as a good producer of polysaccharides A and B types having anti-tumor and anti-angiogenic activities. As a white rot species it can synthesize laccases (EC 1.10.3.2), Mn oxidizing peroxidases (EC 1.11.1.13) and lignin peroxidases (EC 1.11.1.14) (Varela *et al*., 2000; Ko *et al*., 2001; Silva *et al*., 2005). Because of persuasive producer of these enzymes, *G. lucidum* can mutate and decompose various aromatic compounds, particularly lignin, and can play its role in several biotechnological practices. The present study was carried out for the optimization of conditions for laccase production by *Ganoderma lucidum* using effluent of pulp and paper industry. Study was also aimed at partial purification and characterization of the enzyme.

MATERIALS AND METHODS

Collection and maintenance of *Ganoderma lucidum*: Fruiting bodies of *G. lucidum* used in this study were collected from Chhanga Manga Forest and stored in a sterile plastic bag. For isolation of mycelium inner sterile tissue of the fruiting body was cultured on to the modified Tein & Krik's medium aseptically and stored at 4 ^{o}C. For further studies massive production of pure culture was done on the same medium.

Laccase activity assay: Pure fungal culture was

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qualitatively analyzed for laccase production, using ABTS [2, 2' azino-di-(3 ehtyl benzo thiazoline-sulphonate)] as an oxidizing agent added in malt extract agar (MEA) medium. Laccases oxidized ABTS to pale brown complex giving a visual confirmation (Ding *et al.,* 2012).

Collection of effluents: The effluents of paper and pulp industry required in this experiment were obtained from Century Pulp and Paper, Jambar Khurd, Bhaipheru, Multan road, District Kasur. This effluents were collected in plastic bottles, brought to the laboratory and filtered by using muslin cloth and stored in refrigerator at 4 ^{o}C till further required.

Quantitative analysis for laccase production: Fifty mililitres of sterilised industrial effluent medium was poured into 250 mL conical flask and inoculated with 5 fungal discs of 5 mm diameter each. The flasks were incubated on rotary shaker at 180 rpm and 28 ± 2 ^{o}C for 7 days. After every 24 h, 2 mL fermented broth was sampled and centrifuged at 5000 rpm for 25 minutes. Supernatant containing crude enzyme was used for estimation of laccase activity.

The assay mixture contained 0.5 mM ABTS, 2.8 mL of 0.1 M sodium acetate buffer (pH 4.5) and 100 µL of culture supernatant and incubated at room temperature for 5 minutes. Absorbance was recorded at 420 nm in spectrophotometer against a suitable blank (Ding *et al*., 2012).

Optimization of different growth and nutrient parameters: In this experiment, the effect of inoculum size,, temperature, pH, carbon and nitrogen sources was studied for laccase production.. Different inoculum sizes were tested ranging from 3–9 mycelial discs of 5 mm diameter 50 mL^{-1} of medium. The effect of temperature on laccase production was determined by inoculating the 5 mycelial discs of *G. lucidum* culture in the flasks containing 50 mL of medium, incubated at different temperatures i.e. (10, 15, 20, 25, …, 50 °C) for 16 days on shakers at 180 rpm. The effect of pH on laccase production was carried out by incubating the culture flasks containing 50 mL of the sterilised industrial effluent inoculated with 5 mycelial discs of *G. lucidum* at different pH ranging from 4 - 8. These flasks were incubated at 35 °C for 16 days on electrical shaker at 180 rpm. The role of incubation period was investigated for optimum enzyme production by incubating the whole set up at 180 rpm for 8 to 26 days. Three different synthetic carbon sources i.e., glucose, maltose, cellulose (ranging from 1.5–5% (w/v) were added in the fermentation medium to be optimized for enhanced biosynthesis of laccase enzyme. The role of 4 different nitrogen sources i.e., peptone, yeast extract, ammonium chloride and urea (ranging from 0.5–3% (w/v) was investigated for best enzyme biosynthesis. Each experiment was performed in triplicate.

Purification of laccase: The crude laccase was purified from culture broth containing effluent of paper and pulp industry and lignocellulosic substrate using two $(NH4)_2SO_4$ precipitation steps. Forty percent saturated solution of $(NH4)_2SO_4$ was used to remove hybrid proteins from the fermentation broth, and then 70% saturated ($(NH4)_2SO_4$ was used to precipitate laccase from the supernatant obtained in the first step. The protein precipitate was dissolved in 50 mM (pH 3) sodium acetate buffer, dialyzed in same solution to remove $(NH4)_2SO_4$ and the desalted enzyme solution was applied to DEAE FF 10/16 column pre equilibrated with pH 7.0 sodium phosphate buffer solution (Buffer A). The column was washed with buffer A and the absorbed protein were eluted using a linear gradient of 0.0 - 1.0 mol L^{-1} NaCl in buffer A over 40 min at flow rate of 5 mL min $^{-1}$. The active fractions were pooled and dialyzed against buffer A, concentrated to 2 mL by lyophilization and passed though a 2.5 × 100 cm Sephadex S-100 column equilibrated with buffer A. The column was washed at the rate of 1 mL min^{-1} with buffer A and the active fractions were pooled. Finally the active fractions were concentrated by lyophilization, and the purified laccase was used in the subsequent experimentation (Xiao *et al*., 2003).

Effect of pH and temperature on laccase activity: To investigate the effect of optimal pH, laccase activity was measured with a pH ranging from 1.0-11.0 using ABTS as substrate. The pH ascent was prepared using sodium acetate buffer (2.0-7.0) and HCL-KCL buffer (1.0). The effect of temperature on laccase activity was analyzed over a temperature ranging from 20 to 80 ^{o}C, by incubating the essay mixture at various temperatures for 10 minutes before the addition of enzyme and determination of laccase activity using ABTS as substrate.

Effect of inhibitors on laccase activity: Solutions of 1 mM and 5 mM concentrations of different inhibitors (calcium nitrate, barium chloride, cobalt chloride, strontium chloride, bismuth nitrate, lead chromate, sodium chloride and silver nitrate) were prepared to

investigate their effect on laccase activity. Enzyme solutions along with these inhibitors were incubated at 25 °C for 1 h. Laccase activity was measured afterwards.

RESULTS and DISCUSSION

The major component of wastes released form paper and pulp industry is ligninocellulose, which is composed of cellulose, hemicellulose and lignin (Sanchez, 2009). Lignin acts as a barrier to guard celluloses and hemicelluloses from enzymatic actions. Under natural conditions white rot fungi decompose the lignin fences to gain energy form cellulose, secreting lignin degrading enzymes including laccases. Abundance of nutrients has been reported in agro industrial effluents which can be used for maintenance of microbial growth during enzyme fermentation processes. Effluent of paper and pulp industry has been found rich in phenolic compounds (syringic acid, coumaric acid and ferulic acid) which stimulate the laccase synthesis in white rot fungi (Revankar and Lele, 2006). Different agriculture wastes (fruit peels, wheat bran, rice bran) acting as lignocellulosic substrates have been used by workers to stimulate the synthesis of laccase enzyme (Roalses *et al*., 2007; Sathishkumar *et al*., 2010; Ding *et al*., 2012). In this study effluent of paper and pulp industry act in the same providing lignocellulosic substrate for laccase synthesis.

Effect of incubation period on *G. lucidum* for laccase fermentation: Figure 1 shows the effect of six incubation periods and 16 days was found to be most effective resulting in highest production of laccase (19.78±0.142 U mL^{-1}). After this incubation period there was a gradual decrease in the level of enzyme produced. It has been reported that incubation period and incubation temperature play important role in the synthesis and activity of laccase enzyme because it reaches to its highest value in gradual manner and then falls down. The production shows a drop after reaching its maximum might be due to reduction of micro and macro- nutrients in the fermentation broth with a time interval, hence giving a stress to fungal physiology, inactivating the machinery of enzyme synthesis (Simoes *et al*., 2009; Elshafie *et al*., 2012). Zeng *et al*. (2006) described that production of enzymes by most white rot fungi has been investigated as secondary metabolic event. Forteina *et al*. (1996) reported that *Botrytis cinere* produced noticeable amount of laccase in a short period of 5-7 days but some fungi may take longer periods of 12- 30 days for remarkable production.

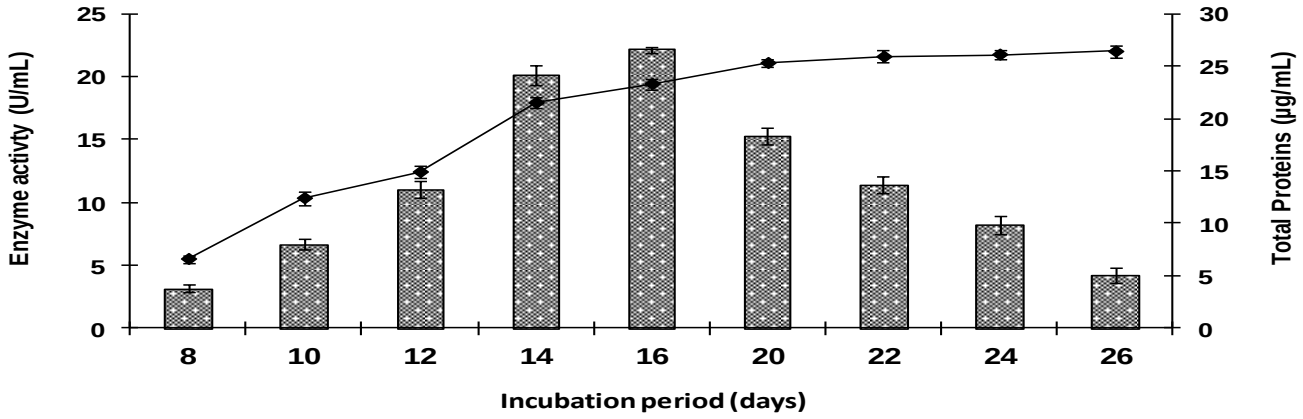


Figure 1. Effect of incubation period on *G. lucidum* for laccase production.

Effect of inoculum size on *G. lucidum* for laccase fermentation: The effect of seven different inoculum sizes is shown in Figure 2. The highest (19.78±0.292 U mL^{-1}) amount of enzyme was produced by 13 disks per 50 mL of medium. Further increase in inoculums size showed a decrease in the level of enzyme formation. Consequently the size of inoculum was maintained at 13 discs for the further experiment. Elshafei *et al*. (2012) used 2-7 agar plugs of *Penicillium martensii* 14 mm diameter for inoculation of laccase production medium for laccase synthesis and then optimized inoculums size at 5 discs.

Effect of initial pH on *G. lucidum* for laccase fermentation: Figure 3 shows the effect of 5 different pHs on laccase production. pH 5.5 has been selected best for laccase synthesis (19.90±0.123 U mL^{-1}). Above and below of the optimized pH the level of the enzyme produced was noticeably low. Initial pH is another

important parameter that affects the enzyme synthesis, Hossain and Anantharaman (2006) reported that pH value of 4.5 was optimum for maximum ligninolytic activity by *Trametes versicolor*. Most of the fungi prefer slightly acidic pH for their growth and enzyme synthesis. The intact enzyme may contain both positively and negatively charged groups at any given pH. Such ionizable groups are often apparently part of the active site. Variations in the pH of the medium result in change in the ionic forms of the active sites and changes in the activity of the enzymes, and hence the reaction rate (Haltrich *et al.,* 1996). Changes in pH may alter the three dimensional shape of the enzyme. For these reasons, enzymes are very active over a certain pH range.

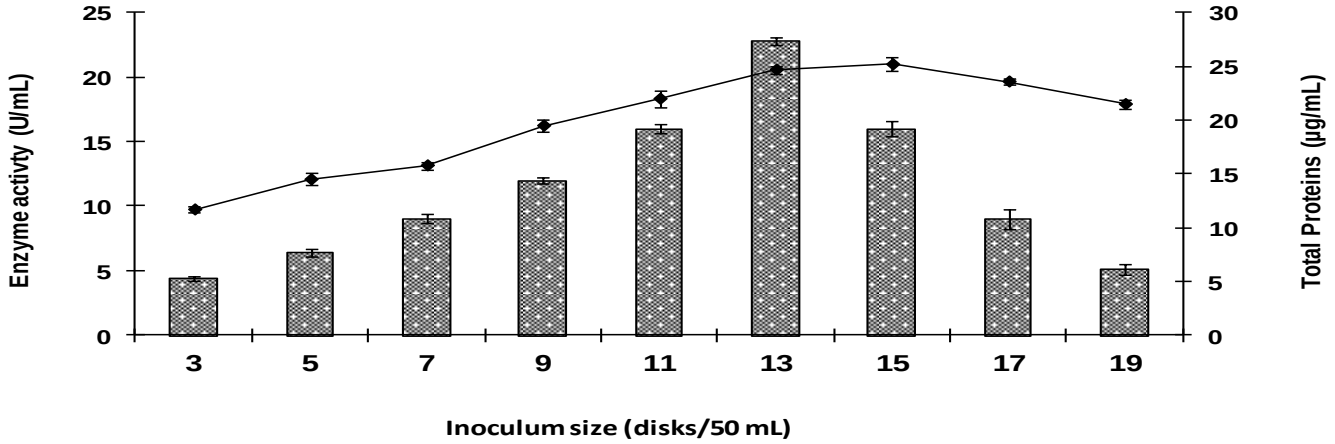


Figure 2. Effect of inoculum size on G. lucidum for laccase production.

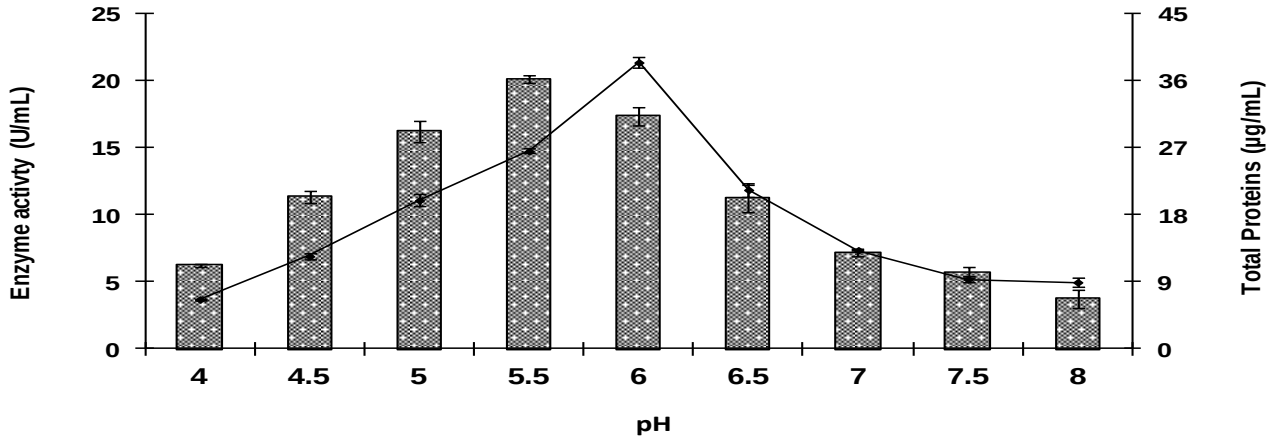


Figure 3. Effect of initial pH on *G. lucidum* for laccase production.

Effect of incubation temperature on *G. lucidum* for laccase fermentation: The effect of temperature on laccase production by *G. lucidum* incubated at various temperatures ranging from 15 °C to 45 °C is executed in figure 4. Highest units of enzyme (19.99±0.164 U mL^{-1}) were produced at 35 °C with a gradual decrease till 45 °C. Total protein content also showed same pattern of increase and then decrease. The results are in accordance with Elshafi *et al*. (2012), who demonstrated that optimum temperature for laccase synthesis by *penicillium martensii* NCR 345 was 30 °C with 7.21 U mg^{-1} with a considerable low level of enzyme above. Zadrazil *et al*. (1999), reported that, temperatures higher than 30 °C caused reduction in ligninolytic enzymes activity whereas the temperature of 40 °C was found to be optimum for maximum laccase enzyme production by Hossain and Anantharaman (2006).

Effect of Carbon source on *G. lucidum* for laccase fermentation: Figure 5 shows the effect of eight different concentrations (1.5-5.0 g 50 mL^{-1}) of glucose, maltose and cellulose on laccase production. Among all the tested concentrations 4.0 g 50 mL^{-1} of glucose was

found best for laccase production whereas maltose and cellulose gave best results at 3.5 g 50 mL^{-1}. The other tested amounts gave diverse responses at different concentrations. It has been reported that carbon source is amongst the important factors in laccase production, and that the addition of suitable amount of sugar to the fermentation media influence laccase synthesis (Teerapatsakul *et al*., 2007). The production for enzyme is linked with the type and concentration of carbon source in the fermentation medium Gawande and Kamat (2000). At high concentrations sugars may fulfill the nutrient requirement of *G. lucidum* for biomass growth, without the obligation to secret laccase to decompose lignin so as to obtain energy from cellulose. Eggert *et el*. (1996) reported that increase in concentrations of glucose can trigger the synthesis of extracellular polysaccharides which can obstruct the extraction of laccase from the culture broth.

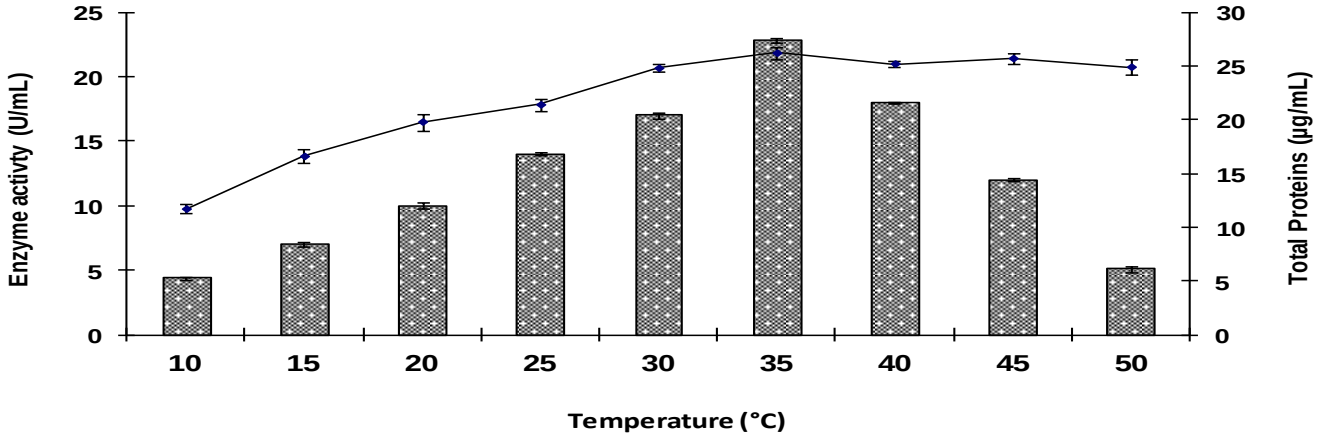


Figure 4. Effect of incubation temperature on *G. lucidum* for laccase production.

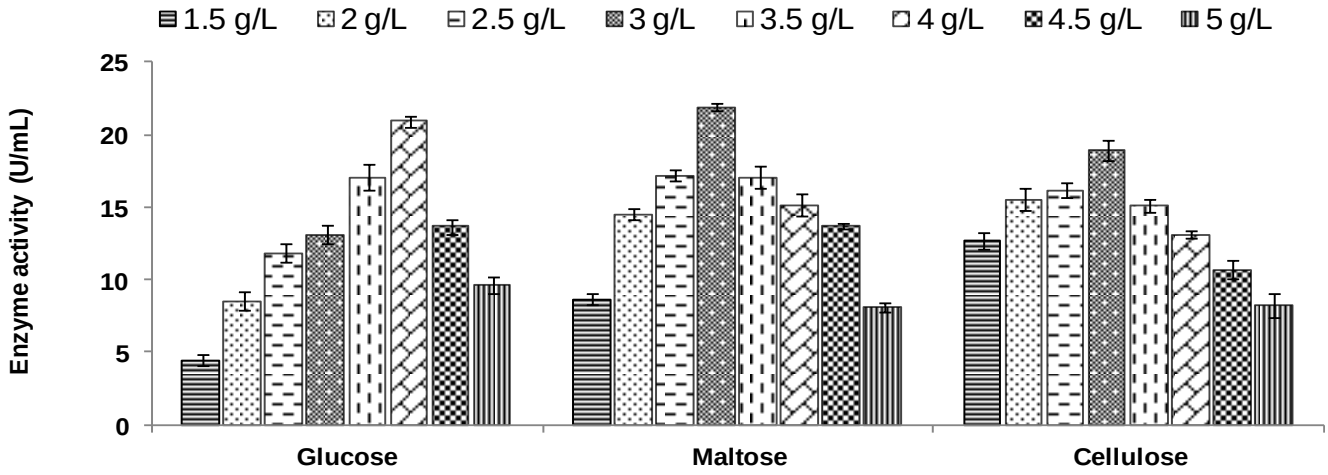


Figure 5. Effect of carbon source on *G. lucidum* for laccase production.

Effect of Nitrogen source on *G. lucidum* for laccase fermentation: The effect of different nitrogen sources (peptone, yeast extract, ammonium chloride and urea (ranging from 0.5–3% w/v) has been shown in Figure 6. Yeast extract, peptone and ammonium sulphate at 2 g 50 mL^{-1} concentration were found to be most productive for laccase enzyme (22.54 U mL^{-1}). Urea gave highest enzyme (19.48 U mL^{-1}) at concentration of 1.5 g 50 mL^{-1}. The type and concentration of nitrogen required for laccase synthesis has been found to be variable for different white rot fungi. *Pycnoporus cinnabarinus*, *Botryosphaeria sp*., gave optimal laccase under limited nitrogen supply (Eggert *et al*., 1996; Vasconcelos *et al*., 2000), whereas *Physisporinus rivulosus* yielded high levels of laccase under nitrogen rich culture conditions (Hilden *et al*., 2007). Ding *et al*. (2012) reported that

laccase synthesis by *G. lucidum* was appreciable at high nitrogen concentrations using yeast extract as nitrogen source. Yeast extract has been known to stimulate laccase synthesis and our results are in concordance with optimal culture condition for *G. lucidum* (Ding *et al*., 2012).

Effect of pH and temperature on laccase activity: The effect of pH value ranging from 1.0-11.0 on laccase activity was monitored (Figure.7). Enzyme showed highest activity at pH 5.0. There was sharp decrease in enzyme activity subsequently, leading to inactivation of enzyme after 8.0. The influence of temperature on laccase activity was revealed with a temperature range 20 to 80 °C, incubated up to 5 hours (Figure 8). The optimal temperature determined was 60 °C. Enzyme activity showed a decline up to 80 °C.

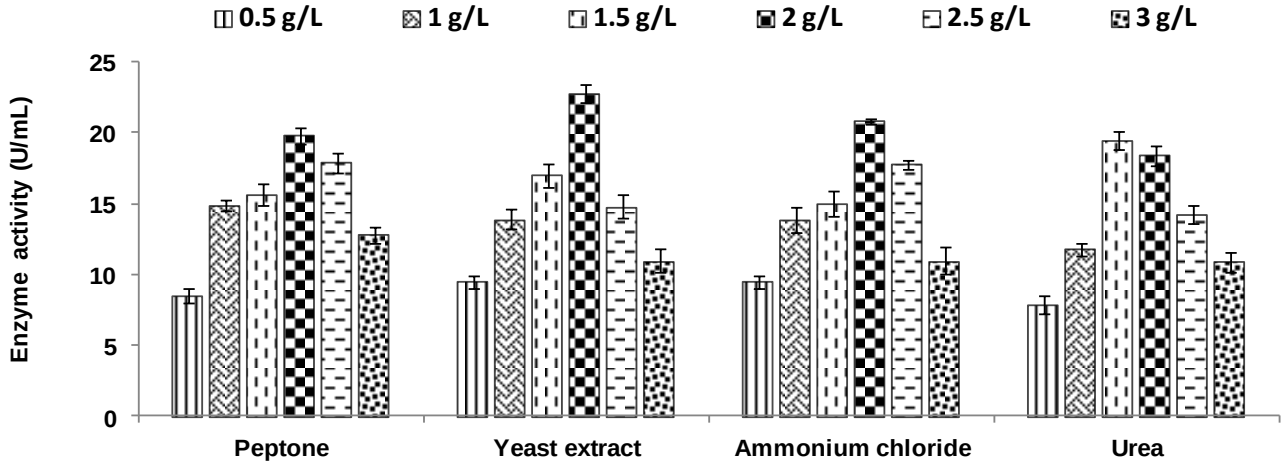


Figure 6. Effect of nitrogen source on *G. lucidum* for laccase production.

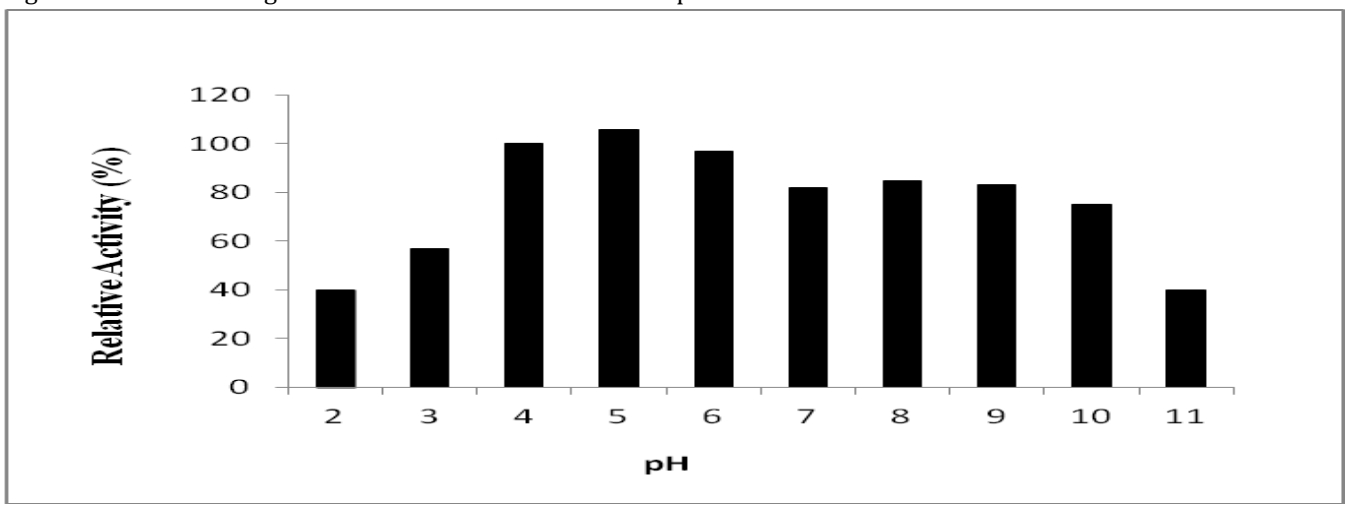


Figure 7. Effect of pH on laccase activity.

Reports show that optimal pH for activity of laccase varies with the variety of substrates. However, when ABTS is used as substrate the optimal exhibited pH lies in acidic range (Halaburgi *et al*., 2011), which is coherent with this study as the optimal pH was 4.0 with a rapid decrease in activity till pH 8.0 with a narrow range of pH stability. Thermal stability of laccase has been reported in *Pycnoporus* sp. (Wang *et al*., 2010), *Cladosporium eryngii* (Halaburgi *et al*., 2011) and *Pleurotus ostreatus* (Liu *et al*., 2009). In this study thermostable laccase has been produced by *G. lucidum* showing stability till 60 °C using submerged fermentation.

Effect of inhibitors on laccase activity: The laccsae activity was strongly inhibited by cobalt at both concentrations (1 mM and 5 mM). Barium and bismuth both showed no activity inhibition at concentrations, However calcium strontium and sodium had no effect on enzyme activity when used in 1 mM concentration, and showed little reduction at 5 mM concentration. Lithium, silver and lead exhibited a retard at both concentrations.

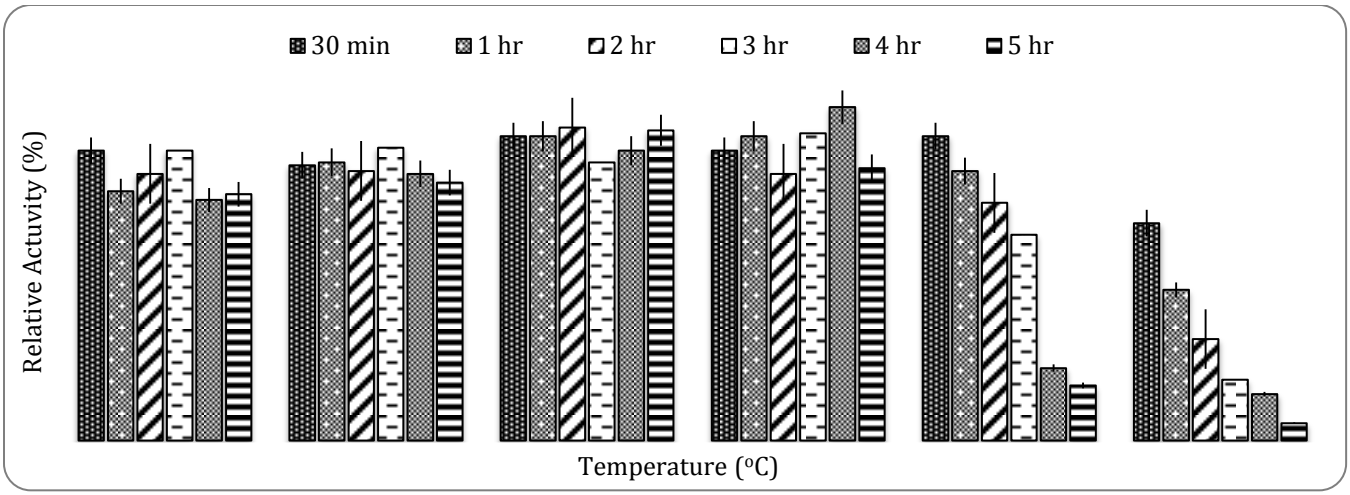
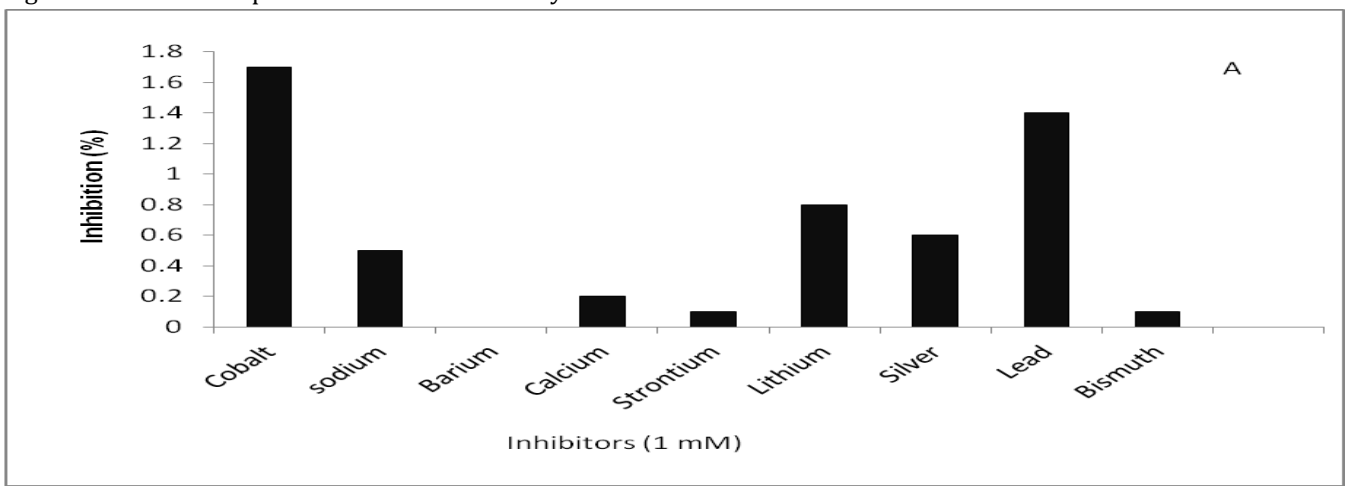
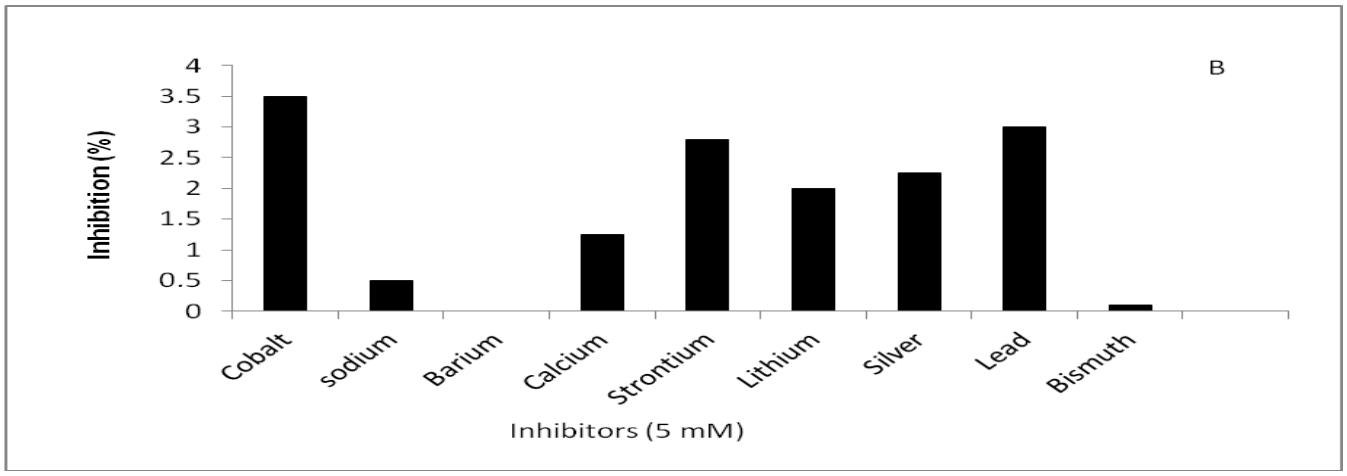


Figure 8. Effect of temperature on laccase activity.





CONCLUSION

The present study concludes that *G. lucidum* has the potential to produce laccase as main ligninolytic enzyme. The effluent of paper and pulp industry acts as a good lignocellulosic substrate for laccase synthesis by the test fungus. The optimized parameters were incubation period of 16 days, inoculums size of 13 discs, initial pH 5, incubation temperature of 30 °C, Glucose, 4.0 g 50 mL^{-1} and 2 g 50 mL^{-1} concentration of yeast extract. Purified enzyme showed remarkable thermostability thus proving *G. lucidum* as better choice for both industry and biotechnological applications.

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