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Evaluation of Lacc134 Oxidoreductase of *Ganoderma multistipitatum* in Detoxification of Dye Wastewater under Different Nutritional Conditions

Eman A. Alhomaidi [1,*], Aisha Umar [2], Salam S. Alsharari [3] and Sami Alyahya [4]

[1] Department of Biology, College of Science, Princess Nourah bint Abdulrahman University, P.O. Box 84428, Riyadh 11671, Saudi Arabia
[2] Institute of the Botany, University of the Punjab, Lahore 54590, Pakistan; ash.dr88@gmail.com
[3] Biology Department, College of Science, Jouf University, P.O. Box 72341, Sakaka 72388, Saudi Arabia; sssharary@ju.edu.sa
[4] Wellness and Preventive Medicine Institute, King Abdulaziz City for Science and Technology, P.O. Box 6086, Riyadh 11442, Saudi Arabia
* Correspondence: eaalhomaid@pnu.edu.sa

Abstract: In the present study, we investigated the effects of different carbon sources (glucose, sucrose, and maltose) on laccase production from mycelium of *Ganoderma multistipitatum* grown on malt extract agar plates. The preliminary screening test was performed on the guaiacol plate, where a maroon brown zone formed after laccase oxidation. A few pure mycelial discs of *Ganoderma* species were transferred into submerged fermentation nutrient broth. The nutrient medium of submerged fermentation at 20 g of glucose revealed the highest laccase activities (2300 U/L) than other carbon sources. The interesting results also shown by inorganic $NaNO_3$ in the production of maximum laccase (7800 ± 1.1 U/L). The organic nitrogen inducer, namely yeast extract, exhibited 5834 U/L laccase activity and a potential source of laccase secretion. The results concluded that C and N inducers enhanced the laccase production. This production process is eco-friendly and effective in the removal of dye from water. Laccase from the cultural broth was partially purified by SDS-PAGE for molecular weight determination, while Native-PAGE confirmed the laccase band after staining with guaiacol. The *Km* and *Vmax* values of Lacc134 were 1.658 mm and 2.452 mM min^{-1}, respectively. The Lacc134 of this study effectively removed the Remazol Brilliant Blue R (RBBR) dye (extensively used in textile industries and wastewater). For dye removal capacity, 2.0 mg, 4.0 mg, 5.0 mg, and 6.0 mg were used, from which 6.0 mg was most effective in removal (85% and 88%) dye concentration in 1st and 2nd h interval treatment, respectively. Total organic carbon (TOC) quantity after dye removal percentage in the first- and second-hour time interval was 62% and 89%, respectively, at 30 g glucose. According to the experimental finding of this study, the breakdown products catalyzed by Lacc134 are less hazardous due to lower molecular weight than the dye itself.

Keywords: carbon; kinetics; laccase; nitrogen; wastewater; wood rotter

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1. Introduction

Ganoderma P. Karst. belongs to Polyporales. The species under this genus are health-oriented and contain numerous pharmacological compounds important in therapeutic effects [1]. A good strategy to increase the productivity of the laccase is a fermentation process. This process optimizes the different carbon and nitrogen sources in the fermentation medium to enhance the laccase activity. The choice of suitable carbon and nitrogen sources is crucial in efficient production at an economical level.

Ganoderma species are successful in the production of laccase. Laccase is an oxidoreductase enzyme abundantly found in white-rot fungi. This "eco-friendly green catalyst" utilized molecular oxygen during the redox degradation of waste products, and the active

site of laccase is flexible to accommodate the multiple substrates (non-specific towards substrate). Laccase biocatalyst is valuable in many biotechnological applications and catalyzes the waste materials, which support the cleanliness of environments [2]. However, the application of this enzyme in biotechnological processes demands the mass production of laccase at a low cost. Therefore, this study is oriented toward the search for a new and efficient laccase producer, i.e., *G. multistipitatum*. The carbon sources are important in the production of the ligninolytic enzyme, during the secondary metabolism under the limited conditions of nitrogen [3].

Fungi can discolor and completely mineralize the dyes [4]. Most industrial lines utilize the synthetic dyes (textile, dyeing, pharmaceutic, cosmetic, and food industries) day by day [5]. Basidiomycetes can degrade the bio-wastes [6], where *Ganoderma* species are a white-rot fungus that efficiently break the synthetic dyes, because they produce the laccase [7]. Recalcitrant after industrial applications are discharged into the aquatic environment leads to pollution. Enzymes are effective alternatives to minimize the "pollution" [8]. Forlaccase catalytic efficiency, low substrate specificity, and minimum reaction time are required. The parameters for the reaction are simple and do not produce the harmful byproducts [9]. The efficiency of color removal depends upon pH, nutrient load, C/N ratio, treatment time, aeration, and fungal biomass. [10]. The high catalytic potential of this enzyme has the ability to treat wastewater of industries, and biotransformation of the dyes [11]. Laccase efficiently decolorizes the different dyes due to their broad substrate specificity. Therefore, in view of dye decolorization efficiency, many researchers studied the various laccases [12–15].

In this study, laccase extracted from *G. multistipitatum* successfully decolorized the dye and cleaned the environment from pollution. Thus, the aim of this study was to evaluate the significance of various nitrogen and carbon sources for laccase production in an eco-friendly manner from *Ganoderma multistipitatum* with an application of environmental cleanliness.

2. Materials and Methods

2.1. Species Collection, DNA Extraction, Sequence Alignment, and Molecular Phylogeny

The specimens used in this study were collected in 2019 from Abha City, South of Saudi Arabia. The DNA extraction, sequence alignment, and molecular phylogeny were completed in the Department of Botany and Microbiology, College of Science, King Saud University, Riyadh, Saudi Arabia. MEGA10 used the maximum likelihood approach with 1000 replications to create the phylogenetic tree. The tree was rooted by using the *Amauroderma rude* as an outgroup.

2.2. Qualitative Laccase Analysis

Malt extract agar medium was prepared in g/L by adding Malt Extract 7, $MgSO_4 \cdot 7H_2O$ 0.5, K_2HPO_4 0.5, KH_2PO_4 0.5, $ZnSO_4$ 0.005, $MnSO_4$ 0.05, Peptone 2.5, and Glucose 15 in Agar (10 g) at pH 5.0 (sterilized in an autoclave for 20 min at 121 °C). This medium was autoclaved and then 0.02% guaiacol was mixed before solidification of agar medium to evaluate the laccase-producing ability from pure mycelium of the specimen. The replicates (plates) incubated (at 30 °C for 7 days) and the formation of (reddish-brown oxidation) zone on agar plate indicated the ability of this species to release the laccase.

Laccases are similar in action like other phenol-oxidizing enzymes. The working of this enzyme is like a battery, because of electrons storage from individual oxidation reactions in order to reduce the molecular oxygen. The oxidation of four reducing substrate (guaiacol) molecules is necessary for the reduction of O_2 into H_2O. Substrate oxidation by laccase is a $1e^-$ reaction generating a free radical. The initial product of this reaction is unstable and may undergo a 2nd enzyme-catalyzed oxidation. Bonds of the natural substrate (lignin) are cleaved by laccase comprise "Cα-oxidation, Cα-Cβ cleavage and aryl-alkyl cleavage" etc. [15].

2.3. Quantitative Laccase Analysis

Kirk's medium was designed for quantitative analysis of laccase activity with a little modification in the shake flasks [16]. The macronutrients and tracer elements (g/L) of Kirk's medium were taken in shake flasks for the growth of mycelium. The macronutrients with little modifications were mixed in g/L (yeast extract 5, starch 1), while tracers ($MgSO_4 \cdot 7H_2O$ 0.5, NaCl 0.5, $FeSO_4 \cdot 7H_2O$ 0.5, KH_2PO_4 0.046, K_2HPO_4 0.1, $CaCl_2$ 0.5, $ZnSO_4$ 0.02, $CuSO_4 \cdot 5H_2O$ 0.5, H_4PO_4 1.0, Na_4HPO_4 0.05, $MnSO_4$ 0.001, $ZnSO_4$ 0.001) were adjusted at pH 5.0 (incubated at 27 ± 2 °C in static condition for 7 days). A UV spectrophotometer monitored the absorbance at 470 nm (3 min) and the activity was expressed in "U" [17]. The unit of activity is defined as the amount of enzyme that oxidizes 1 µmol substrate per minute.

$$\frac{U}{L} = \Delta Abs470 \times \frac{Vt}{€ \times l \times Vs}$$

where,

€ = 6740 M^{-1} cm^{-1} extinction coefficient of guaiacol
Vt = Total vol. of the reaction mixture (mL)
Vs = Vol. of the sample (mL)
l = Length of the cuvette (1 cm)

2.4. Effects of Nutritional Sources on Laccase Production

In this study, flask liquid medium was altered by varying the amount and type of nutritional sources. The potential nutritional sources were mixed in the flasks (250 mL) to check the laccase activity. Three actively grown discs of pure mycelium via cork borer were taken out and poured into 250 mL flask, which contained liquid broth of 100 mL (pH 5.0) and then incubated on a rotary shaker (27 ± 2 °C) at 100 rpm. The complete medium in 1 L shake flask was autoclaved and cooled before inoculation of mycelial discs. From day 3 to onwards, the medium was dynamically agitated on a shaker (4 days) to optimize the nutrient sources (C and N).

Until each concentration was optimized, the various carbon and nitrogen sources were added one at a time in the ongoing culture. The production medium was amended by three concentrations in g/L (20 g, 25 g, and 30 g) of carbon sources (maltose, glucose, and sucrose). Organic nitrogen sources (g/L) "peptone, beef extract, and yeast extract" (5 g, 10 g, and 15 g) and inorganic (ammonium sulfate, sodium nitrate, and potassium nitrate) were amended in 5 g/L, 10 g/L, and 15 g/L with 3 mycelia discs. The liquid medium was altered by varying the kind and concentration of each nutrient source. The rpm 100 was set for 10 days at 35 °C for maximum laccase production. The above liquid samples were used for the analysis of the best source that exhibited maximum laccase activity. The enzyme activity was determined by 100 mM guaiacol substrate dissolved in 100 mM sodium acetate buffer (pH 5.0). This reaction mixture contained 1.5 mL acetate buffer, 1.5 mL guaiacol, and 1.0 mL of crude enzyme source of the above-mentioned broth.

2.5. Partial Purification of Laccase

Whatman filter No. 1 was used to filter the broth and the filtrate was centrifuged at 13,000× *g* for 15 min at 10 °C. The supernatant was gathered for laccase partial purification. The grounded powder of $(NH_4)_2SO_4$ was thoroughly mixed in cold supernatant until the saturation level was achieved (80%) for protein precipitation [18]. This saturated enzyme assay was incubated overnight at 4 °C and these precipitates were collected by centrifuging at 12,000× *g* for 35 min. The protein pellets were dissolved in 20 mM citrate–phosphate buffer (pH 5.0). The same buffer was used in dialysis at 4 °C for 1 day.

2.6. Laccase Molecular Weight

The protein yield was evaluated by SDS-PAGE, using a Criterion XT gel system (Bio-Rad, Hercules, CA, USA). The estimated protein molecular weight (MW) of laccase was

made against the standard protein markers (29–100 kDa). To assign laccase kDa, PAGE was stained with guaiacol (laccase was visualized by incubating the gel in 50 mM sodium acetate buffer (pH 5.0) containing 100 mM guaiacol).

2.7. Dye Decolorization

Dye decolorization section of this study was conducted in Department of Botany and Microbiology, College of Science, King Saud University, Riyadh, Saudi Arabia. Laccase was used for decolorization of RBBR. The stock solutions (final concentration of 200 mg/L) were prepared by dissolving RBBR in citrate phosphate buffer solutions (pH 5.0). The 1.5 mL of laboratory-based dye solution was added separately onto different amounts of Lacc134 (2.0 mg, 4.0 mg, and 6.0 mg) in Eppendorf. These Eppendorf tubes were incubated at 80 °C (optimized T, where laccase exhibited maximum durability) and 160 rpm for 2 h. The decolorization by laccase was determined by a relative decrease in absorbance (617 nm) at a maximum wavelength for dye by the following formula.

$$D(\%) = \frac{100(C1 - C2)}{C1}$$

where D (%) is the decolorization of dye, C1 is the OD of initial dye system, and C2 is the OD of dye system after incubation with Lacc134. The absorbance was measured at 617 nm and decolorization was expressed in percentages. Control samples without Lacc134 were separately maintained in parallel to experimental samples.

2.8. Estimation of TOC (Total Organic Carbon)

The HACH a TOC (Model: TOC-5000A, Shimadzu, Kyoto, Japan) analyzer equipment of Department of Botany and Microbiology, College of Science, King Saud University, was used to measure TOC concentration (Shimadzu TOC-L total organic carbon analyzer). This equipment comprised a unique combustion catalytic oxidation according to the 5310B Standard method [19]. Readings were performed by a HACH spectrophotometer (430 nm) using a "blanc one COT tube with Milli Q water".

2.9. Km & Vmax Value

The *Km* and *Vmax* values of the Lacc134 were estimated by Lineweaver–Burk plot of 1/V versus 1/S at the different quantity of 1, 2, 3, 5, and 10 (mM) guaiacol concentrations.

2.10. Statistical Analysis

The data collected from various parameters during the presented study were subjected to statistical analysis in computer software, Co-Stat version 3.01. Assays were carried out in triplicate and the values were presented as mean ± standard deviation.

3. Results

3.1. Species Identification by Phylogenetic Analysis

In this study, *Ganoderma* was explored for laccase production. The *G. multistipitatum* sp. nov. was highlighted with black bullets (square and circle) and this species successfully falls in the clade of *Ganoderma*. This species was identified by a phylogenetic method with a 99% bootstrap value (Figure 1). The sequences of *Ganoderma multistipitatum* were deposited in GenBank under accession No. ON032992, ON032991. Morphologically, this species was identical to typical *Ganoderma lucidum*. The species used in this species was dissimilar to other *Ganoderma* species in having verrucose, laccate cinnamon orange to brown basidiomata. Stipe was multistipitate, laccate, maroon brown to blackish brown, and soft with hard crust. Basidiospores are ellipsoid, bitunicate, pigmented, thick, green, echinulae, guttulated, highly thick inter-walled pillars, and apically truncate. Trimitic hyphal system comprised generative, skeletal, and binding hyphae.

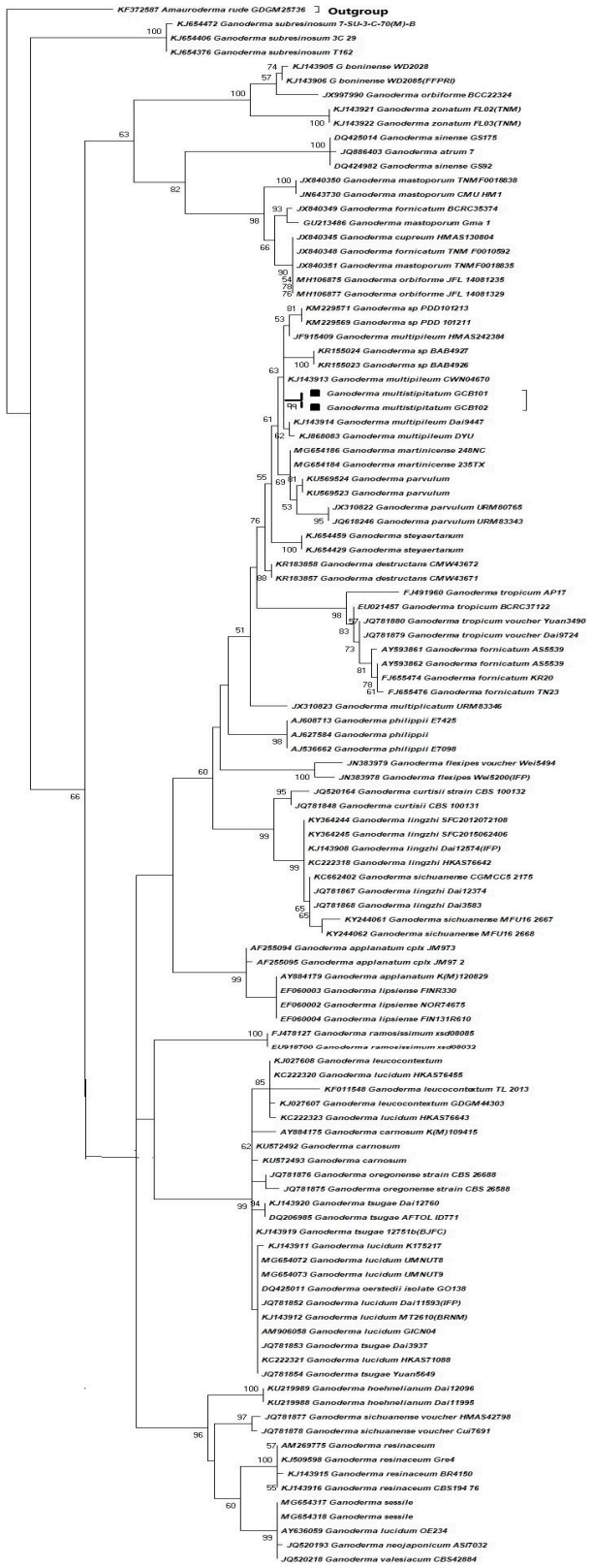

Figure 1. Phylogenetic tree of *Ganoderma multistipitatum* (represented by black box).

3.2. Qualitative Estimation of Laccase

The pure mycelium was obtained on the MEA plate from basidiomata (Figure 2A). The preliminary guaiacol plate test confirmed the presence of laccase by the formation of a

maroon brown to dark purplish zone (Figure 2B). The color intensity of guaiacol increased day by day because laccase was strongest in action. The color zone became intensified after 2 days (Figure 2(B1–B4)). The intensity of the maroon-brown halo was observed after 2, 4, 5, and 7 days. No change was observed after 7 days. This indicated that laccase strongly oxidized the guaiacol substrate.

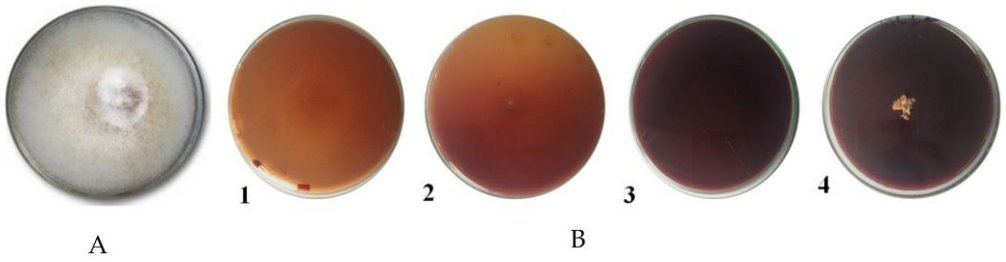

Figure 2. (**A**) Pure mycelium of *G. multistipitatum.* (**B**) qualitative detection of laccase by guaiacol with the passage of time (after 2 (**B1**), 4 (**B2**), 5 (**B3**), and 7 (**B4**) days).

3.3. Effects of Nutritional Sources on Laccase Production

Carbon Sources: Different carbon sources like maltose, glucose, and sucrose were evaluated one by one. The production medium was amended by the three concentrations (20 g, 25 g, and 30 g). The 20 g glucose was a more appropriate concentration for secretion of maximum laccase rather than 25 g and 30 g (Figure 3A). The laccase activity was 2386 ± 0.2 U/L in the fermentation flask. The best secondary inducer was sucrose (25 g) for laccase activity, but the activity declined as the concentration crossed this range. The 20 g and 25 g were more suitable in the case of maltose, while 30 g was not a suitable quantity for laccase activity as compared to control (Figure 3A).

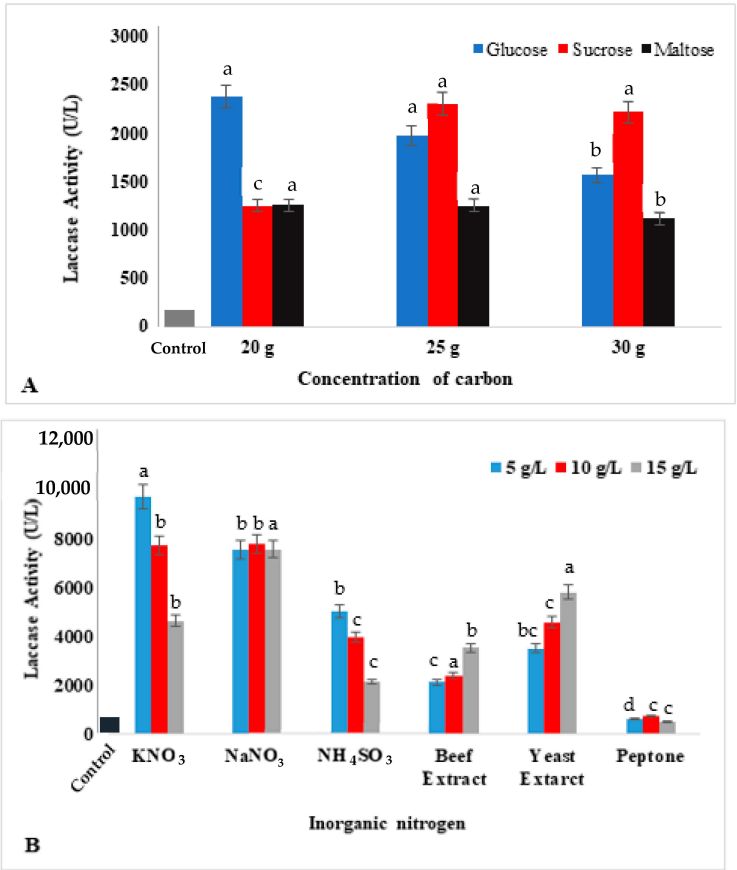

Figure 3. (**A**) Effect of organic carbon sources on the production of laccase (U/L), (**B**) effect of organic

and inorganic nitrogen sources on the production of laccase (U/L). The data collected from each treatment was expressed as mean ± SE and statistically analyzed through analytical software. The means were compared by Least Significant Difference. The statistical analysis was done at the significance level $\alpha = 0.05$ using Co-Stat version 3.01. Similar letters indicated non-significant effects at $p < 0.05$ between different treatments.

Nitrogen Sources: To determine the maximum laccase production, suitable inorganic and organic nitrogen sources were chosen and utilized for the *Ganoderma* species. Inor-ganic ammonium sulfate, sodium nitrate, and potassium nitrate were adjusted in concentrations of "5 g/L, 10 g/L, and 15 g/L" with three mycelia discs. Organic nitrogen sources peptone, beef extract, and yeast extract were incubated for 10 days at 27 °C. The inorganic nitrogen quantities were suitable for laccase secretion rather than organic sources. The 5 g/L KNO_3 facilitated 9750 ± 1.3 U/L laccase activity rather than higher concentrations. The 10 g/L $NaNO_3$ released 7800 ± 1.1 U/L laccase from this species as compared to control (Figure 3B). In this study, $(NH_4)_2SO_4$ was not the best nitrogenous source compared to other inorganic sources. The selected organic sources significantly enhanced the laccase like inorganic sources. The yeast extract was a better extractor of laccase from the mycelium of *Ganoderma* than beef extract and peptone. The laccase activity was 4589 ± 0.5 U/L and 5834 ± 2.2 U/L at 10 and 15 g/L, respectively (Figure 3B). The 15 g/L beef extract was a suitable concentration for laccase secretion. From the organic nitrogen sources yeast extract and from inorganic nitrogen sources $NaNO_3$ were suitable inducers for laccase secretion from this new species.

3.4. Purification and Molecular Weight Determination of Lacc134

In this experimental result, Lacc134 was identified by SDS-PAGE and Native-PAGE (Figure 4). The molecular weight of Lacc134 was ~68 kDa and efficiently removed the dye from water.

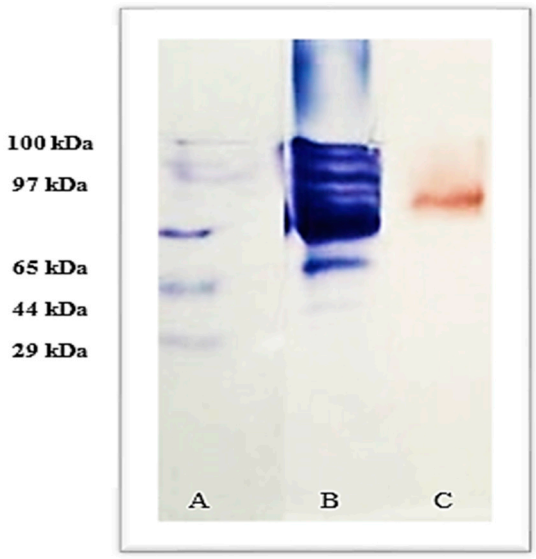

Figure 4. Molecular marker (**A**), SDS-PAGE (**B**), and Native-PAGE (**C**).

3.5. Dye Decolorization by Lacc134

Different amounts of Lacc134 were used to decolorize the RBBR. The Lacc134 (2.0 mg, 4.0 mg, and 6.0 mg) was incubated in a solution of RBBR for 10 repetitions at 1 h. This process was repeated by replacing the decolorized water with 1.5 mL of fresh dye solutions for 2nd next 1 h to check the stability of Lacc134 (Figure 5). The results exhibited that dye was highly decolorized after 1 h incubation for all the amounts of tested enzyme. The higher Lacc134 amount effectively removed the dye than lower amounts. This concluded that the increased amount of Lacc134 gradually increased the color removal. The 2 mg

removed 62% dye from the 1.5 mL solution in first 1 h, while 69% in the second round of 1 h cycle. Similarly, 4.0 mg slightly increased the rate of dye removal (74%) in the first round of 1 h, while 78% in the second 1 h cycle as compared to control. The 6.0 mg was efficient and fast in removal of dye concentration from the water. This higher amount of Lacc134 efficiently removed 85% RBBR in 1 h, whereas 88% in the second 1 h time interval treatment (Figure 5). This study concluded that the maximum quantity of laccase from *Ganoderma* was efficient in the removal of dye RBBR. The efficiency of Lacc134 to decolorize the blue dye makes the enzyme for industrial and biotechnological applications. RBBR is an anthraquinone dye, a second most important class of textile dye that to belongs hazardous and resistant pollutants. The following equation indicated how the dye (RBBR) degraded by laccase into low biodegradable compounds, and water was released as a byproduct during laccase mechanistic action in the following equation.

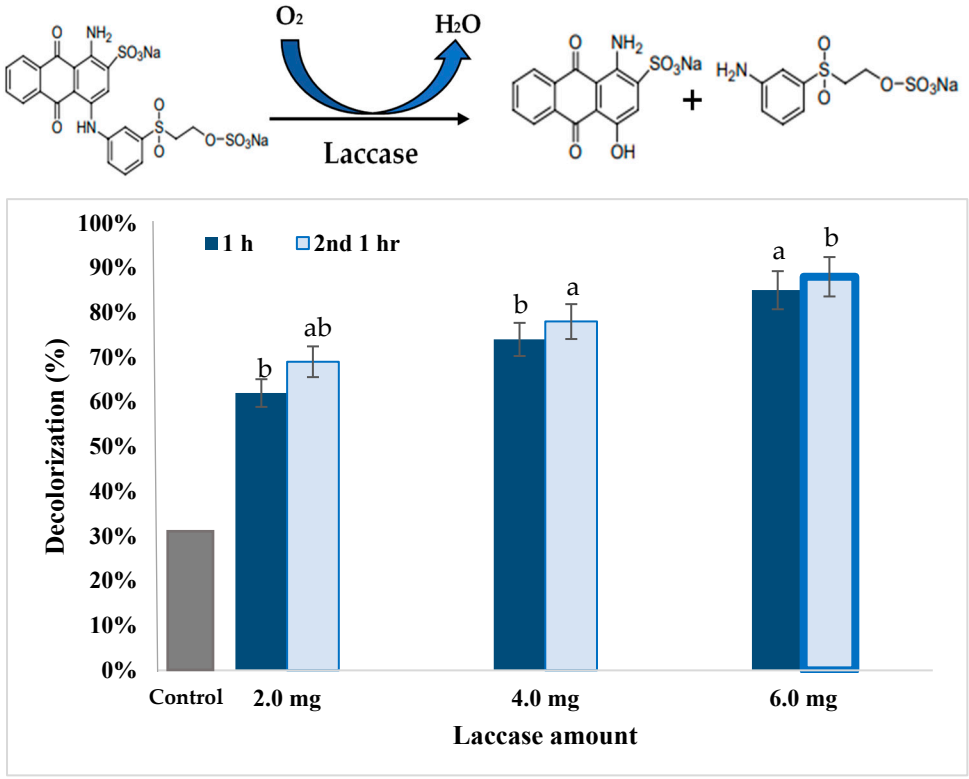

Figure 5. Dye decolorization (%) of RBBR for 2 h. (mg = milligram). The data collected from each treatment was expressed as mean ± SE and statistically analyzed through analytical software. The means were compared by Least Significant Difference. The statistical analysis was done at the significance level $\alpha = 0.05$ using Co-Stat version 3.01. Similar letters indicated non-significant effects at $p < 0.05$ between different treatments.

During RBBR degradation, laccase performed redox, hydroxylation, and deamination reactions. In oxidation reaction of the enzyme-substrate complex, molecular O_2 is required from the atmosphere, and reduction of O_2 into the water takes place. Instead, H_2O_2 is required by other oxidative enzymes. So, this mechanistic action is eco-friendly in nature because oxidation and intramolecular electron transfer are simultaneous to proton transfer that allows O_2 reduction into H_2O. The use of oxygen in the laccase reaction has sparked interest at industrial level; since O_2 can be used as a primary oxidant, being possible to control the injection or decrease of O_2 pressure during the enzymatic reaction, the resulting products formation comprised an amino group in their structure. The formation of low-molecular-weight products after laccase-catalyzed reaction possessed low toxicity level.

3.6. Concentration of TOC after Dye Removal Efficiency

Carbon is widely used in the decontamination of air and wastewater. Its effectiveness in removal of pollutants is superior to many other methods, because of the high quality of effluent, simplicity of design, ease of operation, and insensitivity to toxic substances. In this study, laccase was maximum in the concentration of glucose carbon sources. In this study, the after-effects of TOC were observed in the case of glucose rather than other organic carbon sources. The effect on final concentrations of organic carbon sources was investigated during the action of dye removal. The initial pH of the solution was 5.0 at 30 °C. The adsorption process of Lacc134 with "20 g/L and 25 g/L" carbon doses was determined after a contact time of 1 h. The removal efficiency of color and total organic carbon (TOC) in the dye solution was decreased with increased concentration. The results indicated that 85% color removed and 99.9% TOC was presented in the solution at the initial concentration (20 g/L) of glucose, while 74% color removed and 97.9% TOC was present at 25 g/L. The maximum 30 g glucose concentration removed 62% dye from the solution and 89% TOC left in the solution as compared to control (Figure 6). It appeared that the removal efficiency of color and TOC merely declined, indicating the carbon contents made the high adsorption capacity of dye removal. The color degree and TOC removal of the dye in solution increased sharply with increasing carbon concentration. The reason behind the availability of more surface area at higher carbon dosages. The carbon concentration was a valuable and economical adsorbent with lower cost than commercial activated carbons. In adsorption, the amount of dye adsorbed onto the carbon was dependent on adsorption time and carbon concentration. The 30 g/L of carbon dosage was sufficient for the removal of dye. Over the range of initial concentration, the dye removal efficiency remained above 62% as compared to control, indicating that this carbon concentration has a large adsorption capacity. The laccase efficiently removed the pollutants from wastewater. This pollutant was enzymatically degraded by Lacc134. The maximum degradation efficiency of dye removal was compared to laccase without organic carbon sources, because carbon sources facilitated better transfer of electrons between laccase and substrate molecules once adsorbed onto the pollutant surface.

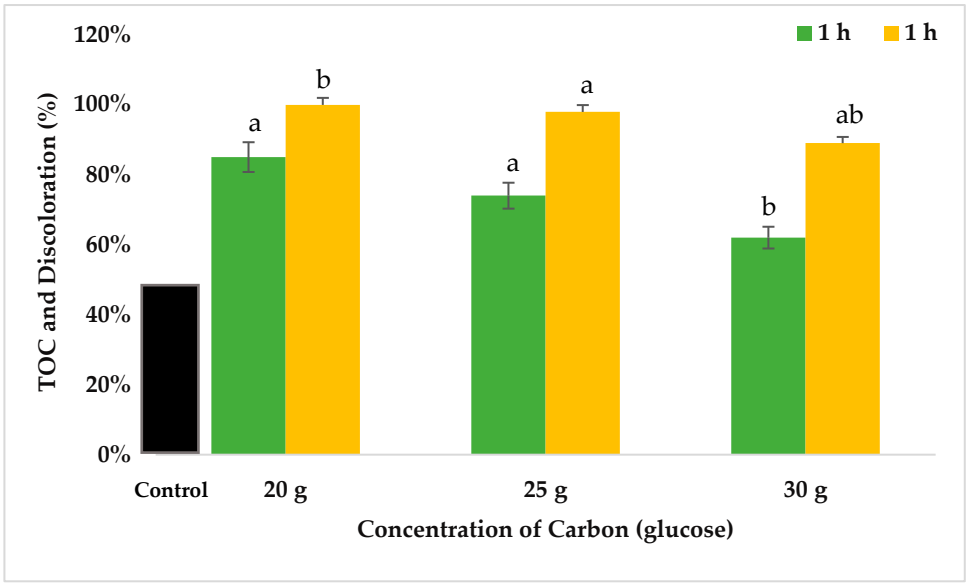

Figure 6. Comparative evaluation of discoloration (% ■), and TOC (total organic carbon) content (% ■). (g = gram). The data collected from each treatment was expressed as mean ± SE and statistically analyzed through analytical software. The means were compared by Least Significant Difference. The statistical analysis was done at the significance level $\alpha = 0.05$ using Co-Stat version 3.01. Similar letters indicated non-significant effects at $p < 0.05$ between different treatments.

3.7. Km & Vmax Value

The kinetic studies *Km* and *Vmax* of Lacc134 were 1.658 mM and 2.452 mM min^{-1}, respectively, under different concentrations of guaiacol dose (Figure 7). These values indicated how efficiently Lacc134 reacted with its substrate.

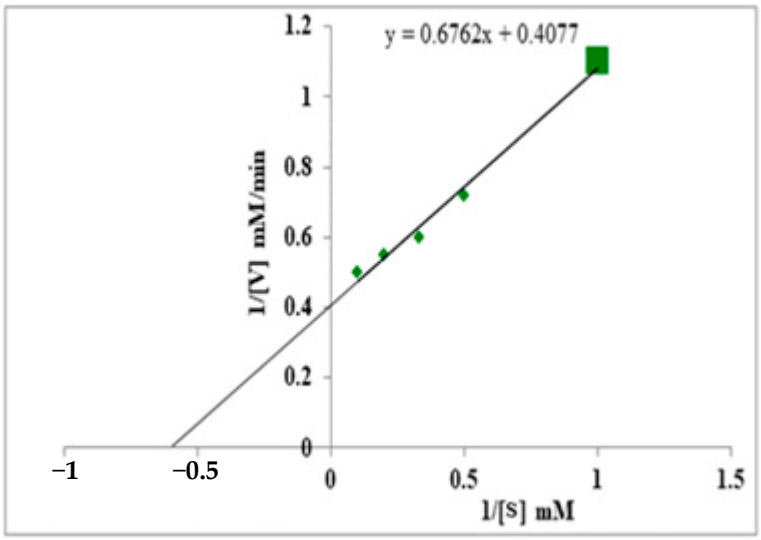

Figure 7. "Lineweaver–Burk plot" of purified "Lacc134" of *G. multistipitatum*.

4. Discussion

Significant inducers of laccase synthesis are carbon sources [20], which symbolize the first sign of growth within 24 h or 6 days [21]. Li et al. [22] explained glucose as a stronger inhibitor of laccase expression and its scarcity in medium improved the activity. On the other hand, they said that "glucose is an important nutrient factor convincing the basidiomycetes to secrete the laccase [22]. The presence of sugar caused the reduction in enzyme yield attribute to repress the catabolites [23]. There have been several reports of low carbon–nitrogen ratios and some prefer high carbon–nitrogen ratios to obtain a better impact on high laccase production with activity [24]. Maximal laccase secretion was possible after the complete depletion of carbohydrates in the medium. Regarding influence, it has been observed that substrates such as glucose, cellobiose, and mannitol carbon sources are used rapidly to increase the high laccase activities as compared to lactose or cellulose [25].

Glucose is substituted by fructose to increase the laccase-specific activity and the laccase synthesis with higher activity accompanied by consumption and complete utilization of glucose. The initial concentration of 20 g/L glucose was consumed very quickly and the remaining concentration was less than 0.5 g/L on the 7th day [26]. Laccase production was repressed by glucose in many species [27]. Similarly, Zhongyang et al. [28] tested the different carbon sources in their experiment. According to them, glucose 20 g/L was the most effective carbon source to stimulate the maximal laccase activity (2564.86 U/L). The other studies explained that the increased sugar concentrations from 20 to 80 g/L lead to a decline in laccase production. *G. multistipitatum* sp. nov. showed a value near to Zhongyang et al. [28] at 20 g/L glucose. Galhaup et al. [29] have the same opinion about the maximum concentration, which repressed the synthesis of laccase in *Trametes pubescens*. On the other hand, it has been reported that a high quantity of glucose triggered the manufacturing of extracellular polysaccharides, which hinder the extraction of laccase from the culture broth.

Hailei et al. [30] showed 1.0 g/L glucose in fermentation broth acted as glucose limitation, which induced laccase production in some isolates of *G. lucidum*. They quoted that glucose concentration consumed rapidly during the exponential growth of mycelium in culture medium. Songulashvili et al. [31] revealed that simple glucose produces very

low laccase, whereas complex substrates highly stimulate the activity in *Ganoderma* species. Moldes et al. [32] exhibited laccase activity 295 U/L in the presence of glucose; this value was very low than the data obtained in this experimental work.

Teerapatsakul et al. [33] used glucose and lactose carbon sources with concentration to evaluate the laccase activity. They concluded that glucose was more efficient than lactose from *Ganoderma* sp. KUAlk4. Glucose is rapidly consumed by the organism for maximum level of laccase [34]. The sequential addition of glucose caused the higher laccase production in *Trametes hirsute T. versicolor* 1666 [35].

Li et al. [22] have the same opinion related to glucose limit in *Ganoderma* isolates. According to Li et al. [22], laccase secreted in 4 days under the availability of "reducing sugar", and as the concentration exceeded the limit (≥3.50 g/L), the cells of *Ganoderma* species faced the state of glucose limit and inhibited the production of the enzyme [36]. *Ganoderma* expressed the laccase during the second phase of growth under limited sources (C and N) [20]. Glucose (2%) was the best carbon source to exhibit 124 U/mL laccase activity [37] from *Pleurotus ostreatus* [38] and *Phellinus noxius* hpF17 [39].

Higher concentration of sucrose exhibited a -ve effect on the production of laccase from *G. lucidum* [40]. This work concluded that excessive sucrose enhanced the production of laccase in *Ganoderma* species. In contrast to this work, the sucrose (20 g/L) was best to enhance the laccase activity (1351.41 U/L) in submerged fermentation [33]. However, the value of Zhongyang et al.'s [33] work was lower than *Ganoderma* used in this study at 20 g sucrose. They also proposed an idea regarding the high concentration of carbon sources satisfying the nutrient demands for biomass growth, not facilitating the mycelium biomass to secrete the maximum laccase. Sivakumar et al. [41] used sucrose and glucose to evaluate the laccase activity in *Ganoderma* species, but the maximum activity was supported by starch.

Laccase activity is influenced by nitrogen concentration and type [25,29]. Organic sources of nitrogen were more efficient than inorganic [40]. The authors have found that nitrogen does not affect the activity and yield of a few fungal species [42]. Several authors reported a low carbon –nitrogen ratio and some prefer a high carbon–nitrogen ratio [24] to obtain a better impact. "N" suppressed and stimulated the activity in numerous species (*Trametes trogii*) [43]. In *Ganoderma* sp. kk-02, nitrogen sources increased the laccase up to 3.5-fold [44].

Nitrogen-rich medium enhanced the laccase production rather than nitrogen-limited medium. The majority of authors reported that the exhaustion of nitrogen source influences laccase production. Synthetic medium with low N (malt extract) facilitated the fabrication of laccase isoforms in *G. lucidum* in basidiomycete NIOCC#2a and *T. gallica*. Nitrogen depletion triggered the laccase secretion in some fungal species, so nitrogen does not affect laccase activity of *Ganoderma* species [45].

In this experimental work, different nitrogen sources were applied to *Ganoderma* species. The literature reported that organic and inorganic nitrogen sources are useful for better production of laccase. Songulashvili et al. [23] revealed that nitrogen sources (inorganic and organic) stimulated *Ganoderma* growth and protein content (10–29%) in fermented biomass.

The source of vitamin in shake flask broth is yeast extract, which is a better nitrogen source [46]. Songulashvili et al. [31] explained that all nitrogen sources enhanced the activity of laccase in *G. lucidum*. Primary yeast extract comprised amino acids, nucleotides, peptides, and other soluble components, which indicated that yeast extract stimulates the laccase production more efficiently than other organic nitrogen sources similarly in this work. Yeast extract supported maximum laccase production than beef extract, peptone, NH_4Cl_2, and $NaNO_3$ from *Ganoderma* species [40]. Yeast extract with higher concentration exhibited +ve effect in the production of laccase of *G. lucidum* [41].

Piscitelli et al. [47] strongly encouraged the results regarding the organic nitrogen sources used in this study. Positive impact was observed on the 7th and 8th day as the concentration of yeast extract and peptone increased, respectively. The literature considered that yeast extract is a better nitrogen source to enhance laccase yield [47]. Teerapatsakul

et al. [33] set 4.0, 6.0, and 8.0 pH with different nitrogen sources to calculate the highest laccase activity (50 U/mL) by utilization of 0.22 g/L yeast extract at pH 6.0 from *Ganoderma* KU-Alk4. These views supported the data of this experiment. Yeast and malt extract released the maximum laccase (906,000 U/L) from *Pleurotus ostreatus* [38].

Peptone was a crucial factor in the efficient production of laccase in this study. Shakhova et al. [35] explained that peptone nitrogen sources for activation of ligninolytic enzymes. Peptone complex composition provides multiple assistances to the fungal cells and cell performance [48]. Peptone concentration increased laccase activity on the 8th day [40]. The peptone nitrogen source produced maximum laccase on the 5th day, while activity declined on the 6th (0.0129 U/mL) and 8th day (0.0104 U/mL) from *T. versicolor* 1666 [35].

Similarly, in the work of Kuhar and Papinutti [49], no significant influence was shown by peptone concentration on laccase activity. In contrast, organic peptone was an appropriate nitrogen source for laccase production (89,020 U/L) [31] from *P. ostreatus*. Inorganic sources of nitrogen have varied impact on the expression of laccase from *Ganoderma* species in submerged broth. Songulashvili et al. [23] revealed that inorganic nitrogen sources stimulated the growth and protein content of *Ganoderma* in fermented broth. The maximal value (75%) of laccase activity (93,840 U/L) was enhanced in *G. lucidum* by supplying the culture medium separately with KNO_3 [31]. In the study of Songulashvili et al. [31], KNO_3 revealed the maximum value might be due to inhibition of culture medium from acidification. The maximal value (75%) of laccase activity (68,000 U/L) was enhanced in *G. lucidum*, when culture medium augmented with NH_4NO_3. The $NaNO_3$ produced maximum laccase on the 5th day (0.0291 U/mL), while activity declined on the 6th (0.0062 U/mL) and zero on the 8th day from *T. versicolor* 1666 [35]. Similarly, the maximal laccase activity (50,320 U/L) was enhanced in *G. lucidum* by $(NH_4)_2SO_4$ [31].

The hallmark sign of industrial wastewater increasing day by day. Different methods, e.g., "physicochemical, chemical, enzymatic and microbiological" are applied for the degradation of dyes [50]. Chemical and physical methods are not eco-friendly. So, many scientists are studying environmentally friendlier biological tools for color removal from dye wastewater. Nowadays, the best approach is the laccase of white-rotters to decolorize the dye [51].

Several experimental studies [52–55] have been performed to investigate the decolorization of an anthraquinone dye by laccase. It has been demonstrated that laccases decolorize anthraquinone dyes more effectively than other types of dyes [56]. Anthraquinone by Zeng et al. [57] is a redox mediator for laccase-catalyzed decolorization.

It has been proved that laccase breaks synthetic colors with a variety of chemical configurations. The type of laccase-producing organism controlled the redox potential of laccases, various substrate specificities, and last but not least the rates of dye biodegradation. Wood-rotting fungi secrete laccases within medium with high-redox potential, while bacteria and some plant species only make laccases of low redox potential. As a generator of laccases with higher redox potential, the white-rot fungus group is particularly intriguing in this regard. A useful method to determine toxicity potential of a synthetic dye is the ability to forecast the development of products after laccase treatment.

Malvis et al. [58] explained that TOC/TN ratio decreased due to the oxidation of organic matter and maximum elimination of "carbon compounds" than "nitrogenous compound". Furthermore, in all cycles of treatments, TOC decreased in the form of complex sub-products [59]. Low TOC concentration indicated the residual organic carbon due to the existence of recalcitrant organic compound. The change in TOC is associated to non-biodegradable organic compounds, which are achieved at the end of wastewater treatment. TOC/TN ratio in all processes decreased within 73 h, which demonstrated a decrease in complex sub-products after dye mineralization.

In this work, Lacc134 retained its decolorization activity against the dye. The Lacc134 gradually increased the color removal, when 2 mg removed 62% dye from the 1.5 mL solution in first 1 h, while 69% in the second round of 1 h cycle, whereas 6.0 mg Lacc134 was efficient in removal of 85% and 88% dye concentration in 1st and 2nd h treatment,

respectively. The reason for this is the different structures of dyes [60]. Overall, Lacc134 exhibited the better performance in terms of textile dye removal. According to the kinetic study, the dramatic decrease in *Km* of Lacc134 was due to an increase in substrate affinity. However, the *Vmax* value of Lacc134 increased the maximum dye decolorization. In this study, the minimum TOC concentration indicated that organic carbon sources in the medium reacted more speedily with Lacc134. The reactiveness of this laccase isolated from *Ganoderma* sp. was maximum due to the presence of medium supplements.

The overall conclusion of this work suggested that Lacc134 is valuable for industrial application and plays an important role in dye decolorization.

5. Conclusions

In this study, *Ganoderma* species was identified by morpho-anatomical and ITS marker, The phylogenetic tree facilitated correct species identification. The laccase was isolated from this species and secretion of laccase was enhanced by organic C and inorganic C and N sources in submerged fermentation flask. The Lacc134 successfully decolorized the dye water. This is a big eco-friendly achievement in detoxification of dye water and industrial applications.

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