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Biodegradation of Chromium by Laccase action of Ganoderma multipileum Abstract Laccase is a fungal enzyme that play a crucial role in bioremediation. The purified laccase from Ganoderma multipileum and its effectiveness in bioremediation of Cr (VI) was determined in this study. Two species of G. multipileum were identified by ITS sequences and their phylogeny was compared with G. multipileum taken from GenBank (KF494997, LC149613, MG739453, MG739455). The fungi were grown on guaiacol substrate for laccase optimization using different environmental and nutritional conditions. Laccase Glacc113 (75 kDa) was partially purified and characterized under different parameters. Glacc113 (GIAPTAD) was confirmed by using a Precise Protein Sequencing System to analyze sequence of N-terminal amino acid. Laccase exhibited maximum optimal activity (1355.5 ± 8.8 U/L) at pH 3.0 and can tolerate the maximum temperature upto 70 ºC. During submerged fermentation, on 7th day after inoculum of 3 fungal discs at 100 rpm yielded maximum laccase. The production of laccase increased by optimization of inorganic and organic nitrogen and carbon sources. The purified laccase from G. multipileum was used to reduce (>94%) 100 µg/mL of Cr (VI) into less toxic chromium (Cr (III). The catalytic kinetic parameters Vmax and Km for guaiacol were 1.817 (mM min-1) and 1.4617 (mM), respectively. This study determined the conditions that enhance production and an ecofriendly approach to bio remediate the Cr (VI) to Cr (III). The purified enzyme exerted maximum durability and reliability for industrial usage also. Key words: Bioremediation, characterization, guaiacol, laccase, nutritional parameters, kinetics Commented [o1]: Rewrite the title for improvement as per yours suggestion Commented [o2]: 1 key word is added as per suggestion 1. Introduction 1 Ganoderm multipileum, commonly known as lingzhi or chizhi belongs to the family Ganodermataceae (Wang et al., 2009; Bhosle et al., 2010). The species of this genus are widely grown on a commercial scale due to its medicinal properties and commonly used in traditional medicines (Zhou et al., 2015). Multiple biological activities of the genus Ganoderma are due to its secondary metabolites including lanostane triterpenoids, meroterpenoids, ergostane steroids and farnesyl hydroquinones. Major lanostanes extracted from G. multipileum are ganoderic acid AM1, ganodermanondiol 24, 25-acetonide, lucidumol A, B, ganoderiol F, ganoderitriol M, ganodermanontriol and 7-oxoganoderic acid (Binh et al., 2018). G. multipileum produces laccase, a ligninolytic and extracellular enzyme belonging to the family oxidoreductase (Alfarra et al., 2013). It

accommodates a broad range of substrates viz: diphenols, polyaromatic amine and iodine as well as phosphates, ketones, ascorbate and lignin (Munk et al., 2017; Rodrigues et al., 2019). Laccase is a metalloenzyme with a wide range of activities such as azodye oxidation, xenobiotic degradation, pollutant detoxification, steroid transformation as well as pharmaceutical products formation and degradation (Tortella et al., 2013; Litwińska et al., 2019). Many other laccase producing wood rotting fungi with multiple applications are Pleurotus sajor-caju, P. ostreatus, P. ostreatus POXA1, Trametes trogii POXL3, Pycnoporous cinnabarius, Coriolus hirsutus, and Ganoderma lucidum (Shin and Lee, 2000; Soden et al., 2002). In low quantities, a few heavy metals are necessary for life, but as concentrations rise, they become poisonous. Their high concentrations cause allergy, carcinogenicity and sometimes inhibit the enzymes activities (Koropatrick and Leibbrandt, 1995). Exposure to environmental or natural concentrations of chromium are hardly hazardous to human health. Natural occurrence in plants, soils and its inclusion in animal feed, Cr (III) is part of the human diet (Pavesi and Josino, 2020). Chromium heavy metal toxicity poses a great threat to the environment. Soil, air and water are heavily contaminated by Cr (VI) released by chrome-plating, steel manufacturing, anti-corrosion agents, leather tannery, textiles, dyes and pigments (Gu et al., 2015). The compounds contain Cr (VI) are mutagenic and carcinogenic; and poses serious injuries to the ecosystem with serious health issues in humans, animals and marine life (Sandana et al, 2015). Cr (VI) easily penetrates the red blood cells (RBCs) due to its bioavailability and gets converted to Cr (III), which sticks to the cellular components of RBC (Shekhawat et al., 2015). 2 It is critical to comprehend in-depth that the reduction conditions in order to reassemble the higher quality of chromium toxicity. A variety of functional groups in fungal species provide great biosorbent capacity in heavy metal remediation. Moreover, fungi grow naturally in heavily polluted environments (Zapana-Huarache et al., 2020). A few fungal species especially laccase from filamentous fungi (Trichoderma viride, Aspergillus flavus, A. fumigates, A. awamori, Fusarium proliferatum, Penicillium radicum, Beauvariabassiana, Phanerochaete chrysosporium, etc.) indicated in literature with great potential for heavy metals bioremediation (Tanvi

et al., 2020 ; Kumar **et al** ., 2019; Hussain **et al** ., 2018; Gola **et al** ., 2016; Shazia **et al** 12

., 2013; Vala, 2010; Joshi et al., 2011; Kamal et al., 2023). Similarly, Streptomyces sp. is a stronger candidate for the remediation of chromium containing effluents (Shazia et al., 2022). Isolates of dark septate endophytic

fungi exhibited an efficient removal capacity (99% **of** 25

50 mg/L) of Cr(VI 25

) (Melati et al.,2023). In literature, there are a few reports on laccase treated chromium tolerant wood rotters e.g., Phlebia brevispora and P. floridensis (white rot fungi) effectively removed the chromium from industrial wastewater (Sharma et al., 2023). In view of the above literature and environmental problem, Ganoderma species are one of the most important ornamental degrader of heavy metals pollutants, but no or a very few work available on this achievement.

The present investigation **was** taken **to investigate the** ability **of** a **laccase** 14

from newly explored wood rotting fungal species to deal the chromium metal and to study the efficiency of metal removal from the liquid medium. This study also suggests that its high potential for effluents bioremediation and biotechnological usage. G. multipileum is a key player in the bioremediation process. The presence of a functional Cr (VI) reducing mushroom is a necessary pre-requisite for developing a bioremediation technique for Cr detoxification (VI). This study drop down the Cr (VI) to a less toxic Cr (III) via a novel purified laccase under less cost-effective and eco-friendly techniques. The findings from this study will be a new report, which will decipher the significance of G. multipileum laccase in

bioconversion of toxic Cr (VI) to less **toxic Cr (III** 34

) state without any pollution.

2: MATERIALS AND METHODS 2.1. Sample collection and Molecular Identification 29

Samples were collected **from**

Pakistan in 2018 during the monsoon season. The samples (Fig. 1A) were dried by using a dehydrator. Modified CTAB method was applied to extract the 3 total genomic DNA of the specimen (

Doyle and Doyle, 1987). The nuclear ribosomal ITS region **was amplified using** the **primers ITS1** (5' CTTGGTCATTTAGAGGAAGTAA'3) **and ITS4** (5'TCCTCCGCTTATTGATATGC'3) (**White et al., 1990** 20

). BioEdit version 7.2.5 used to create the consensus sequences and BLASTn was used to examine homology at the

National Center for Biotechnology Information (NCBI). The sequences generated during **this study were deposited in GenBank** and assigned **accession numbers** 22

. 2.2. Phylogenetic Analysis A dataset of ITS-based accessions was acquired from GenBank based on published literature. To align and edit the sequences, we utilized ClustalX 2.1 and BioEdit (Hall, 1999; Larkin et al., 2007). MAFFT v. 10 (Katoh and Standley, 2013) was used to manually align the downloaded and newly produced sequences at 593 location.

The maximum likelihood technique **with 1000 bootstrap replicates** were used **to** create **the phylogenetic tree using** 5

these sequences representing 15 taxa in MEGA 10.0 software. Tomophagus colossus was selected as an out-group. In the obtained tree less than 50% bootstrap supports were buckled. 2.3. Qualitative Plate Screening of Laccase Production The MEA (

Malt Extract Agar) media was made **in g.L** -1 **by adding** ME **7** g, **Agar 10** 1

g,

MgSO4.7H2O 0.5, K2HPO4 0.5, KH2PO4 0.5, ZnSO4, **0** 36

.005, MnSO4

0.05, Peptone 2.5 and Glucose 15 at pH 5.0 (Fig. 1B	18

) (White et al., 1990). Streptomycin (200 mg.L-1) an antibacterial agent was added and sterilized for 20 min at 121 ºC,

allowed to cool down for 15 min and then	33

add 0.02% guaiacol for laccase screening (Fig. 1B). This media was transferred into Petri plates to get hard. A 2 to 3 mm of pure mycelium fungal disc was inoculated in each plate after solidification of media. All of

the plates were incubated	for 5 days	**at 30 °C. The**	1

laccase-producing G. multipileum was screened by

formation of	a	**reddish brown oxidation zone**	1

. (Fig. 1C). 2.4. Quantitative Analysis of Extracellular Laccase Activity Laccase activity was determined by “Kirk's medium” with little modification (Hall, 1999). For mycelial growth, the macronutrient and trace elements (g.L-1) was kept in the flasks. The macronutrients included (10 g.L-1) glucose, starch and yeast extract, while the trace elements were [MgSO4.7H2O, NaCl, FeSO4.7H2O, KH2PO4 0.046%, K2HPO4 0.1%, CaCl2.2H2O, ZnSO4, CuSO4.5H2O, H4PO4 (1.0%), Na4HPO4 (0.05%), MnSO4 (0.001%), ZnSO4 (0.001%)] (Larkin et al., 2007) regulated at pH 5.0. One-liter medium was autoclaved, allowed to cool and aliquots of 4 100 mL of each was put into 3 different flasks. Each of the flasks was inoculated with mycelial plugs (5 mm) and incubated at “27 ± 2 °C in the static condition for 3 days”. The medium moved gently through shaker to “optimize the nutritional and environmental factors”. Laccase activity was measured using.Liquid broth in a shaking flask. The guaiacol substrate used to determine enzyme activity following Umar & Ahmed (2022). UV Spectrophotometer used to monitor the change in absorbance of the reaction solutions containing guaiacol for 3 min at 470 nm (Sharma et al., 2013). This activity was measured by following formula in triplicate and expressed in U/L by measuring the absorbance for 3 to 5 min (Jhadav et al ., 2009). $\mathbf{U\ Us\ L} = \mathbf{\Delta Abs470} * € * \mathbf{l} * \mathbf{Us}$ 2.5. Optimization of Environmental Conditions The culture growth conditions for hyper-production of

laccase by selected Ganoderma species were optimized by adjusting the pH, temperature, incubation time, quantity of fungal discs, and agitation speed of the media. Hundred milliliter separated from the culture flasks containing two mycelia discs were cultured at varied pH levels for seven days “(3.0, 5.0, 6.0)” and temperature (60 °C, 40 °C and 20 °C). A complete batch was set at different revolutions (50, 100, 150) per min for 7, 10 and 15 days with 2, 3 and 5 mycelial discs to maximize the laccase production. 2.6. Nutritional Conditions and Laccase Production The medium was amended by nature and concentration of the nutritional sources. Actively growing three mycelial discs were plugged out and inoculated in three different flasks comprising fermented broth of pH 5.0 on a shaker with 100 rpm at 35 °C. According to Revankar and Lele (Revankar and Lele. 2006),

after 10 days of post inoculation (dpi), **laccase activity was** calculated. **The**	1

filtrate was used optimization of nutritional conditions. For the carbon optimization, different sources like “1: maltose, 2: glucose and 3: sucrose” at 20 g and 25 g concentration were evaluated. For nitrogen optimization, suitable sources (5

g.L-1 and 10 **g.L-1) of** beef **extract**	13

,

peptone, yeast extract; and inorganic sources like **potassium nitrate, ammonium sulphate** and sodium **nitrate**	35

(“incubated for 10 days at 40 °C”) were selected for this study. 2.7. Laccase Purification 5 Commented [o3]: Heading is shorten as per suggestion Commented [o4]: Removed full stop The best optimized conditions were used to prepare 1000 mL broth. The filtrate was centrifuged

for 15 min at 10 ° **C at “13** ,000 × g. **The** cold **supernatant**	7

thoroughly mixed 60% - 80% NH_4SO_4 saturation level was achieved (Das et al., 2001). The ground powder

was added until the protein was precipitated in the liquid broth. The further protocol followed Umar & Ahmed (2022). 2.8. Determination of Laccase Mmolecular Weight SDS-PAGE (Criterion XT,

Bio-Rad, CA, USA) gel apparatus **was used to** determine **the**	8

yield of expressed protein. Estimated Laccase's molecular weight (MW) was compared to conventional protein indicators (14.3–97.0 KDa).

A native PAGE was performed and stained with guaiacol to assign **the**	1

~67 kDa laccase.

Incubating the gel in 50 mM sodium acetate buffer (pH 5.0) with **100 mM guaiacol** allowed **the**	1

separated protein to be seen. 2.9.

Analysis of "Nitrogent-terminal Amino Acid Sequence" **The** protein **sequence was determined**	44

on the "N-terminal amino acid of laccase" band by the "Precise Protein Sequencing System" (Applied Biosystem). 2.10. Characterization of Laccase The impact

of pH on the partial purified **laccase was** evaluated **at pH** ranges **2**	3

.0–8.0 (

in 50 mM citrate phosphate buffer) and temperature **of**	21

40 °C. Laccase activity and stability was measured after every 15 min. For temperature effect on laccase activity, the protein was “incubated at optimal pH of 3.0 to 5.0”. The thermo stability of the enzyme was measured at 10 °C to 80 °C temperature range. The readings were taken every 10 °C increase in temperature. Various metal concentrations were

used to investigate **the** impact **of metal ions on** laccase **activity**	9

(Cu2+, Ca2+, Zn2+) with sulfate donor in 1, 3, 6, and 9 mM of the solutions. Aliquots of the enzymes,

50 mM citrate–phosphate buffer (pH 3.0), and	21

specific metallic ions were mixed in the right concentrations for enzymatic assays for 30 min at 40 °C. For 10 min, the laccase band (10 µg) was incubated in the above-mentioned solution, 100 mM guaiacol added and assay activity was done at 470 nm. The initial activity before incubation was used to calculate the RA %. Laccase kinetic parameters (Km and Vmax) were resoluted by guaiacol at various concentrations “1 mM, 2 mM, 3 mM, 5 mM and 10 mM in 100 mM”

of citrate–phosphate buffer (pH 3 .0). **After** 6 15 **min**	19

, a spectrophotometer was used to detect the wavelength of the enzyme in the presence of guaiacol. 2.11. Quantification of Chromium Reduction The effect of purified laccase was investigated on Cr (VI) reduction. About 0.5 µg of the partial purified laccase was homogenized in various concentration of Cr (VI) (100, 150, 200, 250, 300 “µg/mL”) in 1.5 mL Eppendorf. The inoculated tubes were

incubated for 120 **min at** 35 ° **C** with 200 **rpm** shaking. **The** cultures **were**	19

taken out of each tube

at 24 h intervals and centrifuged for 15 min at 8000 rpm 23

. The Cr (VI) concentration in the supernatant and

laccase activity was spectrophotometrically measured by guaiacol method. The 4

direct UV-vis spectrophotometer scanned at 1100 nm was used to recognize the most profound Cr(VI) absorption wavelength. The following formula (Mousavi et al., 2023) was used to calculate the Cr (VI) removal ratio (percentage): Removal ratio (%) = [(A0 − At)/A0] ×

100 where A0 is the Cr (VI) conc. of individual treatment without laccase and 4
At is the Cr (VI) concentration of each treatment measured in the

presence of laccase along the time. 2.12. Statistical Analysis The collected data from various parameters were analyzed. The vertical error bars represent the ± standard deviation (SD) less than 5% of triplicate assays. Statistical analysis was calculated by using 1-way ANOVA in SPSS18.0 software using Duncan's

LSD test at 5% level of significance. 3. Result and Discussion 3.1 26

. Molecular Phylogeny Phylogenetic analysis of the sequenced ITS region with other G. multipileum species from GenBank produced four major clades (A, B, C and D) with Tomophagus clossus as the outgroup. Two of our newly sequenced G. multipileum (MW349830 and MW349829) nexted with other Ganoderma species in clade A with 73% branch support (Fig. 2). Clade A comprised mainly of G. multipileum specie nexting with G. lucidium, G. parvulum, G. martinicense and G. destructants. No G. multipileum was found in clades B, C and D. These three clades were dominated by other 7 Ganoderma species. Fungal species in clades B and D had very high branch supports (88-99%), while clade C had 69-98% branch support. In this study, G. multipileum was collected from tropical region of Pakistan. Species of this study analyzed by sequencing, molecularly identified and their phylogenetic relatedness with Ganoderma species from the other regions were conducted. Our sequenced were closely matrixed to Nepalian and Chinese G. multipileum. Furthermore, all G. multipileum formed a close cluster in the phylogenetic analysis was an indication of close evolutionary

emergence. 3.2. Effect of Environmental Conditions on Laccase Production The screening experiment is dependent on inexpensive method. Previously, Plate-method was reported, where guaiacol utilized for laccase detection and quick visual expression. The laccase potential of G. multipileum was sorted out by using a preliminary differential screening procedure (by using appropriate growth media). The formation of brown,

intense brown and reddish brown color below **and around the fungal colony** a **positive** indicator **of guaiacol oxidation** 1

(Vantamuri et al., 2015). The development

of reddish brown color below **the fungal colony** 42

of this study was analogous to other reports for the screening of laccase producing in Ganoderma species (Kiiskinen et al.,2004) . Laccase synthesis is influenced by culture circumstances, which include differences in the kind and concentration of available nutrients that drive laccase formation (Lorenzo et al., 2002). Extracellular laccase is formed in a very small proportion by default, but this can be enhanced by improving the fermentation parameters

such as medium components, temperature, **carbon-nitrogen ratio** , and **aeration rate** 38

(An et al., 2020). Temperature is a substantial environmental aspect for exudation of laccase isozymes (Li et al ., 2016). In this work, temperature at which laccase produced at its best in G. multipileum (G113) was 30 °C (Fig. 3). At this temperature, 789 U/L ± 5.4 of the enzyme produced. The activity

decreased as the temperature rose **from** 35 ° **C to** 40 ° **C** 40

. Laccases in fungi play a function,

as phenol oxidases prefer the temperature between **30** and 55 ° **C** for **catalytic activity** 1

.

In this study, the optimal temperature for laccase activity **was** between 25 ° **C** 31

to 30 °C reported from other studies as well. In other fungal species like P. ostreatus, Cyathus bulleri, Trametes modesta, Phlebia brevispora, the optimal temperature for highest isozyme studies is 30 °C and 35 °C for T. versicolor (Snajdr and Baldrian. 2007). Ganoderma lucidum MDU-7 secreted laccase isozymes in comparable patterns at 25 and 8 30 °C, with additional isoforms with larger molecular mass seen at 35 °C, in contrast to Ganoderma sp. kk-02 (25 °C) (Kumar et al ., 2017). Temperature

is a key factor in regulating **the development of** 39

fungi, when grown **with malt extract alone** . Laccase **activities were highest in cultures of** T. versicolor **and** 27

R. vitreus (Reyes et al., 2021). The excretion of laccase experienced a slight decline at 40 °C. The fungal laccase showed greater production at pH 4-6/3.6-5.2. The “optimum pH for guaiacol (phenolic compounds) was 4.0 to 7.0” (Fig. 3). Ganoderma species secreted the maximum laccase when the pH was 5.0. The pH 5.0 was more promising than pH 3.0 and 6.0. The laccase activity was minimum at 3.0 pH in this species and optimum at 5.0 pH (Fig. 3). A reduction in activity was observed at pH 3.0 (1355.5 ± 8.8 U/L) and pH 6.0 (605 ± 3.9 U/L), respectively. The fungal laccase exhibits the highest solidity in “acidic pH (pH 4-6/3.6-5.2)”. It also acts as phenol oxidases under acidic medium (Hailei et al., 2013). The polypeptide mobility enlarged at “pH 3.0 to 5.0”(Bonomo et al., 2001), while no activity was seen at neutral pH. Laccase isozyme regulatory patterns (G. lucidum MDU-7) at pH 5.2 have recently been published, which drop sharply as pH climbed from 5.0 to 7.0 in various studies. Shrestha et al. (Shrestha et al., 2016) studied G. lucidum-CDBT1 to see maximal level of laccase secretion (92 U/mL) by adjusting the pH. Fomitopsis pinicola FP58527 SS1 secreted several

laccases and two of them (FpLcc1 and FpLcc2) were acidic at pH 3.5 for guaiacol. At pH 5.0, the activation impact is substantially stronger than pH 3.0 (Csarman et al., 2021). On the seventh day of incubation, G. multipileum produced the maximum laccase. The production level started on 4th day, but reached at peak on 7th day. The 80%

laccase production was observed on 7th day of	41

inoculum, but the activity declined as the days increased from 10 to 14 days (Fig. 3). D'Souza et al. (2004) studied four Ganoderma species and found that they have laccase activity ranging from 0.6 to 49.5 U/L. Comparatively, Trametes under submerged fermentation released higher “extracellular laccase 9000–20000 U/L” on the 7th to 14th day than Ganoderma species (Moldes et al., 2004). At the 10th day, the maximum laccase produced in the liquid medium was 0.59 U/ml. G. lucidum produced laccase in just six days (Fang et al., 2015). Wehaidya et al., 2018 observed maximum production on 7th day, however, the activity lowered in Polyporus durus ATCC 26726 at this time point. The authors suggested that reduction in 9 production may be due to nutrient depletion or proteolytic enzymes that may have caused cell digestion by autolysis. Inoculating “5 mycelial” discs of G. multipileum in shake flasks exhibited maximum activity of laccase (2598 ± 0.7 U/L). No significant difference observed in activity, when 2 and 3 fungal discs were used (Fig. 3). Also, no significant difference was observed by using three and five discs, but 5 mycelial discs was greater than the use of two discs. The highest production of laccase in G. multipileum was on 10th day at 50 rpm (Fig. 3). The secretion levels were 3204.4 ± 9.3 U/L at 50 rpm, whereas 498.5 ± 1.4 U/L and 253.3 ± 5.41 U/L at 100 and 150 rpm, respectively. G. multipileum showed more secretion of laccase at 100 rpm (Fig. 3). Higher rpm imply to improve the oxygen transport to G. lucidum mycelium in the fermenting broth. Because of the stirring situation, the highest enzyme ability in shake flask fermentation resulted in the creation of tiny pellets (Li et al., 2016). 3.3. Effects of Nutritional Variations on Laccase Yield The laccase activity at 25 and 20 (g.L-1) sucrose concentrations were 2310 ± 1.5 UL-1 and 1525 ± 1.3 U L-1, respectively. Laccase yield in G. multipileum significantly declined with maltose addition (no significant difference was observed by using

20 g.L -1 **and 25 g.L** -1 maltose **in**	1

the culture) (Fig. 4A). For the nitrogenous sources, 10 g.L-1 of the organic beef extract showed significant increase in laccase activity (Fig. 4B). The yeast extract had a greater impact on laccase production than the beef extract at 10 g.L-1. The laccase secretion was also improved by the yeast extract (5 g.L-1) (Fig. 4B).

Organic peptone did not significantly influence the laccase activity at the two tested concentrations (Fig. 4B). For the inorganic nitrogen, the laccase activity was significantly increased at 5 g.L-1 KNO3 (Fig. 4B). However, for NaNO3, the laccase activity was slightly more improved at the “10 g.L-1” concentration than “5 g.L-1”. For (NH4)2SO4, the activity was more enhanced in

5 g.L-1 than10 **g.L-1**	43

concentration (Fig. 4B). Laccase activity is also influenced by the amount and kind of carbon sources used, as well as the mushroom species (Claudia et al., 2013). Similarly, different carbon sources have been tested in some experiments, where “20 g.L-1 glucose” was effective to stimulate maximum activity (Zhang, 2012), similarly the findings of this work. 10 Furthermore, “

laccase activity is reliant **on** nature **and** concentration **of nitrogen sources in wood** decaying **fungi**	1

(Elisashvili et al., 2008). Organic nitrogen was more effectual than the inorganic sources7. Also, nitrogen found to have less effects on the enzyme activity, but it could affect the laccase yield in some fungal species (Kunamneni et al., 2007). Some authors have been suggested that low carbon-nitrogen ratio for high laccase production, while others shown the high carbon-nitrogen ratio exhibited a higher yield (Dong et al., 2005). Zhang (2012) used different nitrogen sources to evaluate the laccase from G. lucidum (Garzillo et al., 2001). This result agreed with Teerapatsakul et al., 2007 for Ganoderma species. Many natural laccase-mediators including proteins and many other factors are secreted by mushrooms in shake flasks culture (Papinutti et al., 2008). 3.4. Purification, Identification and N-terminal Sequences of Laccase Separately, a complete culture broth setup (1000 mL) was created under optimal conditions. The best concentration (80%) for laccase production was ammonium sulphate, which yielded 65% laccase.

Protein (Glacc 113) **molecular weight of** ~75. **0 kDa was estimated by SDS- PAGE (Fig. S1. A) and Native PAGE (Fig. S1. B). A brown band of** ~75. **0 kDa in a lane was stained by guaiacol, which** designated **the** laccase **of**	1

G. multipileum extract. The molecular weight (75 kDa) of Glacc113 of G. multipileum was quite similar to

other wood rotting fungal laccases. Single protein band was appeared on SDS-PAGE (Fig. S1. A), which mean Glacc113 comprised only N-glycosylation. The N-terminal “amino acid” sequence was same (GIAPTAD) exhibited closest similarity to wood rotting fungi (Table 1). Laccases ranging from 30 to 300 kDa e.g., isoforms from G. lucidum were reported to be 40 kDa to 68 kDa (D’souza et al., 1999). Kuhar and Papinutti (2014) reported isozyme in G. lucidum, while GILCCI of G. lucidum was 58 kDa (Sun et al., 2012). From literature findings, the molecular mass of laccase ranges from 34–85 kDa, 50–80 kDa (Thitinard et al., 2012), 55–90 kDa, 50–100 kDa, 40–66 kDa (Amit et al., 2017) and 38.3 kDa in Ganoderma sp (Manavalan et al.,2013). In other fungal species, the molecular mass is

45 and 90 kDa for **C. versicolor** , 61.7 **kDa** for **Mycena purpureofusca**	1

and 66 kDa in Lentinus squarrosulus (Shujing et al., 2013; Mukhopadhyay and Banerjee, 2015). The Glacc113

show 7–10% glycosylation . The **glycoproteins lose** their **activity, when**	3

carbohydrate moieties are removed, so that enzyme proteins denature first to eliminate the carbohydrates from the fungal laccase. This is impossible to estimate the deglycosylated proteins 11 activity, when

endoglycosidase H (which removes all glycosylations	3

) or

N-glycosidase F (which removes N-glycosylation	3

) applied to the Glacc113. Carbohydrate moieties of Glacc113, each moiety of protein exhibited identical band. Furthermore,

the N-terminal amino acid **sequence of** Glacc113 wad **similar to other**	3

wood rotter laccases. 3.5. Characterization of Laccase In this experiment, pH profile aided in the identification of Ganoderma species. The uppermost relative activity (%) was found in the pH of 3.0-5.0, while dropped at pH 6.0-8.0. At pH 3.0, highest laccase activity was observed under standard conditions. The purified laccase stability index was retained at pH 3.0-5.0. The relative activity of Glacc113 was 77.12% at pH 3.0 (Fig. 5A). As the pH changed from acidic to basic, the relative activity decreased. The purified laccase's stability plummeted at the pH range of 6.0-8.0. The purified laccases was analyzed at temperature range 10-80 °C to evaluate its tolerance and maximal activity after incubation for 60 min at pH 3.0 (Fig. 5B). Favorable stability temperature range of the laccase was 40–70 °C for 60 min. At 80 °C, this activity was nearly inactivated.

Laccase activity increased dramatically **from** 60 **to** 70 ° **C** , then declined **at** 70 ° **C** 1

, according to the temperature tolerance profile. Laccase has been characterized by many scientists to check its stability. Reported optimal pH for laccases in literature is 2.0–3.0 (Garzillo et al., 2001). Thermostable laccase has been reported in Pycnoporus sp., P. ostreatus and G. lucidum (Wang et al., 2010).Thermal transitions (87 and 92 °C) in laccase have been examined in Coriolus hirsutus and C. zonatus using scanning calorimetric curves (Koroleva et al.,2001). The optimum, stable and inactivated temperatures of laccase in Trametes sp. LS-10C were “40 °C, 20 °C and > 60 °C”, respectively (Li et al., 2016). The purified laccase from Ganoderma species was constant at 30 °C and retained 100% residual activity after 150 min. Sharma et al. (2013) shown the optimal temperature in Ganoderma species (purified laccase) was 50 °C. Similarly, the optimum temperature in G. lucidum was 50 °C and 70 °C, whereas uppermost laccase activity established at 25 °C (Sandana et al., 2015). 3.6. Effects of Ion Modulators on Laccase Activity The purified laccase gave 100 % relative activity with the addition of 1 mM Cu2+, also 9.0 mM CuSO4 significantly increased the laccase relative activity. The maximum laccase relative activity 12 (174.4 %) obtained in Ganoderma Glacc113 at 9 mM CuSO4 (Fig. 5C). All the selected concentrations given 100% relative activities. The highest concentration of Ca2+ (9 mM) had a pronounced effect on Glacc113. The RA increased abruptly from “1 mM to 9 mM”. All the selected concentrations of Ca2+ exerted more than 100% positive effect on this species (Fig. 5C). For Zn2+modulators, 9.0 mM ZnSO4 considerably increased (136 %) the laccase RA, whereas sharply decreased at 6 mM to 1 mM (Fig. 5C). Metallic ions regulate the manifestation of laccase in fungi and mushrooms. The tolerance of fungi to metal ions in laccase expression is an outstanding property. Murugesan et al., 2009 explained the effects of some metal ions in laccase expression as a major obstacle for practical application in biotechnology industries. Effect of metal ions' has two research thoughts.

According to Okamoto et al.,2000, metallic ions induce changes in enzyme. Liu et al.,2020 found that

adding 12.5 mg.L-1 Cu2+ to a G. lucidum containing medium provided the most **laccase stimulation and increased laccase activity by**	11

1.6 times. The highest

laccase activity from P. ostreatus in basal media	15

with and without seven different metal ions e.g., Cu2+ (Media 1), Mn2+ (Media 2), Cu2+ and Mn2+ (Media 3), Fe2+ (Media 4), Mn2+ and Fe2+ (Media 5), Fe2+ and Cu2+ (Media 6), and Cu2+, Mn2+, Fe2+ (Media 7) were increased

approximately 21.5-fold, 4.7-fold, 14.9-fold, 16.9-fold, 4.0- fold, 11.0-fold, 12.7-fold, and 24.8-fold higher	15

(An et al., 2020). Peniophora lycii a white-rot basidiomycete fungus was studied for laccase synthesis under copper induction (Glazunova et al., 2020). Laccase from T. hirsuta exhibited specific activity was 978.34 U/mg. At 4–6 pH and temperatures ranging from 20–40 °C, the laccase remained stable for 16 h. Except for Fe2+ and Hg2+, the isolated enzyme displayed substantial stability for 10 metal ions (10 mM). Laccase activity was up to 142% greater in Cu2+-treated cells than in control cells. 3.7. Kinetic Studies The kinetic characteristics of the Glacc113 were evaluated by using guaiacol to assess the effect of substrate concentration on "laccase activity" (470 nm). Guaiacol concentration range was 1 mM, 2 mM, 3mM, 5mM

and 10 .0 **mM in 100** .00 **mM citrate–phosphate buffer (pH 3.0**	1

). Km value is different form laccase to laccase. Lineweaver-Burk plot values were generated after adjusting kinetic information (data) to hyperbolae of Michaelis Menten's equation. The effect of 13 substrate on laccase

activity, and the Km 1.4617 **mM and Vmax 1** .817 mM **min** 6

-1 of G. multipileum is presented in figure 6. The Km was 400 ± 60 µM and Kcat was 80.20 ± 1.59/s for guaiacol (Navada and Kulal, 2021). As the temperature lowered from 28 to 4 °C or increased upto 40 °C in Cerrena unicolor, the increasing quantities of copper and manganese in the medium induced the biggest change in laccase gene expression, and three laccase transcripts were considerably affected (Pawlik et al., 2021). P. ostreatus LAC-Yang1 demonstrated a high resilience to severely acidic conditions and a high level of

stability under strongly **alkaline conditions (pH 9–12**). This **LAC-Yang1 also** shown **a** high resistance **to inhibitors (EDTA, SDS), metal ions (Mn2+, Cu2+, Mg2+, Na+, K+, Zn2+, Al3+ , Co2+), and metal ion mixtures** 2

(Liu et al., 2021). The Km (mM) values of purified laccase of Pleurotus sajor-caju, P. ostreatus, P. ostreatus POXA1, T. trogii POXL3,G. lucidum, G. lucidum GaLc3 (pH 5.0) were 2.50, 0.28, 0.09, 0.03, 0.107 and 0.037, respectively (Soden et al., 2002). Pycnoporous cinnabarius (pH 4.0) used guaiacol for Km value and T. hirsutus Km value was 10.9 µM10. The highest Km of laccase was 0.107 mM from G. lucidum (Zinnai et al., 2013). Production of an extracellular

laccase from Phoma herbarum KU4 was reported 23

in submerged fermentation (1590 U/mL). The

Km, Vmax and Kcat of laccase was **0.216 mM, 270.27 U/mg and 506.69 s−1, respectively** 2

(Debnath et al., 2021). 3.8. Percentage Inhibition of Cr Concentration by Purified Glacc13 In the environment,

chromium is found in two oxidation states: Cr(III) and Cr(VI). The “Cr(VI)” **is highly** 24

carcinogenic, toxic, and mutagenic (Hamilton et al., 2018). This is widely use in multiple applications e.g., metal plating and tanneries. The Cr(VI) is an alarming contaminant of the environment (Peng et al., 2018). Chromium enter in the environment

by weathering of Cr- containing rocks 16

, leaching of soils, and direct ejection from industrial processes. Under all pH values of water,

the forms of Cr(VI) chromate and dichromate are very **soluble** 16

.

The concentration of Cr in soils differ **according to** 16

sediments, rocks and increase through anthropogenic deposition (

Kimbrough et al., 1999). Chromium **in soil** presents **a** mixture **of both Cr(III) and (VI). In aquatic environment, soil or sediment** , this **Cr undergoes a variety of transformations** 10

(Kimbrough et al., 1999). 14 The MnO2 and dissolved oxygen (

oxidants) present in the "soil can oxidize Cr(III) to Cr(VI 28

) (Wang et al., 2010).

Cr(VI) is **more** movable **in soil and** has a **higher** environmental **toxicity** 16

. Laccase of G. multipileum effectively eliminated (>94%) the chromium concentration (“100 µg/mL”). The eradication of Cr (VI) decreased from 72.18% to 14.46% as Cr (III) concentration increased from 150 µg/mL to 300 “µg/mL”. At lower Cr (VI) concentrations of 100.00, 150.00, and 200.00 g/mL, a full decrease of Cr (VI) was seen for 20, 40, and 80 h, respectively (Fig. 7). As the concentration of Cr (III) increased, the time taken was maximum for Cr (VI) to reduce completely. After 120 h of incubation, G. multipileum was able to completely decreased the Cr (VI) at a concentration of 250 g/mL (82.3 percent) (Fig. 7). In this study, laccase of G. multipileum effectively eliminated the Cr (VI) at 100 µg/mL. Increased Cr (VI) concentration, the effectiveness of Glacc113 reduced at 150 µg/mL to 300 µg/mL. The maximum Cr (VI) alter the physiological reactions and metabolic activities as well as reducing the growth of the living organism. Cr (VI) is toxic and mutagenic at 100

µg/mL concentration (**Liu et al** ., 2020). Oves **et al** .,2013 **reported that** 32

different Cr (VI) concentration could affect the growth of microbes. Trametes hirsuta TH315 eliminated the Cr (VI) (>96%) at 0.5 and 1 mM (Liu et al., 2020). The removal ratio decreased from 76.78% to 16.56% at 2 to 5 mM concentration of Cr (VI), respectively (Baldrian. 2003). The heavy metals toxicity directly or indirectly influenced the growth

and cellular components by **the generation of free radicals** 4

. The study has identified the conditions that enhance the optimal production of laccase in G. multipileum and also the purified laccase by N-terminal amino acid sequences. G. multipileum has been successfully identified by using ITS markers. “The laccase produced in this work has interesting characteristics like thermo stability at higher temperature and acidic pH also with ability to reduce the toxic level of chromium”. Conclusion and Future Prospects 15 During this investigation, a new species of Ganoderma multipileum was discovered, and its laccase was found to effectively remediate chromium, a hazardous agent. This discovery opens up a new avenue for industrial and biotechnological applications. However, this study is limited to the lab due to a lack of resources. Therefore, researchers worldwide are focusing on 'greener' technology- based concepts. The laccase found in this study can be used in advanced biotechnical tasks like the "Fungal Fuel Cell" due to the limited availability of organic reservoirs of bioenergy, biofuel, and bio products. Furthermore, the findings of this study can determine the life cycle analysis of various pretreatment strategies for power generation. ‘Life cycle assessment (LCA)’ aims to understand the environmental performance of bio

production from lignocellulosic feedstock. This analysis includes the use of raw materials, treatment processes, purification steps, energy consumption and generation rates, and any waste produced during production. The pretreatment of biomass in the life cycle analysis of biofuel production is given less emphasis than other stages. While a generalized comparison of various fungal production strategies may not be conclusive, there are global efforts to develop economically viable methods for commercial biofuel synthesis through innovative technological advances. To obtain practical and validated results, it is necessary to focus on optimizing and analyzing a single type of feedstock. The life cycle analysis of multiple production strategies, developed over several years of research, can often assist in creating sustainable and feasible synthesis pathways. Table 1. N-terminal amino acid sequences of laccse of Glacc113 and some other wood rotting fungi Sr No. Wood Rotting Fungi N-terminal amino acid sequences References 1 Ganoderma multipileum GIAPTAD This work Ganoderma lucidum GIGPT Ko et al., 2001 2 Trametes versicolor 951022 GIGPVAD Han et al., 2005 3 Trametes versicolor ATCC 20869 laccase II GIGPVAD Bourbonnais et al., 1995 4 Trametes versicolor ATCC 20869 laccase I AIGPVAS Bourbonnais et al., 1995 5 Trametes villosa I AIGPVAD Yaver et al., 1996 16 454 455 456 6 Basidiomycete PMI SIGPVAD Han et al., 2005 7 Phlebia radiata SlGPVTD Saloheimo et al., 1991 8 Coriolus hirsutus GICTKAN Shin and Lee, 2000 9 Pleurotus ostreatus POXAI AlGPTGD Palmieri et al., 1997 10 Phellinus ribis AlVSTPL Min et al., 2001 11 Agaricus bisporus DTXKTFN Perry et al., 1993 12 Ceriporiopsis subvermispora AIGPVTD Fukushima and Kirk, 1995 13 Pycnoporus cinnabarinus AIGPVAD Eggert et al., 1996 14 Coriolus hirsutus AIGPTAD 131 17 B C 457 458 459 Figure 1. Pictures showing Ganoderma multipileum (CM10): A. Basidiome, B. Pure culture and 460 C. Guaiacol plate medium (bottom view) (Photos taken by Aisha Umar). 461 18 A B C D Outgroup 462 19 463 464 Figure 2. Phylogenetic tree of G. multipileum (CM10, CM101)

and related species based on ITS 465 **sequences generated by maximum likelihood** 1 **method in MEGA 10** .0. Tomophagus colossus **was** 466 **chosen as** the **outgroup. Bootstrap values (>50%) are shown at the branches (Constructed by** 467 **Aisha Umar**). a a **b**

b b c A a pH a **B** Temperature **a a b b C** 17

No. of days D No. of discs a b c E Agitation speed 468 20 469 Figure 3. Optimization of culture growth conditions (A. pH; B. Temperature; C. No. of Days; D. 470 No. of Fungal Growth Discs; E. rpm= revolutions per min) for maximum laccase production

ab a **ccbAab** a **a** 33 **Babcc** d **c**	17

bb 4)2 4 Figure 4. Optimization of nutritional conditions for laccase production: A-Organic carbon sources and B-organic and inorganic nitrogen sources. 21

ABaa cc bc **aababa**	30

1 3 6 9 477 22 C Concentration (mM) 478 Figure 5. Determination of

the effect of environmental parameters **on the activity of the purified**	37

479 laccase, Glacc113: A-Effect

of pH, B-Effect of temperature	19

, and C-Effect of metallic ions. 480 481 482 483 484 1/[V] mM/min -1 -0.5 1.6 y = 0.8044x + 0.5503 1.4 1.2 1 0.8 0.6 0.4 0.2 0 -0.2 0 0.5 1 1.5 1/[S]

mM Figure 6. **The Lineweaver-Burk plot of purified** "Glacc113" **of G**	18

. multipileum Cr µg/ml % Inhibiton Cr Concentration (µg/mL) hours 60 70 80 40 20 Figure 7. Removal of Cr(VI) concertation by Glacc113 23 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100 101 102 103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120 121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136 137 138 139 140 141 142 143 144 145 146 147 148 149 150 151 152 153 154 155 156 157 158 159 160 161 162 163 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 179 180 181 182 183 184 185 186 187 188 189 190 191 192 193 194 195 196 197 198 199 200 201 202 203 204 205 206 207 208 209 210 211 212 213 214 215 216 217 218 219 220 221 222

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