# Study the role of bacterial laccase enzyme for detoxification of residual organic pollutants in pulp and paper mill wastewater after secondary treatment

**SUMMARY OF**

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**Summary**

A recent study revealed that complex organic pollutants are retained as residual organic pollutants in discharged pulp and paper mill wastewater, even after secondary treatment. Pulp and paper mill wastewater is a threat to environment for its safe disposal due to presence of residual organic pollutants, cellulose, hemicellulose, lignin, pectin, carbohydrate, extractives resins, fatty acids, terpene alcohols, phenolic compounds. The most critical reaction in the bleaching stage is chlorine oxidation, and chlorinated organic compounds or adsorbable organic halides are the main problems with the wastewater materials. The toxic effects of these by-products on the environment were analyzed in the wastewater. Various studies have documented the toxic/lethal effects on daphnia, shrimp, and planktons in the water bodies receiving wastewaters of the pulp and paper industry. Lignin is a major colourant of pulp and paper mill wastewater. It has also been reported that lignin has net negative charges; therefore, various heavy metals strongly binds with lignin to form large organo-metallic complex molecules. Among the different physicochemical and biological processes available for the treatment of pulp and paper mill wastewater. The wastewater after secondary treatment becomes dark brown colour due to complexation of lignin along with organic and inorganic residual organic pollutants present in pulp paper mill wastewater. The discharged wastewater after secondary treatment causes environmental problems in the aquatic and terrestrial ecosystem which ultimately affect aquatic flora and fauna. Therefore, adequate treatment of the pulp and paper mill wastewater is warranted prior to its safe discharge into environment. Hence, the detailed chemical properties of various pollutants present in pulp paper mill wastewater before and after secondary treatment have not yet to be elucidated in detail.

The above information has been systematically elaborated in the first chapter of the thesis as introduction. Subsequently, the second chapter has mentioned objectives of the thesis. Further, chapter three is the review of literature on the topic that has been elaborated pulp and paper mill wastewater treatment through the ligninolytic enzyme such as laccase. Ligninolytic enzymes play a key role in the degradation and detoxification of lignocellulosic waste in environment. The major ligninolytic enzymes are laccase, lignin peroxidase, manganese peroxidase, and versatile peroxidase. The structurally laccase is isoenzymes with monomeric or dimeric and

glycosylation levels (10–45 %). This contains four copper ions of three different types. The enzyme catalyzes the overall reaction: 4 benzenediol + $O_2$ to 4 benzosemiquinone + $2H_2O$. While, lignin peroxidase is a glycoprotein molecular mass of 38–46 kDa containing one mole of iron protoporphyrin IX per one mol of protein, catalyzes the $H_2O_2$ dependent oxidative depolymerization of lignin. The manganese peroxidase is a glycosylated heme protein with molecular mass of 40–50kDa. It depolymerizes the lignin molecule in the presence of manganese ions. The versatile peroxidase has broad range substrate sharing typical features of the manganese and lignin peroxidase families. Although laccase enzyme has broad range of industrial applications especially the degradation and detoxification of lignocellulosic waste discharged from various industrial activities, its large scale application is still limited due to lack of limited production. Further, the extremophilic properties of laccase enzyme indicated their broad prospects in varied environmental conditions.

Chapter four has mentioned the physico-chemical analysis and identification of various residual organic pollutants from the bleached and unbleached paper mill wastewater and both sources showed endocrine-disrupting compounds. The result has revealed that bleached paper mill wastewater showed high BOD (225.0 ± 2.24), COD (543.04 ± 1.22), TS (3280.0 ± 1.32), DS (3110.0 ± 2.42), fixed solid (2900.0 ± 2.35), chloride (2350.0 ± 1.14), total phenol (13.195 ± 0.52), sulfate (713.1 ± 1.11), nitrate (210.08 ± 2.32), phosphate (2.56 ± 0.12) and lignin (578 ± 0.13 mg $L^{-1}$) levels with alkaline pH (7.68 ± 0.21), and dark brown colour. In addition, several major heavy metals were detected, including Fe (1.8598 ± 0.90), Pb (0.2550 ± 0.12), Cr (0.3058 ± 0.01), Cu (1.3814 ± 0.16), Cd (0.08632 ± 0.13) and Zn (0.0945 ± 1.21 mg L−1). While the result has revealed that unbleached paper mill wastewater showed high BOD (112.0 ± 1.14), COD (413.5 ± 0.81), TS (698.0 ± 2.34), DS (584.0 ± 1.82), fixed solid (566.0 ± 1.12), chloride (1740.0 ± 1.10), total phenol (11.691 ± 0.82), sulfate (316.71 ± 0.81), nitrate (47.17 ± 1.10), phosphate (1.1 ± 0.20) and lignin (285 ± 0.20 mg $L^{-1}$) levels with alkaline pH (7.95 ± 0.16), and dark brown colour. In addition, several major heavy metals were detected, including Fe (0.4232 ± 1.02), Pb (0.1360 ± 0.42), Cr (0.0834 ± 0.11), Cu (0.8971 ± 0.20), Cd (0.219 ± 0.10) and Zn (0.0408 ± 0.82 mg $L^{-1}$). The GC-MS analysis of bleached paper mill wastewater revealed the presence of toxic residual organic pollutants i.e. Silanol, trimethyl-, triester with boric

acid ($H_3BO_3$) (CAS); D-LACTIC ACID-DITMS; Dodecane, 1-iodo; Decane, 1-iodo; Tetracosane; Heptacosane; Pentan-1,3 dioldiisobutyrate, 2,2,4- trimethyl; Octadecane, 1-iodo; Heneicosane; Hexadecanoic acid, trimethylsilyl ester; Eicosane; Diethyl 3,4-dihydro-2-naphthyl-phosphonate; Hexadecane, 2,6,10,14-tetramethyl; 1,2,3,4,5-Pentaisopropylbis(cyclopentadienyl) cobalticimium (Cobaltocene); Methyl ester of [1'R-[1'à,4'á,8'à(E),8a'á]]-3-(8'-ethyl-1,2,2',3',6',7',8,8a'- octahydro-1 -methyl-2-oxospiro[3H-indole-3,1'(5'H)-inndolizin-8'-yl)-2-propenoic acid N-oxide; 2-(1-Methyl-1H-2-pyrrolyl)quinolone; Cyclotrisiloxane, hexamethyl; 5,11,17,23-Tetra-t-butyl-25,26,27,28-tetrahydroxycalix-4-arene and a-Fluoro-(p-methyl)chalcone. While, unbleached paper mill wastewater i.e. 2-Phenyl-N-propyl-4-quinazolinamine, Sulfurous acid, 2-ethylhexyl hexyl ester, 7,8-Dimethyl-4 trifluoromethyl-(1H,5)benzodiazepine, Nonadecane, Dodecane, 1-iodo, 3,6-Dioxa-2,7-disilaoctane, Tricosane, 7,7-diphenyl-3,5-dioxo-7-hydroxyheptanenitrile, Tetradecane, Eicosane, Hexadecanoic acid, trimethylsilyl ester, Octadecanoic acid, trimethylsilyl ester, 4-(Chloromethyl)-3-methyl-5-phenylisoxazole, N(2)-[2,5-di(t-butyl)phenyl]-N(1)-ethyl-3,4:9,10- perylenetetracarboxydiimide, and (3R)-3-Phenyl-2,3-dihydro-1H-isoindol-1-one. The toxicity test with *Phaseolus aureus* seed germination showed inhibition of seed germination and alpha-amylase activity >25 % in bleached and unbleached paper mill wastewater. The LC50 of *Tubifex tubifex* was noted of >50 % after 48 hours incubation test. This revealed that the wastewater discharged from bleached paper mills is more toxic than unbleached paper mill waste this might be due to the use of more chemicals during bleaching and pulping process.

Chapter five of the thesis has mentioned the isolation, screening and identification of laccase producing bacteria from pulp paper mill sludge samples. The 26 isolated bacterial strains were purified on the MSM agar plate by the streak plate technique. All bacterial strains were screened for laccase production on modified B and K agar medium containing 5 mM guaiacol. The three bacterial strains (i.e. BL1, BL6 and BL9) demonstrated laccase activity. The study revealed that the identified potential laccase-producing bacterial strains *Bacillus* sp. AKRC01, *Bacillus aquimaris* AKRC02 and *Bacillus cereus* AKRC03.

Based on the potentiality of isolated laccase producing bacterial strains were optimized environmental and nutritional conditions which constitute chapter six of my thesis. The result revealed that screening of suitable agro residues waste (potato peel,

banana peel, sawdust, pea peel, wheat bran, orange peel, and rice bran) for maximum laccase production. Among these, rice bran supported the maximum laccase production. The *Bacillus* sp. AKRC01 were under submerged fermentation state using rice bran as the substrate. The optimized environmental conditions such as incubation period 96 hours, temperature 35 $^0$C and pH 8.0 were obtained maximum enzyme production 3.832 U/mL. In addition, optimized the carbon and nitrogen sources such as 2.0 % glucose (4.967 U/mL) and 1.0 % peptone (6.236 U/mL) resulted in maximum enzyme production with rice bran as an effective agro-waste substrate. While, *Bacillus aquimaris* AKRC02 maximum laccase production optimized environmental conditions (incubation time 120 h; 4.58 U/mL), 35 $^0$C; 6.624 U/mL) and pH 7.0; 10.142 U/mL) and nutritional sources (glucose 1.0 %; 14.164 U/mL and peptone 0.5 %; 18.124 U/mL) significantly enhanced the laccase production.

As per the objective, the purified laccase using culture supernatant was collected by centrifugation of 24 h grown culture of *Bacillus* sp. AKRC01 and *Bacillus aquimaris* AKRC02 at 5000rpm for 20 min. The elution profile of purified laccase showed a well-resolved single peak of enzyme activity. Approximately 38.08-fold purification was achieved with the specific activity of 228.34 U/mg Extracellular laccase was purified from a culture of *Bacillus aquimaris* AKRC02 in optimized conditions for enzyme production. The laccase enzyme was purified from bacterial culture and determined molecular weight by denaturing 10 % SDS-PAGE performed. The molecular weight of laccase produced by *Bacillus* sp. AKRC01 in the presence of rice bran was found to be 61 kDa and *Bacillus aquimaris* AKRC02 was determined to be 65 kDa. Thermo stability of purified laccase was incubated in various temperature ranges (25, 35, 45, 55, 65, 75, and 85 $^0$C) for different time intervals (0, 2, 4, 6, 8, 10, 12, 14, and 16 h) to determine the thermal stability. The residual laccase activity was calculated by evaluating the oxidation of guaiacol at 100 mM acetate buffer pH 5.0 at 465 nm. Purified laccases of *Bacillus aquimaris* AKRC02 were stable at 45 $^0$C for 8 h with an enzyme activity loss of just 50 % in comparison to the enzyme activity at the optimum temperature. About 28 % of its activity was maintained by the enzyme even after 16 h at 45 $^0$C. The half-life of laccases at higher temperatures of 55 and 65 $^0$C was approximately 6 h. The activity of the enzyme dramatically decreased and displayed about 90 % loss of activity at a higher temperature of 85 $^0$C after 16 h. At 25 and 35 $^0$C, the laccase was very stable with a minimum loss of 5 % in its activity,

even after 16 h. After 16 h, there was approximately 76, 84, 88 % loss in enzyme activity at 55, 84, 88 $^0$C, respectively entailing the enzyme stability at the optimal temperature (35 $^0$C).

Chapter eight has described Study the effect of the copper (Cu) and iron (Fe) nanoparticles on the production of bacterial laccase. The nanoparticles were synthesised and characterised through XRD, XPS, SEM, TEM and FTIR analysis. After-effects of laccase production were observed in different concentration NPs and immobilized laccase with magnetic nanoparticles. Under mild conditions, an effective technique for improving catalytic activity and stability of immobilised laccase via condensation reaction with magnetically separable and reusable magnetite-supported copper (nanocat- Cu/FeO) nanoparticles. Immobilizing a *Bacillus aquimaris* AKRC02 laccase in magnetic multiwalled carbon nanotubes (Cu/FeO), an excellent substrate for immobilising enzymes, resulted in a new magnetically separable laccase immobilised system. According to our findings, Cu/FeO nanoparticles had a high loading amount of bovine serum albumin (BSA, 436 mg/g support) and laccase activity recovery of 86.45 % after immobilisation. It should be noted that the immobilised laccase's temperature and pH stabilities, as well as tolerances to organic solvents, were significantly higher than those of the free laccase. Furthermore, after 10 reuses, the immobilised laccase maintained 54 % of its original activity. After 16 days, immobilised laccase had 59 % storage stability. The use of an immobilised system to catalyse the phenolic lignin model compounds syringic acid and guaiacol was studied. Because of the high catalytic effectiveness for guaiacol, using immobilised laccase to catalyse lignin is a very promising method for further application in the wood industries.

Further, as per the objective of the study, the laccase producing bacterial degradation of PPMW. The physic-chemical analysis and identification of various residual organic pollutants were present in PMMW before and after secondary treatment. The result has revealed that i.e. (mg/L$^{-1}$) i.e. pH (8.1 ± 0.12), Total solid (1946.0 ± 1.02), Dissolved Solid (1784.0 ± 1.24), COD (752.0 ± 0.78), BOD (380.0 ± 0.45), Lignin (416.0 ± 5.12), phosphate (34.0 ± 0.46), nitrate (695.5 ± 1.05), total phenol (29 ± 0.95) and heavy metals i.e. Fe (5.6982 ± 1.02), Ni (0.956 ± 0.58) and Zn (3.2346 ± 0.89). However, a sharp reduction pH (7.64 ± 0.11), Total solid (314.0 ± 1.12), Dissolved Solid (652.0 ± 2.15), COD (186.0 ± 0.16), BOD (52.0 ± 0.58),

Lignin (30.0 ± 1.02), phosphate (14.5 ± 0.21), nitrate (112.75 ± 0.12), total phenol (6.27 ± 0.00) and heavy metals i.e. Fe (3. 1462 ± 1.00), Ni (0.6217 ± 0.91) and Zn (1.1462 ± 0.98) was noted. The main focus of this work is to study the potential laccase producing bacterium *Bacillus cereus* AKRC03 (accession no. MN720581.1) for biodegradation and toxicity reduction from pulp paper mill wastewater. The laccase producing *Bacillus cereus* AKRC03 exhibited up to 78.67 % of decolorization and degradation capability for hazardous residual organic pollutants at different nutritional (glucose: 1.0 %, peptone: 0.5 %) and environmental conditions (pH: 7.0, temperature: 37 $^{0}$C, agitation: 180 rpm, incubation period: 120 h). The absorption peak of the UV-Vis spectral scan identified the decolorization and degradation pattern for pollutants present in wastewater during treatment. Furthermore, the transformation of major residual organic pollutants was exhibited through GC-MS analysis. These were 1-dotriacontanol, 1-heptadecanol, tricosane, 1-(2-hydroxyethoxy) tridecane, and n-[(methylphenyl) methylene]-2-methyl-2-propanamine n-oxide. While silane, (dodecyloxy)trimethyl, (1á,3á,4à)-3,4-bis[dimethyl(4-methylphenyl)silyl]cyclo pentan-1-yl acetate, (2S,3R)-(3-tetradecyloxiranyl)methanol, hexadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester, and 2,4,6-tri[4,5-(methylenedioxy)phenyl]-s-triazine appeared as metabolic product after degradation of wastewater. The reduction of toxicity was measured up to 70 % for treated wastewater with *Phaseolus mungo* L seeds and *Tubifex tubifex* worms. From these findings, it is concluded that the isolated bacterium may be used in the bioaugmentation process for the further detoxification and degradation of discharged pulp paper mill wastewater for environmental safety.

**Conclusion**

The study concluded that pulp and paper mill wastewater after secondary treatment contains various residual organic pollutants which showed aquatic and terrestrial toxicity such as *Phaseolus aureus, Phaseolus mungo* L seeds and *Tubifex tubifex.* The isolate laccase producing *Bacillus* sp. AKRC01, *Bacillus aquimaris* AKRC02 and *Bacillus cereus* AKRC03 from pulp and paper mill sludge samples. Extracellular laccase was purified from a culture of *Bacillus* sp. AKRC01 and *Bacillus aquimaris* AKRC02 in optimized environmental and nutritional conditions for enzyme production. The molecular weight of extracellular purified laccase produced by *Bacillus* sp. AKRC01 in the presence of rice bran was found to be 61 kDa and

*Bacillus aquimaris* AKRC02 was found to be 65 kDa through SDS-PAGE. In addition, the effects of laccase production were observed in different concentrations of copper (Cu) and iron (Fe) NPs and immobilized laccase with magnetic nanoparticles. Under mild conditions, an effective technique for improving catalytic activity and stability of immobilised laccase via condensation reaction with magnetically separable and reusable magnetite-supported copper Cu/FeO nanoparticles. It should be noted that the immobilised laccase's temperature and pH stabilities, as well as tolerances to organic solvents, were significantly higher than those of the free laccase. Furthermore, after 10 reuses, the immobilised laccase maintained 54 % of its original activity. Therefore, the laccase producing *Bacillus cereus* AKRC03 exhibited up to 78.67 % of decolorization and degradation capability for hazardous residual organic pollutants at different nutritional and environmental conditions. The transformation of major residual organic pollutants was exhibited through GC-MS analysis such as 1-dotriacontanol, 1-heptadecanol, tricosane, 1-(2-hydroxyethoxy) tridecane, and n-[(methylphenyl) methylene]-2-methyl-2-propanamine n-oxide. While silane, (dodecyloxy)trimethyl, (1á,3á,4à)-3,4-bis[dimethyl(4-methylphenyl)silyl]cyclo pentan-1-yl acetate, appeared as metabolic product after degradation of wastewater. The reduction of toxicity was measured up to 70 % for treated wastewater with *Phaseolus mungo* L seeds and *Tubifex tubifex* worms.

Based on work, nine original research papers have been published in high impact journals and three research papers are under consideration. While five conference papers were presented at national and international conferences. Besides, three book chapters are published in firm of reputed publishers such as Elsevier and Springer.