Universal Journal of Environmental Research and Technology
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Available Online at: www.environmentaljournal.org
Volume 2, Issue 1: 58-64

Open Access **Research Article**

Bacterial Lignin Peroxidase: A Tool for Biobleaching and Biodegradation of Industrial Effluents

Bholay A. D., Borkhataria Bhavna V., Jadhav Priyanka U., Palekar Kaveri S., Dhalkari Mayuri V., P. M. Nalawade*

Department of Microbiology, K.T.H.M. College, Nashik, M.S, India
* Department of Environmental Sciences, K.T.H.M. College, Nashik, M.S, India

Corresponding author: adbholay@gmail.com

Abstract:

Lignin, the nature's plastic is the major pollutant from paper-pulp mill effluent due to its intense unaesthetic brown color, hydrophobicity and poor mechanical properties, tends to be a recalcitrant compound. Textile dye-based industries release colored effluents due to presence of large amount of mixture of dyes which is also hazardous. Microbial extracellular lignin peroxidase enzymes have a potential to degrade lignin and a wide range of complex aromatic dyestuffs. From various environmental niches eleven isolates were screened for lignolytic activity, out of which two bacterial isolates *Pseudomonas aeruginosa* and *Serratia marcescens* were able to decolorize 44% to 49% of lignin. The studies on biobleaching of paper-pulp mill effluent gave 60% to 75% color reduction and in case of textile dye- based effluent 50% to 58% decolourization was observed. The heterogeneous combination of lignin peroxidases from mixed consortia gave 80% to 85% color reduction in treatment of paper-pulp mill effluent and 70% to 75% decolourization in treatment of textile dye-based effluent which is significantly high. This system of lignin peroxidase may be efficiently used in biobleaching and biodegradation of effluents from respective industries.

Keywords: Lignin Peroxidase, Decolourization, Paper-pulp mill effluent, Textile dye-based effluent.

1.0 Introduction:

The lignocellulosic material of plants consists of three main components, namely cellulose, hemicellulose and lignin. After cellulose, lignin is the second most abundant renewable biopolymer in nature. It is the most abundant aromatic polymer in the biosphere. It is an essential part of the plant cell wall, imparting rigidity and protecting the easily degradable cellulose from attack by pathogens. (Prasongsuk S. *et al* 2009) Due to its complicated structure and nonhydrolysable bonds, lignin is more difficult to break down than cellulose or hemicellulose. Lignin surrounds cellulose in the plant cell wall forming a matrix, which itself is resistant to degradation. (Donna C. R. *et al* 2000) Lignin biodegradation is responsible for much of the natural destruction of wood in use, and it may have an important role in plant pathogenesis. On the other hand, potential applications utilizing lignin-degrading organisms and their enzymes have become attractive, because they may provide environmental friendly technologies for the paper-pulp and various other industries. (Cullen D. *et al* 2011).

In biosphere, a wide variety of species are involved in lignin degradation including fungi and bacteria. To date, only a few groups of organisms are capable of degrading complex lignin polymers, and they are best exemplified by the white rot fungi and others such as *Phanerochaete chrysosporium, Streptomyces viridosporus, Pleurotus eryngii, Trametes trogii, Fusarium proliferatem, Agaricus, Erwenia, Copricus, Mycema, Sterium.* (Martin A. 1977) Till now, all the basics and applied research work has centered mostly on fungi. In case of industrial applications, the use of fungi is not feasible due to the structural hindrance caused by fungal filaments, requirement of particular culture conditions such as humidity, aeration, temperature and pH which are not compatible with industrial processing environments, requirement of long lag period and thus degrade lignin very slowly, additional food source to support the fungal growth like glucose and nitrogen, stability of fungi is not good in practical treatment under extreme environmental and substrate conditions, such as higher pH, oxygen limitation, and high lignin concentrations. (Crawford D. L. *et al* 2004). Bacteria are worthy of being studied for their lignolytic potential due to their immense environmental adaptability and biochemical versatility. There is wide range of examples where bacteria like *Pseudomonas aeruginosa, Serretia marcescens, Nocardia, Arthrobacter, Flavobacterium, Micrococcus, Xanthomonas* have been identified as

lignocellulosic-degrading microorganisms. (Kalyani D. C. *et al* 2008) These include various environmental niches like compost soil, rhizosphere soil, cow dung, lake water containing decomposing plant material, decaying bark and also termite gut where bacteria may be present involved in lignocellulosic degradation as symbionts. (Yang J. *et al* 2006)

Therefore, identification of bacteria having lignin oxidizing enzymes would be of significant importance. (Timonthy D. H. *et al* 2011) The lignin peroxidase activity is associated with primary growth of bacteria and thus the delignification process is presumed to be the result of primary metabolic activity and not dependent upon other factors such as stress to induce production. (Crawford D. L. *et al* 2004) Thus, degradation of lignin by bacteria confers a new understanding that may be of tremendous industrial significance. Some of the industries that discharge highly colored effluents are paper and pulp mills, textiles and dye-making industries, alcohol distilleries and leather industries. Each of these industrial effluents creates some specific problem besides producing aesthetically unacceptable intense coloring of soil and water bodies. (Jin X. C. *et al* 2007) Moreover they lead to anaerobic conditions in aquatic ecosystems due to blockage of light to lower depths and cessation of photosynthesis. This results into death of aquatic life thereby producing foul smelling toxic waters. Oxidoreductive enzymes play an important role in degradation and transformation of polymeric substances. The partially degraded or oxidized products can easily be taken up by microbial cells where they are completely mineralized. (Duran *et al* 2000).

Lignin-degrading enzymes are one such group of oxidoreductive enzymes, which have practical application in bioremediation of polluted environment. (Castillo M. P. *et al* 1997) Lignin degrading enzymes- Lignin Peroxidase enzyme (LiP) and Manganese-dependent Lignin Peroxidase enzyme (MnP) both are essentially extracellular in nature due to the large and complex structure of lignin which cannot enter the cell for intracellular action. It has been shown that LiP attacks both phenolic and nonphenolic aromatic residues, and the latter give rise to cation radicals that fragment spontaneously. MnP catalyzes the oxidation of Mn(II) to Mn(III) which in turn can oxidize phenolic substrates. For the peroxidase activity of both the enzyme the presence of extracellular hydrogen peroxide is essential. (Archital F. S. *et al* 1992) Lignin peroxidases are useful in the treatment of colored industrial effluents and other xenobiotics as it has bioremediation potential to decolourize the effluents. (Buzzini A. P. *et al* 2006) For colored effluents, bioremediation is measured by estimating the reduction in color units of effluents.

1.1 Aim and Objectives:

- Isolation, characterization and acclimatization of lignin degrading bacteria.
- Check for lignolytic activity and enzyme assays for lignin peroxidase and manganese dependent lignin peroxidase enzymes.
- Determination of extend of lignin degradation by estimating the lignin degrading products (phenols).
- Application of bacterial lignin peroxidases in biobleaching of coloured industrial effluents from paper pulp industry and textile dye based industry.

2.0 Materials and Methods:

2.1 Alkali Lignin Preparation: The sources used for extraction of lignin were dried bark grinded to powder, dried straw grass and newsprint paper as depicted in *figure 1* below.

Figure 1: Powdered Lignin source

In 100 gm of lignin source 50ml of 1% sulfuric acid was added and heated for 20mins for acid pretreatment and allowed to cool to obtain the lignocellulosic mass. Further 100ml of 4% sodium hydroxide was added and heated for 30 mins. The dark brown coloured alkali lignin was filtered and autoclaved at 15 lbs for 10 mins. (Allen C. D. et al 1983).

2.2 Isolation and Acclimatization: The sample sources used for the isolation of lignin degrading bacteria were lake water containing decomposing plant material, rhizosphere soil with cow dung, compost feedstock, scraps of decaying bark and crushed termites obtained from the decaying bark. The medium used for enrichment, isolation and acclimatization of the lignin degrading bacteria were 0.3% lignin broth (soil extract + 0.3% lignin), soil extract agar with 0.3% lignin and 0.5 % lignin broth (soil extract + 0.5% lignin) respectively.

Antifungal used was Fungdid B (clostrimazole + beclomethasone).

2.3 Lignolytic Activity: All the isolates were freshly inoculated in 100ml of 0.5% lignin broth in 500ml baffled erlenmeyer flask and an uninoculated control was also kept. All the flasks were incubated at 30^0C at 120rpm for 5days in a shaker incubater and absorbance was taken on each day. The percent decolourisation of 0.5% lignin for each isolate was calculated as (A_{465} on 1^{st} day – A_{465} on 5^{th} day/ A_{465} on 5^{th} day of control) x 100. (Prabhu P. C. *et al* 2005).

2.4 Enzyme Assay: The isolates were assayed for the presence of Lignin Peroxidase enzyme (LiP) and Manganese-dependent Lignin Peroxidase enzyme (MnP).

2.4.1 Lignin Peroxidase enzyme (LiP):

A spectrophotometric assay method for the determination of lignin peroxidase activity is based on the demethylation of the methylene blue dye. The method can be efficiently used for the quantification as its sensitivity is close to the veratryl alcohol assay. Methylene blue is used as a substrate and the enzyme lignin peroxidase demethylates methylene blue in the presence of H_2O_2 (inducer). The final product is a tri-demethylated methylene blue derivative, Azure C and the reaction occurs at pH-4. Methylene blue demethylates to Azure C (luco). Enzyme activity can be measured as percent decolourization of the methylene blue dye. All the isolates were freshly inoculated in 100ml of 0.5% lignin broth in 500ml flask and were incubated at 30^0C at 120rpm for 5days in a shaker incubater to obtain a heavy bacterial growth. About 10ml of the culture broth of the isolates was taken in cooling centrifuge tubes and the culture broths were centrifuge at 4^OC at 7000 rpm in cooling centrifuge. After centrifugation the tubes were kept in ice bath without disturbing it and enzyme assay was carried out using the supernatant. Assay Protocol- In 1ml of 50mM Sodium Potassium Tartarate (pH-4) buffer, 0.1 ml of 0.1mM H_2O_2 inducer was added to which 32 µM methylene blue as substrate and 10 µl of enzyme solution was added. The solution was incubated for 1hr at RT and A_{650} was measured. The results were interpreted as the percent decolourization of the methylene blue dye by the enzyme as compared to the control tube calculated as (A_{650} for control – A_{650} for test / A_{650} for control) x 100. (Denise B. M. *et al* 1996).

2.4.2 Manganese-Dependent Lignin Peroxidase (MnP) Enzyme Assay:

Manganese-dependent lignin peroxidase (MnPs) oxidize phenolic compounds to phenoxy radicals by oxidation of Mn(II) to Mn(III) with H_2O_2 as an oxidant. Mn (III) is chelated by organic acids (e.g. oxalate or malate in nature). Chelated Mn (III) oxidizes phenolic lignin compounds to phenoxy radicals that degrade spontaneously (Hofrichter *et al* 2002). MnP oxidizes a wide range of compounds from lignin to polycyclic aromatic hydrocarbons (Castillo M. P. *et al* 1997). MnP activity was monitored with phenol red at 30^0C. Reaction mixture contained 25 mM lactate, 0.1 mM $MnSO_4$, 1 mg/ml of bovine serum albumin , 0.1 mg/ml of phenol red, and 0.5 ml of culture filtrate in 20 mM sodium succinate buffer (pH 4.5) in a total volume of 1 ml. The reaction was initiated by the addition of H_2O_2 to final concentration of 0.1 mM and was stopped after 1 min with 50 µl of 10% NaOH, and A_{610} was measured. Control assays of phenol red oxidation in the absence of Mn^{2+} were carried out by omitting $MnSO_4$ from the reaction mixture. MnP activity was calculated by subtracting the value for phenol red-oxidizing activity in the absence of Mn^{2+} from the value for the activity obtained in the presence of manganese. Activity was expressed as the increase in A_{610} per minute per milliliter. (Ryuichiro K. *et al* 1994). Enzyme activity = (A_{610} for test assay) – (A_{610} for control assay).

2.5 Characterization of Potent Isolates: The two potent lignin degrading bacterial isolates i.e. W and Q obtained based on the lignolytic activity and enzyme assay results were characterized and identified. Colony characteristics, Gram staining, motility, biochemical tests and enzyme profiles were observed to identify the isolates using the Bergey's manual of Determinative Bacteriology 9^{th} edition and also by comparing them with standard microorganisms *Pseudomonas aeruginosa* ATCC 19154 and *Serratia marcescens* NCIM 2919.

2.6 Lignin Degrading Products (Phenols):

Lignin is a complex aromatic polymer containing phenyl units and degradation of lignin leads to the accumulation of phenolic components and a number of simple aromatic compounds. Phenol estimation was done by Folin- ciocalteau method. (Oliveria P. L. *et al* 2009) For phenol estimation – In 1 ml of the centrifuged culture broth of respective isolates, 1ml D/W and 5 ml alkaline copper solution was added and incubated at RT for 10mins then added 0.5ml folin ciocalteau reagent. After incubation at RT for 30mins A_{615} was measured. The phenol concentrations present in culture broth were calculated from the standard graph.

2.7 Biobleaching of Effluents:

Paper-pulp mill industries releases effluent having characteristics dark brown colour and textile dye-based industries also releases highly coloured effluents due to the mixture of dyes. (Singh P. *et al* 2004) In case of paper-pulp effluent the color is due to lignin while in case of textile dye- based effluent it's due to various dyes mainly of azo group. Both lignin and azo group dyes have a complex structure and are considered as recalcitrant. (Livin L. *et al* 2004) The lignin peroxidase enzymatic system can be applied for biobleaching of these effluents. The lignin peroxidases belong to the oxidoreductases group which can oxidize large number of complex structure as it is not specific in action and act on a wide range of substrates. It can bring about oxidative breakdown of many azo dyes- Brilliant Green, Congo Red, Remazol Brilliant Blue R, Poly R-478, Reactive Red, and Golden Yellow (Abadulla E. et al 2000). The diluted (2:1) effluents from respective industries were grouped into six flasks. Fungdid B was added to each flask as W, Q, mixed consortia and control. The crude extract of the lignin peroxidases enzymatic system of the isolates were added to the respective flasks, and the rate of decolourization were monitored. The activity was calculated using the formula after measuring the absorbance of the respective centrifuged effluents samples for consecutive 5 days. The color reduction percentage (%) was measured as (Abs on 1st day – Abs on 5th day/ Abs on 5th day) x 100. (Palmieri G. *et al* 2005).

3.0 Results and Discussion:

3.1 Alkali Lignin Preparations: The crude lignin was extracted from natural sources of lignin so as to obtain maximum lignin degrading microbes from various sources. The extraction was done by alkaline delignification method. After alkaline delignification by 4% Sodium hydroxide of lignocellulosic biomass, a dark colour lignin i.e. alkali lignin was obtained (*figure 2*) which was filtered and autoclaved. This alkali lignin was stored in a air tight bottle and used for further experiments.

3.2 Isolation and Acclimatization: Total 11 isolates were obtained from various sources and their lignolytic activity was checked. The lignin agar plates showed decolourization of lignin after incubation as shown in the *figure 3*.

Figure 2- Alkali Lignin

Figure 3: Decolourisation of lignin plates

Total 11 isolates from the different sample sources were obtained, and their colony characteristics were observed. The isolates were further acclimatized to higher concentration i.e. from 0.3% to 0.5% for 1 month.

Table 1: Isolates obtained from different sources

Source	Isolates
Compost and dung sample	C1, C2, C3 and C4
Soil sample	DS and Q
Lake water	W and O.S
Bark sample	B1, B2 and B.S

3.3 Lignolytic Activity:

The decolourisation of lignin by the isolates was significantly enhanced after acclimatization of the isolates at 0.5% lignin for 1month. Before 1 month the lignolytic activity was in the range of 22% to 37%. The lignolytic activity of the isolates was enhanced after acclimatization and was in the range of 30% to 49% (*figure 4*). Among all the isolates highest activities were obtained for W and Q which were 48.99% and 45.14% respectively. After the acclimatization the activity was enhanced significantly.

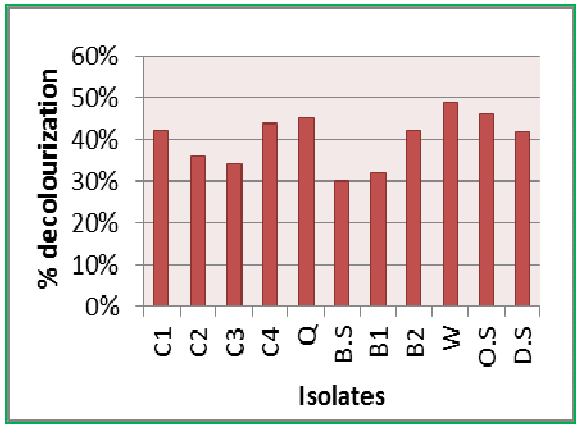


Figure 4: Lignolytic activities in terms of percent decolourization

3.4 Enzyme Assay:

3.4.1 *Lignin Peroxidase Enzyme Assay:*

The A_{650} were measured and enzyme activity was calculated for each isolate. The lignin peroxidase enzyme activity of the isolates was in the range of 30% to 76% (*figure 5*). Among all the isolates highest activities were obtained for W and Q which were 75.67% and 59.45% respectively.

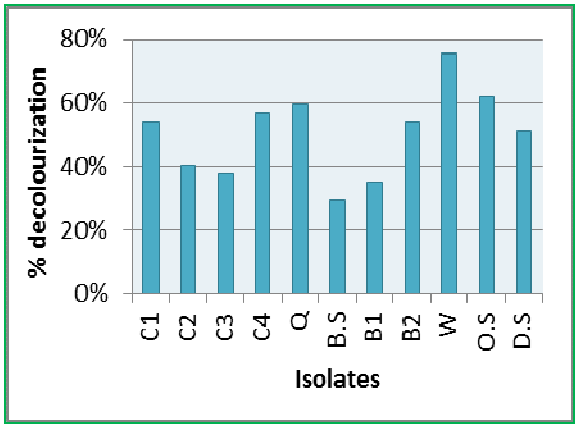


Figure 5: LiP enzyme assay

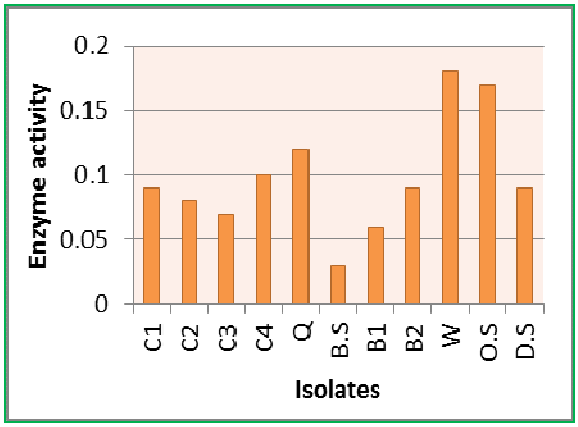


Figure 6: MnP enzyme assay

3.4.2 *Manganese-Dependent Lignin Peroxidase Enzyme Assay:*

The Manganese-dependent Lignin Peroxidase enzyme activity as increase in A_{610} per minute per milliliter of the isolates was in the range of 0.03 to 0.18 *(figure 5).* Among all the isolates highest avtivities were obtained for W and Q which were 0.18 and 0.12 respectively.

3.5 Characterization of Potent Isolates:

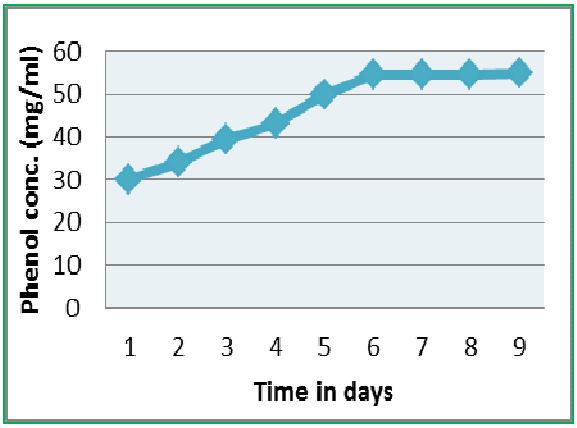
Among the 11 isolates, 2 bacterial isolates W and Q showed highest results which were selected for characterization and for further application. As per Bergey's manual of Determinative Bacteriology 9th edition and comparing with standard microorganisms *Pseudomonas aeruginosa* ATCC 19154 and *Serratia marcescens* NCIM 2919 respectively. It was concluded that the isolate W and Q were *Pseudomonas aeruginosa*, and *Serratia marcescens* respectively.

3.6 Lignin Degrading Products (Phenols):

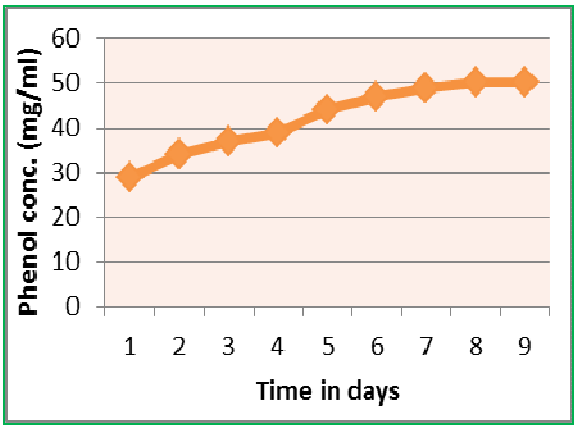
The concentrations of phenol were almost same till 48 hours but it changed as per the lignolytic activity of the isolates. After incubation the early increase showed that the lignin was degraded into various phenolic compounds. The concentration of phenol at 9th day for *Pseudomonas aeruginosa* was 55mg/ml and *Serratia marcescens* was 53mg/ml (Oliveria P. L. et al 2009).

3.7 Biobleaching of Effluents:

The application of the present invention of the lignin peroxidase enzymes as crude extracts of the culture broth was studied on biobleaching of paper pulp mill and textile dye- based effluents. *Pseudomonas aeruginosa* and *Serratia marcescens* were individually applied and a mixed consortium of the two isolates was also applied. The result for the mixed consortium was high than the individual isolates. The λmax of the controls for paper- pulp mill effluent and textile dye-based effluent was recorded as 465nm and 515nm respectively. The absorbances from 1st day to 5th day decreased and the decolourization efficiencies were calculated for each isolate and for the consortium.



(a)



(b)

Figure 7: Conc. of phenol vs time for the isolate
(a) Pseudomonas aeruginosa and
(b) Serratia marcescens

Treatment of paper-pulp mill effluent gave 60% to 75% color reduction and in case of textile dye- based effluent 50% to 58% decolourization was observed. The heterogeneous combination of lignin peroxidases from mixed consortia gave 80% to 85% color reduction in treatment of paper-pulp mill effluent and 70% to 75% decolourization in treatment of textile dye-based effluent which is significantly high. It was concluded that the treatment of both the effluents with heterogeneous combination of mixed consortium gave maximum decolourization.

4.0 Conclusion and Future Trends:

1) The application of lignin peroxidases as an enzymatic method for biobleaching of paper-pulp and textile-dye based effluents showed significant colour removal.
2) Therefore, this treatment can be adopted and applied at industrial level for decolourisation of various coloured effluents.
3) The lignin peroxidase enzymatic system including the enzymes lignin peroxidase and manganese-dependent lignin peroxidase can be used in variety of applications.
4) The crude extract of the enzymes from the culture broths can be used directly or the purified enzyme can be immobilized and used repeatedly on large scale. (Arulmani M. *et al* 2008)

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