ISSN: 2230-9926

Available online at http://www.journalijdr.com

IJDR

International Journal of Development Research
Vol. 08, Issue, 08, pp. 22419-22424, August, 2018

ORGINAL RESEARCH ARTICLE OPEN ACCESS

BIOREMEDIATION OF INDUSTRIAL EFFLUENTS BY LIGNINOLYTIC MICROBES

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ARTICLE INFO

Article History:

Received 17th May, 2018
Received in revised form
06th June, 2018
Accepted 08th July, 2018
Published online 31st August, 2018

Key Words:

Lignin, Lignin-peroxidase,
Manganese-peroxidase,
Laccase, COD,
Methylene blue.

ABSTRACT

Lignin is a complex biopolymer which forms the hard part of woody tissues. It's high molecular weight and rigid nature makes it recalcitrant for digestion. Lignin is exudated to access other compounds like cellulose, hemicellulose etc., for industrial production of bioethanol, paper etc., Paper pulp industries being one of the major wood processers remove lignin through various thermochemical methods like sulphonation, chlorination etc., to give a soft texture for paper. The resulting effluents are rich in recalcitrant lignin derivatives which pollutes the receiving water bodies making it toxic to aquatic life. Bioremediation is the use of biological agents like microorganisms to break down environmental pollutants. The biodegradation of lignin can be carried out by microbes such as bacteria and fungi which possess lignin degrading enzymes like Lignin peroxidase (LiP), Manganese peroxidase (MnP), Laccase. Ligninolytic enzymes have bioremediation potential to degrade the industrial effluents and other xenobiotic wastes. In this study the ligninolytic bacteria were studied for the presence of ligninolytic enzymes and were tested for application in bioremediation by analyzing their potential in reducing Chemical Oxygen Demand (COD) of industrial effluents. The isolates showing effective reduction could be exploited for bioremediation.

Citation: Madhumita Ghosh Dastidar, Akshaya Simha, N., Ananth Koushik, B., Aradhya, D., Harishmitha, N., Nayana, S., Nidhish, P., Shakthi Vignesh, S., Siri, G. and Tavisha, V. K. 2018. "Bioremediation of industrial effluents by ligninolytic microbes", *International Journal of Development Research*, 8, (08), 22419-22424.

INTRODUCTION

In India, pulp and paper manufacturing is one of the eldest and leading industry which produced about three million metric tons per annum finished products (Malaviya *et al.,* 2007). These effluents contain many organic compounds, derived from lignin, which are responsible for their brown colour, increasing water temperature and decreasing photosynthesis rate of the phytoplanktonic community (Pedroza *et al.,* 2007). All these organic compounds are toxic to aquatic organisms and resistant to microbial degradation, resulting in a decrease of the ecological value of natural systems surrounding the pulp mill (Clesceri *et al.,* 1999; Ali *et al.,* 2001). The raw wastewater from paper and board mills causes pollution and increases chemical oxygen demand (COD) values.

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Thus, wastewater from the industry needs to be treated to reduce any possible impacts on the aquatic environment (Buyukkamaci *et al.,* 2010). The conventional treatment methods, such as aerated lagoons and activated sludge plants are ineffective in removing colour and phenolics. In most cases, this effluent (raw or treated) is discharged into the rivers, stream or other water bodies; resulting in high BOD, COD and also causing problems to community and environment. In many developing countries farmers are irrigating their crop plants with water bodies which might be severely exposed to industrial effluents. This leads to risks of bioaccumulation of toxicants especially at various level of the food chain. Thus, it is important to treat the industrial effluents before their final discharge (Yang *et al.,* 2008). Despite the fact that, wide range of physical and chemical treatment methods (electrocoagulation, ozonation, adsorption, advanced oxidation, and ultrafiltration) or combination of different methods in series are available for the treatment of effluent,

but they are more energy intensive and suffer from residual effects. Thus, there is still a need for energy efficient, affordable and environment friendly technologies (Yang *et al.,* 2008; Raj *et al.,* 2014). In recent years the biotechnological approaches based biological treatment came in to scenario in present treatment systems, in which wide variety of microorganisms including fungi, actinomycetes, and bacteria as well as enzymes have been implicated but the recent research has been focused on with ligninolyic fungi (white rot and brown rot) due to their powerful lignin-degrading enzyme system (Malaviya *et al.,* 2007; Kumar *et al.,* 2012; Jiang *et al.,* 2010). A large number of enzymes from a variety of different plants and microorganisms have been reported to play an important role in an array of waste treatment applications. Enzymes can act on specific recalcitrant pollutants to remove them by precipitation or transformation to other products. They also can change the characteristics of a given waste to render it more amenable to treatment or aid in converting waste material to value-added products. Enzymes are the major proteins which catalyses various biochemical reactions. Lignin, a toxic and a recalcitrant molecule, is degraded by microbial enzymes into non-toxic molecules. The fungi and the bacteria are the two classes of micro-organisms which are intensively involved in the biodegradation of lignin. The lignin-degrading bacteria represent mainly three classes: Actinomycetes, α-Proteobacteria and γ-Proteobacteria (Huang *et al*. 2013). The widely distributed ubiquitous group of microorganism occurs both in terrestrial and aquatic habitats (Srinivasan, Laxman and Deshpande 1991). 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 peroxidase (LiP), also known as ligninase or diarylopropane oxygenase was first reported in 1983. It is part of the extracellular enzyme system of the white-rot fungus Phanerochaetechrysosporium (Aitken, M. D *et al* 1994). LiP was shown to mineralize a variety of recalcitrant aromatic compounds and to oxidize a number of polycyclic aromatic and phenolic compounds. LiPÏs role in lignin depolymerization has also been confrmed (Venkatadri, R. and Irvine, R. L.1993). Manganese peroxidase (MnP), produced by Phanerochaetechrysosporium has also been observed to catalyze the oxidation of several mono-aromatic phenols, and aromatic dyes, but these reactions depend on the presence of both divalent manganese and certain types of buffers. In fact, MnPcatalyzes the oxidation of Mn(II) to Mn(III) in the presence of Mn(III) stabilizing ligands. The resulting Mn(III) complexes can then carry out the oxidation of organic substrates.16 However, the enzymeÏs requirement for high concentrations of Mn(III) makes its feasibility for wastewater treatment applications doubtful (Aitken, M. D *et al* 1994).

Laccase is produced by several fungi and seems capable of decreasing the toxicity of phenolic compounds through a polymerization process. Also because of its relative non-specificity, it can induce the cross-coupling of pollutant phenols with naturally- occurring phenols. In fact, laccase can oxidize phenolic compounds to their corresponding anionic free radicals which are highly reactive (Breen, A. and Singleton, F. L. 1999). The white rot fungi and other fungi such as Phanerochaetechrysoporium, Streptomyces viridosporus, Pleurotuseryngii, Trametestrogii, Fusarium proliferatem, Agaricus, Erwenia, Copricus, Mycema and Swterium (Alexender, 1977) are involved in lignin degradation. But the enzymes of fungi are not suitable for commercial lignin degradation because with respect to environmental factors like pH, oxygen and concentration of lignin the enzymes act unstable (Crawford and Muralidhara, 2004). Therefore bacterial enzymes which are extracellular preferable (Renugadevi *et al.,* 2011). Lignocellulytic enzymes also have significant potential application in various industries including chemicals, fuel, food, brewery and wine, animal feed, textile and laundry, pulp and paper, and agriculture (Howard *et al.,* 2003).

MATERIALS AND METHODS

The available ligninolytic bacterial isolates were further inoculated in Minimal salt medium containing lignin (MSML) broth for performing various assays. MSML broth contained potassium dihydrogen phosphate(3g), disodium hydrogen phosphate(6g), sodium chloride(5g), ammonium chloride(2g), magnesium sulphate(0.1g), in 1000ml of distilled water (pH 7) along with 1% lignin extract. (Chandra R *et al.,* 2008). Lignin extract was prepared from saw dust as lignin source. 5ml of 1% sulphuric acid was added to 10g of saw dust. This mixture was heated in hot air oven at 80°C for 20 minutes. It was then cooled and boiled with 100ml of 4% sodium hydroxide solution for 30 minutes. The dark brown coloured alkaline lignin extract solution was filtered and autoclaved. (Bholy *et al.,* 2012). The isolates were then screened for ligninolytic activity quantitatively using methylene blue dye reduction test.

Methylene blue dye reduction test

The ligninolytic enzymes being oxidative in nature can decolourise the basic dyes by reducing them. Hence, the isolates can be screened for ligninolytic activity based on methylene blue dye reduction test. All the isolates were inoculated in MSML broth containing methylene blue dye and incubated at 37°C for 48 hours. Absorbance was measured at 600nm in the intervals of 24 hours. The percentage of decolourisation was calculated using the equation,

$$\% \text{ of decolourisation} = \frac{C_O - C}{C_O} \times 100$$

Where,

C_0=Absorbance of Control
C= Absorbance at the intervals of 24 hours

Enzyme Assay

The screened isolates were assayed for the activity of Lignin peroxidase, Manganese-dependent lignin peroxidase and Laccase enzymes. Firstly, the isolates were inoculated in 25ml of nutrient broth and were incubated at 37°C for 48 hours. These newly grown cultures were used for further experiments.

Lignin Peroxidase Assay

This assay is based on demethylation of malachite green dye by lignin peroxidase enzyme. Here, malachite green is used as substrate for the enzyme in presence of H_2O_2 as inducer. Isolates were inoculated in 1% lignin broth and were incubated at 30□ and 120rpm for 6 days.10ml of culture broth was taken from these and was centrifuged at 7000rpm at 4□. Then the tubes were placed in ice water bath and supernatant was used

as crude enzyme for the assay. The assay reaction mixture contained 1ml of 50mM sodium potassium tartarate buffer (pH : 4.5) containing 32µM malachite green as substrate and 10µL of enzyme solution. One test tube without enzyme was used as control. To these, 0.1ml of 0.1mM H_2O_2 inducer was added. This solution mixture was incubated for an hour at room temperature and absorbance was measured at 650nm (A_{650}). The enzyme activity was interpreted as percentage of decolourization of malachite green dye by enzyme with respect to control using the given formula.(Denise B M *et al*, 1996).

$$\text{Percentage of decolourization} = \frac{A_{650}\ of\ control - A_{650}\ of\ test}{A_{650}\ of\ control}$$

Manganese-dependent Lignin Peroxidase (MnP) Assay: It is a colorimetric assay based on the oxidation of phenol red by the enzyme in presence of manganese and hydrogen peroxide as inducer. MnP catalyses the oxidation of Mn(II) to Mn(III), which in turn oxidises phenolic substances (Phenol red).In this assay, the reaction mixture contained, 28mM Lactate, 0.1mM $MnSO_4$, 1mg/mL of Bovine Serum Albumin (BSA), 0.1mg/mL of phenol red, 0.5 mL of culture filtrate in 20mM sodium succinate buffer (pH: 4.5) in a total volume of 1mL.Firstly, H_2O_2 inducer, in the final concentration of 0.1mM was added to this mixture to initiate the reaction. The reaction was stopped after a minute with 50µL of 10% NaOH. Test tubes with reaction mixture devoid of $MnSO_4$ (Hence, Mn^{2+}) was used as Control tube. Absorbance was measured at 610nm.Manganese peroxidase activity is expressed as the difference in the value of phenol red-oxidising activity in the absence of Mn^{2+} and value of activity obtained in presence of Mn^{2+}.The increase in absorbance at 610nm per minute per millilitre, indicates the presence of manganese dependent lignin peroxidase activity (Ryuichiro K. *et al*, 1994).

Laccase assay

Laccase enzyme activity can be assayed using Tannic acid and α-Napthol as substrates. The newly grown culture on agar plates were inoculated into laccase production medium and incubated at 37□ at 200rpm for 48 hours. Laccase production medium contained (g/L of distilled water) Dextrose, 10;Peptone, 5; NaCl, 5; Beef extract, 3;$MgSO_4.7H_2O$, 1;$CaCO_3$, 0.2; $FeSO_4.7H_2O$, 1;$ZnSO_4.7H_2O$, 0.9; $MnSO_4$, 0.2and $CuSO_4$, 0.195. Stock solutions of salts were prepared separately and autoclaved. Now the respective salt solutions were added to the medium.1ml of isolates grown in laccase production medium were transferred to test tubes containing 0.3% tannic acid and were incubated at 35□ for 1hour at 200rpm. Presence of laccase activity is denoted as brownish colour change in the test tube. 1ml of 24hours old culture of isolates were added to 1ml of 0.5% α-naphthol taken in the test tubes and were incubated at 35□ for 48 hours at 200rpm. Test tube containing 1ml of uninoculated media in place of isolates was used as bank. Presence of laccase activity was seen as the purple colour change in the test tubes. The laccase activity was quantitatively measured colorimetrically at 540nm.

Analysis of reduction in Chemical Oxygen Demand: Chemical Oxygen Demand (COD) is the measure of oxygen consumed by water during the decomposition of organic matter and the oxidation of inorganic chemicals. This test is mainly used to quantify the amount of oxidizable pollutants in water contaminated by domestic wastes and industrial effluents. The amount of organics in water is estimated by the ability of oxidation of strong chemical oxidant such as Potassium permanganate or Potassium dichromate. Water sample containing industrial effluents are treated with a known volume of oxidizing agent in acidic medium. The concentration of organic content in water sample is calculated by measuring the amount of oxidant remaining in the solution after oxidation by titration. COD is expressed as mg/L of oxygen consumed in the solution. 50ml of waste water sample containing effluents was taken in three 100ml conical flasks along with three distilled water blanks. 5ml of 0.1N $K_2Cr_2O_7$ solution was added to each of the six flasks and were incubated in water bath at 100□ for 1 hour. After incubation, they were allowed to cool for 10 minutes. Then, each flask was added with 5ml of 10% Potassium iodide solution, followed by 10ml of 2M H_2SO_4 solution. The contents in the flasks were titrated against 0.1M Sodium thiosulphate solution until pale yellow colour appears. Then, 1ml of 1% freshly prepared starch solution was added as indicator turning the solution blue. This mixture is once again titrated against 0.1M Sodium thiosulphate solution until the blue colour disappears completely. COD per litre of the water sample was calculated using the following formula:

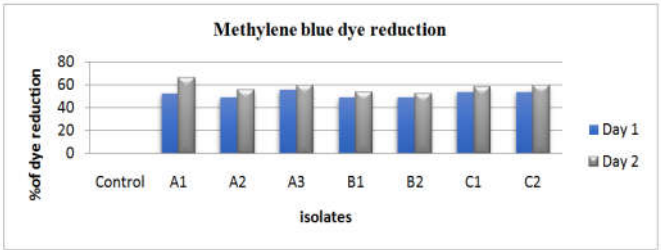
$$\text{COD of sample} = \frac{8 \times C \times (B - A)}{S}\ \text{(mg/litre)}$$

Where,

C = concentration of titrant(mol/L)
A = volume of titrant used for blank (mL)
B = volume of titrant used for sample (mL)
S = volume of water sample taken (mL)

Further, 10% of each ligninolytic isolate was inoculated in the waste water sample containing effluents and incubated at room temperature in dark chamber for 48 hours. The COD was calculated after 48 hours and change in COD was analysed. The decrease in the value of COD indicates that the oxygen was utilized by microorganisms to degrade organic matter in the effluents. (K. R. Aneja)

RESULTS

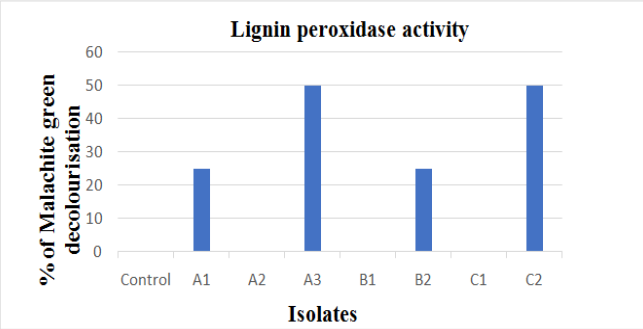


Graph 1. Results of Dye reduction test

All the isolates were able to reduce methylene blue dye owing to its ligninolytic activity. The reduction activity increased for every 24 hours of incubation. The isolateA1 has shown greater dye reducing ability compared to other isolates.

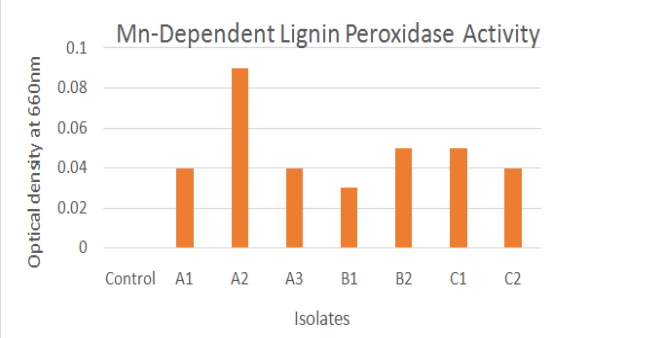
RESULTS OF ENZYME ASSAY

Lignin Peroxidase (LiP) activity: Among the isolates subjected to lignin peroxidase enzyme assay, only few showed LiP activity. Isolates C2 and A3 have shown a greater LiP activity, whereas A2, B1 and C1 showed no activity.



Graph 2. Results of lignin peroxidase assay

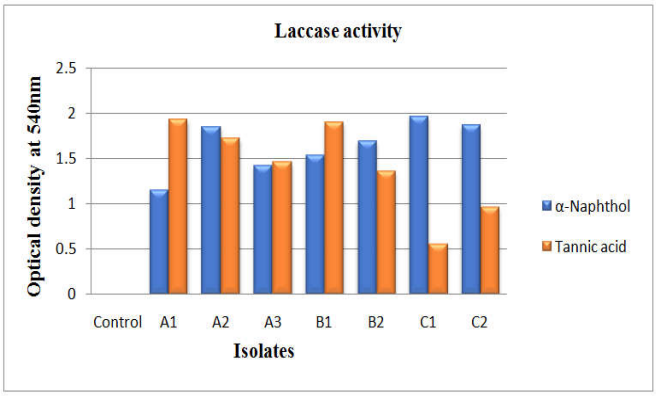
Mn-dependent lignin peroxidase (MnP) Activity



Graph 3. Results of Manganese dependent lignin peroxidase assay

All the isolates showed MnP activity. Isolate A2 has shown highest activity whereas, isolates A1, C2 and A3 has shown a moderate activity.

Laccase Activity

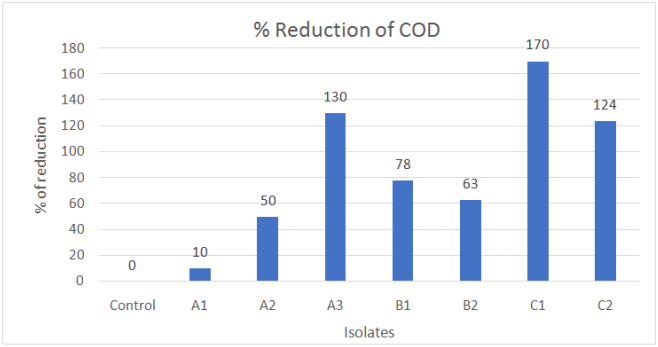


Graph 4. Results of laccase activity assay

Each isolate showed varied laccase activity with respect to the substrates α-Naphthol and Tannic acid. When the substrate is α-Naphthol, C1 and C2 has shown greater activity. The activity of A1 and B1 is higher when the substrate is Tannic acid.

COD Assay

All the isolates were able to reduce COD of the effluents. The isolates C1, A3 and C2 have shown excellent reduction in COD, whereas the other isolates showed moderate reduction.



Graph 5. Percentage of Reduction in COD

DISCUSSION

Lignocellulose compound degrading bacteria were isolated from the soil sample collected from the premises of pulp and paper industry. The dye decolorization of lignin was studied to know ligninolytic potential. Dyes usually have a complex aromatic molecular structure, which makes them stable and resistant to biodegradation. The azo dyes with chromophore group are largest class of commercially produced dyes. In dye decolourization, azo linkage is split either by reduction, the reaction used by some bacterial consortia under anaerobic conditions, or by oxidation, the reaction used by ligninolytic white rot fungi. Researchers found that lignolytic enzymes such as lignin peroxidase, manganese peroxidase and laccase were responsible for dye decolourization. (Moldes *et al.,* 2003).Some actinomycetes and anaerobic bacteria have also dye degrading activity, by utilizing lignin degrading enzymes (Lee *et al.,*1999).The isolated bacterial cultures capable of degrading azo dyes was reported in 1970s with Bacillus subtilis (Chen *et al.,*1982), then Aeromonas hydrophila followed by Bacillus cereus (Rodriguez A *et al.* 1996). Numerous bacteria capable of dye decolorization, either in pure cultures or in consortia, have been reported (P. Kumar *et al.,* 2009). All the isolates were able to reduce methylene blue dye owing to its ligninolytic activity. The reduction activity increased for every 24 hours of incubation. In this study the isolate A1 has shown greater dye reducing ability compared to other isolates. More than 50% dye reduction were observed after 24 hrs by isolates and more than 60% reduction were observed after 48hrs.Telke *et al.,*2010 reported bacterial dye decolorization by oxidoreductive enzymes, including laccase, azoreductases and NADH-DCIP reductase. The laccases and laccase like activity have been demonstrated in other bacteria such as Bacillus halodurans, Bacillus licheniformis, Bacillus subtilis, E coli, Nitrosomonas europea, Streptomyces coelicolor, Streptomyces griseus and Thermus thermophilus (Arora *et al.,* 2002). The experiments were conducted to ensure that the lignolytic enzymes were produced by these bacterial isolates. The lignin peroxidase activities were present in A_1, A_3, B_2 and C_2.The Mn Peroxidase were observed in all isolates. The substrate alpha naphthol and tannic acid was studied previously as a substrate for laccase. In presence of alpha naphthol and tannic acid all the isolates showed positive results. In literature it was mentioned that ABTS and guiacol was used as a substrate in laccase enzymatic assay. Other researchers also reported the presence of peroxidase and laccase in lignolytic bacteria (Shi *et al.,* 2013). Some other reports are also on bacterial lignin degrading enzymes isolated lignin degrading bacteria from palm oil plantation soil by using a selective nutrient media medium. Bacteria were found

to produce all three lignin degrading enzymes peroxidase and laccase (Arora *et al.,* 2002) by the strain Klebsiella aerogenes NCIM 2098 (K. aerogenes) of family Enterobacteriaceae and find effective in lignin removal. Chandra *et al*. (2007) degraded Kraft lignin by three bacterial isolates which were able to reduce 69% color, 40% lignin and total substrate by 50% after 48 h. On the other hand, Chandra et al. (2009) used Bacillus cereus (ITRC-S6) and Serratia marcescens (ITRC-S7) for reducedingcolor (45-52%), lignin (30-42%), BOD (40-70%), COD (50-60%), in 7-day period supplementing the sample with glucose and peptone. Similarly, Raj et al. (2007) demonstrated that Bacillus sp. was able to remove 61%, 53%, 82% and 78% of color, lignin, BOD and COD within 6 days of incubation by adding co-substrate glucose and peptone. Gupta *et al*. (2001) employed two strains of Aeromonas formicans were able to remove 70% to 80% of COD, and lignin's while the color around 85% in 8 days. Tyagi, S. *et al.* showed degradation and decolourization of pulp and paper effluent which ultimately reduce high load of BOD, COD, TS, TDS and total suspended solids (TSS) after 168 h of incubation by addition of glucose and peptone as additional nutrient source. By using sulphate reducing bacteria, Hao and Man (2006) were able to remove COD up to 70 -75% after 3 weeks and increase to 82 - 88% by subsequent aerobic treatment for 48 h. Researchers are now focusing on utilizing, and improving these enzymes for use in various industry such as pulp and paper, biofuel and bioproduct industries.

Conclusion

The results of the study showed that indigenous bacterium isolated from pulp and paper industry effluent was capable of using lignin as the sole carbon source and reducing the chemical oxygen demand (COD). The lignin degrading bacteria were positive for lignin degrading enzymes like Lignin Peroxidase, Mn dependent lignin peroxidase and laccase activity. Further the ligninolytic activity was correlated with methylene blue dye reduction activity of bacteria upto48hrs.Since bioremediation of lignin rich pulp and paper mill effluent has emerged as a challenge for the environmentalists, the bacterium can play a lead role due to its significant bioremediation potential.

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