# PRODUCTION OF BACTERIAL LIGNINOLYTIC ENZYMES AND THEIR POTENTIAL APPLICATION IN DECOLORIZATION OF DYES

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Lignin is a complex, three-dimensional aromatic polymer, consisting of dimethoxylated, monomethoxylated and non-methoxylated phenylpropanoid subunits, and is recalcitrant in nature. In this study, the isolation of lignin degrading bacteria was carried out from decaying wood samples. Ligninolytic activity was evaluated by growing bacterial isolates on lignin monomers and by the degradation of indicator dyes. Out of 16, the bacterial isolates L8, L12, L13 and L15 exhibited growth on monomers *viz*., vanillin, veratryl alcohol, phenol, guaiacol and lignin, with efficient dye decolourization. Further, the production of ligninolytic enzymes (laccase, lignin peroxidase and manganese) was estimated for the 4 selected bacterial isolates. The maximum enzymatic activity response was noted in the case of isolate L15, with the best growth on every lignin monomer.

***Keywords***: lignin degradation, decolourization, Azure-B, Toluidine blue, ligninolytic enzymes

## INTRODUCTION

With the progress of science and technology, many organic pollutants are released into the environment from agricultural activities and through the waste effluents of various industries, such as chemical factories, oil refineries, paper factories and sugar mills. The continuous discharge of organic pollutants into water bodies can cause harmful carcinogenic, mutagenic and teratogenic effects.[1] With increasing environmental awareness, environmental protection agencies have set the goal for reducing harmful pollutants and seeking eco-friendly solutions. Recent research in biodegradation has revealed that using lignocellulolytic enzymes or microorganisms could serve as the basis for the development of new, environmentally friendly technologies to be applied in various fields.[2]

Presently, millions of tons of lignin and its derivatives are generated as effluents from the oil, pulp and paper, and sugar industries, in addition to the lignocellulosic wastes resulting as by-products from agricultural and forestry practices.[3] Lignin is a complex, three-dimensional aromatic polymer, consisting of dimethoxylated, monomethoxylated and non-methoxylated phenylpropanoid subunits.[4] It is a recalcitrant compound, of an intense brown colour, hydrophobicity and poor mechanical properties.[5] Its presence in industrial effluents is highly polluting, causing dark coloration and toxicity to aquatic life. Therefore, lignin degradation in industrial wastewater before its release into the environment is essential. Although there are various physical and chemical methods to achieve this, the biological one, involving microorganisms and enzymes possessing degradative potential towards lignin, has recently gained much research interest.[6] For example, actinomycetes (*Rhodococcus*, *Nocardia* and *Streptomyces* species), alfa-proteobacteria (*Sphingomonas*) and gamma-proteobacteria (*Pseudomonas*) have been demonstrated to have great lignin-degrading ability.[7] Also, ligninolytic enzymes could provide a more specific and effective alternative for lignin depolymerization.[8]

There are four major groups of lignin modifying enzymes (LMEs), which are responsible for the degradation of lignin, *i.e*., laccase, lignin peroxidase, manganese dependent (MnP), versatile peroxidase (VP).[9-11] These enzymes can act on a wide range of aromatic compounds, such as methoxybenzenes and various other non-phenolic compounds. In addition, laccase acts as a mediator and aids in generation of stable radicals during the process of enzymatic oxidation. The process of lignin degradation by using LMEs is further enhanced by the cooperative action of several accessory enzymes, which may include glyoxal oxidase, aryl alcohol oxidase, also known as veratryl alcohol oxidase, pyranose 2-oxidase (glucose 1-oxidase), cellobiose/quinone oxidoreductase, and cellobiose dehydrogenase. Various other peroxidases, such as heme peroxidases, form a superfamily of enzymes responsible for numerous biosynthetic and degradative functions.

Lignin modifying enzymes (LMEs) have found application in the paper and pulp industries due to their great decolorizing and detoxifying properties. For instance, the manganese peroxide enzyme has been used for dye wastewater decolourization.[12] It undergoes the mechanism of peroxide-dependent degradation. Using lignin modifying enzymes in bioremediation is environmentally friendly and involves low operating costs. Thus, considering the need for removing numerous dyes that are recalcitrant in nature from industrial effluents, the present study was focused on isolating potential ligninolytic bacterial strains and determining their dye degradation potential.

## EXPERIMENTAL

### Materials

#### *Sample collection*

Soil samples were collected from wood decomposing areas from the three villages, namely, Jamsher Khas, Bajuha Khurd and Kadian Wali, of Jalandhar, Punjab, India. The samples were collected and stored in air-tight polythene bags, with holes in them, at 4 °C.

#### *Chemicals*

$K_2HPO_4$, $KH_2PO_4$, $MgSO_4$ and $NH_4NO_3$ salts for preparing the minimal salt medium were purchased from Sigma Aldrich, and yeast extract was provided by Loba Chemie Pvt. Ltd. The kraft lignin used was manufactured by Sigma Aldrich. All the dyes and the aromatic monomers utilised in experiments were purchased from Loba Chemie Pvt. Ltd.

### Isolation of lignin degrading bacteria

The lignin degrading bacteria were isolated on plates of minimal salt medium after incubation for 7 days at 37 °C. The medium was prepared with the following composition: $K_2HPO_4$ (4.55), $KH_2PO_4$ (0.53), $MgSO_4$ (0.5), $NH_4NO_3$ (5) and yeast extract (0.1) g/L, supplemented with kraft lignin (0.5%). The isolated bacterial cultures were maintained at 4 °C on the mineral salt medium.[13]

### Growth of lignolytic bacterial isolates on lignin monomers

In order to assess the growth of the bacteria on aromatic monomers, 1% of lignin, vanillin, phenol, veratryl alcohol and guaiacol were added separately to individual mineral salt medium plates. The contents were mixed properly and autoclaved at 121 °C, 15 lbs for 15 min. Then, bacterial isolates were streaked on the prepared supplemented medium plates and the plates were incubated at 37 °C for 7 days.

### Dye decolourization assays with bacterial isolates

The decolourization of various dyes, such as Azure-B (AZB), Remazol brilliant blue R (RBBR), Congo red, Toluidine blue and Indigo carmine, was performed using the isolated strains as an indicative method to verify their ligninolytic capacity. The dye decolourization assays were performed using two methods: in solid medium and in liquid medium.[14]

#### *Solid medium decolourization assay*

Minimal salt medium containing 40 mM glycerol was used with the following dyes: AZB (100 mg/L), RRBR (50 mg/L), Indigo carmine (50 mg/L) and Congo red (50 mg/L), Toluidine blue (25 mg/L). The dyes were added individually and buffered with phosphate buffer saline.[15] The components were mixed and autoclaved at 121 °C for 15 min. After autoclaving, the plates were prepared, inoculated by streaking and then incubated for 7 days at 37 °C. After 7 days, the plates were observed to determine decolourized regions in the medium.[16]

#### *Liquid medium dye decolourization assay*

Luria broth was autoclaved at 121 °C, 15 lbs for 15 min. Then, the medium (20 mL) was inoculated with bacterial isolates (1%) and incubated for 24 h, prior to dye addition at 37 °C.[15] After 24 h of incubation, the optical density was measured. Then, the dyes (Azure-B, Indigo carmine, Congo red, Toluidine blue) were added separately to each test tube as per the following concentration: AZB – 100 mg/L, Indigo carmine and Congo red – 50 mg/L, Toluidine blue – 25 mg/L. After that, the optical density of each sample was measured every 24 h, at the following wavelengths: for Congo red – at 470 nm, Azure-B – at 650 nm, Indigo carmine – at 615 nm, Toluidine blue – at 635 nm, for 7 days.[17]

The data were collected for each selected isolate and the percent decolourization was calculated by using the formula:

Decolourization (%) = $(A_o-A_t/A_o) \times 100$ (1)

where $A_o$ = initial absorbance or absorbance before treatment, $A_t$ = absorbance after time 't' of the treatment.

**Liquid state production of ligninolytic enzyme**

Under aseptic conditions, the selected bacterial isolates were inoculated in nutrient broth and were incubated for 48 h at 37 °C. The re-inoculation of bacterial isolates (1%) grown in nutrient broth was done in mineral salt medium supplemented with kraft lignin (0.5%) and incubated for 7 days in an incubator shaker at 37 °C. The enzyme was extracted by adding 50 mL of 0.1M citrate buffer (pH 5) to the culture. The crude enzyme was filtered under aseptic conditions, using Whatman filter paper, and the supernatant was harvested by centrifugation at 8000 rpm for 10 min at 4 °C.[18]

**Enzymatic assay of isolated bacteria**

A basic enzyme assay consists of three major components, *i.e.* an enzyme, a substrate and a detection method, which are necessary for a single catalytic reaction.[19] In the case of the ligninolytic enzymatic activity, there are three lignin modifying enzymes: lignin peroxidase, manganese peroxidase and laccases, therefore, three different assays were performed.

***Lignin peroxidase assay***

In this case, one unit of enzyme activity is expressed as an optical density decrease at 651 nm of 0.1 units per minute per mL of the culture filtrate (supernatant extracted). In this assay, 3 mL of reaction volume was prepared, containing 1 mL of 0.1M citrate buffer (pH 5), 0.5 mL of 0.16 mM Azure B, 0.5 mL of culture filtrate and 0.5 mL of 2 mM hydrogen peroxide, and was incubated at 25 °C for 2 h.[20]

***Manganese peroxidase assay***

In this case, the enzymatic activity assay utilizes phenol red as substrate and optical density is measured at 610 nm. In this assay, the reaction mixture contained: 250 µL of enzymatic extract, 0.1% phenol red (50 µL), 250 mM sodium lactate (100 µL), 2 mM manganese sulphate (25 µL), 0.5% BSA (100 µL), 0.2 mM hydrogen peroxide (25 µL) and 0.1M citrate buffer of pH 5 (0.5 mL);[21] it was incubated at 25 °C for 2 h.

***Laccase assay***

Guaiacol was used as substrate in this assay. 3 mL of reaction volume was prepared, containing 1.5 mL of 10 mM acetate buffer (pH 5), 1 mL of guaiacol and 0.5 mL of enzyme extract to be added; it was incubated at 25 °C for 2 h and optical density was measured at 450 nm.

***Determination of enzyme activity***

The enzymatic activity was expressed in units/mL/min of enzyme extract, and calculated by the following formula:[22.]

Enzyme activity = (Absorbance of test/min – Absorbance of blank/min)*(d.f.) / [(0.1)*(0.5)] (2)

where 0.1 = the change in absorbance/min per unit of enzyme at its specific pH in a reaction volume, 0.5 = volume of enzyme (mL). The estimation of the enzymatic activity (laccase, lignin peroxidase and manganese) was done with selected bacterial isolates.[23]

## RESULTS AND DISCUSSION

**Isolation and culture of ligninolytic bacteria**

Sixteen bacterial isolates were obtained from the soil sample collected from different sites (Kadian Wali, Bajuha and Jamsher Khas) of Jalandhar, Punjab, India. Morphological characterization of the isolates was also performed. Out of 16 bacterial isolates, 10 showed gram-positive reaction, with raised, convex or flat colony surface. Screening of the bacterial isolates was performed for determining their ligninolytic activity. The lignin degrading bacteria were isolated and cultured on minimal salt medium supplemented with kraft lignin.

Earlier, it has been observed that the microorganisms were able to grow on low and high molecular weight monomers of lignin. For example, *Pandoraea norimbergensis* and *Pseudomonas* species were found to have potential to grow on lignin monomers.[4] Accordingly, in the present study, the growth of the obtained isolates on lignin and its aromatic monomers (*i.e.* phenol, guaiacol, veratryl alcohol and vanillin) was assessed.

After incubation for 7 days at 37 °C, all the 16 bacterial isolates showed good growth on lignin added as a carbon source to the culture medium. As regards its aromatic monomers, veratryl alcohol has proved to be favourable to the growth of a maximum of 12 bacterial isolates, phenol was preferred by 10 bacterial isolates, and vanillin – by only 7 bacterial isolates (Table 1, Fig. 1). The least growth was observed on guaiacol: out of 16, only 3 bacterial isolates showed growth. Also, four isolates, namely, L8, L12, L13 and L15, exhibited growing ability on at least three different lignin monomers, of which isolate L15 showed growth on all the selected lignin monomers, *i.e.* phenol, guaiacol, veratryl alcohol and vanillin, as well as on lignin.

**Dye decolourization assays**

***Solid medium dye decolourization assay***

The extensive use of synthetic dyes in the textile industries, due to their easy manufacture and cheap production, causes serious environmental problems. Moreover, as their chemical structure is resistant to breakdown, this leads to their accumulation in the environment, threatening not only ecosystems, but also human health, as most of the dyes are mutagenic, toxic and carcinogenic agents. Earlier reports have stated that microbial degradation of dyes can be performed under aerobic as well as anaerobic conditions.[26-27] Moreover, previous studies have reported on the decolourization ability of ligninolytic bacteria and fungi towards synthetic dyes.[27] Accordingly, in present study, the degradation ability of the obtained isolates was examined towards a number of synthetic dyes (Azure-B (AZB), Remazol brilliant blue R (RBBR), Congo red, Toluidine blue, Indigo carmine). After 7 days of incubation of the plates at 37 °C, the results showed highest decolourization of Congo red, Toluidine blue and Indigo carmine. Clear streaks of decolourization were observed on the plates (Fig. 2). Nine bacterial isolates succeeded in decolorizing Toluidine blue, eight were successful on Indigo carmine, six on Congo red, and only three bacterial isolates could degrade Azure-B and Brilliant blue dyes.

Table 1
Growth of bacterial isolates on lignin monomers*

| Isolate no. | Lignin | Phenol | Guaiacol | Veratryl alcohol | Vanillin |
|---|---|---|---|---|---|
| L1 | + | - | - | + | + |
| L2 | + | - | - | + | + |
| L3 | + | + | - | + | - |
| L4 | + | + | - | - | - |
| L5 | + | + | - | + | - |
| L6 | + | - | - | + | - |
| L7 | + | - | + | - | - |
| L8 | + | + | - | + | + |
| L9 | + | + | - | - | + |
| L10 | + | - | - | + | - |
| L11 | + | + | - | + | - |
| L12 | + | + | - | + | + |
| L13 | + | + | - | + | + |
| L14 | + | - | + | - | - |
| L15 | + | + | + | + | + |
| L16 | + | + | - | + | - |

*'+' stands for observed growth and '-' means no growth

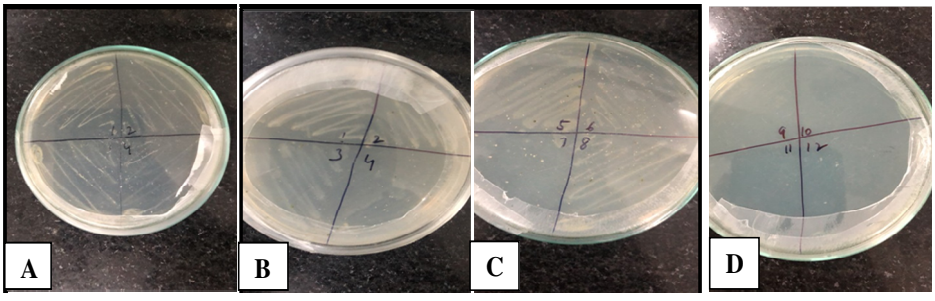
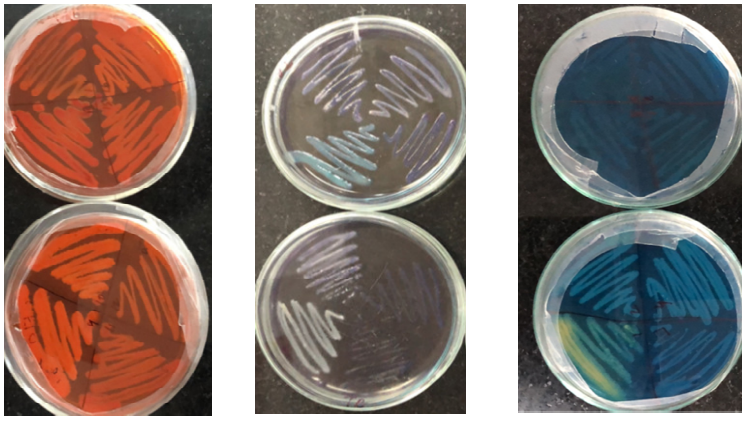



Figure 1: Growth of bacterial isolates on lignin and its monomers: veratryl alcohol (a), vanillin (b), lignin (c), guaiacol (d) after incubation of 7 days at 37 °C



(a) (b) (c)

Figure 2: Decolourization of Congo red, Toluidine blue and Indigo carmine dyes by ligninolytic bacterial isolates after incubation of 7 days at 37 °C

On the basis of the results obtained in the experiments described above regarding the growth of the bacterial isolates on the lignin monomers and the solid state dye decolourization assay, four isolates, namely, L8, L12, L13 and L15, were selected for further testing. These four isolates showed maximum growth on most of the monomers tested and maximum decolourization of the dyes on solid medium.

***Liquid medium dye decolourization assay***

The four selected isolates were further examined for their ability to decolorize synthetic lignin like dyes in liquid medium. The percentage decline of decolourization was calculated after every 24 h for about 5 days at 37 °C.[18] In the case of Congo red, the maximum percentage decolourization was observed for bacterial isolate L8, *i.e.* 97%, while the minimum percentage decline was recorded for bacterial isolate L12, *i.e.* 84% after incubation for 24 h. After 144 h, the maximum percentage decline of decolourization was observed for bacterial isolate L8 and the minimum – for L15 (Fig. 3a). In the case of Azure-B, the maximum percentage decolourization was observed in bacterial isolates L8 and L13, *i.e.* 93%, and the minimum – for L15, *i.e.* 90% after incubation for 24 h (Fig. 3b). Regarding the degradation of Indigo carmine, the maximum percentage decline was noticed for bacterial isolate L15, *i.e.* 93%, and the minimum – for L12, *i.e.* 88%, after incubation for 24 h. At the end of the experiment, *i.e.* after 144 h, the maximum percentage decolourization was recorded for bacterial isolate 13 and the minimum – for L12 (Fig. 3c). In the case of Toluidine blue, the maximum percentage decolourization was observed for bacterial isolate L15, *i.e.* 96%, and the minimum – for L12, *i.e.* 91%, after incubation of 24 h. At the end of the experiment (after 144 h), the maximum percentage decolourization was recorded for bacterial isolate L15, *i.e.* 89%, and the minimum – for L13, *i.e.* 86% (Fig. 3d). It was observed that initially, within the first 24 hours of the assay, the rate of decolourization was high, but slowly decreased after that for all the four isolates with respect to each of the dyes used in the present study (Fig. 3).[24-25]

Previous studies have reported that it is difficult to culture bacterial strains by using dyes as the sole source of energy. No decolourization or short duration decolourization may be caused by the requirement of labile carbon and nitrogen sources.[30] With supplementation of the carbon source, for example, with glucose, and of the nitrogen source, as with a yeast extract, extended decolourization of dyes may be possible. Thus, medium composition is directly related to the bacterial dye decolourization efficiency. Also, azo dye decolourization needs an electron donor substrate in the medium, which can induce dye degrading enzymes.[26]

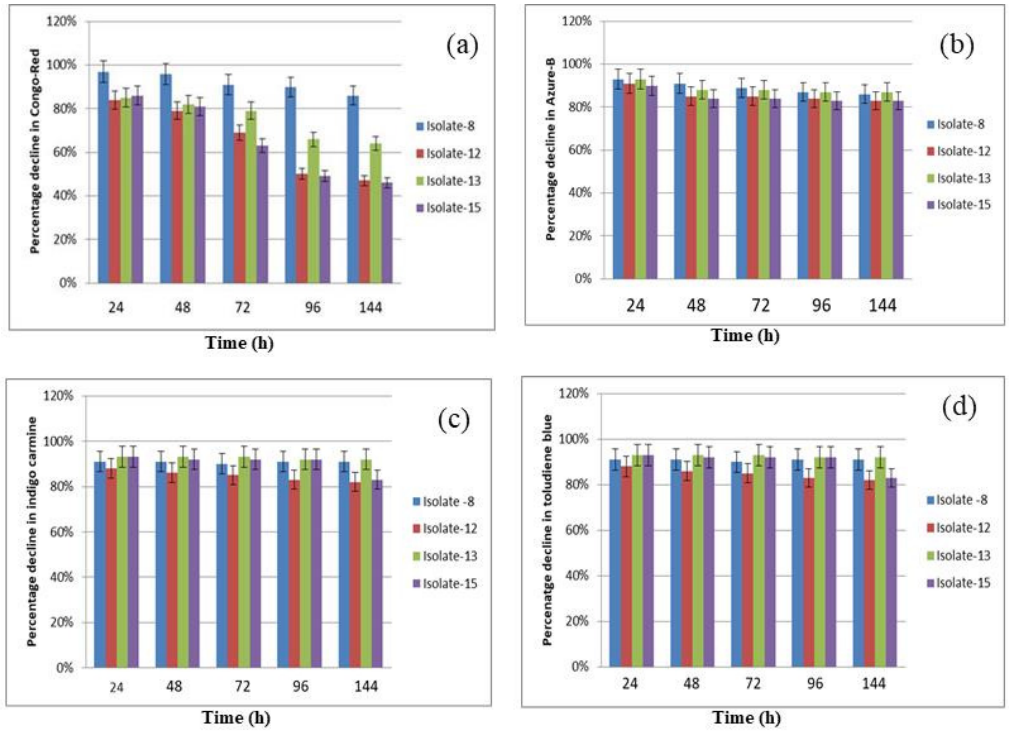



Figure 3: Decolourization percentage of ligninolytic indicator dye; *i.e.* Congo red (a), Azure-B (b), Indigo carmine (c), and Toluidine blue (d) of selected isolates L8, L12, L13 and L15

**Enzymatic activity of isolated bacteria**

The estimation of the enzymatic activity (laccase, lignin peroxidase and manganese) was performed for the four selected bacterial isolates, *i.e.* L8, L12, L13 and L15. The enzyme activity was expressed in units/min/mL of the enzyme extract. It has been observed previously that the enzymatic activity of manganese peroxidase, lignin peroxidase and laccases occurs in presence of the specific substrates, such as phenols, polycyclic aromatics and polyamines.[27] In the present study, higher laccase activity was recorded in bacterial isolates L12, L13 and L15 after an incubation period of 15 min, in contrast to bacterial isolate L8. After an incubation period of 30 min, a decline in laccase enzyme activity was observed in all 4 bacterial isolates. After the incubation period of 2 h, a significant drop in laccase activity was noted for L8, as compared to the rest (Fig. 4a).

In the case of the lignin peroxidase enzyme, higher enzymatic activity was observed in bacterial isolate L15 after an incubation period of 15 min, compared to the other bacterial isolates, while after 30 min of incubation, a decline in lignin peroxidase activity occurred in all the 4 bacterial isolates. After completion of the experiment, *i.e.* after 2 h, the maximum decline of enzymatic activity was recorded in bacterial isolate L15 and the minimum – in L13 (Fig. 4b).

For manganese peroxidase activity, it was noted that higher enzymatic activity was exhibited by bacterial isolate L13 after an incubation period of 15 min, as compared to the other isolates. Interestingly, after 30 min of incubation, the manganese peroxidase activity increased (Fig. 4c) in all the 4 bacterial isolates. After completion of the experiment, *i.e.* 2 h of incubation, the maximum increase in enzyme activity was noted for bacterial isolate L13 and the minimum increase – for L8.

Azure-B dye is the most suitable for testing the ability of the isolates for degrading synthetic dyes, as this dye is not affected by MnP or laccases alone. However, it requires high redox potential agents, like lignin peroxidase. According to the results obtained from the liquid medium dye decolourization assay, all isolates have shown quite high ability to decolorize Azure-B. Similarly, lignin peroxidase activity was also found in all the isolates, with maximum activity recorded for L15.[4] Accordingly, it has previously been observed that in bacteria *Acinetobacter calcoacetius* and *Streptomyces viridosporus*, LiP was able to attack lignin monomers.[28] On the other hand, RBBR is an indicator of laccase

activity. In our study, only isolate L12 showed growth on the RBBR containing plate. However, high laccase activity was found in L12, L13 and L15. Hence, our results demonstrate a positive correlation between the production of ligninolytic enzymes and synthetic dye decolorization.

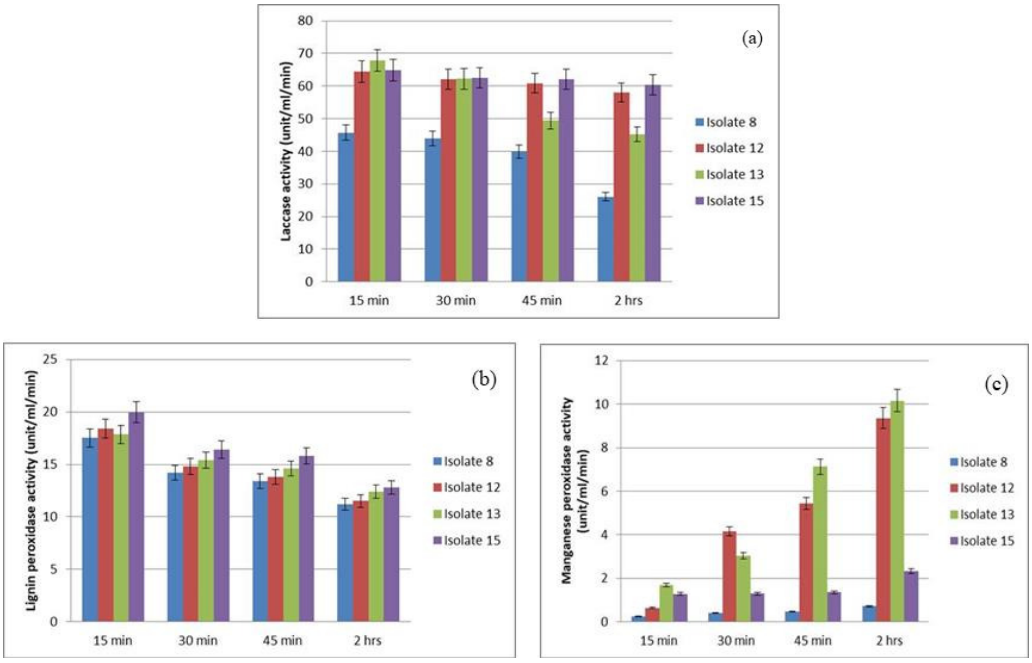



Figure 4: Laccase (a), lignin peroxidase (b) and manganese peroxidase (c) activities of selected bacterial isolates L8, L12, L13 and L15

Overall, according to the findings of this study, the best enzymatic activity was noticed in L15, which also showed growth on every lignin monomer. Thus, it can be concluded that L15 isolate has all the characteristics that recommend it as a lignin degrader and as a decolorizing agent, and could be used in bioremediation of industrial dye-polluted effluents.

## CONCLUSION

Increasing environmental awareness and concerns about the release of industrial synthetic dyes into water bodies, have directed research towards the treatment of these recalcitrant dyes by different methods. This study was carried out with the aim to isolate bacterial ligninolytic enzymes in order to attain compounds that can degrade various dyes. Sixteen isolates were obtained from soil samples collected from different sites. The isolates were grown on various lignin monomers. All the isolates were assayed for decolourization of various dyes. Out of sixteen, we reported four bacterial isolates that can be used to degrade various classes of dyes, including azo dyes, which are recalcitrant to degradation. Among these isolates, L15 was able to grow on all the tested lignin monomers, and was found to be the most efficient ligninolytic enzyme producer and dye decolourizer.

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