JETIR.ORG ISSN: 2349-5162 | ESTD Year : 2014 | Monthly Issue

JOURNAL OF EMERGING TECHNOLOGIES AND INNOVATIVE RESEARCH (JETIR)

An International Scholarly Open Access, Peer-reviewed, Refereed Journal

Isolation of Laccase producing microorganisms and production of extracellular laccase enzyme and its potential application in decolorization and degradation.

Dr. Reena G. Desai[1], Riya V. Lad[2], Khushi P. Shah[3]

[1]Head of the department, Microbiology.

[1,2,3]Dolat-Usha Institute of applied sciences and Dhiru-Sarla Institute of management and commerce, Valsad, India.

Veer Narmad South Gujarat University, Surat, India.

***Abstract*:** This study has been undertaken to investigate the determination of Laccase and its application in bioremediation. Laccases are a family of copper-containing oxidases with important applications in bioremediation and other various industrial and biotechnological areas. Laccase producing bacteria were isolated from local regions of Valsad district using an enrichment culture technique. The bacterial isolates were grown on Luria Bertani agar medium containing 0.5 mM guaiacol for detection of extracellular laccase enzyme. The bacterial isolates produced reddish brown coloration around the colonies was further purified and plate assayed on the same media for laccase enzyme production. In liquid culture, the highest laccase production (0.048 U/ml) was achieved on the 24-48 h of incubation at room temperature and pH 7.0. The Luria Bertani broth medium was optimized with different carbon sources as well as nitrogen sources and gives maximum laccase activity at carbon source glucose (16.66 U/ml) and nitrogen source fallen leaves (33.20 U/ml). Dye decolorization were done with Reactive blue 171 at different concentrations 100ppm, 200ppm and 300ppm and dye were decolorized 86.61%, 78.08% and 76.30% respectively. Bioremediation of pesticides like Cypermethrin and Chlorpyriphos was done which was 71.73% and 56.73% respectively.

***IndexTerms* – Isolate, sources, laccase, Guaiacol, Bioremediation, Pesticide**

I. INTRODUCTION

Recent focus of global countries is shifting towards concerns over environmental protection. In developing countries, the discharge of untreated effluents is checked by the regulations of environmental legislations. Due to the higher cost involved in the removal of dyes industries face the problem of treatment in industrial effluent containing dyes since the effluents consists of various chemical groups such as azo, anthraquinone and triphenylmethane dyes. The harmful effect of untreated effluents is seen adversely in various biotic environments ranging from phytoplanktons, aquatic organisms, zooplanktons, and human beings. In humans, the carcinogenic dyes present in effluents released can cause skin allergies, dermatitis, lung problems such as asthma, and problems in nervous system. Out of 72 toxic chemicals found in textile effluents, 30 chemicals cannot be removed easily besides the color of the dye interrupts with aquatic environmental balance by interfering with photosynthesis of phytoplankton.

Textile effluents consist of more than 10,000 different chemicals where 70% of these chemicals employed in dyeing industries are azo dyes. Azo dyes are resistant to degradation and decomposition because of their higher stability because of one or two strong azo bonds between chromophore and auxochrome molecules. Laccases have been extensively studied for their degradation of azo dyes. These enzymes are multicopper phenol oxidases that decolorize azo dyes through a highly nonspecific free radical mechanism forming phenolic compounds, thereby avoiding the formation of toxic aromatic amines. Over long periods of time, there can be a coupling between the reaction products, and even polymerization.

II. MATERIALS AND METHODS

Isolation of bacterial isolates

All the bacteria were isolated from soil contaminated with saw dust from saw mill Kalwada (Sabina *et al.,* 2020), bio deteriorated paper from paper and pulp industry Gundlav (Dhiman and Poonam shrirkot, 201**5**) and garage surface soil from garage in Valsad (Lisnawati *et al.,* 2016), on Luria Bertani agar medium (Evangelos Topakas *et al.*,2019).

Screening of laccase producing bacteria

All collected isolates were cultured on Petri plates containing sterilized Luria Bertani agar (LB) supplemented with 0.5 mM Guaiacol and adjusted at pH 7. These Petri plates were incubated at room temperature for 24-48 h and then screened for reddish brown colored colonies of laccase producers (Hemaraju S. and Narasegowada P. 2018).

Composition of medium include (g/l): Peptone (1 g), Yeast extract (0.5 g), NaCl (1 g), Agar (2.7 g), Guaiacol (0.062 g) in 100 ml distilled water.

Fermentation process for growth and enzyme production

Standard Luria Bertani medium was prepared for the inoculum of the isolates were cultivated into 250 ml flasks containing 100 ml of productive liquid medium (Hemaraju *et al*., 2018) which contained the following: 10 g Tryptone, 5 g Yeast extract, 10 g sodium chloride, 0.5 mM guaiacol and pH 7, and then incubated at room temperature for 24-48 hours. Bacterial growth and enzyme activity were assayed periodically.

Guaiacol assay method for laccase assay

Oxidation of guaiacol has been reported for laccase assay by (Enas A. Hassan[b] Elshahat M.Ramadan[b],2016) Guaiacol assay method. The reddish-brown color developed due to oxidation of guaiacol by laccase is used to measure enzyme activity at 450 nm. The reaction mixture can be prepared: (a) Guaiacol 1 ml (b) Sodium acetate buffer 3 ml (c) Enzyme source 1 ml.

A blank was also prepared that contains 1 ml of distilled water instead of enzyme. The mixture was incubated at room temperature for 15 min and the absorbance was read at 450 nm using UV spectrophotometer. Enzyme activity was expressed as International Units (IU), where 1 IU is the amount of enzyme required to oxidize 1 µmol of guaiacol per min. The laccase activity in U/ml is calculated by this formula:

$$\text{Enzyme activity} = \frac{A \times V}{t \times e \times v}$$

Where, E.A = Enzyme activity, *A* = Absorbance, *V* = Total mixture volume (5ml), *v* = enzyme volume (1 ml), *t* = incubation time, *e* = extinction coefficient for guaiacol (5,250 µM/cm).

Effect of different carbon sources, nitrogen sources, temperature, pH and incubation period on enzyme production

In order to record the optimum temperature, pH value and incubation period for laccase enzyme production, the productive medium was prepared and inoculated by standard inoculum of isolates as mentioned before. Four carbon sources, i.e., glucose, molasses, sucrose and waste fruit peels were applied. Five different nitrogen sources, i.e., urea, yeast extract, human urine, animal urine and fallen leaves were investigated. Two different temperatures, i.e., room temperature and 37 °C were investigated. Also, different levels of initial pH values ranged from 5-7 were applied. The proper time for the maximum laccase production was detected during 168 h fermentation period on productive medium. One flask containing 100 ml medium was taken as a sample periodically every 24 h, and centrifuged for getting laccase enzyme activity in supernatant.

Characterization of enzyme activity

Effect of temperature and pH of buffer during the oxidation of guaiacol reaction was studied. Temperature was studied by incubating the enzyme mixture containing enzyme, guaiacol and sodium-acetate buffer at different carbon sources, nitrogen sources, temperatures and pH. After incubation for 15 min, the absorbance of enzyme catalyzed reaction was recorded. Then the optimum temperature and pH of the enzyme activity were detected (Enas A. *et al.,* 2016).

Extraction of crude laccase enzyme

The supernatant of cultivated culture was saturated by 60%,70% and 80% ammonium sulfate to recover the extracellular protein enzyme and then centrifugate at 10,000 rpm for 15 min. The pellet was dissolved in 10 mM phosphate buffer (pH 6.5). The sample was dialyzed by a large volume of 10 mM phosphate buffer (pH 6.5) using dialysis membrane. Dialyzed product was kept in the refrigerator at 4 °C (Enas A. *et al.,* 2016).

Application of laccase enzyme to decolorize dye

Different concentrations of Reactive blue dye like 100 ppm, 200 ppm and 300 ppm were prepared in Luria Bertani broth and extracellular laccase were inoculated in this broth containing dye. Control sample was prepared in parallel by adding acetate buffer instead of laccase under the same conditions. All measurements were done in triplicate. The absorption spectrum of pigment

was measured by Spectrophotometer at the specific wavelength 620 nm. The effect of pigment decolorization was determined by the decrease in absorbance under the maximum wavelength of the dye. The efficiency of decolorization was expressed in terms of decolorization percentage (%) (Enas *et. al.*, 2016).

$$\text{Decolorization (\%)} = \frac{\text{(Initial absorbance - Observed absorbance)}}{\text{Initial absorbance}} \times 100$$

Application of laccase enzyme in pesticides degradation

Different concentrations of pesticides like 100ppm, 200ppm and 300ppm of Chlorpyriphos and Cypermethrin were prepared in Luria Bertani broth and extracellular laccase were inoculated in this broth containing pesticides along with controls. The absorption was measured by spectrometer and efficiency of decolorization was expressed in terms of degradation percentage as above.

III. RESULTS AND DISCUSSION

Enrichment of samples

Samples like saw dust, garage soil sample and sample from paper and pulp industry waste were collected from local region of Valsad were enriched in nutrient broth (pH-7) and this broth showed turbidity after 24-48 hours at room temperature.

Isolation and screening for extracellular laccase producing bacteria

A total of 8 laccase producing strains were isolated from the selected 3 different environmental samples. The extracellular laccase activity was detected in the three isolates namely S, G and PS3. Isolate S and isolate G were both Gram-positive rod-shaped bacteria while isolate PS3 was a coccobacillus. These all three isolates gave brown colored colonies on Luria Bertani agar plates which containing 0.5 mM guaiacol. The strain PS3 produced maximum laccase (33.20 U/ml) followed by G (18.22 U/ml) and S (16.38 U/ml).

Enzyme activity and partial purification of crude enzyme

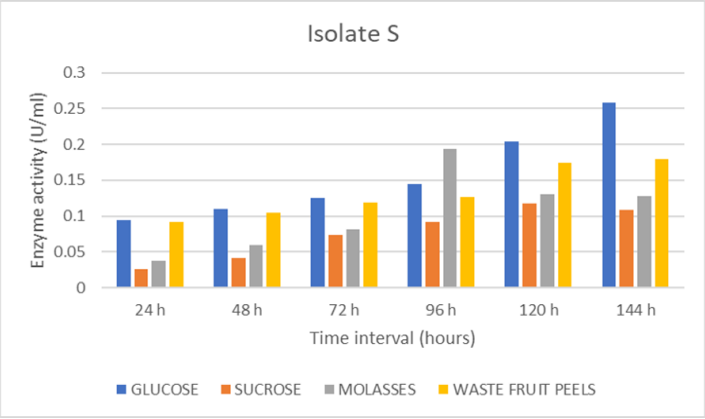
Isolated six strains were checked for enzyme activity by guaiacol assay method. The selected strain PS3 showed maximum laccase activity (0.048 U/ml) followed by S (0.045 U/ml) and G (0.027 U/ml). The development of pale-yellow color shows the production of laccase in guaiacol assay method. Crude enzyme obtained was precipitated within the range of 60-80% of ammonium sulphate. At 70% ammonium sulphate saturation, the highest activity was obtained (0.124 U/ml) after 1 h incubation in refrigerator for saturation.

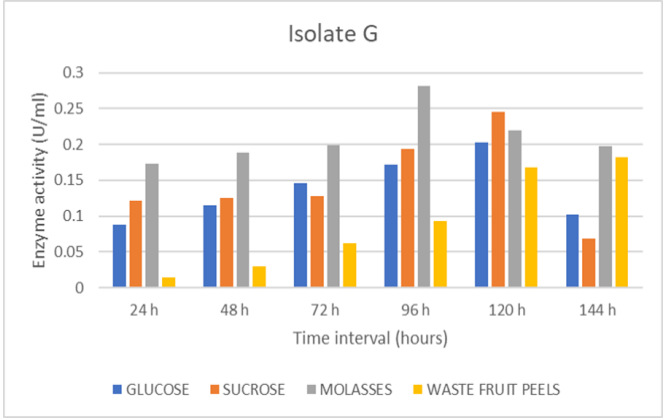
Effect of incubation period on the extracellular laccase activity

The isolated strains were inoculated in the growth medium to know at how much hour of incubation the maximum laccase production was achieved. Isolated strain PS3 (33.20 U/ml) showed maximum laccase production until 168 hours of incubation. Whereas, S (12.88 U/ml) showed maximum growth till 144 hour and then their growth rate was started to decline. Isolated strain G (18.22 U/ml) showed their growth till 144 hours, then their growth rate was decline.

Effect of carbon sources on the extracellular laccase activity

The type of carbon source in the medium plays a major role in the production of laccase enzyme. Luria Bertani medium (pH-7) were optimized with different carbon sources like glucose, sucrose, molasses and waste fruit peels which having 0.5 % concentration. Among all the given carbon sources used in present study, the carbon source glucose showed maximum laccase production for the strain PS3 (26.66 U/ml) and S (16.38 U/ml) except G showed maximum laccase production with sucrose (15.55 U/ml).





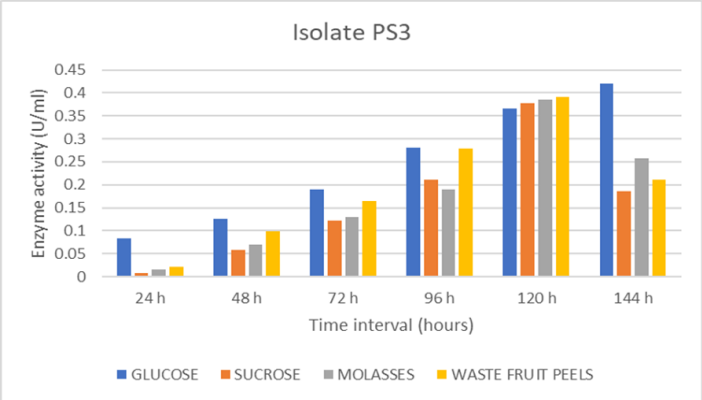


Figure 1: Effect of carbon sources on isolates

Effect of different nitrogen sources on the extracellular laccase activity

The nitrogen is primarily metabolized to produce amino acids, proteins, nucleic acid and cell wall components in microbes. Luria Bertani medium (pH-7) with guaiacol concentration 0.5 mM were optimized with different nitrogen sources like yeast extract, urea, human urine, animal urine as well as fallen leaves which having 0.5 % concentration. Above all the nitrogen sources PS3 as well as G produce maximum laccase with fallen leaves with enzyme activity 33.20 U/ml and 21.84 U/ml respectively except S showed maximum production 12.88 U/ml with animal urine as a nitrogen source.

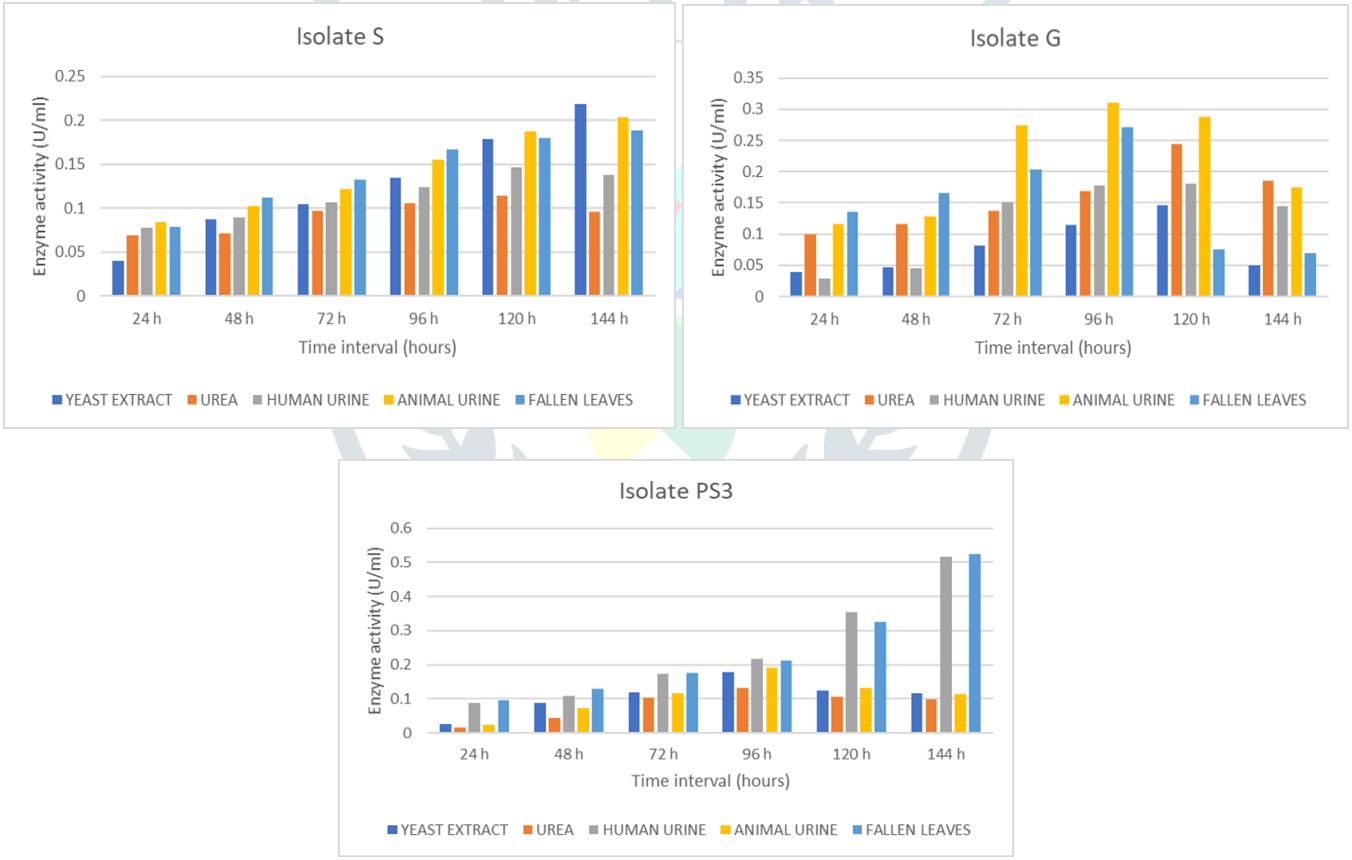
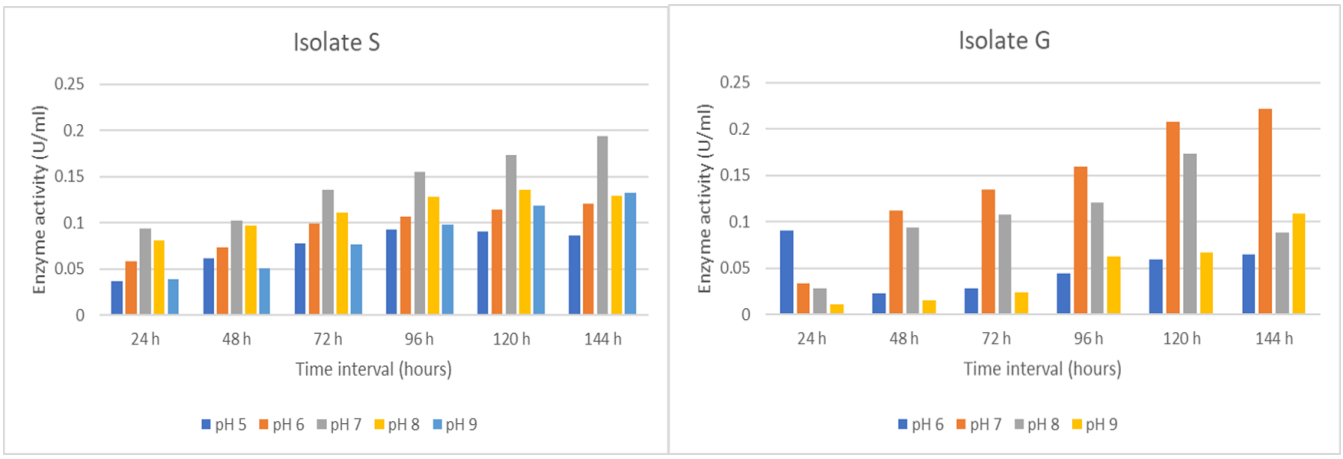


Figure 2: Effect of nitrogen sources on isolates

Effect of different pH on extracellular laccase activity

The pH optima for the extracellular laccase produced by the strains namely S, G and PS3 were determined by over the range of 5 to 10. The highest laccase activity found with the pH 7 for the all three isolates were 31.80 U/ml,8.69 U/ml and 7.92 U/ml for PS3, S and G, respectively. Growth of isolate S was inhibited with increased pH at pH 10. For isolate G pH-5 as well as pH-10 was not favorable.



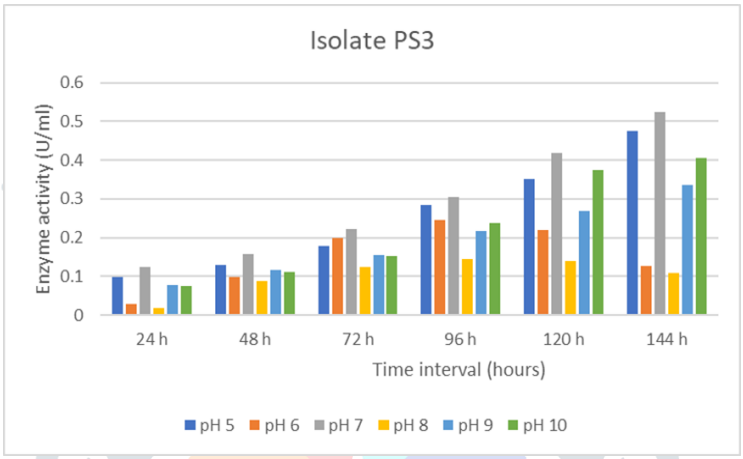


Figure 3: Effect of pH variation on isolates

Effect of different temperature on extracellular laccase activity

One of the key factors determining the suitability of the enzyme laccase in industrial application is its thermostability. Hence, the thermostability of the laccase produced by the isolated strains was assessed by incubating the isolates at two different temperatures room temperature and 37˚c. All of the isolated strains showed growth at room temperature. When strains incubated at 37˚c, the growth of isolates were inhibited and the only color change of the medium was observed.

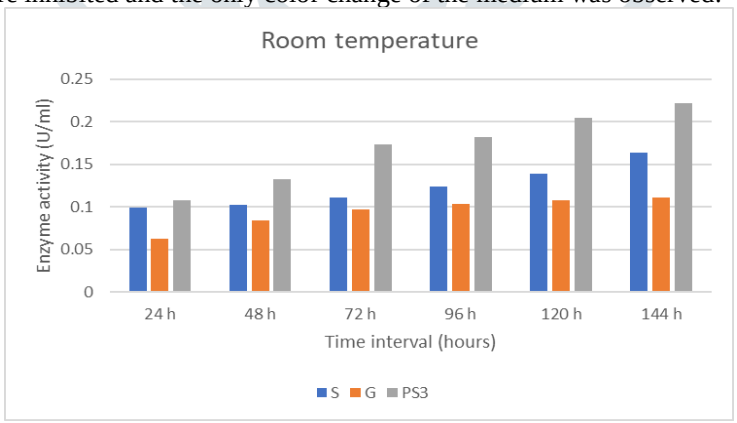


Figure 4: Effect of room temperature on isolates

Effect of incubation condition on extracellular laccase activity

Incubation condition also play major role in growth of microorganisms. All the six isolated strains S, G and PS3 showed their maximum growth only when they incubated at room temperature in static condition compare with shaking condition.

Enzyme activity and partial purification of extracellular laccase after optimized condition

Isolated six strains were optimized with different factors like pH range from 10 and temperature like room temperature and 37˚c, different carbon and nitrogen sources like glucose, sucrose, molasses as well as waste fruit peels and yeast extract, urea, human urine, animal urine as well as fallen leaves, respectively. The maximum laccase production achieved after optimization for the strain PS3 (26.66 U/ml), G (18.22 U/ml) and S (16.38 U/ml).

Comparison of unoptimized and optimized conditions

From the below table, it was clearly observed that enzyme activity of all isolates was much higher in optimized conditions compared to unoptimized conditions.

Table 1: Comparison of all isolates in two different conditions

Isolates	Unoptimized condition	Optimized condition
S	0.045 U/ml	16.38 U/ml
G	0.027 U/ml	18.22 U/ml
PS3	0.048 U/ml	26.66 U/ml

Partial purification of laccase enzyme

Partial purification of laccase enzyme was done with ammonium sulphate precipitation method. Crude enzyme obtained from optimized fermentation medium was precipitated with the range of 60-80% of ammonium sulphate. At 70% ammonium sulphate saturation, the highest activity was obtained (0.199 U/ml) after 1 h of incubation.

Application of extracellular laccase enzyme in selective dye decolorization

Decolorization of reactive blue 171 dye was carried out with three different concentrations like 100ppm, 200ppm and 300ppm. At 100ppm concentration the highest dye colorization of 80.78% was given by isolate G, followed by isolate S with 74.03% decolorization. Consortium of all isolates and isolate PS3 gave 61.29% and 46.95% decolorization, respectively. At 200ppm concentration, isolate S gave the highest dye decolorization which was 78.08%. This was followed by isolate G with 74.06%, consortium gave 70.65% and lastly isolates PS3 showed 52.71% decolorization. At 300ppm concentration, highest dye decolorization achieved by PS3 isolate which was 76.30%.

Table 2: Decolorization of Reactive blue 171 by all isolates and consortium

Isolates	100ppm	200ppm	300ppm
S	74.03%	78.08%	54.02%
G	80.78%	74.06%	61.68%
PS3	46.95%	52.71%	76.03%
Consortium	61.29%	70.65%	54.33%

Application of extracellular laccase enzyme in selective pesticide degradation

Degradation of two selective pesticides Chlorpyriphos and Cypermethrin was carried out using three different concentrations like 100ppm, 200ppm and 300ppm. Maximum degradation of Chlorpyriphos at 100ppm concentration was given by isolate PS3 which was 56.73%, followed by consortium degrade 53.02%, isolate G and S showed 32.41% and 29.77% degradation, respectively. At 200ppm concentration, Cypermethrin degradation was 58.74% by isolate PS3 which was followed by isolate G that was 54.21%. Then isolate G and consortium had given 25.97% and 25.25% degradation, respectively. The highest degradation of Chlorpyriphos at 300ppm concentration was 32.34% which was given by isolate PS3, followed by 32.05% with isolate G which was almost similar to PS3. Then consortium gave 30.50% degradation that was nearly similar to isolate G and isolate S gave 26.45% degradation. At 300ppm concentration, Cypermethrin pesticide was degrade the highest by isolate PS3. The isolate S showed 36.05% degradation. This was followed by 35.24% degradation by isolate and 37.33% degradation by consortium of all isolates.

Table 2: Degradation of selected pesticides by all isolates and consortium

Isolates	Chlorpyriphos			Cypermethrin		
	100 ppm	200ppm	300 ppm	100 ppm	200 ppm	300 ppm
S	29.77%	35.86%	26.45%	32.12%	25.97%	36.05%

G	32.41%	27.89%	32.05%	22.75%	54.21%	35.24%
PS3	56.73%	47.43%	32.34%	71.73%	58.74%	39.03%
Consortium	53.02%	24.88%	30.05%	42.36%	25.25%	37.33%

IV. CONCLUSION

In conclusion, the present review provides comparative information of laccase producing bacteria in unoptimized and unoptimized condition and their applications in decolorization as well as degradation. If this research satisfies these requirements in the future, the use of Laccase will represent a valid means for the purification of textile effluents and other pollutants in the ecosystem.

V. REFERENCES

1) Ambika V., Karuna D. & Poonam S. 2015. Optimization for Laccase by *Pseudomonas putida LUA15.1* using Response Surface Methodology. Journal of pure and applied microbiology 9(2): 1247-1256.
2) Andrea Z., Barbara G., Astrid R. & Artur C. P. 2005. Degradation of Azo dyes by *Trametes villosa* Laccase over long periods of oxidative conditions. Applied and environmental microbiology 71(11): 6711-8.
3) Enas A. Hassan, Elshant M. Ramadan, Rehan A. Abd El Monssef 2019. Production of laccase enzyme for their potential application to decolorize fungal pigments on aging paper and parchment. Annals of agricultural sciences 61(1). DOI: 10.1016/j.aoas.2015.11.007.
4) Evangelos T., Anastasia Z., Stefan S., Jasmina N. R., 2019. Applications of Microbial Laccases: Patent review of the past decade (2009-2019). Catalysts 9(12): 1023. DOI: 10.3390/catal9121023.
5) Hemaraju S., Narasegowda P. N., 2018. Screening and characterization oh high laccase producing *Enterobacter sp.* From Devarayanadurga Forest Soil, Tumkur District. International journal of pure and applied Bioscience 6(4): 203-213.
6) Lisnawati, Nunik A., Nita A., Harist N. R., Dwi Yulian F. S. 2016. Biochemestrical and environmental applications: Utilization of laccase enzyme as Bioremediation in Petroleum industry waste by reducing Polyaromatic Hydrocarbon. International journal of advances in science, engineering and technology, pp. 124-130.
7) Ramsay, J. A., Nguyen, T., 2002. Decolorization of textile dyes bye *Trametes versicolour* and its effects on dye toxicity. Biotechnol. Lett. 24, 1757-1761.