Available online at www.ijpab.com

DOI: http://dx.doi.org/10.18782/2320-7051.2672

ISSN: 2320 – 7051

Int. J. Pure App. Biosci. **5 (2):** 913-922 (2017)

Research Article

International Journal of Pure & Applied Bioscience

OPEN ACCESS

Biodegradation of Malachite Green by Extracellular Bacterial Laccase and Its Phytotoxicity Studies

Ambika Verma*, Karuna Dhiman and Poonam Shirkot

Department of Biotechnology, Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan-173230 (H.P.), India

*Corresponding Author E-mail: ambikaverma9@gmail.com

Received: 11.03.2017 | Revised: 25.03.2017 | Accepted: 26.03.2017

ABSTRACT

Laccase producing bacteria, Pseudomonas putida LUA15.1 was isolated from rice rhizospheric soil samples of paddy fields of Una district, Himachal Pradesh (India). Malachite green (60 mg/l) was 87.7% decolorized within 48 hrs by Pseudomonas putida strain LUA15.1. Purification of laccase enzyme was done by using ammonium sulfate precipitation, dialysis, gel filtration chromatography and ion exchange chromatography and 92.47% decolorization of malachite green (60 mg/l) was observed by purified laccase of Pseudomonas putida LUA15.1. UV-Visible absorption spectrum showed the decolorization of malachite green, and phytotoxicity studies revealed the degradation of malachite green into non-toxic products by purified laccase of Pseudomonas putida LUA15.1. Seed germination, length of plumule and radicle of Phaseolus aureus were significantly affected by malachite green than its degradative metabolites indicating less toxic nature of degradation metabolites as compared to dye. It was therefore concluded that Pseudomonas putida LUA15.1 and its extracellular laccase has a good potential for use in the treatment of industrial effluent containing malachite green.

Key words: *Decolorization, Pseudomonas putida, malachite green, phytotoxicity, Phaseolus aureus*

INTRODUCTION

Industrialization is requisite to a nation's economy because it serves as a medium for the development. The extensive usage of synthetic dyes in industries such as textile, paper, plastics, printing, leather, cosmetics, and pharmaceuticals has led to releasing of industrial effluents containing various toxic products into the environment[1,2]. Many of these products are problematic because of their persistence, low biodegradability and high toxicity. Many reports indicate that textile industry effluent have toxic effect on the germination rates and biomass concentration of several plant species which play important ecological functions such as providing the habitat for wildlife, protecting soil from erosion and providing huge bulk of organic matter that is significant to soil fertility.

Cite this article: Verma, A., Dhiman, K. and Shirkot, P., Biodegradation of Malachite Green by Extracellular Bacterial Laccase and Its Phytotoxicity Studies, *Int. J. Pure App. Biosci.* **5(2):** 913-922 (2017). doi: http://dx.doi.org/10.18782/2320-7051.2672

The toxicity of effluent is because of the presence of dyes or their products which are mutagenic or carcinogenic. Thus, the wastewater must be treated before releasing into the natural environment. Among various physicochemical and biotechnological techniques, the enzymatic removal of synthetic dyes is the most preferred method due to its efficiency at high and low pollutant concentration over a wide range of pH and temperature, low energy required, minimal impact on ecosystem, and less sludge production in the decolorization process[3,4,5]. Biological processes present eco-friendly and cost competitive alternatives to abiotic treatment. Amongst the methods used in biological treatment of wastewater containing dyes, the microbial decolorization and degradation of dyes has been of considerable interest[6,7]. Bacterial treatment offers a cheaper and environment friendlier alternative for color removal in textile effluents[8]. The potential of microbes to degrade synthetic dyes have been linked with the production of enzymes during degradation[9,10]. The three major classes of these enzymes include laccases, lignin peroxidases and manganese dependent peroxidases. Laccase has been shown to be of critical importance to the dye-degrading potentials and various recent studies have demonstrated the versatility of laccases as industrial biocatalyst[7,10-13]. In this study, the ability of laccase from *Pseudomonas putida* LUA15.1 to degrade Malachite Green was investigated and the phytotoxicity experiments were also performed *in vitro* and *in vivo* on *Phaseolus aureus* to evaluate the toxicity of untreated and laccase treated dye.

MATERIALS AND METHODS

Microbial Strain

The microbial culture used in study was *Pseudomonas putida* LUA15.1, isolated from rice rhizospheric soil samples of paddy fields of Una district, Himachal Pradesh (India).

Decolorization of Malachite Green by *Pseudomonas putida* LUA15.1

Pseudomonas putida LUA15.1 was tested for its ability to decolorize malachite green. To determine the effect of dye concentration on decolorization activity of *Pseudomonas putida* LUA15.1, decolorization assays with varing initial dye concentrations between 20-100 mg/l were carried out. A loopful of microbial culture was inoculated in each of five 250 ml flasks containing 100 ml TY broth and incubated at 28^{o}C for 24 hrs. After 24 hrs of incubation, malachite green was added in each flask at concentrations of 20, 40, 60, 80 and 100 mg/l and 3 ml of the culture media was withdrawn at six hour time intervals between 0 hr and 48 hrs, from each flask respectively. Aliquots were centrifuged at 5000 rpm for 15 minutes to separate the bacterial cell mass, clear supernatants were used to measure the decolorization and decrease in color intensity of malachite green was observed at 620 nm. Spectral analysis of the samples was performed using UV-Vis Spectrophotometer (Simadzu UV-Vis 1800, Japan). Abiotic controls (without microorganism) were always included. Dye decolourization was expressed in terms of percentage calculated according to the equation.

$$\text{Decolourisation (\%)} = \frac{A_0 - A_t}{A_0} \times 100$$

Where, A_0 is an initial absorbance of malachite green and A_t is final absorbance after each time intervals.

Enzyme Assay

Pseudomonas putida LUA15.1 was inoculated into the Tryptone Yeast (TY) broth and flask was kept on a rotary shaker at 150 rpm for 24 hrs at 28^{o}C. The culture supernatant was obtained by centrifugation of overnight culture of *Pseudomonas putida* LUA15.1 at 10,000 rpm, for 10 mins at 4^{o}C and used for the enzyme assay. Laccase activity was measured by monitoring the oxidation of ABTS[14]. The reaction mixture was prepared by adding 0.5 ml of the enzyme solution on top of the ABTS (3 mM) substrate dissolved in 0.1 M

acetate buffer (pH = 4.5) and then it was incubated at 32°C. The oxidation of ABTS was determined by monitoring the increase in absorbance at 420 nm. And the one unit of a laccase activity was defined as the required amount of enzyme to oxidize 1 µmol of ABTS/min ($\varepsilon 420 = 36{,}000\ M^{-1}\ cm^{-1}$).

Optimization of Culture Conditions for Maximum Laccase Production

Pseudomonas putida LUA15.1 was further investigated to study effect of different factors such as incubation temperature, pH and incubation time on laccase enzyme production. The pH range was optimized using TY medium adjusted from 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 separately and temperature range for incubation investigated varied from 20-50^{o}C where as effect of different incubation times was studied for 24, 48, 72, 96, 120 and 144 hrs. In all cases optical density was monitored by using a double beam UV/VIS scanning spectrophotometer.

Purification of Laccase Enzyme

All steps of purification were performed at a temperature of 4^{o}C using 100 mM sodium phosphate buffer, pH 6.5. Techniques used for the purification of laccase enzyme were ammonium sulfate precipitation, dialysis, gel filtration chromatography and ion exchange chromatography. The enzyme preparations at various stages of laccase enzyme purification were analyzed for protein concentration and enzyme activity.

Decolorization of Malachite Green by Laccase Enzyme

In this study, it was investigated that whether the crude and purified laccase enzyme from *Pseudomonas putida* LUA15.1 can be used in the degradation of malachite green used in industry or not. For this purpose, malachite green was solublized in distilled water to the final concentration of 60 mg/l. The reaction mixture (6.0 ml) contained 2.0 ml acetate buffer (pH: 4.6), 2.0 ml of dye solution and 2.0 ml of laccase enzyme preparations followed by incubation at 37^{o}C for 0-48 hrs. Control sample was run in parallel without addition of laccase enzyme. The decolorization percentage was determined spectrophotometrically as the relative decrease of absorbance at each maximal absorbance wavelength of the dyes.

***In vitro* phytotoxicity study**

The effect of malachite green and its degradative metabolites on germination and early seedling growth of *Phaseolus aureus* was evaluated under *in vitro* conditions. The original dye solution and dye solutions degraded with crude and purified laccase enzyme preparations, were used for phytotoxicity studies. Seeds of *Phaseolus aureus* were sterilized first followed by dipping five seeds each in original dye solution, dye treated with crude enzyme preparation, dye treated with purified laccase enzyme preparation and in distilled water for about 4 hrs before transferring to the surface of the paper in petri dish. The seeds were germinated separately in sterile petri dishes, layered with sterile filter paper. The phytotoxicity study was carried out at room temperature in relation to *Phaseolus aureus* seeds (5 seeds per plate) by watering with respective solutions separately. Seeds germinated in water irrigated petri dish were used as a control. Length of plumule (shoot), radicle (root) and germination (%) were recorded for one week.

***In vivo* phytotoxicity study**

The effect of malachite green and its degradative metabolites on germination and early seedling growth of *Phaseolus aureus* was also evaluated under *in vivo* conditions. Similarly, the original dye solution and dye solutions degraded with crude and purified laccase enzyme preparations, were used for phytotoxicity studies. Five seeds of *Phaseolus aureus* were sown per pot at equidistance, at a uniform depth of 5 times diameter of the seed. The phytotoxicity study was carried out in relation to *Phaseolus aureus* seeds (5 seeds per pot) by watering with respective solutions separately *viz.*, original dye solution, dye treated with crude laccase enzyme preparation, purified laccase enzyme preparation and

distilled water. Seeds germinated in water irrigated pot were used as a control. Length of plumule (shoot), radicle (root) and germination (%) were recorded for one month.

RESULTS AND DISCUSSION

Decolorization of Malachite Green by *Pseudomonas putida* LUA15.1

The laccase producing bacterial isolate *Pseudomonas putida* LUA15.1 was tested for its ability to decolorize malachite green and it was found that this bacterial isolate was able to degrade this dye effectively (Figure-1). The strain demonstrated 87.70% decolorization of malachite green at 60 mg/l concentration, within 48 hrs (Figure-2). Absorbance values decreased from 1.86 at 0 hr to 0.78 at 24 hrs and further 0.24 at 48 hrs and significant decolorization of 53.76 % was observed after 24 hrs and 87.09% decolorization of malachite green was observed after 48 hrs (Figure-3). Thus, it have been depicted that bacterial isolate *Pseudomonas putida* LUA15.1 can degrade textile dyes successfully. It was also observed that decolorization efficiency increased with increasing concentration of malachite green up to 60 mg/l and over 87.70% decolorization was observed at 48 hrs at 60 mg/l of malachite green concentration (Figure-2). The initial increase in decolorization as the concentration increased may be due to induction of enzymes involved in decolorization[10]. It has been shown that dyes that act as inducers of enzyme production in a culture medium are in turn decolorized by the enzymes, and the highest inducer is decolorized highest[15]. Significant induction in DCIP reductase and MG reductase activities were observed during decolorization of malachite green by *K. rosea*[16]. When the concentration was increased to 100 mg/l however, decolorization efficiency dropped to 46.15%, indicating that malachite green may be toxic to the organism at the higher concentration levels. It has been shown that rate of decolorization of malachite green was decreased with increasing concentration of malachite green and also inhibits the growth of bacterium, which indicates toxicity of malachite green at higher dye concentration[10,16,17], while Cha et al.[18] and Youssef et al.[19] have also reported similar observation for inhibition of fungal growth at higher concentration of malachite green.

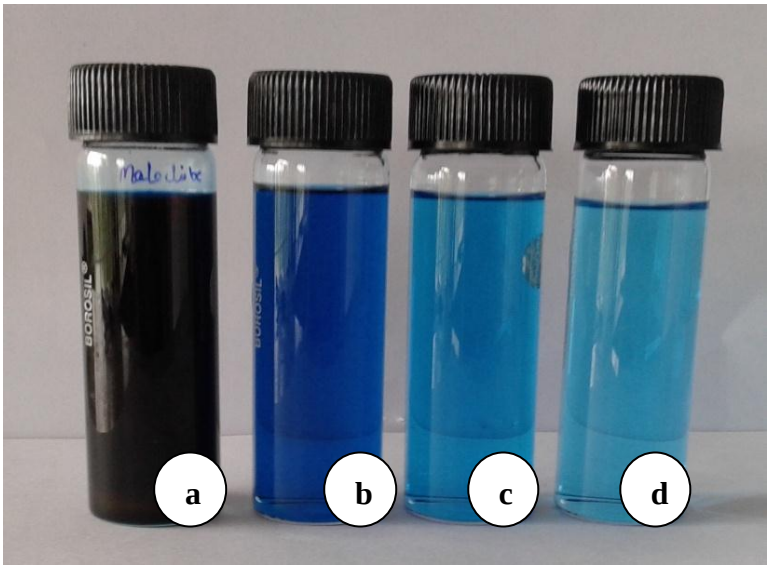

Fig. 1: Malachite green dye degradation a) Dye b) Dye + *Pseudomonas putida* LUA15.1 c) Dye + Crude laccase enzyme preparation d) Dye + purified laccase enzyme preparation respectively from left to right

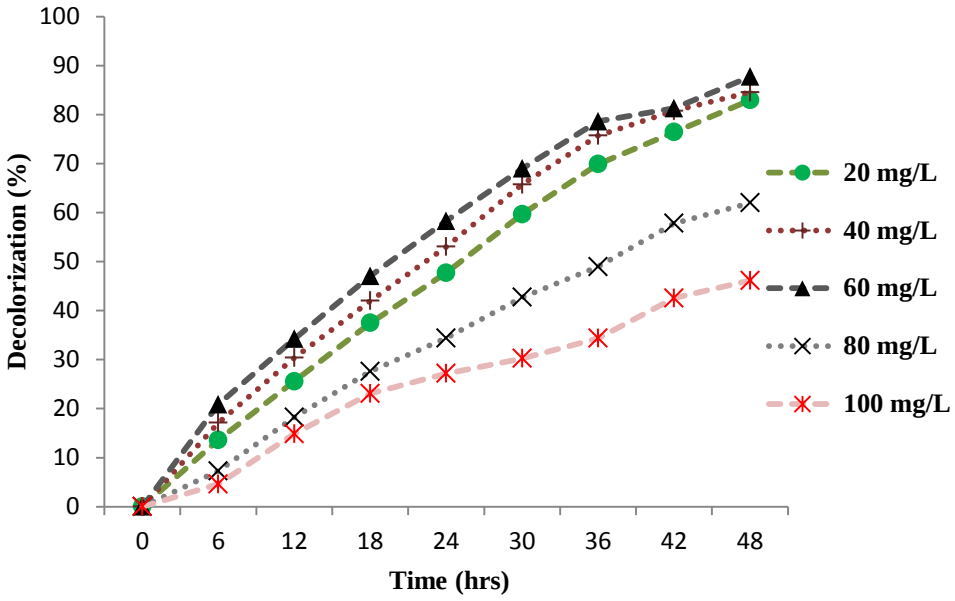

Fig. 2: Decolorization of various concentrations of malachite green by *Pseudomonas putida* LUA15.1

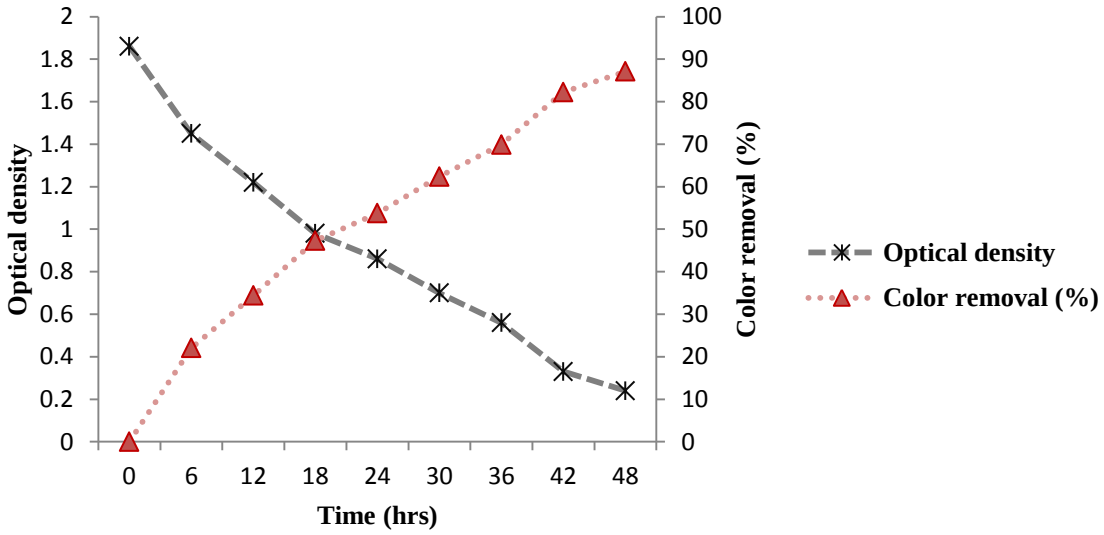

Fig. 3: Decolorization of malachite green (60 mg/l) by *Pseudomonas putida* LUA15.1

Optimization of Culture Conditions for Maximum Laccase Production

The isolate exhibited maximum growth OD of 1.51 at wavelength of 540 nm after 96 hrs, where as maximum extracellular activity of 58.10 U/l was observed after 24 hrs of incubation period. Maximum growth OD of 1.52 and maximum extracellular activity of 58.15 U/l was observed at pH: 7.0. The selected bacteria showed a maximum growth OD of 1.51 and maximum extracellular activity of 58.18 U/l at an incubation temperature of 28^{o}C. All these optimal conditions were used for malachite green

degradation by *Pseudomonas putida* LUA15.1 and observed 87.70% malachite green degradation after 48 hrs. Saravanakumar and Kathiresan[12] achieved 89% degradation of malachite green under optimal conditions of temperature 30^{o}C, pH of 5.8 and incubation period of 10 days.

Purification of Laccase Enzyme from *Pseudomonas putida* LUA15.1

Crude extracellular enzyme extract was subjected to ammonium sulphate (0-90%) saturation and it was found out that maximum laccase enzyme activity was detected in 50-90 percent level of saturation. The enzyme preparation at this stage was treated as ammonium sulphate fraction (ASF). The ASF (proteins precipitated after the overnight dialysis of 50-90% ammonium sulphate cut) exhibits the highest rate of precipitation and purified the enzyme 20.56 times with a 68.17% yield and was applied on Sephadex G-100 column equilibrated with sodium phosphate buffer (pH 6.5), purified the enzyme 28.30 times with a 32.69% yield. And in the last step, the enzyme fractions obtained and concentrated from Sephadex G-100 column were applied to the DEAE sephadex ion exchange column. It was found that laccase fractions were eluted with 1.0 M NaCl gradient which further purified laccase with 48.49 times purification and 10.08% yield (Table-1). Kumar et al.[20] developed simple cost effective and scalable purification procedure to purify extracellular laccase from *P. ostreatus* and obtained 161% yield with 27.8 fold purity, which has been found to have an enormous potential to degrade textile dyes.

Table 1: Purification of laccase from *Pseudomonas putida* LUA15.1

Steps	Total Enzyme Activity (U)	Total soluble protein (mg)	Specific activity (U/mg protein)	Fold purification	Percent yield
Crude extract	57.5	10833	0.00530	1.0	100
Ammonium sulphate precipitation	39.2	358	0.109	20.56	68.17
Gel filtration chromatography	18.8	125	0.150	28.30	32.69
Ion exchange chromatography	5.8	22.5	0.257	48.49	10.08

Decolorization of Malachite Green by Laccase Enzyme

Both crude and purified laccase enzyme preparations were investigated for malachite dye decolourization ability. Two ml each of laccase enzyme preparations were used in the decolourization study. It was found that 76.34% of decolourization of malachite green was observed after 24 hrs of incubation and then after 48 hours of incubation, 89.24% of decolourization was observed with crude laccase enzyme (Figure-4). Similarly, 83.33% of decolourization was measured after 24 hrs of incubation and it decolourized up to 92.47% after 48 hrs of incubation with purified laccase enzyme preparation (Figure-5). Thus it was observed that crude as well as purified laccase enzyme preparation also have ability to effectively degrade the textile dyes. The involvement of bacterial laccases in the decolorization of malachite green has also been reported[10,16] while Saravanakumar and Kathiresan[12], have also reported decolorization of malachite green by marine *Trichoderma* sp.

Malachite green was also reported to be efficiently decolorized with purified laccase[20].

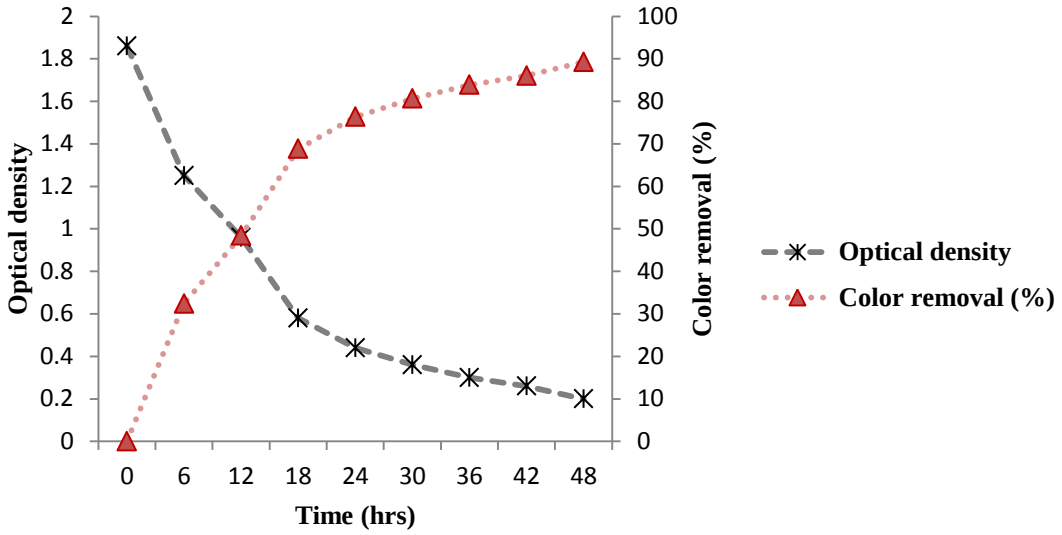

Fig. 4: Decolorization of malachite green (60 mg/l) by crude laccase enzyme of *Pseudomonas putida* LUA15.1

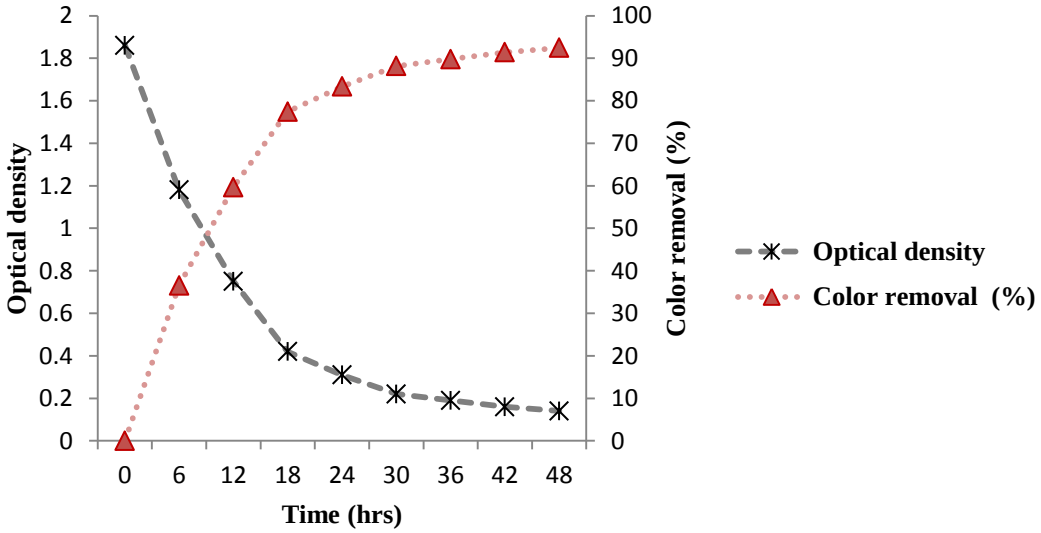

Fig. 5: Decolorization of malachite green (60 mg/l) by purified laccase enzyme of *Pseudomonas putida* LUA15.1

In vitro and *In vivo* Phytotoxicity Studies

Seed germination and plant growth bioassay are the most common technique used to evaluate the phytotoxicity. Thus, it was of primary aim to assess the phytotoxicity of the dye and its metabolites after degradation by crude laccase and purified laccase enzyme preparations. No germination of seeds of *Phaseolus aureus* was observed *in vitro* with malachite green treatment and degradation metabolites by crude laccase as compared to its degradation metabolites by purified laccase and distilled water (Figure-6, Table-2). The length of plumule and radicle were significantly affected by malachite green than its degradative metabolites (Table-2), indicating less toxic nature of degradation metabolites as compared to dye. Germination percentage of seeds (*Phaseolus aureus*) in pots irrigated with dye and its degradation

metabolites by crude and purified laccase were compared with water control and found to be 20%, 40%, 40% and 60% respectively, indicating toxic nature of the dye (Figure-7, Table-2) also showed similar toxicity of malachite green with severely affected plumule and radicle growth of *Phaseolus aureus*. Toxicity of malachite green on *Phaseolus aureus* was summarized in the table-2. Toxicity in terms of germination and growth of seeds dipped or irrigated with purified laccase enzyme treatment was less than crude enzyme and native dye compound. Parshetti et al. also showed the germination of *T. aestivum* was less with malachite green treatment compared to its degradation products and plain water[16]. Kumar et al[20]. reported 100 % seed germination with water whereas seed germination has been found to be inhibited when seeds were treated with malachite green.

Table 2: Phytotoxicity study of malachite green and its degradation metabolites on seed germination and growth of *Phaseolus aureus* under *in vitro* and *in vivo* conditions

Parameter studied	*Phaseolus aureus* *(In vitro)*			
	Water	**Malachite green**	**Malachite green treated with crude laccase enzyme**	**Malachite green treated with purified laccase enzyme**
Germination (%)	100	-	-	80
Plumule (cm)	2.92	-	-	1.9
Radicle(cm)	0.98	-	-	0.8
Parameter studied	*Phaseolus aureus* *(In vivo)*			
	Water	**Malachite green**	**Malachite green treated with crude laccase enzyme**	**Malachite green treated with purified laccase enzyme**
Germination (%)	60	20	40	40
Plumule (cm)	30.33	5.1	22.5	29
Radicle(cm)	5	1.5	5.5	6

Mentioned values in the table are mean of all germinated seeds in three sets.

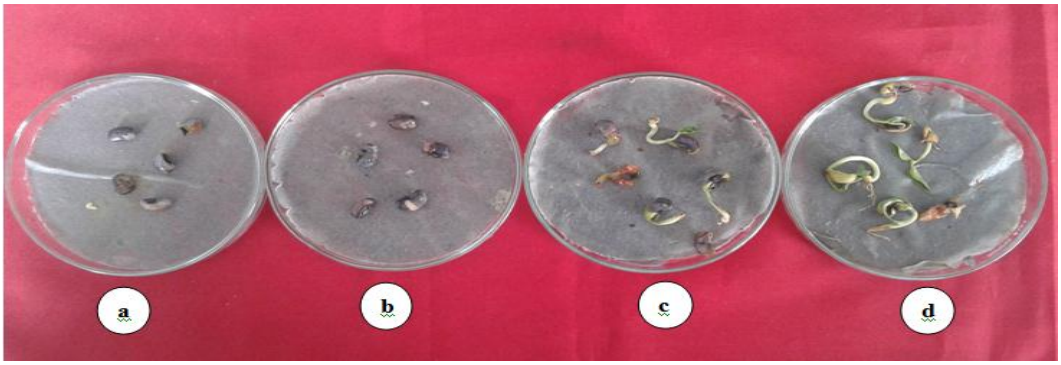

Fig. 6: Germination of seeds of *Phaseolus aureus* under *in vitro* conditions irrigated with a) malachite green dye b) Malachite green dye treated with crude laccase enzyme c) Malachite green dye treated with purified laccase enzyme d) water control, respectively from left to right

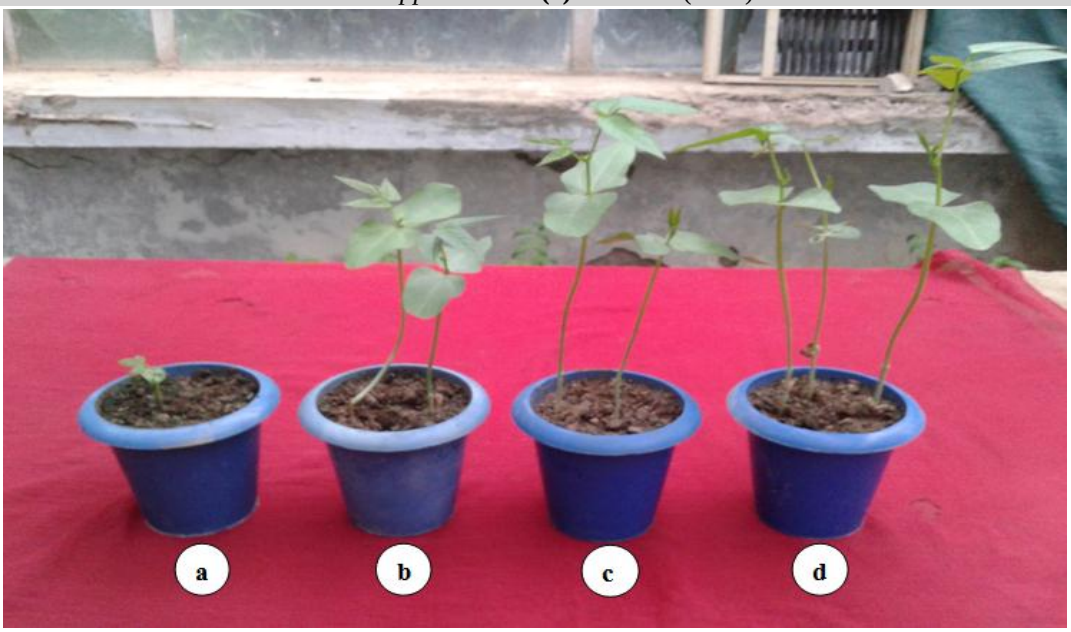

Fig. 7: Germination and growth of *Phaseolus aureus* under *in vivo* conditions irrigated with a) malachite green dye b) Malachite green dye treated with crude laccase enzyme c) Malachite green dye treated with purified laccase enzyme d) water control, respectively from left to right

CONCLUSION

The strain *Pseudomonas putida* LUA15.1 and its extracellular laccase, decolorize and degrade malachite green successfully. The obtained results display that the use of *Pseudomonas putida* LUA15.1 and its laccase has an enormous potential to degrade malachite green. Seed germination, length of plumule and radicle of *Phaseolus aureus* were significantly affected by malachite green than its degradative metabolites indicating less toxic nature of degradation metabolites as compared to dye. It was therefore concluded that *Pseudomonas putida* LUA15.1 and its extracellular laccase has a good potential for use in the treatment of industrial effluent containing malachite green. So this strain and enzyme can be used for treating textile wastewaters, particularly for water recycling.

Aknowledgement

The author acknowledges the financial assistance received from Department of Biotechnology at Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh to carry out the whole part of this research work.

REFERENCES

1. Ashrafi, S.D., Rezaei, S., Forootanfar, H., Mahvi, A.H., and Faramarzi, M.A., The enzymatic decolorization and detoxification of synthetic dyes by the laccase from a soil-isolated ascomycete, *Paraconiothyrium variabile*. *Int. Biodeter. Biodegr.*, **85:** 173–181 (2013).
2. Mirzadeh, S.S., Khezri, S.M., Rezaei, S., Forootanfar, H., Mahvi, A.H., and Faramarzi, M.A., Decolorization of two synthetic dyes using the purified laccase of *Paraconiothyrium variabile* immobilized on porous silica beads. *J. Environ. Health Sci. Eng*., **12:** 9-12 (2014).
3. Gholami-Borujeni, F., Faramarzi, M.A., Nejatzadeh-Barandozi, F., and Mahvi, A.H., Oxidative degradation and detoxification of textile azo dye by horseradish peroxidase enzyme. *Fresenius Environ. Bull.,* **22:** 739–744 (2013).
4. Gholami-Borujeni, F., Mahvi, A.H., Nasseri, S., Faramarzi, M.A., Nabizadeh, R., and Alimohammadi, M., Enzymatic treatment and detoxification of acid orange 7 from textile wastewater. *Appl. Biochem. Biotechnol*., **165:** 1274–1284 (2011).
5. Asadgol, Z., Forootanfar, H., Rezaei, S., Mahvi, A.H., and Faramarzi, M.A., Removal of phenol and bisphenol-A catalyzed by laccase in aqueous solution.

J. Environ. Health. Sci. Eng., **12:** 93-98 (2014).

6. Barathi, S., and Arulselvi, P.I., Decolorization, Degradation and Toxicological analysis of textile dye effluent by using novel techniques-Review. *Int. J. Sci. Res. Man.,* **3:** 2118-2136 (2015).
7. Shah, K., Biodegradation of azo dye compounds. *Int. Res. J. Biochem. Biotech.*, **1:** 5-13 (2014).
8. Olukanni, O.D., Osuntoki, A.A., and Gbenle, G.O., Textile effluent biodegradation potentials of textile effluent-adapted and non adapted bacteria. *Afr. J. Biotech.*, **5:** 1980-1984 (2006).
9. Moturi, B., and Singara-Charya, M.A., Decolorization of crystal violet and malachite green by Fungi. *Sci. World. J.*, **4:** 28-33 (2009).
10. Olukanni, O.D., Adenopo, A, Awotula, A.O., and Osuntoki, A.A., Biodegradation of Malachite Green by Extracellular Laccase Producing *Bacillus thuringiensis* RUN1. *J. Basic Appl. Sci*., **9:** 543-549 (2013).
11. Sahasrabudhe, M., and Pathade, G., Biodegradation of azo dye C.I. Reactive Orange 16 by an actinobacterium *Georgenia* sp. CC-NMPT-T3. *Int. J. Adv. Res.*, **1:** 1-19 (2013).
12. Saravanakumar, K., and Kathiresan, K., Bioremoval of synthetic dye malachite green by marine *Trichoderma* sp. *Springer Plus*, **3:** 1-12 (2014).
13. Rezaei, S., Tahmasbi, H., Mogharabi, M., Ameri, A., Forootanfar, H., Khoshayand, M.R., and Faramarzi, M.A., Laccase-catalyzed decolorization and detoxification of Acid Blue 92: statistical optimization, microtoxicity, kinetics, and energetic. *J. Env. Health Sci. Eng.*, DOI 10.1186/s40201-015-0183-1 (2015).
14. Faramarzi, M.A., and Forootanfar, H., Biosynthesis and characterization of gold nanoparticles produced by laccase from *Paraconiothyrium variabile*. *Coll. Surf. B Bioint.,* **87:** 23-27 (2011).
15. D'Souza, D.T., Tiwari, R., Shah, A.K., and Chandralata, R., Enhanced production of laccase by a marine fungus during the treatment of colored effluents and synthetic dyes. *Enzyme Microb. Technol.*, **38:** 504-511 (2006).
16. Parshetti, G., Kalme, S., Saratale, G., and Govindwar, S., Biodegradation of malachite green by *Kocuria rosea* MTCC 1532. *Acta Chim. Slov.,* **53:** 492–498 (2006).
17. Lal, N., and Srivastava, A.K., Decolorization of malachite green by newly isolated *Bacillus Strain* MTCC-3330. *Arch. Environ. Sci*., **5:** 71-76 (2011).
18. Cha, C., Doerge, D.R., and Cerniglia, C.E., Biotransformation of malachite green by the Fungus *Cunninghamella elegans. Appl. Environ. Microbiol*., **67:** 4358-4360 (2001).
19. Youssef, A.S., El-Sherif, M.F., and El-assar, S.A., Studies on the decolorization of malachite green by local isolate *Acremonium kiliense. Biotechnology,* **7:** 213-223 (2008).
20. Kumar, V.V., Sathyaselvabala, V., Premkumar, M.P., Vidyadevi, T., and Sivanesan, S., Biochemical characterization of three phase partitioned laccase and its application in decolorization and degradation of synthetic dyes. *J. Mol. Cat. B: Enzy.*, **74:** 63–72 (2014).