ISSN 2348-313X (Print)
International Journal of Life Sciences Research ISSN 2348-3148 (online)
Vol. 7, Issue 2, pp: (186-193), Month: April - June 2019, Available at: www.researchpublish.com

Enhanced decolourization and degradation of crystal violet by *Ganoderma lucidum* and *Pleurotus* sp. MAK-II crude extracts: antimicrobial and phytotoxicity analysis of product

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***Abstract*: Several enzyme extracts, crude and purified, have been used in the decolourization and degradation of various dyes including crystal violet. But, not much research has been carried out to analyse the efficiency of synergistic effect of enzyme extracts from two different organisms with different enzymatic profile. This study attempted to evaluate the efficiency of *Ganoderma lucidum* and *Pleurotus sp.* MAK-II crude extracts, separately and together, on decolourization and degradation of crystal violet. The combined extracts of both fungi achieved better decolourization and degradation of CV dye (98%; 30°C; pH 4.5; 100 rpm) than the individual enzyme extracts. This was evident from the UV-visible spectroscopy analysis and FTIR analysis. The antimicrobial studies suggested that the dye degraded by combined extracts does not have significant antimicrobial activity against tested five soil bacterium and it does not pose any danger to soil microbe when released into environment. Further, the combined extracts yielded better sprouting and seedling growth on *Phaseolus vulgaris* L. allowing its usage in agricultural purposes.**

***Keywords*: Crystal violet; Degradation and decolourization; *Ganoderma lucidum*; *Pleurotus* sp. MAK-II; Phytotoxicity.**

1. INTRODUCTION

Nowadays, the usage of chemical dyes has become inevitable in various industries, mainly paper, textile, leather, food, plastic, and cosmetic industries [1,2]. These dyes are produced in large quanta which amounts to one million tons per year of which nearly more than 15% of the total dye is released as effluent [3,4]. Indiscriminate release of effluents with such huge amount of dyes from these industries results in serious environmental problems including water pollution that ultimately affects the life on the planet. Triphenylmethane dyes are the third largest produced dyes in the world, after azo and anthraquinone dyes [5]. Crystal violet is a cationic triphenylmethane dye derived from aniline [6]. It has been used as a constituent in topical solutions for skin ailments, colorant in textile dyeing, microbial stain, bacteriostatic agent, hair colorant, and as an additive in poultry feeds to discourage fungal growth [7−10]. CV, like other chemical dyes, is produced with the aim of long stability to ensure the long term quality of the product. Therefore, degradation of CV is naturally challenging using conventional methods and so it is termed as “rebellious” molecule [11]. When the CV reaches ground water and consumed by humans, it causes several complications which include nausea, vomiting, increased heart rate, and cancer [12,13]. Therefore, detoxifying these dyes before discharge into environment requires serious attention to maintain the equilibrium state of the ecosystem.

Present techniques that are used for dye removal or degradation involves ozonation, ultrafiltration, adsorption, oxidation, photocatalysis, and biological process [14−17]. Biological process involves usage of several bacterial, fungal and algal isolates to remediate the dye effluents [18−20]. CV, for example, has antibacterial and antifungal activity and this limits the usage of live microbial system for remediation process [21]. The microbial culture filtrates containing extracellular remediation enzymes serve as better and fruitful alternative for this situation [22]. Ligninolytic enzymes such as laccase (Lac), manganese peroxidase (MnP), and lignin peroxidase (LiP) are well known for their ability to degrade natural recalcitrant molecule lignin. *Ganoderma lucidum* is a well known white rot fungi that houses all these ligninolytic and thermostable enzymes required for remediation process. These enzymes were also reported to degrade synthetic recalcitrant compounds and dyes [23,24]. Purified laccase from white rot fungi *Pleurotus* sp. MAK-II was used for biodegradation of various recalcitrant dyes [22,25]. Usage of purified enzymes for biodegradation is infeasible in large scale and hence it is practicable to use the culture filtrates of *G. lucidum* and *Pleurotus* sp. for the decolourization and biodegradation of crystal violet.

In the present study, we examined the effect of addition of culture filtrates of *G. lucidum* and *Pleurotus* sp. MAK-II on the discoloration and degradation of CV dye. The discoloration was monitored using UV-visible spectrophotometer and the degradation product was confirmed using Fourier-transform infrared spectroscopy (FTIR). Further, the degraded product was analysed for its antibacterial activity and phytotoxicity activity, in terms of rate of seed germination, to determine its suitability for release into the environment.

2. MATERIALS AND METHODS

Chemicals and cultures

Crystal violet (CV) and 2,4-dimethoxyphenol (DMP) were purchased from TCI Chemicals (India) Pvt. Ltd. Guaiacol was purchased from HiMedia Laboratories (India) Pvt. Ltd. All other chemicals used were of analytical grade. Seeds of common bean (*Phaseolus vulgaris* L.) purchased from local market of Chennai, Tamil Nadu, India were used for phytotoxicity experiments. The *Ganoderma lucidum* RMK1 (Genbank Accession: MH553170) was isolated from Kumarakom Bird Sanctuary, Kottayam, Kerala, India. *Pleurotus* sp. MAK-II culture, which was already isolated by our laboratory, was used in this experiment. The cultures were maintained on Malt extract agar and subcultured every 15 days and stored in dark condition at room temperature.

Medium composition and enzyme production

Both *G. lucidum* and *Pleurotus* sp. MAK-II cultures were cultivated in basal medium, amended with 1% wheat bran, with composition (g/L): glucose (20), $MgSO_4 \cdot 7H_2O$ (0.5), KH_2PO_4 (0.46), K_2HPO_4 (1), yeast extract (2) and peptone (2). Micronutrients were added in the following composition (g/L): $ZnSO_4 \cdot 7H_2O$ (0.001), $FeSO_4 \cdot 7H_2O$ (0.005), $CaCl_2 \cdot 2H_2O$ (0.06), $CuSO_4 \cdot 7H_2O$ (0.005), $MnSO_4 \cdot H_2O$ (0.005). About six mycelia disks of 6 mm diameter from the periphery of growing culture was used to inoculate 100 ml of basal medium and incubated under shaking condition at 28 °C for 10 days at 150 rpm. After the incubation time, the medium was filtered with eight fold muslin cloth and resulting filtrate was centrifuged at 12000 rpm at 4 °C for 15 mins and the supernatant was collected and stored at −20 °C until further use.

Preliminary plate assay

Qualitative laccase assay was carried out on Malt extract agar (g/L): malt extract (15), agar (20), pH 5.5; amended with 5 mM guaiacol. Dye degradation assay was also carried out by adding CV dye at final concentration of 50 mg/L instead of guaiacol. A mycelial disk of 6 mm diameter from *G. lucidum* and *Pleurotus* sp. MAK-II was placed at the centre of separate agar medium plates and incubated in dark at room temperature.

Enzyme assay

Ligninolytic enzymes were quantified using the modified method of Field et al. 1996 [26]. Laccase activity was determined by measuring the increase in absorption due to the oxidation of DMP to coerulignone at 469nm (ε_{469} = 27.5 $mM^{-1}cm^{-1}$) and 30 °C. The reaction mixture of 1 ml contained 5 mM DMP in 100 mM Na-tartrate (pH 4.5) and culture filtrate. Subsequently the peroxidase assay was carried out under the above said conditions modified only by addition of H_2O_2 and $MnSO_4$ to the final concentration of 1 mM. One unit of enzyme activity is defined as1 µmol reaction product formed per minute. Total peroxidase activity was obtained by subtracting the final absorbance value from the laccase activity results.

Spectrophotometric analysis of decolourization and degradation

The decolourization assay was carried out in 250 ml Erlenmeyer flasks with 50 ml of CV at 100 mg/L. In three different flasks, the dye solution was added with 5 ml of *G. lucidum* supernatant, 5 ml of *Pleurotus* sp. MAK-II to separate flasks and 2.5 ml of each extract in another flask and incubated at 30 °C. For control, 5 ml of sterile distilled water was added to another flask with same dye concentration and incubated under same conditions as above. Periodically, the change in absorbance was monitored using UV-visible spectrophotometer at 590 nm and expressed in terms of percentage decolourization as per the following formula

$$\%\, Decolorization = 100 \times \frac{(A_0 - A_t)}{A_0}$$

Whereas, A_0 denotes absorbance value at initial dye concentration, A_t denotes absorbance value of dye at the time (t) being analysed. The sample drawn for spectrophotometric analysis was heated for enzyme inactivation to stop the activity of the enzymes.

The degradation of CV was analysed using FTIR. The undegraded CV and the CV degraded using combined extract were added to pure KBr and made into uniformly thin pellets and the vibrational spectra of samples were recorded using Bruker Tensor 27 IR spectrophotometer.

Influence of static and shaking conditions on decolourization

The influence of static and shaking conditions on the decolourization of CV dye was carried out in 250 ml Erlenmeyer flasks containing 50 ml of CV at 100 mg/L. The culture filtrates were added in different proportions as mentioned earlier and the flasks were incubated at room temperature under static and shaking conditions (100 rpm). The decolourization levels were analyzed spectrophotometrically at regular intervals and recorded as percentage decolourization.

Influence of physicochemical characteristics on decolourization

The influence of different temperatures and pH on CV degradation was carried out in 250 ml Erlenmeyer flasks containing 50 ml of sterile CV at 100 mg/L concentration. The quantity and the order of addition of two supernatants were done as mentioned above. The flasks were incubated under shaking conditions (100 rpm) at different temperatures such as 25, 30, 35, 40, 45 °C. Similarly dye degradation at different pH ranging from 3 to 9 was also examined under shaking condition. The decolourization was monitored spectrophotometrically at regular intervals and the results were recorded as percentage decolourization.

Antibacterial studies against rhizosphere bacteria

Rhizosphere bacteria were isolated from the soil around the roots of healthy tomato plant grown at an organic farm near Chennai, India. Five different bacterial isolates (RS1-RS5) that showed distinct colony morphology were chosen for antibacterial studies. The study was carried by disk diffusion method in nutrient agar medium. Sterile disk were loaded with 5 µl of (100 mg/L) dye, *G. lucidum* supernatant treated dye, *Pleurotus* sp. MAK-II supernatant treated dye, and dye treated with supernatant of both organisms. Negative control contained 5 µl sterile water and the positive control contained 5 µl of tetracycline (50 mg/ml).

Phytotoxicity studies against *Phaseolus vulgaris* L.

Phytotoxicity analysis was carried out using *P. vulgaris* L. seeds. About 10 seeds were placed in petridishes containing filter paper saturated separately with untreated CV solution (100 mg/L), CV solution treated with *G. lucidum* supernatant alone, CV solution treated with *Pleurotus* sp. MAK-II supernatant alone, CV solution treated with both supernatants. Control experiment was carried out simultaneously where the filter paper was saturated with sterile water. The phytotoxicity was evaluated in terms of germination rate and the length of radicules with reference to the measurements made in control (water treated) seeds.

Statistical analysis

All the experiments were conducted in triplicate and the obtained data were expressed as means±standard deviations. The *t* test was carried out to assess the differences among the means and the values with $p \leq 0.05$ were considered significant.

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3. RESULTS AND DISCUSSION

Organism selection and enzyme production

In this study, we selected two potential white rot fungi, *G. lucidum* and *Pleurotus* sp. MAK-II due to their ability to produce variety of extracellular ligninolytic enzymes. Preliminary screening for extracellular enzyme production was carried out on MEA amended with guaiacol. Both the organisms showed dark reddish brown zone of coloration on third day of incubation, confirming the potential and early production of ligninolytic enzymes (Figure 1). Further, the organisms were also screened for their ability to decolorize CV dye on agar medium. Both the organisms were capable of decolorizing the CV dye amended at 50 mg/L concentration (Figure 2). The *G. lucidum* produced laccase (39.8 U/mL) and peroxidase (14.6 U/mL) whereas *Pleurotus* sp. MAK-II produced laccase and peroxidase to about 27.3 and 16.7 U/mL respectively. Hence, the organisms were used for the further decolourization studies.

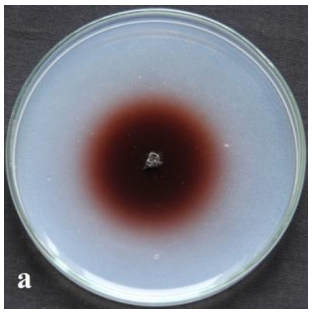

Fig. 1. Screening of *G. lucidum* (a), *Pleurotus sp.* MAK-II (b) for ligninolytic enzymes on guaiacol amended MEA medium.

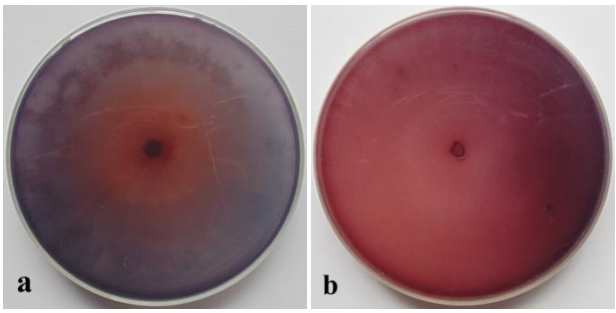

Fig. 2. CV dye decolourization on MEA agar plate using *G. lucidum* (a), *Pleurotus sp.* MAK-II (b).

Effect of different factors on decolourization

The efficiency of supernatants on decolourization of CV dye was executed both under static and shaking conditions (100 rpm) at room temperature. The shaking conditions favoured better decolourization (83%) when compared to static conditions (58%) in the combined extracts and even in then individual extracts. Bharagava et al. 2018 [27] also found similar observations in decolourizing crystal violet with *Aeromonas hydrophila*. The possible explanation for this could be that the shaking condition might have provided the constant supply for the enzyme activity. The pH is one of the factors that favour enzyme activity and enzyme stability. Among the pH tested, pH 4 and 5 favoured the highest degradation in the combined extract and even in individual extracts (Figure 3a). The stability and action of peroxidases contributed in large in this particular range of acidic pH. Among the different temperatures, 30°C favoured highest decolourization (Figure 3b) and the pattern of decolourization did not steeply decline because of the presence of thermostable enzymes, as observed by Manavalan et al. 2015 [25]. In general, the combined extracts of *G. lucidum* and *Pleurotus* sp. MAK-II possessed better decolourization potential than the individual extracts, possibly due to the synergistic action of different classes of ligninolytic enzymes.

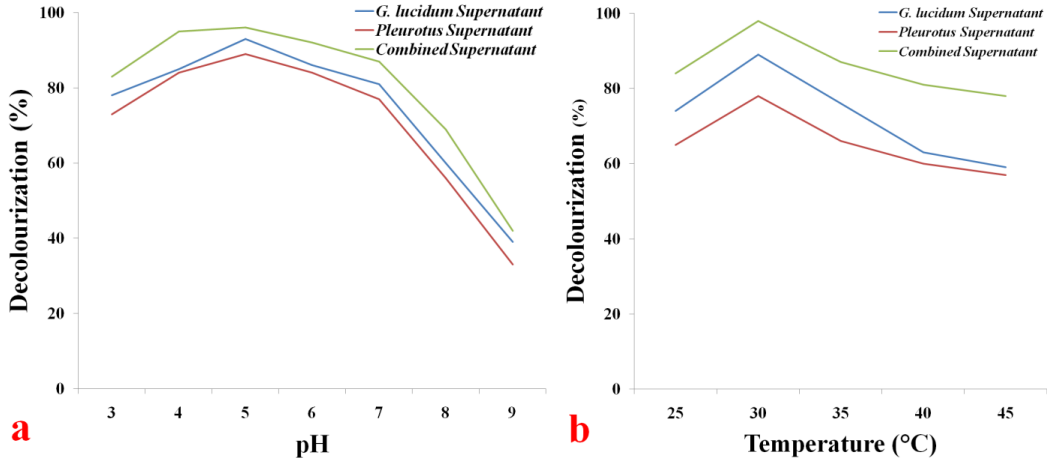

Fig. 3: Optimization of dye decolourization using individual extracts of *G. lucidum* and *Pleurotus sp.* MAK-II and their combined extracts at different pH (a) and temperature (b).

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International Journal of Life Sciences Research ISSN 2348-3148 (online)
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Spectrometric analyses

The decolourization of CV dye (treated and untreated samples) was confirmed using UV-Visible spectroscopic analysis at 590 nm (λ_{max}). UV-Visible Spectroscopic analysis of CV dye solution showed a maximum absorbance at 590 nm and further analysis showed decrease in absorbance for the samples that were withdrawn during the course of degradation study. In our experiment, the obtained result suggests that the decolourization of dye solution was due to degradation of CV rather than simple decolourization, in conccurance with observations made by Chen et al. 2003 [28].

The FTIR spectrums of both degraded and undegraded CV are shown in Figure 4. The untreated CV exhibited, between 1500 and 500, the peaks for monosubstituted, disubstituted and trisubstituted benzene rings. According to Bharagava et al. 2018 [27] the peak at 940.13 $^{cm-1}$ may indicate organo phosphorous; the peak at 832.87 may indicate 1,2,3- trisubstituted benzenes, primary amines or 1,2,5-trisubstituted benzenes; and the peak at 758.10 $^{cm-1}$ may indicate the presence of 1,2,3-trisubstituted benzenes, monosubstituted benzenes ortho-substituted benzenes. Further, the peaks such as 911.73, 832.87, 743.01 $^{cm-1}$ are the characteristics of the out of plane bending of ring hydrogen. Besides few exceptions, the FTIR spectra of degraded CV showed significant difference from the spectral characteristics undegraded CV. The absence of some characteristic peaks such as at 743.01 $^{cm-1}$ and 721.94 $^{cm-1}$ indicated the absence of benzene rings in the spectra of degraded CV. Similar findings were also made by Mota et al. 2015 [29].

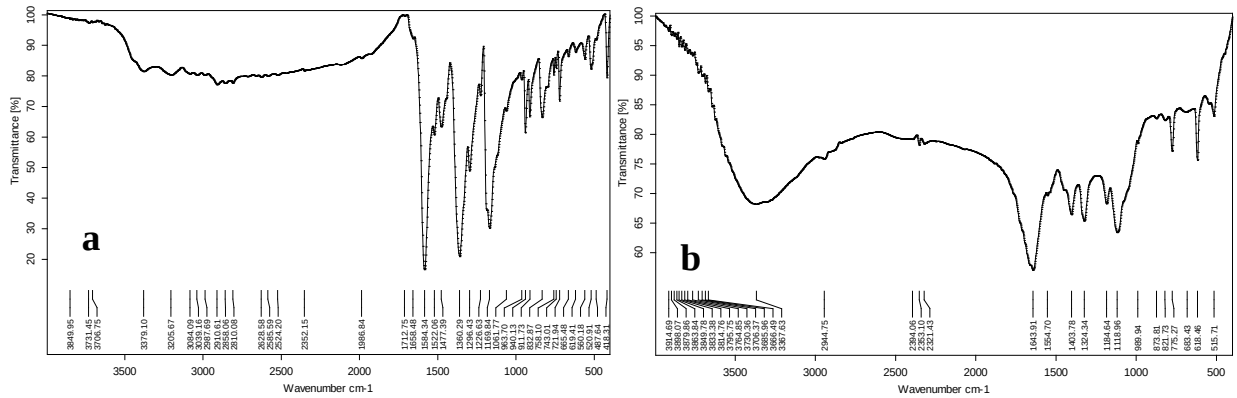

Fig 4: FTIR analysis of untreated CV (a) and degraded product after treatment with combined extracts of *G lucidum* and *Pleurotus sp.* MAK-II (b).

Antibacterial activity of the degraded product

In our study, we studied the bactericidal effect of CV (degraded and undegraded) was using disk diffusion method and the results obtained were expressed in Table 1. The CV dye solutions that were treated with enzyme extracts showed reduced antibacterial effect than the untreated CV solution. The dye solution treated with *G. lucidum* supernatant showed better toxicity reduction than the dye solution treated with *Pleurotus* sp. MAK-II, compared to untreated CV. But, the CV dye solution treated with both supernatants showed almost negligible activity (on isolates B1, B2, and B4) or nil activity (on isolates B3 and B5). According to Maley et al. 2013 [30] CV solution is particularly efficient in inhibiting the growth of gram-positive bacteria and *Candida* sp. whereas it is moderately effective against the gram-negative bacteria.

Table 1: Antimicrobial activity* against rhizosphere bacterial isolates using untreated CV dye, dye treated with *G. lucidum*, dye treated with *Pleurotus sp.* MAK-II extract, and dye treated with both extracts, sterile water (negative control), tetracycline (positive control).**

Bacterial isolate	Control (+)	CV treated with *G. lucidum* extract	CV treated with *Pleurotus sp.* MAK-II extract	CV treated with both extract	Untreated CV
RS1	28	12	16	10	18
RS2	18	14	15	11	16
RS3	23	11	14	-	15
RS4	25	13	16	10	17
RS5	18	13	14	-	15

*the antimicrobial activity activity (diameter zone of inhibition) is expressed in centimetres.

**the negative control did not show any visible difference on grow of the bacterial culture.

***the hyphen indicates lack of any visible clear zone of inhibition of cell growth.

ISSN 2348-313X (Print)
International Journal of Life Sciences Research ISSN 2348-3148 (online)
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Phytotoxicity of the degraded product against *Phaseolus vulgaris* L.

The phtotoxicity of crystal violet and its degraded product after the treatment with two enzyme extracts separately and together were analyzed and the results were tabulated (Table 2). In this study, the untreated CV, and the CV treated with extracts of *G. lucidum*, *Pleurotus sp.* MAK-II and both showed 60%, 75%, 65%, and 90% seed germination respectively. This indicates that the CV treated with both extracts has significantly high germination rate.

Table 2: Phytotoxicity analysis against *P. vulgaris* L. using different enzyme extracts.

Treatment	Germination %			Average radical length (cm)	Phytotoxicity %
	24 h	48 h	72 h		
Untreated CV	20	40	60	1.7±0.4	56.41
G. lucidum treated CV	30	50	75	3.5±0.2	10.25
Pleurotus sp. MAK-II treated CV	20	40	65	2.9±0.3	25.64
Combined extract treated CV	40	65	90	3.8±0.4	02.54
Control	40	60	95	3.9±0.6	-

Besides inhibiting the germination of seeds, both the enzyme treated and untreated CV had shown their effect on seedling growth but with marked differences. The untreated CV had severely inhibited the seedling growth (56%). Among the enzyme treatment, CV treated with both *G. lucidum* and *Pleurotus sp.* MAK-II extracts showed better seedling growth (98%), followed by CV treated with *G. lucidum* extract (90%) and *Pleurotus sp.* MAK-II extract (75%) separately. Augmentation of the seedling growth and germination by the combined extract treated CV solution may possibly attribute to two reasons. Firstly, the enhanced degradation may have reduced the toxicity and secondly, the possible presence of cellulases may have assisted in breaking of cell walls for making way for better germination percentage. Earlier Parshetti et al. 2011 [31] and Khandare et al 2011[32] have made similar observations with regard to enhanced seed germination and seedling growth respectively.

4. CONCLUSION

The results of this study indicate that the usage of combined extracts of *G. lucidum* and *Pleurotus sp.* MAK-II has improved the decolourization potential dye to the synergistic effect of various laccases and peroxidases. Further, the antimicrobial and phytotoxicity analyses confirm the products of combined treatment are less toxic than the individual extracts treated dye solution. Hence, this study concluded that the usage of combined enzymatic extracts of different organisms has significantly reduced the toxicity levels of the dye solutions and it can be utilized for the industrial applications to further reduce the toxicity levels of dye effluents ensuring cleaner environment.

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