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Extraction and quantification of phenolic compounds from *Prunus armeniaca* seed and their role in biotransformation of xenobiotic compounds

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Abstract–The current research project has been devoted to isolating new low cost and eco-friendly phenolic compounds from fruit seeds, peels and vegetables to reduce the atmospheric pollution. Natural phenolic compounds were extracted from different fruit seeds and agriculture waste: *P. armeniaca*, *P. persica*, *P. domestica* and *Triticum aesativum*. The total phenolic content was quantified, and the maximum value (1 mL extract having 1,933 μg) was found in *P. armeniaca* seed extract. Phytochemical screening showed that *P. armeniaca* seeds contain higher amount of alkaloid, tannins, saponins and flavonoid. *P. armeniaca* seeds enhanced the biotransformation of reactive yellow dye up to 69.89% with maximum laccase (322.45 IU/mL) production. Biodegradation of reactive yellow was only 23.34% without natural redox mediator at sixth day of incubation. Use of *P. armeniaca* seed stimulators resulted in maximum laccase activity (894.4 IU/mL) with 99.5% rate of removal. UV-Vis, HPLC & FTIR analysis confirmed the transformation of parent dye into various new products. Phytotoxicity study indicated 0% germination index of *Avena sativa* seeds with reactive yellow, whereas 83% germination index having 100% seed germination while 83% root elongation with treated sample. Thus, the study revealed that the natural phenolic compounds could serve as high potential redox mediators for enhanced laccase-mediated decolorization of reactive yellow dye.

Keywords: Enzyme Catalysis, Natural Redox Mediators, Phytotoxicity, Hazardous Pollutants

INTRODUCTION

Billions of hazardous pollutants are produced annually by the chemical, agricultural, oil, paper, textile and other industries. Human exposure to industrial wastes has led to various health hazards ranging from headaches, nausea, lung, and skin irritations, to serious ailments like congenital malformations etc. [1,2]. The highly toxic nature of these compounds, such as dyes released by textile industries, generates an intimidation to environmental protection [3,4]. Bioremediation is a remarkable tool [5] that exploits the metabolic potential of microorganisms to clean up the environmental pollutants to the less toxic forms with less contribution of chemicals, energy and time [6].

The efficiency of microbial decolonization depends on the consistency and the activity of the selected microorganisms. Consequently, a huge number of genus has been tested for the decolonization and mineralization of various dyes in recent years [7].

White rot fungi due to production of ligninolytic enzymes have been considered for their ability to break down ecological pollutants, for example sulfonphthalein DDT, lindane, dioxin and benzopyrene and dyes etc. [8]. Among these white rot fungi, the Basidiomycete, *P. ostreatus* belongs to a subclass of lignin-degrading microbes that synthesize laccase, MnPs and veratryl alcohol oxidase but no LiPs [9]. The extensively cultured species-oyster mushroom *P. ostreatus* inhabits 14% of the worldwide marketplace and ranks third in world trade [10]. *Pleurotus* and *Corilopsis* produce no clear lignin peroxidase or manganese peroxidase, excluding sufficient extracellular laccase [11] signifying that amongst the various degrading enzymes, laccase play a key role [12]. Laccase having wide range of redox mediators can enhance the rate of decolorization and extend the range of dyes that can be decolorized [13-15]. However, synthetic redox mediators are costly, lethal, and diminish the enzyme activity at higher concentration. Therefore, enzymes of WRF mediated by natural phenolic compounds can act on non-specific recalcitrant noxious waste by converting them into the other products and permitting a better final treatment of the waste with reduced treatment cost. Owing to their redox properties which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers, naturally occurring phenols are considered as the potential alternatives for the synthetic redox mediators [16]. Therefore,

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present efforts have been designed to extract and quantify the phenolic compounds from various fruit seeds. The extracted phytoconstituents, steroids, alkaloid, tannins, and flavonoids, are applied for biotransformation of xenobiotic compounds as natural redox mediators.

1. Experimental Work

Reactive yellow, acid fuchsin, basic fuchsin and methylene green dyes were purchased from Sigma Chemicals Co., USA. All other chemicals and reagents in use were of analytical grade and were used without any further purification.

2. Extraction and Estimation of Phenolic Contents

Five grams of crushed seeds (*P. armeniaca*, *P. persica* and *P. domestica)* was mixed with 100 ml of 65% (v/v) ethanol (methanol and acetone) and then sonicated for 30 min at 50 °C in an ultrasonic water bath independently. The mixture was immediately filtered under vacuum and then the solvent was evaporated by incubating at 50 °C in an oven [17]. Then subjected to quantifying the phenolic content by Folin-Ciocalteu method [18]. Seeds extract was also subjected to various chemical tests to identify chemical constituents by following the method of [19-22] for alkaloids, tannins, steroids, flavonoids, respectively.

3. Optimization of Process Parameters

Different process parameters were optimized by using the classical approach of varying one parameter at a time and maintaining the optimizing at constant level. To determine the most suitable inoculum level for effective biotransformation and enhance synthesis of Laccase by *P. ostreatus* mediated by natural phenolic compounds, shake flask experiments were conducted at different initial inoculum level (V/V) from 2 to 10%. In the subsequent experiments, the flasks adjusted at optimum inoculum level were incubated at different initial pH (3-5) values using 1 M HCl/1 M NaOH to find the effective decolorization by the fungus. In the next trials, the effects of different synthetic mediators were compared with that of natural one to select the best redox mediator for maximum decolorization of dyes by *Basidiomycete*.

4. UV-visible spectral analysis

The removal of color was determined by UV-visible spectrophotometer at the maximum wave length of dye. The decrease in absorbance showed that there was decolorization, which expressed in percentage along with control without fungus. Treatments were performed in two different sets and the obtained data was expressed with mean±S.E. The removal of color in percentage was calculated from the following formula:

$$\text{Removal color } \% = \frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}} \times 100$$

Note that initial absorbance is the absorbance of target textile dye before degradation, while final absorbance is the absorbance of the target textile dye after degradation

5. Enzyme Assays

Laccase activity was determined by using azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; Sigma, USA) as substrate at 405 nm. One unit of enzyme activity was defined as the amount of enzyme that oxidized 1 μmol of ABTS per min. Manganese peroxidase activity was examined by the addition of $MnSO_4$ as a substrate in sodium melonate buffer in the presence of H_2O_2. Manganese ions Mn^{+3} form a complex with melonate, which absorbs at 270 nm (ε_{270} 115,900 M^{-1} cm^{-1}) $MnSO_4$ [23]. Lignin peroxidase activity was determined by the oxidation rate of veratryle alcohol to veratraldehyde in sodium acetate buffer of pH 3 in the presence of H_2O_2 [24] against control. Absorbance was monitored at 310 nm after 10 min time interval.

6. High Performance Liquid Chromatography Analysis (HPLC)

The transformation products of reactive yellow dye were monitored through reverse-phase HPLC (Agilent 1100 series, Agilent, Waldbronn, Germany). The control as well as treated samples was vortexed, then filtered through 0.45 μm syringe filter. The filtrate (10 μL) was then injected to HPLC column ZORBAX SB C-18 and eluted using 65% aceto-nitrile at flow rate of 1 mL per minute through UV detector at 495 nm and scanned in the range of 200-700 nm.

7. Fourier Transformed Infrared Analysis (FTIR)

Decolorization was monitored by UV-Vis spectroscopic analysis (Hitachi U-2800), whereas biodegradation was monitored by high performance liquid chromatography (HPLC) and Fourier transform infrared spectroscopy (FTIR). To carry out this FTIR analytical technique, 100 mL treated sample was centrifuged at 10,000 g and metabolites were extracted from supernatant using equal volume of ethyl-acetate. The extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness in oven. The bio-transformed reactive yellow was characterized by FTIR (Shimadzu, Spectrum one) and compared with control dye. The FTIR analysis was done in the mid-IR region of 500-4,000/cm with 35 scan times.

8. Phytotoxicity Test

Phytotoxicity test was performed to assess the toxicity of treated sample at the concentration of 100 ppm according to [25] on the seeds of *A. sativa*. Growth of *A. sativa* watered with transformed water was compared with the growth of *A. sativa* watered with simple tap water after four days of incubation.

RESULTS AND DISCUSSION

1. Screening of Different Reactive Dyes by Basidiomycetes Under Static and Shaking Conditions

The transformation capability of *P. ostreatus* was checked by addi-

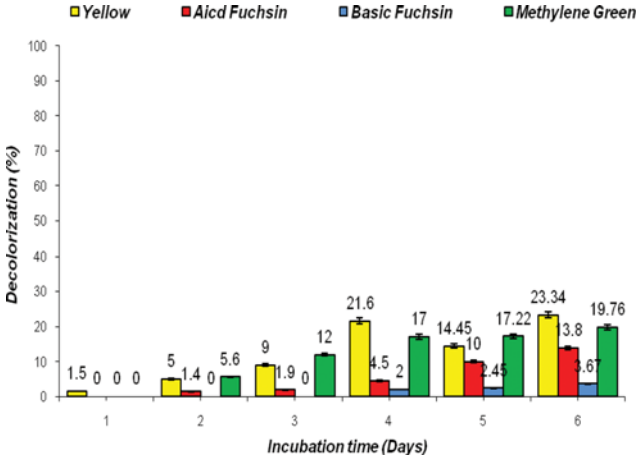

Fig. 1. Effect of incubation time on the biotransformation of dyes.

tion of four different reactive dyes, methylene green, acid fuchsin, reactive yellow and fuchsin basic, at each concentration of 100 ppm at shaking condition for six days. Maximum decolorization (23.34%) of reactive yellow was achieved after six days of incubation (without natural phenolic compounds) followed by methylene green 19.76%, acid fuchsin 13.8% and very poor decolorization of basic fuchsin 3.67% was recorded (Fig. 1). Maximum laccase activity (105.29 IU/mL) was observed in the flask containing reactive yellow dye while minimum laccase activity (15.98 IU/mL) was achieved in the flask containing basic fuchsin after six days of incubation; whereas there was negligible production of LiP and MnP in all the flasks containing all the tested dyes (Fig. 2).

Similarly, the decolonization of all the tested dyes was studied under static condition for six days and results clearly indicated that the rate of decolonization was very slow as compared to under shaking condition, and the ability of microbes to transform the xenobiotic compound was different for structurally different compounds.

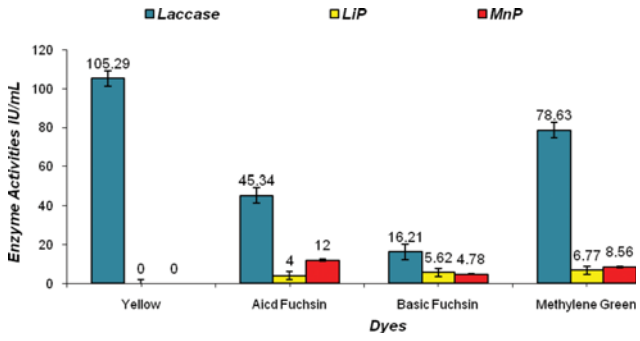

Fig. 2. Production of LMEs after 6th day of incubation during the decolorization of acid fuchsin, basic fuchsin, reactive yellow and methylene green textile dye.

Variation in the removal of color might also be due to the physiological discrepancy of different WRF [26]. It is also reported that the variation of structurally different dyes was not simple to elucidate [27]. The biodegradation step required the destruction of the chromophore; thus, the slow decolorization rate of some dyes could be attributed to the complexity of their chromophores; however, the overall complexity alone was not a sign of the difficulty of degradation of a particular dye. The enzyme system of WRF responsible for the removal of color as well as the appearance of these enzymes may also vary among the fungal strains depending on the dye present in the medium [28,29]. The degree of dye decolourization is mainly due to the breakdown of the aromatic ring of the dye, which relies on the substituents of the ring including amino, 2-methoxyphenol, acetamide, phenol, as well as other easily degradable functional groups [30].

2. Extraction and Quantification of Phenolic Contents of Various Fruit's Seeds

Phenolic compounds of different fruits seed (*P. armeniaca*, *P. persica*, *P. domestica* and agriculture waste; *T. aesativum*) were extracted using methanol, ethanol, DMSO, respectively. The total content of phenolic compounds was quantified as ferulic acid equivalent. Quantification made with different volumes (50-150 µL) of apricot's extract, which indicated the best co-relation (R^2=0.9883) to the concentration of phenolic component in the extract and maximum phenolic content (1,933 µg/ml), was found in ethanolic extract of *P. armeniaca* seeds.

3. Chemical Tests for the Confirmation of Phenolics in *P. armeniaca* Seeds Extract

The confirmation of various polyphenolic compounds (Tables 1 and 2) in seed of *P. armeniaca* is given in Fig. 3. Phytochemical screening showed that *P. armeniaca* seeds contain higher amount of alkaloid, tannins, and flavonoid (Tables 1 and 2).

Table 1. Test for the confirmation of phenolic compounds

Sr. no	Experiments	Observations	Inference
1	1.0 ml *P. armeniaca* seeds extract +10% aqueous solution of NaOH	Formation of Brown colour precipitation	Phenolic compounds present [31]
2	0.5 g *P. armeniaca* seeds extract +2.5 ml Fecl3 Soln+1 ml water	Green color appeared	Phenolic compounds present [32]
3	0.5 g *P. armeniaca*' seeds extract +Br water	Colour of Br discharge and ppt appeared	Phenolic compounds present [33]

Table 2. Phytochemical screening of *P. armeniaca* seeds extract [19-22]

4	Alkaloids	2 ml of MeoH extract +1 ml of 5% HCL +6 drops of wagner reagent	Formation of orange-brown precipitate indicates the presence of alkaloids	++
5	Tannins	2 ml of MeoH extract+2 ml of Fecl3	Apprance of Green colour indicates the presence of Tannins	++
6	Steroids	0.5 g *P. armeniaca*' seeds extract+acetic anhydride------ cool down then add 0.5 ml chloroform+1 ml H_2SO_4	No ring formation indicates the absence of steroids.	−−
7	Flavonoids	2 ml of MeoH extract+5 ml of Conc.HCL +0.5 g of Mg Ribbon	Appearance of magenta color demonstrated the presence of flavonoids.	++

−−=Absent, ++=present

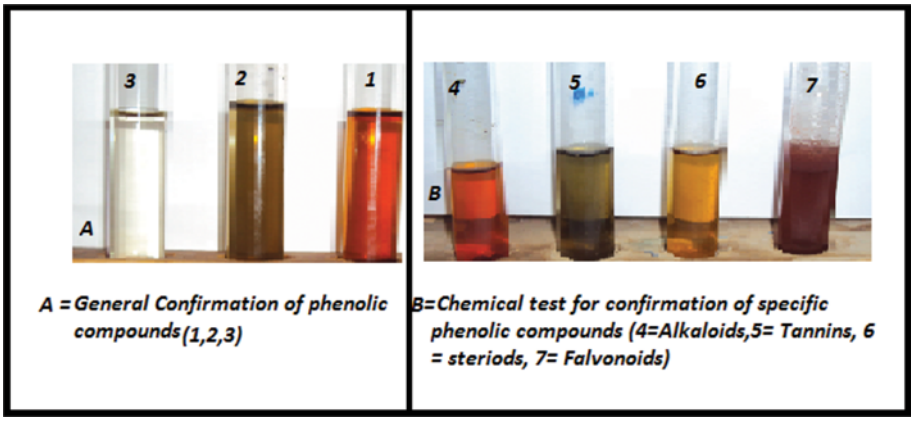

Fig. 3. Phytochemical screening of seeds extract of *P. armeniaca*.

4. Effect of Low Cost Natural Phenolic Compounds on the Biotransformation of Reactive Yellow Dye

Various fruit seeds--*P. armeniaca*, *P. persica*, *P. domestica* and agriculture waste; *T. aesativum*--can serve as potential natural mediators for laccase catalyzed biodegradation. The next target was to evaluate this extract (*P. armeniaca seeds*) as redox mediator for laccase (major enzyme of *P. ostreatus*) mediated reactive yellow biotransformation. The final volume 100 mL of reaction mixture contained reactive yellow dye (100 ppm), 2 mL fungal spore, 200 micro liter of phenolic extract and was incubated in temperature (30 °C) controlled shaker at 120 rpm.

It was noted that 69.89% of color removal was observed after sixth day of incubation along with maximum production of laccase having activity 322.45 IU/mL when the reaction was stimulated by natural phenolic compounds of *P. armeniaca* seeds (Figs. 4 and 5).

Whereas, least removal of reactive yellow dye (10.9%) was observed when the fungal culture was mediated by plum's seeds extract, co-related with minor production of Laccase (72.5 IU/mL) (Fig. 5). Thus, use of natural redox mediators *(P. armeniaca* seeds) significantly enhanced the rate of biotransformation of reactive yellow.

5. Effect of Inoculum Size on the Biotransformation of Xenobiotic Compound

Biotransformation potential of *P. ostreatus* for reactive yellow was investigated using different levels of inoculum. In this trial the decolorization flasks were autoclaved, inoculated with different volumes of inoculum (2, 4, 6, 8 and 10%) containing 10^6-10^8 spore's mL^{-1} and incubated at 30 °C in an orbital shaker at 120 rpm. It was observed that dye decolonization and laccase production by *P. ostreatus* gradually increased with increase in inoculum size; the results indicated that the enhanced color removal was observed with 8% (V/V) spore suspension after third day of incubation. Results depicted in Fig. 6 clearly indicated that maximum dye

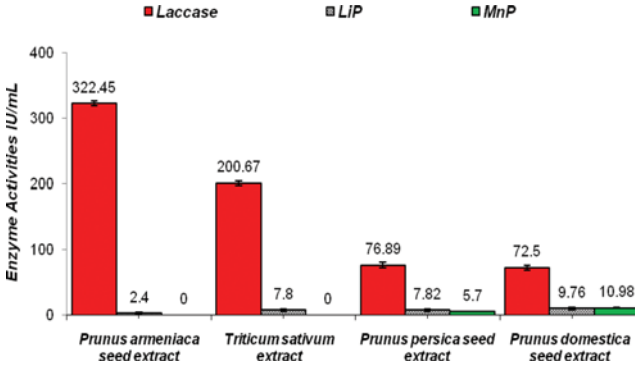

Fig. 5. Effect of natural phenolic compounds on the production of LMEs.

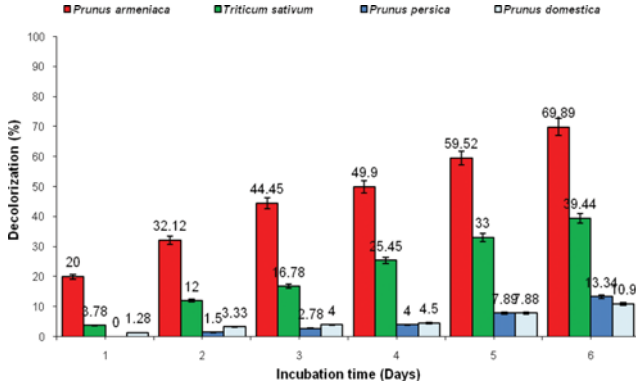

Fig. 4. Effect of natural polyphenol on the biotransformation of reactive yellow textile dye.

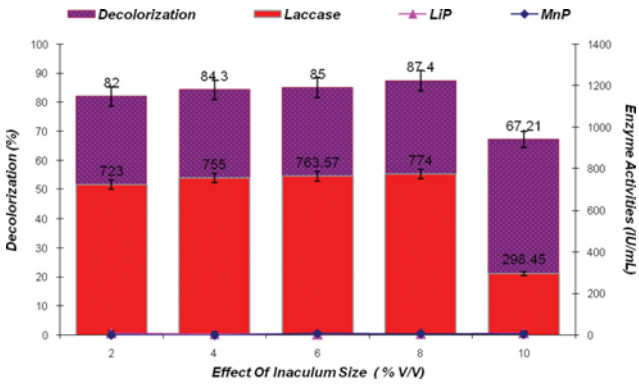

Fig. 6. Effect of inoculum size on the biotransformation reactive yellow and synthesis of LMEs.

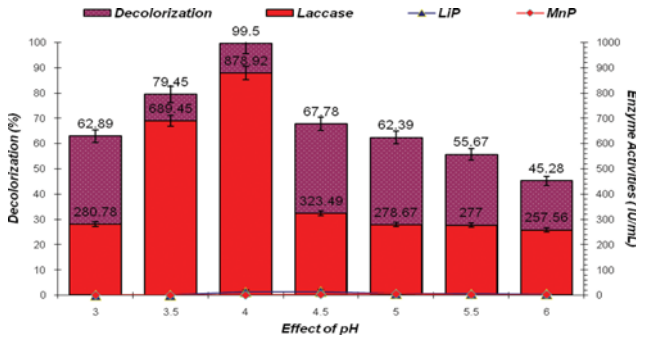

Fig. 7. Effect of pH on the biotransformation and synthesis of LMEs.

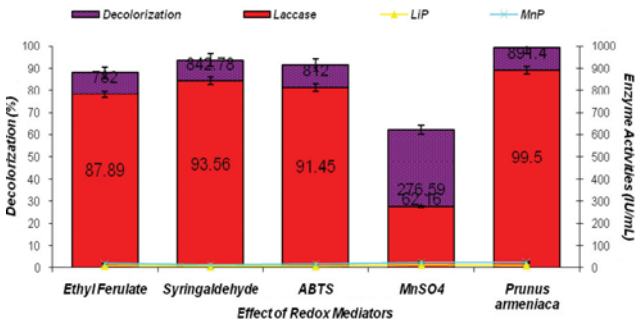

Fig. 8. Effect of natural and synthetic redox mediators on biotransformation of reactive yellow and synthesis of LMEs.

removal (87.4%) co-related with laccase activity (774 IU/mL). [34] reported that an inoculum level up to 10% was adequate for decolorization of textile water, and it has been also reported that inoculum has a crucial role in the secretion of laccase because an inoculum in lower size may not be able to initiate growth and, on the other hand, high level of inoculum causes maximum color removal [30].

6. Effect of pH on the Biotransformation of Xenobiotic Compound

Experiment was performed at pre-optimized conditions to elucidate whether the change of different pH conditions can enhance the rate of biotransformation of xenobiotic compound, which is ultimately co-related with the formation of laccase production; the results indicated (Fig. 7) that with increase in pH (3-4.0) of the culture medium, the decolorization potential and enzyme synthesis of the fungus was also increased and peaked at pH 4.0 (95.5%) after 48 hr of incubation co-related with maximum production of laccase (878.92 IU/ml).The fungus could cause 67.78% dye removal at pH 4.5 (pH of the screening trial) and 62.39% at pH 5. Laccase was the major enzyme secreted by *P. ostreatus* for degradation of reactive yellow with minor activities of MnP and LiP (Fig. 7). Lignolytic enzyme (laccase) formation and dye removal increased with an initial increase in pH and showed a parabolic decline thereafter. Maximum activity of laccase, noted in the culture filtrate harvested from the most decolorized medium (pH 4.5), suggested it had the predominant role in the biotransformation of dye. A sharp decrease in activity of laccase was observed beyond optimum pH [35].

7. Effect of Synthetic and Natural Redox Mediators on the Biotransformation of Dye

Enzyme mediator systems are critical bioremediation agent, as reaction rate could be enhanced in the presence of these stimulators. Effect of various synthetic redox mediators on biotransformation of reactive yellow was performed at pre-optimized condition; and these were compared with natural stimulator. The results depicted in Fig. 8 revealed that fungus showed maximum biotransformation of reactive yellow (99.5%) and enhanced laccase activity (894.4 IU/mL) in the presence of natural high potential redox mediator *P. armeniaca,* followed by syringaldehyde (93.56%), ABTS (91.45%), ethyl ferulate (87.89%). Least color removal was observed with $MnSO_4$. Thus, natural redox mediators of *P. armeniaca* did not inhibit laccase activity, which is actually responsible for the enhancement of color removal. It has been reported that the presence of natural mediators did not inactivate *P. cinnabarinus* laccase [36]. The ability of the laccase-mediator approach in biodegradation response depends on redox reversibility of the radical-substrate reaction, furthermore balance between the stability and reactivity of the mediator radical which, in addition, should not hinder enzyme activity [37].

8. Extraction and Analysis of Metabolites

One hundred mL of decolorized sample was taken, centrifuged at 10,000 rpm, and extraction of metabolites was carried out from supernatant using equal volume of ethyl acetate. The extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness in an oven. The dried metabolites were dissolved in minimum quantity of solvent; the next target was the further confirmation of the transformation of reactive yellow dye into different products by passing all the treated and control sample through HPLC column. There was only one major peak at 1.354 min in control sample (Fig. 9(b)).

Whereas, when the rate of removal was 99.5%, the biodegradation of the parent compound was observed with seven detectable peaks at retention time 1.503, 1.620, 1.957, 2.083, 2.392, 2.650 and 2.915 min; however, major peaks were observed at 1.503 and 1.620 min (Fig. 9(b)). Comparison of FTIR spectrum of control

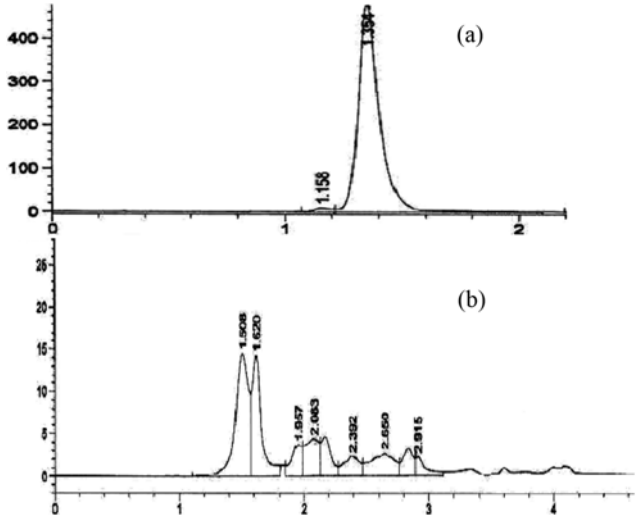

Fig. 9. (a) HPLC Chromatogram of original reactive yellow dye. (b) HPLC Chromatogram of treated reactive yellow.

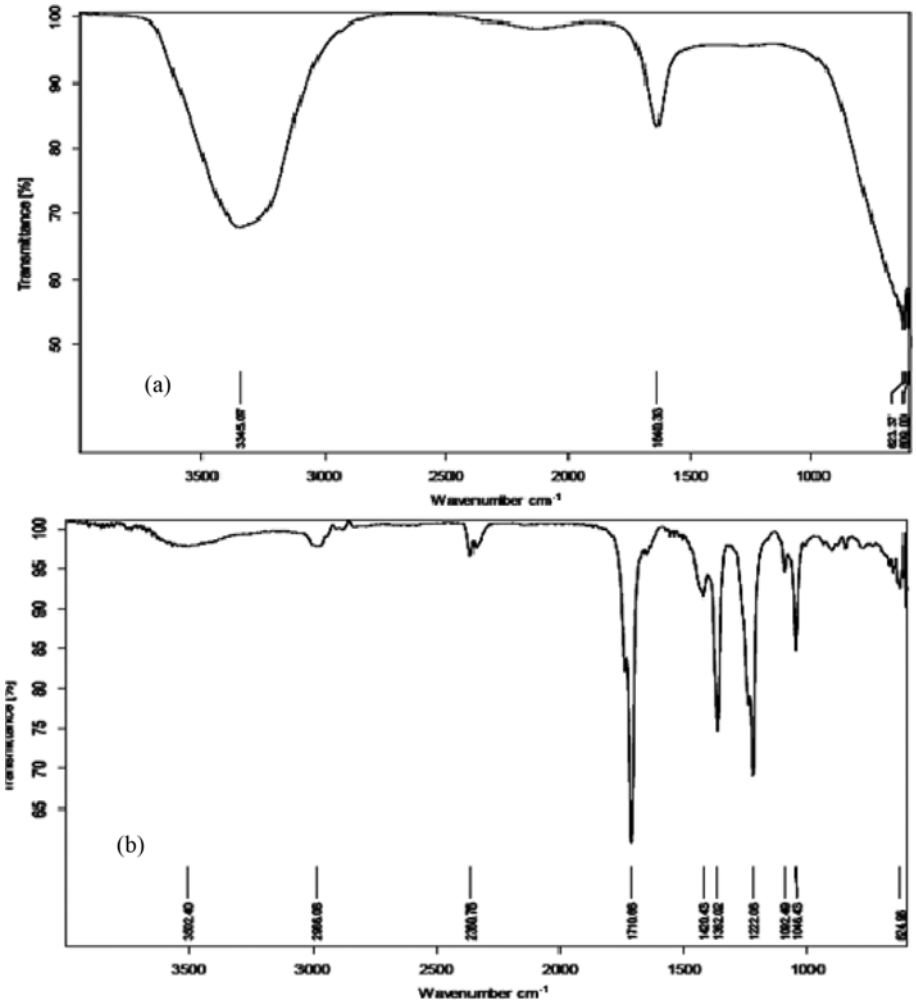

Fig. 10. (a) FTIR Spectrum of original reactive yellow. (b) FTIR Spectrum of treated reactive yellow.

dye with metabolites extracted after biotransformation, evidently pointed out the biodegradation of the parent dye compound by *P. ostreatus* mediated by redox mediators (Fig. 10(a) and 10(b)).

Results of FTIR analysis of the treated sample showed the absence of peak at 1,640 cm^{-1}, indicating that the breakdown of azo bond might be due to the action of oxidoreductase enzyme (Lacc) of high redox potential. The absence of peaks at 609 and 623 cm^{-1} showed the loss of aromatic nature of the xenobiotic compound.

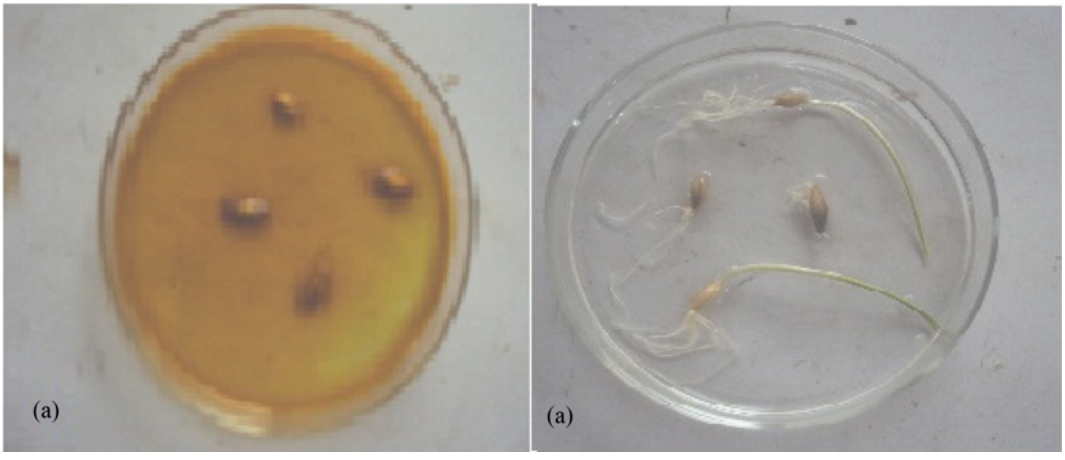

Fig. 11. (a) Germination of seed in original dye (100% Germination inhibition). (b) Germination of seed in treated dye (0% Germination inhibition).

The peaks at 1,046 cm^{-1} and 1,092 cm^{-1} indicated the production of primary and secondary alcohol. A new peak at 1,222 cm^{-1} represented C-O stretching, whereas at 1,362 cm^{-1} stand for aliphatic NO_2 stretching. Peaks at 1,420 and 1,710 were observed for CH_2 deformation and C=O stretching, respectively, while O-H stretching was observed at 3,502 cm^{-1}; whereas at 2,962 cm^{-1} stands for C-H stretching (Fig. 10(b)). All these new peaks clearly indicated that parent Xenobiotic compound was completely transformed into different fragments.

9. Phytotoxicity

It was of prime curiosity to gauge the phytotoxicity of the dye and its extracted metabolites after biotransformation. Relative sensitivities towards the reactive yellow (100 ppm) and its biotransformation products in relation to *A. sativa*seeds (Fig. 11(a) and 11(b)), show 100% germination inhibition for *A. sativa* seeds having zero germination index (Fig. 11(a)) When these seeds were treated with metabolites (100 ppm) formed after biotransformation of reactive yellow dye, there was 16.67% germination inhibition having germination index 83% along with seed germination 100% and root elongation 83% (Fig. 11(b)). Hence, the phytotoxicity experiment clearly indicated that biotransformation of xenobiotic reactive yellow resulted in its detoxification. Therefore, the treated effluent can be used as fertile-irrigation as the biodegradation products did not drastically interfere with the germination of plant seed.

CONCLUSION

Polyphenols are associated with reduction of heart diseases by inhibiting in-vitro oxidation of low-density lipoproteins possess anti-ulcer, anti-mutagenic, anti-inflammatory activity and anti-carcinogenic properties as well as the natural redox stimulator for the removal of recalcitrant and xenobiotic compounds. Phenolic compounds include tannins, alkaloids, and flavonoids which present in higher concentration in *P. granatum* peels compared to *P. armeniaca*, *P. domestica* and *T. aesativum*. Natural phenolics of *P. granatum* peels seemed to stimulate laccase production, which ultimately plays a key role in the biotransformation of Reactive yellow textile dye. The rate of transformation of Reactive yellow was more than threefold as compared to xenobiotic compound treated with only *P. ostreatus*. Transformation of reactive yellow dye into various different products was confirmed by HPLC and FTIR analysis. Phytotoxicity test confirmed the detoxification of reactive yellow. Thus, the natural phenolic compounds could serve as high potential redox mediators for enhanced laccase-mediated decolorization of reactive yellow dye.

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