

Bioremediation of textile dye solutions, textile dye mixtures and textile effluents by laccase from *Aureobasidium pullulans* (de Bary) G. Arnaud (1918) (Fungi: Ascomycota)

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Abstract. The environmental impact of textile waste water is detrimental to all living species in such habitat and hence there is the need to seek novel bioremediation alternative means to alleviate the effect of these dyes on aquatic life. This study investigated the potentials of *Aureobasidium pullulans* (de Bary) G. Arnaud (1918) (Fungi: Ascomycota) in bioremediation of waste water through its production of laccase. Both the fungi and its secreted laccase (crude and purified) were used to decolorize textile dyes, textile dye mixtures and textile waste-water effluent. *A. pullulans* laccase was able to decolorize malachite green (79.9%), allura red (57.5%), tartrazine (21.5%) and methylene blue (4.5%) after 2 h of incubation. The crude *A. pullulans* laccase was able to decolorize the textile waste water up to 35% after 3 h incubation and it was able to decolorize the blue + green + red dye mixtures more specifically. The purified laccase was able to specifically decolorize malachite green. The fungi was able to absorb the textile dyes when it was inoculated into a laccase culture medium containing the dyes or textile waste water effluent. There was approximately 91% decolorization of malachite green, 71% decolorization of allura red, 23% methylene blue and 38% tartazine after three day incubation *A. pullulans* when inoculated into the textile water effluent was able to decolorize the textile waste water effluent up to 80% after 5 days of incubation. Manganese peroxidase played additional role in the decolorization as it was detected in the crude filtrate (1.2 units/mL/min) but no lignin peroxidase activity was detected. It can be deduced that *A. pullulans* is a potential fungi useful for bioremediation of textile waste-water polluted environment.

Keywords: Textile dyes, Bioremediation, Laccase, *Aureobasidium pullulans*, Textile effluents.

Introduction

Discharge of wastewater containing synthetic dyes especially polyaromatics and their carcinogenic health effects as an environmentally important problem, persuaded environmental engineers to develop new techniques for treatment of such hazardous compounds (Gholami-

Borujeni et al., 2011). Bioremediation utilizes natural processes to control pollution problems caused by xenobiotics. These xenobiotics may contain several aromatic ring systems substituted by electron withdrawing groups such as azo, halogen and nitro groups (Kandelbauer and Guebitz, 2005). These groups are present in dyes and it makes them toxic and

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recalcitrant to conventional methods of degradation such as chemical coagulation, flocculation and precipitation techniques meant for the removal of the dye colors from waste waters (Shertate and Thorat, 2014). To reduce toxicity level, several remediation techniques are applied namely microbial degradation (application of fungal and bacterial strains capable of adsorbing or degrading of different dye groups) and enzyme remediation has been considered as a novel concern in this field during last decades (Saratale et al., 2011).

Consequently, microbial degradation or enzymatic attacks leads to complete mineralization of these groups present on the dyes making them less toxic and production of less coloured effluents (Kandelbauer and Guebitz, 2005). The ability of laccases to decolourise even dyes of the same class varies and it solely depends on the biological nature of the source of the microorganisms (Almansa et al., 2004). In this study, *Aureobasidium pullulans* (de Bary) G. Arnaud (1918) a black-yeast-like fungus, was isolated from soil containing decayed plant litters. This fungi has been reported to possess many biotechnological application. It is known to produce several industrially relevant hydrolytic enzymes, polysaccharide (pullulans) and antimycotic agent, aureobasidin A (Gostinčar et al., 2014). *A. pullulans* was able to secrete laccase and the enzyme produced was used in the decolorization of several dyes, dye mixtures and textile waste water effluents. There have been no previous report on the utilization of the fungi or the enzyme in bioremediation of textile waste water effluents. The free enzyme and the fungi were both used in the decolorization process, hence this study would provide information on the utilization of *A. pullulans* in bioremediation of waste water effluents

Materials and method

Microorganism and maintenance

A. pullulans was isolated from soil containing decayed plant litters. It was identified on the basis of the appearance of

polymorphic colonies formed on agar plates, lactophenol in cotton blue test and light microscopy. The fungi was periodically sub cultured every 2 weeks.

Crude enzyme preparation

The laccase production for *A. pullulans* was carried by inoculating a well grown fungus (agar plugs of (5.0 mm) in a sterilized medium that consists of (g/L): Peptone (3.0), glucose (10), KH_2PO_4 (0.6), ZnSO_4 (0.001), K_2HPO_4 (0.4), FeSO_4 (0.0005), MnSO_4 (0.05), MgSO_4 (0.5) (Kiiskinen et al., 2002). In order to monitor the production of laccase in the liquid culture medium, 1 mL aliquots of the culture medium were withdrawn at regular intervals of 24 h and was centrifuged at 4000 xg for 10 min and was analyzed for activity of laccase using guaiacol as the substrate by the method provided in the assay section. Extracellular secretion of laccase in the liquid culture medium by the fungi was determined by plotting the enzyme activity of the growth medium against the number of days after inoculation of the fungal mycelia. Each point on the curve is an average of three measurements.

Purification of laccase

For purification of laccase, the fungi was grown in 100 mL culture flasks, each containing 50 mL sterilized optimal growth medium. Maximum activity of laccase appeared on the 6th day of inoculation of the fungal mycelia. On the 6th day, all the cultures in the 10 flasks were pooled together; mycelia were removed by filtration through double layers of cheese cloth. The culture filtrate (200 mL) was then lyophilized and the powdered enzyme reconstituted with 10 mM M Tris HCl buffer, pH 7.2. The reconstituted crude enzyme (3 mL) was layered on a 1 cm x 10 cm column of DEAE-Sephadex A-50 which had previously been equilibrated with 10 mM M Tris HCl buffer, pH 7.2. Fractions (2 mL) were collected at a flow rate of 20 mL/h. Laccase activity in the fractions were determined and the protein concentrations monitored at 280 nm. All laccase active fractions were pooled and

dialysed against 80% glycerol in 10 mM acetate buffer, pH 5.0.

Assay for laccase, lignin peroxidase and manganese activity

Laccase activity was determined using 2-methoxyphenol (guaiacol). One unit of laccase activity is defined as the amount of laccase that catalyzed the oxidation of one micromole of guaiacol per minute (Kiiskinen et al., 2002). Lignin peroxidase activity in the crude was determined by the hydrogen peroxide-dependent veratraldehyde (3, 4-dimethoxybenzaldehyde) formation ($\square_{310\text{nm}}$ and extinction coefficient of $9,300 \text{ M}^{-1} \text{ cm}^{-1}$) from 2mM veratryl alcohol (3, 4-dimethoxybenzyl alcohol) in 100 mM sodium tartrate buffer (pH 3) in the presence of 0.4 mM H_2O_2 (Tien and Kirk, 1983). Manganese peroxidase activity in the cell free solution was determined by the modified method of Wariishi et al. (1992). One unit of Manganese peroxidase activity corresponds to the change in absorbance per minute at 25 °C.

Dye decolorization potentials of crude and purified laccase

Decolorization potentials of the crude and purified laccase was determined in reaction mixture that consists of 0.5 mL of the enzyme, 2.0 mL of the dye solution (concentration 120 mg/L) and 2.5 mL of 0.1 M citrate buffer (pH 4.5). The reaction mixture was incubated at room temperature without agitation for 30 min. 1.0 mL of the mixture was taken and decrease in absorption was monitored for 3 h. Percentage decolorization (%) was calculated using formula below:

$$\text{Decolorization (\%)} = ((A_C - A_0)/A_C) \times 100$$

Where:

A_C : absorbance of control which consist of 0.5 mL of the denatured enzyme broth, 2 mL of the dye solution and 2.5 mL of citrate buffer.

A_0 : observed absorbance which consists of 0.5 mL of the pure enzyme, 2 mL of the dye

solution and 2.5 mL of citrate buffer.

Negative control (reaction mixture without enzyme) was prepared as reference to compare decolorization percentage of treated sample (Foorontafar et al., 2012).

Textile dye decolorization potentials of *A. pullulans* in laccase culture medium

The potentials of the fungi to act in bioremediation of textile waste water were carried out by inoculating the organism in a laccase culture medium (Kiiskinen et al., 2002) that contained 120 mg/L quantity of the dyes. The culture flasks were incubated at 27 °C and samples were withdrawn every 24 h and centrifuged at 4000 rpm. The rate of decolorization was monitored at each dye wavelength. Percentage (%) decolorization was calculated as earlier described.

Textile dye mixtures decolorization potentials of cell free solution

The potentials of the crude enzyme to mediate in decolorization of mixtures of several textile dyes was determined by incubating the crude enzyme with the dye mixtures in final concentration of 40 mg/L. The dye mixtures were scanned using a UV-visible spectrophotometer from 200-800 nm to obtain the maximal absorption. All dye mixtures contained malachite green. The rate of decolorization was monitor decolorization at each dye mixture wavelength. Percentage decolorization was calculated as earlier described.

Decolourization potentials of *A. pullulans* laccase on textile waste water effluent

Characterization of the textile waste water effluent

The textile waste water effluents used in this study was collected from a textile manufacturing industry in Lagos, Nigeria in a sterile container. The pH and temperature were immediately determined. Other characteristics such as odour and colour were observed. The effluents was

scanned from 200 nm to 800 nm. The waste water effluents were used without further pre-treatment for the decolourization experiments. The peak obtained served as the utilized wavelength for absorption determinations.

Textile waste water effluent decolourization by A. pullulans laccase

Textile waste water effluent decolourization potential of the crude *A. pullulans* laccase was determined in reaction mixture that consists of 2.0 mL of the textile waste water effluent, 2.5 mL of 0.1 M citrate buffer (pH 4.5) and 0.5 mL of the crude laccase. The reaction mixture was incubated at room temperature in static condition for 30 min. 1.0 mL of the mixture was withdrawn and decrease in absorption was monitored for 3 h. Percentage (%) decolourization was calculated using formula below:

$$\text{Decolorization (\%)} = [(A_C - A_O)/A_C] \times 100$$

Where:

A_C : Is the absorbance of control (at 482 nm) which consist of 2 mL of the textile effluent, 2.5 mL of citrate buffer and 0.5 mL of the denatured crude *A. pullulans* laccase.

A_O : Is the observed absorbance (at 482 nm) which consist 2 mL of the dye solution, 2.5 mL of citrate buffer and 0.5 mL of the crude *A. pullulans* laccase.

Negative control (reaction mixture without enzyme) were prepared as reference to compare decolourization percentage of treated sample (Gomaa et al., 2011).

Textile waste water effluent decolourization by A. pullulans

The potentials of the fungi to act in decolourization of textile waste water was carried out by inoculating the organism in a laccase culture medium [peptone (0.3 g), glucose (1 g), KH_2PO_4 (0.06 g), ZnSO_4 (0.0001 g), K_2HPO_4 (0.04 g), FeSO_4 (0.00005 g), MnSO_4 (0.005 g), MgSO_4 (0.05 g) per liter] prepared using the textile waste water effluent that had been sterilized. The culture flasks were incubated at 27 °C and one milliliter (1.0 mL) were withdrawn every 24 h and centrifuged at 4000 rpm for 5 min. Percentage (%) decolourization was calculated as described above.

Results

The cell free solution was able to decolourise malachite green ($79.9 \pm 0.05\%$), allura red ($57.5 \pm 0.07\%$), tartrazine ($21.5 \pm 0.07\%$) and methylene blue ($4.5 \pm 0.08\%$) and after 2 h of incubation (Figure 1). The fungi was able to decolourise the textile dyes in a laccase producing medium after three days in the following decreasing order malachite green > allura red > tartazine > methylene blue (Figure 2). The cell free supernatant was able to specifically decolorize the dye mixtures (blue + green + red) more than it decolorized the other dye mixtures (Figure 3). The textile waste water had a turquoise blue colour and the pH and temperature of the waste water are 6.0 and 48 °C, respectively. The wavelength of maximal absorption was 486 nm. The textile waste water was decolourized up to 40% (Figure 4) by the *A. pullulans* laccase after 3 h. There was up to 80% decolourization by the fungi after three days incubation (Figure 5 and Figure 7). The purified enzyme was also specifically able to decolorized malachite green more efficiently than the other dyes used in the study (Figure 6).

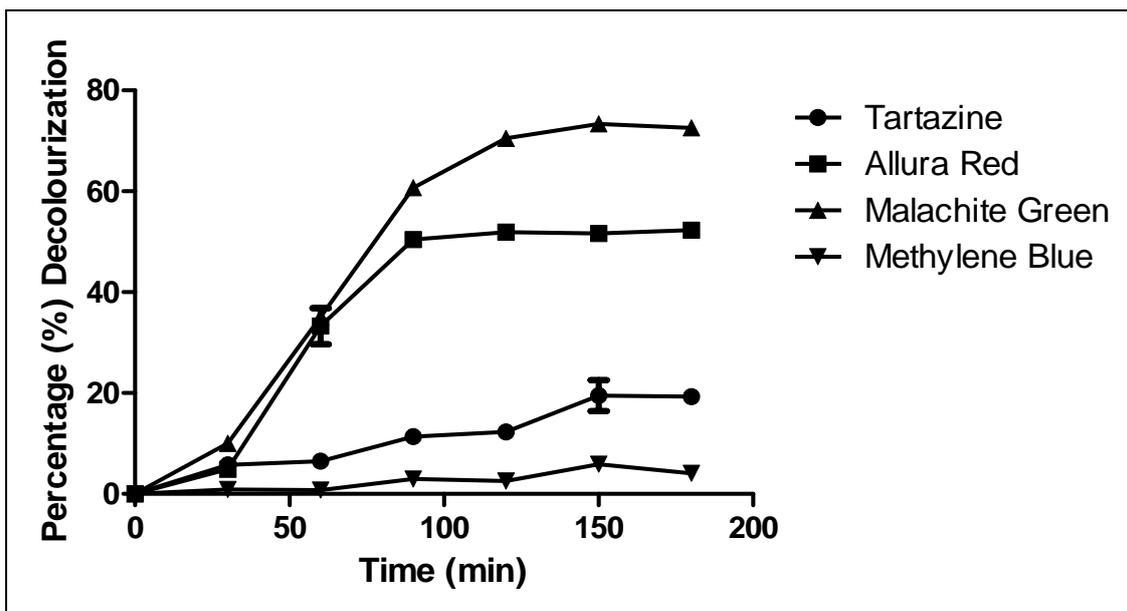


Figure 1. Decolorization of several textile dyes by extracellular laccase from *A. pullulans*.

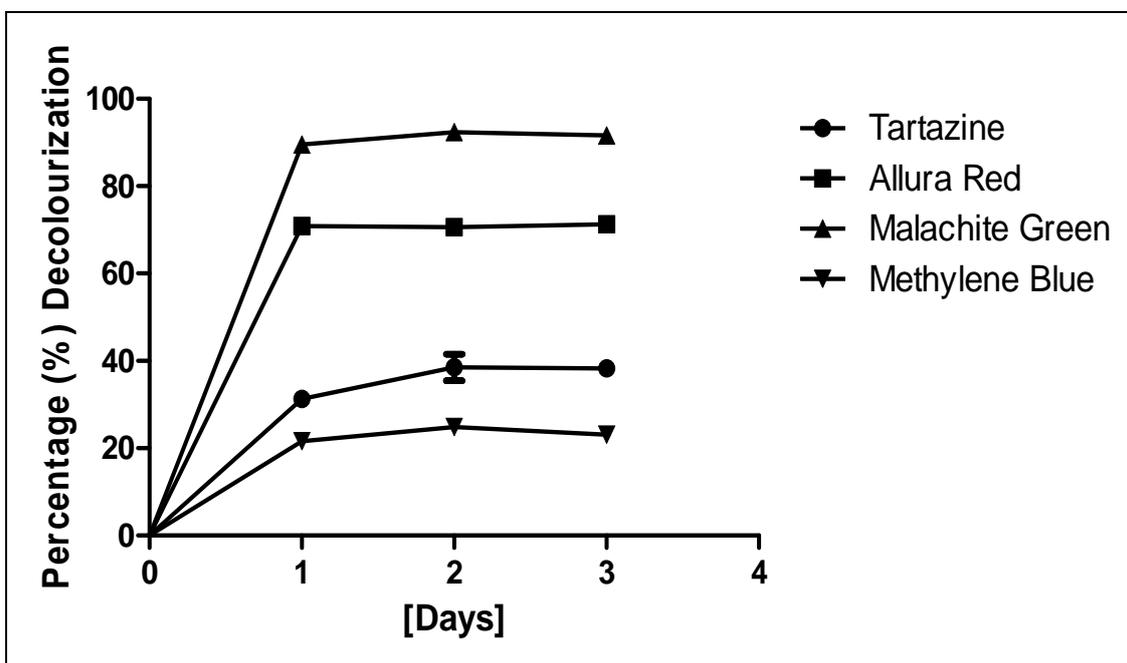


Figure 2. Decolorization of several textile dyes by *A. pullulans* in laccase culture medium.

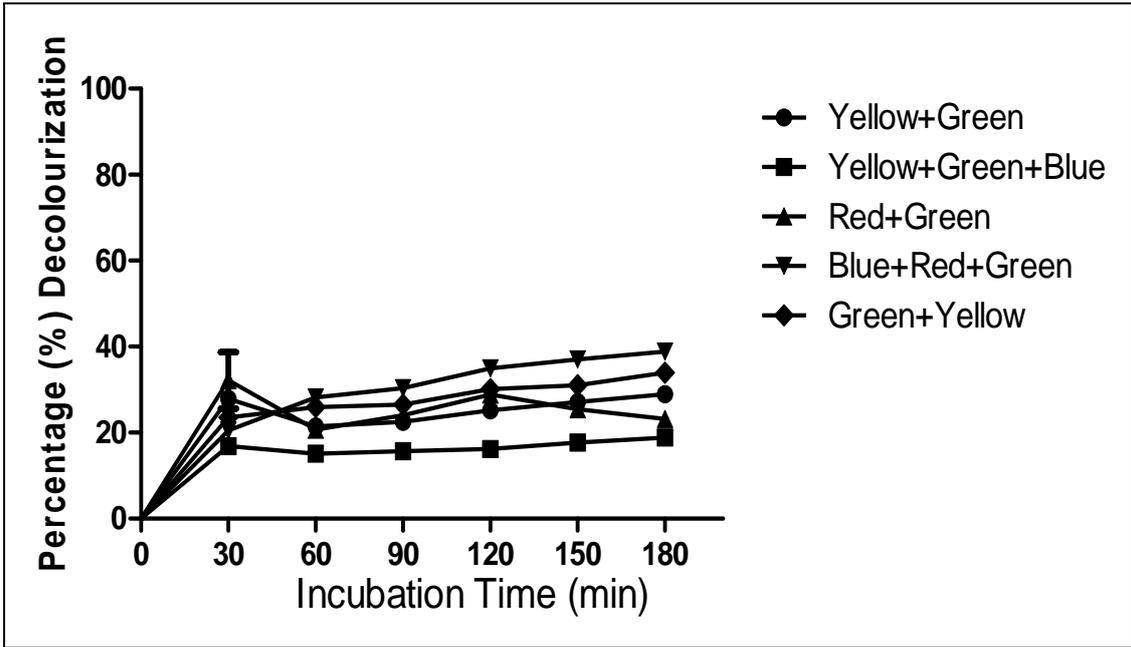


Figure 3. Dye decolorization of textile dye mixtures by cell free solution of laccase obtained from *A. pullulans*.

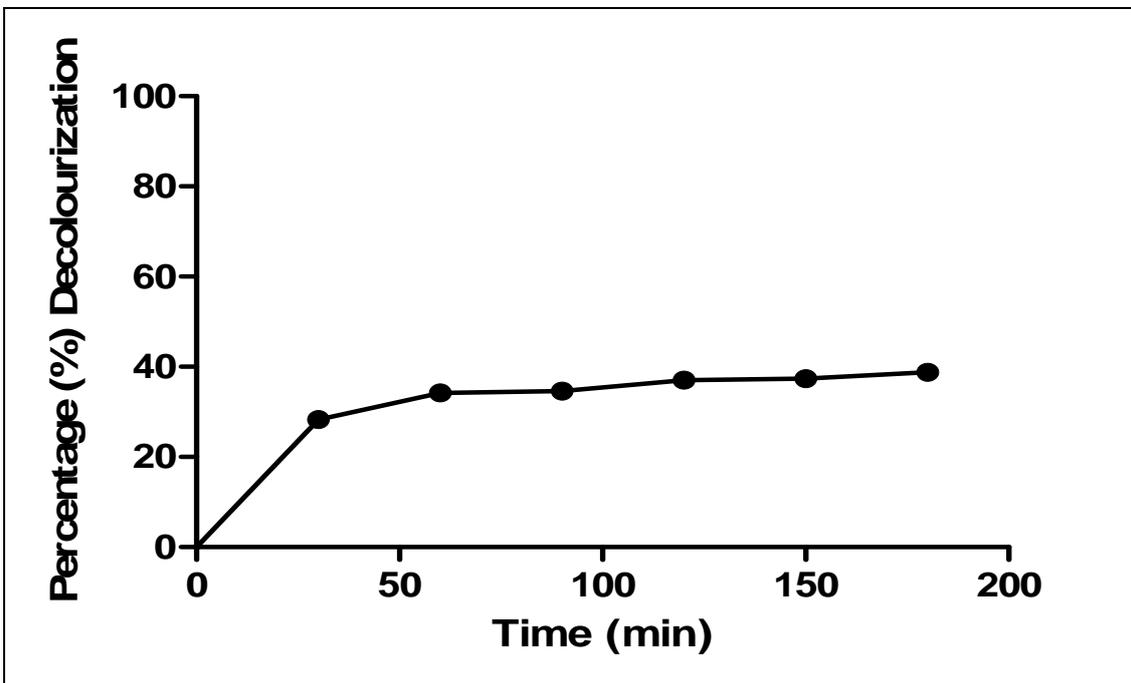


Figure 4. Dye decolorization of textile waste water by crude *A. pullulans* laccase. Textile waste water, 0.1 M citrate buffer, pH 5.0 and crude *A. pullulans* laccase was added. The rate of decolorization was monitored by taking the absorbance every 30 min.

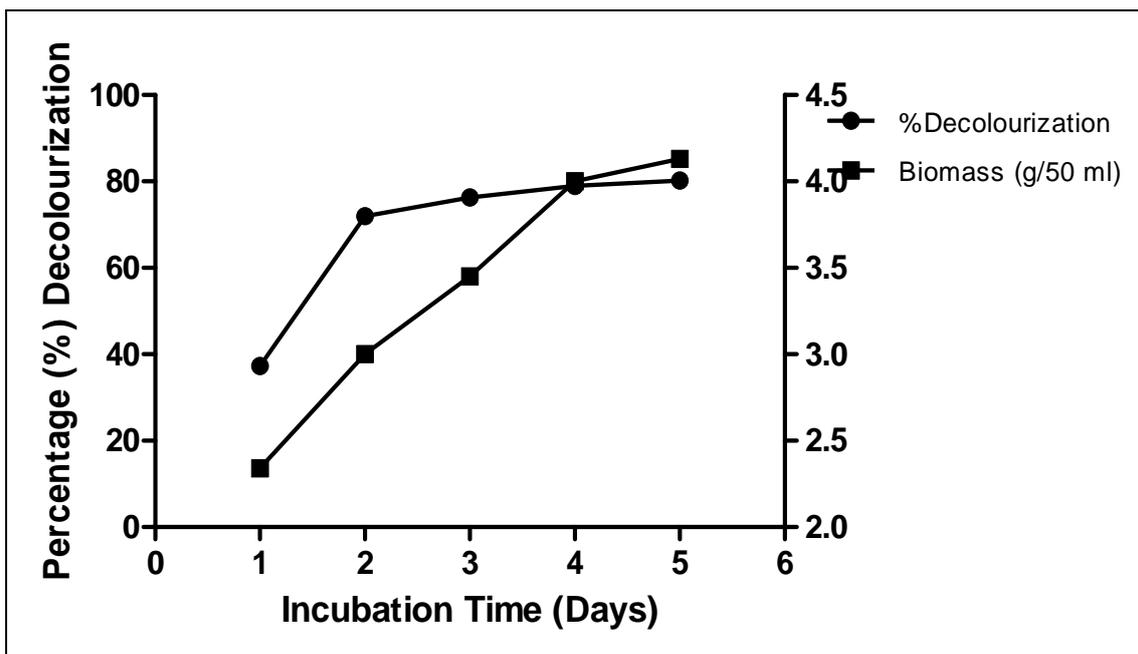


Figure 5. Decolorization of textile waste water effluents by *A. pullulans*, inoculated into a laccase culture medium containing the textile waste-water. The rate of decolorization was monitored by taking the absorbance every 24 h.

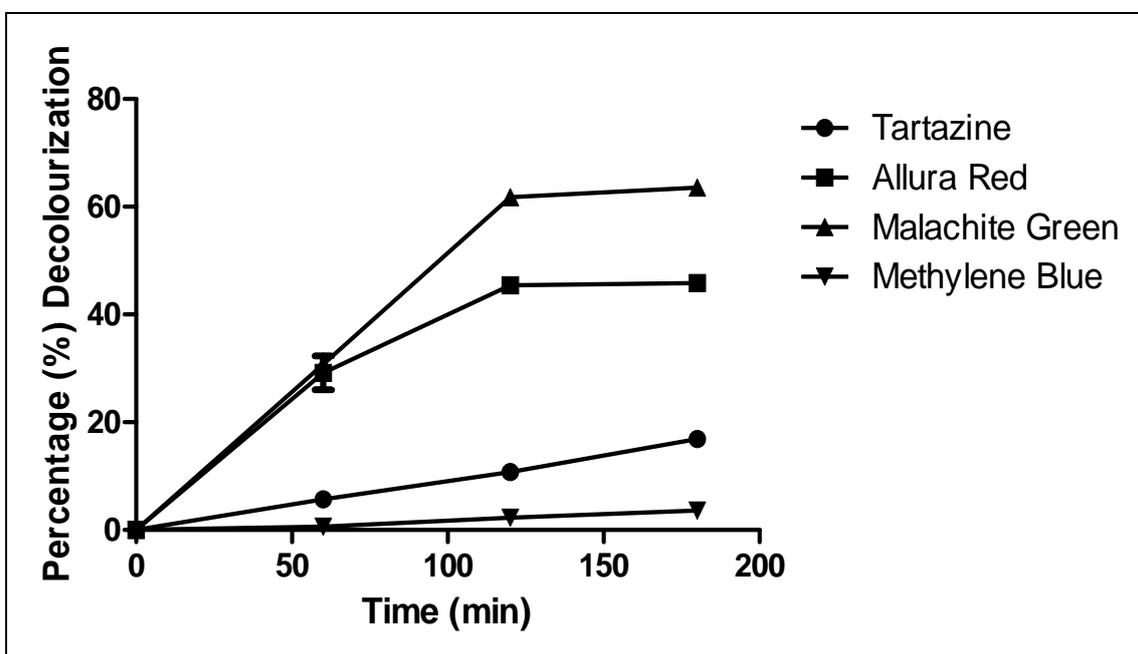


Figure 6. Decolorization of several textile dyes by purified laccase from *A. pullulans*.

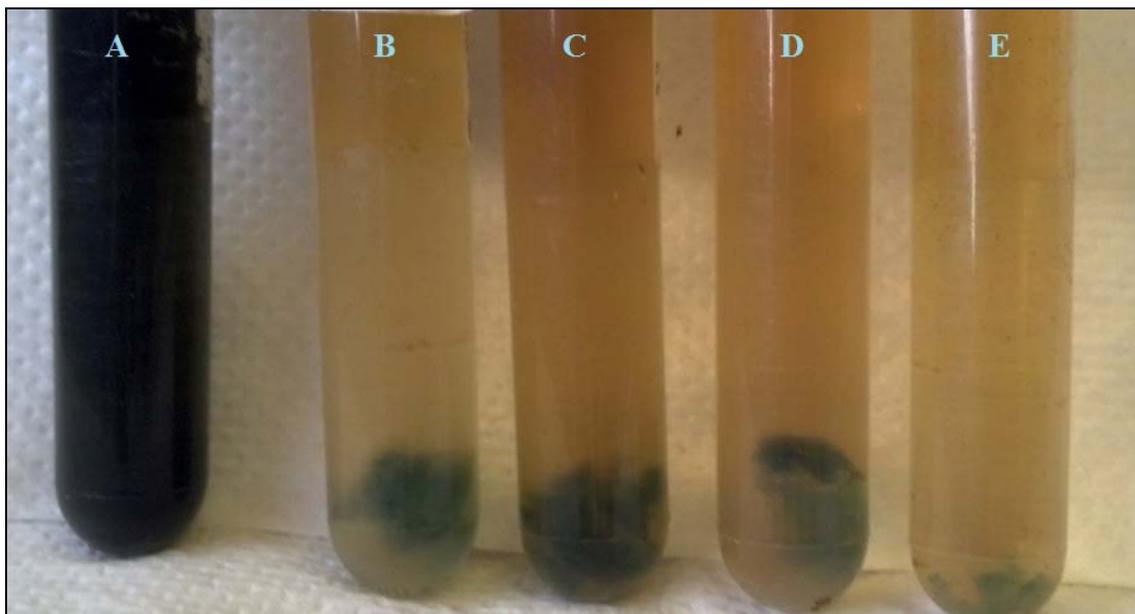


Figure 7. The decolorization of textile waste water effluents by *A. pullulans* by absorption of the dyes. (A) Textile waste water effluent; (B) After 24 h of inoculating the organism into the textile waste water effluents; (C) After 48 h of inoculating the organism into the textile waste water effluents; (D) After 72 h of inoculating the organism into the textile waste water effluents; (E) After 96 h of inoculating the organism into the textile waste water effluents.

Discussion

This study investigated the possibility of utilizing laccase produced by *A. pullulans* in the bioremediation of textile waste-water.

Laccase from *A. pullulans* was able to decolourize malachite green at 80% (Figure 1). This was comparable to the decolourization at 60.5% of malachite green (with initial concentration of 60 mg/L) in presence of laccase from *P. variabile* (Forootanfar et al., 2012) in 15 min while there was 98% of malachite green decolourization using laccase of *Ganoderma* sp (Zhuo et al., 2011).

In comparison to other classes of dyes the triphenylmethane dyes are resistant to enzymatic treatment and requires time for decolorization (Sivakumar et al., 2010). The inability of the crude *A. pullulans* laccase to efficiently decolourize methylene blue and tartrazine may be eliminated by inclusion of laccase mediators such as hydroxybenzotriazole (HBT) which is a synthetic laccase mediator that assists in laccase oxidation of different substrates by facilitating electron transfer from O_2 to

laccase substrate (Zhuo et al., 2011). The inoculation of the fungi to the laccase culture medium containing 120 mg/L of the used textile dyes caused a removal of the dyes. There was approximately 91% decolourization of malachite green, 71% decolorization of allura red, 23% methylene blue and 38% decolourization of tartrazine after three day incubation (Figure 3).

The organism was also specific in the decolourization of the dyes utilizing malachite green more efficiently than other dyes after three day incubation under static conditions. This is similar to laccase from *Trametes* sp. which was malachite green specific (Maalej-Kammoun et al., 2009). Also a mixture of the textile dyes was decolourised by the crude laccase by more than 40% and 60% at 80 mg/L and 40 mg/L of the dye mixtures respectively (Figure 3). The crude laccase was able to decolourise the textile waste water effluents by up to 40% (Figure 4) while *A. pullulans* in the laccase culture medium was able to decolourise the textile waste water effluent by up to 80% (Figure 5).

The autoclaved biomass showed minimal decolourization suggesting that

viable cells are required for maximum decolourization. The majority of colour was removed when viable cells were cultivated. Adsorption is a common fungal dye removal process which involves the attachment of the dye molecule to functional group present on the fungal wall (Coulibaly et al., 2003). In this study, the fungal biomass did acquire the colour of the textile dyes and the textile dye waste water (Figure 7).

The majority of colour was removed when viable cells were cultivated. This indicated that dye absorption onto fungal biomass represents the mode of removal by *A. pullulans*. The decolorization is not based solely on laccase but indication exists of manganese peroxidase assisted decolorization of textile dyes. The detection of the presence of manganese peroxidase in the culture medium is indicative of the lignolytic enzyme production by the fungi. This was evident as there was reduced decolorizing properties by the purified enzyme. There was no lignin peroxidase detected in the cell free solution.

Conclusion

The findings of this study suggests that *A. pullulans* could be potentially employed in the bioremediation of textile dye waste water polluted environment. There is still need to study the effects (if any) on mediators on the decolourization potentials of laccase by this fungi.

Conflicts of Interests

Authors declare that they have no conflict of interests.

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