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DYE DECOLOURIZATION: LACCASES BY THE BEST FUSANT STRAIN OF *TRAMETES HIRSUTA*

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ABSTRACT

About 100,000 commercial dyes are manufactured by various industries worldwide including several varieties of dyes such as acidic and basic dyes [1]. These dyes are important chemical pollutants released into the environment especially from the textile industry. The enzymatic catalysis or hydrolysis could serve as a more environmentally benign alternative than the currently used chemical processes for the same or may be better result. In this work decolorization of the dyes has been used as a test for checking any improvement in the laccases production by the fusant strains of the test fungi *Trametes hirsuta*. The improved strain (fusant) of *Trametes hirsuta* was able to decolourize dye in 9 days of incubation at 37˚C. Among the dyes (0.02%W/V) used for decolourization test (malachite green, orange G, eriochrome black T, basic fucshin, congo red and crystal violet), the purified immobilized enzyme of *Trametes hirsuta* was able to decolourize Malachite green and crystal violet to the maximum extent in 15 days. The rate of decolourization activity was observed to be higher in free laccases when compared to the immobilized enzyme at optimum conditions (pH5.0 and 70°C).

Key words: Dyes, decolourization, Laccases, *Trametes hirsuta*.

1. INTRODUCTION

The current environmental problems are mainly due to the extensive disposal of pollutants in the environment. Many reports have been obtained from the release of hazardous compounds such as polycyclic aromatic hydrocarbons (PAH), pentachlorophenols (PCP), polychlorinated

biphenyls (PCB), 1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane (DDT), benzene, toluene, ethylbenzene, xylene (BTEX) and trinitrotoluene (TNT) in the environment that have carcinogenic or mutagenic effects on the living beings. Reports are available showing that fungal laccases can neutralize a wide variety of hazardous chemicals [2, 3]. This has generated a great interest in using them for bioremediation.

Diverse chemical reagents, ranging from inorganic compounds to polymers and organic products are used for wet processing of textiles which also consumes large volumes of water. Most of them are resistant to fading on light exposure, water treatment or even chemical treatment. The effluents from textile industries with such resistant dyes when released in the nearby water bodies, these dyes are harmful chemicals with cytotoxic and cytomutagenic effects on various living organisms in the water bodies capable of moving through food chain and ultimately harming human beings too [4].

Laccases have become a group of very important enzymes due to their use in a variety of applications, including the chemical, agricultural, paper, textile, fuel, food and cosmetic sectors. They are equally sought after as an enzyme for industrial production and as a tool for the purpose of bioremediation [5, 6, 7]. Laccases show direct involvement in the degradation of various dyes and xenobiotic compounds [8]. Resarchers have studied various aspects involved with the identification of the organisms producing the enzyme as well as purification, characterization and optimization of the enzyme production and applications [7, 9, 10].

The potential of laccases in degrading dyes of diverse chemical structure [11] including synthetic dyes, currently employed in the industry [12] has provided an attractive solution to the above problem. Beside decolorization of textile effluents, it is used to bleach textiles and even to synthesize dyes [13].

2.1 MATERIALS AND METHODS

2.1.1 Laccases production

The ability of laccase production by the selected fusant strain of *T.hirsuta* was determined qualitatively and quantitatively.

2.1.1.2 Qualitative estimation of laccases production

The best fusant of *T.hirsuta* was inoculated in the screening medium containing 0.02%

Guaiacol and incubated at 30°C for 5 days. In order to assess any change in laccases production, the mutant and fusant strains and their respective parent fungal cultures were also examined under similar conditions.

2.1.1.3 Quantitative estimation of laccase production

All the strains of the test fungus (wild, mutant and fusant) were examined for laccase production quantitatively in the best production medium M4+WB+G with pH 3.0 [14]. 10mm plugs of each strain were inoculated in a series of production medium (100ml) and incubated at 35ºC in an orbital shaking incubator for 9 days.

After incubation the quantitative difference in laccase production among the wild, mutant and fusants was deliberated by calculating enzyme activity.

2.1.2 Purification and characterization of laccases

Purification of laccases was carried out by ammonium sulfate precipitation followed by dialysis membrane and gel filtration chromatography (Anion exchange chromatography).

The absorbance of the reconstituted enzyme solution was measured with a spectrophotometer at 465nm using Guaiacol as a substrate.

The concentration of protein in the crude samples and the purified samples of enzyme produced by the wild and mutant strains of *T.hirsuta* in the production medium was calculated by Folin Lowry method of protein estimation.

2.1.3 Immobilization of laccases

The partially purified laccases in buffer solution (Elution buffer) was mixed with 2% sodium alginate solution in 1:1 ratio. The Laccase-alginate mixture was added drop wise into 0.2 M solution of calcium chloride with continuous shaking at 4°C. As the drop of laccase-alginate solution get mixed with $CaCl_2$ solution immediately Na + ions of Na-alginate were replaced by the Ca^{+2} ions of $CaCl_2$ solution, which finally resulted in formation of Ca-alginate beads entrapping enzyme within. The beads thus formed were washed 3-4 times with deionized water and finally with 50M Tris-HCl buffer of pH 7.5. These beads were dried and weighed for further studies like characterization of its activity as done for the partially purified enzyme before immobilization [15].

2.1.4 Characterization of purified laccases

The enzyme purified from the culture supernatant through various steps and immobilized

enzyme was then characterized as below.

2.1.4.1 Effect of pH on the activity of purified Laccases

The effect of pH on the activity of purified enzyme (free enzyme and immobilized enzyme) was studied by measuring the enzyme activity at various pH in the range of 3-9, using Guaiacol as substrate. Buffer solutions (20mM) of different pH values were used which includes acetate buffer (pH 3.0, 4.0, and 5.0), phosphate buffer (pH 6.0, 7.0, and 8.0) and Tris HCl (pH 9.0). The purified enzyme was incubated for 1 h in buffers with varying pH values at 30°C and then remaining enzyme activity was measured under standard conditions.

2.1.4.2 Effect of temperature on the activity of purified laccases

Laccases activity of the purified enzyme (free enzyme and immobilized enzyme) was determined after 1 hour of incubation at 0°C to 90°C temperature and then remaining activity was measured under standard conditions.

2.1.5 Application of laccases- dye decolorization

To investigate the effect of improved strain on the production of decolorizing enzymes, the wild, mutants and fusants of both test fungi were inoculated in malt extract medium containing 0.02% of dye (Malachite green) and incubated at 37°C for 9 days and the zone of decolorization was measured after every 3 days of time interval. Similarly the effect of decolorization by the partially purified free and immobilized enzyme (1ml) was investigated by incubating 1ml of the enzyme (obtained from the better strain among the two) in 0.02% of dye (Malachite green, Orange G, Eriochrome black T, Basic Fucshin, Congo red and Crystal violet) at optimum pH and Temperature of enzyme activity.

2.1.5.1 Decolorization assay

As described by Yatome *et al.* (1993) [16], the decolorizing activity was expressed in terms of percent decolourisation calculated according to the following formula where the decrease in absorbance was monitored at A_{420} nm for malachite green.

$$D\% = 100 \times \frac{(A_{ini} - A_{fin})}{A_{ini}}$$

Where D = decolourisation, A_{ini} = initial absorbance, and A_{fin} = final absorbance of dye, after incubation time.

2.1.6 Statistical assay

All the reports were evaluated by using statistical software Minitab 16 and Portable IBM SPSS Statistics v19.

3.1 RESULTS AND DISCUSSION

3.1.1 Qualitative estimation of laccase production

The fusant strain obtained from the test fungus exhibited higher growth rate. There was 34.7% increase in growth rate by PF (fusant strain of *T.hirsuta*) when compared to the parent wild strains and 6.5% increase in growth rate by the fusant strains of *T.hirsuta* when compared to the mutant strain (PM).

The fusant strains were also observed to produce more laccases as per the plate screening assay in 5 days of incubation when compared to their respective wild and mutant strains. The colored zone of Guaiacol oxidation around the colony which was 0.5cm by PR was increased 140% by PM (mutant strains of the test fungi). Similarly 380% increase in the diameter of the colored zone by the respective fusant strain (PF) was observed in 5 days of incubation (Fig.1).

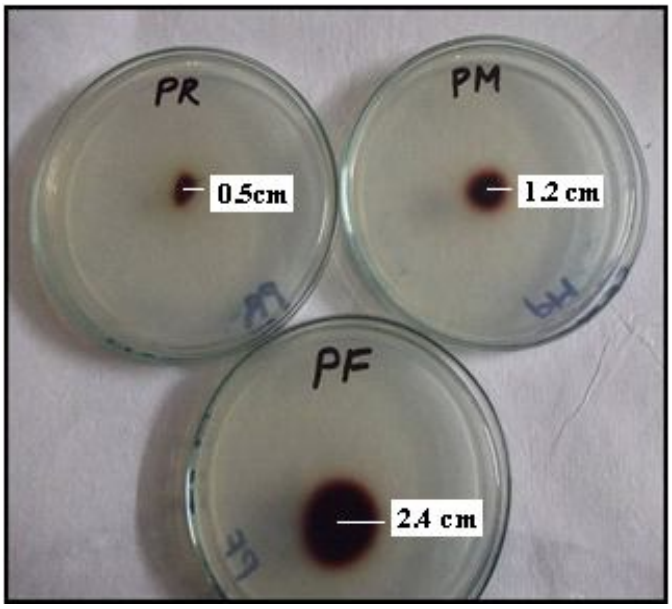

Fig.1 *T.hirsuta*- Qualitative estimation of laccase Production by wild, mutant and fusant.The diameter of zone of guaiacol oxidation indicates the improvement of the strain for laccases production. PR= Wild, PM= Mutant (PH2), PF=Fusant.

3.1.2 Quantitative estimation of laccase production

A comparison of the quantitative assay of laccases showed higher laccases production by the fusant strains when compared to the wild and mutant. There was only approximately 8.9% increase in productivity by both the mutant strains (PM) but approximately 64% increase in enzyme production was observed by the fusant strains of *T.hirsuta* (PF) when compared to their wild parents (Fig.2). Khattab *et al.* (2012) [17] also reported up to 111.85% increase in protease enzyme production in the fusant as compared to the higher yielding parent. A majority of the fusants tested gave better results than either of the two parents.

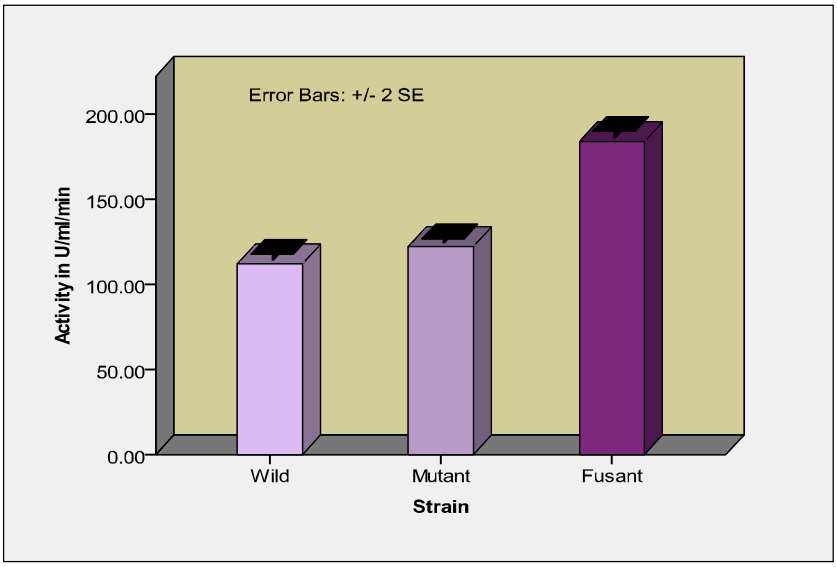

Fig.2 *T.hirsuta* -Laccases production by wild, mutant and fusant

The wild, mutant and fusant strains of the test fungi inoculated in laccases production medium (M4+WB+G) with pH 3.0 for 9 days in an orbital shaking incubator (120rpm) at 35°C. Enzyme activity calculated in U/ml/min.

3.1.3 Purification of Laccases obtained from the best producers

A three step purification procedure (ammonium precipitation, membrane dialysis and anion exchange chromatography) was used for purifying laccases produced by the fusant of *T.hirsuta*. It resulted in an increase of 24.3% laccases activity as compared to the crude extract. Though the total protein content (mg) in the sample was decreased, the specific activity (U/mg) and purification fold was found to be increased after every step of purification (Table 1).

Table.1 laccases purification Table-*T.hirsuta*

Purification method	Volume ml	Total protein mg	Activity Units	Total Activity (units*ml)	Specific Activity (units/mg)	Fold Purification	% yield
Culture Filtrate	80	27.25	185	14800	6.8	1.0	100
85% (NH4)2SO4 Precipitation	**40**	**18**	170	**6800**	**9.4**	1.4	45.9
Membrane Dialysis	**30**	**7.8**	162	**4860**	**20.8**	3.1	32.8
DEAE Cellulose Chromatography	**20**	**1.5**	180	**3600**	**120**	17.6	24.3

A three step purification procedure resulted in 24.3% yield with 17.6 fold purification

3.1.4 Immobilization of Laccases

The laccases enzyme in partially purified form was successfully entrapped in Calcium-alginate beads. The rate of enzyme activity was found to be slower. Full activity was achieved in 10 minutes of incubation, where as the same was achieved within 3minutes by the respective free enzyme under similar conditions.

3.1.5 Effect of physical factors on the activity of purified free and immobilized Laccases

The optimum pH for the enzyme activity for purified free enzyme and immobilized enzyme produced by the best strains of the test fungi was 5.0 but the rate of reaction was decreased by approximately 48% in *T.hirsuta* after immobilization (Fig.3).

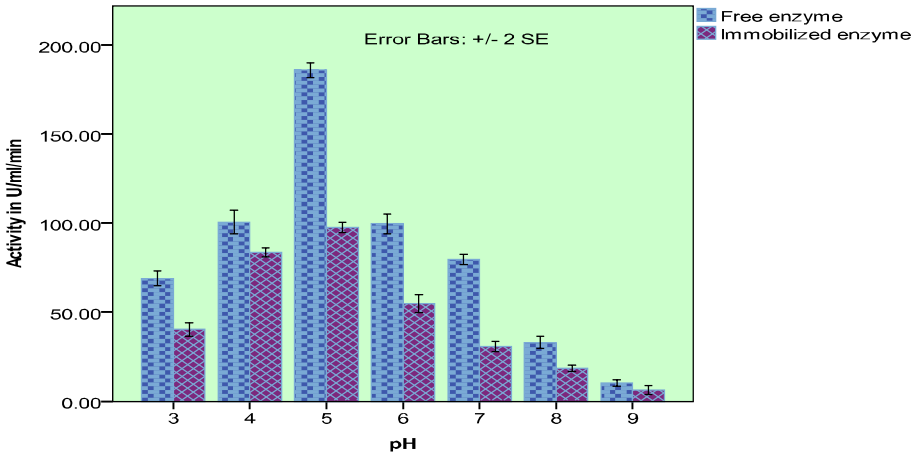

Fig.3 *T.hirsuta*- Effect of pH on the activity of purified free and immobilized Laccases

Though the optimum pH for enzyme activity for both free and immobilized enzyme is 5.0, the rate of reaction was decreased in case of immobilized enzyme.

The optimum temperature for enzyme activity for the purified free and immobilized enzymes produced by the best strain of the test fungi was found to be 70°C. Similar to the observation earlier, the rate of reaction was decreased by 22.7% in *T.hirsuta* after immobilization (Fig.4).

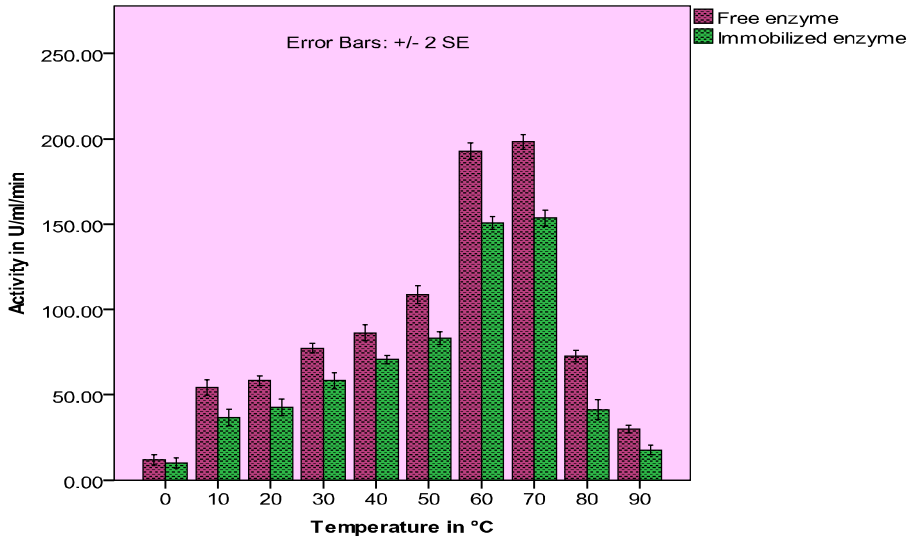

Fig.4 *T.hirsuta*- Effect of Temperature on the activity of purified free and immobilized Laccases.

Though the optimum temperature for enzyme activity for both free and immobilized enzyme is 70°C, the rate of reaction was decreased in case of immobilized enzyme.

3.1.6 Application of laccases- dye decolourization

About 100,000 commercial dyes are manufactured by various industries worldwide including several varieties of dyes such as acidic and basic dyes [1]. These dyes are important chemical pollutants released into the environment especially from the textile industry. The ability of some of the enzymes in decolorizing and bleaching such dyes may contribute positively in the environment sustenance. Such enzymes have wide applications including textile dye bleaching, pulp bleaching and bioremediation. The enzymatic catalysis or hydrolysis could serve as a more environmentally benign alternative than the currently used chemical processes for the same or may be better result. Various higher fungi such as *Trametes hirsuta, Thelephora sp.* and *Pleurotus florida* have been evaluated for their ability to decolorize

various dyes like blue CA, black B133, corazol violet SR, orange G, congo red and amido black 10B [18, 19, 20, 21].

In this work decolorization of the dyes has been used as a test for checking any improvement in the laccases production by the mutant and the fusant strains of the test fungi.

3.1.6.1 Qualitative estimation of dye decolourization ability

The improved strain (fusant) of *Trametes hirsuta* was able to decolourize dye in 9 days of incubation at 37˚C (Fig. 5). The enzyme obtained from fusant strain of *Trametes hirsuta* was used for further quantitative investigation of dye decolourization.

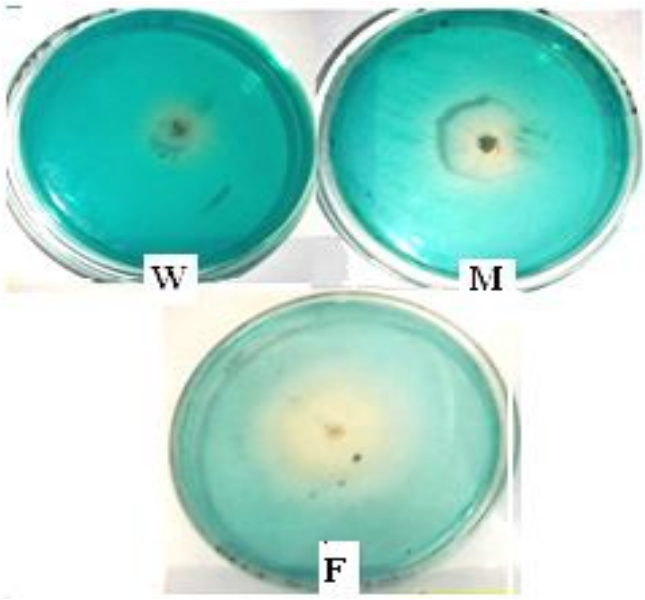

Fig.5 Decolourization of Malachite green by *T.hirsuta.* The strains of T.hirsuta observed for dye decolourization (0.02%) after 9 days of incubation at 37°C. W=Wild, M=Mutant, F=Fusant

3.1.6.2 Quantitative estimation of dye decolourization ability

Among the dyes (0.02%W/V) used for decolourization test (malachite green, orange G, eriochrome black T, basic fucshin, congo red and crystal violet), the partially purified immobilized enzyme of *Trametes hirsuta* was able to decolourize Malachite green and crystal violet to the maximum extent in 15 days (Table.2).

Table 2. Dye Decolorization property of immobilized Laccases

Dye	Type		Percentage of Decolorization			
		Day3	Day6	Day9	Day12	Day15
Malachite green	Aryl methane	22.2%	70%	87%	91%	98%
Fucshin basic	Aryl methane	0%	15%	38%	53%	67%
Crystal Violet	Aryl methane	28%	32%	60%	70%	96%
Congo Red	Azo dye	10%	28%	44%	62%	75%
Eriochrome Black T	Azo dye	05%	20%	45%	58%	65%
Orange G	Azo dye	20%	31%	56%	69%	80%

Each dye was prepared with concentration 0.02% and incubated with the immobilized enzyme sample (1ml) for various days. Absorbance was monitored spectrophotometrically (fuschin basic=540nm, crystal violet=565nm, congo red=550nm, eriochrome black T=525nm and orange G=476nm).

The rate of decolourization activity was observed to be higher in free laccases when compared to the immobilized enzyme. It resulted in 100% decolorization of malachite green with free enzyme in only 7 days of incubation where as only 85% was reported with the immobilized enzyme.

But 98% of decolourization activity was achieved by the immobilized enzyme after 15 days of incubation at optimum conditions (pH5.0 and 70°C) (Fig. 6). Similarly 96% of Crystal violet (0.02%W/V) was decolourized by the immobilized enzyme in 15 days of incubation but only 90% of the dye was decolourized by the free enzyme in 7 days of incubation at optimum conditions of enzyme activity (Fig. 7).

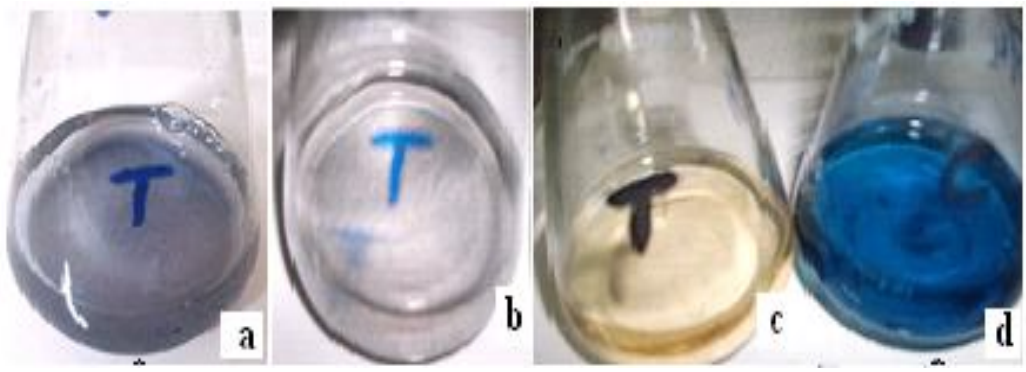

Fig.6Decolorization of malachite green by free and immobilized laccase

a) **Decolorization by immobilized enzyme in 7 days of incubation**
b) **Decolorization by immobilized enzyme in 15 days of incubation**
c) **Decolorization by free enzyme in 7 days of incubation**
d) **Control flask with 0.02% malachite green**

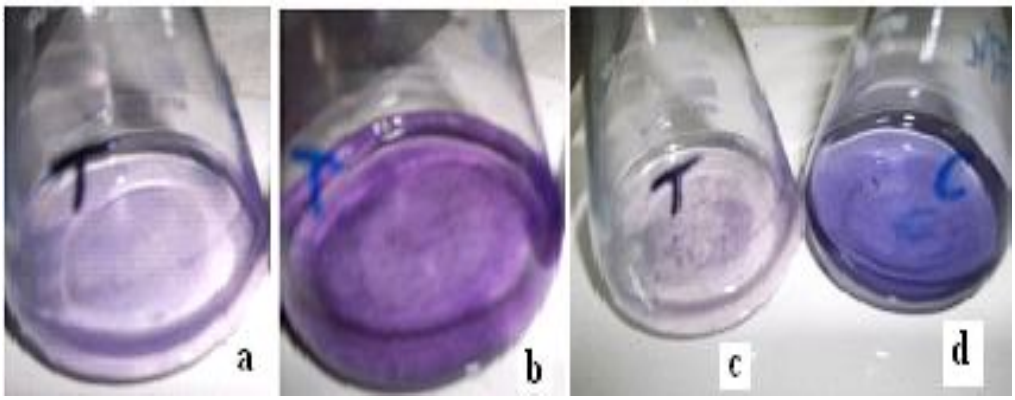

Fig.7 Decolorization of Crystal violet by free and immobilized laccase

a) **Decolorization by free enzyme in 7 days of incubation**
b) **Decolorization by immobilized enzyme in 7 days of incubation**
c) **Decolorization by immobilized enzyme in 15 days of incubation**
d) **Control flask with 0.02% crystal violet**

CONCLUSION

The improved strains of the test fungus were observed exhibit to increased enzyme production in the cheap formulated production medium[14], the strains were further subjected to intraspecific protoplast fusion with in their mutated strains. As a result there was 64% increase in laccases production by the fusant strains of *T.hirsuta* when compared to the wild parent. The efficiency of the laccases production by the fusant strains was analyzed by applying them in one of the most important applications of laccases “dye decolourization”.

100% and 85% of decolorization of malachite green and 90% and 50% decolourization of crystal violet by the partially purified free and immobilized laccases respectively was observed with in 7days of incubation at pH 5.0 and incubation temperature of 70°C.

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