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THE USE OF BACTERIAL LACCASE IMMOBILIZED ON AGRO-WASTE AS A POTENTIAL ECOFRIENDLY TECHNIQUE OF AZO DYE DE-COLORIZATION

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ABSTRACT

In the modern world, the effluent of the textile industry contains azo dyes, which are considered an alarming threat to the environment. Crystal violet, malachite green, reactive red are the most common dyes which are wieldy used in textile industries. Due to the sophisticated structure of these dyes, they are mutagenic and carcinogenic to both humans and aquatic life. A viable alternative is an affordable and eco-friendly bioremediation technique using bacteria or their enzyme that can degrade the azo dyes. Azo dye contaminated samples were collected from the industrial area of Ahmedabad and Jetpur (Gujarat, India). Forty three isolates with various morphologies were employed in the dye de-colorization experiment. Among them four most promising isolates (E11, S32, S12 and J24) were selected which de-colorize the all three dyes. Ago-waste materials with MBSS medium were used to produce laccase enzymes from S12. Laccase was partially purified with the help of pre-chilled acetone. Partially purified laccase was immobilized in sodium alginate beads with rice husks and check the de-colorization efficiency of our experimental dyes. From the results, immobilized laccase can degrade 55.29% crystal violet and 55.10% malachite green dye in 4hr of incubation. Because of its incredible de-colorization ability, immobilized laccase has quite the potential as a cost-effective, biologically beneficial strategy for treating the textile effluents.

KEY WORDS : Azo dye, Textile effluent, Immobilized laccase, Agro-waste, Eco-friendly

INTRODUCTION

Textile industry releases wastewater that contains unused dyes and chemicals that are extremely harmful and mutagenic to human and aquatic life (Makwana, 2020). The complex chemical structure and synthetic nature of azo dyes make them more resistant to degradation. Crystal violet, malachite green, reactive pink and reactive red are the most common dye which are widely used in textile industries of Ahmedabad and Jetpur were studied in this research work.

According to WHO 17 – 20% water pollution is caused by dyes which are widely used in textile industries. We must remove these azo dyes from the water bodies in order to prevent harmful or adverse effects of dye effluent on the environment as well as the well-being of humans and animals.

There are numerous physicochemical processes available, including ozonization, membrane filtration, precipitation, coagulation, adsorption, electrochemical destruction, and so on (Verma *at el.*, 2012). Such procedures are quite expensive and also produce considerable amount of sludge, which causes further pollution (Shah *et al.*, 2012).

As a result, a cost-effective and eco-friendly potential alternative is a biological approach that includes microbial degradation and bacterial immobilized enzyme. Many bacteria have ability to degrade the azo dye from the water bodies (Bhattacharjee *et al.*, 2014). The azo dye can also be degraded or decolorized by bacterial enzymes such as laccase, azoreductase, lignin peroxidase, manganese peroxidase, etc. Many reports show that the laccase enzyme has a great potential of removal of dyes from the effluent (Rodriguez *et al.*, 1999).

Laccases (EC.1.10.3.2, p-diphenol/dioxygen oxidoreductase) belong to the enzyme group called polyphenol oxidases (PPO) containing multicopper and widely distributed in higher plants, fungi, algae and bacteria (Tuncay and Yagar, 2020). The disadvantage of applying free enzyme can be eliminated by immobilizing the enzyme on solid supports. The immobilized enzyme has many advantages including increase shelf life, reuses, easy to recover etc. Many natural and synthetic supports are available for the immobilization of the enzyme. One of the best natural support systems for the immobilization of enzyme is rice husks. It is an agricultural waste and readily available in our area. It also contains a lignocellulosic structure (Ndazi *et al.,* 2007).

Furthermore, there is a need to find an inexpensive, simple and ecological alternative to enzyme immobilization for large-scale textile dye wastewater treatment.

MATERIALS AND METHODS

Sample collection

Azo dye contaminated water and soil sample collected from the industrial area and river of Ahmedabad and Jetpur, India. Samples were in the form of liquid untreated effluent, treated effluent and soil. All the samples were collected in sterile plastic bottles & boxes and preserved at 4 ^{o}C in refrigerator.

Isolation of bacterial isolates

Take 1g soil from the contaminated soil sample and added into 100ml sterile distilled water flask and put it into the environmental shaker for 30min. Than serially dilute the sample from 10^{-4} to 10^{-7} by using serial dilution method. 100µl sample from each dilution tubes were spread onto N-agar plate by spreading plate methods (Rajwade, 2022). All plates were put into incubator for 24hr at 37 ^{o}C. After 24hr morphological different microbial colonies appeared on agar medium were selected for further screening analysis. Selected isolates were purified by streaking on N- agar plate. Streaking was done thrice in four flame method. The purified cultures were preserved in a refrigerator by 35% glycerol preservation technique. In this study, reactive pink, reactive red, malachite green and crystal violet were employed for the examination of dye degradation at a later stage.

Primary screening

The initial screening involved adding a loopful of bacterial culture to 25 ml of Bushnell Hass Broth (BHB is composed of $MgSO_4$ 0.20g^{-1}, $CaCl_2$ 0.02g^{-1}, KH_2PO_4 1.00g^{-1}, K_2HPO_4 1.00g^{-1}, NH_4NO_3 1.00 g^{-1} and $FeCl_3$ 0.05 g^{-1}), with yeast extract, crystal violet, and malachite green dye solution in different 100ml conical flasks. The environment shaker was set to 30 °C and 120 rpm. Strains that showed higher de-colorizing potential were selected for further experimentation.

Secondary screening and cell morphology

Secondary screening was carried out using reactive red dye in Bushnell Hass broth. A loopful of the selected isolate was then transferred into test-tubes and put it into the environmental shaker at 30 ^{o}C and 120rpm. De-colorization of dye solution checked visually after 24-72 hr incubation. Most potential bacterial isolates selected based on dye degradation capacity for further processes. Gram staining and negative staining carried out for selected most promising bacterial isolates using standard procedure.

Dye degradation potential of bacterial isolate with respect to dye concentration

The decolorizing activity of the selected isolates was studied by using crystal violet and malachite green dye at different initial concentrations varying from 5-100 mg^{-1}. After 24hr of incubation complete dye degradation was examined through naked eyes (Mechichi *et al.,* 2006).

Production of Laccase enzyme

Exactly, 2g of wheat bran and rice husk was taken in different flasks and moistened with 100ml of Mineral Basal Salt Solution (MBSS composed of Peptone 3g^{-1}, Dextrose 10g^{-1}, K_2HPO_4 0.4g^{-1}, KH_2PO_4 0.6g^{-1}, $MnSO_4$ 0.5g^{-1}, $FeSO_4$ 0.0005g^{-1}, $ZnSO_4$ 0.01g^{-1}). The flasks were sterilized, cooled to room temperature, and inoculated with selected isolate and incubated for 24hr. Than purified the enzyme with the help of pre-chilled acetone. Check the dye degradation capacity of crude laccase enzyme in malachite green dye solution. Control experiments where the dyes were incubated with free laccase in solution were performed in order to evaluate the extent of biodegradation involved in the de-colorization process and its dependence on substrate structure (Muthukumarasamy *et al.,* 2015).

Laccase immobilization

Laccase was immobilized within rice husks and sodium alginate (Na-alginate) beads. The required amount of sodium alginate was dissolved in distilled water under stirring condition. 2g rice husk was added in sodium alginate solution, mixed and cool-down the solution. 2 ml of extracted laccase solution was added to the sodium alginate with rice husk solution and mixed under gentle mixing. Beads were formed by dropping sodium alginate solution containing laccase through a sterile pipette or syringe fitted with a needle into 100 ml chilled $CaCl_2$ solution. After 60 min of incubation, the beads were removed from the $CaCl_2$ solution. After completing immobilization, the beads were washed twice with distilled water and drained. All immobilized beads were stored at 4°C until further use (Ratanapongleka and Punbut, 2018).

To evaluate the effectiveness of the dye de-colorization process, all of the de-colorization experiments for the malachite green and crystal violet dyes were carried out with immobilized S12 laccase onto rice husks. By measuring the decrease in absorbance at absorption maxima (ë max), the rate of de-colorization was calculated as a percentage. As a reference, the un-inoculated dye solutions were used. A total of 2 ml of reaction mixture was kept at different time intervals, and the samples were centrifuged at 10,000 rpm for 10 min to separate biomass. The concentration of crystal violet and malachite green dye was determined by absorbance at 580 and 620 nm respectively (Kumar *et al.*, 2012). The spectrophotometer was used to measure absorbance. The following formula was used to calculate the de-colorization activity (%) of immobilized enzyme (Cristóvãoa *et al.*, 2011; Rajeeva and Lele, 2011).

Dye Degradation (%) = Initial OD " Final OD/ Initial OD ×100

RESULTS

Sample collection

Samples were collected from total 3 different sites of Jetpur and Ahmedabad industrial area (Table 1). The effluent colors of all samples were black due to a mixture of different chemicals and dye. So, the collected textile effluent samples had revealed a high load of pollution indicators.

Isolation and primary screening for azo dye degradation

Different morphological colonies with the ability to thrive on a N-agar plate were identified during the isolation of bacterial isolates. Bacterial isolates were first screened for de-colorizing azo dye in 100ml flasks containing Bushnell Hass broth with 0.25% yeast extract, crystal violet (0.01%) and malachite green (0.01%) dye solutions. Select most potential isolates which show the de-colorization of dye solution after 24-48 hr incubation at 30 °C (Pokharia and Ahluwalia, 2013).

Secondary screening and cell morphology of selected isolates

Out of 43 isolates thirteen isolates were capable to decolorize the crystal violet and malachite green dye (0.01%) at different rate of de-colorization. Thirteen isolates showing reactive red dye de-colorization were selected for further studies in BHB for three days (Table 2). After primary screening and

Table 2. Reactive red degradation reading after 24hr, 48hr and 72hr of incubation

Sr. No.	Isolates ID	Degradation of reactive red		
		24hr	48hr	72hr
1	E11	-	-	+
2	E12	-	+	+
3	E15	-	-	+
4	E22	-	-	+
5	S11	-	+	+
6	S12	-	-	+
7	S24	-	+	+
8	S32	-	+	+
9	P12	-	+	+
10	P32	-	+	+
11	J14	+	+	+
12	J24	+	+	+
13	J25	+	+	+

Table 1: Geographical Location of sampling site for isolation of dye degrading microbes

Sr. No.	Sampling site	Geographical location	Sample type	Sample ID
1.	Ahmedabad (river)	22°58′51.3"N 72°32′36.3"E	Effluent Water	E1 & E2
2.	Jetpur (industrial area)	21°46′08.3"N 70°38′00.9"E	Treated and untreated effluent water	P1-P3 &S1-S3
3.	Jetpur (river)	21°45′40.5"N 70°37′47.3"E	Dye contaminated Soil	J1-J3

secondary screening four isolates decolorize all three dyes (crystal violet, malachite green and reactive red) within 72hr at 30 °C (Kalme *et al.,* 2007). The Gram's staining and negative staining experiment indicated that one was gram positive and three were gram negative. All four were designated as E11, S32, S12 and J24.

Dye degradation potential of selected isolates

To determine optimum dye concentration, each selected isolate were transferred in the crystal violet and malachite green dye solution which contain varied concentration in the range of 5–100 mg^{-1}. The assay was performed at 25 °C for 24hr. From all four selected isolates S32 and P12 have ability to degrade 100mg^{-1} malachite green dye within 24hr (Fig. 1). However in higher concentration, dye degradation rate was remarkably reduced. The complete de-colorization of 5mg^{-1} crystal violet using S12 and J24 was complete upon continued incubation for 24hr Bushnell Hass broth medium (Khehra *et al.,* 2006).

Fig. 1. 100 mg^{-1} malachite green dye degradation by P12 & S32 isolates

Laccase isolation and purification from S12

The MBSS with rice husk and wheat bran was used as media constituent for the laccase production in fermentation media. A cell free extracellular liquid was recovered by centrifugation. The protein contents of crude extract and precipitate were found to be 0.65±0.99, 0.68±0.5mg^{-ml} and 0.42±1.1mg^{-ml}, respectively (Muthukumarasamy *et al.,* 2015).

De-colorization assay of crude laccase enzyme

In de-colorization experiment of crude lacasse activity the optical density (O.D.) of malachite green dye was reduced by 15% after 30 min of incubation recorded at 620 nm. After 4hr incubation 70.83% and 90% dye degradation is observed by crude laccase enzyme of wheat bran and rice husk respectively (Fig. 2). It can be seen from Table 3 that the ability of rice husk crude laccase enzyme has potential to give more and rapid de-colorization of malachite green dye.

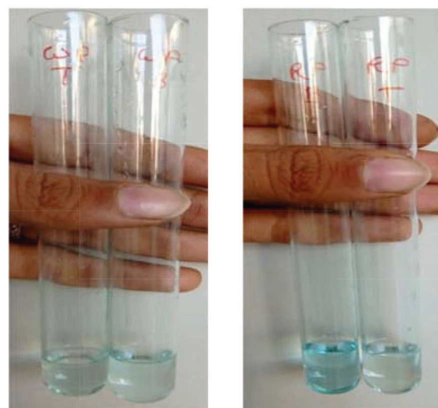

Fig. 2. Malachite green dye degradation by crude laccase enzyme

The immobilization of S12 laccase onto rice husks

This objective of this work was to immobilize commercial laccase employing an efficient, effortless, and economical method for the deterioration of textile dyes. In order to preserve enzyme activity and to achieve high immobilization efficiency, the gelling agent (sodium alginate, rice husks, glutaraldehyde and $CaCl_2$ solution) should be studied (Daâssi *et al.,* 2014). S12 laccase was immobilized onto rice husks using adsorption method, which is rapid, cost-effective and eco-friendly (Fig. 3).

Dye degradation efficiency of immobilised laccase

Two synthetic dyes crystal violet and malachite green were selected as model dyes to study the de-colorization ability of the S12 laccase immobilized

Fig. 3. Rice husk + Na- alginate Immobilized laccase enzyme (S12)

Table 3. Percent dye degradation of malachite green by crude laccase enzyme after 4hr of incubation.

	Laccase from agro-waste containing MBSS fermentation media	
	Wheat bran	Rice husk
Blank	0.000	0.000
Initial OD	0.024	0.022
Final OD	0.007	-0.004
Dye degradation (%)	70.83	90

into rice husk Na-alginate beads.

Malachite green and crystal violet dyes showed a decrease in absorbance value, indicating that immobilized laccase enzyme catalyzed dye-degradation at different time intervals (1hr, 2hr, 3hr & 4hr). Fig. 4 and 5 show the results of immobilized laccase enzyme's de-colorization of the crystal violet and malachite green dyes. With immobilized laccase, 55.29% crystal violet and 55.10% malachite green dye degraded just after 4hr of incubation (Fig. 6).

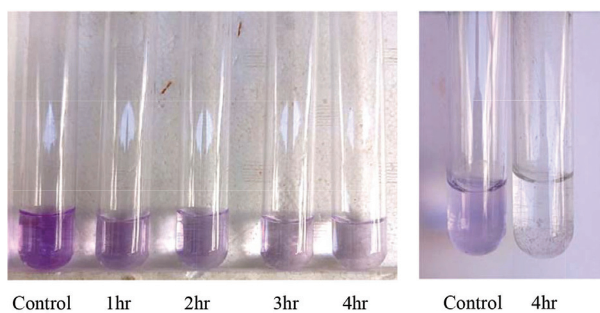

Fig. 4. Crystal violet dye degradation by immobilized laccase at different time intervals

The work also suggested that using these kind of immobilized bacterial laccase for de-colorization of azo dye in industries at large scale level will definitely prevent the water pollution and keep the environment clean.

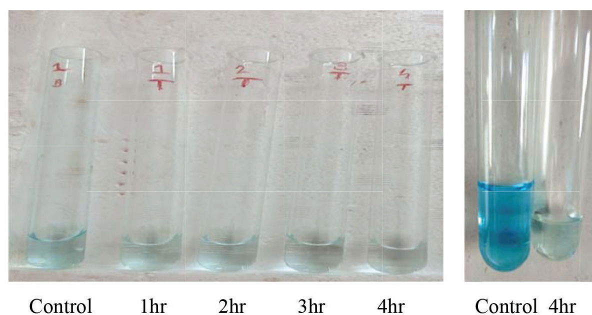

Fig. 5. Malachite green dye degradation by immobilized laccase at different time intervals

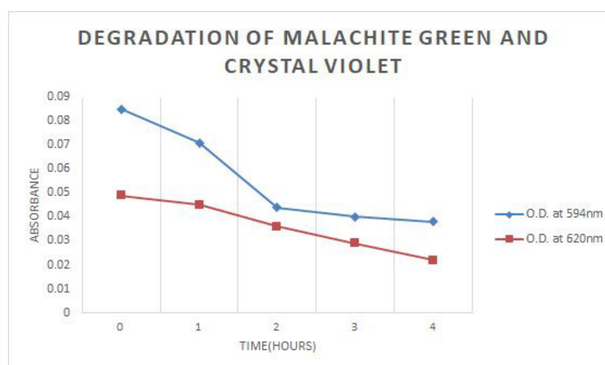

Fig. 6: Azo dye degradation an hourly interval of time

DISCUSSION

The treatment of water and soil contaminated with azo dyes remains a major engineering challenge. In many studies, it has been observed that bacteria isolated from various sources such as dye-contaminated soil and textile dyeing wastewater are able to effectively decolorize and degrade these dye pollutants, which improves water quality (Moyo *et al.*, 2022).

Bacillus subtilis isolated from dye-contaminated sites was observed to have higher dye de-colorization efficiency (78.5%) compared to *Bacillus cereus*.

Immobilized laccase enzyme can be cost effective and eco-friendly approach to remove azo dye contaminated water discarded by dyeing industries. Laccase used in the degradation of mixed azo dyes showed 58.4% in 72hr (Sridharan *et al.*, 2021). During out study it was observed that rice husks immobilized remove 55.29% of crystal violet and 55.10% malachite green dye. Purified laccase immobilized would be a cheap and eco-friendly approach for dye bioremediation process.

CONCLUSION

In this study most potential azo dye degrading bacteria were isolated from textile dye contaminated sites of Ahmedabad and Jetpur, Gujarat, India. S32 and P12 were de-colorizing 100 mg^{-1} malachite green dye and S12 and J24 were give complete de-colorization of 5 mg^{-1} crystal violet dye. Immobilized laccase was also used for de-colorization and degradation of the crystal violet and malachite green dye. Laccase production was carried out on the wheat bran and rice husk as substrate using eco-friendly approach. Study results showed that crude

laccase decolorize malachite green dye 70.83% by wheat bran and 90% by rice husks after 4hr of incubation. While rice husk and sodium alginate mediated immobilized enzyme decolorize dye at constant rate and remove 55% of dye after 4hr at 30 ^{o}C. This eco-friendly approach is effectively used as an alternative to the physico-chemical processes of textile effluents, as they have a high potential for being able to decolorize or degrade azo dyes.

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