Biological Method for Degradation of an Azo Dye: Recycling and Reuse of Treated Waste Water for Leather Processing

J.Kanagaraj*, T.Senthilvelan, M.Vinodh Kumar, R.C.Panda and A.B.Mandal

*Corresponding author,

Leather Processing Division, CSIR- Central Leather Research Institute, Adyar, Chennai, 600020, India.
Ph: 044-24911386 (Off), 044-244530630 (Res), Fax : 91-044-24911589, Email: jkraj68@yahoo.co.uk

Abstract

Dyeing is an important unit operation in leather processing. Various types of dyes are used to color the leather. Amongst them, azo dyes known for their versatile ranges are very often carcinogenic and less biodegradable. The degradation of the dyes is a challenging task and requires suitable microorganisms/enzymes to degrade the same. Laccase is an enzyme that helps to degrade the azo dyes. The enzyme was isolated from *Micrococcus luteus* strain and showed optimal growth conditions at pH 7.0, temperature 37°C and incubation duration of 72 h. The enzyme also exhibited better activity in dextrose, yeast and acetone medium. The enzyme showed maximum degradation efficiency of 91.3% for the CI Acid Black 234. The results of degradation efficiency were analyzed by various analytical tools. The mass spectra analysis showed the conversion of azo dye into new intermediates such as 2,7,8 triamino naphthalen 1-ol, 7,8-diamino naphthalen-1-ol, benzene 1,4-diamine, benzene 1,2,4 triamine, 4-amino benzene thiol. COD and BOD results showed the reduction of 93% and 90% for the dye. The treated dye effluent was reused for dyeing process in leather making. The leather obtained by this method showed comparable strength and other organoleptic properties.

Key words: *Micrococcus luteus*, Laccase, Mass-Spectra, BOD and COD reduction.

Introduction

Azo dyes are important synthetic dyes that are widely used in industry due to their relatively simple synthetic methods and almost unlimited number and types of substituents. These are aromatic compounds having -N=N- groups and forming largest class of synthetic dyes used in commercial applications. The azo groups are generally connected to benzene and naphthalene rings, but can also be attached to aromatic heterocyclic or aliphatic groups (Kanagaraj and Mandal 2012). The azo dyes used in the leather industry not only cause pollution problems but are also toxic to the natural living beings in the water. Hence removal of the dye from effluent is the major task. The general structure of the azo dye molecule can be Ar-N=N-R. Degradation of dye using biological methods is the eco-friendly one and has the advantages over other techniques (Kanagaraj and Panda 2011). Various researchers have reported the degradation of dyes using biological methods. In our study the enzyme Laccase obtained from *Micrococcus luteus* was used for the degradation of dye and the treated liquor was reused in dyeing process of leather manufacture.

Material and Methods

Isolation of bacteria

The *Micrococcus luteus* was isolated from wood decayed environment soil by serial dilution technique and plated on nutrient agar medium under aseptic conditions. The plates were incubated at 37˚C for 24 h. Pure cultures of the isolates were maintained on nutrient agar slants at -4°C. Then the bacterium was identified by the standard morphological and biochemical tests.

Production of Laccase enzyme

The Laccase production was carried out in Erlenmeyer flasks (250 mL) containing 100 mL of the following liquid medium (in g.L^{-1}): starch-20, yeast extract-2.5, KH_2PO_4-1.0, Na_2HPO_4-0.05, $MgSO_4$ -0.5, $CaCl_2$-0.01, $FeSO_4$-0.01, $MnSO_4$-0.001, $CuSO_4$-0.002, pH- 7.0, at 37˚C. The isolated bacterial culture was inoculated in Laccase production media. The culture flask was then incubated at 37˚C in orbital shaker for 72h at 80 rpm. After 3 d of incubation, the culture was then centrifuged at 10,000g for 10 min at 4˚C. The supernatant liquid collected was used as crude enzyme solution and was assayed for enzyme activity (Shraddha et al. 2011).

Enzyme assay

All enzyme (Laccase) assays were carried out in a quartz cuvette with a total reaction volume of 1 ml. The activity of the enzyme was assayed by measuring the decrease in the optical density for the azo dye with a Shimadzu UV—1601PC UV–Vis spectrophotometer. This method was based on the oxidation of ABTS at 420 nm with an absorbance coefficient value (36 000 M-1 cm-1). The reaction mixture consisted of 1.5 ml sodium acetate buffer (1 mM, pH 5.0), 1.5 ml ABTS (0.5 mM) and 1.5 ml culture medium. One unit enzyme activity was defined as the amount of enzyme that oxidizes 1µ mole of ABTS per minute at 37°C. The activities were expressed in U/ml.

Dye degradation

The degradation experiments were carried out in conical flasks containing C.I Acid Black 234 dye. 20 mg of dye was taken in separate conical flasks and 5 ml of enzyme solution was added to all the flasks. It was then incubated at 37˚C for 72 h. Control was performed without enzyme treatment (Zenga et al. 2011). The % degradation achieved was determined by UV–Vis spectrophotometer by monitoring optical density at 420 nm, and then calculated as follows

$$\text{Degradation (\%)} = \frac{C_O - C_1}{C_O} \times 100$$

where, C_0- initial absorbance; C_1- final absorbance

Mass Spectrometry

The sample after dye degradation was collected, lyophilized (to remove the water content in the samples) and the various intermediate metabolites formed are investigated by Mass (ESI)-spectra analyses. The sample was further dissolved in acetonitrile solvent and then subjected to Mass (ESI)-spectra (Kanagaraj et al. 2012).

Estimation of Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD)

The control and experimental sample were collected from various stages and analyzed for the pollution loads such as BOD, COD following the standard methods. The results are expressed in parts per million (ppm) of the sample.

Recycling and reuse of treated dye liquor for dyeing process in leather making operation and study the various leather properties

The treated dye liquor was reused in dyeing process of leather processing. The chrome tanned leathers were taken for this study. The leathers were rechromed with 100% water, 5% self basified chrome powder and basified to the pH of 4. Further the leathers were washed and neutralized to pH of 5.5 and again washed. Then the neutralized leathers were processed with 100% treated liquor (obtained after treatment of enzyme with dye), 13% retanning agents (acryic 3%, 5% Filling syntan, 5% Wattle extract), 1% dye. The samples were fatliquored with fatliquoring agents 14% (Synthetic 5%, semi synthetic 5%) and finally fixed with 1% formic acid. The spent liquors were collected and the dye exhaustion property was studied.

Results and discussion

During primary screening numerous colonies were identified, from which individual colonies were picked out and streaked in different plates. Finally more than 30 colonies were obtained and subjected to guaiacol hydrolysis, out of which one isolate was found to possess the capacity to produce Laccase. The culture was isolated from wood decayed environment soil and identified as *Micrococcus luteus* and the strain was used for Laccase production.
The activity of the crude enzyme, and purified enzymes were assayed. The total activity and specific activity of the enzymes were tabulated. The total protein content of the crude extract was 197.6 mg, ethanol precipitated enzyme was 120.2 mg and ammonium sulphate precipitated enzyme was 83.5 mg (Table 1). It can be seen from Table 1 that an increase in activity is attributed to the purification of the enzyme. Using ice cold ethanol method, activity was found to grow up to 10.7 times over that of crude extract. Similarly in the case of sulphate the activity increased to 12.7 times over crude extract due to the application of pure coenzyme.

Table 1. Enzyme purification

Sample	Total volume (ml)	Amount of protein (mg)	Enzyme Activity (U/ml)	Purification factor (fold)	Yield (%)
Crude enzyme	500	197.6	0.81	1	100
90% Ice Cold Ethanol method	10	120.2	10.7	2.6	93
Ammonium sulfate precipitation followed by dialysis	50	83.5	12.7	5	91

Dye degradation studies

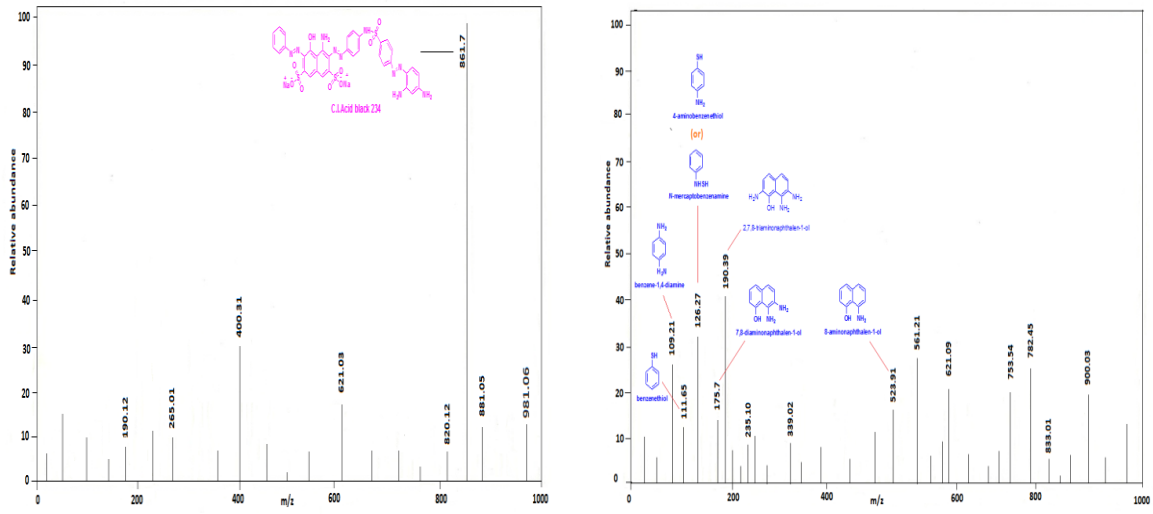
By using Laccase enzyme the dye, C.I Acid Black 234 was degraded at the maximum duration of 96 h. The percentage of degradation was found to be 92.2 % while the control sample showed degradation of 78% (Table 2). Several studies reveal that Laccase is a common type of oxido-reductase enzyme that is generally found in higher plants and fungi but recently it is reported to be also present in some bacteria such as *S. lavendulae*, *S. cyaneus*, and *Marinomonas mediterranea*. In plants, Laccases are found in cabbages, turnip, potatoes, pears, apples, and other vegetables. In fungi, Laccases appear more than the higher plants. Laccase from the *Monocillium indicum* was the first Laccase to be characterized from Ascomycetes which showed peroxidase activity. *Pycnoporus cinnabarinus* produces Laccase as ligninolytic enzyme while *Pycnoporus sanguineus* produces Laccase as phenol oxidase (Schuckel et al. 2011).

Table 2. Degradation of dyes

Different dyes	Time duration (mg/h)										% of degradation
	Dye conc.	enzyme	12h	24h	36h	48h	60h	72h	84h	96h	
C.I. Acid Black 234	20 mg	5ml	18.7	15.3	10.4	8.1	4.8	3.1	2.1	1.52	91.3
Control	20 mg	nil	19.3	18.1	17.1	14.3	11.6	8.1	5.5	3.0	78.0

Mass spectra analysis

The control and experimental samples were analyzed by ESI- Mass spectroscopy after treatment of the dye for identification of intermediate products (Fig.1). The mass spectra analysis showed the conversion of azo dye into new intermediates such as 2,7,8 triamino naphthalen 1-ol (190.39 m/z), 7,8-diamino naphthalen-1-ol (175.7 m/z), benzene 1,4-diamine (109.21 m/z), benzene 1,2,4 triamine, 4-amino benzene thiol (126.27 m/z), N^1-mercaptobenzenamine (125.19 m/z) and 8-amino naphthalen 1-ol (523.91 m/z).



(a) (b)

Figure 1. Mass (ESI) -Spectral analysis of control (a) and treated dye samples (b)

Recycling of treated liquor for dyeing process in leather making

The treated liquor was reused for dyeing process and the result obtained in the experiment was observed. The dye at the level of 1% was used and subsequently retanned (10% in various compositions was added) and fatliquored with 8% Fatliquor (synthetic and semi synthetic). The resultant effluent was analyzed for the dye uptake and presented in the Table 3. The experimental leathers showed better dye uptake in comparison to control sample. The % exhaustion of dye was increased from 90 to 94% for the experimental sample. The possible reason for better uptake of dye may be due to the fact that amide groups can increase hydrogen bonds with the side chain groups of polar amino acids of collagen.

Table 3. % Dye exhaustion

Experiments	Amount of dye used	% Dye exhaustion
Experiment	1%	94%
Control	1%	90%

Chemical Oxygen Demand (COD) and Total Organic Carbon (TOC) analysis

COD and BOD analysis for the experimental samples and control samples were carried out to understand the rate of COD and BOD generated and the reduction rate in the experimental samples. The results are presented in the Table 4. It is evident from the Table that the COD showed the reduction of 93% in comparison with the reduction of 71% in the control sample. The BOD results show reduction of 90% in comparison with reduction of 78% in the control sample.

Table 4. Estimation of BOD and COD values

Sample	COD		BOD		% reduction	
	Before degradation	After degradation	Before degradation	After degradation	COD	BOD
C.I. Acid Black 234	8,530	580	3710	360	93	90
Control dye	7,450	2150	2425	530	71	78

Physical strength properties of leather

The physical strength properties are also important for assessing the quality of leather made by using the treated dye liquor. The results of the strength properties are presented in Table 5. The experimental leather shows comparable tensile strength, distension and load at grain crack characteristics in comparison to control. The other properties such as elongation at break and tear strength in experimental leather are comparable to control samples.

Table 5. Physical strength properties of softy upper leather

Parameters	Experiment	Control
Tensile strength (kg / cm^2)	220±4.0	215±5.0
Elongation at break (%)	62±4.0	60±3.0
Tear strength (kg / cm thickness)	55±8	55±4.0
Load at grain crack (kg)	36±1.0	36±1.0
Distension at grain crack (mm)	12±0.4	12±0.3

Conclusion

The enzyme Laccase obtained from the strain *Micrococcus luteus* was used for the degradation of the azo dye, C.I. Acid Black 234. The enzyme was found to be very effective for the degradation of the dye at the optimum parameters of pH 7.0, temperature 37°C and incubation duration of 72 h. The enzyme degraded the dye at the level 91.3% at duration of 72 h. The treated liquor used for dyeing process showed improved uptake of dye at the level of 94% in comparison with 90% of the control sample. The organoleptic and strength properties of the experimental leather are comparable to the control sample. The present method is a viable and effective method for the biodegradation of C.I. Acid Black 234 dye and treatment of dye effluent in leather processing.

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