Biodegradation of an Azo Dye By Using Azoreductase Enzyme And Its Relevance in Leather Manufacture

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Abstract

Azo dye (C.I. Acid Blue 113) used in leather dyeing generates enormous amount of dye waste water. This necessitates the development of efficient waste water treatment methods. The azo dye was degraded by *Shigella boydii* which secretes an extra cellular enzyme of azoreductase. The optimum activity of enzyme was observed at pH 7 and 32°C in 76 h. The maximum activity of enzyme was 0.0014U/µl under standard assay conditions and protein concentration was found to be 792.3µg /ml of enzyme. The maximum rate of dye degradation was achieved at 96% and 92% for 100 & 200mg/L of dye. The COD and TOC values were reduced up to 87 & 88% for the dye sample. The FT-IR analysis of treated sample showed the transformation of azo linkage into N_2 or NH_3 or incorporated into complete biomass. The presence of aromatic amine in the degraded sample indicated the presence of azoreductase activity. The mass spectra analysis showed the conversion of the azo dye into new intermediate metabolites such as aniline, naphthalene-1,4-diamine, 3-aminobenzenesulfonic acid, naphthalene-1-sulfonic acid, 8-aminonaphthalene-1-sulfonic acid, 5, 8-diaminonaphthalene-1-sulfonic acid. The treated waste water was reused for dyeing process of the upper leather and the results indicated comparable leather properties with that of conventional one.

Key words: *Shigella boydii*, Azoreductase enzyme, FT-IR, Mass-spectrum, GC-MS, COD and TOC reduction.

Introduction

Dyeing is one of the important operations in leather processing. Different types of dyes are used but azo dyes are toxic and carcinogenic. Azo dyes one of the most important and largest class of synthetic colorants have the azo structure of (–N=N–) and used for different commercial applications. Removal of dye is mandatory and several treatment methods are adapted to reduce the dye pollution (Kanagaraj and Panda 2011; Kanagaraj and Mandal 2012). The adapted conventional dye treatment methods are fentons reagent, ozonation method, photochemical, NaOCl method, cucurbituril, electrochemical destruction, activated carbon, peat, wood chips, silica gel, membrane filtration, ion exchange and electro kinetic coagulation, etc. Microorganisms and its enzymes are used for treatment of dyes and don't produce secondary pollution problem and are eco-friendly. Many organisms (bacteria, fungi and yeasts) have been used for the dye degradation studies by researchers and resulted economically very feasible. Sometimes microorganisms cannot be employed successfully for dye degradation due to its higher

concentration and heavy metals present in the dye effluent. Under these conditions the microbial extracellular enzyme applied can effectively degrade and mineralize the dyes.

All the dyes are have high structural variations and can be degraded by only few microorganisms and enzymes using common degradation mechanism. In our present study the azoreductase enzyme extracted from *Shigella boydii* was used for the biodegradation studies, which showed better degradation efficiency for C.I. Acid Blue 113.

Material and Methods

Microorganism used for dye degradation studies

The bacterial strain *Shigella boydii* was obtained in the product development lab at leather processing division, CLRI, Chennai, India. The organism was inoculated into nutrient agar plates containing azo dye and incubated for 48 h for screening the organism for dye degradation studies. The obtained pure culture was maintained in nutrient agar medium slant at 37°C as well as sub cultured from time to time to regulate its viability in the laboratory and stored at -4°C. All microbial media and chemicals used in this study were purchased from Hi-media chemicals and Sigma-Aldrich in Mumbai, India.

Biodegradation study

Basal media was used for dye degradation studies. The media comprised of (g/L): K_2HPO_4 -1.6, KH_2PO_4 -0.2, $(NH4)_2SO_4$ -1.0, $MgSO_4.7H_2O$-0.2, $FeSO_4$ $7H_2O$-0.01, NaCl-0.1, $CaCl_2.2H_2O$-0.02, glucose-3.0, yeast extract-1.0 and different conc. of C.I. Acid Blue 113 (100, 200, 300,400 and 500 mg/L) and kept in an incubator (at 120 rpm) for 96 h in different pH and temperature conditions. The samples were withdrawn from the culture flasks at every 12 h intervals and centrifuged at 10000 rpm for 10 min. The supernatant was taken for the measurement of dye degradation and enzyme activity by UV-VIS spectrophotometer at its respective λ_{max} 322 nm (Hitachi U-2000 spectrophotometer). The UV-Vis spectroscopy study was carried out (ranges 200-750 nm) for both control and experimental samples and recorded the changes in the spectrum (Viral and Kunjal 2012). The degradation efficiency was expressed as per the following equation;

$$\text{Degradation (\%)} = \frac{(\text{Initial Absorbance} - \text{Final Absorbance}) \times 100}{\text{Initial Absorbance}}$$

Purification of enzyme

The extracellular enzyme was partially purified by ammonium salt precipitation method followed by dialysis by using suitable buffer (0.1M and 0.01M phosphate buffer pH 7). The partially purified enzyme was further concentrated by lyophilization method and its molecular weight and protein concentration were measured by SDS-PAGE and Lowry's method.

Enzyme assay (azoreductase)

The reaction mixture (1 ml) containing 25 mM potassium phosphate buffer (pH 7.0), different concentrations of the C.I. Acid Blue 113 (17 and 31 μM), different concentrations of NADH (0.1 mM, 0.5 mM) and culture supernatant of 100 μl was used for the assays. Enzyme was denatured by boiling followed by addition of a few drops of HCl was used as a control. One unit of enzyme activity is defined as the amount of enzyme that catalyzed the degradation of 1 μM of azo dye/minute. Enzyme reactions were carried out aerobically at room temperature and the reactions were initiated with the addition of enzyme. All reactions were carried out in triplicate. A time course experiment was carried out for 2 min and readings were acquired every second by Hitachi U-2000 spectrophotometer at 322 nm by measuring the decrease in the optical density for C.I. Acid Blue 113 (Kanagaraj et al. 2012).

Analysis of biodegraded dye sample by GC-MS, Mass (ESI) and FT-IR

The samples (both control and experiment) were lyophilized after extraction from the culture flasks to remove the moisture content in the samples and the residues were dissolved in acetonitrile for carrying out GC-MS analysis and Mass (ESI)-Spectral analyses. FT-IR analyses of the samples were done as KBr pellets.

Measurement of pollution load

The control and experimental dye samples (after biodegradation) were analyzed for measurement of pollution load in the form of COD and TOC by adopting the standard procedures.

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

The treated dye liquor was reused in dyeing process of leather processing operation. The chrome tanned leathers were taken for this study. The leathers were rechromed conventionally with 100% water, 5% self basified chrome powder and finally basified to the pH of 4. Further the leathers were washed and neutralized to the pH of 5.5 and again washed. Then the neutralized leathers were treated with 100% treated liquor (obtained after treatment of degradation of dye), 13% retanning agents (acryic 3%, filling syntan 5%, wattle extract 5%), 3% dye, Orange dye (Luganil FBNR), 10% fatliquoring agents (Synthetic 5%, semi synthetic 5%) and finally fixed with 1% formic acid. The spent liquors were collected and the dye exhaustion property was studied. The leathers were further analyzed for color measurement studies and physical strength properties. All color measurements were carried out using Gretag Macbeth Spectrolino Spectrophotometer with the measurement geometry of $45^o/0^o$ and L, a, b, c, H and ΔE were obtained using the standard procedure. Samples for various physical tests from experimental and control crust leathers were obtained as per IULTCS method.

Results and discussion

The micro organism was screened based on the degradation capacity of strain on the azo dye. The culture was grown in the nutrient agar media and streaked on the petriplates containing dye (Fig.1a and b). The strain degraded the dye and is shown in Fig. 1c. The formation of colorless

zones around the colony indicates that the isolate is able to utilize azo dye as the source of nitrogen. Zone of degradation around the colonies is clearly seen and the same strain is grown and the enzyme was extracted from the strain. The suitable organism secreting active enzymes can use for dye degradation studies.

Fig. 1a.

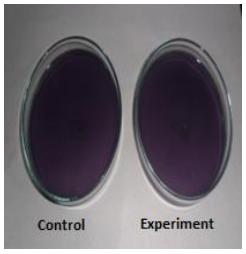

Fig.1b.

Fig. 1c.

Figure 1. Screening of microorganism for dye degradation study (Plate assay) (a. Micro organism used for dye degradation study, b) Screening of microorganism for dye degradation study (0 h incubation, c) Zone of clearance observed after 48h)

The bacterial strain *Shigella boydii* was obtained from the tannery environment and its growth at different atmospheric conditions were studied. The optimum activity of *Shigella boydii* was observed at pH 7.0, temperature 32°C and duration of 48 h. The activity shows decreasing trend on both side of the optimum pH and the results clearly indicate that better bacterial growth usually occurs at the pH of 7-8 for the particular species (Fig.2). It has been found that *Bacillus Cereus* also showed optimum pH of 7.3 and *E.coil* and *Pseudomonas luteola* also exhibited optimum activity at pH 7 (Kanagaraj et al. 2012). The activity increases with temperature from 22°C to 37°C with maximum activity attained at 32°C. The rise in temperature resulted in the reduction of the activity and this may be due to the fact that the bacterial culture is more sensitive to temperature. Similarly the activity increases with increase in duration from 12 h to 48 h and the maximum activity is recorded at 48 h and thereafter the activity decreases which may be due to the fact that the strain belongs to the category of mesophillic type.

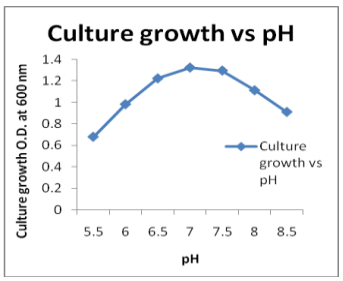

Fig. 2a.

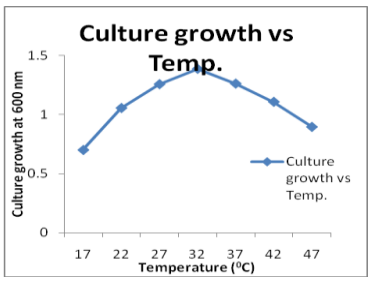

Fig. 2b.

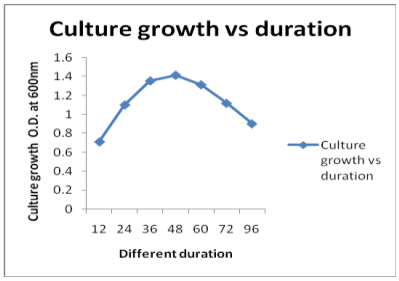

Fig.3c.

Figure 2. Culture growth condition at different atmospheric conditions

The enzyme was extracted from the bacterial strain by adopting the suitable procedures and was purified by 90% ice cold ethanol method and ammonium sulfate precipitation followed by dialysis (Table 1). The results clearly indicate that ammonium sulfate precipitation followed by

dialysis shows purification factor of 5 with yield of 91%. The purification of enzyme by ice cold ethanol method also shows purification factor of 2.6 and percentage yield of 93%. The molecular weight of the enzyme was found out by SDS-PAGE method and presented in Fig.3. The molecular weight of the enzyme was 24 k Da. The other researchers also confirmed the molecular weight of the enzyme was between 22-25 k Da.

Table 1. Enzyme purification

Sample	Total volume (ml)	Amount of protein (µg)	Enzyme Activity (U/mg)		Purification factor (fold)	Yield (%)
			17µM	31 µM		
Crude enzyme	500	792.3	0.0014	0.0025	1	100
90% Ice Cold Ethanol method	10	345.5	2.29	2.93	2.6	93
Ammonium sulfate precipitation followed by dialysis	50	137	3.586	4.143	5	91

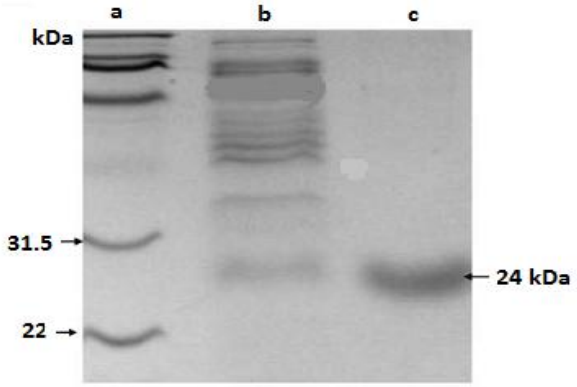

Figure 3. Molecular weight of the enzyme analyzed by SDS-PAGE.

Table 2 shows the rate of dye degradation by Azo reductase enzyme. The enzyme at the level of 50 ml was taken and mixed with dye at various concentrations and incubated for 76 h. The dye degradation experiment conducted by other researchers show that there was a decrease in degradation rate for shake/agitation mode of experiments and hence the present study was conducted under static conditions. It is seen from the Table that dye concentration at the level of 100 mg/l gave the dye degradation level of 96%. It is further evident that the rate of degradation decreases with the increase in concentrreation of dye. The rate of degradation is 89% for the dye concentartion at the level of 500 mg/l in comparison with 82% of dye degradation rate for the control sample (Ong et al. 2011). The mechanism of dye degradion is due to the breakdown of azo dye to amides by azoreducatse enzyme in the experimental sample. Azoreductase is the key enzyme responsible for the reductive azo dye degradation in bacterial species. The presence of oxygen normally inhibits the azo bond reduction activity, since aerobic respiration may dominate the use of the NADH, thus impeding electron transfer from NADH to the azo bonds (Fig. 4). In addition, different models for the non specific reduction of azo dyes or reduced flavins through the cell membrane, or that describe the extracellular reduction of azo dyes by anaerobic bacteria, were

recently suggested (Khalid et al. 2011; Enayatzamir et al. 2010). These results suggest that azo dye reduction is a strain specific mechanism that could perform by an azoreductase enzyme.

Table 2. Application of enzyme for degradation of dye

Conc. of dye (mg/l)	Enzyme conc. (%)	pH (phosphate buffer)	Dye degradation measurements (mg/l)					
			0h	24h	48h	60h	76h	% dye degradation
100	5	7	100	75	51	24	4	96
200	5	7	200	127	76	35	16	92
300	5	7	300	235	164	72	27	91
400	5	7	400	347	220	116	40	90
500	5	7	500	432	291	113	55	89

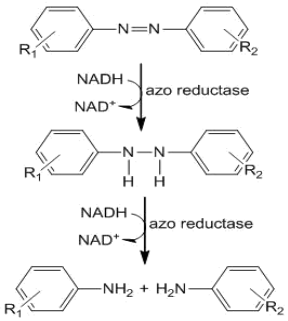

Figure 4. Schemes for the breakdown of azo dyes by azo reductase enzyme

UV-Vis Spectrophotometric analysis of dye

The degraded dye sample was subjected to UV-Vis analysis at 322 nm for the change in absorbance that directly relates to degradation rate. The results are presented in Fig. 5.There are two peaks observed at 300 nm and 550 nm for the untreated dye. These peaks are not present in the experimental sample, indicating complete degradation of the dye sample. In the UV spectra, the peaks observed at the region of 220 and 320 nm indicate the presence of benzene and naphthalene rings respectively. The formation of new peak at 400nm suggests that the reductive destruction of the azo conjugated structure of the azo dye.

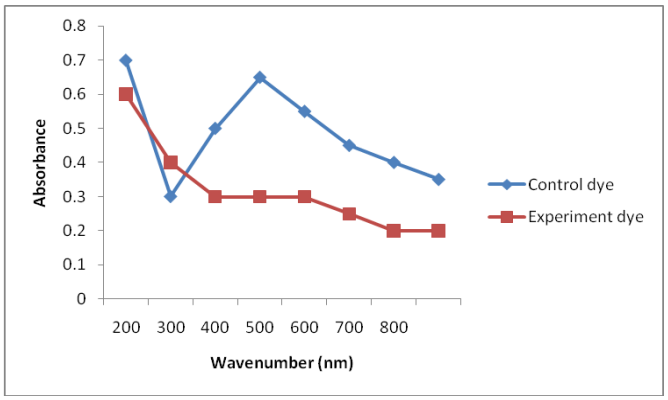

Figure 5. UV-Vis spectrophotometric study of dye degradation

GC-MS analyses

The dye sample treated with azoreductase was analyzed by gas chromatography for finding out intermediate metabolites formed during degradation. The peaks observed at various retention times reveal the respective compounds of the degraded dye sample (Fig. 6). The GC-MS analysis was carried out for both control and experiment samples. GC-MS spectra of experimental sample show various peaks at different retention time that correspond to the various intermediates. The GC-MS spectra show the retention time such as 9.45, 11.183, 12.536, 18.4, 19.77, 21.25, 29.93, and 31.39 for the degraded experimental dye sample. The peaks that show various retention time corresponds to formation of various intermediate products of experimental dye sample such as hexadecane (226), tetradecane (198 m/z), Heptadecane (240 m/z), naphthalene(128 m/z), octadecane (254 m/z), pthalic acid (166 m/z), dibutyl phthalate (278 m/z), diisooctyl adipate (368 m/z), pyridine-3-carbaxamide (122 m/z). These peaks are not present in the control sample.

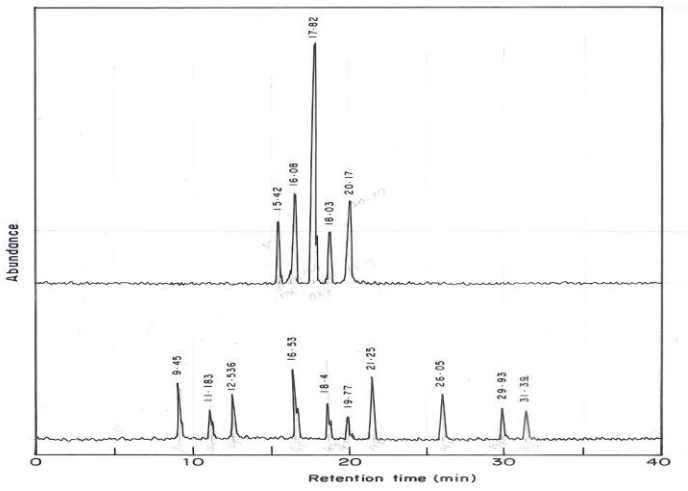

Figure 6. GC-MS analysis of dye before and after biodegradation

Mass Spectra analyses

The dye sample before and after biodegradation was analyzed for Mass (ESI)-Spectral investigation (Fig. 7). The spectra show various peaks that correspond to m/z of particular compounds present in the sample. The spectra show peak at 682.65 in control sample that corresponds to molecular weight of the pure dye. The particular peak represents the presence of pure dye in control sample. The other spectrum taken from the experimental one shows that the respective peak was absent in the biodegraded dye sample (experiment), that indicates the pure dye was degraded by organism or enzyme. The other peaks shown in the experimental sample corresponds to the presence of secondary metabolites with m/z 94.45, 159.02, 173.19, 209.23, 224.25, and 239.75. The corresponding peaks indicate the presence of intermediate metabolites such as aniline, naphthalene-1,4-diamine, 3-aminobenzene sulfonic acid, naphthalene-1-sulfonic acid, 8-aminonaphthalene-1-sulfonic acid, 5, 8-diaminonaphthalene-1-sulfonic acid respectively.

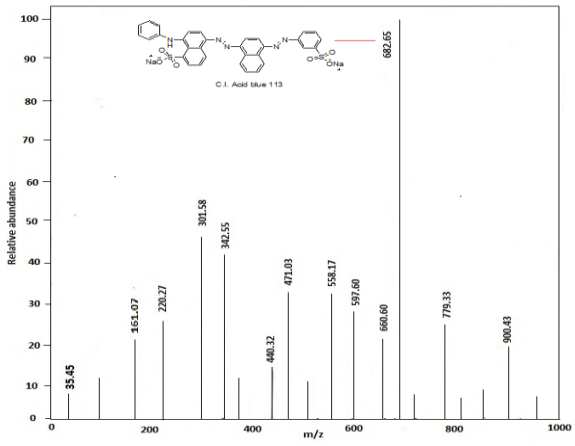

Fig. 7a.

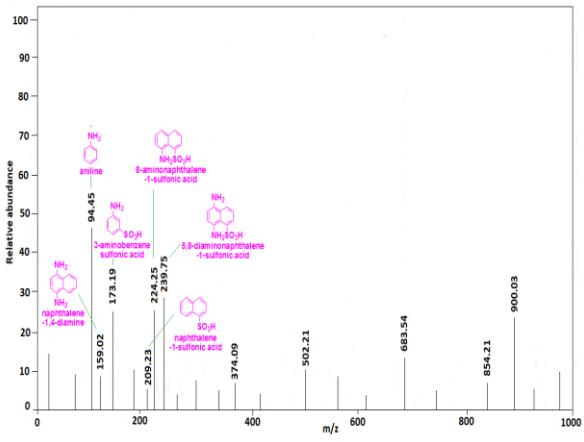

Fig. 7b.

Figure 7. Mass (ESI) spectra analysis of dye samples, before (7a) and. after biodegradation (7b).

FT-IR analyses of the sample

The control and experiment dye samples were analyzed by FT-IR (Fig. 8). FT-IR spectra analysis is very useful in detecting the compounds that are transformed from azo linkages into biomass. The main reason for carrying out FT-IR analyses was to know the shift of band that corresponds to N=N in azo dye. The bands located within the range of 1599-1564 cm^{-1} and 1305 cm^{-1} are due to the azo linkages of –N=N- on aromatic structure and –N=N- stretching in α substituted compounds respectively. These peaks decreased during the treatment of *Shigella boydii*, confirming the previous UV-vis results about azo linkage disruption (Fig. 8b). The fact that no new peaks appeared between 3300-3500 cm^{-1} attributed to azo bonds and OH groups in position α relative to the azo linkage (Fig. 8b). Similarly no new peaks appeared in the region between1340 and 1250 cm^{-1} (Fig. 8b) corresponds to -NH_2 suggested that the azo linkage could be transformed into N_2 or NH_3 or incorporated in to the biomass. The band observed at the region of 3443 cm^{-1} corresponds to intermolecular hydrogen bonded O-H stretching. The band observed at 1641 cm^{-1}, 1497 cm^{-1} and 1405 cm^{-1} represents C=C stretching. The peak observed at 1642 cm^{-1} is due to the conjugation of C=C and C=O groups, suggesting that this peak could belong to a

carbonyl groups in a carboxylic acid, ketone, ester or conjugated aldehyde groups attached to an aromatic ring. Further from the spectra, it is seen that absence of new peaks between 1345 cm^{-1} relating to NH_2 groups.

Recycling of treated liquor for dyeing process in leather making

The treated liquor was reused for dyeing process in leather making and the results were observed. The experimental leathers showed better dye uptake in comparison to control sample. The % exhaustion of dye was increased to 98% in comparison with 90% for the control sample. The reason for the improved uptake of dye may be due to the presence of amide groups that play main functional group of interaction with dye compounds. These amide groups can form hydrogen bonds with the side chain groups of polar amino acids like aspartic acid, glutamic acid, lysine and arginine. Also it is possible that hydrophobic interaction and ionic interaction of the collagen also play major roles in improving the efficiency of dye uptake.

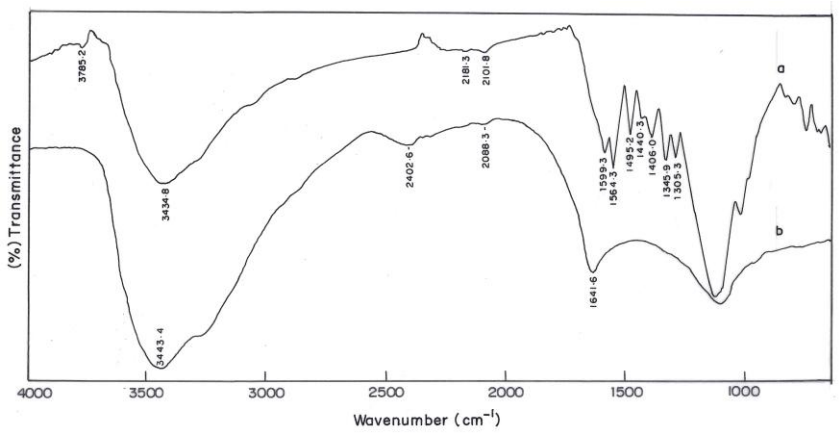

Figure 8. FT-IR analysis of dye before and after biodegradation

Color matching results for the leather

The Fig. 9 shows color matching of dyed crust leather for the experiment and control samples. L, a, b, c are color coordinates. Δa, Δb, Δc are red-green, yellow-blue color difference and chromaticity difference respectively. The experimental sample shows lesser lightness as compared to control sample indicated by ΔL. The experiment leather sample is darker in shade and towards redder component. The chromaticity of the experiment leather is high and the hue shows decreasing trend in the experiment leather sample. The magnitude of the overall color difference in the experiment leather is high. The overall results how that the experiment leather sample is darker, redder in comparison with control sample. This may be due to the fact that experiment leather had better dye uptake as compared to the control sample.

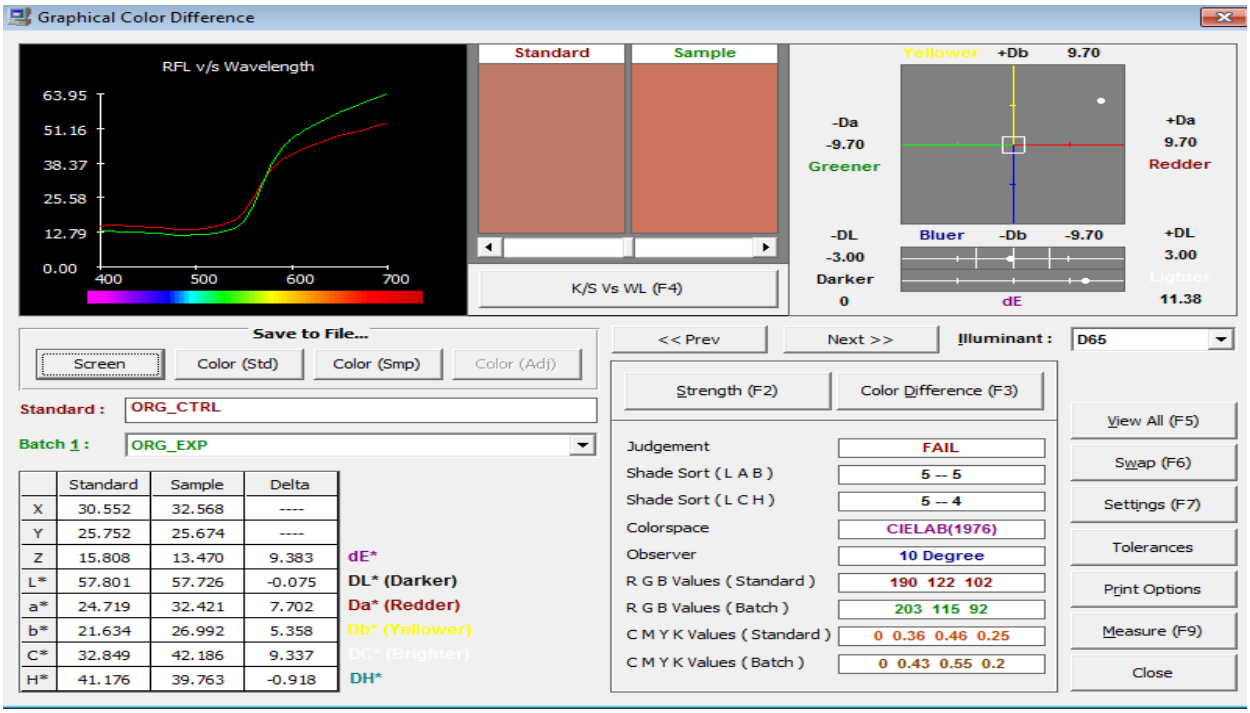

Figure 9. Graphical color matching of leather after dyeing process in both control and experiment sample

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

COD and TOC analysis for the experimental samples and control samples were carried out to understand the rate of COD and TOC generated and the reduction rate in the experimental samples. The results are presented in the Tables 3 and 4. It is evident from the Table that the COD showed a reduction of 87% in comparison with the reduction of 71% in the control sample. The TOC analysis also showed similar results of the reduction rate of 88% in the experiment in comparison with 64% of the control sample. The COD results are in agreement with % dye degradation rate for the experimental sample that more than 80% of dye degradation and COD removal have been achieved in the present investigation.

Table 3. Measurement of COD level in both control and experiment samples at different time intervals

Sample	0h	24h	48h	72h	96h	%of COD reduction
Experiment (ppm/l)	9,568	6,730	5560	4866	1220	87
Control (ppm/l)	9,047	6210	4971	4013	2620	71

Table 4. Measurement of TOC level in both control and experiment samples at different time intervals

Sample	0h	24h	48h	72h	96h	%of TOC reduction
Experiment (ppm/l)	956	632	460	381	110	88
Control (ppm/l)	947	612	481	405	340	64

Organoleptic properties of full chrome crust leather

Organoleptic properties of experimental leathers are important because they give overall quality of leather. The properties of leather were evaluated manually by visual assessment in comparison to control leather sample. The results of experiment 4 are presented in Fig. 10a. Figs.10b and 10c show control and experiment sample respectively. The softness of the experimental leathers (full chrome upper leather) was better than the control samples. Similarly the fullness property was better in control sample. The general appearance and grain tightness properties of the experimental leather were comparable to the control sample.

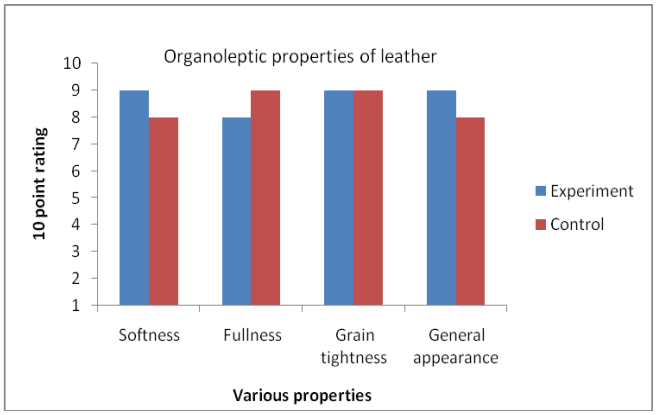

Fig. 10a.

Fig. 10b.

Fig. 10c.

Figure 10. Organoleptic properties of full chrome crust leather

Physical strength properties of leather

The physical strength properties are also important for assessing the quality of leather made by treated dye liquor. The results of the strength properties are presented in Table 5. The results show that the physical strength properties of the experiment leather were comparable to the control leather sample. The experimental leather shows comparable tensile strength, distension and load at grain crack characteristics in comparison to control. It is significant to observe that percentage elongation at break and tear strength in experimental leathers are comparable to control samples.

Table 5. Physical strength properties of softy upper leather

Parameters	**Experiment**	**Control**
Tensile strength (kg / cm^2)	210±4.0	205±5.0
Elongation at break (%)	60±4.0	61±3.0
Tear strength (kg / cm thickness)	52±8	50±4.0
Load at grain crack (kg)	33±1.0	30±1.0
Distension at grain crack (mm)	12±0.4	10±0.3

Conclusion

The bacterial strain of *Shigella boydii* was obtained from Tannery Division, CLRI and testified in laboratory for degrading azo dye. It secretes the Azo reductase enzyme and the enzyme was purified and used for degrading the dye C.I. Acid blue 113. The enzyme degraded the C.I. Acid blue 113 at the level of 96% for the duration of 76 h. The mass spectra analysis shows the formation of various intermediate metabolites during dye degradation. The intermediates are aniline, naphthalene-1,4-diamine, 3-aminobenzene sulfonic acid, naphthalene-1-sulfonic acid, 8-aminonaphthalene-1-sulfonic acid, 5, 8-diaminonaphthalene-1-sulfonic acid at respective m/z of 94.45, 159.02, 173.19, 209.23, 224.25, and 239.75. FT-IR results confirm the absence of peaks in the region between1340 and 1250 cm^{-1} correspond to $-NH_2$ suggesting that the azo linkage could be transformed into N_2 or NH_3 or incorporated in to the biomass. The treated liquor used for dyeing process shows that the uptake of dye was better in the experimental sample in comparison with the control sample. The organoleptic and strength properties of the leather were also comparable in the experimental leather. The present method of biodegradation of dye is a viable and eco-benign method for treating the azo dye in the dye house effluents.

References

1. Khalid, A., Batool, S., Siddique, M.T, Nazli, Z.H, Mahmood, R.B.S., Arshad, M., 2011, Decolorization of remazol black-B azo dye in soil by fungi, Soil Environ, 30(1), 1-6p.
2. Viral, S., Kunjal, P., 2012, Degradation of azo dye acid blue 158 by soil microbes, Res. J. Biotech. 7(2), 50-57p.
3. Ong S-T, Keng, P-S., Lee, W-N., Ha, S-T., Hung, Y-T., 2011, Dye Waste Treatment- Review, Water, 3, 157-176p.
4. Enayatzamir, K., Alikhani, H., Yakhchali, B., Tabandeh, F., Rodríguez-Couto, S., 2010, Decolouration of azo dyes by Phanerochaete chrysosporium immobilized into alginate beads, Environ. Scie. Pollut. Res. 17, 145-153p.
5. Kanagaraj, J., Mandal, A.B., 2012. Combined biodegradation and ozonation for removal of tannins and dyes for the reduction of pollution loads, Environ. Sci. Pollut. Res. 19, 42-52p.
6. Kanagaraj, J., Panda, R.C., 2011. Modelling of dye uptake rate, related interactions, and binding energy estimation in leather matrix using protein based nano particle polymer, Industrial Engineering & Chemistry Research 50 (22), 12400-12408.
7. Kanagaraj, J., Senthilvelan, T., Mandal, A.B., 2012, Biological method for decolourisation of an azo dye: Clean technology to reduce pollution load in dye waste water, Clea. Technol. Environ. Policy 14, 565-572p.