Original Article

DECOLOURIZATION OF TRIPHENYLMETHANE DYES AND DYE INDUSTRY EFFLUENT BY *STAPHYLOCOCCUS AUREUS* ISOLATED FROM DYE CONTAMINATED SITE

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ABSTRACT

Objective: The objectives of the present study were a) to isolate and screen bacteria for dye removal from synthetic solution b) to optimize various variables such as pH, static/shaking and initial dye concentration on degradation of triphenyl methane dyes namely basic violet 3 and basic green 4 by isolated *Staphylococcus aureus* c) to analyse enzymes involved in the biodegradation of triphenylmethane dyes d) to treat real leather dyeing wastewater with newly isolated strain of *Staphylococcus aureus* e) to characterize untreated and treated leather dyeing wastewater f) to study the effects of real and treated effluent on plants and *Rhizobium*.

Methods: Isolation of bacteria from sludge was carried out by spread plate method and the bacteria was identified by morphological and biochemical characterization. The isolated bacterium was screened for dye decolorization potential of triphenylmethane dyes basic violet 3 and basic green 4 The effects of parameters were studied by varying pH (from 3 to 9), temperature (from 15-45 °C), and initial dye concentration (from 10-500 mg/l). The enzyme involved in biodegradation was studied in intracellular extract. Real leather dyeing wastewater was treated with the bacteria and characterized. The treated wastewater was tested on plants and *Rhizobium* for toxicity.

Results: Dye decolorization potential of bacteria *Staphylococcus aureus* isolated from wastewater for leather dyes basic violet 3 and basic green 4 were evaluated. Dye decolorization using bacteria was found to be dependent on physicochemical parameters (shaking, pH and initial dye concentration). Enzymes NADH-DCIP reductase and MG reductase were found to play dominant role during biodegradation of synthetic dyes. Application oriented studies using growing bacteria in pure cultures were carried out with leather dyeing wastewater collected from DKS prime tanners. Analysis of raw leather dyeing wastewater showed high pollution load in terms of color, Total solids, Total suspended solids, Total dissolved solids and Biological oxygen demand whereas the leather dyeing wastewater treated with pure culture of *Staphylococcus aureus* showed considerable decrease in Total solids, Total suspended solids, Total dissolved solids and Biological oxygen demand values which were within the permissible limits. Phytotoxicity and microbial toxicity studies confirmed the non-toxic nature of treated leather dyeing wastewater.

Conclusion: Our study proved that *Staphylococcus aureus* can serve as a potential remediation agent for the treatment of leather dyeing wastewater.

Keywords: Leather dyeing waste water, Crystal Violet, Malachite Green, *Staphylococcus aureus*

INTRODUCTION

The use of synthetic dyes is increasing in recent years. These dyes are mainly consumed in the textile, tannery, pharmaceutical, paper, plastic and cosmetic industries [1]. The leather dyeing industry consumes large quantities leather contaminated soil and produces large volumes of contaminated from different steps in the dyeing and finishing processes [2]. Leather dyeing contaminated soil derived from the leather and dyestuff activities can provoke serious environmental impact in the neighbouring receptor water bodies because of the presence of toxic synthetic dyes, chlorolignin residues and dark coloration [3]. Colour is first contaminant to be recognized in wastewater and has to be removed before discharging into water bodies or on land. A very small amount of dye in water (10-50 mg/l) affects the aesthetic value, water transparency and gas solubility of the water bodies [4, 5]. Due to rapid industrialization, dyes have become one of the main sources of severe water pollution as it can be widely used. The release of dyes into our surrounding water bodies has toxic effect on human health and marine life.

The most acutely toxic dyes for fish are basic dyes, especially those with a triphenylmethane structure. Fish also seem to be relatively sensitive to many acid dyes [6]. Over the past decade many microorganisms capable of decolorizing triphenylmethane dyes at lab scale level have been reported [7-11]. The triphenylmethane dye, basic violet 3 (crystal violet) used as a commercial textile dye, is a recalcitrant molecule indicating that it is poorly metabolized by microbes and consequently is long lived in environment [12].

Basic violet 3 has been found in water streams as a consequence of improper chemical waste disposal from dyeing industries. Because of its low cost, its effectiveness as an antifungal agent for commercial poultry feed and its ready availability, the general public may be exposed to the dye and its metabolites through the consumption of treated poultry products. Basic Violet 3 is a mutagen, a mitotic poison and clastogens possibly responsible for promoting tumour growth in some species of fish [13]. The carcinogenic effects of basic violet 3 in mice and rodents have also been reported [14, 15]. Therefore, there are both environmental and animal health concerns regarding the bioaccumulation of basic violet 3.

Malachite green (basic green 4) is an organic compound that is used as a dyestuff and has emerged as a controversial agent in aquaculture. Malachite green is traditionally used as a dye for materials such as silk, leather, and paper. It is highly toxic to mammalian cells, carcinogenic and can cause skin irritation. Therefore, removal of malachite green from effluent is essential to protect the environment. There are various physical and chemical wastewater treatment methods that can be applied for leather dying contaminated soil treatment such as flocculation, coagulation, adsorption, membrane filtration, precipitation, irradiation and ozonation [4, 16]. But the applicability of those methods is limited due to various limitations [17].

Over the past decades, biological decolorization has been investigated as a method to transform, degrade or mineralize dyes [4]. Moreover, such decolorization and degradation is an environmentally friendly and cost effective alternative to chemical decomposition processes [18-20]. Application of bioremediation in wastewater treatment is a prominent technology, which was successfully used to treat various organic effluents and dye effluents [21].

Hence, the present investigation was carried out to revitalize the role of bacteria isolated from sludge of leather dyeing effluent for the removal of synthetic triphenyl methane dyes *viz.* (crystal violet) basic violet 3 and (malachite green) basic green 4 from leather dyeing wastewater with the following objectives.

• Isolation, identification and screening of the bacteria based on dye removal potentiality.

• Optimization of various variables such as pH, static/shaking and initial dye concentration on degradation of triphenyl methane dyes *viz.* crystal violet and malachite green by *Staphylococcus aureus*.

• Analysis of enzymes involved in the biodegradation of triphenyl methane dyes.

• Treatment of real leather dyeing wastewater with newly isolated strain of *Staphylococcus aureus.*

• Characterization of untreated and treated leather dyeing wastewater.

• Phytotoxicity and Microbial toxicity of real and treated effluent.
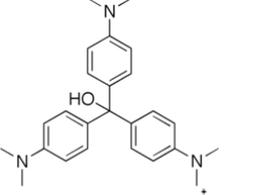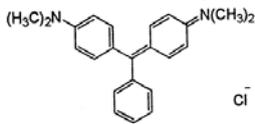
MATERIALS AND METHODS

Microbiological media

Complete mineral media was composed of Peptone 5g/l, yeast 3g/l, glucose 2g/l, sodium chloride 5g/l, dipotassium hydrogen phosphate 5g/l, potassium dihydrogen phosphate 5g/l, magnesium sulphate 1g/l. pH was adjusted to 7. The pH of the media was adjusted to 7.0 with 0.1N HCl and 0.1N sodium hydroxide solutions. The media ingredients were purchased from Himedia Ltd., Mumbai.

Dyes

The synthetic dyes *viz.* basic violet 3 (crystal violet) and basic green 4 (malachite green) were used in this study. The dyes were chosen because of their extended use in leather dyeing. The dyes were purchased from Himedia Ltd., Mumbai. Stock solution of dyes were prepared at 1000 mg/l, and diluted before use. The details of the dyes used in the study are given in table 1.

Table 1: Details of the dyes used in the study

Dyes	Class	Color Index. No	λmax (nm)	Chemical structure
Basic Violet 3	Cationic triphenyl methane	C. I 42555	588 nm	
Basic Green 4	Cationic triphenyl methane	C. I 42000	615 nm	

Chemicals

NADH, DCIP were purchased from Himedia Ltd., Mumbai. Tartaric Hydrogen peroxide solutions, methyl red, malachite green, were obtained from SRL chemicals, India. All chemicals used were of the highest purity available and of an analytical grade.

Sample collection

Sludge samples were collected from reservoir of colored leather dyeing from DKS prime tanners, and preserved in sterilized polyethylene bottles. Raw leather dyeing wastewater was collected from No 11, SIDCO industrial estate Sipcot, Ranipet, Tamil Nadu, India.

Isolation of bacteria

Isolation of bacteria from sludge was carried out by spread plate method [22]. One gram of sludge was suspended in 50 ml of mineral media and incubated at 28 °C for 48 h at 120 rpm. After 48 h, 1 ml of the broth was serially diluted (10^{-1} to $10^{-}[10]$ dilutions). 50 µl of each dilution was plated onto Nutrient agar plates and incubated at 37 °C for development of colonies. The isolates were purified and maintained on Nutrient agar slants at 4 °C.

Identification of dye decolorizing bacteria

Dye decolorizing bacteria was identified by morphological and biochemical characterization.

Screening of bacteria for dye decolorization

Screening was performed by modification of method adapted by Charumathi [22]. A loop full of bacterial cultures were inoculated separately in 250 ml Erlenmeyer flasks containing 100 ml of complete mineral media and incubated at 120 rpm in an orbital shaker. An aliquot (2 ml) of bacterial cultures in their exponential phase were inoculated into complete mineral media containing two synthetic dyes *viz.* basic violet 3 and basic green 4 at a concentration of 10 mg/l. The decolourization performance of bacterial culture was monitored under shaking condition. An aliquot (5 ml) of culture media was withdrawn at the different time intervals. The aliquots were centrifuged at 8000 rpm for 15 min to separate the cell biomass. The supernatants were used to determine decolourization by measuring the change in the absorbance of the culture supernatants at the maximum absorption wavelength (λ_{max}) of the respective dyes using UV-Visible Spectrophotometer (Biochrom Libra Make, Cambridge,UK). Abiotic controls (without micro-organisms) were also included. All decolorization experiments were performed in triplicates. Growth of the microorganism in dye containing medium was measured by the gravimetric method after drying at 40 °C until constant weight was obtained. The dye decolorization percentage was calculated as follows

$$\text{Decolorization \%} = \frac{A_0 - A_f}{A_0} \, x100$$

Where, A_0-initial absorbance of dye (mg/l)

A_f-final absorbance of dye (mg/l)

The specific growth rate of bacteria was calculated as follows

$$\mu = \frac{\ln\left(\dfrac{X_{t_2}}{X_{t_1}}\right)}{t_2 - t_1}$$

Where, $t_2 > t_1$

μ-specific growth rate (1/h)

X^{t_2} and X^{t_1} –biomass dry weight at time points (t_2 and t_1) respectively (g/l).

Decolorization of dyes under different physico-chemical conditions

The decolorization experiments were carried out in 250 ml Erlenmeyer flasks with slight modification of method followed by Charumathi [22]. Cells of *Staphylococcus aureus* in exponential growth phase were harvested and inoculated into 10 mg/l of dye containing growth medium. The samples were withdrawn at regular intervals and centrifuged at 8,000 rpm for 5 min. The supernatants were subjected to UV-Visible spectral analysis and the pellets were dried to constant weight for growth measurements. The effect of initial pH on decolorization of dyes by *Staphylococcus aureus* was studied by varying the pH of the growth medium from 3 to 9. The effects of temperature on decolorization of dyes by bacterial species were studied by varying the temperature from 15-45 °C. The influence of initial dye concentration on decolorization of dyes was also studied by varying the dye concentration from 10-500 mg/l.

Preparation of samples for enzyme assays

Staphylococcus aureus grown in complete mineral medium were harvested before and after biodegradation by centrifugation at 10,000 rpm for 15 min. The cell pellet was crushed and washed twice with 0.03M sodium phosphate of pH 6.8 and centrifuged at 10,000rpm for 10 minute at 4°C. Then the washed pellet resuspended in 5 ml of 0.2M phosphate buffer of pH 6.8. Ten ml of cell suspension was ground for 10 min in pestle and mortor with 2g alumina and 0.1M of 9:1 mixture of toluene and acetone. The suspension was added to 8 ml of phosphate buffer and centrifuged at 15,000 rpm for 10 min at 4°C. The supernatant obtained was used for the study of intracellular enzyme activities.

Enzyme assays

All enzyme assays were performed at 30 °C where reference blanks contained all components except the enzyme. All assays were run in triplicate and average rates were calculated and one unit of enzyme activity was defined as a change in absorbance unit per min per ml of enzyme.

NADH-DCIP reductase activity was determined in an assay mixture containing 50 µM DCIP, 28.57 mmol NADH in 50 mmol potassium phosphate buffer (pH 7.4) and 0.1 ml of enzyme solution in total volume of 5 ml. The DCIP reduction was calculated using the extinction coefficient of 19 mmol/cm [23]. MG reductase activity was determined in reaction mixture containing 323 µM Malachite green, 28.57 mmol NADH in 50 mmol potassium phosphate buffer (pH 7.4) and 0.1 ml enzyme solution in a total volume of 5.0 ml. Absorbance of remaining malachite green was calculated using the extinction coefficient of 8.4×10^{-3} mmol/cm [24]. Azo reductase activity was determined by monitoring the decrease in the methyl red concentration at 440 nm in a reaction mixture of 2.2 ml containing 152 µM methyl red, 50 mmol sodium phosphate buffer (pH 5.5) and 20 µM NADH [25].

Treatment of leather dyeing wastewater using pure culture of growing *Staphylococcus aureus*

The leather dyeing wastewater was supplemented with complete mineral media. 1 ml of pure culture of *Staphylococcus aureus* was added to 100 ml of diluted (1:2, 1:3, 1:4 and 1:5) and undiluted autoclaved leather dyeing wastewater. The pH was maintained at 7. The decolorization of leather dyeing wastewater was monitored at regular time intervals using UV-Visible Spectrophotometer. The decolorization % of leather dyeing wastewater was calculated as

$$\text{Decolorization \%} = \frac{A_0 - A_f}{A_0} x100$$

Where, A_0-Initial absorbance at λmax; A_f =Final absorbance at λmax

Characterization of untreated and real leather dyeing wastewater

Parameters adopted to study pollution levels using standard methods were color, smell, pH, Biological oxygen demand (BOD), Chemical oxygen demand (COD), Total suspended solids (TSS), Total dissolved solids (TDS) and Total solids (TS).

Color

The colour was determined by visual comparison of the sample with distilled water. About 20 ml of the sample and 20 ml of distilled water were taken in two separate wide mouth test tubes. The results were tabulated by comparing the color of the sample with distilled water visually [26].

PH-electrometric method

The pH of the samples was recorded by electrometric method.

Total solids (TS)

Total solids include total suspended solids (TSS) and total dissolved solids (TDS). A known volume of the well mixed sample (50 ml) was measured into a pre weighed dish and evaporated to dryness at 103 °C on a water bath. The evaporated sample was dried in an oven for about an hour at 103–105 °C and cooled in desiccators and recorded for constant weight. Total solids were determined by using the following formula [26].

$$\text{Total solids (mg/l)} = \frac{W_1 - W_2}{\text{Sample volume (ml)}} x1000$$

TS-Total solids (mg/l)

W_1-Weight of dried residue+dish (mg)

W_2-Weight of empty dish (mg)

Total suspended solids (TSS)

Total suspended solids are the portions of solids that usually retained on the filter paper. Suspended solid consists of silt, fine particles of organic and inorganic matter. For TSS analysis, known amount of sample was filtered through the pre-weighed filter paper. The filter paper was dried at 103–105 °C. TSS was determined by using the following formula [26],

$$\text{TSS (mg/l)} = \frac{\text{Final Wt} - \text{Initial Wt}}{\text{Amount of sample taken}} x1000$$

Where TSS–total suspended solids (mg/l)

Total dissolved solids (TDS)

Dissolved solids are solids that are in dissolved state in solution. The difference in weight of total solids (W_1) and total suspended solids (W_2) expressed in the same units gives total dissolved solids (TDS) [26].

$$\text{TDS} = W_1 - W_2$$

TDS-Total dissolved solids (mg/l)

W_1-Weight of total solids+dish

W_2-Weight of total suspended solids

Biological oxygen demand (BOD)

The samples were collected in BOD bottles, to which 2 ml of manganous sulphate and 2 ml of potassium iodide were added and sealed. This was mixed well and the precipitate was allowed to settle down. At this stage 2 ml of concentrated sulphuric acid was added, and mixed well until all the precipitate got dissolved. The sample (203 ml) was measured into conical flask and titrated against 0.025 N sodium thiosulphate using starch as an indicator. The end point was the change of colour from blue to colourless. The reduction in dissolved oxygen concentration during the incubation period yields a measure of the biological oxygen demand [27].

$$\text{Dissolved Oxygen (mg/l)} = \frac{(0.025)(1000\text{ml})(\text{Volume of sodium thiosulphate})}{200}$$

Biological oxygen demand (BOD) is the amount of oxygen required by microorganisms for stabilizing biologically decomposable organic matter in water under aerobic conditions. The sample having pH of 7.0 was determined for first day DO. Various dilutions were prepared to obtain about 50% depletion of DO using sample and dilution water. The samples were incubated at 20 °C for 5 d and the

5th day DO was determined. BOD was calculated using the following formula:

$$BOD \ (mg/l) = \frac{(DO_1 - DO_5) - (B_1 - B_2) \ (Volume \ of \ diluted \ sample)}{Volume \ of \ sample \ taken}$$

BOD-Biological Oxidation Demand (mg/l)

DO_1-1st day DO of diluted sample

DO_5-5th day DO of diluted sample

B_1-1st day DO of control

B_5-5th day DO of control

Toxicity studies

Phytotoxicity

Phytotoxicity tests were performed in order to assess the toxicity of the real and treated leather dyeing effluent on plants. The phytotoxicity study was carried out on three kinds of seeds commonly used in the Indian agriculture *Vigna mungo, Vigna radiate* and *Pisum sativum* by watering 10 ml sample of real and treated leather dyeing effluent. Toxicity effect was measured in terms of percent germination and lengths of shoot and root after 7 d.

Microbial toxicity

Microbial toxicity studies were carried out for nitrogen fixing bacteria *Rhizobium* sp. The nutrient medium containing 1.5% agar was used for the study. Toxicities of the real and treated leather dyeing effluent were studied. Toxicity was measured in terms of zone of inhibition (diameter in cm) after 24 h of incubation at 30 °C.

RESULTS AND DISCUSSION

Isolation and identification of the bacteria

A bacterium was isolated from the leather dye contaminated soil. The bacterial isolate was identified as *Staphylococcus aureus* based on morphological and biochemical characterization (table 2).

Table 2: Morphological and biochemical characterization of bacterial isolate

S. No.	Test	Result
1.	Gram staining	Positive
2.	Motility	Non-Motile
3.	Indole test	Negative
4.	Methyl red test	Positive
5.	Voges proskaeur test	Positive
6.	Citrate utilization test	Positive
7.	Triple sugar iron test	Negative
8.	Co-agulase test	Positive
9.	Oxidase test	Negative
10.	Catalase test	Positive

Screening of bacteria for dye degradation

The ability of *Staphylococcus aureus* to decolourise two dyes *viz.* basic violet 3, basic green 4 was evaluated in shaking condition. *Staphylcoccus aureus* could decolorize the dyes effectively only when the medium was supplemented with carbon sources. So complete mineral media of following composition: Peptone 5g/l, yeast 3g/l, glucose 2g/l, sodium chloride 5g/l, dipotassium hydrogen phosphate 5g/l, potassium dihydrogen phosphate 5g/l, magnesium sulphate 1g/l. pH was adjusted to 7 was used for all decolourization experiments.

Fig. 1 shows the decolourization of dyes under shaking condition by growing *Staphylococcus aureus.* The bacteria took 24 h for 100 % decolourization of tested dyes. Fig. 2 shows the growth pattern of *Staphylococcus aureus* in presence of triphenylmethane dyes. The growth pattern of *Staphylococcus aureus* was slightly different from those of control and the lag phase delayed upto 6 h for both the dyes. The specific growth rate was found maximum (0.132 h⁻¹) in absence of dyes. Growth rate was lower (0.127 h⁻¹) in presence of basic violet 3 as compared to basic green 4 (0.130 h⁻¹).
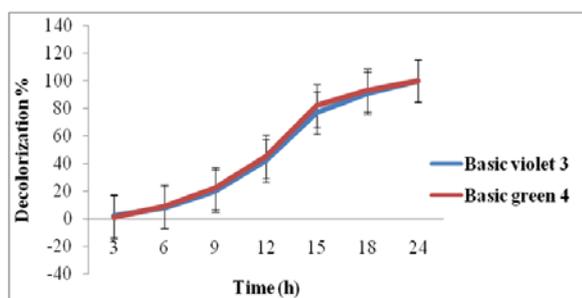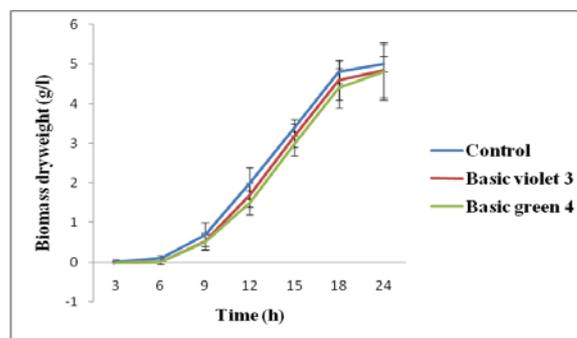

Fig. 2: Growth curve of *Staphylococcus aureus* in presence of basic violet 3 and basic green 4. pH: 7; Initial dye concentration: 10 mg/l
All the experiments were conducted in triplicates. The results were expressed as mean±SEM; n=3

The beginning of the exponential growth phase of bacteria coincided with the onset of decolourization of dyes. It was also observed that the decolourization was faster during exponential growth phase of bacteria and slowed down in the stationary phase. Thus, it was evident from the present study that the decolourization of dyes by basic violet 3 and basic green 4 bacteria was dependent on cell biomass and on actively growing cells.

Decolourization under different physico-chemical condition

Decolourization under shaking and static condition

The effect of static and shaking environment on decolourization of dye were tested by incubating the bacteria, containing dyes 10 mg/l. The process of decolourization was monitored for 24 h under shaking and static condition (fig. 3). 100 % decolourization of dye was achieved under shaking condition with specific growth rate (0.127 h⁻¹) in presence of basic violet 3 and (0.130 h⁻¹) in presence of Basic green 4, but under the static condition, only 41% and 43%

Fig. 1: Decolourization of dyes by *Staphylococcus aureus.* pH: 7; Initial dye concentration: 10 mg/l
All the experiments were conducted in triplicates. The results were expressed as mean±SEM; n=3

decolourization was obtained with specific growth rate (0.043 h^{-1}) in presence of Basic violet 3 and (0.045 h^{-1}) in presence of basic green 4 (fig. 4). This indicates the decolourization process is oxygen dependent. Therefore, shaking conditions were adopted in the following experiments.
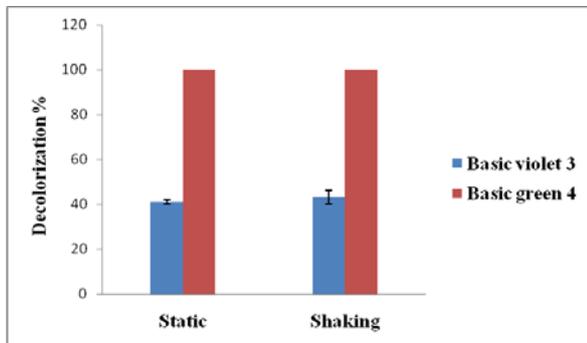

Fig. 3: Decolourization of dyes by *Staphylococcus aureus* under static and shaking condition. pH: 7; Initial dye concentration: 10 mg/l

All the experiments were conducted in triplicates. The results were expressed as mean±SEM; n=3
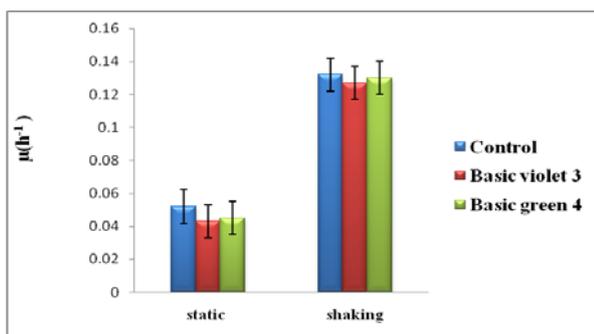

Fig. 4: Specific Growth rate of *Staphylococcus aureus* presence of basic violet 3 and basic green 4. pH: 7; Initial dye concentration: 10 mg/l

All the experiments were conducted in triplicates. The results were expressed as mean±SEM; n=3
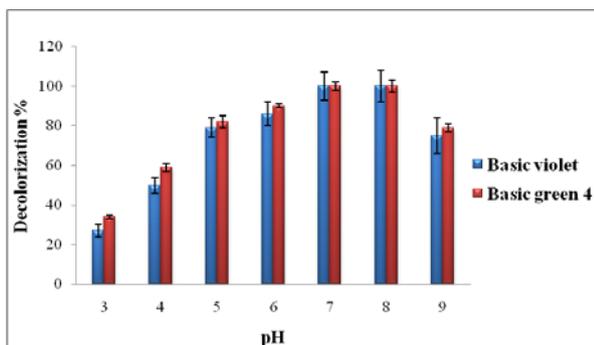

Fig. 5: Decolourization of dyes by *Staphylococcus aureus* under different pH. pH: 7-9; Initial dye concentration: 10 mg/l

All the experiments were conducted in triplicates. The results were expressed as mean±SEM; n=3

Decolourization under different pH

The effect of pH on the dye decolourization was given in the fig. 5. The dye decolourization was found to increase with increase in pH

up to 8 and with further increase in pH the decolourization % decreased. The maximum dye decolourization was shown at pH 7 and 8. Fig. 6 shows the specific growth rate of *Staphylococcus aureus* in different pH condition. The highest specific growth rate of 0.127 h^{-1}and 0.130 h^{-1}was observed at pH 7 in presence of basic violet 3 and basic green 4 respectively. The results showed lower growth rate at acidic pH values.
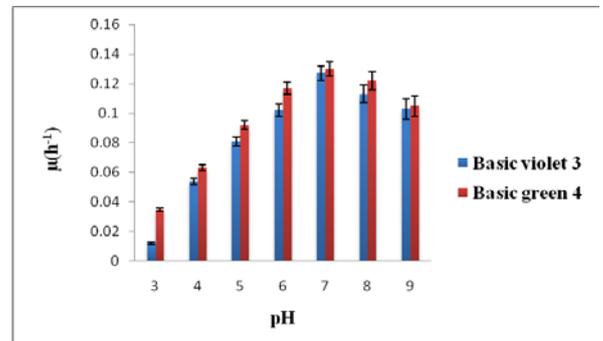

Fig. 6: Specific Growth of *Staphylococcus aureus* in presence of Basic violet 3 and Basic green 4 under different pH. pH: 7-9; Initial dye concentration: 10 mg/l

All the experiments were conducted in triplicates. The results were expressed as mean±SEM; n=3.

Decolourization at different initial dye concentration

The percent decolourization of basic violet 3 and basic green 4 by *Staphylococcus aureus* was carried out at different dye concentrations from 10 mg/l to 500 mg/l. Fig. 7 Shows the effect of initial dye concentration on decolourization of dyes by *Staphylococcus aureus*. The results of our study showed that the bacteria could effectively decolorize basic violet 3 and basic green 4 with 100% decolourization at 10 mg/l concentration. With increasing initial dye concentration from 10 mg/l to 200 mg/l, the decolourization decreased considerably from 100% to 33 % for basic violet 3 and from 100% to 39% for basic green 4. This could be attributed to the toxicity of the dyes to the growing bacterial cells at higher dye concentration.

No decolourization was observed at dye concentrations≥300 mg /l. Specific growth rate of *Staphylococcus aureus* in the absence and presence of increasing concentrations of dyes are given in fig. 8. It was observed that *Staphylococcus aureus* could tolerate up to 200 mg/l of dyes with specific growth rate of 0.012h^{-1} (basic violet 3) and 0.016h^{-1}(basic green 4). Further increase in dye concentration, completely inhibited the bacterial growth.
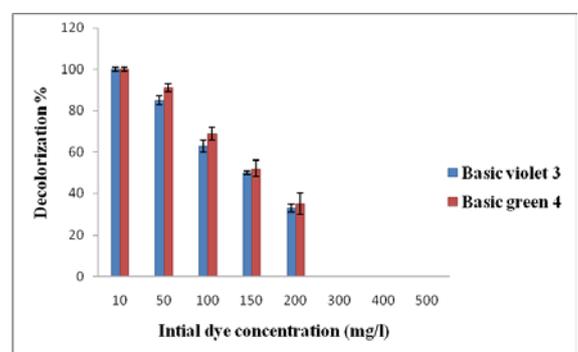

Fig. 7: Decolourization of dyes by *Staphylococcus aureus* under different initial dye concentration. pH: 7; Initial dye concentration: 10-500 mg/l

All the experiments were conducted in triplicates. The results were expressed as mean±SEM; n=3
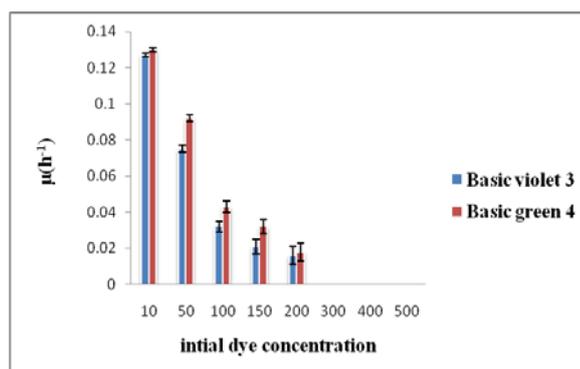
Fig. 8: Specific growth of *Staphylococcus aureus* in presence of basic violet 3 and basic green 4 under different initial dye concentration. pH: 7; Initial dye concentration: 10-500 mg/l
All the experiments were conducted in triplicates. The results were expressed as mean±SEM; n=3

Enzyme assays

The data shown in table 3 represents the enzymatic activities present in the intracellular extract before and after degradation of basic violet 3. Reductive enzymes *viz.* NADH-DCIP reductase, MG reductase and azo reductase activities were observed in intracellular extract before degradation. Activity of MG reductase was observed in the extract before degradation and after degradation significant increase in the MG reductase activity (201.00±2.135 µmol of Malachite green reduced/ml/min) was observed. We also observed significant induction in the activities of NADH-DCIP reductase (350.00±1.06 µmol of DCIP reduced/ml/min) and azo reductase activity was slightly increased (5.85±1.5 µmol of Methyl red reduced/ml/min) in the intracellular extracts obtained after degradation. It is evident from our results that MG reductase and NADH-DCIP reductase were involved in degradation of tested dye.

Table 3: Enzyme activities during degradation of basic violet 3 by *Staphylococcus aureus*

Enzymes	Enzyme activity before degradation	Enzyme activity after degradation
NADH-DCIP reductase [a]	32.3±2	350.00±1.06
MG reductase [b]	0.32±0.09	201.00±2.13
Azo reductase [c]	0.54±0.05	5.05±1.5

[a]µmol DCIP reduced/ml/min; [b]µmol malachite green reduced/ml/min; [c]µmol methyl red reduced/ml/min, All the experiments were conducted in triplicates. The results were expressed as mean±SEM; n=3.

The data shown in table 4 represents the enzymatic activities present in the intracellular extract before and after degradation of basic green 4. Reductive enzymes *viz.* NADH-DCIP reductase, MG reductase and azo reductase activities were observed in intracellular extract before degradation. Activity of azo reductase was negligible in the extract before degradation and after degradation slight increase in the azo reductase activity was observed. We also observed significant induction in the activities of NADH-DCIP reductase (361.08±2.0 µmol of DCIP reduced/ml/min) and MG reductase (232.56±1.5 µmol of Malachite green reduced/ml/min) in the intracellular extracts obtained after degradation. Our results show that NADH-DCIP reductase and azo reductase are involved in degradation of triphenyl methane dye tested.

Table 4: Enzyme activities during degradation of basic green 4 by *Staphylococcus aureus*

Enzymes	Enzyme activity before degradation	Enzyme activity after degradation
NADH-DCIP reductase [a]	32.3±2	361.08±2.0
MG reductase [b]	0.32±0.09	232.56±1.5
Azo reductase [c]	0.54±0.05	56.13±2.0

[a]µmol DCIP reduced/ml/min; [b]µmol malachite green reduced/ml/min; [c]µmol methyl red reduced/ml/min, All the experiments were conducted in triplicates. The results were expressed as mean±SEM; n=3.

Treatment of leather dyeing wastewater using growing *Staphylococcus aureus*

In the present study, treatment of real leather dyeing wastewater by growing *Staphylococcus aureus* was conducted in batch mode. The UV-visible spectra of leather dyeing wastewater containing pure culture of *Staphylococcus aureus* showed 25% decolorization at the absorbance maxima 420 nm. So the wastewater was diluted to different ratios (1:2; 1:3, 1:4 and 1:5) and treated with pure culture of *Staphylococcus aureus. Table* 5 shows the decolourization % of raw and diluted leather dyeing waste water. A continuous decrease in the absorbance maxima at 420 nm was observed as a function of time at all dilution ratios. The dilution factor 4 was found to be optimum for complete decolourization of real leather dyeing wastewater.

Table 5: Treatment of real leather dyeing wastewater by growing *Staphylococcus aureus*

Decolourization %	Raw wastewater	Diluted waste water			
	25±2.5%	1:2	1:3	1:4	1:5
		66±1.5%	86±2.1%	100%	100%

All the experiments were conducted in triplicates. The results were expressed as mean±SEM; n=3.

Characterization of untreated and treated leather dyeing wastewater

Physico-chemical status of colored wastewater samples collected from DKS prime tanners showed considerably high pollution load in terms of pH, TSS, TDS and BOD compared to permissible limits according to Pollution Control Acts [28]. The presence of color in the wastewater reduces the light penetration thereby limiting the biological activity in it which ultimately reduces the self purification ability of the ecosystem [29]. Table 6 shows the results of physico-chemical characterization of raw and treated leather dyeing wastewater. High pH in the wastewater limits the microbial growth which is an important source of bioremediation in water ecology [4]. Maximum limits of Total Solids, TDS and TSS allowed in liquid industrial wastewater are 2200, 2100 and 100 mg/l respectively [28]. But in this study, wastewaters examined were having the values greater than the permissible limit.

Biological oxygen demand (BOD) is the amount of oxygen required for the biodegradation of organic matter. Analytical study showed that BOD value of effluent was quite high as the permissible limit of BOD is 30 mg/l according to Pollution Control Acts [28]. The high levels of BOD are indications of low available oxygen for the utilization of organic matter by microorganisms, in a way, describing the pollution strength of the waste waters.

Therefore, it can be concluded from the analysis that the wastewater generated by DKS prime tanners is highly polluted with dyes and other pollutants. The physico-chemical characteristics of the leather dyeing effluent treated with growing *Staphylococcus aureus* were studied. Sample collected after batch operation revealed almost zero color. The pH of the treated leather dyeing effluent was approximately 6.4 and the decrease in other parameters *viz.* TS, TDS, TSS and BOD of the treated effluent were within the values of permissible limits.

Table 6: Characterization of untreated and treated leather dying wastewater

S. No.	Parameter	Before treatment	After treatment	CPCB permissible limits
1	Color	Black	colorless	
2	Temperature	28 °C	35 °C	
3	pH	8.2±0.2	6.4±0.1	
4	Biological oxygen Demand (mg/l)	120±2.0	7±1.3	30
5	Total dissolved solids (mg/l)	3425±5.8	320±.5	2100
6	Total suspended solids (mg/l)	600±3.0	30±2.0	100
7	Total solids (mg/l)	4025±7.0	350±8.9	2200

All the experiments were conducted in triplicates. The results were expressed as mean±SEM; n=3.

Toxicity studies

Phytotoxicity

Phytotoxicity tests were performed in order to assess the toxicity of the real leather dyeing wastewater and treated leather dyeing wastewater on plants. The phytotoxicity study was carried out on three kinds of seeds commonly used in the Indian agriculture *Vigna mungo, Vigna radiata* and *P*isum *sativum* by watering 10 ml sample of real leather dyeing wastewater and treated leather dyeing wastewater. Toxicity effect was measured in terms of percent germination and lengths of shoot and root after 7 d.

The relative sensitivity of the three plants seeds *viz. Vigna mungo*, *Vigna radiata* and *Pisum sativum* towards real leather dyeing wastewater and treated leather dyeing wastewater are presented in fig. 9. For all three plant seeds, the germination % and the shoot and root lengths of the plants were reduced due to toxicity of dyes present in leather dyeing wastewater (table 7). On the other hand, no germination inhibition was observed in seeds watered with treated leather dyeing wastewater. The growth of the plants was also not affected when watered with treated leather dyeing wastewater.

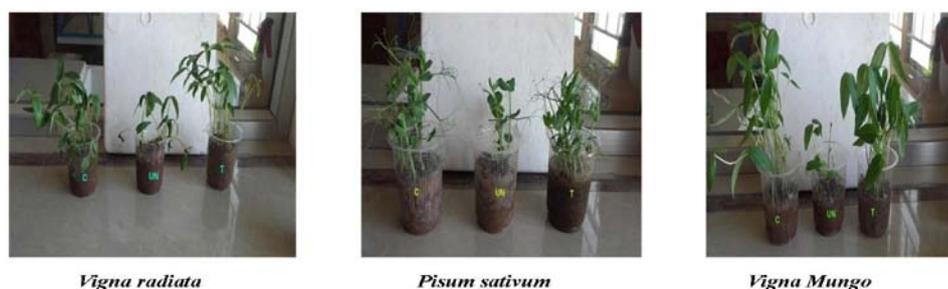*Vigna radiata*   *Pisum sativum*   *Vigna Mungo*

Fig. 9: Phytotoxicity of the real leather dyeing wastewater and treated leather dyeing wastewater. C-control, UN-untreated effluent, T-treated effluent
All the experiments were conducted in triplicates. The results were expressed as mean±SEM; n=3

Table 7: Phytoxicity study of untreated and treated leather dyeing wastewater

Parameter studies	Vingo mungo			Vinga radiata			Pisum sativum		
	water	Untreated waste water	Treated waste water	water	Untreated waste water	Treated waste water	water	Untreated waste water	Treated waste water
Germination (%)	100	50±1.5	100	100	40±2	100	100	30±1.5	100
Shoot length (cm)	17.3±1.2 cm	6±2.1 cm	16.2±1 cm	17±0.5 cm	4±0.5 cm	15±1.0 cm	17±0.5 cm	10±1.2 cm	15±1.3 cm
Root length (cm)	5±1.1 cm	1.5±0.2	4±1.3 cm	5±1.1 cm	1±0.2 cm	5.5±1.5 cm	4±1 cm	2.5±0.5 cm	6.5±0.8 cm

All the experiments were conducted in triplicates. The results were expressed as mean±SEM; n=3.

Microbial toxicity

Microbial toxicity studies were carried out for nitrogen fixing bacterium *Rhizobium* sp. The nutrient medium containing 1.5% agar was used for the study. Toxicity was measured in terms of zone of inhibition (diameter in cm) after 24 h of incubation at 30 °C. Toxicity of real leather dyeing wastewater and treated leather dyeing wastewater were tested on ecologically important bacteria *Rhizobium* sp. using well diffusion method (fig. 10). The results showed significant growth inhibitory zones around the real leather dyeing wastewater containing well on nutrient agar plates cultured with *Rhizobium* sp. However, treated leather dyeing wastewater did not show any inhibitory zone on nutrient agar plates cultured with *Rhizobium* sp. The values of inhibitory zones are given in table 8.

Fig. 10: Microbial Toxicity of untreated and treated leather dyeing effluent on *Rhizobium*

All the experiments were conducted in triplicates. The results were expressed as mean±SEM; n=3

Table 8: Microbial toxicity of untreated and treated leather dyeing wastewater

Bacteria	Inhibition zone (cm)	
	Untreated waste water	Treated waste water
Rhizobium	0.8±0.1	NI

NI-no inhibition**,** All the experiments were conducted in triplicates. The results were expressed as mean±SEM; n=3.

CONCLUSION

Biodegradation studies of Triphenylmethane dyes *viz.* basic violet 3 and basic green 4 were conducted using *Staphylcoccus aureus* isolated from leather dyeing effluent. Biodegradation was found to be maxima at conditions such as pH 7, shaking and 100 mg/l of dye concentration. MG reductase, NADH-DCIP reductases were found to play dominant role in biodegradation. Treatment of diluted real leather dyeing wastewater with *Staphylcoccus aureus* showed complete decolorization. Phytotoxicity and microbial toxicity studies confirmed the non-toxic nature of treated leather dyeing wastewater. The following conclusions may be drawn based on the results of the present investigation:

• *Staphylcoccus aureus* can be employed as a potential remediation agent for decolorization of leather dyeing wastewater containing dyes *viz.* basic violet 3 and basic green 4.

• Dye decolorization was dependent on physicochemical parameters such as pH, shaking condition and initial dye concentration.

• Enzymes *viz.* NADH-DCIP reductase and MG reductase were found to play the dominant role during biodegradation.

• Application oriented studies were carried out with real leather dyeing wastewater using pure culture of *Staphylcoccus aureus*. On dilution, the leather dyeing wastewater was decolorized completely.

• Phytotoxicity and microbial toxicity studies confirmed the non-toxic nature of *Staphylcoccus aureus* treated leather dyeing wastewater and thus could be used for irrigation.

Therefore, the suitability of *Staphylcoccus aureus* as potential remediation agent for the treatment of textile wastewater was confirmed.

CONFLICT OF INTERESTS

Declared none

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