Microbiological removal of crystal violet dye by Bacillus subtilis ETL-2211

MP Shah*, KA Patel, SS Nair

Abstract

Introduction
The growth of the world population, the development of various industries and the use of fertilisers and pesticides in modern agriculture have overloaded not only the water resources but also the atmosphere and the soil with pollutants. In the last few decades, the handling of wastewater appeared to be one of the most important environmental issues. The textile industry, which is one of the largest consumers of water in the world, produces wastewater comprising various recalcitrant agents such as dyes, sizing agents and dying aids. Therefore, care should be taken when releasing these types of wastewater into the environment. The aim of this study is to discuss the microbial removal of crystal violet dye by Bacillus subtilis ETL-2211.

Materials and methods
Isolation and identification of dye decolourising bacterial isolate from textile dye effluent was carried out. The isolates of B. subtilis ETL-2211 were isolated from the effluent samples collected from textile industries of Ankleshwar, Gujarat, India. Different parameters were used for optimising the conditions for maximum decolourisation effect by the bacterial isolate.

Results
The temperature (40°C), pH (8.00), biological oxygen demand (220 mg L⁻¹), chemical oxygen demand (700 mg L⁻¹), total suspended solids (2800 mg L⁻¹), total dissolved solids (7500 mg L⁻¹), and colour were more than the prescribed fresh water limits. A potential bacterial strain was isolated and selected from the textile effluent on the basis of rapid azo dye Crystal violet (100 mg L⁻¹) decolourisation and later identified as belonging to B. subtilis based on phylogenetic and phenotypic characterisation. Effects of physicochemical parameters (such as pH and temperature) on the Crystal violet decolourisation by the B. subtilis ETL-2211 were studied.

Conclusion
Decolourisation was effective at pH 8, 35°C with starch and peptone as carbon and nitrogen sources and in static condition. This decolourisation potential increased the applicability of this micro-organism in dye removal.

Introduction
The growth of the world population, the development of various industries and the use of fertilisers and pesticides in modern agriculture have overloaded not only the water resources but also the atmosphere and the soil with pollutants. In the last few decades, the handling of wastewater appeared to be one of the most important environmental issues. The textile industry, which is one of the largest water consumers in the world, produces wastewater comprising various recalcitrant agents such as dyes, sizing agents, and dying aids. Therefore, care should be taken when releasing these types of wastewater into the environment. In the disposal of textile wastewater, colour is very important due to the aesthetic deterioration as well as the obstruction of penetration of dissolved oxygen and sunlight into natural water bodies. The degradation of the environment due to the discharge of wastewater from industrial sources is a real problem in several countries. This situation is even worse in developing countries such as India where little or no treatment is carried out before the discharge.

In spite of the many steps taken to maintain and improve the quality of surface and groundwater, the quantity of wastewater generated by these industries continues to increase. Municipalities and industries continue to increase and they are confronted with an urgent need to develop safe and feasible alternative practices for wastewater management. Bioremediation is a pollution-control technology that uses natural biological species to catalyse the degradation or transformation of various toxic chemicals to less harmful forms. Xenobiotic compounds are not naturally available and hence the locally occurring micro-organisms cannot readily degrade them. Hazardous materials may render harm to humans, livestock, wildlife, crops, or native plants through handling, ingestion, application to land, or other distributions of the contaminated materials into the environment. The textile industry leaves about 50% of the textile azo dyes in free state to be discharged in the factory effluent and eventually into the surrounding environment. Azo compounds constitute the largest and the most diverse group of synthetic dyes and are widely used in a number of industries such as textile, food, cosmetics, and paper printing. The reactive azo dyes-containing...
effluents cause serious environmental pollution. Therefore, industrial effluents containing azo dyes must be treated before being discharged into the environment to remove the toxicity. This study aims to investigate the potential of bacterial cultures isolated from industrial dye effluent for decolourisation of a textile dye, Crystal violet. Dye decolourisation by bacterial cultures was optimised with respect to various nutritional sources (carbon and nitrogen) and environmental parameters (temperature and pH).

**Materials and methods**

The protocol of this study has been approved by the relevant ethical committee related to the institution in which it was performed.

**Sampling and analysis of the effluent**

Ankleshwar is one of the most industrialised cities in India. It is known as the chemical hub and was chosen for effluent sample collection. The effluent sample was collected from the centre of the area. Standard procedures (Spot and Grab) were followed during sampling. The temperature and pH were determined at the sampling site. The pH was determined by using a pH meter (Cyber scan pH meter) and temperature with a laboratory thermometer. The sample was transported to the laboratory at 4°C in accordance with the standard methods. The physico-chemical parameters, such as colour, biological oxidation demand (BOD) chemical oxygen demand (COD), total suspended solids (TSS) and total dissolved solids (TDS), were determined as soon as the sample was brought to the laboratory. Sample colour was analysed by using a spectrophotometer (Shimadzu UV-1700). BOD was determined by employing the evaporation method using a dissolved oxygen meter while COD was measured by using the COD instrument directly.

**Chemicals**

The textile dye, Crystal violet ($\lambda_{max} 523$ nm) was obtained from Ankleshwar Textile Industries, Ankleshwar. Nutrient broth (g L$^{-1}$, peptone: 5, meat extract: 1, yeast extract: 2, NaCl: 5, pH: 7) and a stock solution of the dye (1000 mg L$^{-1}$) were prepared in de-ionised water and used for all studies.

**Isolation, screening and identification of dye decolourising bacteria from effluent**

The textile effluent was collected in sterile collection tubes from the sludge and the wastewater of the ditches at industrial site located in Ankleshwar Textile Industries, Ankleshwar. The sample collected from the textile mill was screened for azo dye (Crystal violet) decolourising bacterial strains by inoculating 10 mL of sludge solution into 250-mL Erlenmeyer flask containing 100 mL nutrient broth. The flasks were incubated at 35°C under shaking conditions (140 r.p.m.). After 48 h of incubation, 1 mL of the culture broth was appropriately diluted and plated on nutrient agar (g L$^{-1}$, peptone: 5, meat extract: 1, yeast extract: 2, NaCl: 5, agar: 15, pH: 7.0) containing 20 mg L$^{-1}$ Crystal violet. The morphologically distinct bacterial isolates showing clear zones around their colonies due to decolourisation of dye were selected for further studies. The pure culture stocks of these isolates were stored at 4°C on nutrient agar slopes and these isolates were screened for their ability to decolourise Crystal violet in liquid culture. The screening process in liquid media was carried out by inoculating a loop full of cultures exhibiting clear zones into nutrient broth containing Crystal violet under static conditions. After 24 h of incubation, 1 mL of cell suspension was transferred to fresh nutrient broth containing Crystal violet to screen the strains with colour removing ability. The screening procedure in liquid medium was continued until complete decolourisation of the broth was obtained. A small amount of de-colourised broth was transferred to nutrient agar plates containing Crystal violet (50 mg L$^{-1}$). The bacterial isolate that tolerated higher concentration of the Azo dye was isolated by the streak plate method. The azo dye-decolourising bacteria was identified from several aspects including morphology characters, biochemical tests as described in *Bergey's Manual of Determinative Bacteriology* (indole, methyl red, Voges–Proskauer test, citrate, catalase, oxidase, nitrate reduction test, hydrolysis of casein, starch, urea and gelatin). Assimilation of various sugars such as d-glucose, d-fructose, galactose, mannitol and d-maltose as a sole carbon source was determined by inoculating the isolate into carbohydrate broth supplemented with respective carbon source. After incubation the tubes were incubated at 37°C for 24–48 h.

**Analyses of 16S rRNA sequences**

Genomic DNA of the isolate was extracted with a GenElute DNA extraction kit from Sigma. The 16S rRNA gene of isolate was amplified using the universal primers 8F (5′-AGAGTTTGATCCTGGCTCAG) and 1541R (50-AAAAAGGTTGATCCTGGCTCAG-CCGA-3′). The amplification was done by initial denaturation at 95°C for 5 min followed by 10 cycles of 93°C for 1 min, 63°C for 1 min, 71°C for 1.5 min; 20 cycles of 93°C for 1 min, 67°C for 1 min, 71°C for 2 min and final extension at 71°C for 5 min. The purified polymerase chain reaction product was sequenced in both directions using an automated sequencer by Bangalore Genei (India). The phylogenetic relationship of the isolate was determined by comparing the sequencing data with sequences of some members of the genus bacillus available through the GenBank database of the National Centre for Biotechnology Information. The gene sequences of each isolate obtained in de-ionised water and used for all studies.

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in this study were compared with known 16s rRNA gene sequences in the GenBank database.

Decolourisation assay
The decolourising activity was expressed in terms of the percentage decolourisation by the modified method described previously. The decolourisation process was carried out using shaking culture and static culture by inoculating 1 mL of pre-cultured (O.D 0.85-1) Bacillus subtilis ETL-2211 into 100 mL of sterilised nutrient broth in a 250-mL Erlenmeyer flask and incubated on a rotary shaker (130 r.p.m.) for 24 h. Filter sterilised (0.22 μm) Crystal violet (100 mg L⁻¹) was added to the culture and incubated in shaking conditions at 140 r.p.m. and in static conditions at room temperature for decolourisation to occur. At regular intervals, 4 mL samples were withdrawn aseptically and centrifuged at 8500 r.p.m. for 15 min. The cell-free supernatant was used to determine the percentage decolourisation of Crystal violet. Decolourisation of dye was determined by monitoring the decrease in absorbance at the maximum wavelength of Crystal violet (λmax 523 nm) by using a ultraviolet-visible spectrophotometer (UV-1700 Pharmaspec, Shimadzu, Japan). The un-inoculated dye medium supplemented with respective dye was used as a blank. Decolourisation activity (%) was calculated by the following formula and all assays were done in triplicates:

% Decolourisation: \[
\frac{\text{Initial absorbance} - \text{final absorbance}}{\text{Initial absorbance}} \times 100
\]

Decolourisation of crystal violet under different culture conditions
The decolourisation efficiency of B. subtilis ETL-2211 strain was compared over a wide range of pH (5–9) by adjusting the pH with hydrochloric acid or sodium hydroxide. Decolourisation at different temperatures (room temperature, 35°C, 37°C, 40°C, 45°C, and 50°C) was carried out by adjusting the pH to 8. Varying carbon sources 1% each (dulcitol, starch, maltose, sucrose, dextrose, mannitol, D-xyllose, lactose, and mannose) and nitrogen sources 1% each (urea, potassium nitrate, sodium nitrate, malt extract, ammonium sulphate, ammonium nitrate, ammonium chloride, and peptone) were used to check the decolourising potential of the strain. All the flasks were incubated in static conditions at pH 8 and at 35°C.

Results
Physico-chemical characterisation of textile effluent
The effluent sample collected from a small-scale textile industries, Ankleshwar, Gujarat, India was dark black in colour, with a pungent smell and indigo in appearance. The pH slightly above neutral level and was within the permissible limits (Table 1). The temperature of the effluent was high. TSS and TDS in the textile effluent were very high. The solids present in ground water, besides affecting the growth of the plants directly, also affect the soil structure, permeability and aeration, indirectly affecting the plant growth. The COD and BOD values were within the permissible limits in the effluent sample. Different bacterial strains isolated from the textile effluent were screened for their ability to decolourise the textile azo dye (Crystal violet) and the potential strains were characterised morphologically and biochemically.

Isolation and identification
The isolated organism was analysed morphologically, biochemically and genetically to identify the species and genus. The isolated organism was identified as B. subtilis. The study started by screening for potential textile azo dye decolourising bacteria isolated from the textile industry effluent. Colonies surrounded by a nearly decolourised zone were isolated and then tested for dye removal capability using submerged culture. Strains isolated from the white colonies were inoculated in 100 mL of nutrient broth in a 250 mL conical flask and incubated at 35°C under static conditions. One strain exhibiting highest decolourising activity was chosen for further studies. The gram staining test showed the isolate to be non-motile, gram-positive, spore-forming and rod-shaped bacteria. The spore was terminal located and ellipsoidal in shape. Biochemical characterisation of the isolate revealed it to be negative for indole, methyl red test, Voges-Proskauer, citrate, catalase, oxidase test and nitrate reduction test. The isolate showed a negative result for the hydrolysis of casein, gelatin, starch and urea. The strain utilised various sugars, D-maltose, D-glucose, D-fructose, mannitol and galactose as substrates.

Table 1  Physico-chemical characterisation of the textile effluent collected from Textile Industries, Ankleshwar

<table>
<thead>
<tr>
<th>S. No</th>
<th>Parameter (units)</th>
<th>Effluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Colour (–)</td>
<td>Dark black</td>
</tr>
<tr>
<td>2</td>
<td>Temperature (°C)</td>
<td>38</td>
</tr>
<tr>
<td>3</td>
<td>pH (–)</td>
<td>8.0</td>
</tr>
<tr>
<td>4</td>
<td>TDS (mg l⁻¹)</td>
<td>7500</td>
</tr>
<tr>
<td>5</td>
<td>TSS (mg l⁻¹)</td>
<td>2800</td>
</tr>
<tr>
<td>6</td>
<td>COD (mg l⁻¹)</td>
<td>700</td>
</tr>
<tr>
<td>7</td>
<td>BOD (mg l⁻¹)</td>
<td>220</td>
</tr>
</tbody>
</table>
sole carbon sources and was found to be positive. From the results obtained by the sequence analysis of 16S rRNA and the phylogenetic tree (Figure 1), the organism was identified as *B. subtilis* and it is used for further studies.

**Effect of pH and temperature on decolourisation**
The decolourisation efficiency of *B. subtilis* ETL-2211 was compared across a wide range of pH (5–9). The maximum decolourisation (90%) was recorded at pH 8. At neutral pH, the strain exhibited a percentage decolourisation of 80%. It was 45% and 40% at pH 6 and 9, respectively. The percentage decolourisation decreased markedly at pH 5 (10%) due to acidic conditions (Figure 3). The optimum pH for growth and decolourisation was found to be 8. The dye decolourisation activity of the strain was found to decrease with increasing incubation temperature. Highest decolourisation was achieved at 35°C (95%) and least percentage decolourisation at room temperature (25%). At 37°C, 85% decolourisation was noted followed by 70%, 55% and 25% at 40°C, 45°C and 50°C, respectively, at the end of 24 h incubation (Figure 2). No specific decolourisation was observed in shaking conditions (140 r.p.m.).

**Effect of different carbon and nitrogen sources on Crystal violet decolourisation**
Results of Crystal violet decolourisation with different carbon (Figure 4) and nitrogen sources (Figure 5) are depicted. Dextrose resulted in better decolourisation efficiency with 92% followed by starch (80%) and mannose (65%) at the end of 24 h incubation period. The decolourisation efficiency decreased with dulcitol (52%), mannitol (45%), lactose (35%), d-xylose (30%) and sucrose (25%). Least decolourisation was observed with maltose. Maximum decolourisation with nitrogen sources was achieved with peptone (90%) and least was with malt extract (15%). Urea and ammonium sulphate exhibited good decolourisation with 75% and 65%. The decolourisation efficiency decreased markedly with ammonium nitrate (50%), sodium nitrate (25%), potassium nitrate (22%) and ammonium chloride (15%).

**Discussion**
Throughout India, grave concern and constant attention has been given to the treatment of industrial effluent from textile and dye-manufacturing units. Several researchers have demonstrated the possibility of utilising micro-organisms for biotreatment of textile wastewater. In India, most textile units are scattered and/or operated from private homes. Therefore, it is necessary to collect and treat the waste in common effluent treatment plants. Microbiological methods are quite simple to use and the cost of operation is low. Industrial effluent is not stable and it often varies to a wide range depending on the process practiced. South Asian countries are experiencing severe environmental problems due to rapid industrialisation. This phenomenon is very common where the polluting industries such as textile dyeing, leather tanning, paper and pulp processing, and sugar manufacturing.
thrive as clusters. Among these the textile industries are large industrial consumers of waters as well as producers of wastewater. The effluent discharged by this industry leads to serious pollution of groundwater and soils and ultimately affects the livelihood of the poor. The physico-chemical characterisation of the collected textile effluent sample from textile industries of Ankleshwar showed a high load of pollution indicators. Colour is contributed to a water body by the dissolved compounds (dyes and pigments). The effluent colour was dark black due to the presence of various dyes and chemicals used in the dyeing process. The pH of the study sample was slightly alkaline when compared with the acidic pH of the dyeing effluent in a previous study. The pH of the effluent alters the physico-chemical properties of water which in turn adversely affects the aquatic life, plant and humans. The soil permeability gets affected resulting in polluting underground resources of water. The temperature of the effluent was high in comparison with the temperature of another effluent in one study. High temperature decreases the solubility of gases in water, which is ultimately expressed as high BOD/COD. The values of BOD and COD were within the permissible limits in the present sample in comparison to the very high values of BOD and COD in another effluent study. TDS and TSS values of effluent sample were higher than the permissible limits. Sediment rate is drastically increased because of the high value of TDS, which reduces light penetration into water, and ultimately decreases photosynthesis. The decrease in photosynthetic rate reduces the dissolved oxygen level of wastewater, which results in decreased purification of wastewater by micro-organisms. The current sample exhibited high values of heavy metals, which were of the same order of magnitude reported in another effluent sample.

**Figure 3:** Effect of pH on Crystal violet decolourisation.

**Figure 4:** Effect of carbon source on Crystal violet decolourisation.

**Figure 5:** Effect of nitrogen source on Crystal violet decolourisation.

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The nutrients of the surrounding soils are depleted as a result of high value of heavy metals thereby affecting soil fertility. High chloride contents are harmful for agricultural crops and using wastes containing high chlorides for irrigation purposes might turn harmful\(^{23}\). The majority of textile effluent samples have permissible limits of sulphate ions. The effluent showed phenolic contents greater than 0.1 ppm, which is though the permissible limit. These compounds are still very toxic to fishes even at very low concentrations\(^{23}\). The bleaching and dyeing processes are the main cause of pollutants, which include caustic soda, hypochlorite and peroxides. The isolation of different micro-organisms from the effluent sample collected from the textile industries of Ankle-shwar indicates natural adaptation of micro-organisms to survive in the presence of toxic chemicals and dyes. Interest in the bioremediation of pollutants using bacteria has intensified in recent years, as many researches demonstrated the efficacy of bacterial bioremediation over fungal and actinomycetes. Many bacteria capable of reducing azo dyes reported were isolated from textile effluent contaminated sites\(^{23}\). A strain of bacterium B. subtilis ETL-2211 with strong decolourising ability was isolated from textile effluent to decolourise the textile azo dye Crystal violet (100 mg L\(^{-1}\)) within 24 h in aerobic and static conditions. The reason for the decreased decolourisation under shaking conditions could be competition of oxygen and dye compounds for the reduced electron carriers under aerobic conditions. The percentage of decolourisation of Crystal violet by B. subtilis ETL-2211 strain under static conditions was 90% within 24 h of incubation which was equal to a similar study but with 35 h of incubation period\(^{24}\). In another study conducted with Pseudomonas putida, Pseudomonas fluorescence, Bacillus cereus and Stenotrophomonas acidaminiphila to decolourise Acid Red 88 showed their efficiencies at 35%, 31%, 40% and 50%, respectively\(^{25}\). Under aerobic conditions azo dyes are generally resistant to attack by bacteria\(^{26}\). The optimal pH for complete decolourisation of Crystal violet was at 8 which is slightly in accordance with Cosmarium spp. decolourising malachite green at pH 9\(^{27}\) and Klebsiella pneumonia RS-13, which completely degraded Methyl Red in the pH range of 6–8\(^{28}\). Optimal growth temperature was found to be 35°C, which is consistent with the highest decolourisation temperature in our study. Maximum potential of Pseudomonas sp. to decolourise malachite green, fast green was noticed at 37°C\(^{29}\). Vibrio logei and Pseudomonas nitroreducens showed the highest Methyl Red degradation activity at 30–35°C\(^{30}\). Dextrose and peptone were found to be most effective carbon and nitrogen sources for decolourisation of Crystal violet in the present study compared with lactose and yeast extract in another similar study for decolourisation of Everzol Red RBN\(^{31}\).

**Conclusion**

The study concluded that pH, temperature, various carbon and nitrogen source have a significant influence on dye removal efficiency by B. subtilis ETL-2211. This shows that the isolated bacterium has enormous potential to degrade the textile dyes and resolve the problem of unnecessary dyes present in the effluents of textile industries. Further, pilot-scale studies are required with these strains for actual industrial applications, and detailed study is needed to explore the mechanism involved. Although decolourisation is a challenging process to both textile industry and wastewater treatment, the result of these findings and literature suggest a great potential for bacteria to be used to remove colour from dye wastewaters. The bacterial strain B. subtilis ETL-2211 showed decolourising activity through a degradation mechanism rather than adsorption. This observation has established that the bacteria are adaptive in nature and can degrade contaminants. The ability of the strain to tolerate, decolourise azo dyes at high concentration gives it an advantage for treatment of textile industry wastewaters. However, potential of the strain needs to be demonstrated for its application in treatment of real dye bearing wastewaters using appropriate bioreactors. Application of traditional wastewater treatment requires enormous cost and continuous input of chemicals, which becomes uneconomical and causes further environmental damage. Hence, economical and eco-friendly techniques using bacteria can be applied for fine-tuning of wastewater treatment. Biotreatment offers easy, cheaper and effective alternative for colour removal of textile dyes.

**Acknowledgement**

Authors are immensely grateful to the management of Enviro Technology Limited, Ankleshwar, Gujarat, India for allowing us to carry out such a noble work for the sustainable environment.

**Abbreviations list**

BOD, biological oxidation demand; COD, chemical oxygen demand; TDS, total dissolved solids; TSS, total suspended solids.

**References**


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