Microbial degradation and decolourisation of Reactive Black by an application of Pseudomonas stutzeri ETL-79

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Abstract
Introduction
The textile industry in India is one of the oldest industries. It provides direct employment to nearly thirty million people. Wastewater from the textile industries is a complex mixture of many polluting substances such as organo-chlorine-based pesticides, heavy metals, pigments and dyes. The dye that has been chosen for this study is Reactive Black. This study focused on microbial degradation and decolourisation of Reactive Black by an application of Pseudomonas stutzeri ETL-79.

Materials and methods
The decolourising bacterium was identified as P. stutzeri ETL-79 based on physiological and biochemical characteristics as well as the 16S rRNA sequence. In this study, the effects of P. stutzeri ETL-79’s decolourising ability, for example, glucose concentration, temperature, pH and dye concentration, were investigated.

Results
The results show that under 40°C, pH 7.0, glucose concentration 0.2% and concentration of Reactive Black of 30 mg/L, the decolourisation rate in 24 and 48 hours was 86.3% and 93.2%, respectively.

Conclusion
The study concluded that pH, temperature and various carbon sources have a significant influence on the dye removal efficiency by P. stutzeri ETL-79. This shows that the isolated bacterium has enormous potential to degrade the textile dyes and resolve the problem of contamination by unnecessary dyes present in the effluents of textile industries. Further pilot scale studies need to be conducted with such strains for actual industrial applications, and detailed studies are needed to explore the mechanisms involved. Although decolourisation is a challenging process for both textile industry and wastewater treatment, the result of these findings and literature suggests a great potential for such bacteria to be used to decolourise dye wastewaters. P. stutzeri ETL-79 showed a decolourising activity through a degradation mechanism, rather than adsorption. This observation has established that bacteria are adaptive in nature and that they can degrade contaminants. The ability of the strain to tolerate and decolourise azo dyes at a high concentration gives it an advantage for treatment of textile industry wastewaters. However, potential of the strains needs to be demonstrated for their application in treatment of real dye-containing wastewaters using appropriate bioreactors. Application of traditional wastewater treatment involves 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 used for fine-tuning of wastewater treatment. Bio-treatment offers an easy, cheap and effective alternative for colour removal of textile dyes.

Introduction
The textile industry is one of the oldest industries in India with over 1000 industries. Taking into account the volume and composition of effluent, the textile wastewater is rated as the most polluting among all in the industrial sectors. In general, the wastewater from a typical textile industry is characterised by high values of BOD, COD, colour and pH. It is a complex and highly variable mixture of many polluting substances ranging from inorganic compounds and elements to polymers and organic products. Incomplete use and washing operations give the textile wastewater a considerable amount of dyes. The untreated textile wastewater can cause rapid depletion of dissolved oxygen if it is directly discharged into the surface water sources due to its high BOD value. The effluents with high levels of BOD and COD values are highly toxic to biological life. The high alkalinity and traces of chromium, which is employed in dyes adversely affect the aquatic life and also interfere with the biological treatment processes. It induces persistent colour coupled with organic load leading to disruption of the total ecological/symbiotic balance of the receiving water stream. Dyes with striking visibility in recipients may lead to reduced light penetration in aquatic environment which will significantly affect the photosynthetic activity. The high concentration of nitrogen in the textile industrial effluents can cause the eutrophication of closed water bodies. In addition, coloured water is objectionable as it can spoil the beauty of water environments. In view of the earlier mentioned adverse effects, the textile industry effluent should be discharged after proper treatment. The dyes are stable to light, heat and oxidizing agents, and it is difficult to remove the dyes from effluents.
This makes the effective and economic treatment of the effluents containing various dyes an important environmental problem. Traditionally, both physical and chemical methods such as coagulation, ozonation\textsuperscript{9}, precipitation, adsorption by activated charcoal, ultrafiltration, nanofiltration\textsuperscript{12}, electrochemical oxidation, electrocoagulation\textsuperscript{13,14}, etc. were used in the treatment of the textile industrial effluents\textsuperscript{2}. But both the methods have many shortcomings\textsuperscript{9,15,16}. Chemical methods like coagulation often produce excess amount of chemical sludge, which create problems in its disposal. Physical methods like adsorption by activated charcoal often need high capital investment. Hence, most of the physical and chemical methods of effluent treatment are not accepted by the industries due to their high cost, low efficiency and inapplicability to a wide variety of dyes. Currently, much research has been focused on the biodegradation of the industrial effluents\textsuperscript{9,17,18}. It mainly shows interest towards the pollution control using bacteria, fungi in combination with physicochemical methods\textsuperscript{19,20}. The biomass can absorb the chromophores and also these chromophores can be reduced in low redox potential environments. The attractive features of biological treatment are low cost, renewable and regenerative activity, and little or no secondary hazard\textsuperscript{21-23}. The conventional biological processes are not effective because the dye content in the textile effluent is toxic to the microorganisms used\textsuperscript{24,25}. \textit{In situ} degradation of the effluent is a novel method under the biodegradation process. In this method, the microorganisms are isolated from the site of pollution and the same microorganisms can be used for the treatment of the effluent\textsuperscript{26,28}. The current study has evaluated the potential of isolated bacterial strain from textile effluent for their decolourisation efficiency of the textile dye, Reactive Black under \textit{in vitro} conditions and optimisation of the factors influencing the process.

**Materials and methods**

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

**Dyes and samples**

The Reactive Black dye used for this biodecolourisation study was obtained from the local textile industry in Ankleshwar, Gujarat. Heavily dye-contaminated soil and wastewater samples were obtained from surrounding areas of the textile industries and wastewater treatment factory in Ankleshwar, respectively. All samples were collected in sterile plastic containers.

**Isolation of Reactive Black dye resistant and decolourising bacteria**

Soil samples were used as inoculum and 1 g of soil was added into 50 ml of sterile modified base mineral medium (BMM) in flasks and Reactive Black was added as the sole carbon source to give a final concentration of 30 mg/L. One litre of BMM contained 5.17 g KHPO\textsubscript{4} 1.70 g KH\textsubscript{2}PO\textsubscript{4} 1.63 g NH\textsubscript{4}Cl and 10 ml of a salt solution. One litre of the salt solution contained 8.5 g MgSO\textsubscript{4} 5 g MnSO\textsubscript{4} 5 g FeSO\textsubscript{4} 0.3 g CaCl\textsubscript{2}. The initial pH value of media was 7.2\textsuperscript{27}. The inoculated flasks were agitated on an orbital shaker at 130 rpm at 32°C for 72 hours. Then 2 ml of the culture medium was transferred to another 50 ml of fresh culture medium and cultivation was carried out under the same conditions for two to three times. The obtained suspensions were streaked on the nutrient agar plates. The medium composition is BMM, 30 mg/L of Reactive Black and 1.5% of agar. All the plates were incubated at 32°C for 48 hours. The morphologically distinct bacterial isolates showing a clear decolourisation zone were selected and preserved on Reactive Black dye-containing nutrient agar slant at 4°C.

**Identification of an organism**

The isolate with the largest decolourisation zone for the dye was selected and streaked on Reactive Black-containing nutrient agar for further purification and study. The purified isolate was used to inoculate on several media for biochemical testing. The physiological and biochemical characteristics of the Reactive Black decolourisation organisms were examined using standard procedures\textsuperscript{29}. The utilisation of some substrates as carbon sources and nitrogen sources by the isolate were performed based on Bergey’s Manual of Determinative Bacteriology\textsuperscript{29}. Genomic DNA was isolated from the pure culture pellet using consensus primers and partial 16S rRNA genes were amplified by PCR using forward primer (5′-GACCAGATAACATTCCACAACA-CAGG-3′), reverse primer (5′-CCGTTTTCCAGGCAGCACTA-3′) and internal primer (5′-CAGCGACCCGGTAAATAC-3′). The amplified 16S rRNA gene was sequenced by Bangalore Geneti (Bangalore, India). The obtained sequence data were aligned and analysed for identification and finding the closest homology for the isolate. The next closest homology was found with \textit{Pseudomonas stutzeri} and it was designated as \textit{P. stutzeri} ETL-79.

**Reactive Black decolourisation quantification**

The dye decolourisation quantification studies were performed in a cultivated medium. The cultivation was centrifuged at 8000 g for 20 minutes; the supernatant was scanned for absorbance maxima on a UV-VIS spectrophotometer (Shimadzu, Japan). Decolourisation study in aqueous medium was performed by inoculating 15% of (v/v) actively growing strain ETL-79 to another fresh 100 ml
of medium containing Reactive Black dye in a 250 ml flask. All the tests were done in triplicates. One set of the flasks without inoculation was kept as control. After incubation, 10 ml of the inoculated and uninoculated samples were centrifuged at 8000 g for 20 minutes and the supernatant was analysed at 540 nm wavelength and decolourisation rate was calculated. Viable cell counts during Reactive Black decolourisation was followed from previous reports.

Decolourisation experiments
The Reactive Black stock solution of 3000 mg/L was prepared and using the required concentrations of dye, the solution was diluted. To study the effect of temperature (15°C–50°C), pH (3.0–10.0), dye concentration (15–500 mg/L), glucose concentration (0%–0.5%) in each experiment, one factor was changed, with the other factors remaining constant. The effects of these factors were evaluated by measuring the Reactive Black decolourisation rate and in triplicate.

Results
Isolation of Reactive Black decolourisation bacteria
There were 18 morphologically distinct isolates showing decolourisation zones from 4 to 10 mm on nutrient agar containing 30 ppm Reactive Black. The isolated one, designated as ETL-79, was with a larger decolourised zone (10 mm) for a colony size of 4–5 mm, and was selected for further studies. The other six isolates showed decolourisation zones between 4 and 7 mm and were resistant to 150 mg/L of Reactive Black, which may be the results of heavy contamination with dyes due to the consequence of natural adaptation of the organism as the samples from which the bacterial isolate was obtained were contaminated heavily with dyes. The strain ETL-79 had higher efficient decolourisation activity and could degrade the synthetic dye Reactive Black.

Identification of the ETL-79 isolate
The 16S RNA gene sequences were compared by using BLAST similarity searches, and the closely related sequences were obtained from GenBank. On the basis of morphological and biochemical analysis in combination with phylogenetics analysis, the strain ETL-79 was identified as *P. stutzeri* and designated as *P. stutzeri* ETL-79 (Figure 1).

Effect of temperature on Reactive Black decolourisation by *P. stutzeri* ETL-79
Temperature plays an important role in microbial growth and enzyme activity and is one of the most important parameters taken into consideration in the development of biodecolourisation processes. The temperature range was 15°C–50°C. The results (Figure 2) reveal a high decolourisation percentage at 35°C and 40°C. At 40°C and after 48-hour cultivation, the decolourisation percentage was 93.2%, while it was only 81.2% and 58.3% at 35°C and 30°C, respectively. The viable cell count was evaluated during Reactive Black decolourisation. Cell density was about 106 cfu/mL at the beginning of the tests. At 40°C cell density increased rapidly and the number of alive bacteria in the medium increased.

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could reach 108 cfu/mL, more than that under the condition at 35°C (1.3 × 107 cfu/mL) and 45°C (1.1 × 107 cfu/mL). ETL-79 strain could not stay alive when temperature exceeded 50°C, and the Reactive Black was not decolourised because of that. Therefore, the strain Reactive Black was a facultative heat-resistant bacteria and the optimum decolourisation temperature could be determined as 40°C.

**Effect of initial pH on Reactive Black decolourisation**

*P. stutzeri ETL-79*

To test the effect of pH on Reactive Black decolourisation by *P. stutzeri* ETL-79, the temperature was maintained constant (40°C) for 48 hours and pH was varied from 3 to 10. Figure 3 shows that decolourisation percentage of Reactive Black was higher at pH 6–8. The highest decolourisation rate could be obtained at pH 7 for 48 hours, and the decolourisation rate also reached over 65% at pH 6 and 8, while the decolourisation rate was 32.5% at pH 5 and was almost zero at lower (3 and 4) or higher (9 and 10) pH values. The variation of the amount of viable bacteria under different pH was also tested and the results show (Figure 2) that there was no bacterial growth at pH values under 6 and above 9, and the highest densities of viable bacteria were obtained between the pH range 6–8.5.

**Effect of the glucose concentration on Reactive Black decolourisation**

*P. stutzeri ETL-79*

To test the effect of glucose concentration on Reactive Black decolourisation by ETL-79 isolate, temperature and pH were kept constant at 40°C and pH 7, for 48 hours, and glucose concentration varied from 0% to 0.5%. Even though *P. stutzeri* ETL-79 had the ability to use Reactive Black as the sole carbon source, it grew slowly. But when glucose was added, the growth rate increased as well as the decolourisation rate. Figure 4 shows that decolourisation rate of Reactive Black was higher at the range 0.2%–0.3%. The highest decolourisation rate is 92.5% when the glucose concentration is 0.2% while the rate is 59.8% and 82.6% when the glucose concentration is 0.1% and 0.3%, respectively. When the glucose concentration was over 0.3%, the decolourisation of Reactive Black rapidly decreased. Bacteria densities for a determined glucose concentration (Figure 3) varied greatly in the range 0%–0.5% with the highest value reached at 0.5% (109 cfu/mL). *P. stutzeri* ETL-79 can use glucose as a carbon and energy source. Thus, it provided sufficient electrons for reductive conditions through the cleavage of the azo bond of Reactive Black. The increase of glucose concentration decreases the decolourisation rate of Reactive Black and it could be assumed that when there is enough glucose in the medium *P. stutzeri* ETL-79 prefers to use glucose instead of the dye Reactive Black.

![Figure 3: Effect of pH on decolourisation of Reactive Black.](image)

![Figure 4: Effect of glucose concentration on Reactive Black decolourisation.](image)
Effect of Reactive Black concentration on decolourisation
*P. stutzeri* ETL-79

For the study of the effect of Reactive Black concentration on decolourisation, temperature, pH and glucose concentration were constant at 40°C, 7 and 0.2%, respectively for 48 hours, while Reactive Black varied from 15 to 500 mg/L. Figure 5 shows that decolourisation rate decreases with the increasing Reactive Black concentration.

UV-Vis absorption spectra

Figure 6 shows that main visible absorption peaks of Reactive Black decreased after 24 hours and almost disappeared at 48 hours, which indicated that the azo groups were broken. Moreover, it was also concluded that the ring was opened during the decolourisation of Reactive Black because its absorbance value at 330 nm was decreased at 24 hours and almost disappeared at 48 hours. It has been reported that the oxidation of azo bonds led to decolourisation of dye solutions with the formation of primary aromatic amines and the formation of aromatic amines has also been reported in the natural anaerobic degradation of azo dyes. The azo bond of Reactive Black was broken and the ring was opened during decolourisation of Reactive Black by *P. stutzeri* ETL-79 in this study. This would lead to formation of aromatic amines in the medium. This is in accordance with previous reports.

Discussion

The aim of this work was to biologically degrade the dyes, which is by using bacteria that can survive in the conditions imposed by the effluent. The bacterium that was isolated from the effluent was identified to be *P. stutzeri* ETL-79. Industrial effluent is not stable and it varies often in a wide range depending upon the process practiced. South-Asian countries are experiencing severe environmental problems due to rapid industrialisation. This phenomenon is very common where the polluting industries like textile dyeing, leather tanning, paper and pulp processing, sugar manufacturing, etc. thrive as clusters. Among these, the textile industries are large industrial consumers of water 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 physicochemical characterisation of the collected textile effluent sample from Ankleshwar Textile Industries, Ankleshwar, Gujarat, India 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 black due to a mixture of various dyes and chemicals used in the dyeing process. The pH of the tested sample was slightly alkaline when compared to the acidic pH of the dyeing effluent in a previous study. The pH of the effluent alters the physicochemical properties of water which in turn adversely affects aquatic life, plants and humans. The soil permeability gets affected resulting in polluting...
Research study

The temperature of the effluent was high in comparison with the temperature of another effluent in one study\(^\text{14}\). 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 one effluent study. Sediment rate is drastically increased because of high value of TDS which reduces the light penetration into water and ultimately decreases photosynthesis. The decrease in photosynthetic rate reduces the DO level of wastewater which results in decreased purification of wastewater by microorganisms\(^\text{35}\). The current sample exhibited high values of heavy metals which were of the same order of magnitude reported in another effluent sample\(^\text{36}\). 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 if such wastes containing high chlorides are used for irrigation purposes\(^\text{37}\). The majority of the textile effluent samples have permissible limits of sulphate ions. The effluent showed phenolic contents greater than 0.1 ppm which, though is the permissible limit of the phenolic compounds, are still very toxic to fish even at very low concentrations\(^\text{38}\). The bleaching and dyeing process are the main causes of pollutants, which include caustic soda, hypochlorite and peroxides. The isolation of different microorganisms from the effluent sample collected from the Ankleshwar Textile Industries, Gujarat, India indicates natural adaptation of microorganisms 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\(^\text{39}\). The effluent sample collected from Ankleshwar Textile Industries, Ankleshwar, Gujarat, India was black in colour with a pungent smell and pH of slightly above neutral level and was within the permissible limits. 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 plant growth. The 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 and the potential strains were characterised morphologically and biochemically for identification. The main benefit of employing this technique is that the culture has an optimal temperature of 32°C and optimum pH of 7. In addition to this, the inherent advantages of the microorganism, like rapid growth, less space requirement, etc. makes this an efficient method for treatment of the textile industrial effluent. The massive mobilisation of organic pollutants in natural resources or the introduction of xenobiotics into the biosphere leads to the persistence of a number of chemicals in the biosphere and thus constitutes a source of contamination. Often these organic pollutants comprise a potential group of chemicals, which can be dreadfully hazardous to human health and are resistant to degradation. As they persist in the environment, they are capable of long-range transportation, bioaccumulation in human and animal tissue and biomagnifications in food chains. Biodegradation is used to describe the complete mineralisation of the starting compound to simpler ones like CO\(_2\), H\(_2\)O, NO\(_3\) and other inorganic compounds. Microorganisms play an important role in the field of environmental science by degrading and transforming toxic compounds into non-toxic or less toxic forms. Microbes found in natural water and soil have a broad ability to utilise virtually all naturally and some synthetically occurring compounds as their sole carbon and energy sources thus, recycling the fixed organic carbon back into harmless biomass and carbon dioxide resulting in a clean-up of the environment. Azoreductase can catalyse the reductive cleavage ofazo bonds, which is the key enzyme expressed in azo dye-degrading bacteria. In the last decade, enzymes with azoreductase activity have been identified and characterised from many bacteria, such as P. chrysosporium, Xenophilus azovorans KF46F, Pigmentiphaga kullae K24, Enterococcus faecalis, Staphylococcus aureus\(^\text{40-44}\). Azoreductase has low substrate specificity and can break the dye molecules in the high-affinity electron azo bond, producing colourless aromatic amines utilising NAD(P)H as an electron donor \textit{in vitro}\(^\text{45,46}\). However, the involvement of intracellular azoreductases in bacterial decolourisation has been doubted in recent years due to their high polarities and complex structures, many azo dyes are difficult to diffuse through cell membranes. In this study, the azo dye Reactive Black was degraded by \textit{P. stutzeri} ETL-79. The other 18 isolates showed decolourisation zones of 4–7 mm and they were resistant to 150 ppm Reactive Black concentration. The temperature, pH, concentration of Reactive Black, glucose content could change the strain ETL-79 biodecolourisation rate and its rate reached 93.2% in 48 hours, under the condition of 40°C, pH 7.0, glucose concentration 0.2% and anaerobic culture, which is one of the fastest reported biodecolourisation organisms for Reactive Black. All these results showed that \textit{P. stutzeri} ETL-79 had higher activity...
of the azo dye decolourisation than previous reports. Therefore, *P. stutzeri ETL-79* is a potential candidate for degrading and bioremediating azo dye wastewater and polluted soil. The next step, we will purify its azoreductase and test its physicochemical properties of the purified azoreductase, which may open new possibilities for its biotechnological applications and allow the use of *P. stutzeri ETL-79* in the treatment of azo dyes in industrial effluents. Furthermore, we will also construct gene engineering bacteria to be used in a continuous process for the degradation of azo dyes in industrial effluents.

**Conclusion**

The study concluded that pH, temperature and various carbon sources have a significant influence on the dye removal efficiency by *P. stutzeri ETL-79*. This shows that the isolated bacterium has enormous potential to degrade the textile dyes and resolve the problem of contamination by unnecessary dyes present in the effluents of textile industries. Further pilot scale studies need to be conducted with such strains for actual industrial applications, and detailed studies are needed to explore the mechanisms involved. Although decolourisation is a challenging process for both textile industry and wastewater treatment, the result of these findings and literature suggests a great potential for such bacteria to be used to decolourise dye wastewaters. *P. stutzeri ETL-79* showed a decolourising activity through a degradation mechanism, rather than adsorption. This observation has established that bacteria are adaptive in nature and that they can degrade contaminants. The ability of the strain to tolerate and decolourise azo dyes at a high concentration gives it an advantage for treatment of textile industry wastewaters. However, potential of the strains needs to be demonstrated for their application in treatment of real dye-containing wastewaters using appropriate bioreactors. Application of traditional wastewater treatment involves 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 used for fine-tuning of wastewater treatment. Bio-treatment offers an easy, cheap and effective alternative for colour removal of textile dyes.

**References**

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