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BIODEGRADATION OF REACTIVE DYE REACTIVE VIOLET 5 BY BACTERIA ISOLATED FROM DYE CONTAMINATED SOIL

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ABSTRACT

The Pseudomonas aeruginosa GSM3 isolated from dye contaminated soil was able to degrade Reactive Violet 5 completely as a sole source of carbon (300 mgL⁻¹) within 20 h under static condition. The organism exhibited good decolorization ability in the pH ranges from 5.0 to 9.0 and temperature from 30 to 40°C respectively. The optimum degradation was observed at 37°C and pH 7. The maximum concentration of Reactive Violet 5 (800 mgL⁻¹) was degraded up to 58% within 68 h. The culture showed remarkable ability to decolorize repeated additions of dye, with decrease in time up to 10 h at fifth dye aliquots addition and also tolerate high salt concentration up to 6 gL⁻¹ NaCl. Continuous decrease in optical density at 558 nm on incubation, increased the colorless bacterial biomass and no change in color on changing pH of decolorized filtrate depicted that decolorization was due to biodegradation. Thus, the isolate P. aeruginosa GSM3 could be good candidate organism for the biotreatment of textile industry effluents.

KEYWORDS: Reactive Violet 5, Reactive dye, Decolorization, Biodegradation and Pseudomonas aeruginosa GSM3.

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INTRODUCTION

Synthetic dyes are most widely used in textile dyeing, paper printing, color photography, pharmaceutical, food, cosmetic, and leather industries. Since 1856, over 10^5 different dyes have been produced worldwide with an annual production of over 7×10^5 metric tons. The textile industry is one of the greatest generators of liquid effluent pollutants, due to the high quantities of water used in the dyeing processes. It is estimated that 280,000 tonnes of textile dyes are discharged in such industrial effluent every year worldwide. All dyes do not bind to the fabric depending on the class of the dye. Its loss in wastewaters could vary from 2% for basic dyes to as high as 50% for reactive dyes, resulting in severe contamination of surface and ground waters in the vicinity of dyeing industries. Textile wastewaters are highly colored because they are typically discharged with a dye contain in the range 10–200 mgL^{-1} and many dyes are visible in water at concentrations as low as 1 mgL^{-1}. Effluents from the textile industries are characterized by extreme fluctuations in many parameters such as chemical oxygen demand (COD), biochemical oxygen demand (BOD), pH, color, and salinity. The wastewater composition will depend on the different organic-based compounds, chemicals, and dyes used in the industrial dry and wet-processing steps.

Discharge of textile industry effluents into rivers and lakes results into reduced dissolved oxygen concentration, thus creating anoxic conditions that are lethal to resident organisms. Many reports indicate that textile dyes and effluents have toxic effect on the germination rates and biomass of several plant species, whereas plant play many important ecological function such as providing the habitat for a wildlife, protecting soil from erosion, and providing bulk of organic matter that is so significant to soil fertility. Many synthetic azo dyes and their metabolites are toxic, carcinogenic, and mutagenic. Therefore, treatment of textile industrial effluents containing azo dyes and their metabolites becomes necessary prior to their final discharge to the environment. Existing physicochemical methods for color removal are expensive, commercially unattractive and greatly interfered by other wastewater constituents or generate waste products that must be difficult to handled. The microbial decolorization and degradation of aromatic compounds has been of considerable interest since it is inexpensive, eco-friendly, and produces a less amount of sludge.

The decolorization potential of the microbes depends on the adaptability and the activity of selected microorganisms. Many microorganisms capable of degrading reactive dyes, including bacteria, fungi, actinomycetes, and algae. Pure fungal cultures have been used for the treatment of textile industry effluent has some disadvantages, including long growth cycle, low pH requirement for the optimum activity of enzymes, the long hydraulic retention time for complete decolorization and requirement of longer time for decolorization. In contrast, it is reported that many potential bacteria having ability to degrade or mineralize several textile dyes. However, comprehensive solutions for Reactive azo dye removal are far from reality, which calls for continued search for new organisms and technologies. In this study a bacterium Pseudomonas aeruginosa GSM3, capable of degrading Reactive Violet 5 was isolated and the effects of different parameters (such as static, shaking, temperature, pH, initial dye concentration, salt concentration and repeated addition of dye aliquots) on dye decolorization by an isolated bacterium was investigated.

MATERIALS AND METHODS

Dyes and chemicals

Reactive dye Reactive Violet 5 has been most commonly used in dyeing and textile industries. Moreover, it is a sulfonated polycyclic aromatic compound and hence it was selected as a model dye (Figure 1). This dye was gifted from...
Colors India Inc. Pvt. Ltd. Ahmedabad, India. All required chemicals were procured from Sigma-Aldrich, India and Hi-Media, Mumbai, India. All chemicals used were of the highest purity available and of an analytical grade.

**Culture medium**

The mineral salts medium (MSM) containing (gL⁻¹): Na₂HPO₄.2H₂O (12.0), KH₂PO₄ (2.0), NH₄NO₃ (0.50), MgCl₂.6H₂O (0.10), Ca(NO₃)₂.4H₂O (0.050), FeCl₂.4H₂O (0.0075) and 10.0 mL of trace element solution containing (mgL⁻¹): ZnSO₄.7H₂O (10.0), MnCl₂.4H₂O (3.0), CoCl₂.2H₂O (1.0), NiCl₂.6H₂O (2.0), Na₂MoO₄.2H₂O (3.0), H₃BO₃ (30.0), CuCl₂.2H₂O (1.0); pH 7.0. The MSM was supplemented with 0.1% (w/v) of yeast extract was used to see the change in the degradation efficiency of bacterium. This was blended with different concentrations of Reactive Violet 5 was used throughout the study as a test and without culture as a control for decolorization studies. When required, 1.9% (w/v) of agar was added into the media to solidify. All the media were sterilized at 121°C for 20 min before use.

**Isolation, screening and identification of dye degrading microorganism**

The isolation, screening and identification of a dye degrading bacterium was done as earlier reported by Bheemaraddi et al.²⁰.

**Decolorization studies of Reactive Violet 5 in liquid medium**

The decolorization experiments were performed in 250 ml Erlenmeyer flasks containing 100 ml of sterilized MSM broth with yeast extract (0.1% w/v) and 100 mgL⁻¹ Reactive Violet 5. They were inoculated with 5 ml of *P. aeruginosa* GSM3 culture broth in test and without inoculum acts as control. The flasks were incubated at 37°C under static as well as shaking (120 rpm) conditions. The 5 ml of cultures were withdrawn at different time intervals (4 h) from both flasks and supernatant was collected by centrifuging at 10,000 rpm for 15 min. The supernatants were used to check the optical density (OD) at 558 nm using UV–Vis spectroscopy (Systronics AU-2700).

**Effect of different parameters on dye decolorization**

Effects of various parameters were conducted using varying in initial pH values ranges from 4 to 10 keeping temperature constant at 37°C. Similarly, calculate the optimum temperature for maximum dye decolorization using varied temperatures ranging from 20 to 50°C with 5°C intervals keeping optimum pH 7.0 and incubating under static conditions. By the optimization study optimum pH (7.0) and temperature (37°C) were selected for further studies using different physicochemical factors such as initial dye concentration (100–800 mg L⁻¹), NaCl concentration (1–6%) and continuous addition of Reactive Violet 5 on dye decolorization by *P. aeruginosa* GSM3.

**Decolorization mode of Pseudomonas aeruginosa GSM3 on Reactive Violet 5**

Decolorization of dyes by *P. aeruginosa* GSM3 was determined by measuring absorbance of culture supernatants at 558nm using UV-visible spectroscopy, and percent decolorization was calculated as mentioned by Dave and Dave²¹,²⁰.
Dye decolorization may take place by adsorption\(^2^2\) or degradation\(^2^3\). In the case of adsorption, dyes are only adsorbed onto the surface of bacterial cells, whereas new compounds come into being when dyes are degraded by bacterial enzymes during the degradation process. In adsorption, examination of the absorption spectrum will reveal that all peaks decrease approximately in proportion to each other. If the dye removal is attributed to biodegradation, either the major visible light absorbance peak will completely disappear or new peaks will appear\(^2^4\). Dye adsorption can be also easily judged by an evidently colored cell pellet, whereas those retaining their original colors are accompanied by the occurrence of biodegradation\(^2^5\). A bacterial cell suspension was withdrawn at regular intervals and supernatant were used to check the optical density (OD) at 558 nm using UV-visible spectroscopy and similarly cell pellet washed with methanol was used to confirm decolorization is not due to surface adsorption.

RESULTS AND DISCUSSION

**Acclimatization studies on Reactive Violet 5 degradation by Pseudomonas aeruginosa GSM3**

To enhance the bacterial growth and dye decolorization efficiency of *P. aeruginosa* GSM3, we added 0.1% (w/v) yeast extract to MSM broth as a co-substrate. We observed that complete decolorization of 100 mgL\(^-1\) Reactive Violet 5 in MSM medium within 16 hours as compared to 52 hours without yeast extract under static condition. Similar observations have been reported by Khalid *et al.*\(^2^6\) However, they used 0.4% of yeast extract as a cosubstrate for the growth of azo dye decolorizing organisms. Earlier, Isik and Sponza\(^2^7\) showed that yeast extract could be used as growth supplement for azo dye degrading bacteria. In our study 0.1% yeast extract enhances the dye degradation efficiency of this organism by three times and reduced the time period to one third. So, in this study further decolorization experiments was performed using MSM broth supplemented with 0.1% (w/v) yeast extract as a cosubstrate for *P. aeruginosa* GSM3 growth.

**Optimization of abiotic parameters**

We optimized the static/shaking, temperature, pH, and Initial dye concentration for the maximum decolorization of Reactive Violet 5 by *P. aeruginosa* GSM3. Figure 2 shows that complete decolorization of added 100 mgL\(^-1\) Reactive Violet 5 within 16 h under static condition as compared to only 18% decolorization in the culture flask incubated in the shaking condition, Hence, in this study static conditions were maintained to investigate bacterial decolorization.

![Figure 2](image)

*Effect of static and shaking conditions on decolorization of Reactive Violet 5.*

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In the temperature optimization study, dye decolorization activity of *P. aeruginosa* GSM3 was found to increase with increase in incubation temperature from 20 to 37°C. Further increase in temperature to 40°C, decolorization efficiency was decreased by 2% and 25% decreased at 45°C, and 31% at 50°C (Figure 3). Optimum temperature for this organism was in the range of 30 to 40°C and similar finding was reported by Sheth and Dave\textsuperscript{12}. The decrease in decolorization at high temperature can be attributed to the decline in microbial activity that led to the inactivation of the enzyme and eventually the no decolorization of dye\textsuperscript{28}.

![Figure 3](image)

**Figure 3**

*Effect of temperature on decolorization of Reactive Violet 5.*

Similarly the optimization of pH for maximum decolorization of Reactive Violet 5 by this bacterial strain was determined over a wide range of pH 4.0 to 10.0 with an interval of pH 1. The isolate showed maximum of 100% decolorization at neutral pH 7.0 at 37°C (Figure 4). Increase in the either side of neutral pH, the percentage of decolorization was decreased steadily from 93% to 31% on alkaline side from pH 8 to 10 however, steep decline in percent decolorization from 90 to 33% on acidic side at pH 6 and 4 respectively. More than 50% of decolorization was observed in the pH range of 5 to 9. Sheth and Dave\textsuperscript{12} also found that pH 7.0 was optimum for the decolorization of Reactive Red BS (C.I.111) by *Pseudomonas aeruginosa* NGKCTS. Chan and Kuo\textsuperscript{29} reported that the neutral pH would be more favorable for decolorization of the azo dyes and is suitable for industrial applications.

![Figure 4](image)

**Figure 4**

*Effect of pH on decolorization of Reactive Violet 5.*
The decolorization efficiency of Reactive Violet 5 by *P. aeruginosa* GSM3 was studied by increasing initial dye concentration (100–800 mgL⁻¹). We observed that the percentage of decolorization was decreased slowly with increasing dye concentration (Figure 5). It could effectively decolorize up to 300 mgL⁻¹ Reactive Violet 5 (100%) within 20 h and is decreased to 58%, when dye concentration increased to 800 mgL⁻¹ and decolorization time increases from 20 to 68 h respectively. The decrease in percentage of decolorization and enhanced time period at high dye concentration may be attributed to the inhibitory effects of high dye concentration and azo dyes usually contain one or more sulfonic acid groups on aromatic rings, which act as detergents to inhibit the growth of microorganisms. Similar type of observations was reported by Jain *et al.* for same dye Reactive Violet 5 using bacterial consortium SB4.

**Figure 5**

*Effect of initial dye concentration on decolorization performance of Pseudomonas aeruginosa GSM3.*

Our results on optimization studies revealed that *P. aeruginosa* GSM3 is a mesophilic (optimum of 37°C), facultative anaerobe (static incubation) and also decolorized Reactive Violet 5 more efficiently at neutral pH 7. These optimum parameters were maintained throughout this study on decolorization of Reactive Violet 5 using an isolate *P. aeruginosa* GSM3. Jain *et al.* reported that complete decolorization of dye Reactive Violet 5 at 37°C and pH 7 under static condition, in case of bacterial consortium SB4. Similar types of cultural conditions were adopted for the degradation of azo dyes using mesophilic bacterial strains such as *Pseudomonas* sp. SUK1, *Pseudomonas aeruginosa* NGKCTS and *Pseudomonas aeruginosa* BCH. The reason for decreased decolorization under shaking condition was due to most of the azo dye degrading bacteria required reduced oxygen tension may be the mechanism of microbial degradation of azo dyes involves the reductive cleavage of azo bonds (−N=N−) with the help of azoreductase, while the presence of oxygen usually inhibits the azo bond reduction activity since aerobic respiration may dominate utilization of NADH; thus impeding the electron transfer from NADH to azo bonds.
Decolorization of repeated addition of dye aliquots
The ability of *P. aeruginosa* GSM3 to decolorize repeated additions of Reactive Violet 5 (100 mgL$^{-1}$ each time) up to ten cycles was tested. There was 100% decolorization up to 5$^{th}$ aliquots with decreasing time taken for respective percent decolorization from 16 h to 10 h respectively. From the 6$^{th}$ cycle onwards percent decolorization is also reduce gradually from 95% to 58% (10$^{th}$ cycle). Similarly time taken for respective percent decolorization was increases from 14 h for 7$^{th}$ cycle to 42 h for 10$^{th}$ cycle (Figure 6). Repeated use of microbial cells for decolorization has been reported by other Researchers$^{12,13}$. Our observations and earlier reports made by others, the decrease in the efficiency might be due to nutrient depletion, decrease in viable cells and inhibition of enzyme systems gradually$^{11}$. Thus, *P. aeruginosa* GSM3 shows the ability to decolorize repeated addition of dye aliquots, which is significant for its commercial application.

Effect of salt concentration on decolorization of Reactive Violet 5
Effluents from the both dye manufacturing and dye consuming industries generally contains chloride salts of sodium and potassium which are most widely used for salting out of dyes and discharged along with unused dyes. Hence, the present investigation was undertaken to study the effect of salt concentration (1–6%) on decolorization of Reactive Violet 5 by the *P. aeruginosa* strain GSM3 was carried out. Isolate showed significant decolorization in the presence of all salt concentrations selected. At 1%, 2%, 3%, 4% of salt concentration, the percentage of decolorization of Reactive Violet 5 was of 100%, 100%, 98% and 92% respectively after 16 h of incubation. Further, increase in salt results in decreased percentage of decolorization. Furthermore, it was stated that sodium concentration higher than 3 g/L can cause inhibition of most the bacterial metabolism$^{31}$. But on contradictory, in the presence of 5% and 6% salt drops dye decolorization to 81% and 67% respectively. Inhibition to microorganisms by high salt concentration may cause plasmolysis or loss of activity of cells$^{32}$. Hence, *P. aeruginosa* GSM3 has great potential application in the bioremediation of azo dyes at higher salt concentration.
Decolorization mode of *Pseudomonas aeruginosa* GSM3 against Reactive Violet 5

Spectrophotometric analysis (400–800 nm) of supernatants at different intervals of incubation time showed visible decolorization and decrease in the dye concentration from batch culture. Reduction in the optical density of decolorized media observed at 558 nm as compared to the no change in the peak of the control medium through the period (16 h) of incubation (Figure 8). The significant changes in the visible spectra showed that the molecular structure of Reactive Violet 5 changed evidently after decolorization.

The present results indicate that the color removal by *P. aeruginosa* GSM3 may be due to biodegradation not by adsorption and it was confirmed by colorless cell pellet obtained upon centrifugation. Further colorless cell pellets were dissolved in methanol and analyzed by UV-visible spectroscopy. There is no absorbance at the 558 nm of methanol dissolved cell pellets indicates that decolorization was due to biodegradation and also we are not providing carbon source in media so that our organism can able to utilize dye as a sole source of carbon. These results provided the obvious evidence of
biodegradation of Reactive Violet 5 by an isolate *P. aeruginosa* GSM3 and also supported the earlier conclusion that decolorization by bacteria is mainly due to biodegradation, rather than inactive surface adsorption\textsuperscript{25}.

**CONCLUSION**

An isolate *P. aeruginosa* GSM3 has potential to degrade Reactive Violet 5 as a sole source of carbon under static condition. This isolate also showed decolorization of Reactive Violet 5 under continuous addition of dye fractions in ongoing decolorization experiments and also withstand the higher salt concentration up to 4% without change in efficiency of percent degradation of dye. Therefore, this isolate may prove to be a candidate organism for the treatment of textile industrial effluents containing reactive dyes especially Reactive Violet 5. However the complete degradation pathway of Reactive Violet 5 by this bacterium is in progress.

**CONFLICT OF INTREST**

Conflict of interest declared none.

**ACKNOWLEDGEMENT**

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ISOLATION AND CHARACTERIZATION OF AN AZO DYE REACTIVE RED 2 DEGRADING BACTERIA FROM DYE CONTAMINATED SOIL

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ABSTRACT

A bacterial strain GSM3, capable of decolorizing an azo dye Reactive Red 2 was isolated from dye contaminated soil sample collected from Ammu dyeing industry, Bellary, India. Based on phenotypic characteristics and 16S rDNA sequencing, strain GSM3 was identified as *Pseudomonas aeruginosa* GSM3. Isolate GSM3 showed 97% decolorization of Reactive Red 2 (300 mgL⁻¹) within 10 h, while maximally it could decolorize 2 gL⁻¹ of dye within 62 h with 75% decolorization in carbon free mineral salts medium supplemented with 0.1% (w/v) of yeast extract as a cosubstrate under static condition. The isolate has shown good decolorization activity over a wide range of pH from 5.0 to 9.0 and temperature 30°C to 45°C respectively. However, highest decolorization was observed at 37°C and pH 7.0. UV-vis analyses and colorless bacterial cells suggested that the isolate GSM3 exhibited decolorizing activity through biodegradation, rather than inactive surface adsorption. Thus, the isolate GSM3 had great potential to be applied in biodegradation of dye effluents treatment.

KEYWORDS: Reactive Red 2, Azo dye, Decolorization, Degradation and *Pseudomonas aeruginosa* GSM3
INTRODUCTION

In 2000, it was estimated that $5 \times 10^{10}$ kg of fibres were produced worldwide, which consumed more than $8 \times 10^8$ kg of different dyes and pigments. In 2005, the global market size for dyes, pigments and intermediaries was US$ 23 billion. In terms of total volume, the global dyestuff production was $3.4 \times 10^{10}$ kg, which accounted for annual global sales of nearly US$ 6 billion. Pollution problems due to textile industry effluents have increased in recent years. From the available literature it can be estimated that approximately 75% of the dyes discharged by textile processing industries belong to the classes of reactive (36%), acid (~25%) and direct (~15%) dyes. In these classes, azo dyes are the diverse group of synthetic organic compounds accounts for the majority of all textile dyestuffs produced and are the most extensively used in a number of industries such as textile dyeing, paper, food, leather, cosmetics and pharmaceutical industries. The effluents from these industries are complex, containing a wide variety of dyes and other products, such as dispersants, acids, bases, salts, detergents, humectants, oxidants, etc. Discharge of these colored effluents into rivers and lakes results into reduced dissolved oxygen concentration, thus creating anoxic conditions that are lethal to resident organisms. Many reports indicate that textile dyes and effluents have toxic effect on the germination rates and biomass of several plant species, whereas plants play many important ecological function such as providing the habitat for a wildlife, protecting soil from erosion, and providing bulk of organic matter that is so significant to soil fertility. In addition, azo dyes also have an adverse impact in terms of total organic carbon (TOC), biological oxygen demand (BOD) and chemical oxygen demand (COD). Many synthetic azo dyes and their metabolites are toxic, carcinogenic and mutagenic. Therefore, the treatment of industrial effluents containing aromatic compounds becomes necessary prior to their final discharge to the environment. Existing physical/chemical technologies for color removal are very expensive and commercially unattractive. Biological processes provide an alternative to existing technologies because they are more cost-effective, environmental friendly and do not produce large quantities of sludge. Many microorganisms belonging to the different taxonomic groups of bacteria, fungi, actinomycetes and algae have been reported for their ability to decolorize and degrade reactive azo dyes. The key features like faster growth rate, facultative nature and high adaptability are the desirable qualities of bacterial community for the bioremediation when compared fungal cultures. The isolated Pseudomonas species are ecofriendly, since they have been studied for biological control. However, comprehensive solutions for reactive azo dye removal are far from reality, which calls for continued search for new organisms and technologies. In the present investigation, we have reported the isolation and identification of potential bacterial strain from dye contaminated soil capable of degrading Reactive Red 2. The various abiotic parameters such as initial dye concentration, temperature and pH have been optimized to achieve maximum dye decolorization.

MATERIALS AND METHODS

Dyes and chemicals
Azo dye Reactive Red 2 was gifted from Colors India Inc. Pvt. Ltd. Ahmedabad, India (Figure 1). Other chemicals and media components used in the study were of analytical grade and were obtained from Hi-Media, Mumbai India.
Culture medium
The mineral salts medium (MSM)\(^{17}\), containing (g/L): \(\text{Na}_2\text{HPO}_4\cdot2\text{H}_2\text{O}\) (12.0), \(\text{KH}_2\text{PO}_4\) (2.0), \((\text{NH}_4)\text{2NO}_3\) (0.50), \(\text{MgCl}_2\cdot6\text{H}_2\text{O}\) (0.10), \(\text{Ca(NO}_3)_2\cdot4\text{H}_2\text{O}\) (0.050), \(\text{FeCl}_2\cdot4\text{H}_2\text{O}\) (0.0075) containing 10 mL (v/v\(^{-1}\)) of trace element solution and 0.1% (w/v\(^{-1}\)) of yeast extract was used. This was blended with different concentrations of Reactive Red 2 was used throughout the study. The trace element solution containing (mg/L): \(\text{ZnSO}_4\cdot7\text{H}_2\text{O}\) (10.0), \(\text{MnCl}_2\cdot4\text{H}_2\text{O}\) (3.0), \(\text{CoCl}_2\cdot2\text{H}_2\text{O}\) (1.0), \(\text{NiCl}_2\cdot6\text{H}_2\text{O}\) (2.0), \(\text{Na}_2\text{MoO}_4\cdot2\text{H}_2\text{O}\) (3.0), \(\text{H}_3\text{BO}_3\) (30.0), \(\text{CuCl}_2\cdot2\text{H}_2\text{O}\) (1.0); pH 7.0. When required, 1.9% (w/v\(^{-1}\)) of agar was added into the media to solidify. All the media were sterilized at 121°C for 20 min before use.

Screening, isolation and identification of dye decolorizing bacterial strain
Dye contaminated soil sample collected from Ammu dyeing industry, Bellary, India was brought to lab. Ten gram of soil weighed out from the soil sample and agitated in 50 mL of normal saline at 180 rpm for 30 min, then allowed the soil to settle down for 30 min. For initial screening 10 mL of supernatant was transferred to 100 mL MSM broth containing 100 mg/L of Reactive Red 2 as a sole source of carbon incubated at 37°C for 15 days. The flask was looked for the change in the initial color and turbidity of the MSM broth. Then 10 mL of culture broth from the decolorized cultured flask transfer to 100 mL of fresh MSM broth containing 100 mg/L of dye and incubated for one week under static conditions. From the decolorized culture 0.5 mL was taken out and inoculated onto the agar plates of MSM containing Reactive Red 2 (100 mg/L) and incubated the plate at 37°C until prominent dye degrading bacterial colonies appeared. A total of five morphologically distinct colonies were observed and were streaked separately, once again on the MSM agar plate containing dye. From the isolated colonies pure cultures were prepared. A total of five cultures were preserved at −20 in (15% w/v\(^{-1}\)) glycerol for further use. All the five pure cultural isolates were tested for their capacity to degrade Reactive Red 2 (300 mg/L) in 100 mL of MSM medium as a sole source of carbon and incubated up to one week. Further experiments were carried out with 250 mL Erlenmeyer flasks containing 100 mL MSM supplemented with Reactive Red 2 (300 mg/L) and (0.1% w/v\(^{-1}\)) yeast extract as a cosubstrate to enhance the bacterial growth rate and decolorization efficiency.

16S rDNA sequencing
The 16S rDNA fragment was amplified and from the pure genomic DNA of \textit{Pseudomonas aeruginosa} GSM3 sequenced at Royal Life Sciences Pvt. Ltd., Hyderabad, India. Taxonomic analysis was conducted with the BLAST program on the NCBI website (http://www.ncbi.nlm.nih.gov) and alignment of the sequences was analyzed by using CLUSTALW program V1.82 at European
bioinformatics site (www.ebi.ac.uk/clustalw). After removing ambiguities and crosschecked refined sequences was submitted to the GenBank, accession number is JF510526. To see the phylogenetic position of the bacterial isolate phylogenetic tree was constructed by employing neighbor–joining method\textsuperscript{18}, using Kimura–2–parameter distances in MEGA 5 software\textsuperscript{19}.

**Decolorization studies of Reactive Red 2 in liquid medium**

The dye decolorization experiments were performed in 250 ml Erlenmeyer flasks containing 100 ml of sterilized MSM broth with yeast extract (0.1% w/v) and 300 mgL\textsuperscript{-1} Reactive Red 2. They were inoculated with 5 ml of culture broth in test and without inoculum acts as control. The flasks were incubated at 37°C under static as well as shaking (120 rpm) conditions till the decolorization was completed. The 5 ml of cultures were withdrawn at different time intervals (2 h) from both flasks and supernatant was collected by centrifuging at 10,000 rpm for 15 min. The supernatants were used to check the optical density (OD) at 538 nm using UV–Vis spectroscopy (Systronics AU-2700). All experiments were run in triplicates and average value was calculated.

**Optimization of abiotic parameters**

Effects of abiotic parameters on the decolorization study were conducted using initial pH values ranges from 4 to 10 keeping temperature constant at 37°C. Similarly, calculate the optimum temperature for maximum dye decolorization using varied temperatures ranging from 20 to 50°C with 5°C intervals keeping optimum pH 7.0 and incubating under static conditions. By the optimization study, optimum pH (7.0) and temperature (37°C) were selected for further study such as initial dye concentrations (300–2000 mgL\textsuperscript{-1}) on dye decolorization were investigated.

**Decolorization assay**

Decolorization of dyes by *Pseudomonas aeruginosa* GSM3 was determined by measuring absorbance of culture supernatants at 538 nm using UV-visible spectroscopy. Percent decolorization was calculated as mentioned by Dave and Dave\textsuperscript{20}.

\[
\text{Decolorization} (\%) = \frac{I - F}{I} \times 100
\]

Where I = Initial absorbance and F = Absorbance of decolorized sample.

**Decolorization manner of Pseudomonas aeruginosa GSM3 on Reactive Red 2**

Decolorization of dyes may take place by adsorption\textsuperscript{21} or Degradation\textsuperscript{22}. In the case of adsorption, dyes are only adsorbed onto the surface of bacterial cells, whereas new compounds come into being when dyes are degraded by bacterial enzymes during the degradation process. In adsorption, examination of the absorption spectrum will reveal that all peaks decrease approximately in proportion to each other. If the dye removal is attributed to biodegradation, either the major visible light absorbance peak will completely disappear or new peaks will appear\textsuperscript{23}. Dye adsorption can be also easily judged by an evidently colored cell pellet, whereas those retaining their original colors are accompanied by the occurrence of biodegradation\textsuperscript{24}. A bacterial cell suspension was withdrawn at different intervals (2 h) of time from both flasks and supernatant was collected by centrifuging at 10,000 rpm for 15 min. The supernatants were used to check the optical density (OD) at 538 nm using UV–Vis spectroscopy. After centrifugation, cell pellet was dissolved in methanol to confirm the decolorization due to biodegradation or by surface adsorption.
RESULTS AND DISCUSSIONS

Isolation and identification of dye decolorizing bacteria

A total of five morphologically distinct kinds of colonies were observed on the MSM agar plates containing 300 mgL⁻¹ Reactive Red 2 preinoculated with dye degraded culture. Among all the isolates, one isolate (GSM3) showed rapid decolorization (97%) of Reactive Red 2 within 10 h and remaining isolates were showed decreased percent decolorization (up to 40 to 84%) even after 3 days of incubation. The organism showing highest decolorization was selected and identified by various conventional identification methods. The isolated bacterium was gram negative, motile and the results biochemical tests and sugar utilization tests were given in the Table 1. The isolated organism identified as Pseudomonas aeruginosa. The identity of this isolate was confirmed by 16S rDNA analysis of 1460 base pairs and it was identified as Pseudomonas aeruginosa strain GSM3. The sequence was deposited in Gene Bank with accession number JF510526. Phylogenetic analysis of Pseudomonas aeruginosa strain GSM3 using MEGA5 software can be seen from Figure 2. The nearest homology genus-species was found to be Pseudomonas aeruginosa strain NF-1.

![Phylogenetic tree of the isolate GSM3 and related organisms were aligned based on 16S rDNA sequences (neighbor–joining tree). Scale bar: number of nucleotide changes per sequence position. The number at nodes shows the bootstrap values obtained with 1000 resampling analyses. Paracoccus sp. YM3 has been taken as out group. Numbers in bracket represent GenBank accession numbers.](image)

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Table 1

Morphological, biochemical and physiological characterization of Pseudomonas aeruginosa strain GSM3.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Results</th>
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<tbody>
<tr>
<td>Gram’s staining test</td>
<td>Gram negative</td>
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<tr>
<td>Shape</td>
<td>Rods</td>
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<tr>
<td>Motility</td>
<td>Motile</td>
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<tr>
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<td>+</td>
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<td>Oxidase</td>
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<td>Urease</td>
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<td>Citrate utilization</td>
<td>+</td>
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<td>Mannose</td>
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<td>Trehalose</td>
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<td>Glucose</td>
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<td>Lactose</td>
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<td>Mannitol</td>
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<td>Sorbitol</td>
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<td>Dulcitol</td>
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<td>Inositol</td>
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<td>Arabitol</td>
<td>+</td>
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<td>Erythritol</td>
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<tr>
<td>α-Methyl-D-glucoside</td>
<td>+</td>
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<tr>
<td>Rhamnose</td>
<td>–</td>
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<td>Cellubiose</td>
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<td>α-Methyl-D-mannoside</td>
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<tr>
<td>Malonate utilization</td>
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<tr>
<td>Sorbose</td>
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(+) - Positive, (–) – Negative.

Acclimatization studies

To enhance the bacterial growth rate and decolorization efficiency of the isolate GSM3, we added 0.1% (w/v) yeast extract to MSM broth as a co-substrate. We observed that 97% decolorization of 300 mg L⁻¹ Reactive Red 2 in MSM medium within 10 h as compared to 28 h without yeast extract under static condition. Similar observations have been reported by Khalid et al.²⁵ However, they used 0.4% of yeast extract as a co-substrate for the growth of azo dye decolorizing organisms. Earlier Sponza Isik²⁶ showed that yeast extract could be used as growth supplement for azo dye degrading bacteria. In our study 0.1% yeast extract enhances the dye degradation efficiency and reduced the time period to one third. So, in this study further decolorization experiments was performed using MSM broth supplemented with 0.1% (w/v) yeast extract as a cosubstrate.

Optimization of abiotic parameters

We optimized the pH, temperature and static/shaking conditions for maximum decolorization of Reactive Red 2 by the isolate GSM3. Figure 3 shows that 97% decolorization of added Reactive Red 2 within 10 h under static condition compared to only 16% decolorization in the culture flask incubated in the shaking condition. Hence, in this study static conditions were maintained to investigate bacterial decolorization.
Effect of static and shaking conditions on decolorization of Reactive Red 2

In the temperature optimization study, the dye decolorization activity of *Pseudomonas aeruginosa* GSM3 was found to increase with increase in incubation temperature from 20 to 37°C (Figure 4). Further increase in temperature to 40°C, decolorization was decreased by 1% and 14% decreased at 45°C. Decolorization activity was significantly suppressed at 50°C. The decrease in dye decolorization at high temperature can be attributed to the decline in microbial activity that led to the inactivation of the enzyme and eventually the no decolorization of dye.

Effect of temperature on decolorization of Reactive Red 2

Similarly, the optimization of pH for maximum decolorization of Reactive Red 2 by a bacterial strain was determined over wide range of pH 4.0 to 10.0 with an interval of pH 1. The isolate showed maximum of 97% decolorization at neutral pH 7.0 at 37°C (Figure 5).
either side of neutral pH, the percentage of
decolorization was decreased steadily from 94% to 10% on alkaline side from pH 8 to 10 however, steep decline in percent decolorization from 95 to 20% on acidic side at pH 6 and 4 respectively. More than 84% of decolorization was observed in the pH range of 6 to 8. pH has a major effect on the efficiency of dye decolorization, and the optimal pH for color removal is often between 6.0 and 10.0\textsuperscript{28}. Jadhav et al\textsuperscript{29} also found that pH 7.0 was optimum for decolorization of Direct Orange 39 by \textit{Pseudomonas aeruginosa} BCH. The isolated strain capable of decolorizing Reactive Red 2in a wide range of pH, a desirable characteristic, i.e., in contrast with common decolorizing bacteria that have a narrow pH range\textsuperscript{30,10}.

Figure 5

\textit{Effect of pH on decolorization of Reactive Red 2}

Our results on optimization studies revealed that \textit{Pseudomonas aeruginosa} GSM3 is a mesophilic (optimum of 37°C), facultative anaerobe and also decolorized Reactive Red 2 more efficiently at neutral pH 7. These optimum parameters were maintained throughout this study on decolorization of Reactive Red 2. Kalyani et al\textsuperscript{10} reported that maximum decolorization of same dye Reactive Red 2 of about 96% was observed under static condition, in case of bacterial strain \textit{Pseudomonas} sp. SUK1. Similar types of cultural conditions were adopted for the degradation of azo dyes using mesophilic bacterial strains such as \textit{Pseudomonas} sp. SUK1, \textit{P. aeruginosa} NGKCTS, \textit{P. aeruginosa} BCH, and \textit{Pseudomonas} sp. LBC1\textsuperscript{10,11,29,31}. The reason for decreased decolorization under shaking condition was due to most of the azo dye degrading bacteria required reduced oxygen tension may be the mechanism of microbial degradation of azo dyes involves the reductive cleavage of azo bonds (–N=N–) with the help of azoreductase, while the presence of oxygen usually inhibits the azo bond reduction activity since aerobic respiration may dominate utilization of NADH; thus impeding the electron transfer from NADH to azo bonds\textsuperscript{10}.

\textbf{Effect of initial dye concentration on decolorization of Reactive Red 2}

The decolorization efficiency of Reactive Red 2 by \textit{Pseudomonas aeruginosa} GSM3 was studied by increasing initial dye concentration (300–2000 mg L\textsuperscript{-1}). We observed that the percentage of decolorization was decreased slowly with increasing dye concentration above 300 mg L\textsuperscript{-1} (Figure 6). It could effectively decolorize up to 300 mg L\textsuperscript{-1} Reactive Red 2 (97%) within 10 h
and is decreased to 75%, when dye concentration increased to 2000 mg L\(^{-1}\) and decolorization time increases from 10 to 62 h respectively. The decrease in percentage of decolorization and enhanced time period at high dye concentration may be attributed to the inhibitory effects of high dye concentration and azo dyes usually contain one or more sulfonic acid groups on aromatic rings, which act as detergents to inhibit the growth of microorganisms\(^{10}\).

![Figure 6](image)

**Figure 6**

*Effect of initial dye concentration on decolorization of Reactive Red 2.*

**Decolorization manner of Pseudomonas aeruginosa GSM3 against Reactive Red 2**

Spectrophotometric analysis (400–800 nm) of supernatants at different intervals of incubation period showed visible decolorization and decrease in the dye concentration from batch culture. Reduction in the optical density of decolorized media observed at 538 nm as compared to the no change in the peak of the control medium through the period (10 h) of decolorization (Figure 7). According to Asad et al\(^{15}\) decolorization of dyes by bacteria could be due to adsorption by microbial cells or to biodegradation. In the case of adsorption, the UV–vis absorption peaks decrease approximately in proportion to each other, whereas in biodegradation, either the major visible light absorbance peak disappears completely, or a new peak appears. Dye adsorption can be also easily judged by an evidently colored cell pellet, whereas those retaining their original colors are accompanied by the occurrence of biodegradation\(^{24}\). The present results indicate that the color removal by GSM3 may be due to biodegradation not by adsorption and it was confirmed by colorless cell pellet obtained upon centrifugation. Further colorless cell pellets were dissolved in methanol and analyzed by UV-visible spectroscopy. There is no absorbance at the 538 nm of methanol dissolved cell pellets indicates that decolorization was due to biodegradation.
CONCLUSION

In the present study, a potential Reactive Red 2 decolorizer, *Pseudomonas aeruginosa* GSM3, was isolated from dye contaminated soil. The isolate GSM3 had the significant ability to decolorize high concentration of Reactive Red 2 (2 g/L) under static conditions which will reduce the operational cost. Optimization studies revealed that decolorization occurred over a range of pH, temperature and initial dye concentrations with minimal nutritional requirements. Overall findings suggested that, *Pseudomonas aeruginosa* GSM3 may be employed for the ecofriendly degradation textile waste water treatment. However, the complete degradation of Reactive Red 2 is in progress from this isolate.

ACKNOWLEDGEMENT

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REFERENCES


25. Khalid A, Arshad M and Crowley DE, Accelerated decolorization of structurally different azo dyes by newly isolated


Research Article

Isolation and Characterization of Paracoccus sp. GSM2 Capable of Degrading Textile Azo Dye Reactive Violet 5

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A potential bacterial strain GSM2, capable of degrading an azo dye Reactive Violet 5 as a sole source of carbon, was isolated from textile mill effluent from Solapur, India. The 16S rDNA sequence and phenotypic characteristics indicated an isolated organism as Paracoccus sp. GSM2. This strain exhibited complete decolorization of Reactive Violet 5 (100 mg/L) within 16 h, while maximally it could decolorize 800 mg/L of dye within 38 h with 73% decolorization under static condition. For color removal, the most suitable pH and temperature were pH 6.0–9.0 and 25–40°C, respectively. The isolate was able to decolorize more than 70% of five structurally different azo dyes within 38 h. The isolate is salt tolerant as it can bring out more than 90% decolorization up to a salt concentration of 2% (w/v). UV-Visible absorption spectra before and after decolorization suggested that decolorization was due to biodegradation and was further confirmed by FT-IR spectroscopy. Overall results indicate the effectiveness of the strain GSM2 explored for the treatment of textile industry effluents containing various azo dyes. To our knowledge, this could be the first report on biodegradation of Reactive Violet 5 by Paracoccus sp. GSM2.

1. Introduction

In 1856, the world’s first commercially successful synthetic dye, mauveine, was discovered for practical uses. Over 10,000 different dyes with an annual production of over $7 \times 10^5$ metric tons worldwide are commercially available [1, 2]. Azo dyes are the diverse group of synthetic organic compounds accounting for the majority of all textile dyestuffs produced and are the most extensively used in a number of industries such as textile dyeing, paper, food, leather, cosmetics, and pharmaceutical industries [3]. The amount of dye lost depends upon the class of dye application, varying from 2% loss while using basic dyes to 50% loss in certain reactive sulfonated dyes, leading to severe contamination of surface and ground waters in the vicinity of dyeing industries [4]. In India, an average mill discharges about 1.5 million liters of contaminated effluent per day, which leads to chronic and acute toxicity [5]. Improper textile dye effluent disposal in aqueous ecosystems leads to the reduction in sunlight penetration which in turn decreases photosynthetic activity, dissolved oxygen concentration, and water quality and depicts acute toxic effects on aquatic flora and fauna, causing severe environmental problems worldwide [6]. They can also cause human health disorders such as nausea, hemorrhage, ulceration of the skin and mucous membranes, and severe damage to kidneys, the reproductive system, liver, brain, and central nervous system [7]. In addition, azo dyes also have an adverse impact in terms of total organic carbon (TOC), biological oxygen demand (BOD), and chemical oxygen demand (COD) [8]. Many synthetic azo dyes and their metabolites are toxic, carcinogenic, and mutagenic [9]. Therefore, the treatment of industrial effluents containing azo dyes and their metabolites is necessary prior to their final discharge to the environment.

Various physicochemical methods like adsorption, chemical precipitation and flocculation, photolysis, chemical oxidation and reduction, electrochemical treatment, and ion pair extraction have been used for the removal of dyes from
wastewater [10]. The major drawbacks of these methods have been largely due to the high cost, low efficiency, limited versatility, interference by other wastewater constituents, and the handling of the waste generated [11]. Conversely, biological processes provide an alternative to existing technologies because they are more cost-effective, environmentally friendly and do not produce large quantities of sludge. Many microorganisms belonging to different taxonomic groups of bacteria, fungi, actinomycetes, and algae have been reported for their ability to decolorize azo dyes [12]. Pure fungal cultures have been used to develop bioprocesses for the mineralization of azo dyes, but the long growth cycle and moderate decolorization rate limit the performance of fungal decolorization system [13]. In contrast, bacterial decolorization is normally faster. Bacteria capable of dye decolorization/biodegradation either in pure cultures or in consortia have been reported [11, 14–17]. However, comprehensive solutions for sulfonated azo dyes removal are far from reality, which calls for continued search for new organisms and technologies.

This study aimed to isolate and characterize an efficient bacterial strain, which exhibited the remarkable ability to degrade Reactive Violet 5 as a sole source of carbon. Various physicochemical parameters have been optimized for efficient dye decolorization. The dye degraded products were characterized by ultraviolet-visible (UV-Vis) and Fourier transformed infrared spectroscopy (FT-IR) techniques. Very few reports are available on Reactive Violet 5 degradation. The obtained colonies formed were screened out and further were checked for the purity by streaking twice on agar medium. Finally, purified cultures were individually tested for their dye degrading capabilities in MSM under static condition. The potential isolate was preserved at ∼20°C in 15% (w/v) glycerol and used for further investigation. The potential isolate was selected and preliminarily characterized based on its morphological and biochemical properties [19]. Furthermore, various sugar utilization tests were performed using HiCarbo kit (Hi-Media, India).

2. Materials and Methods

2.1. Dyes and Chemicals. Six textile azo dyes Reactive Violet 5, Reactive Red 2, Reactive Orange 16, Reactive Blue 4, Reactive Black 5, and Reactive Green 19 A were generous gifts from Colors India Inc. Pvt. Ltd., Ahmedabad, India. All these dyes were of industrial grade and are widely used in textile industries. Reactive Violet 5 was used as a model azo dye in this study (Figure 1). All required chemicals were obtained from S.D. Fine chemicals (India) and Sigma-Aldrich, (USA). Biochemical and physiological test kits were obtained from Hi-Media, India. All chemicals used during the study were of analytical grade.

2.2. Culture Medium. The mineral salts medium (MSM) was prepared as per Brilon et al. [18] with some modifications. The MSM consisted of the following constituents (g/L): Na₂HPO₄·2H₂O (12.0), KH₂PO₄ (2.0), NH₄NO₃ (0.50), MgCl₂·6H₂O (0.10), Ca(NO₃)₂·4H₂O (0.050), and FeCl₃·4H₂O (0.0075) with 10 mL of trace element solution per liter. The trace element solution was prepared as follows (mg/L): ZnSO₄·7H₂O (10.0), MnCl₂·4H₂O (3.0), CoCl₂·6H₂O (1.0), NiCl₂·6H₂O (2.0), Na₂MoO₄·2H₂O (3.0), H₃BO₃ (30.0), and CuCl₂·2H₂O (1.0). Further, MSM was blended with different concentrations of Reactive Violet 5 and used throughout the study as a test medium and uninoculated flasks were also incubated as control. The final pH of the medium was adjusted to 7.0 ± 0.2. The MSM with agar (1.9% w/v) was used for isolation and maintenance of pure culture. The media were sterilized at 121°C for 20 min before use.

2.3. Screening, Isolation, and Identification of Dye Decolorizing Bacteria. Textile mill effluent collected from Solapur, India, was brought to the laboratory for isolation of dye degrading bacteria. 10 mL of sample was added to 100 mL of MSM broth containing Reactive Violet 5 (100 mg/L) as a sole source of carbon and incubated at 30°C for 15 days under static as well as shaking conditions. The flasks were checked for change in initial color and turbidity. Then 10 mL of culture broth from the decolorized culture flask was transferred to 100 mL of fresh MSM broth containing Reactive Violet 5 and incubated for one week under static condition. 0.5 mL of decolorized culture was taken out and spread over the agar plates of MSM containing dye and incubated at 30°C until prominent dye degrading bacterial colonies appeared. Further the prominent colonies were streaked onto the MSM agar plates amended with dye and 0.1% (w/v) yeast extract. The obtained colonies formed were screened out and further were checked for the purity by streaking twice on agar medium. Finally, purified cultures were individually tested for their dye degrading capabilities in MSM under static condition. The potential isolate was preserved at ∼20°C in 15% (w/v) glycerol and used for further investigation. The potential isolate was selected and preliminarily characterized based on its morphological and biochemical properties [19]. Furthermore, various sugar utilization tests were performed using HiCarbo kit (Hi-Media, India).

2.3.1. 16S rDNA Sequencing and Analysis. The 16S rDNA fragment was amplified and from the pure genomic DNA of isolated bacterial strain was sequenced at Royal Life Sciences Pvt. Ltd., Hyderabad, India. The genomic DNA was extracted using QIAgen bacteria DNA purification kit according to manufacturer's instructions. The universal primers, namely, a forward primer, Eub27F (5'-AGAGTTTGATCCTGG-3'), and a reverse primer, Eub1492R (5'-ACGGCTACCCTGTATCCTG-3'), were used to amplify bacterial 16S rDNAs by PCR which yielded a product of approximately 1500 bp. After an initial denaturation at 95°C for 10 min, the DNA was amplified during 25 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min and the final extension (72°C) time was 10 min. Then the purified PCR products were run on ABI-PRISM automated sequencer (ABI-3730 DNA Analyzer). A resultant of 1311 bases was compared with nine closely related taxa of the isolate, retrieved from the GenBank database using BLAST (blastn) program on the NCBI website (http://www.ncbi.nlm.nih.gov). The alignment of the sequences was done using CLUSTALW program V 1.6 at European bioinformatics site (http://www.ebi.ac.uk/Tools/msa/). The sequence was refined manually after crosschecking with the raw data to remove ambiguities and submitted to GenBank. To see the phylogenetic position of bacterial isolate evolutionary history was inferred using the neighbor-joining method [20]. The optimal tree with the sum of branch length =
0.28801765 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches [21]. The phylogenetic tree was linearized assuming equal evolutionary rates in all lineages [22]. The clock calibration to convert distance to time was 0.02 (time/node height). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method [23] and are in the units of the number of base substitutions per site. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5 software [24].

2.4. Decolorization Experiment. The dye decolorization experiments were performed in 250 mL Erlenmeyer flasks containing 100 mL of sterilized MSM broth supplemented with yeast extract (0.1% w/v) and Reactive Violet 5 (100 mg/L). We recorded complete decolorization of Reactive Violet 5 in MSM with yeast extract within 16 h as compared to 56 h without yeast extract under the static condition (data not shown). Reports suggest that the inclusion of yeast extract was found to be most effective supplement for growth of azo dye degrading bacteria as well as increasing the dye decolorization efficiency [13, 25]. Therefore all further decolorization experiments were performed using MSM broth supplemented with 0.1% (w/v) yeast extract as a cosubstrate. The flask were incubated at 30°C under static as well as shaking (120 rpm) conditions till the decolorization was completed. The 5 mL of cultures was withdrawn at different intervals for color measurement. The supernatant was collected by centrifuging at 10,000 rpm for 15 min. Decolorization was monitored spectrophotometrically by measuring absorbance of culture supernatant at 558 nm. Growth of bacteria in dye containing medium was determined spectrophotometrically. The cell pellet obtained upon centrifugation of 5 mL culture was resuspended in 5 mL distilled water and its absorbance was studied at 660 nm. The percentage of decolorization was calculated as mentioned by R. Dave and H. Dave [26]:

\[
\text{Decolorization (\%) } = \frac{I - F}{I} \times 100,
\]

where \( I \) = initial absorbance and \( F \) = absorbance of decolorized sample.

2.5. Optimization of Physicochemical Parameters. The decolorization efficiency of Paracoccus sp. GSM2 on Reactive Violet 5 was studied at different pH (4–10) and temperatures values (20–50°C). The obtained optimum pH 7.0 and temperature at 30°C were selected to study the decolorization activity under various physicochemical factors such as initial dye concentration (100–800 mg/L), salt concentration (1–6%), and yeast extract concentration (0.1–2.0 g/L). Further, the decolorization of various azo dyes was studied by incubating MSM containing respective dye with bacterial strain GSM2 under static condition.

2.6. Decolorization and Biodegradation Studies. The Reactive Violet 5 degraded products formed during biodegradation after 16 h of incubation under static condition were studied by following the change in the UV-Vis spectra (200 to 800 nm) using a UV-Vis spectrophotometer (Systronics, AU-2700). To know that the decolorization was due to biodegradation of Reactive Violet 5 was confirmed by FT-IR by analyzing dye degraded products in the decolorized medium. After complete decolorization, culture medium was centrifuged at 10,000 rpm for 15 min to remove the suspended particles. The supernatant was once again centrifuged to ensure the supernatant was free of bacterial cells and was used for extraction of metabolites using an equal volume of ethyl acetate. The extracts were dried over anhydrous Na₂SO₄ and
concentrated in a rotary evaporator. The crystals obtained were dissolved in a small volume of high performance liquid chromatography (HPLC) grade methanol and the same sample was used for FT-IR analysis. The FT-IR analysis of extracted metabolites was done using Fischer Scientific (Nicolet, iH5) Spectrophotometer and compared with control dye in the IR region of 550–4000 cm\(^{-1}\) with 32 scan speed.

3. Results and Discussion

3.1. Isolation, Screening, and Identification. A total of seven morphologically distinct colonies were observed on the MSM agar plates (data not shown). Amongst positive strains subjected to screening, the potential bacterial strain GSM2 showed a rapid and complete decolorization of Reactive Violet 5 within 16 h under static condition and was selected for further study. The selected strain was gram negative, nonspore former, and nonmotile coccoid. Its colony was white to light yellow, round hunch, and slick. The potential strain was identified as *Paracoccus* sp. GSM2 on the basis of 16S rDNA gene sequence and biochemical characteristics (Table 1). The 16S rDNA sequence (1311 base pairs) was deposited in GenBank with accession number JF510527. Its 16S rDNA sequence did not show any similarity to known azo dye degrader and had the greatest similarity to members of *Paracoccus* sp. group. The phylogenetic relationship between the *Paracoccus* sp. GSM2 and other related microorganisms using MEGA5 software can be depicted from Figure 2. *Pseudomonas aeruginosa* CGR has been taken as out-group and the numbers shown in parentheses are accession numbers of different species.

The homology assay result indicated that the *Paracoccus* sp. GSM2 in phylogenetic branch showed maximum similarity (99%) to *Paracoccus* sp. YM3 which is already known for degradation of carbofuran [27]. Few reports are published on degradation of polycyclic aromatic hydrocarbons by *Paracoccus* sp. [28, 29]. To our knowledge, this could be the first report on biodegradation of textile azo dye Reactive Violet 5 by *Paracoccus* sp. GSM2.

3.2. Effect of Static and Shaking Conditions. *Paracoccus* sp. GSM2 showed that 100% decolorization of added Reactive Violet 5 (100 mg/L) within 16 h under static condition when compared to only 16% decolorization was observed under shaking condition, while the growth of bacterium was greater under shaking condition as compared to static condition (Figure 3). To confirm whether this decolorization was due to microbial action or due to change in pH, the change in pH was recorded, which was in the range of 6.0–7.5 at static condition, thus confirming that the biodegradation of dye was due to microbial action. Under aerobic conditions azo dyes are generally resistant to attack by bacteria [30]. Similar findings were reported by other researchers [14, 15]. During dye decolorization in shaking environment electrons released

### Table 1: Biochemical and sugar utilization tests of bacterial strain GSM2.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Bacterial strain GSM2</th>
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<tr>
<td>Catalase</td>
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<td>Oxidase</td>
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<td>Urease</td>
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<td>Rhamnose</td>
<td>+</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>+</td>
</tr>
<tr>
<td>Melezitose</td>
<td>+</td>
</tr>
<tr>
<td>α-Methyl-D-mannoside</td>
<td>–</td>
</tr>
<tr>
<td>Xylitol</td>
<td>–</td>
</tr>
<tr>
<td>ONPG</td>
<td>–</td>
</tr>
<tr>
<td>D-Arabinose</td>
<td>+</td>
</tr>
<tr>
<td>Malonate utilization</td>
<td>+</td>
</tr>
<tr>
<td>Sorbose</td>
<td>–</td>
</tr>
</tbody>
</table>

+: positive, [+] : weakly positive, and –: negative.
by oxidation of electron donors are preferentially utilized to reduce free oxygen rather than azo dyes [31]. Hence, in this study static conditions were maintained in the following experiments.

3.3. Effect of pH. The effect of pH on decolorization of Reactive Violet 5 by Paracoccus sp. GSM2 was determined over a wide range of pH 4.0 to 10.0 with an interval of pH 1. The isolate showed the maximum of 100% decolorization at pH 7.0 at 30°C. Following the increases from either side of neutral pH, the percentage of decolorization decreased steadily from 97% to 40% on the alkaline side while steep decline in percent decolorization from 89% to less than 15% on acidic side was found. More than 88% of decolorization was observed in a wide range of pH 6.0 to 8.0 (Figure 4). Similar optimum pH was observed in the decolorization of the same dye Reactive Violet 5 by bacterial consortium SB4 [14]. Chan and Kuo [32] reported that the neutral pH would be more favorable for decolorization of the azo dyes and is suitable for industrial applications.

3.4. Effect of Temperature. Similarly in the temperature optimization study, the dye decolorization activity of Paracoccus sp. GSM2 was found to increase with increase in incubation temperature from 20 to 30°C. Further increase in temperature, decolorization was decreased by 23% and 44% at 40°C and 45°C, respectively, and almost no activity was found at 50°C (Figure 5). This might be attributed to the adverse effect of high temperature on enzyme activities [33]. Tamboli et al. [16] also found the decrease in dye decolorization efficiency of Sphingobacterium sp. ATM for color removal beyond 30°C, which may be due to the thermal inactivation of the decolorizing enzymes.

3.5. Effect of Initial Dye Concentration. The decolorization performance of Reactive Violet 5 by the Paracoccus sp. GSM2 was studied by increasing initial dye concentration (100–800 mg/L). We observed that the percentage of decolorization was decreased slowly with increasing dye concentration (Figure 6). It could effectively decolorize up to 100 mg/L of Reactive Violet 5 (100%) within 16 h and is decreased to 63%, when dye concentration increased to 800 mg/L and decolorization time increases from 16 h to 38 h, respectively. Lower percentage of decolorization and enhanced time period at high dye concentration may be attributed to the presence of four sulfonic acid groups on Reactive Violet 5 which acts as
detergent exerting inhibitory effect on growth of Paracoccus sp. GSM2 [14].

3.6. Effect of Salt Concentration. Textile industry effluents generally contain chloride salts of sodium and potassium which are most widely used for salting out of dyes and discharged along with unused dyes. Hence, the present investigation was undertaken to study the effect of salt concentration (1–6% w/v) on decolorization of Reactive Violet 5 by the strain GSM2. The organism showed satisfactory decolorization up to maximum of 2% salt concentration in MSM under optimum conditions after 16 h of incubation (Figure 7). Previously De Baere et al. [34] have stated that sodium concentration higher than 3 g/L can cause inhibition of most of the bacterial metabolism. But, contrary to the above statement, we could notice 74% and 45% of decolorization at 3% and 4% of salt concentration, respectively. Which agree with the earlier report [14]. Negligible activity was observed when 6% of salt concentration was employed into the medium. This may be attributed to the inhibition of bacteria at high salt concentration due to plasmolysis or loss of activity of cells [35].

3.7. Effect of Different Concentrations of Yeast Extract. Paracoccus sp. GSM2 was able to degrade Reactive Violet 5 (100 mg/L) efficiently in the presence of yeast extract as a cosubstrate. Among all other nitrogen sources, only yeast extract that served as better nitrogen source for decolorization of Reactive Violet 5 within less time was selected for further experiments (data not shown). Effect of different concentrations of yeast extract (0.1–2.0 g/L) in MSM on the decolorization efficacy of GSM2 was evaluated (Figure 8). Addition of 1 g/L of yeast extract enhanced the decolorization activity and complete decolorization of Reactive Violet 5 was recorded within 16 h. Further increase in yeast extract concentration has no effect on decolorization activity. Thus, to make the process economical 1 g/L of yeast extract concentration was found to be optimum. Similar results were reported by Jain et al. [14] where their findings proposed that yeast extract was essential for regeneration of NADH which acts as an electron donor in azo bond reduction.

3.8. Decolorization of Structurally Different Azo Dyes. Structurally different azo dyes were most widely used in textile processing industries, and, therefore, the effluents from the industry are markedly diverse in composition [36]. Thus, Paracoccus sp. GSM2 was tested for its ability to decolorize five other structurally different azo dyes such as Reactive Red 2, Reactive Orange 16, Reactive Black 5, Reactive Blue 4, and
Reactive Green 19A. The organism effectively decolorized all structurally different azo dyes within 38 h (Table 2). The efficiency was 100% for Reactive Red 2, followed by 99% for Reactive Orange 16, 92% for Reactive Blue 4, 82% for Reactive Black 5, and 73% for Reactive Green 19A. We presume that decolorization of structurally different azo dyes by *Paracoccus* sp. GSM2 within 38 h might be the first. This variation in the decolorization of different dyes might be attributed to the structural differences, high molecular weight, and presence of inhibitory groups like -NO2 and -SO3Na in the dyes [36]. The present study confirms the ability of strain GSM2 to decolorize different azo dyes with decolorization efficiency of more than 70%. Thus, the strain GSM2 could be successfully employed for the treatment of textile industry effluents containing various azo dyes.

### Table 2: Decolorization of structurally different azo dyes by *Paracoccus* sp. GSM2.

<table>
<thead>
<tr>
<th>Reactive dyes</th>
<th>λmax (nm)</th>
<th>% Decolorization</th>
<th>Time (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reactive Red 2</td>
<td>538</td>
<td>100</td>
<td>12</td>
</tr>
<tr>
<td>Reactive Orange 16</td>
<td>416</td>
<td>99</td>
<td>18</td>
</tr>
<tr>
<td>Reactive Blue 4</td>
<td>595</td>
<td>92</td>
<td>22</td>
</tr>
<tr>
<td>Reactive Black 5</td>
<td>597</td>
<td>82</td>
<td>30</td>
</tr>
<tr>
<td>Reactive Green 19A</td>
<td>540</td>
<td>73</td>
<td>38</td>
</tr>
</tbody>
</table>

3.9. Decolorization and Biodegradation Studies. To disclose the possible mechanism of dye decolorization, we also analyzed the degraded products of Reactive Violet 5 by UV-Vis and FT-IR techniques. UV-Vis absorbance of 200–800 nm of Reactive Violet 5 in MSM showed single peak in visible region at 558 nm (λmax) and two intense peaks in UV region near 250 and 325 nm, respectively, correspond to phenyl and naphthyl rings of Reactive Violet 5 (Figure 9) [37]. During decolorization azo bond in Reactive Violet 5 was broken down and peak at 558 nm continuously decreased and completely disappeared within 16 h, without any shift in λmax. Similar observations have been recorded by Jain et al. [14]. According to Asad et al. [12] decolorization of dyes by bacteria could be due to adsorption by microbial cells or to biodegradation. In the case of adsorption, the UV-Vis absorption peaks decrease approximately in proportion to each other, whereas, in biodegradation, either the major visible light absorbance peak disappears completely or a new peak appears. The observation of cell mass showed that *Paracoccus* sp. GSM2 retained their natural color after decolorization of Reactive Violet 5. FT-IR spectrum of control dye with metabolites extracted after complete decolorization (16 h) clearly indicated the biodegradation of the parent dye compound by *Paracoccus* sp. GSM2 (Figure 10). FT-IR analysis of control and decolorized samples showed significant differences in specific peaks of Reactive Violet 5 fingerprint region (550–4000 cm−1). FT-IR spectra of control Reactive Violet 5 show specific peaks for multisubstituted benzene ring, where peaks at 1140.83, 1338.36, 1186.18, and 1549.25 cm−1 corresponded to two SO2H groups, symmetric SO2, and -N=N- (azo bond), whereas parasubstituted azo benzene showed bands near 1433.48 cm−1. Reactive Violet 5 is a metal containing textile azo dye where carbonic ion is bonded with a central copper metal ion giving rise to asymmetrical stretching which was observed near 1614.85 cm−1 [38]. Peak at 1650.89 cm−1 represents the primary amide of the parent structure of Reactive Violet 5. The FT-IR spectra of 16 h extracted metabolites of degraded Reactive Violet 5 showed peaks at 1651.91 and 3252.48 cm−1 which indicates the production of primary amine and secondary amide, respectively, during biodegradation of Reactive Violet 5. Absence of peaks at 671, 721, 763, and 817 cm−1 indicates the breakdown of benzene ring or the loss of aromatic nature of the compound. Jain et al. [14] reported similar kind of benzene ring fission in the same dye Reactive Violet 5 by bacterial consortium SB4. Correspondingly, breakdown of azo bond was confirmed by the absence of spectral peak at 1549.25 cm−1, while the absence of the peaks around 1300 and 1165–1500 cm−1 clearly indicates the degradation of S=O bonds. Peaks at 2972.40 and 2960.03 cm−1 in control Reactive Violet 5 and degraded metabolites, respectively, show the asymmetrical stretching of C–H in CH3. Similar kind of asymmetrical stretching of peak at 2856 cm−1 in control Reactive Violet 5 shows that the asymmetrical stretching for C–H stretching was observed in degradation of the same dye Reactive Violet 5 [39]. On the basis of above results, it can be concluded that *Paracoccus* sp. GSM2 has ability to mineralize Reactive Violet 5 completely.

### Figure 9: UV-Vis spectra of Reactive Violet 5 before and after decolorization by *Paracoccus* sp. GSM2 (a, 0 hour; b, 16 hours).

### 4. Conclusion

The present study showed that a bacterial strain *Paracoccus* sp. GSM2 is capable of degrading Reactive Violet 5 as a sole source of carbon with minimal nutritional requirements under static condition. The potential of this strain has ability to decolorize Reactive Violet 5 in a wide range of pH, temperature, salt, and initial dye concentrations, which is significant for its commercial application. The FT-IR results showed complete loss of the aromatic nature of the dye Reactive Violet 5 by *Paracoccus* sp. GSM2. Furthermore, strain GSM2 had the ability to decolorize five other structurally different azo dyes within 38 h (Table 2). The efficiency was 100% for Reactive Red 2, followed by 99% for Reactive Orange 16, 92% for Reactive Blue 4, 82% for Reactive Black 5, and 73% for Reactive Green 19A. We presume that decolorization of structurally different azo dyes by *Paracoccus* sp. GSM2 within 38 h might be the first. This variation in the decolorization of different dyes might be attributed to the structural differences, high molecular weight, and presence of inhibitory groups like -NO2 and -SO3Na in the dyes [36].
azo dyes indicating its field applicability in the treatment of textile effluents. Therefore, *Paracoccus* sp. GSM2 is the highly promising bacterium that can be used for the treatment of textile industry effluents containing various reactive azo dyes.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

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**References**


