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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**

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INTRODUCTION

In 2000, it was estimated that 5×10^{10} kg of fibres were produced worldwide, which consumed more than 8×10^{8} kg of different dyes and pigments[1]. 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 3.4×10^{10} kg, which accounted for annual global sales of nearly US$ 6 billion[2]. 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[3]. 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[4]. 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[5,6]. 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)[7]. Many synthetic azo dyes and their metabolites are toxic, carcinogenic and mutagenic[8]. 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[9]. 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[10,11], fungi[12], actinomycetes[13] and algae[14] have been reported for their ability to decolorize and degrade reactive azo dyes[15]. 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[16]. 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.

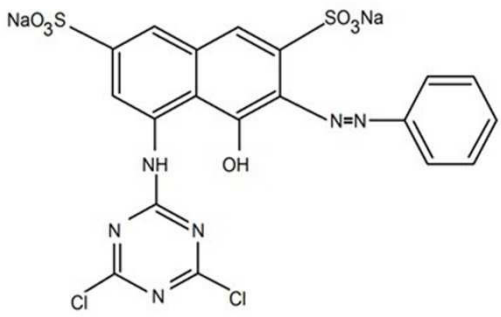

Figure 1
Structure of Reactive Red 2

Culture medium

The mineral salts medium (MSM)[17], containing (g/L): $Na_2HPO_4{\cdot}2H_2O$ (12.0), KH_2PO_4 (2.0), $(NH_4)_2NO_3$ (0.50), $MgCl_2{\cdot}6H_2O$ (0.10), $Ca(NO_3)_2{\cdot}4H_20$ (0.050), $FeCl_2{\cdot}4H_20$ (0.0075) containing 10 mL (vv^{-1}) of trace element solution and 0.1% (wv^{-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): $ZnSO_4{\cdot}7H_2O$ (10.0), $MnCl_2{\cdot}4H_2O$ (3.0), $CoCl_2{\cdot}2H_2O$ (1.0), $NiCl_2{\cdot}6H_2O$ (2.0), $Na_2MoO_4{\cdot}2H_2O$ (3.0), H_3BO_3 (30.0), $CuCl_2{\cdot}2H_2O$ (1.0); pH 7.0. When required, 1.9% (wv^{-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% wv^{-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% wv^{-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 *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[18], using Kimura–2–parameter distances in MEGA 5 software[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^{-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 to10 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^{-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[20].

$$Decolorization\ (\%) = \frac{I - F}{I} \times 100$$

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[21]or Degradation[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[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[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.

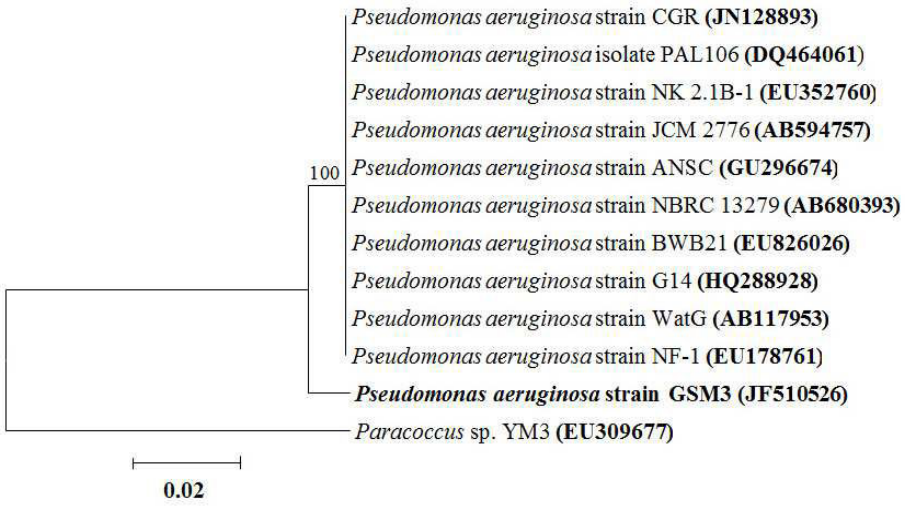

Figure 2

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.

Table 1
Morphological, biochemical and physiological characterization of Pseudomonas aeruginosa strain GSM3.

Characteristics	Results
Gram's staining test	Gram negative
Shape	Rods
Motility	motile
Catalase	+
oxidase	+
Urease	+
Citrate utilization	+
Mannose	–
Trehalose	–
Glucose	+
Adonitol	+
Lactose	–
Mannitol	+
Sorbitol	+
Salicin	–
Dulcitol	–
Inositol	–
Arabitol	+
Erythritol	–
α-Methyl-D-glucoside	+
Rhamnose	–
Cellobiose	–
Melezitose	–
α-Methyl-D-mannoside	–
Xylitol	–
ONPG	–
Esculin hydrolysis	–
D-Arabinose	–
Malonate utilization	+
Sorbose	–

(+) - 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 cosubstrate for the growth of azo dye decolorizing organisms. Earlier Sponza Isik[26] 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.

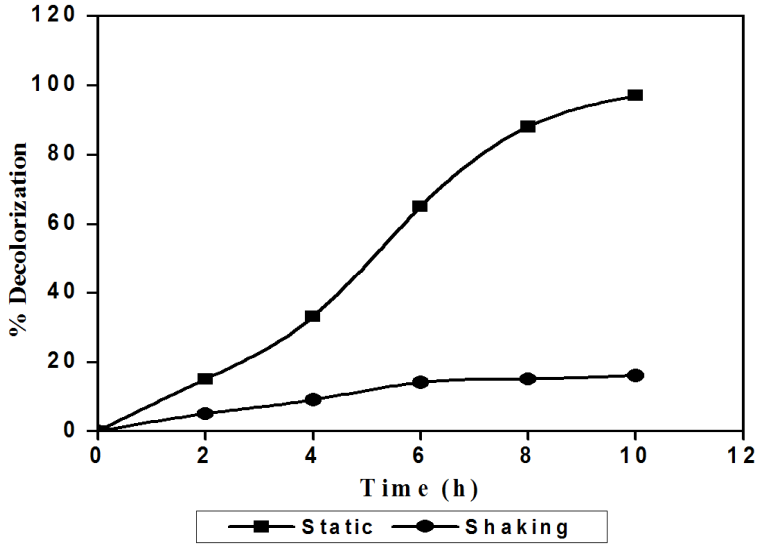

Figure 3
Effect of static and shaking conditions on decolorization of Reactive Red 2

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^{o}C (Figure 4). Further increase in temperature to 40^{o}C, decolorization was decreased by 1% and 14% decreased at 45^{o}C.Decolorization activity was significantly suppressed at 50^{o}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[27].

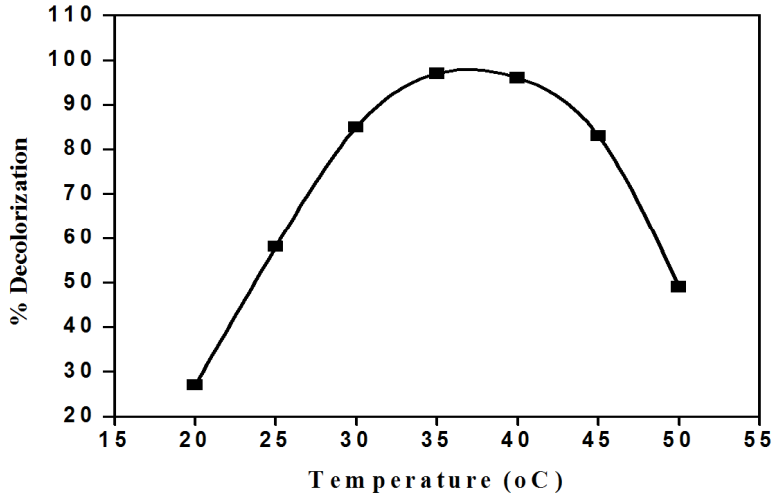

Figure 4
Effect of temperature on decolorization of Reactive Red 2

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^{o}C (Figure 5). Increase in the

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[28]. Jadhav et al[29] also found that pH 7.0 was optimum for decolorization of Direct Orange 39 by *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[30,10].

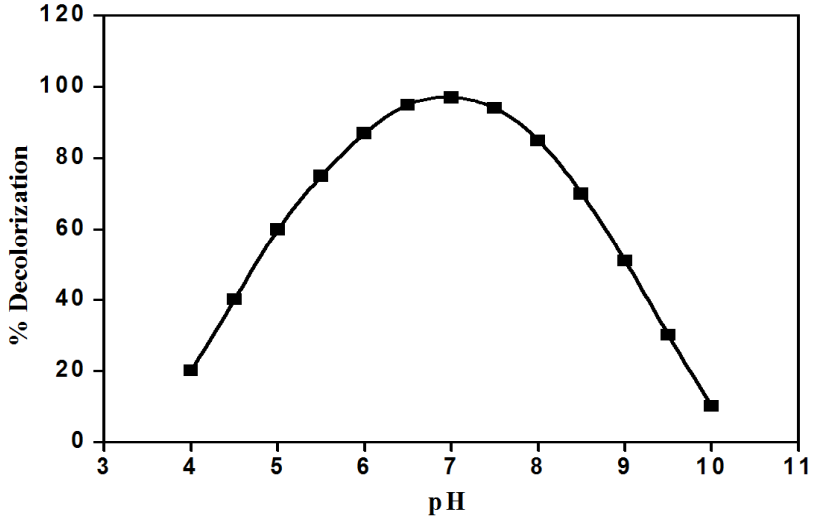

Figure 5
Effect of pH on decolorization of Reactive Red 2

Our results on optimization studies revealed that *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[10] reported that maximum decolorization of same dye Reactive Red 2 of about 96% was observed under static condition, in case of bacterial strain *Pseudomonas* sp. SUK1. Similar types of cultural conditions were adopted for the degradation of azo dyes using mesophilic bacterial strains such as *Pseudomonas* sp. SUK1, *P. aeruginosa* NGKCTS, *P. aeruginosa* BCH, and *Pseudomonas* sp. LBC1[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[10].

Effect of initial dye concentration on decolorization of Reactive Red 2

The decolorization efficiency of Reactive Red 2 by *Pseudomonas aeruginosa* GSM3 was studied by increasing initial dye concentration (300–2000 mg L^{-1}). We observed that the percentage of decolorization was decreased slowly with increasing dye concentration above 300 mg L^{-1} (Figure 6). It could effectively decolorize up to 300 mg L^{-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].

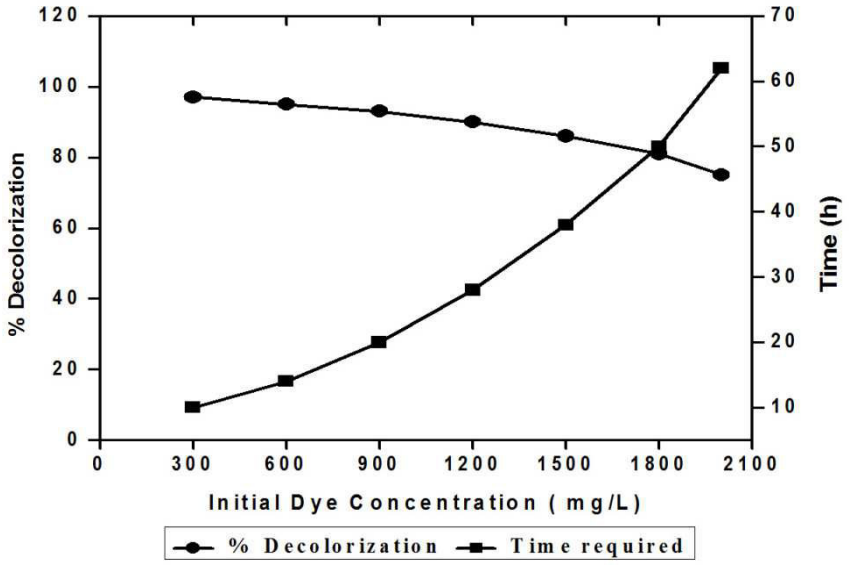

Figure 6
Effect of initial dye concentration on decolorization of Reactive Red 2.

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.

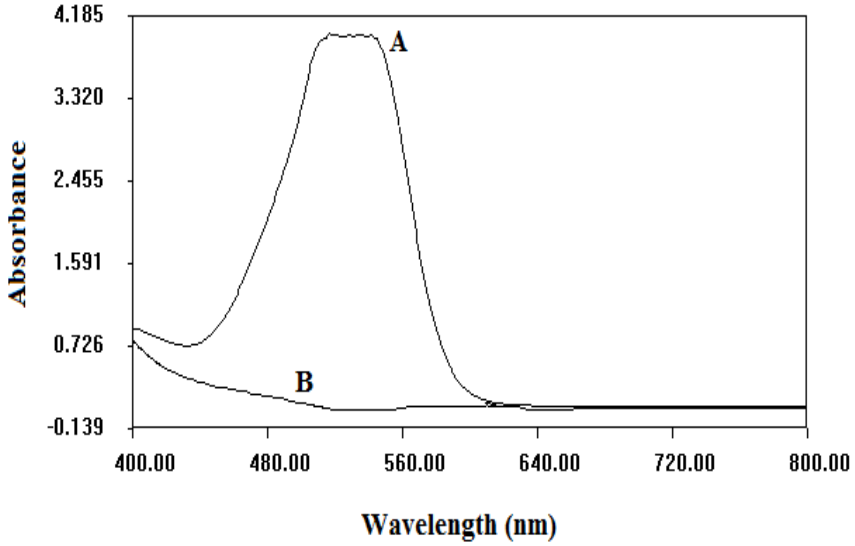

Figure 7
UV–Vis spectra of Reactive Red 2 before and after decolorization by Pseudomonas aeruginosa GSM3 (A, 0 h; B, 10 h).

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.

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