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Original Research Article

A study on Microbial decolourization of Reactive Red M8B by *Bacillus subtilis* isolated from dye contaminated soil samples

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Abstract

Dye Contaminated soil sample and Reactive Azo dye, Red M8B were collected. Soil sample was serially diluted and spread on Nutrient Agar plate containing 1% glucose as enrichment. Colonies isolated on media were further purified and identified based on morphological and biochemical methods. Totally 8 strains were isolated. All the strains were screened by plate assay and conical flask assay. Out of 8 isolates SS4 strain tentatively recognized as *Bacillus subtilis.* This strain was further carried out for several optimizational procedures such as, Dye Concentration, Carbon Sources, Nitrogen Sources, Temperature, pH etc., After inoculation and incubation with above specified strain it shows best optimization in 1% concentration at pH 8-9 and decolourized best at glucose and cellulose as carbon source, Ammonium hydroxide as nitrogen source and at 35°C as best temperature. Crude cell extract were obtained for azoreductase assay and measured spectrophotometricaly. Toxicity assay and TLC were performed. Thus, by this present it is concluded that the bacterial isolates like *Bacillus* sp., can used as a good microbial source for treatment.

Keywords: Dye Pollutuion, Reactive Azo dye, *Bacillus subtilis.*

Introduction

One of the major problems that humans are facing is the restoration of the contaminated environment. Textile dyes contribute as the most important environment-polluting agents. Several classes of such contaminants have been synthesized, and still new products are being synthesized now and then. The textile industry is a large water consumer and produces large volumes of contaminated water. The textile industry generally has difficulty in meeting waste water discharge limits, particularly with regard to dissolved solids, ionic salt, pH, COD, color and heavy metal. Treatment of dye contaminated waste water discharged from the textile and other dye stuff industries is necessary to prevent of soil, surface and ground water. Synthetic dyes and colourants are being increasingly used these days by paper, textile, food, cosmetics, and pharmaceutical industries. Among these, textile industries are the largest consumer of dyes and pigments, accounting for 80 % of total production (Jyoti Kumar Thakur *et al.*, 2014).

Synthetic dyes released into the environment in the form of effluents by textile, leather, food, paper and printing industries cause severe ecological damages. Wastewater resulting from dyeing and finishing processes has an adverse impact in terms of total organic carbon, biological oxygen demand and chemical oxygen demand. Azo dyes are the main constituents of such pollution because of their wide applicability and usages, and therefore, these are present majorly in textile industrial effluents. Moreover their toxicity and resistance to degradation offer great challenge for removal technologies. In many cases the products formed after the degradation

of the parent azo dye molecule are more toxic. These products are mainly in aromatic amine form (Maulin P Shah *et al*., 2013).

Dye, substance used to impart colour to textiles, paper, leather, and other materials such that the colouring is not readily altered by washing, heat, light, or other factors to which the material is likely to be exposed. Dyes differ from pigments, which are finely ground solids dispersed in a liquid, such as paint or ink, or blended with other materials. Most dyes are organic compounds (i.e., they contain carbon), whereas pigments may be inorganic compounds (i.e., they do not contain carbon) or organic compounds. Pigments generally give brighter colours and may be dyes that are insoluble in the medium employed.

Colour has always fascinated humankind, for both aesthetic and social reasons. Throughout history dyes and pigments have been major articles of commerce. Manufacture of virtually all commercial products involves colour at some stage, and today some 9,000 colorants with more than 50,000 trade names are used. The large number is a consequence of the range of tints and hues desired, the chemical nature of the materials to be coloured, and the fact that colour is directly related to the molecular structure of the dye.
The ability of bacteria to metabolize azo dyes has been investigated by a number or research groups .Under aerobic conditions, azo dyes are not readily metabolized, although the ability of bacteria with specialized reducing enzymes to aerobically degrade certain azo dyes was reported. In contrast, under anaerobic conditions many bacteria reduce azo dyes by the activity of unspecific, soluble, cytoplasmatic reductase, known as azo reductases. The anaerobic reduction degrades the azo dyes that are converted into aromatic amines, which may be toxic, mutagenic, and possibly carcinogenic to mammalians. Therefore, to achieve complete degradation of azo dyes, another stage that involves aerobic biodegradation of the produced aromatic amines is necessary.

Bacterial biodegradation of non-azo dyes has only recently been studied. It has been observed that several bacteria can degrade anthraquinone dyes. Aerobic decolorization of triphenylmethane dyes has also been demonstrated. In phtalocyanine dyes, reversible reduction and decolorization under anaerobic conditions have been observed.

The most widely researched fungi in regard to dye degradation are the ligninolytic fungi. White-rot fungi in particular produced enzymes as lignin peroxidase, manganese peroxidase and laccase that degrade many aromatic compounds due to their non-specific activity. Large literature exists regarding the potential of these fungi to oxidize phenolic, non-phenolic, soluble and non-soluble dyes. In particular laccase from *Pleurotus ostreatus, Schizophyllum commune, Sclerotium rolfsii* and *Neurospora crassa*, seemed to increase up to 25% the degree of decolorization of individual commercial triarylmethane, anthraquinonic, and indigoid textile dyes using enzyme preparations. Fungal degradation of aromatic structures is a secondary metabolic event that starts when nutrients (C, N and S) become limiting. The influence of the substitution pattern on the dye mineralization rates and between dye structure and fungal dye biodegradability is a matter of controversy. However, these difficulties are even greater if one considers that complex mixed effluents are extremely variable in composition even from the same factory, as is often the case of the textile industry.

Therefore, in such situations, biological treatment may be a real hope. These methods have the advantages of being environment friendly. Microorganisms have developed enzyme system for the decolourization and mineralization of azo dyes under certain environmental conditions. So, present study was designed to isolate efficient dye decolorizing bacterial strains from the dye contaminated soil. Since the bacterial isolates were originated from the dye contaminated textile wastewater of local industry, so they can easily adapt to the prevailing local environment. Therefore, such bacteria can be used to develop an effective biological treatment system for the wastewaters contaminated with azo dyes.

Materials and Methods

Collection of Samples

The dye contaminated soil sample was collected in sterilized polypropylene bags and brought to the laboratory. Nutrient media was used with slight modification for the enrichment of the culture.

Dye

The dye Reactive Red M8B was used throughout the study

Isolation of Dye Degrading Organisms

1.0g of soil samples were suspended in 100ml sterile water blanks aseptically and various dilutions were made accordingly.

About 0.1 ml of dilutions (10^{-1} to 10^{-5}) was spreaded on Nutrient agar plates containing 1% glucose and then incubated at temperature 37 °C up to 3 - 5 days.

Isolated colonies were picked up and further streaked on Nutrient agar plates.

Purification and initial characterization of isolates were done by repeated streak plate method and staining methods.

All the strains were identified based on the morphology and biochemical characteristics respectively.

Culture media:

Nutrient agar media (Himedia Laboratories) was used for the enumeration and isolation of bacteria from the dye-contaminated soil. Mineral Salt Media and modified with 0.1% of respective dye was used for the decolourization test.

Identification of selected isolates:

The selected isolates were examined for their morphological properties, such as size, shape, cell arrangement and staining properties. Cultural properties including form, colour, elevation, margin, surface of colonies on nutrient agar plate and slant were also recorded. Physiological and biochemical characteristics of the isolates were evaluated by Voges–Proskauer, methyl red, indole, catalase, oxidase, urease, citrate utilization, nitrate reduction, and H2S production tests. The ability of the organisms in fermenting a number of sugars were also performed. The isolates were identified up to species based on comparative analysis of the observed characteristics with the standard description of bacterial strains in Bergey's Manual of Determinative Bacteriology.

Screening of Bacterial Isolates for dye degradation: Inoculum Preparation

The suspension of 2 days old cultures of selected bacteria was used to investigate their abilities to decolourize Reactive Red M8B dye. A loopful of bacterial cultures were inoculated in to LB Broth and incubated at 37 °C for 24 hours.

Dye Decolourization Experiment (Conical Flask Assay):

Dye decolourization experiments were carried out in 100 ml flasks containing 50 ml of MSM with Reactive Red M8B dye (100 mg/l), traces of yeast extract and glucose.

The pH was adjusted to 7±0.2 using sodium hydroxide and hydrochloric acid solution.

Then, the flasks were autoclaved at 121°C for 15 minutes.

The autoclaved flasks were inoculated with 5ml of bacterial inoculum of each isolates.

The flasks were kept in mechanical shaker and incubated at 37°C for 4 days.

Samples were drawn at intervals for observation. 10 ml of the dye solution was filtered and centrifuged to separate the bacterial cell mass.

Decolourization was assessed by measuring absorbance of the supernatant with the help of spectrophotometer at wavelength maxima (λm = 595 nm) of dye.

The percentage of decolourization was calculated from the equation,

$$\% \text{ Decolourization} = \frac{\text{Initial OD - Final OD}}{\text{Initial OD}} \times 100$$

Effect of Dye Concentration

A set of flask containing sterilized Mineral Salt medium with different concentration of dyes (0.1% to 0.5%) was inoculated with selected culture and incubated at 37°C for 5 days and content of the flask were centrifuged at 5000 rpm for 15 min and supernatant was analyzed spectrophotometricaly at 595nm.

Effect of Carbon and Nitrogen Source in the Medium

In this experiment different carbon and nitrogen sources were used keeping the dye concentration same.

Effect on various carbon sources like cellulose, dextrose, fructose, maltose etc. at 1% (w/v) and nitrogen sources sodium nitrate, potassium hydroxide, urea and Ammonium chloride at 1% (w/v) on dye decolourization of Reactive Red M8B in modified MSM was studied for best carbon and nitrogen sources and incubated at 37°C for 5 days and content of the flask were centrifuged at 5000 rpm for 15 min and supernatant was analyzed spectrophotometricaly at 595nm.

Effect of Temperature and pH on Dye Degradation

Effect of various temperatures like 25°C, 30°C, 35°C, 45°C and 55°C and pH such as 5,6,7,8 and 9 on dye decolourization of reactive red M8B on modified MSM was studied for dye degradation.

All the flasks were incubated at 37°C for 5 days and content of the flask were centrifuged at 5000 rpm for 15 min and supernatant was analyzed spectrophotometricaly at 595nm.

Crude Cell extract

To prepare the cell free extract, the bacterial cultures were grown with dyes(100 µg/ml) in 100 ml MSM for 96 hrs at 37°C. Cells were harvested by centrifugation at 9000 for 15 min. Pellet were washed with 0.1mM Phosphate buffer (pH 7) and were suspended in 1ml of the same buffer. Cells were disrupted by lysozyme enzyme. The resultant homogenate was centrifuged at 8000 for 20 min at 4C; the supernatant was used as a crude extract. The activity of azoreductase was determined by slightly modified method of Moutaouakil *et al*. (2003) by measuring optical density of a reaction mixture containing 200µl of 25µM dye, 200 µl of 1mM NADH, 2.07 ml of PBS buffer(pH 7.0) and 300 µl of the crude extract extraction. The reaction was allowed to proceed for 30min at 37°C, and measured at 595 nm. The crude extracts that were heated at 100°C for 30 min acted as control.

Thin Layer Chromatography

The metabolites produced during the biodegradation of dye after decolourization of the medium were extracted with equal volume of chloroform. The supernatant was concentrated with anhydrous Na_2SO_4. TLC analysis was carried out according to Kalyani *et al.*(2008) on silica gel using mobile phase solvent system n-propanol, methanol, ethyl acetate, water and glacial acetic acid(3:2:2:1:0.5) and results were observed under UV illuminator.

Toxicity assay

The biodegraded products were tested for their toxic effect on the agriculturally important soil bacterial flora. *Azotobacter* sp. and *Bacillus cereus* were inoculated on MSM containing agar. Two wells were made on the respective media containing plates and filled with decolourized centrifuged broth. The plates were incubated at 30C for 48 hrs. Zone of inhibition surrounding the well represented the index of toxicity.

Results

Collection of soil sample:

Soil sample was taken from the dye contaminated site of textile industry. Collected soil samples was serially diluted and plated on Nutrient agar plates which was shown in photo 1. After incubation colonies grown on plates were selected, gram stained and identified morphologically and biochemically (Table 1, 2).

Screening of isolates:

In primary screening, only 8 isolates (SS1, SS4, SS6, SS8, ES3, ES4, ES5 and ES6) have the capability of showing visible decolourization of Reactive Red M8B (100mg/l) respectively within 5 days shown in photo 4 .Then these selected bacteria were used in secondary screening. Then the rate of decolourization was calculated by Decolourization assay as shown in the table 3 and photo 5, 6, 6(a) and 6(b). Fig.1 and 2 showed % decolourization of Reactive Red M8B dye respectively in secondary screening by the selected strains. Among selected isolates only two isolates: SS4 (Table – 4; Figure-2) showed up to 60% decolourization within three days

Identification of Isolates:

The selected strains namely SS4 were identified based on their morphological and biochemical and concluded as *Bacillus subtilis*.

Effect on dye concentration:

The selected two strains were further optimized on dye concentration, on which SS4 isolate showed degradation of Reactive red M8B in varying concentration as shown in figure 3and in table-5.Out of 5 different concentration used, 0.1% concentration was degraded most efficiently within 24hrs. The selected isolates SS4 were further used for optimization of various cultural conditions.

Effect on temperature and pH:

The selected isolate showed no decolourization at temperatures like 25°C, 45°C and 55°C in Reactive red M8B dye as shown in photo 9. The isolate SS4 was observed maximum decolourization up to 60% within 248 h at temperature 35°C (Figure 6; table-8).

It was observed that the selected isolates showed no decolourization at pH 5.0 and 6.0 and it was appear at pH 7(Table 9) and it goes on increasing with increase in pH upto 9.0 Figure-7).

Effect of carbon and nitrogen sources:

Effect on different carbon sources such as maltose, cellulose, fructose and dextrose were evaluated on Red M8B decolourization by bacterial isolates (figure-4 and table 6).For studying effect on different nitrogen sources such as $NaNo_3$, NH_4OH, NH_4Cl and Urea were used and incubated with selected strain Table-7; Figure-5

Dye degradation and crude enzyme assay:

Decolourization of reactive azo dyes by bacterial strain has been reported by many researchers. Bacterial degradation of azo dyes is generally considered a specific reaction by azoreductase under aerobic or anaerobic. *Bacillus* was able to reduce a large structural variety of azo dyes.

Toxicity Assay and TLC:

No zone of inhibition observed in surrounding the wells containing decolourized dye water, indicated that the biodegraded or decolourized product was non-toxic to beneficial soil bacteria. TLC results suggested that *Bacillus* Sp. was able to degrade reactive red M8B dye and most of the enzymes could be induced by its degradable substrates .

Table: 1 Colony Characteristics of the Isolates from dye contaminated soil.

S.No	Isolates	Colony morphology on agar plate	Gram reaction	Probable of identification
1.	SS1	Pale, circular, opaque, flat, rough	G +ve, rod, central spores	*Bacillus* species
2.	SS4	Cream, circular ,rough, opaque	G +ve, rod, central spores	*Bacillus* species
3.	SS6	White, circular, opaque, rough	G +ve rods, rod	*Bacillus*
4.	SS8	Cream, circular, rough	G +ve rods, central spores	*Bacillus*
5.	ES3	Smooth ,circular	G-ve short rods	*Pseudomonas* species
6.	ES4	Smooth	G-ve rods	*Pseudomonas* species
7.	ES5	Yellow, Rhizoidal, opaque, rough and raised	G +ve cocci	Micrococcus
8.	ES6	White, circular , rough	G +ve rods	*Bacillus* species

Table:2 Characteristics of *Bacillus subtilis* (SS4):

S.NO:	CHARACTERISTICS	REACTION
1.	Gram staining	Gram positive rod
2.	Shape	Chains in rod
3.	Catalase	Positive
4.	Indole	Negative
5.	Methyl Red	Negative
6.	Voges-Proskauer	Positive
7.	Citrate Utilization	Negative
8.	Endospore staining	Positive
9.	Starch hydrolysis	Positive
10.	Casein hydrolysis	Positive
11.	Gelatin hydrolysis	Positive
12.	Nitrate reduction	Positive

Table: 3 % Degradation of Reactive Red M8B Dye by isolated strains at 5th day of incubation.

S.No	Strain	Dye Degradation (%)
1.	SS1	31.05
2.	SS4	97.13
3.	SS6	81.49
4.	SS8	74.15
5.	ES3	90.67
6.	ES4	82.67
7.	ES5	90.52
8.	ES6	67.40

Figure: 1 % Degradation of Reactive Red M8B Dye by isolated strains at 5th day of incubation.

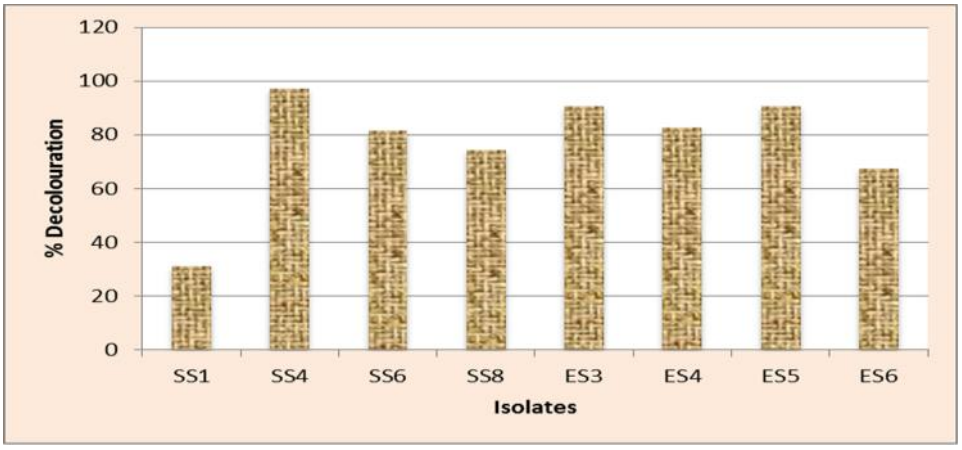


Table: 4 % Dye Degradation for Reactive Red M8B dye by SS4 (*Bacillus subtilis*).

S.No	Days	Dye Degradation (%)
1.	0 day	0
2.	1 day	24.88
3.	2 day	31.05
4.	3 day	68.13
5.	4 day	83.18
6.	5 day	97.35

Figure : 2 % Dye Degradation for Reactive Red M8B dye by SS4.

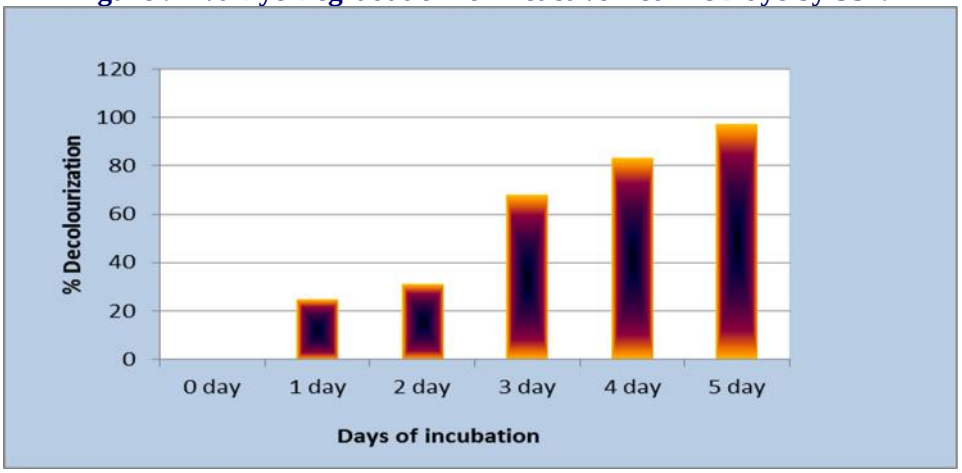


Table: 5 Degradation of dye in different concentration of Reactive Red M8B dye.

S.No	Dye Concentration	Dye Degradation (%)
1.	0.1 %	97.06
2.	0.2 %	75.11
3.	0.3 %	31.35
4.	0.4 %	25.69
5.	0.5 %	09.32

Figure: 3 Degradation of dye in different concentration of Reactive Red M8B dye.

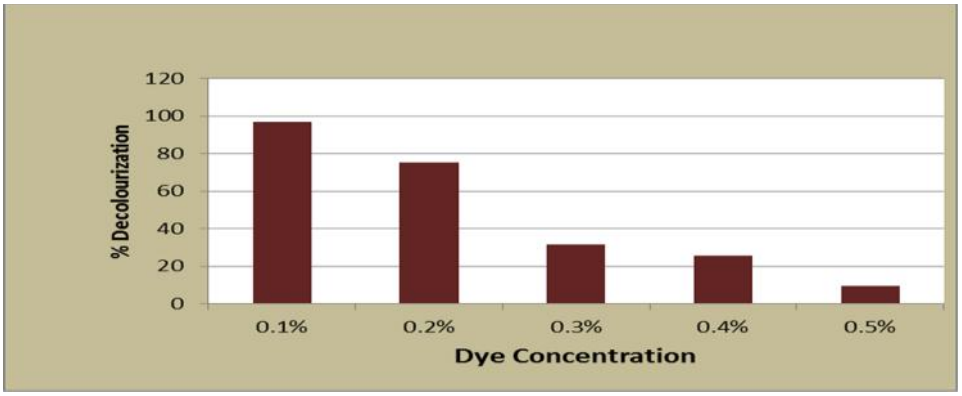


Table: 6 % Degradation of selected isolate at different Carbon sources for Reactive Red M8B dye.

S.No	Carbon sources	Dye Degradation (%)
1.	Maltose	67.69
2.	Cellulose	90.52
3.	Fructose	82.67
4.	Dextrose	18.35

Figure: 4 % Degradation of selected isolate at different carbon sources for Reactive Red M8B dye.

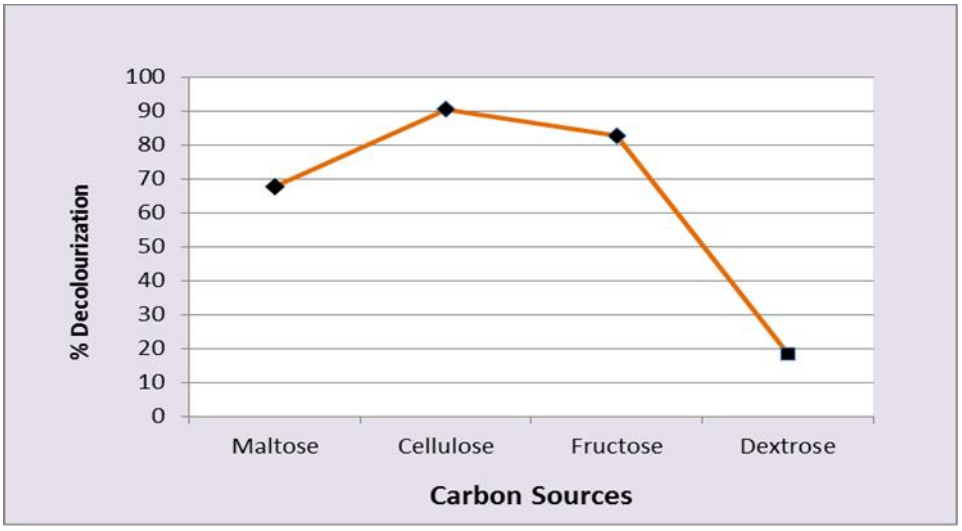


Table: 7 % Degradation of selected isolate at different nitrogen sources for Reactive Red M8B dye.

S.No	Nitrogen sources	Dye Degradation (%)
1.	$NaNO_3$	77.23
2.	NH_4OH	90.45
3.	NH_4Cl	16.59
4.	Urea	35.01

Figure: 5 % Degradation of selected isolate at different Nitrogen sources for Reactive Red M8B dye.

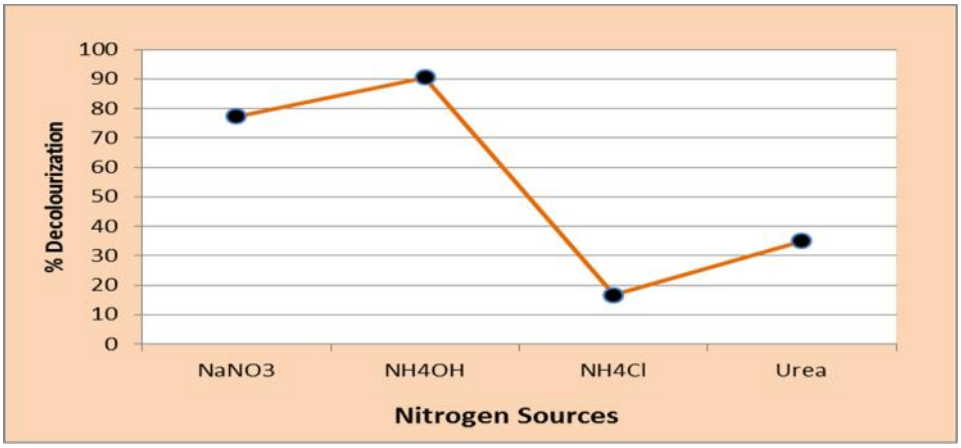


Table: 8 % Degradation of selected isolate at different temperatures for Reactive Red M8B dye.

S.No	Temperatures	Dye Degradation (%)
1.	25° C	36.85
2.	30° C	68.42
3.	35° C	91.55
4.	45° C	32.45
5.	55° C	18.50

Figure : 6 % Degradation of selected isolate at different temperatures for Reactive Red M8B dye.

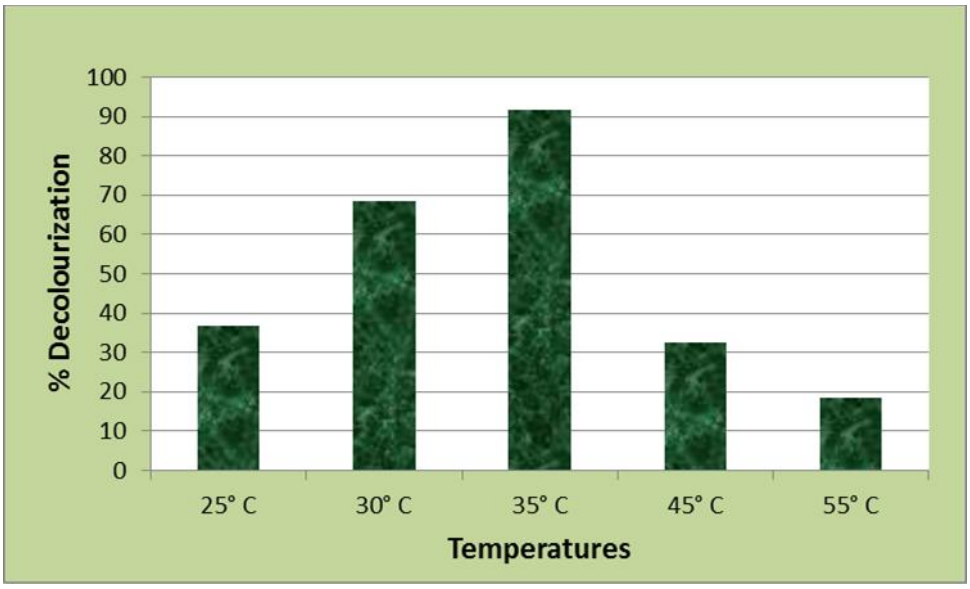
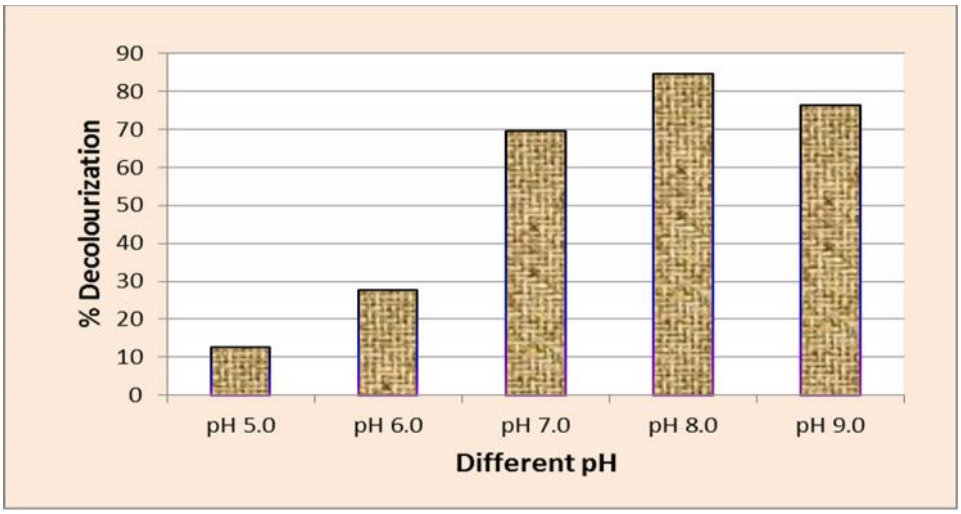


Table: 9 % Degradation of selected isolate at different pH for Reactive Red M8B dye.

S.No	pH	Dye Degradation (%)
1.	pH 5.0	12.62
2.	pH 6.0	27.67
3.	pH 7.0	69.53
4.	pH 8.0	84.58
5.	pH 9.0	76.50

Figure: 7 % Degradation of selected isolate at different pH for Reactive Red M8B dye.



Discussion

Azo dyes are the largest group of dyes. Different varieties of azo dyes are extensively used in the textile, paper, food, cosmetics and pharmaceutical industries. They are the largest and most versatile class of dye, but have structural properties that are not typically removed from water by conventional waste water system. Azo dyes are designed to resist chemical and microbial attacks and to be stable in light and washing. A number of azo dyes including reactive dyes are used in textile dying operations. This leads to effluent streams containing intense colour due to the presence of azo dyes. The removal of azo dyes from effluent is important due to their mutagenicity and carcinogenicity together with their intense colouration. Both physicochemical and biological methods for the removal of dyes have been investigated widely. The isolation of good dye-decolourizing species requires screening, and these isolated strains should have ability to degrade and detoxify textile dyes (Silveira *et al.*, 2009). The present study was focused on decolourization of textile azo dyes and biodegradation of textile dye effluent by using bacteria isolated from textile dye effluent contaminated soil.

Biodegradation of commercially available textile dye Reactive Red M8B was studied against *Bacillus subtilis* which has been isolated from the dye contaminated soil by spread plate method and % decolourization was shown in the figures accompanying the results .Eight different bacteria were isolated from the textile dye contaminated soil. Based on preliminary test, plating on selective media and biochemical test, they were identified as *Bacillus subtilis, Bacillus cereus, Pseudomonas fluorescens, and Staphylococcus aureus.*

Sriram *et al.*, 2013 isolated three different bacterial such as *Bacillus subtilis, E.coli, Pseudomonas fluorescens* for the degradation study. Saranraj *et al*., 2010 isolated 5 different bacterial from the textile dye effluent and identified as *Bacillus subtilis, Proteus mirabilis, Pseudomonas fluorescens, and Staphylococcus aureus*. Based on preliminary tests, plating on selective media and biochemical tests, they were identified as *Bacillus subtilis, Pseudomonas fluorescens, and E.coli.*

The decolourization efficiency of *Bacillus* sps. was studied by measuring the optical density after 5 days of incubation. *Bacillus subtilis* was more effective than other species. Among the isolates SS4 showed upto 60% decolourization within three days. *Bacillus subtilis* was used for optimization of various cultural conditions. The *B. subtilis* showed maximum dye degradation at 1% concentration. The decrease in the decolourisation efficiency was seen to be decreased with increase in the concentration of the dyes. Similar observations have been recorded earlier for decolourization of Turquoise Blue dye by Bhoomi Joshi *et al*., 2013.

Several attempts were made to alter the composition of the medium by altering the carbon source, nitrogen source. The cellulose and glucose were found to be best suitable carbon source for the growth of *B. subtilis.* NH_4OH and Na_2NO_3 were found suitable as nitrogen source while the urea supported the least among the four different types of nitrogen sources used in the present study. Bacterial utilization of azo dyes as a source of carbon, energy and nitrogen source Mukund Chandra Thaur *et al.,* 2012 have been reported by several worker. This study is perhaps one of the few reports in the literature on the utilization of reactive dye as a source of carbon and nitrogen. They were employed as co-substrates, as a requirement for a metabolizable carbon source seems to be obligatory for functioning of dye-decolourizing bacteria.

The bacteria could grow well at 35°C followed by 45°C,and not degraded at 55°C and 25°C. Thus the optimum temperature for the growth and dye degradation for *B. subtilis* was 37°C. The optimum temperature for decolourization was found to be about 37°C. All organisms have their own ability to grow at different temperature and decolourize the dye. It has been reported that *Klebsiella pneumoniae* RS-1 and *Alcaligens liquefaciens* S-1 do no decolourize Methyl Red at 45°C by Wong and Yuen, 1998.

At 5th day of incubation, it was observed that the decolourization percentage was highest at alkaline pH for *Bacillus* sp. at pH 8-9 .Similar results were observed (Mohan *et al.,* 2013) in alkaline pH for *Bacillus* sp. at pH 9 but in *Planococcus* sp. decolourization % was found to be high in acidic pH (6) as compared to other pH ranges. But in the work of Shah *et al.,* 2013, maximum dye decolourization was observed in pH 8 but wide range of activity was seen between pH 6-10.

No zone of inhibition was observed in surrounding the wells containing decolourized dye water, indicated that the biodegraded or decolourized product was non-toxic to beneficial soil bacteria. In one of the investigation from this laboratory reveals that no toxic effects were observed and Similar results were produced by Usman Aftab *et al.,*2011. Azo dyes degradation by microorganisms may utilize many enzymes such as lignin peroxidase, tyrosinase and laccase. TLC results suggested that *Bacillus* Sp. was able to degrade reactive red M8B dye and most of the enzymes could be induced by its degradable substrates. Similar TLC results against Reactive Black 5 and Reactive Yellow 15 treated with *Corynebacterium* sp. were reported Usman Aftab *et al.,* 2011.

In conclusions, the textile, dyeing and finishing industry use wide variety of dyestuffs due to the rapid changes in the customer's demands. Thus by the use of the above isolates sustainable biodegradation of the harmful azo dyes utilized by the dye, textile, paper ink etc. industries can be possible. These methods are not only eco-friendly but also commercially viable even for the small scale industries. A thorough investigation, taking into consideration of certain parameters such as optimization of the dye concentration for the isolates as well as for the dye to be degraded, effect of physicochemical parameters on degradation etc. at large scale is necessary to provide unequivocal evidence for the usefulness of these isolates in sustaining dye degradation capability. Further molecular study on their enzymatic property and degradation process could reveal them as an important textile dye degrader. Thus, by this present it is concluded that the bacterial isolates like *Bacillus* sp., can used as a good microbial source for treatment.

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