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Decolorization of Anthraquinone Vat Blue 4 by the Free Cells of an Autochthonous Bacterium, Bacillus Subtilis

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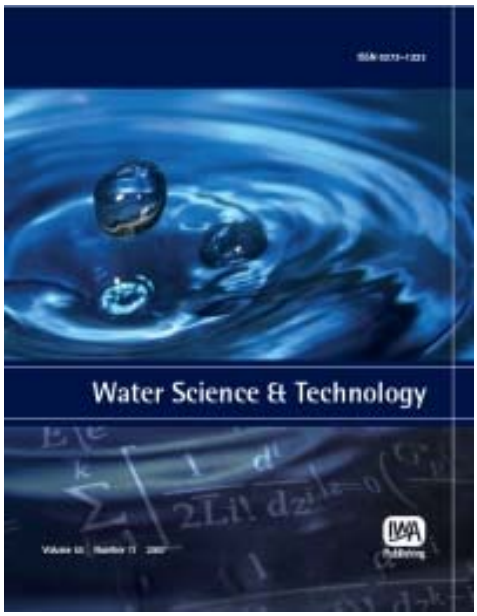

Decolorization of anthraquinone Vat Blue 4 by the free cells of an autochthonous bacterium, *Bacillus subtilis*

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ABSTRACT

Uncontaminated soil, Vat Blue 4 contaminated soil and Vat Blue 4 effluent were screened for heterotrophic bacterial population and the bacterial density were found to be 19.3×10^4 Colony Forming Units (CFU)/gm, 5.5×10^4 CFU/gm and 1.1×10^4 CFU/ml respectively. Student's 't' test analysis affirmed that significant variation prevailed between the three set of 't' tests conducted ($P < 0.001$ to 0.002). The heterotrophic bacterial population of dye contaminated soil comprised of 32.5% of *Pseudomonas* spp. followed by 27.5% of *Bacillus* spp., 15.0% of *Aeromonas* spp., 12.5% of *Micrococcus* spp. and 12.5% of *Achromobacter* spp. The optimum inoculums load, pH and temperature were found to be 5% (10×10^4 counts), 10 and 35°C respectively. Free cells of *B. subtilis* decolorized Vat Blue 4 up to 92.30% after 24 hours of treatment. Total Dissolved Solids (TDS), Biological Oxygen Demand (BOD_5) and Chemical Oxygen Demand (COD) were reduced up to 50.00, 79.60 and 75.40% respectively.

Key words | autochthonous bacteria, *Bacillus subtilis*, decolorization, degradation, Vat Blue 4

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INTRODUCTION

Textile industry plays an important role in the industrial development of India and it is the second largest sector of India's economy next to agriculture. As per the data published by the textile commissioner's office there are 1569 industries in India. Of these 1294 are spinning industries and 275 are composite industries (Series COINDS/59/1999-2000). Dyeing industries are a part of textile industry which involves the dyeing process of garments by means of large consumption of dyestuffs. During the dyeing process a large quantity of water (i.e., 100–200 l/kg of fabric) is used and among that 85 to 95% of water is discharged as waste water. This waste water is highly alkaline having high concentrations of color, TDS, chlorides and sulphates along with Biological Oxygen Demand (BOD_5) and Chemical Oxygen Demand (COD) (Senthilnathan & Azeez 1999). Moreover, majority of the dyes used in the textile industries are difficult to be decolorized (Beydilli *et al.* 2000). There is no universal method for the removal of color from dye waste and the alternatives depend upon the type of dye waste water (McCurdy *et al.* 1992). The characteristics of dye waste water are variable, and many different physical, chemical and biological treatment methods have been employed for its treatment. The effluents are not decolorized by the conventional sewage treatment methods. Various bacteria and fungi are effective in decolorization and hence "bio bleaching" appears to be the only eco friendly and cost effective method to degrade dyes and reduce BOD and COD (Beydilli *et al.* 2000). Manikandan *et al.* (2009) reported two potential strains for bio bleaching of textile dyes. Bacterial strains isolated from the effluent receiving aquatic ecosystems are more effective in degrading the pollutants present in the effluents (Zissi *et al.* 1997). Hence the current study was aimed to isolate few autochthonous bacteria for the complete decolorization of the textile dyes, especially anthraquinone Vat Blue 4.

Vat Blue 4 is one of the most common dye used in the textile dyeing industry to dye silk and cotton and they appear in the dyeing industries effluents of Tamil Nadu. Most of the effluents from textile dyeing industries pollute many of the aquifers, which supply fresh water to the public

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living in and around the dyeing industrial units. Absence of perennial rivers to dilute the dyes to non-hazardous level enhances this problem in Tamil Nadu. Hence biological methods need to be developed in such areas to check the extent of pollution of these dye wastes and their impact on the wastewater receiving aquatic ecosystems. Available literature confirms that the present study on biodegradation of anthraquinone vat dyes is first sort of work and provides first hand information on the degradation of anthraquinone vat dyes commonly used in the dye industry in South India especially Tamil Nadu. Though few studies have been carried out on the biodegradation of the reactive anthraquinone dyes, there is paucity of research on anthraquinone vat dyes. Hence the present study was carried out with the prime objective of investigating the biodegradation of anthraquinone Vat Blue 4 using indigenous bacteria.

MATERIALS AND METHODS

Dye

Navinon Blue, the indanthrone vat dye was discovered by R. Bohn in 1901. It is classified under C.I. Vat Blue 4 and the Colour Index number is 69800. The chemical formula is $C_{28}H_{14}N_2O_4$ and its chemical name is 6,15-dihydroanthrazine-5, 9,14,18-tetrone (www.colourclick.org) (Figure 1).

Analysis of total heterotrophic bacteria and isolation of the dye degrading bacteria

The autochthonous microorganisms were considered to be best suited for bioremediation studies. The effluent discharged from the dyeing unit of Kallidaikurichi of Tirunelveli district, Southern part of Tamil Nadu, India were collected in clean and sterile plastic cans. The soil samples were collected both from uncontaminated and the dye contaminated sites in clean and sterile polythene bags. The samples were kept in an ice box and brought to the laboratory for microbiological analysis. The total heterotrophic bacterial populations of the soil and effluent samples were analyzed by the pour plate technique employing nutrient agar. Morphologically dissimilar and prominent colonies were picked and restreaked on nutrient agar to ensure purity and were identified up to the generic level as per the scheme of Simudo & Aiso (1962). The Bergey's Manual of Systematic bacteriology (1986) was also referred for the identification procedures.

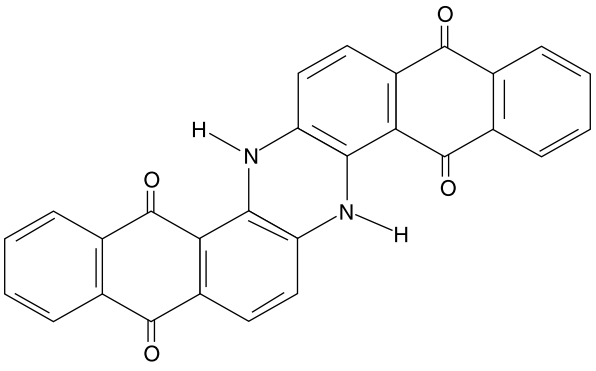

Figure 1 | Structure of Vat Blue 4.

Selection and identification of dye degrading bacteria

The Vat Blue 4 dye was procured from a dyeing industry in Tamil Nadu, India and other chemicals were purchased from Hi-Media Pvt Ltd., India. Different concentrations of Vat Blue 4 (100 to 1,000 mg/l) were prepared and were incorporated individually in the nutrient agar plates. 1.0 ml of the soil suspension was pour plated on the dye incorporated nutrient agar plates and incubated at 37°C for 24 to 72 hours and bacterial growth was observed. The dye degrading bacteria isolated from sample with high concentrations of dye were taken up for further studies. Four bacterial strains of *Bacillus subtilis* were selected based on the efficiency of the dye degradation ability (DS-1, DS-2, DS-3 and DS-4). To evaluate their dye degradation efficiency these isolates were streaked on dye incorporated plates. *B. subtilis,* DS 1 was able to produce clear and distinct zone of clearance of dye around the growth. This strain was selected for further analysis.

Scanning of dye for its absorbance maxima and optimization studies

The absorption maximum for Vat Blue 4 was observed to occur at 650 nm. The degree of decolorization was measured spectrophotometrically by using a UV-Vis spectrophotometer (Bio-Tek Instruments, Milano, Italy).

The nutrient broths (250 ml) incorporated with dye at ten different concentrations (100 to 1,000 mg/l) was inoculated with 24 hour old bacterial culture of DS-1 at 5% (v/v) concentration and incubated at 37°C for 48 hours. Aliquots were withdrawn from the culture broth at 12 hours intervals and centrifuged at 10,000 rpm for 10 min in a tabletop centrifuge and the bacterial cells were removed as pellets. The supernatant was analyzed spectrophotometrically at 650 nm and the decolorization was calculated using the following formula described by Sani & Banerjee (1999):

$$\text{Percentage of decolorization} = \frac{\text{Initial absorbance value} - \text{Final absorbance value} \times 100}{\text{Initial absorbance value}}$$

As a preamble to use DS-1 for decolorization experiments, optimization studies with regard to pH, temperature and inoculum load were also carried out.

Standardization of inoculum load

Five different sets of nutrient broth (250 ml), each incorporated with nine different concentrations from100 to 900 mg/l of Vat Blue 4 was prepared separately. Each set was inoculated with different amounts of inoculums ranging from 1 to 5%. The flasks were incubated at 37°C for 72 hours. At 12 hours interval, the decolorization was monitored spectrophotometerically, using uncoloured broth as control. The optimum amount of inoculum capable of producing maximum percentage of decolorization was taken as standard for further analysis. Further enhancement of inoculum was not attempted in the present study owing to the drastic reduction of pH (<6.9), which may not permit the growth of many commercially important fin and shellfishes.

Determination of optimum pH

The pH of the seven different sets of 250 ml nutrient broths were incorporated with 300, 500 and 700 mg/l concentrations of the dye separately were adjusted to pH 4, 5, 6, 7, 8, 9 and 10 using 0.1 N HCl and NaOH and were inoculated with 5% of 24 hour old bacterial culture of DS-1. All the seven sets were incubated at 37°C for 72 hours. Aliquots were withdrawn from the culture broth at a regular interval of 12 hours and analyzed for decolorization. The optimal pH was assessed on the basis of maximum decolorization.

Determination of optimum temperature

Four different sets of sterile 250 ml nutrient broths were incorporated with 300, 500 and 700 mg/l of Vat Blue 4 and inoculated with 5% of 24 hours old bacterial culture. Each set of inoculated flasks was incubated at different temperatures of 30, 35, 40 and 45°C. The optical density was measured at 12 hours interval up to 72 hours, using uninoculated broth as control. From the optical density, the percentage of decolorization was calculated. The temperature, which affected maximum efficiency of the strain DS-1, was used as another important environmental parameter.

Biodegradation of Vat Blue 4 effluent by free bacterial cells

The wastewaters from the dyeing industry consist of varying concentration of dyes (mg/l). The isolated bacteria were able to grow even in 1,000 mg/l concentration of dyes. Effluent containing Vat Blue 4 was collected from the dyeing unit for conducting bioremediation studies. The effluent was a mixture of anthraquinone dye, inorganic salts and water. Decolorization of Vat Blue 4 by free cells of DS-1 was investigated. The Vat Blue 4 was incorporated with experimental waste water to get the optimum dye concentration (900 mg/l) and no carbon source was added that as an additional source for bacteria. The 24 hours old culture of DS-1 at the concentration of 5% (i.e., 5 ml/ 100 ml; consisting of 10×10^4 counts) was inoculated into the effluent prepared. The physico-chemcial parameters like pH, TDS, BOD_5 and COD of both the raw and treated effluents were assessed using the standard procedures (APHA 1995). The rate and extent of color removal were analyzed after 24 hours adopting the standard procedures of Sani & Banerjee (1999).

Table 1 | Microbial density of both contaminated and uncontaminated sediment soil with special reference to bacteria

No	Uncontaminated soil (CFU/gm × 10^4)	Mean value of CFU/gm × 10^4 in un-contaminated soil	Contaminated soil (CFU/gm × 10^4)	Mean value of CFU/gm × 10^4 in contaminated soil	Effluent (CFU/ml × 10^4)	Mean value of CFU/ml × 10^4 in effluent
1	19.4	19.3 ± 1.25	6.3	5.5 ± 0.917	1.2	1.1 ± 0.01
2	20.5		4.5		1.0	
3	18.0		5.7		1.1	

RESULTS

Isolation and identification of dye degrading bacteria

The values of total heterotrophic bacterial population of the uncontaminated soil, dye contaminated soil and effluent were determined by adopting pour plate technique (Table 1). With the view of understanding the distribution of the heterotrophic bacteria in the study area, comparison was made between the uncontaminated and contaminated soil, between uncontaminated soil and effluent and between contaminated soil and effluent. Student's 't' test analysis affirmed that significant variation prevailed between the three set 't' tests conducted (Table 2). In the present study, a total number of forty bacterial strains were isolated and identified from the dye contaminated soil sample. The bacterial genera of dye contaminated soil were dominated by 32..5% of *Pseudomonas* spp. followed by 27.5% of *Bacillus* spp., 15.0% of *Aeromonas* spp., 12.5.0% of *Micrococcus* spp. and 12.5% of *Achromobacter* spp., as shown in Figure 2. In the present study, anthraquinone dye resistant bacterial strains were isolated using nutrient agar incorporated with Vat Blue 4 dye at various concentrations ranging from 100 to 1,000 mg/l and the results are furnished in Table 3. Among the four strains, DS-1 exhibited normal growth up to 900 mg/l concentration and it exhibited minimum growth at 1,000 mg/l and produced distinct zone of clearance of dye around their growth up to 1,000 mg/l and it was identified as *Bacillus subtilis*.

Optimum inoculum load

The optimal amount of inoculum required for the degradation of Vat Blue 4 was determined. Among the different inoculum concentrations, 5% inoculum was able to bring about the maximum decolorization of Vat Blue 4 dye. Within 72 hours of inoculation at the amount of 5%, total decolorization was observed up to 900 mg/l of Vat Blue 4.

Optimum pH

Among the various pH tested (4, 5, 6, 7, 8, 9 and 10) DS-1 had the maximum decolorization at pH 10 for all the three concentrations such as 300, 500 and 700 mg/l (Figure 3) tested. At pH 10, DS-1 brought about 100% decolorization at 300, 500 and 700 mg/l after 12, 24 and 36 hours respectively.

Optimum temperature

The temperature optima of the efficacy of DS-1 strain in dye degradation of various concentrations such as 300, 500 and 700 mg/l were assessed in 30, 35, 40 and 45°C temperatures and the results were presented in Figure 4. The highest rate of decolorization was found to be at 35°C and complete degradation of Vat Blue 4 could be recorded at 24 hours.

Biodegradation studies

Data presented in Table 4 depict the ability of biodegradation and decolorization of anthraquinone dye by the free

Table 2 | Comparison of microbial density between uncontaminated and contaminated soil along with that of effluent

No	Variable 1	Variable 2	df	't' value	Level of significance
1	Mean microbial load in soil (Uncontaminated)	Mean microbial load in soil (Contaminated)	4	15.397	$P < 0.001$
2	Mean microbial load in soil (Un-contaminated)	Mean microbial load in effluent	4	25.079	$P < 0.001$
3	Mean microbial load in soil (Contaminated)	Mean microbial load in effluent	4	8.266	$P < 0.002$

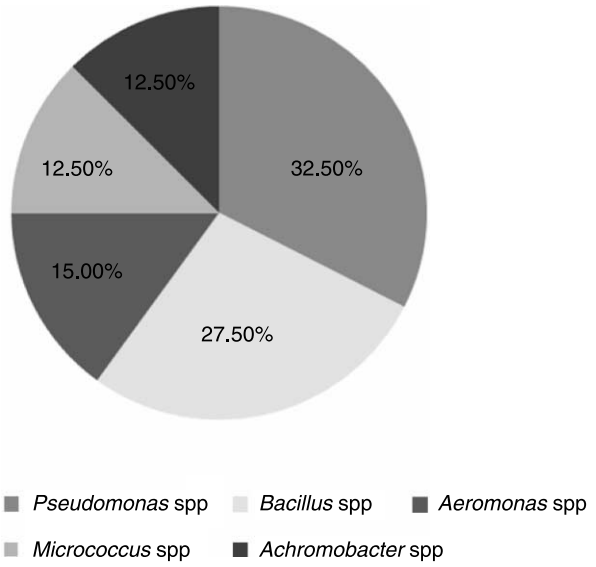

Figure 2 | Percentage prevalence of different bacterial genera of the dye contaminated soil.

cells of *B. subtilis*. The initial pH of the effluent prepared using Vat Blue 4 was 8.5. The free cells of DS-1 reduced the pH to 7.46 after 24 hours of treatment.

DISCUSSION

The mixed bacterial cultures for bio bleaching were isolated from the effluent of dyeing industries and sludge discharge site of the effluent treatment plants of dyeing industries. Forty bacterial strains were isolated and identified in the present study from the dye contaminated soil sample. The bacterial genera of dye contaminated soil were dominated by 32..5% of *Pseudomonas* spp. followed by 27.5% of *Bacillus* spp., 15.0% of *Aeromonas* spp., 12.5.0% of *Micrococcus* spp. and 12.5% of *Achromobacter* spp. Mariappan *et al.* (2003) recorded an appreciable amount of Total Heterotrophic Bacterial Population (THBP) in the azo dye contaminated soil ranging from 13.2×10^7 to 32×10^7. A higher number of total viable bacterial count (8×10^6 CFU/ml) was reported for the azo dye-contaminated water of river Bhadar of India by Doctor *et al.* (1998). The occurrence of high bacterial load in the dye-contaminated soil may be either due to the enrichment of the dye degradable population or the dilution of the dye which might have lowered the toxicity of the dye. Sharma *et al.* (2004) collected various soil and sludge samples from the vicinity of textile dyeing industries and waste disposal sites and identified five bacterial isolates belonging to the genera of *Bacillus*, *Alkaligenes* and *Aeromonas*. From the dye contaminated water of river Bhadar different bacterial species such as *E. coli* (80.0%), *Pseudomonas aeruginosa* (15.0%) and *Klebsiella pneumoniae* (5.0%) were identified by Doctor *et al.* (1998). Manikandan *et al.* (2009) reported Pseudomonas *pudita and B. subtilis* as potential strains for biobleaching of textile dyes.

The necessity of optimization experiments especially in dye degradation was formerly reported by Vyas & Molitoris (1995). In the present investigation, the maximum inoculation attempted was 5% (5 ml/100 ml). The DS-1 had the maximum decolorization at pH 10. Boyd & Pillai (1984) reported that pH ranging from 6.5 to 8.5 was essential for the survival and growth of most of the cultivable fishes. Hence, the maximum limit of 5% was not further enhanced in the bioprocessing of the effluent as the higher inoculum concentration was found to decrease the pH below 6.9 (data not shown). In the case of temperature, highest rate of decolorization was found to be at 35°C and complete degradation of Vat Blue 4 could be recorded at 24 hours. Grigsby *et al.* (1996) reported an enhanced degradation of azo dyes at 37°C by native micro flora. Mariappan *et al.* (2003) recorded maximum degradation of the azo dye orange–G by *Pseudomonas* sp SACO1 at 37°C and

Table 3 | Total dye resistant bacterial population in the dye contaminated sediment samples

Concentration of Vat Blue 4 (mg/l)	Bacterial load (CFU/gm × 10^4)
100	TNTC
200	310
300	240
400	180
500	140
600	70
700	25
800	14
900	2
1000	1

TNTC—Too Numerous To Count.

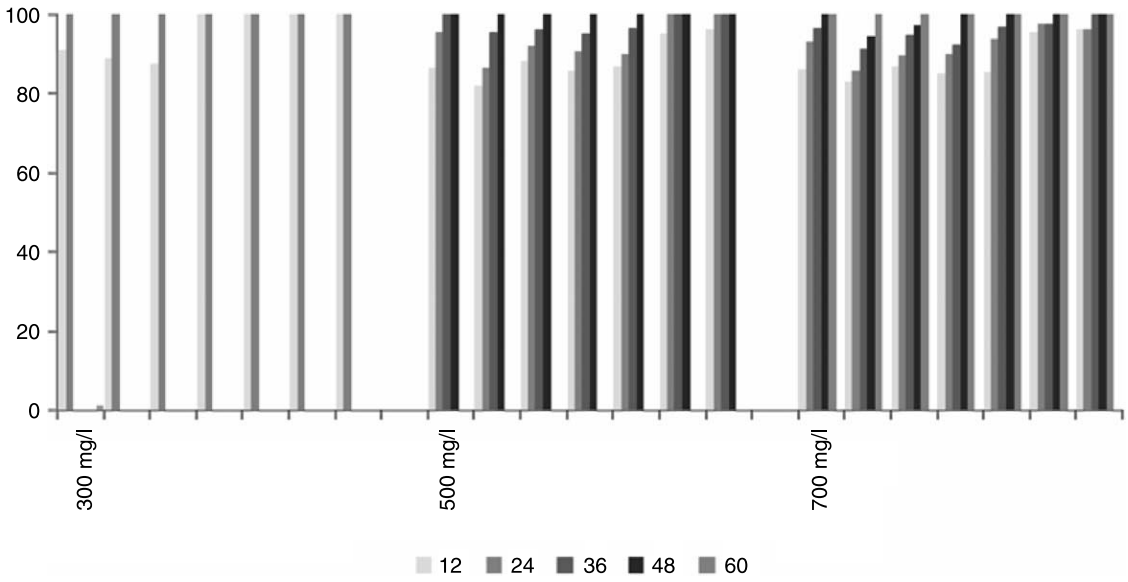

Figure 3 | Decolorization of Vat Blue 4 by DS 1 at different time intervals and at different pH.

Escherichia sp SACO 2 at 44°C. Zimmermann *et al.* (1982) also employed neutral pH for using *Pseudomonas* KF 46 to degrade azo dyes. Similar pH dependent dye degradation efficiency of *Bacillus* sp. and *Pseudomonas* sp. were reported by Mariappan *et al.* (2003). They reported that the maximum decolorization was brought about by *Bacillus* sp., at a pH of 10 and by *Pseudomonas* sp., at pH 8.

Among the several isolates DS-1 was found to be the potential strain with synergestic mode of bio bleaching the colored effluents. Sani & Banerjee (1999) effected the decolorization of synthetic effluents of azo dye by growing cells of *Kurthia* sp. in case of Magenta, nine-hour-old broth of *Kurthia* sp. was used for decolorization. Kulla *et al.* (1983) reported that the free cells of *P. aeruginosa* brought about 100% decolorization of Orange I at 40 hours and Orange II at 72 hours at 50 to 100 mg/l concentrations. *P. alkaligenes* and *P. mendocina* have been reported to degrade 4 di and tri phenyl methane up to 78% in 15 hours at a concentration similar to that of the real effluent (Sarnaik & Kanekar 1999). At a concentration of 10 mg/l

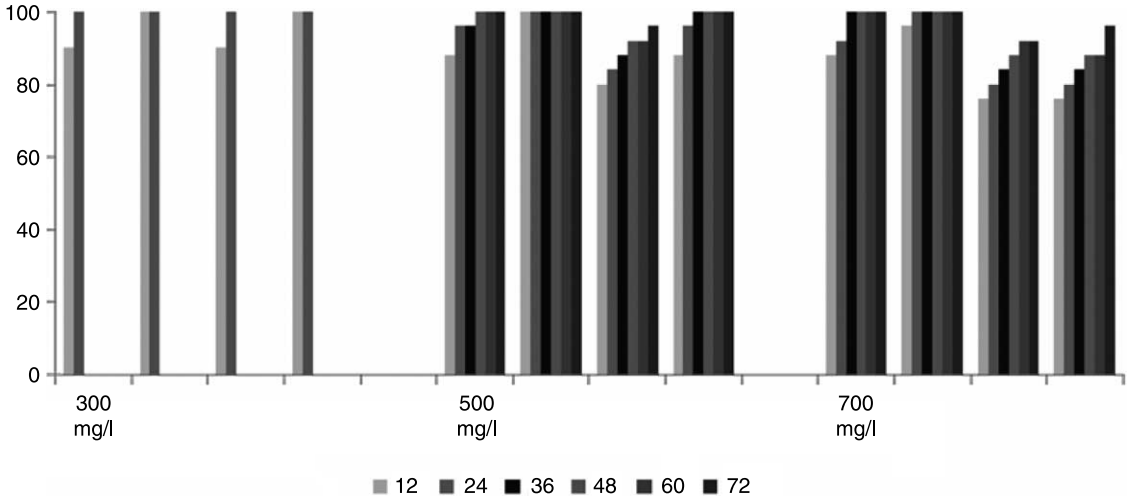

Figure 4 | Decolorization of Vat Blue 4 by DS 1 at different time intervals and at different temperature (°C).

Table 4 | Decolorization of effluent of anthraquinone dye Vat Blue 4 by free cells of *B. subtilis* (DS 1) (No. of samples 5)

Parameters	Control	Treated with B. subtilis (Mean value)	Extent of reduction (%)
pH	8.5	7.46	87.76
TDS* (mg/l)	3215	1610	50.07
BOD5* (mg/l)	3670	2920	79.56
COD* (mg/l)	8254	6220	75.36
Color (optical density value)	1.95	0.15	92.30

*In the case of TDS, BOD_5 and COD the quantities removed after treatment are furnished.

C. I. Acid Orange 12 and C. I. Acid Orange 20 have been reported to be decolorized by free cells of *P. cepacia* 13 NA in eight hours under anaerobic condition (Ogawa *et al.* 1986). Manikandan *et al.* (2009) reported 80-90% of decolorization of textile dyes by two potential strains such as *Pseudomonas pudita and Bacillus subtillus.* Wuhrmann *et al.* (1980) reported that the free cells of *Bacillus cereus* effected appreciable color removal of 78% of nine azo dyes within 15 hours under anaerobic conditions. Various azo dyes at a concentration of 0.1 mM have been observed to get anaerobically degraded up to 90% within 8 to 20 min by *P. stutzeri* (Yatome *et al.* 1990). In reactors, COD removal of 92.00% of orange dye and 94.00% of black dye have been registered by Manu & Chaudhari (2002). In the present study COD and colour removal was reduced and Georgiou *et al.* (2005) reported 8 to 40% of COD reduction in anaerobic process adopted by *Pseudomonas* sp. COD removal of 84% in Congo red and 85% in Direct Black within five days ws reported by Isik & Sponza (2003). Manikandan *et al.* (2009) reported 2-3 fold decrease of BOD and COD during biobleaching by *P. pudita* and *B. subtilis.* Sani & Banerjee (1999) recorded 90% of COD reduction. Under anaerobic treatment condition along with the usage of a fluidized bed reactor, Sen & Demirer (2003) recorded 82% reduction of COD, 94.50% of BOD_5 and 59% reduction of color. Most of the research works conducted so far with textile dyes were mainly concerned with BOD, COD and color removal. However, there is brevity of information in these studies regarding the fate of reduction of TDS and pH. The permissible pH limit for the discharge of textile industry effluent is to the tune of pH 9 (Lee 2003). It warrants mention here that during the process of biodegradation this pH limit was never observed to surpass the stipulated hyper pH condition.

CONCLUSION

The prelude inquiry on the occurrence of anthraquinone vat dye resistant bacterial population and their decolorization potential provide vital basic data on the use of native micro flora in anthrquinone dye degradation. The significant role of autochthonous bacteria in bioremediation has been emphasized in the present study. Thus, *B. subtilis* DS-1 isolated in the present study affirmed as a promising contender for the biodegradation of dye industry effluent. Further studies on analytical aspects of the biotransformation and fate of metabolites would make it possible to utilize those bacteria for bioremediation of Vat Blue 4 polluted habitats.

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