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Exploring indigenous bacterial isolate from dye effluent sites for decolorization of Blue HERD dye

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ABSTRACT

Dye contaminated sites are the efficient source for isolating bacteria capable of dye decolorization and degradation. The effluent and soil samples were collected from industrial zone where such effluents are discharged. Collected samples were enriched in nutrient media containing 100 mg/l Blue Herd dye. Isolate was selected based on zone of clearance on soild media containing dye. Based on biochemical and 16S rDNA comparisons the isolate is identified as *Bacillus subtilis UAS1*. The isolate showed maximum decolorization at 37 °C temperature, pH 7.0, capable of removing 92% dye at 20 mg/l concentration and significantly remove at higher subsequently added dye concentration up to 200 mg/l. The decolorization was highest in presence of Sodium Molybdate, less with Tungastate, Silver Chloride, Zinc Sulphate, whereas no decolorization in presence of Copper sulphate. The strain *B. Subtilis* UAS1 also exhibits significant reduction in the COD values; this suggests its potential and significance in dye removal.

Key words: *Dye decolorization, Blue Herd dye, Bacillus subtilis* UAS1.

Introduction

Textile industry is a rapidly emerging industrial sector in India. The textile industry uses different raw materials such as cotton, woolen and synthetic fibers (Holkar *et al*., 2016). Dyes are used for various colouring purposes in textiles, leather, paper, food, cosmetics, medical treatment and analysis (Godlewska *et al*., 2012). Use of dyes is one of the largest industries that increase the environment pollution (Feng *et al.,* 2014). Global contamination by release of large amount of potentially toxic, mutagenic agent and xenobiotic compounds has created very serious problems for survival of existing living beings (Pandey *et al*., 2015). These are a significant source of pollution and affect on aquatic life; however, their recalcitrance makes their removal using conventional wastewater treatment plants difficult (Garcia *et al.,* 2013). The discharge of azo dyes into the water bodies leads to problems, this majorly affects light penetration and oxygen transfer into water bodies, thus influencing the aquatic life (Hussain *et al.,* 2013).

Azo dyes are a type of synthetic dyes characterized by one or more azo groups (–N N–). Near about 50% dyes produced annually are azo dyes and approximately 1 million tons of dyes are produced throughout the year. The most important chromophores in azo dyes are carbonyl, nitro and quinoid groups. Azo and anthraquinone are the most important chromophoric groups from which textile dyes are synthesized (Holkar *et al.,* 2014). There are numerous methods used for treatment of dye containing effluents. The physical and chemical methods associated with formation of the secondary toxic by-products (Hadibarata *et al.,* 2014). Textile wastewater hashigher BOD, COD value, as well as colour of water body and toxicity. So the major con-

cern for the treatment, is removal of colour i.e. either adsorption removal or breakage of chromophore group.

Nowadays microbiological methods are gaining more importance because they are cost saving, effective and ecofriendly. The products of degradation are mostly non toxic or comparatively less toxic to nature. Soil comprises huge diversity of microorganisms and is a resource for variety of microorganisms performing diverse functions. The bacteria from area where effluents are being discharged can be a potential resource of bacteria capable to withstand and utilize effluent components. With this context in present study *Bacillus subtilis* UAS1 isolate from effluent soil explored for removal of Blue Herd dye in laboratory condition and suggest its suitability for textile effluent treatment.

Materials and Methods

Sample collection

The dye containing wastewater samples were collected from two distinct points of Solapur MIDC where effluents of various textile industries are discharged. The samples were collected in pre-sterilized plastic bottles and soil samples were collected in zip locked plastic bags. The samples were brought to the laboratory and processed within 24 hrs or otherwise stored at 4 °C for further use.

Chemicals and Culture Medium

The media chemicals used in the study were procured from Hi Media laboratories.

Isolation and screening of azo dye degrading bacteria

Isolation of the Blue Herd (BH) decolorizing bacterial isolates was carried out in nutrient broth medium containing (g/l): Peptone (5.0), Nacl (5.0) Yeast Extract (2.0) Beef Extract (1.0) with pH-7.0, supplemented with Blue Herd (BH) dye (100mg/lit). A 100 ml medium in separate 250 ml flasks was mixed with sample in ratio, sample and nutrient medium (1:9) whereas for soil 1gm was added to 100 ml nutrient medium and mixed properly and incubated at 30 °C. After visible turbidity 10 ml of this was transferred to a fresh 90 ml of nutrient medium containing BH and incubated under same conditions. After 2 cycles of such enrichment, 100 µl broth culture was inoculated on nutrient agar plate containing BH (100 mg/l) and incubated at 30°C. Colonies obtained after incubation were resuspended into the nutrient brothwith BH dye and incubated at 30 °C under static conditions, aliquots 10 ml were regularly taken, at 24, 48 and 72 hrs interval, centrifuged (6000 rpm, 10 min) and subjected to spectrophotometric detection (Agilent Carry60 UV) at 610 nm to detect the decolourization. The extent of decolorization was calculated as below:

Formula

$$\% \text{ Dye decolorization} = \frac{(\text{Absorbance in control} - \text{Absorbane in test})}{\text{Absorbance in control}} \times 100$$

Absorbance in control: absorbance obtained for uninoculated media containing dye.

Absorbance in test: absorbance obtained for inoculated media containing dye after incubation.

Bacterial Identification

The isolate was identified based on morphological, cultural, biochemical and molecular characteristics. The phylogenetic description was determined using 16S rDNA nucleotide sequencing at National Centre for Microbial Resource (NCMR), National Centre for Cell Science, Pune. For the phylogenetic identification, the total genomic DNA of isolate was obtained as per Sambrook *et al.* (1989). The 16S rRNA gene was amplified from the total chromosomal DNA using universal specific primer. The 16S rRNA nucleotide sequence obtained was compared with database sequences by using BLASTn programme available at NCBI server(http://www.ncbi.nlm.nih.gov/BLAST). The closely related sequences obtained in Blast search were selected and used for phylogenetic tree using MEGA5. The nucleotide sequence of isolate was submitted to the GenBank database to obtained accession number.

Effect of physico-chemical parameters on decolorization

The effect of physicochemical factors was determined as per one variable at a time (OVAT) approach. The effect of parameters on dye decolorization was studied by preparing and incubating medium at different conditions such as temperature 20-60 °C, static and shaking condition, pH (4.0-10.0), dye concentration (10-100 mg/l), media components (various nitrogen and carbon sources), inoculum size (1-10%) and metals. The potential of the isolate to decolorize reactive azo dye i.e. Blue Herd was

assessed in separate liquid medium with 20 mg/l dye concentration respectively.

COD Determination

The decolorization medium was subjected to Chemical Oxygen Demand analysis at different time intervals. COD was determined using Hatch COD reactor for this a reaction was prepared in hatch tube containing 1 ml of $K_2Cr_2O_7$, 3 ml of $AgSO_4$ with H_2SO_4 and a pinch of $HgSO_4$. After incubation of 24 hrs for given isolate in dye containing medium, the contents were centrifuged at 8000 rpm for 10 minutes to remove cell growth from the medium. The supernatant, 2ml with appropriate blank (media without dye) was added to the COD reaction. The mixture was kept in COD reactor at 150 °C for 90 minutes for complete oxidation. Then reaction volume made to 15ml by adding 9 ml distilled water. This was further titrated against ferrous ammonium sulphate using ferroine indicator. The chemical oxygen demand (COD) was determined as per the standard methods for the waters and wastewaters (APHA, 1995).

Effect of Metals

Effect of various metal ions over decolourization was studied at 1mM metal concentration in nutrient medium containing 20 mg/l dye. The selected isolate was supplemented with (1mM/100 ml Conc.) Ag^{+2} (as Silver Chloride), Zn^{2+} (as Zinc Sulphate), Cu^{2+}(as Copper Sulphate) (as Sodium Tungastate) and Sodium Molybdate. The pH of the growth medium was 6.5 and it was incubated under static conditions at 37 °C. The same medium without inoculum was kept as Acontrols. The aliquot (5 ml) of the culture media was withdrawn at different time intervals; centrifuged at 8000×g for10 min. Decolorization was measured by noting the absorbance of supernatant at 610 nm.

Repeated dye addition

The effect of repeated dye addition on the dye decolourization by *Bacillus subtilis* UAS1 was studied in a sequentially batch reactor way. The nutrient medium containing 20 mg/l dye was inoculated after 24 hours' time interval decolorization of the dyewas measured and then dye (20 mg/l) was repeatedly added to a batch of culture (100 ml). The percentage dye decolorization and time were noted after each cycle dye addition.

Statistical analysis

The reported results are the average value obtained from three independent experiments. Statistical analysis was performed using Microsoft Excel.

Results and Discussion

Total 12 morphologically distinct colonies were isolated from effluent and soil sample. Isolates were selected based on their ability to remove dye in solid media detected in terms of zone of clearance formation on dye containing solid media, the decolorization potential of each isolate was further examined in dye containing liquid nutrient broth (Guadie *et.al* 2017). The isolate UAS1showed highest decolorization amongst the obtained distinct colonies and thus selected for further study.

Bacterial Identification

Based on maximum decolorizing potential for azo dyes the isolate was selected, identified by morphological, biochemical and 16s rRNA gene sequencing. The 16s rDNA gene sequence comparison with database sequences using Blastn program available at NCBI server showed 99% similarity with *Bacillus subtilis*. The sequence was submitted to Genbank database and assigned with accession number MW927221. The closely related sequences obtained in blast search were retrieved and used for describing phylogenetic relationship. Phyogenetictree of the 16 SrDNA sequence is as shown in fig. 1.

Effect of physico-chemical parameters on decolorization

Effect of Temperature

Temperature is one of the most important factors affecting the growth and activity of microorganisms. The decolorization of BH by isolate *Bacillus subtilis UAS1* was studied at various temperatures (30,37,45 and 55 °C) similar to reported studies (Karunya *et al.* 2014). The maximum of decolorization was found to be at 37°C which decreased slowly towards lower temperature where as the decrease was rapid at higher temperatures.

Similar reports were seen by Karunya *et al.* (2014) with few individual and mixed bacterial cultures. Several studies reported a higher decolorizationinin the range 30 to 40 °C, below and above to this temperature, growth along with extent of decolorization

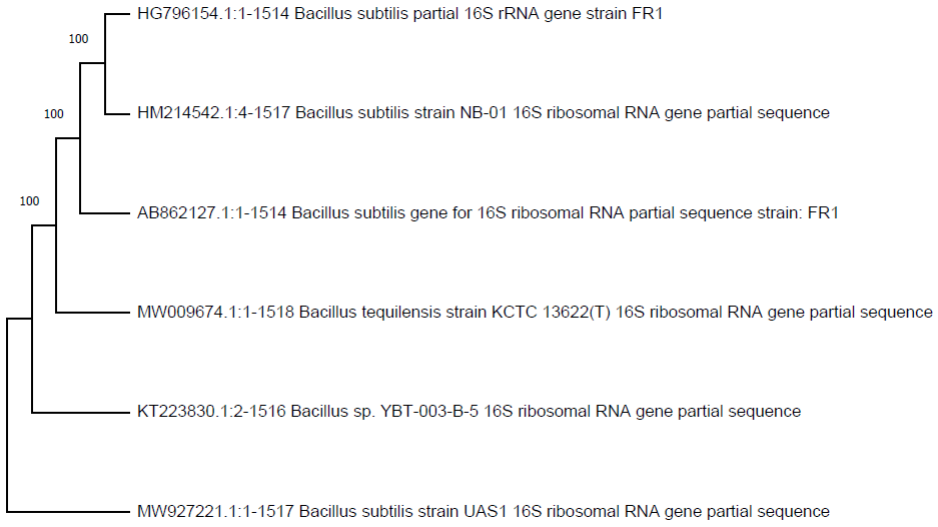

Fig. 1. Phylogenetic Tree of the strain *B. subtilis* UAS1

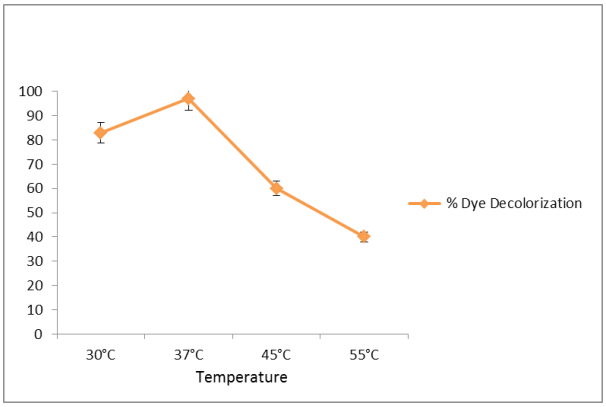

Fig. 2. Decolourization dye by *B. subtilis UAS1* at various Temperature

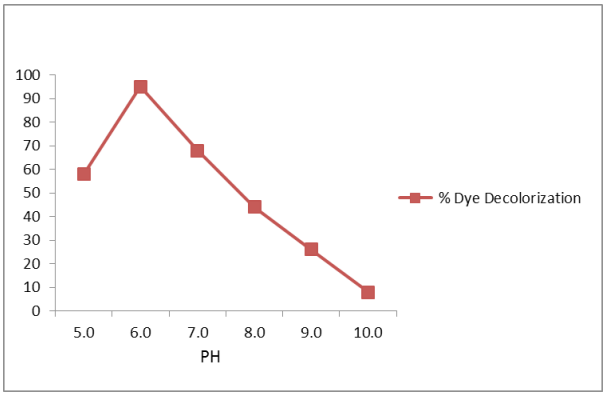

Fig. 3. Decolourization of dye by *B. subtilis UAS1* at various pH

found suppressed (Guadie *et al.* 2017). Maximum decolorization for the used dyes was observed at 35 °C ± 2 and least decolorization was observed at 45 °C (Chawla *et al.* 2014; Roat *et al.* 2016). The results of present study for Blue Herd dye are in accordance to the other studies for *Bacillus subtilis UAS1* for other dyes (Kumar *et al.*, 2015).

Effect of PH on dye decolorization

Initially with the increase in pH from 5.0 to 7.0, decolorization efficiency increased and maximum occurred at pH 6.0. Further increase in pH from 7.0 to 9.0 showed negative effect on decolorization. Some studies suggest maximal decolourization by bacteria at acidic pH (Kilany, 2017) and similar results found in this study, the isolate *Bacillus subtilis UAS1* showed maximal decolourization at pH 6.0.

Effect of Different Media Components on dye decolorization

The effect of different sugars and organic components for dye decolorization activity such as Glucose, Mannitol, Maltose Fructose, Malt extract, Peptone, Meatextract, Beef Extract and Yeast Extract, were tested and Beef Extract, Yeast extract, Meat Extract, Peptone, and Malt Extract were shown good decolorization. The concentration of these components was 1g/l with 20 mg/l concentration of BH dye. Amongst these components Beef extract shown higher decolorization followed by yeast extract, Meat extract and Peptone but in presence of Sugars there is no decolorization activity. Results of this

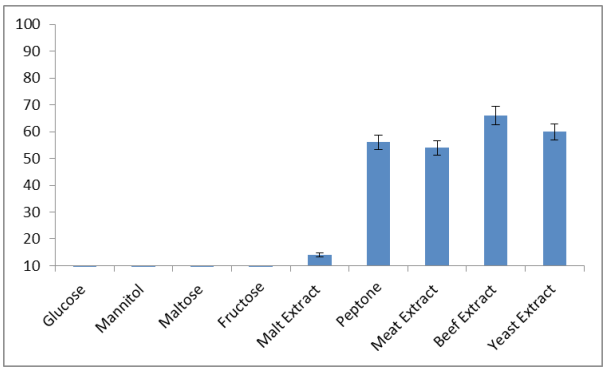

Fig. 4. Effect of Different media Components on decolourization dye by *B. subtilis UAS1.*

study are in accordance to already reported studies. Similar results are reported about the different carbohydrates and organic compounds for decolorization, organic compounds showed higher decolorization than the sugars (Birmole *et al.,* 2019).

Effect of Dye Concentration on dye decolorization

The dye concentration directly affect the rate of dye removal by various microorganisms, this have been studied by a number of researchers. The increase concentration results in more time required for decolorization and prolonged high concentration may retard the decolorization. The enzyme(s) to function efficiently at very low concentrations that may be present in some textile waste water (Kalme *et al.,* 2006).

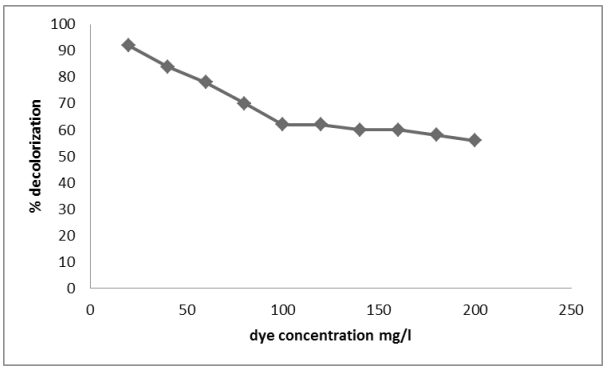

Fig. 5. Decolourization performance of *B. Subtilis UAS1* at different dye concentration

In this study maximum decolorization was found at 20 mg/l (92%) dye concentration whereas lowest at 200 mg/l (56%).

Effect of Static and Shaking Condition on decolorization

The growth of the isolate *B. subtilis UAS1* was higher in shaking condition as compared to static condition. At static condition though the growth is less but decolorization i.e. 92% was noted whereas shaking condition showed very less decolorization (Fig. 1). According to Ladde *et al.* (2014) the increase decolorization rate in static condition might be due to the involvement of enzyme azoreductase which functions well in decolorization. The result of this study are similar tomost of the bacterial decolorization where no decolorization was observed at aerobic conditions (Khehra *et al.*, 2005; Dawkar *et al.*, 2008). Azo reductase is the key enzyme responsible for the reductive cleavage of azo dyes, and the presence of aerobic condition inhibits its azo bond reduction activity (Ladde *et al.,* 2014). Thus the decolorization in static condition is higher in comparison to shaking condition though growth of microorganism is more at shaking than static condition (Kalme *et al.,* 2006).

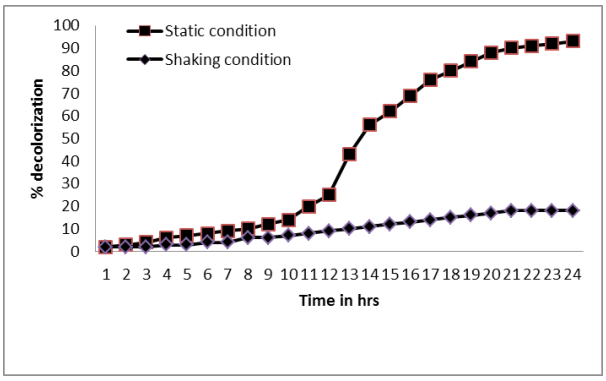

Fig. 6. Decolourization performance of *B. subtilis* UAS1 at Static and Shaking

Effect of Metals on dye decolorization

Textile effluents often contain some amounts of heavy metals which make them more toxic. Some metals ions can be beneficial to the microorganisms but some can be harmful. The direct impact is on the proteins or enzymes because these forms complexes with protein molecules which render them inactive, in some cases they have positive effect. *Bacillus subtilis UAS1* was grown in the presence of different heavy metals; Copper sulphate, Sodium Tungstate, Silver Chloride and Sodium Molybdate (1mM/100 ml) in the nutrient medium containing 20 mg/l BH dye to evaluate their effects on decolorization. The

results obtained showed an increased decolorization in presence of Sodium Molybdate and Sodium Tungastate, a positive effect where as for other used metals there is less or no decolorization (Fig. 7).

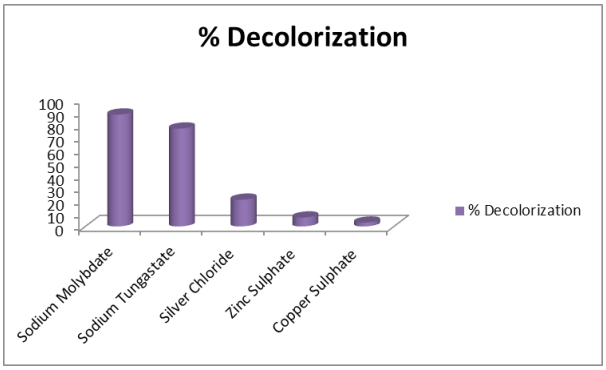

Fig. 7. Effect of Metals on Dye decolorization by *B. subtilis UAS1.*

Effect of Inoculum size

The number of growing cells has profound influence over the activity. The effect of different inoculum size of *Bacillus subtilis* UAS1 on the decolorization of Blue herd dye 20 (mg/l) indicated that the increase in the inoculum size from 1-10% progressively accelerate the decolorization process. Shankara *et al* reported that increase the inoculumn size decrease the time for decolorization process (Shankara *et al.,* 2020). There is similar percent decolorization obtaind at each inoculum (1 t 10 %) only the time required decreases with increase in inoculum.

Dye decolorization with COD reduction

The pollutant can be measured in terms of COD, in present study a treatment to synthetic preparation with BH dye 20 mg/l by *Bacillus subtilis UAS1* for 24 hrs showed significant decrease in the COD values.

Table 1. COD Reduction

Time	COD reduction in %	Decolorization%
24 hrs	64	92
48 hrs	73	93
72 hrs	79	94

The COD reduction was 64% found after 24 hr and further reduce upto 79% after 72 hrs treatment. Various studies with different organism reveal similar reduction with industrial effluents (Kornaros and Lyberatos (2006), Jadhav *et al.* (2010) reported 80% removal of COD during biological treatment of wastewaters from a dye manufacturing company. also showed the reduction in COD. Bacterial treatment to various organic components after utilization or transformation shows reduced COD values, a significant decrease of more than 60% in COD can be found in 24 hours of treatment, this highlights the significance of the isolate obtained in this study. Similar results for Orange red dye by bacterial consortium (Jagwani *et al.,* 2013) and RBHRD by *Comamonas* sp.

Repeated dye Addition

Repeated dye decolorization of BH was studied under optimized condition (Jadhav *et al.,* 2010). The intention of this study was to evaluate the efficiency of bacterial isolate of this study to decolorize BH dye by repeated addition of 20 mg/l of BH dye at interval of 24 hrs. The extent of decolorization further detected after 24 hrs time interval, the result obtained are quite promising as shown in Fig. 8. The reduction in the decolorization during repeated dye addition may be due to the toxicity of dye or lesser cell growth for uptake of increased dye concentration (Sumathi and Manju, 2000). Though there is decreased decolorization with increase in cycle for dye addition but the decolorization obtained at higher concentration suggest that isolate is capable to withstand and decolorize dye for longer duration. This is in accordance to other studies for different organism (Chang and Kuo, 2000; Jadhav *et al.,* 2011).

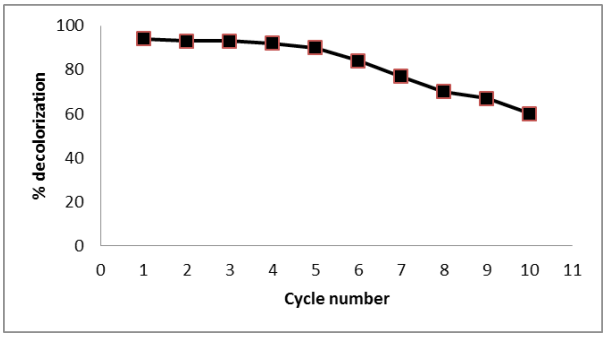

Fig. 8. Decolorization performance of *B. subtilis* UAS1 after each cycle with repeated dye addition (20 mg/l).

Conclusion

The selected isolate in present study identified as *Bacillus subtilis* UAS1. It showed maximal removal of removal of dye 20 mg/l at 37 °C and pH 6.0. The *Ba-*

cillus sp is reported for decolorization potential; in this study we reported strain from industrial site and for BH dye decolorization. The isolate also showed significant decrease in COD in laboratory nutrient medium conditions. It is capable of 90 % removal in repeated dye addition. Thus can be a potential source for further use and study.

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