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Bioremediation of Recalcitrant Textile Azo Dye - Methyl Orange by *Bacillus subtilis* BRTSI-3 Isolated from Textile Effluents

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A B S T R A C T

Textile effluent samples collected from dye contaminated area of Ranipet, Vellore have been used for isolating bacterial strains. Among various bacterial isolates, BRTSI-3 was selected and was further characterized using morphological and biochemical analysis. 16S rDNA sequencing confirmed the strain BRTSI3 as *Bacillus subtilis* (NCBI accession number MH412808). The culture conditions for maximizing bacterial biomass were found to be optimized at 35^{o}C and pH 8.0. *Bacillus subtilis* effectively decolorized methyl orange in nutrient broth within 48 h of incubation. Spectrometric methods such as UV- Vis spectrophotometry and FTIR were used for assessing the decolorization extent of methyl orange by BRTSI3. FTIR results confirmed the breakdown of methyl orange by bacterial metabolites. The investigation proved that the microorganisms found in textile effluent are capable of decolorizing and degrading the azo compounds of textile effluent.

Introduction

Colors play a major role in day to day life. To fulfill the need of the customers, commodities are colored in different shades and patterns. Dyes and dyestuffs were used to impart color in pharmaceutical, textile and food industries (Ayed *et al.*, 2011). More than 10,000 dyes are commercially available in the market. About 60 % of commercially available dyes are azodyes. Azo dyes are distributed in three different classes namely monoazo, diazo and triazo (Weber and Adams, 1995). These dyes are considered to be highly recalcitrant molecules, as they are very difficult to degrade by microorganisms and as a result, pose a serious threat to environment leading to water and soil pollution affecting flora and fauna.

About 10-15 % of dyes used in textile industries do not fix to the fibers, and discharge as waste into the treatment plant or into the environment directly and causes environmental pollution (Cetin *et al.*, 2008). Higher organic or inorganic load with intense heat, color, alkali or acidic nature of the effluent convert them into highly recalcitrant.

Numerous literature sources provide us knowledge about removal of azodyes by the

means of physical, chemical and biological methods. Microorganisms were found to be the ideal candidates in the field of bioremediation. Microbes such as fungi, algae, bacteria and actinomycetes were used as an alternative for physio-chemical treatment methods. Bioremediation has proved to a feasible process for removal of hazardous dye from the ecosystem. According to Jadhav *et al.,* (2008), microbial consortium containing *Bacillus* sps. and *Galactomyces geotrichum* showed effective degradation of Brilliant blue G dye. *Bacillus* species was reported to decolorize methyl orange effectively (Ali, 2005).

Detoxification studies including phytotoxicity and microbial toxicity assay also proved that the degraded compounds are nontoxic when compared to the parental azo compounds (Parshetti *et al.,* 2010). Work done by Shah *et al.,* (2013) proved that *Pseudomonas* sps was found to decolorize methyl orange efficiently and also proved that the strain can tolerate higher concentration of dye which makes them a right choice for their exploration in the textile effluent treatment plant. In the present study, bacterial strain isolated from effluent sample of textile industry was characterized by morphological, biochemical and molecular sequencing (16SrRNA). The efficacy of isolate in decolorizing methyl orange was performed quantitatively by using UV-Vis spectrophotometry and FTIR analysis.

Materials and Methods

Sampling Sites

The sampling area in this study was the textile industries and dyeing units located in and around Arani, Thiruvannamalai District, Tamil Nadu, India. The effluent samples from both textile industries and dyeing units were characterized by its dark color and extreme turbidity.

Azo Dye Used

The commonly used textile azo dye, Methyl Orange used in this study was procured from a local textile dyeing unit. Stock solution was prepared by dissolving 1 g of azo dye in 100 ml distilled water. The dye solution was sterilized by membrane filtration. All the chemicals used in this study were of the highest purity available and of an analytical grade.

Isolation and Screening of Bacterial Strains Decolorizing Methyl Orange

The effluent samples were serially diluted and spread over nutrient agar medium containing 50 ppm of azo dye. pH was adjusted to 7.0 before autoclaving and incubated at 37°C for 5 days. Colonies surrounded by halo (decolorized) zones were picked and streaked on nutrient agar plates containing azo dyes. The plates were re-incubated at 37°C for 3 days to confirm their abilities to decolorize Methyl Orange.

Decolorization Assay using UV-visible spectrophotometer

A loopful of bacterial culture was inoculated in 100 ml of nutrient broth and incubated at 150 rpm at 37°C for 24 h. Then, 1 ml of 24 h old culture of BRTSI 3strain was inoculated in 100 ml of nutrient broth containing 50 ppm of Methyl Orange and re-incubated at 37°C till complete decolorization occurs.

Suitable control without any inoculum was also run along with experimental flasks. 1.0 ml of sample was withdrawn every 12 h and centrifuged at 10,000 rpm for 15 min. Decolorization extent was determined by measuring the absorbance of the culture supernatant at 470 nm respectively, using UV-visible spectrophotometer, according to Hemapriya *et al.,* (2010).

Decolorization efficiency (%) = Dye (i) – Dye (r) / Dye (i) X 100

Where, Dye (i) refers to the initial dye concentration and Dye (r) refers to the residual dye concentration. Decolorization experiments were performed in triplicates.

Characterization and 16S rDNA Analysis of BRTSI-3 strain

Strain BRTSI-3 was characterized morphologically and biochemically according to Bergey's manual of systemic bacteriology. The 16S rDNA sequence of the isolates were amplified via the polymerase chain reaction (PCR), using two universal primers: the 16S forward primer and the 16S reverse primer, which yielded a product of approximately 1.5 kb. The purified PCR product was directly sequenced using Big Dye Terminator version 3.1 cycle sequencing kit. The nucleotide sequence analysis was done at BLAST-n site at NCBI server www.ncbi.nlm.nih.gov/BLAST. The alignment of the sequences was done using CLUSTAL W program VI.82 at European Bioinformatics site (www.ebi.ac.uk/clustalw). The analysis of 16S rDNA gene sequence was done at Ribosomal Data Base Project (RDP) II (http://rdp.cme.msu.edu). The phylogenetic tree was constructed using the aligned sequences by the neighbour joining method using kimura-2 parameter distances in MEGA 2.1 software.

Optimization of culture conditions

100 ml of nutrient broth was inoculated with loopful culture of BRTSI-3 in different conical flasks. All the flasks were incubated at different pH (4, 5, 6, 7, 8 and 9) and different temperature ranges (20, 25, 30, 35, 40, 45 and 50 ^{o}C) for 24 h. Following incubation, the bacterial growth was monitored in above mentioned flasks to check the optimum pH and temperature for maximizing bacterial biomass. The optimum culture condition where maximum growth was observed and was maintained for further studies.

FTIR Analysis of Decolorized Samples

The biodecolorized azo dye samples were characterized by FTIR spectroscopy (JASCO). The analysis results were compared with the control dye. The FTIR analysis was done in the mid IR region (400-4000 cm^{-1}) with 16 scan speed. The samples were mixed with spectroscopically pure KBr in the ratio (5:95). The pellets were fixed in sample holder and then analyzed (Saratale *et al.,* 2009).

Results and Discussion

16S rDNA Analysis of BRTSI-3 Strain

BRTSI-3 strain exhibited remarkable efficiency in decolorizing methyl yellow (Fig 1). The morphological and biochemical characteristics of the strain BRTSI-3 that exhibited maximum decolorization efficiency towards Methyl Orange is shown in Table 1. A total of 1153 bases sequence of PCR amplified 16S rDNA gene was determined from the isolate BRTSI-3.

In the phylogenetic analysis, the sequence formed a cluster with in *Bacillus sps* with 92 % identity, thus confirming the isolate as *Bacillus subtilis* Strain BRTSI-3 (Fig. 2) and phylogenetic tree constructed was shown in Fig. 3. The obtained sequence was submitted to GenBank with the accession number MH412808.

Growth optimization: Influence of temperature and pH

Incubation time played a significant role in maximizing the biomass of *Bacillus* sp. strain BRTSI-3.

Fig.1 Decoloization of Methyl Orange by BRTSI-3 Strain (Control and Test Sample)

Fig.2 PCR amplified 16S r RNA sequence of the isolate BRTSI-3

>CONTIG B
TCGACCGGAAGGATGGGAGCTTGCTCCCTAATGTTAGCGGCGGACGGGAGAGTAA
CACGTGGGTCACCTGCCTGTAAGACTGAGATAACTCCGGGAAACCGGGGCTAATA
CCGGATGGTTGTTTGAACCGCATGGTTCGAACATAAAATGTGGCTTCGGCTACCAC
TTACACATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGG
CAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACG
GCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACAAAAGTC
TGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTG
TTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACCA
GAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAATCG
TTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTG
AAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAG
AAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGA
ACACCAGTGGCGAAGGCGACTCTCTGGTCTGTACTGACGCTGAGGAGCGAAAGCG
TGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAACGATGAGTGCT
AAGTGTTAGGGGTTCCGCCCTTATGCTGCAGCTAACGCATTAGCACTCCTCTGGGG
AGACAGTCGCAGACTGAAATCAAGGAATTGACGGGGGCCCCACAACCGTGGAGAT
GTGGTTAATTCGAGCACGCGAAAACCTTACAGGTCTGACACCTCTGACATCTAAA
AAAGGACGTCTCTTCGGGGCAAATGACAGTGTGCATGGTGTCGTCGCTCAGTCCG
AAATGTGGGTTAGTCCCCACAACGCGCCCTGATCTAGTGCCGCATCATTGGGCCTC
TAAGGGATGCCGGACAACCGGAGGAGGGGGGAGACTCCATTTCTTGCCCTTTGAC
TGGGTACCCGGCTCATGGACAACAAAGGGGCGAACCCGGGTAGCCATCCC

Fig.3 Phylogenetic tree of the isolate BRTSI-3

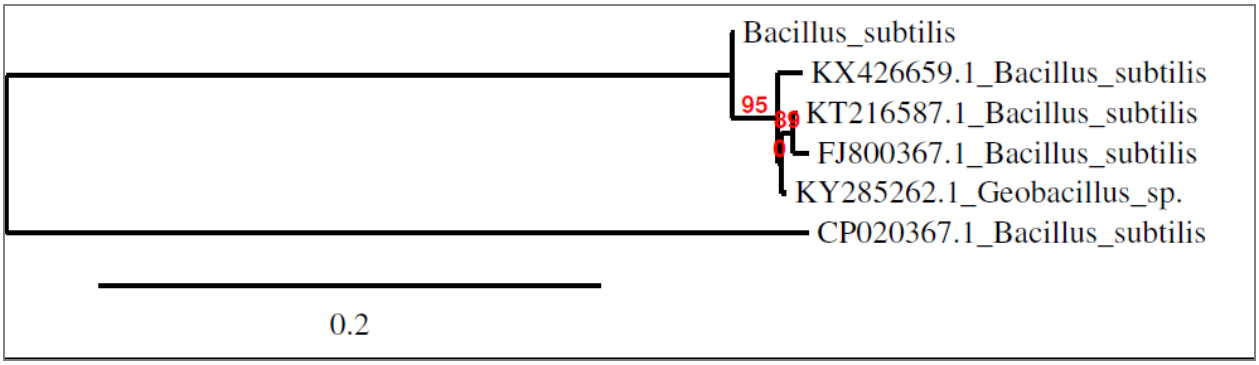

Fig.4 Effect of Temperature on the biomass of *Bacillus subtilis* strain BRTSI-3

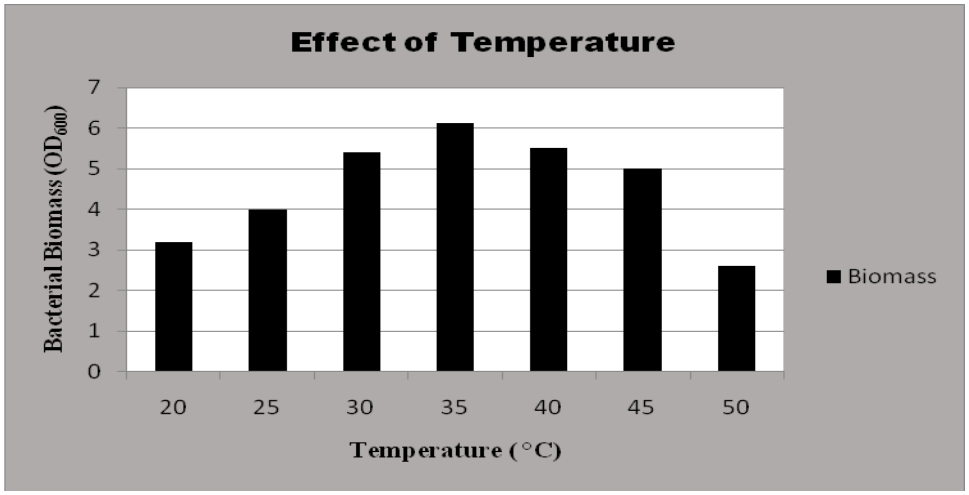

Fig.5 Effect of pH on the biomass of *Bacillus subtilis* strain BRTSI-3

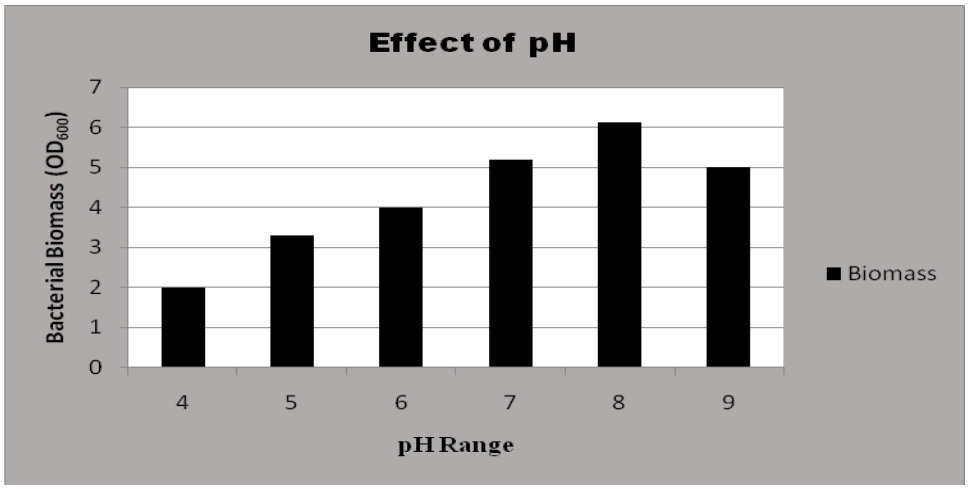

Fig.6 FT-IR spectra of decolorized Methyl Orange

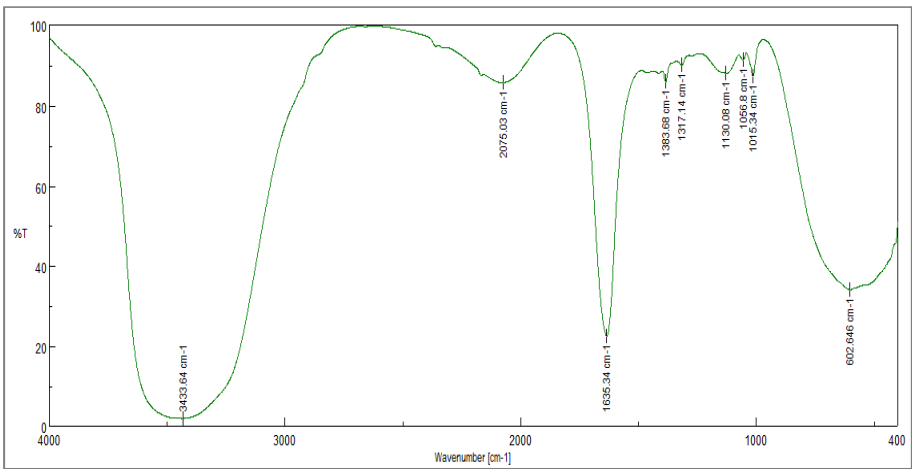

Table.1 Morphological, Physiological and Biochemical Characteristics of strain BRTSI-1

S. No	Test	Observations
1	Morphology	
	Grams staining	Positive
	Cell shape and arrangement	Rods arranged singly/pairs
	Motility	Motile
2	Colony Characters on Nutrient agar	
	Colony morphology	Round
	Colony size	2 -2.5 mm
	Colony elevation	Raised
	Colony edge	Entire
	Pigmentation	Nonchromogenic
3	Sugar Fermentation	
	Lactose	Positive -Acid
	Maltose	Positive -Acid
	Sucrose	Positive -Acid
4	Enzyme Reaction	
	Urease Production	Negative
	Nitrate Reductase	Positive
	Oxidase	Negative
	Coagulase	Negative
	Catalase Activity	Positive
5	H_2S Production	Positive
6	Gelatin	Positive (24 h)

Temperature was found to be directly proportional to bacterial growth till 35^{o}C and inversely proportional to bacterial growth above 35^{o}C. Thus, maximum growth was observed at 35^{o}C. Optical density was found to be 0.56 at 610 nm (Fig. 4). Temperature level above and below 35 ^{o}C drastically reduced the bacterial growth. However, growth rate of BRTSI-3 strain gradually increased with increase in pH level, reaching its maximum growth (biomass) at pH 8.0 whereas, the bacterial growth was found to be reduced at pH level greater than 8.0 (Fig. 5).

Decolorization studies using UV-VIS spectrophotometry

Visible color change was observed in the test flask after 24 h of incubation, which may be either due to biosorption or degradation of methyl orange present in the culture media. The Test and Control sample was centrifuged at 4000 rpm for about 15 min and the resultant supernatant was subjected for UV-Visible spectroscopy. Absorbance peaks of control and decolorized sample evidently showed the decolorization of methyl orange.

FTIR analysis

FTIR analysis enables to study the degradation of methyl orange by bacterial metabolites (Fig. 6). This study clearly indicated the interaction of bacterial molecules in degrading azo dye methyl orange. O-H stretch at 3433cm^{-1} indicates the presence of carboxylic acid group. Vibration at 1635cm^{-1} denotes the presence of amide class of compounds. Stretches between 1317 cm^{-1} to 1015 cm^{-1} represents the presence of

alkyl halides (C-F stretch). Degradation of methyl orange was confirmed by referring the control peaks reported by Chen *et al.,* (2008).

Economically feasible and eco-friendly strategies are inevitably required to degrade dye-contaminated wastewater discharged from various industries. In the present study, bacterial strain BRTSI-3 isolated from textile effluent sample was characterized by means of morphological, biochemical and 16S rDNA sequencing. The strain BRTSI-3 was found to be *Bacillus subtilis* (NCBI accession number MH412808). The bacterial growth was found to be optimized at 35^{o}C and pH 8.0. The bacterial culture was inoculated in nutrient broth with methyl orange for detecting the degradation rate. UV-Vis spectrophotometry results indicated the decolorization of methyl orange by bacterial metabolites. FTIR results confirmed the breakdown of the azo dye by bacterial metabolites. Thus this work may provide a reasonable basis for development of an effective bioremediation process for the safe remediation of dye pollutants present in textile effluents.

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