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Isolation, Identification and Characterization of Novel Azo Dye Degrading Bacteria from the Industrial Effluents of Raipur City, Chhattisgarh

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ABSTRACT

Various chromophores are used to make our day-to-day life colorful. Dyes that are used at a large scale are made using these chromophores. The dyes, especially azo dyes are recalcitrant to the degradation due to the presence of aromatic rings in their structure. Several methods have been developed to reduce the harmful impacts of these dyes on the environment. However, none of the processes is safe and fully effective. In this study, we used bacteria as a bioremediation agent and optimized the various parameters for the bacteria to degrade the dye at its maximum ability. It was found that the isolated bacteria were *Aneurinibacillus* sp. and it completely decolorized methyl orange at a concentration of 20 $mg.L^{-1}$ after 4 days of incubation. The optimum pH for the functioning of bacteria was 5 and the activity decreased as the pH increased. It was also observed that the addition of glucose and yeast extract increased the dye degradation significantly.

INTRODUCTION

Colors play an important role in our daily life. Dyes and dyestuffs are used to impart colors in the pharmaceutical, textile, printing, and food industries. Due to the presence of aromatic rings in their chemical structure, they are very stable and able to resist most degrading factors such as high temperatures, surfactants, and sunlight (Banat et al. 1996). Due to such high stability, they are tenacious. The most used dyes among all the dyes are azo dyes, which are characterized by nitrogen to nitrogen double bonds. Since the synthesis of azo dyes is cheaper and easy, they have a large spectrum of colors and have higher stability as compared to their natural counterparts they account for a large proportion of dyestuffs used in various industries (Chang & Kuo 2000). During and after the coloring process huge amounts of these dye effluents are discharged, which pollute local terrestrial habitats, rivers, and other aquatic bodies. These dyes released in the environment break down into their constituting chemicals such as amines, which have various harmful impacts such as mutagenic effects and chemical toxicity (Weisburger 2002, Xu et al. 2007). These dyes discharged in the water bodies get accumulated in them and hinder the sunlight penetration which in turn will affect various aquatic fauna and flora. A critical decrease in the photosynthetic ability of the aquatic plants and dissolved oxygen level has been observed (Vandevivere et al. 1998). In addition to these, changes in the levels of various water quality parameters such as chemical oxygen demand, total organic carbon, and biochemical oxygen demands were also observed (Saratale et al. 2009).

Several physicochemical techniques such as membrane filtration, electrochemical techniques, ozonization, coagulation, and ion pair extractions have been used to treat these dye-containing effluents. However, there are various major roadblocks to these proposed methods such as they are expensive, complicated, time-consuming, not fully effective and when used produce a large amount of secondary pollution (Churchley 1994, Daneshvar et al. 2004, Forgacs et al. 2004, López-Grimau & Gutierrez 2006). Thus, the need for economical and safe elimination of these recalcitrant dyes was felt. The process of bioremediation using various bacterial isolates was then focused upon for better results and as hoped they yielded results that were cost-efficient and environment friendly at the same time (Ali et al. 2009, Senan & Abraham 2004). There have been many recent studies on the different bacterial isolates having the potential for dye degradation and have reported a large number of microorganisms having the potential for dye decolorization.

However, several attempts made by the researchers for the degradation of dyes have not yielded complete degradation or decolorization. In this context, the present study emphasizes the potential of a bacterial strain isolated from local industrial waste effluent of the dyeing industry for the degradation efficiency of $C_{14}H_{14}N_3NaO_3S$ (methyl orange). It aims to optimize various cultural conditions (pH, dye concentration) and nutritional (carbon source) parameters for maximization of Methyl Orange dye decolorization which can be useful in providing an alternate method to accomplish dye degradation.

MATERIALS AND METHODS

Study Site

Three sites of the Raipur city in Chhattishgarh state of India i.e. Khans dry cleaner in Banzari road, Anshali cards printing in Phool chowk Lorapara, and M.I. industry polymers in Urla were chosen as the site for sample collection as they extensively use the dyes.

Collection of Sample and Physicochemical Characterization

Samples in the form of liquid untreated effluent were collected in sterilized sealed plastic bottles from different sites and stored at 4°C in the refrigerator. The tests for the characterization of the sample were performed on the same day of collection. The samples were analyzed and characterized for various parameters such as pH, color, temperature, odor, chemical oxygen demand (COD), biochemical oxygen demand (BOD), dissolved oxygen (DO), total dissolved solids (TDS) and total suspended solids (TSS) using standard methods.

Isolation and Screening of Dye Degrading Micro-organisms from Dye Effluents

The bacterial isolates present in the dye effluents were isolated by serial dilution pour plate technique on nutrient agar containing different dye concentrations of 30 mg.100mL^{-1}, 40 mg.100mL^{-1} and 50 mg.100mL^{-1}. 0.1mL of serially diluted sample from three different concentrations (10^{-5}, $10^{-6,}$ and 10^{-7}) were spread over the solid medium. The plates were then put in an incubator for twenty-four hours and the temperature was maintained at 37 degrees Celsius. After the incubation period, the colonies showing a clear zone on agar plates were selected for further studies.

Dye Degradation Study

Mineral salt media (MSM) consisting of 2.35 g sodium dihydrogen orthophosphate dehydrate, 0.07 g magnesium sulfate heptahydrate, 0.14 g of calcium chloride, 1 mg ferric chloride and 1000 mL distilled water was used for the degradation test. Based on the screening process four isolates were selected for the methyl orange decolorization study. The dye decolorization experiment was carried out in 100 mL MSM with different concentrations of each dye, i.e. 10, 20, 30, 40 and 50 mg.L^{-1}. Inoculum of the isolates, pre-incubated at 37°C for a day were taken out in the volume of 2mL and were inoculated in flasks containing MSM along with different dye concentrations. The flasks were then kept in the mechanical shaker and incubated at 37°C for 3 days. During the incubation period, the samples were withdrawn periodically and the absorbance was measured at the lambda max of methyl orange, i.e. 465 nm, before taking the absorbance the sample was centrifuged at ten thousand rpm for fifteen minutes. The MSM medium which was uninoculated and dye-free was used as blank. The tests were performed in a set of three and the results were compared with the blank.

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

The dye degradation activity of the isolates was calculated and expressed in the terms of decolorization percentage. At lambda max, a decrease in the absorbance of the sample over time was monitored and the degradation efficiency was calculated from the following equation. The graph of time v/s OD was also plotted.

Optimization of Factors

The micro-organism showing the highest decolorization efficiency was then taken and different parameters such as pH, presence, and absence of carbon source, and yeast extract were optimized. Since the previous study the maximum dye decolorization was observed in the sample containing dye at the concentration of 30 mg.mL^{-1} so, this sample was then subjected to the different optimization experiments. pH values were optimized at 4, 7 and 9. To test the effect of the presence of glucose, 1% glucose was added to the media. Two flasks containing 50 mL of each sample without glucose were also prepared as blank. To test the effect of yeast extract on the decolorization activity, 0.05 g of yeast extract was added to 50 mL of MSM, and an uninoculated flask was used as a blank. And after the incubation period (24 h at 37^{o}C) absorbance was taken at 530 nm.

Bacterial Growth Curve and Generation Time

20 µL of isolate 1 showing the highest dye degradation potential was then inoculated in 200 mL autoclaved optimized media and then incubated in a shaking incubator at 37°C for one week. The medium was then taken out and optical density (OD) was measured at 620 nm every 30 minutes. Bacterial

generation time was calculated from the graph plotted between OD v/s Time in MS Excel 2013. The formula used for extraction of generation time was LN(2)/B. The value of B was obtained from the graph equation.

Identification of Bacteria

Isolate 1 (ShivaniSI) showing the maximum potential for dye degradation was then subject to various biochemical tests for identification as per Bergy's manual. Apart from biochemical tests, the 16s rRNA analysis was also done by Chromous biotech lab, Bangalore, India. For the rest three isolates, only biochemical tests were performed.

RESULTS AND DISCUSSION

Collection of Samples

Fig 1. shows the samples collected from various places. The green bottle contains a sample from Anshali Shadi and Cards, pink from MI polymers, and dark blue from Khan's drycleaners Raipur. All the three places in involved directly or indirectly in the processing and release of azo dyes. The samples from these places contain extreme concentrations of the azo dyes along with other coloring compounds and hence the effluents show bright colors.

Fig. 1: Samples collected from various places.

Physiochemical Characterization of Samples

Various physicochemical investigations done on the sample collected from above mentioned three locations are shown in Table 1. The samples were subjected to the pH estimation, and their temperature was checked. In addition to these to check the level of contamination of water chemical oxygen demand (COD), total suspended solids (TSS), and total dissolved solids (TDS) were analyzed. All the three samples gave high values for COD, TSS and TDS suggesting that the sources are highly polluted. Also, all the samples were alkaline, and the temperature varied between 22°C and 28°C.

Isolation and Screening of Dye Degrading Microorganisms

Fig. 2 shows the isolation of the bacteria based on clear zone formation in the dye-loaded media. The bacteria were plated on the agar with dye in it. The colonies that were able to grow and degrade the dye by the production of extracellular enzymes, as seen in the third image from the top, were selected for further dye degradation experiment.

Dye Decolorization Study

Decolorization percentage of methyl orange by Isolate 1 for different days is presented in Table 2. The isolate 1, showing the highest growth in the dye supplemented medium was further checked for its ability to degrade the higher concentrations of dye in its environment. The different aliquots of media were made and supplemented with different dye concentrations. The samples were taken out every 24 hours and checked for the degradation of dye by the bacterial inoculum. The isolates showed high degradation on the third day of inoculation in all the four concentrations.

Fig. 3 shows the degradation of methyl orange by the bacteria. The methyl orange gives a peak absorbance at 465 nm. The gradual decrease in the values of optical density at 465 nm indicates the degradation of methyl orange due to the action of the bacterial enzyme system and metabolic activities.

Table 1: Physicochemical investigations done on the samples collected from different locations.

S. No.	Site of sample collection	Nature of sample	Color	pH	Temperature	COD [mg.L^{-1}]	TSS [mg.L^{-1}]	TDS [mg.L^{-1}]
1	MI polymers, Urla, Raipur C.G.	Liquid	Light pink	8.2	28°C	1120	14200	28000
2	Khans dry cleaner Banzari road Raipur, C.G.	Liquid	Dark blue	10.7	26°C	2560	8000	19600
3	Anshali Shadi and cards Phool Chouk Raipur, C.G.	Liquid	Green	8.5	22°C	1040	11000	14000

Optimization of Different Factors

Effect of pH: The bacterial isolates on the first day of isolation were able to degrade only a small percent of the dye present in the sample this can be due to the sudden shock that they experienced when transferred from media to dye-containing media. However, after acclimatizing well in the media they showed good efficiency on the 2nd day at pH 5 suggesting the bacteria to be acidophilic. The effect of pH on the percent decolorization of dye is shown in Table 3.

Effect of carbon source: Bacteria like any other living being needs a carbon source for their proper functioning and supplementing the media with carbon sources like simple sugars can help in high energy generation in a short time. This easy energy can be used by bacteria to more efficiently metabolizing the compounds and give higher degradation efficiency as seen in Table 4. The efficiency on 3rd day increased to 94.17% just due to the addition of glucose.

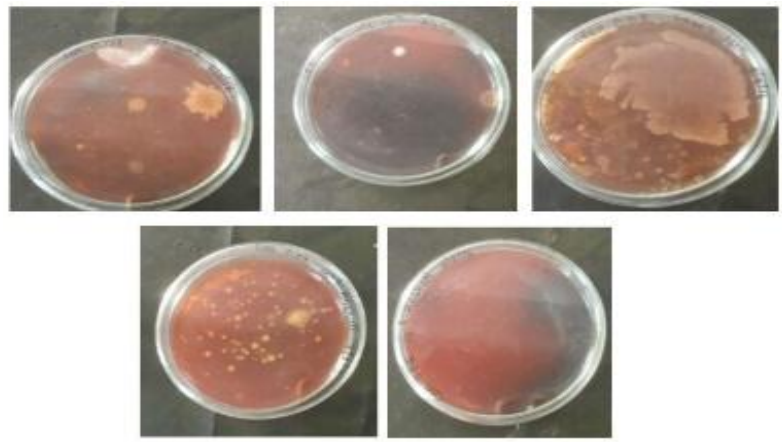

Fig. 2: Isolation of the bacteria based on clear zone formation in the dye-loaded media.

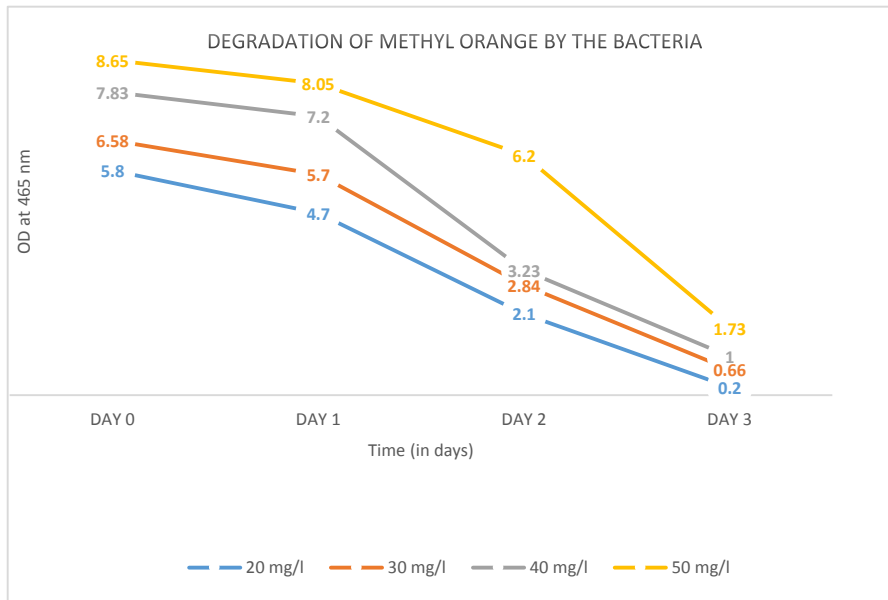

Fig. 3: Degradation of methyl orange by the bacteria.

Effect of yeast extract and glucose addition to MSM: The efficiency of the isolate was further checked by supplementing the media with yeast extract which functions as a nitrogen source. It was observed that the addition of yeast extract reduced the dye degradation time to nearly about 24 hours as compared to 3 days with only glucose and 5 days without yeast extract and glucose (Table 5).

Bacterial Growth Curve and Generation Time

The generation time of the bacteria was calculated using the formula LN(2)/B. The calculated generation time for the bacteria was found to be 32 minutes.

The initial 60 minutes show the bacteria in the lag phase and getting ready to divide. A slight increase in the population number can be observed from 90 minutes to 120 minutes. After 120 minutes the bacteria enters the log phase which lasts for about 240 minutes. After the log phase due to the depletion of the nutrients and accumulation of the secondary metabolites the growth comes to a standstill phase known as the stationary phase. In this phase, the number of cells produced equals the number of cells death. This phase lasts for about 420 minutes post which the death phase sets in and cells eventually begin to die at a much higher rate than they are being generated due to the extreme depletion of the nutrients (Fig. 4).

Identification of the Microorganisms

Biochemical tests for the identification of bacteria: The bacterial isolates were subjected to the biochemical tests and on their ability to produce some chemical or degrade the other, they were classified into groups using Bergey's manual for systematic bacteriology. The isolate 1 that was selected for further studies showed positive gram staining, positive MR test and negative VP test, the bacterial strain produced catalase enzyme when H_2O_2 was added. The strain however was unable to utilize the citrate added in the sample also it showed zero starch hydrolysis suggesting no production of amylase. The strain was positive for indole production, gelatin liquefaction, TSI agar test, nitrate reductase test and oxidase test (Table 6).

Table 2: Decolorization percentage of methyl orange by Isolate 1.

Days	Dye concentration (in $mg.L^{-1}$)			
	20	30	40	50
1 day	18.12%	12.56%	8.14%	7.06%
2 day	63.75%	56.73%	50.64%	21.80%
3 day	96.87%	89.90%	87.53%	80%

Table 3: Effect of pH on the percent decolorization of dye.

Days	pH 5	pH 7	pH 9
1st day	4.23%	0.15%	4.14%
2nd day	80.15%	78.11%	60.38%

Table 4: Comparative Percent decolorization (degradation) of sample on the addition of a carbon source.

Days	Without glucose	With glucose
1st day	55.15%	89.32%
3rd day	68.55%	94.17%

Identification Through 16s rRNA Technique

Aligned sequence data of sample Shivani S1 (isolate 1) (A613mo)(1424bp)

GCCTATACATGCAAGTCGAGCGGACCAATGAA-
GAGCTTGCTCTTCGGCGGTTAGCGGCGGACG
GGTGAGTAACACGTAGGCAACCTGCCTGTACGACT-
GGGATAACTCCGGGAAACCGGAGCTAAT AC-
CGGATACTTCTTTCAGACCGCATGGTCTGAAA-
GGGAAAGACTTTTGGTCACGTACAGATG-
GG CCTGCGGCGCATTAGCTAGTTGGTGGGG-
TAACGGCCTACCAAGGCGACGATGCGTAGCCGACCT
GAGAGGGTGATCGGCCACACTGGGACTGAGA-
CACGGCCCAGACTCCTACGGGAGGCAGCAGTA
GGGAATCTTCCGCAATGGACGAAAGTCTGACG-
GAGCAACGCCGCGTGAACGATGAAGGTTTTC
GGATCGTAAAGTTCTGTTGTTAGGGAAGAAC-
CGCCGGGATGACCTCCCGGTCTGACGGTACCTA
ACGAGAAAGCCCCGGCTAACTACGTGCCAG-
CAGCCGCGGTAATACGTAGGGGGCAAGCGTTGT
CCGGAATTATTGGGCGTAAAGCGCGCGCAGG-
CGGCTTCTTAAGTCAGGTGTGAAAGCCCACGG
CTCAACCGTGGAGGGCCACTTGAAACTGG-
GAAGCTTGAGTGCAGGAGAGGAGAGCGGAAT-
TCC ACGTGTAGCGGTGAAATGCGTAGAGATGTG-
GAGGAACACCCGTGGCGAAGGCGGCTCTCTGGC
CTGTAACTGACGCTGAGGCGCGAAAGCGTGG-
GGAGCGAACAGGATTAGATACCCTGGTAGTCC
ACGCCGTAAACGTTGAGTGCTAGGTGTTGGG-
GACTCCAATCCTCAGTGCCGCAGCTAACGCAATAA
ACAAGCGGTGGAGCATGTGGTTTAATTCGAAG-

Table 5: Effect of yeast extract and glucose added in MSM in the percent degradation.

Sr. No.	Media	Time of Degradation
1	MSM + Yeast extract + Isolate 1.	After 24 hours
2	MSM +Glucose + Yeast extract + Isolate 1	After 24 hours
3	MSM + Glucose + Isolate 1	After 3 days
4	MSM + Isolate 1	After 5 Days

CAACGCGAAGAACCTTACCAGGGCTTGACAT
CCCGCTGACCCTCCTAGAGATAGGAGCTCTCT-
TCGGAGCAGCGGTGACAGGTGGTGCATGGTTG
TCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA-
GTCCCGCAACGAGCGCAACCCTTGTCCTTAGTT
GCCAGCATTTAGTTGGGCACTCTAGGGAGACTGC-
CGTCGACAAGACGGAGGAAGGTGGGGATG
ACGTCAAATCATCATGCCCCTTATGTCCTGGGC-
TACACACGTGCTACAATGGATGGAACAACGG
GCAGCCAACTCGCGAGAGTGCGCGAATCCCT-
TAAAACCATTCTCAGTTCGGATTGCAGGCTG-
CA ACTCGCCTGCATGAAGCCGGAATCGCTAG-
TAATCGCGGATCAGCATGCCGCGGTGAATACGTTC
CCGGGTCTTGTACACACCGCCCGTCACACCAC-
GAGAGTTTGCAACACCCGAAGTCGGTGAGGTA
ACCGCAAGGAGCCAGCCGCCGAAGTTGGCACTC-
CGCCTGGGGAGTACGGCCGCAAGGCTGAAACT
CAAAGGAATTGACGGGGACCCGC

The aligned sequence of the bacterial strains showed a close and high similarity (99%) with the isolates of *Aneurinibacillus* strains (Table 7).

The phylogenetic tree was constructed on the basis of the aligned sequences to check for the evolutionary history of the bacterial strain. The phylogenetic trees also tell the close relative of the species. Our strain was more closely related to Aneurinibacillius migulanus strain RD 16s ribosomal RNA. It was also genetically related to other Aneurinibacillius strains although through a distant relative. Based on this observation our isolate was identified as Aneurinibacillius strain (Fig. 5.

OBSERVATIONS AND DISCUSSION

Various physicochemical parameters shown in Table 1 were analyzed for proper management of effluent. The pH of effluents from all three sites fell under the alkaline category ranging from 8.2 to 10.7. PH is an important factor in the

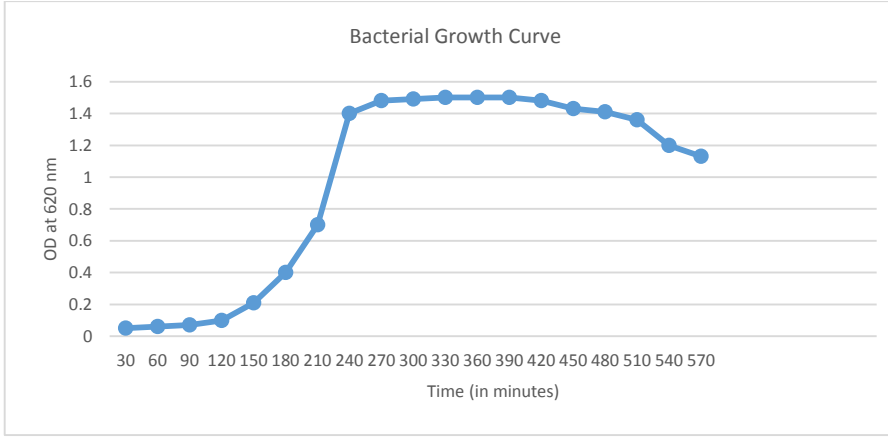

Fig. 4: Bacterial growth curve is taken at 620 nm after a week of incubation every 30 minutes.

Table 6: Results of the different biochemical tests done for the identification of the isolates.

S.No.	Name of test	Isolate No. 1 (A613mo)	Isolate No. 2 (K69mo)	Isolate No. 3 (K611tb)	Isolate No. 4 (K74tb)
1	Gram staining	+ ve	+ ve	+ve	+ve
2	MR-VP test	MR +ve, VP - ve	MR +ve, VP - ve	MR – ve, VP + ve	MR – ve, VP - ve
3	Catalase test	+ve	-ve	-ve	+ve
4	Citrate utilization	-ve	-ve	+ve	+ve
5	Indole production	+ve	+ve	+ve	+ve
6	Amylase production	-ve	+ve	+ve	+ve
7	Gelatin liquification	+ve	+ve	+ve	+ve
8	TSI agar test	+ve	+ve	+ve	+ve
9	Nitrate reductase	+ve	+ve	+ve	+ve
10	Oxidase test	+ve	+ve	+ve	+ve

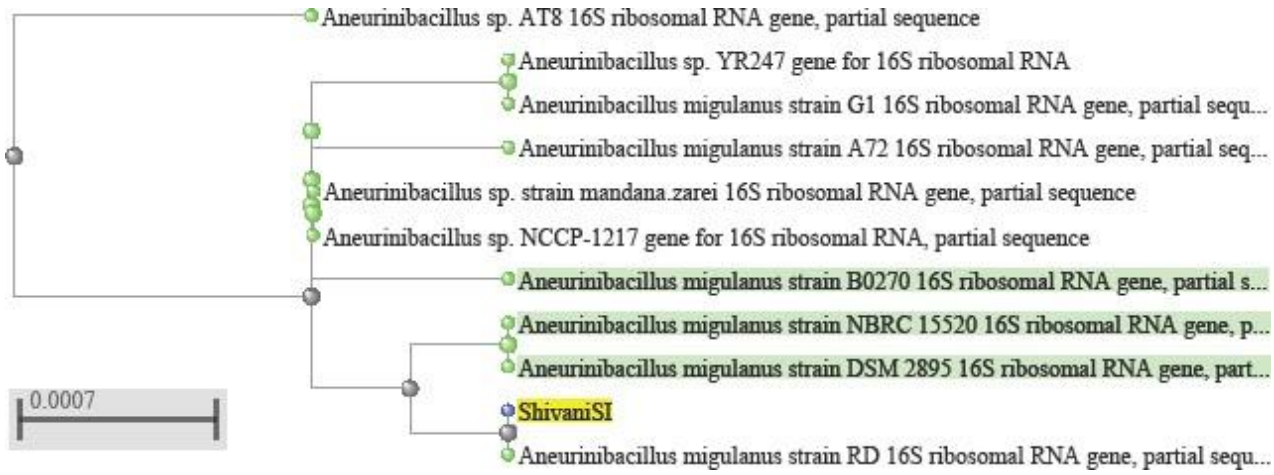

Fig. 5: Phylogenetic analysis based on the sequence alignment.

degradation of dye as it affects microbial growth & solubility of various chemicals and their reactions (Fernández-Calviño & Bååth 2010, Krishnan et al. 2017). The color of the effluent is the primary indicator of any water body being contaminated by the dye. The samples collected from the three study sites varied in the colors blue, pink, and Green (Fig. 1). The samples displayed high values of COD which indicates a huge amount of industrial pollutants in them (Lee & Nikraz 2015). This may be due to the various types of chemicals being used in different steps of the dyeing process. The highest TDS and TSS recorded were 28000 $mg.L^{-1}$ and 14200 $mg.L^{-1}$ respectively. High TDS and TSS values of the effluent affect the potability of the water body if untreated effluent is disposed of in it (Tariq et al. 2006).

Four different bacterial colonies were isolated from the effluents based on the production of clear zones observed (Fig. 2). Based on decolorization potential one isolate was chosen for further study. It was identified as *Aneuranibacillus* sp. through biochemical tests (Table 6) and 16s rRNA sequencing analysis (Table 7, Fig. 5). Its generation time was calculated using the standard growth curve by taking absorbance at 620nm (Fig. 4).

The maximum decolorization percentage (96.87%) of methyl orange was observed in a concentration of 20 $mg.L^{-1}$ after 3 days of incubation by *Aneurinibacillus sp* and almost complete decolorization was attained on the 4^{th} day (Fig. 3). Table 2 indicates that an increase in the concentration of methyl orange decreases the dye degrading capacity of the bacteria. Decolorization of methyl orange dye by *Aneuranibacillus* strain decreased from acidic (80.15%) to neutral (78.11%) pH range (Table 3). To enhance dye decolorization efficiency additional carbon source in the form of glucose was added to the sample. The addition of glucose to the sample led to 94.17% decolorization on the 3rd day (Table 4). It was observed that adding the yeast extract increased the decolorization potential, which is denoted by the complete decolorization of the sample after 24 hours while the addition of only glucose to the MSM required 3 days for complete decolorization. However, SM without any supplementation requires 5 days for complete decolorization (Table 5).

Previous studies done on various strains of *Aneuranibacillus* have explored its capacity in the biodegradation of various substances such as polylactic acid plastic, kraft

Table 7: Results of the sequence alignment for isolate No. 1.

S.No.	Organism's name	Accession No.	% match
1	*Aneurinibacillus migulanus* Strain RD	KX083693.1	99%
2	*Aneurinibacillus migulanus* Strain NBRC 15520	NR_ 113714	99%
3	*Aneurinibacillus migulanus* Strain DSM 2895	NR_112214.1	99%
4	*Aneurinibacillus* sp. YR274	LC110197.1	99%
5	*Aneurinibacillus migulanus* Strain B0270	NR_036799.2	99%
6	*Aneurinibacillus migulanus* Strain G1	JQ337949.1	99%
7	*Aneurinibacillus* sp. AT8	FJ821593.1	99%
8	*Aneurinibacillus migulanus* Strain A72	GU397386.1	99%
9	*Aneurinibacillus* sp. Strain *mandana.zarei*	KY270877.1	99%
10	*Aneurinibacillus* sp. NCCP-1217	LC065244.1	99%

lignin, collagen, polyethylene, and polypropylene (Chaisu et al. 2012, Murai et al. 2004, Raj et al. 2007, Skariyachan et al. 2018). But it is the first of its kind study done on evaluating the potential of *Aneuranibacillus* sp. in the biodegradation of Azo dyes. The possible mechanism by which the *Aneuranibacillus* sp. degrades the azo dye is by cleavage of the azo bond (-N=N-) with the help of H_2O_2 produced during its metabolic activity. The azo dye degradation by the H_2O_2 has already been established (Jaafarzadeh et al. 2018). However, further studies need to be done to understand the full mechanism of dye degradation by *Aneuranibacillus* sp.

CONCLUSION

In this study bacterial strains having dye decolorizing capacity were isolated from colored effluents. The results indicate that this bacterium can be employed for the remediation of effluent from dye industries. Further studies are needed to elucidate the mechanism of dye degradation, the intermediates formed and the dye degradation potential of polluted waters in situ. Also, there is a possibility that the efficacy of reclamation of water bodies may enhance by the use of bacterial consortium.

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