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Research Article

EVALUATION OF AZO DYE ORANGE G DECOLORIZATION AND DEGRADATION BY BACTERIA ISOLATED FROM TEXTILE EFFLUENT

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ABSTRACT

The bacteria were isolated and identified from textile effluent, and their ability to decolorize the hazardous azo dye Orange G was evaluated. After incubating the isolates for 24 hours in mineral salt medium supplemented with 100 ppm Direct Orange G, their decolorizing activity was measured spectrophotometrically. *Lysinibacillus sp.* AUBTP7 (AK1), *Bacillus muralis* strain1Y118 (AK2), and *Bacillus thuringiensis* strain N3 (AK3) were identified as the bacterial strains. *Bacillus thuringiensis* strain N3 (AK3) was the most efficient bacteria at decolorizing Orange G (100ppm), removing 93% of the color in 24 hours at 36°C temperature. Study of HPLC chromatogram of Orange G and degradation products suggests the formation of new compounds. As a result of this research, it has been revealed that some bacteria live in textile effluents and use dyes as a source of energy and nourishment, implying their usefulness in the treatment of industrial effluents.

Keywords: HPLC, Azo dye, Orange G, Decolourization, MS media.

1. INTRODUCTION

One of India's oldest and largest employments has been the textile sector. Commercially, azo dyes are widely applied in various textile industries since they are cost-effective, versatile, cheap to implement, and damage-resistant. These dyes are resistant to a wide variety of pH values, as well as extreme temperatures and light [1,2]. For reuse of treated water, colour removal and treatment of hazardous by-products are critical. The majority of azo dyes are poisonous, carcinogenic, and mutagenic, posing a threat to human health and the environment [3, 4], until now, azo dye wastewater has been regarded as one of the most difficult to treat types of dye wastewater [5]. These azo dyes are difficult to degrade and develop as a stable compound in the environment due to their complex aromatic chemical structure and synthetic origin [6]. As a result, developing an effective treatment for azo dye wastewater has become a major focus of research. Bioremediation techniques are designed to recycle water and prevent pollution by permanently removing pollutants from anthropogenic activity [7]. Biological decolorization and degradation of azo dyes is advantageous because it is a less expensive, environment friendly and efficient solution for a wide range of azo dyes, with less sludge and higher efficiency [8]. Because they have adapted to survive under difficult environments, microbes isolated from dye-contaminated locations are the most promising for wastewater treatment [9, 10]. The focus of this research was to i) Isolate native bacterial strains from a textile effluent capable of degrading azo dye, Orange G ii) Analyse and optimize the biodegradation capabilities of such strains for azo dye, Orange G, remediation applications, and iii) laboratory application of using such bacterial strains to remove colours from dye-contaminated textile wastewater effluent iv) Analysis of the transformation products using HPLC revealed that different bacterial strains may have ability to degrade and form new products.

2. MATERIAL AND METHODS

2.1. Effluent sample collection and Dye

In the Gaya industrial area, wastewater samples were collected from seven different areas. The samples were taken to a lab where they were examined for physico-chemical properties. The sample collecting sites were designated by the numerals S1 through S7.Orange G, an azo dye, was procured from Loba Chemie Pvt. Ltd in Mumbai, India, and a stock solution was prepared.

2.2. Isolation and enrichment of dye de-colorizing bacteria

The enrichment of textile industry liquid effluent led to the isolation of three morphologically distinct bacterial isolates. Three bacterial isolates were individually tested for their ability to decolorize azo dye Orange G at a concentration of 0.02 percent. Bacteria decolorizing azo dye Orange G were enriched in nutrient broth from textile industry effluent using a successive enrichment technique under aerobic conditions.100ppm dye sample was dissolved in 100ml distilled water to make the stock solution. The effluent samples were serially diluted and disseminated on agar medium with 100ppm orange G. Before autoclaving, the pH was adjusted to 7.0 and incubated at 37°C for 5 days. Colonies surrounded by halo zones were selected and streaked on azo dye-containing minimum agar media.The pure cultures were maintained on dye-containing nutrient agar slants at 4°C.

2.3. Determination of optimum growth conditions

Different culture conditions, such as pH, temperature, and dye concentration, influence bacterial optimum growth. The culture medium was adjusted to a pH range of 5.0 to 9.0, the incubation temperatures were varied from 25 to 45°C, and the dye concentration was varied from 100 to 500 ppm.

2.4. Decolourization activity test

For decolourization assay, loopful of bacterial culture was inoculated in an Erlenmeyer flask containing 100 ml of nutrient broth and incubated at 150 rpm at 30°C for 24 hours. Then, 1 ml of the bacterial isolates, 24 h old culture was inoculated in 100 ml of nutrient broth containing 100 ppm of Orange G and re-incubated at 30°C until complete decolourization occurred. Along with the experimental flasks, a suitable control without any inoculum was also run. Every 24 hours, 1 ml of sample was taken and centrifuged for 15 minutes at 10,000 rpm. According to study [11] the decolorization extent was determined by measuring the absorbance of the culture supernatant at 475nm using a UV-visible spectrophotometer.

Decolourization efficiency (%) = Dye (i) - Dye (r)/Dye (i)*100

Where, Dye (i) refers to the initial dye concentration and Dye (r) refers to the residual dye concentration.

Decolourization experiments were performed in triplicates.

2.5. Identification of dye-degrading bacteria by 16S rDNA gene sequence

Forward primers 5'-TGGTAGTCCACGCCCTAAC-3' and reverse primers 5'-CTGGAAAGTTCCGTGGATGT-3' were used to amplify the bacteria's internal transcribed spacer region. Amplification procedures were performed in a 24-liter reaction mixture (Sigma-Aldrich) containing 100 ng of genomic DNA, 1 X PCR buffer (15 mM $MgCl_2$), 0.2 mM each of the dNTPs, 2 M of each primer, and 0.5 l of Taq DNA polymerase. The programme included an initial denaturation phase at 94°C for 5 minutes, followed by denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 45 seconds, and a final extension step at 72°C for 2 minutes in a Mastercycler-Gradient (Eppendorf, Germany). For 35 cycles, steps 2, 3, and 4 were repeated. Electrophoresis of PCR products in 1.0 percent (w/v) agarose gels in 1 X Tris-Borate-EDTA (TBE) buffer and ethidium bromide staining Biologia Research India Pvt Ltd, New Delhi, purified and sequenced the amplified PCR products. To authenticate bacterial sp. and get closely related phylo-genetic sequences, the sequence was placed into the National Centre for Biotechnology Information's BLAST nucleotide search software. MegaX software was used for phylogenetic analysis and tree construction.

2.6. Statistical analysis

All the values are the means of three independent observations and indicate the standard deviation. The parametric statistic programme, version 1.01, was used to calculate the statistics (Lundon Software Inc. Chagrin Falls, OH, USA).

3. RESULTS AND DISCUSSION

3.1. Isolation of dye decolorizing bacteria

By plating dye decolorizing bacteria into an agar solidified MS medium supplemented with dye from textile industry effluents, the bacteria were identified. Isolation, screening, and identification of bacterial strains capable of decolorizing textile dyes revealed that 03 bacterial isolates, designated AK1 to AK3, were capable of decolorizing Orange G.AK3 was found to be the superior strain with the highest decolorization efficiency out of three isolates (93 percent).

The isolates AK1, AK2, and AK3 were identified as *Lysinibacillus sp*. AUBTP7 (AK1), *Bacillus muralis* 1Y118 (AK2), and *Bacillus thureingenesis*(AK3), respectively, based on morphological, cultural, and biochemical characteristics of the strains and a comparison with "Bergey's manual of determinative Bacteriology".

Table 1: Decolourization of Orange G dye by bacterial isolates

DYE	ISOLATES		
	AK 1	AK 2	AK3
Orange G	87%	90%	93%

(a)Control

(b) *Lysinibacillus sp.* AK 1

(c) *Bacillus sp.* AK 2

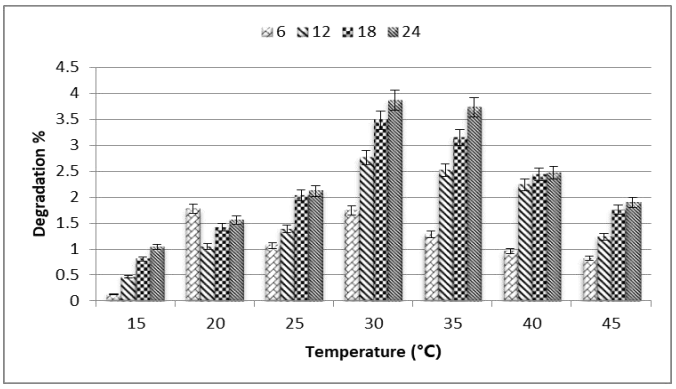
(d) *Bacillus sp.* AK 3

Fig. 1: Decolourization of Orange G by different bacteria in 24 hrs

3.2. Optimization of Dye decolorizing Ability of AK1,AK2 and AK3 Isolates

3.2.1. Effect of Temperature

The temperature can have influence on the decolorization process since the activity of the bacterial enzymes is sensitive to the temperature. Hence, the dye decolorization was studied in the temperature range from 15 to 45°C (Fig.2). At 15°C, strains AK1, AK2 and AK3 decolorized 32.34, 36.2 and 38.16 % dye in 24 hrs and at 45°C, strains AK1, AK2 and AK3 decolorized 27.25, 32.8 and 36.73 % dye respectively in 24 hrs. Whereas at 30°C the decolourization rate was maximum i.e. 78.21, 79.55 and 84.7% dye was decolorized by the strains AK1, AK2 and AK3 respectively. The optimum temperature for decolorization of Orange G was 30°C. The strains exhibited good decolorizing ability in quite wide temperature range. The decolorizing capacity increased gradually from 15 to 30°C and then declined due to thermal denaturation.



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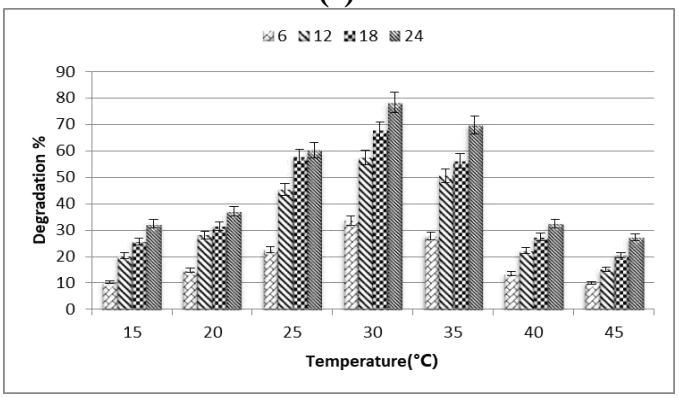

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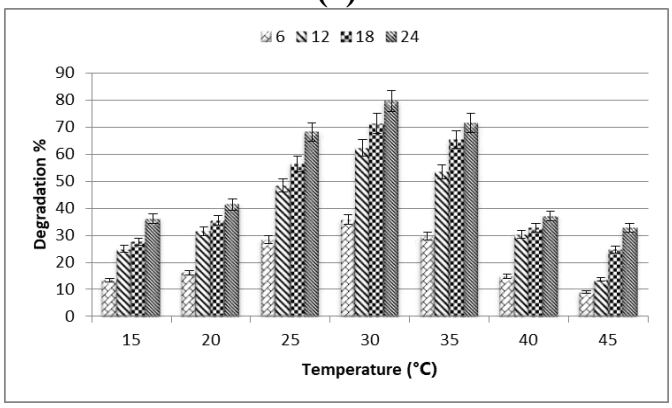

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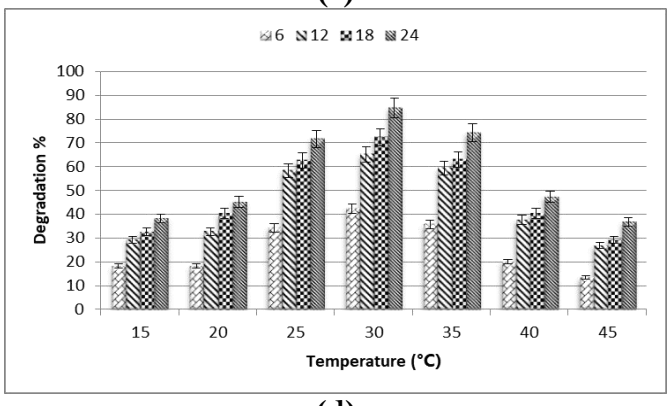
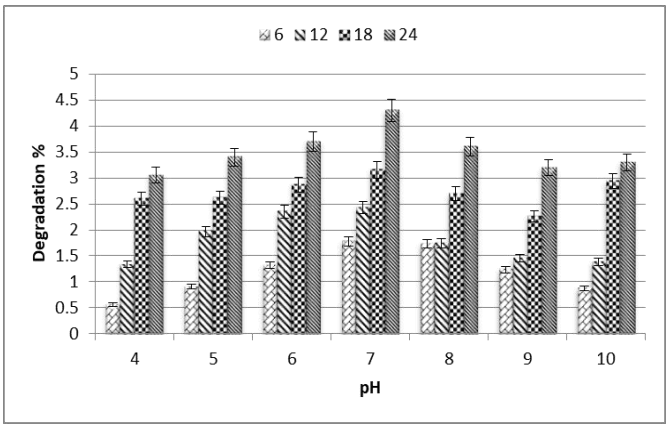


(d)

Fig. 2: Decolourization of Orange G by (a) Control (b) AK1, (c) AK2 and (d) AK3 at different temperatures.

3.2.2. *Effect of pH*

The textile effluents have varying pH from acidic to alkaline conditions. The strains AK1, AK2 and AK3 showed maximum decolorization at pH 7 (Fig.3). Strain AK1 decolorized 37.2, 62.9, 73.7, 82.9, 62.4, 48.8 and 42.1% dye at pH 4, 5, 6, 7, 8, 9 and 10 respectively in 24 hrs. Strain AK 2 decolorized 45.29, 73.4, 76.7, 88.6, 66.8, 53.7 and 49.05 % dye respectively at pH 4, 5, 6, 7, 8, 9 and 10. Strain AK 3 decolorized 53.36, 77.06, 79.5, 91.9, 68.3, 57.6 and 52.1% dye at pH 4, 5, 6, 7, 8, 9 and 10 respectively.



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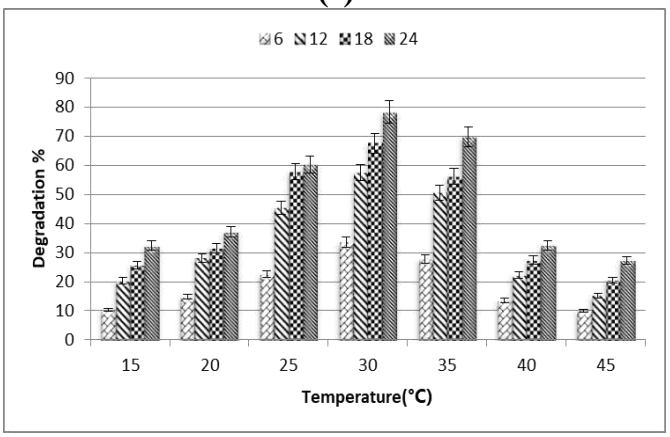

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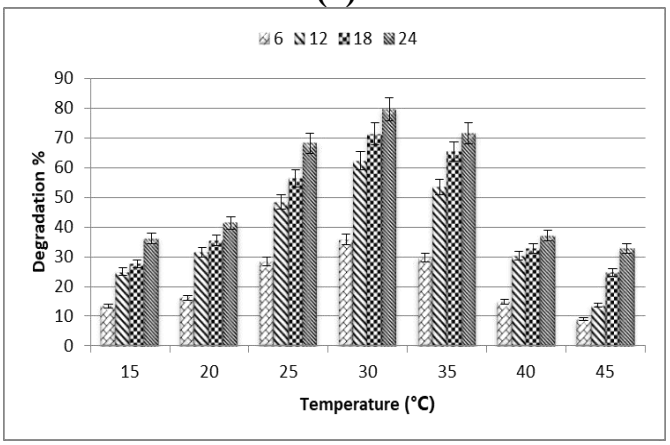

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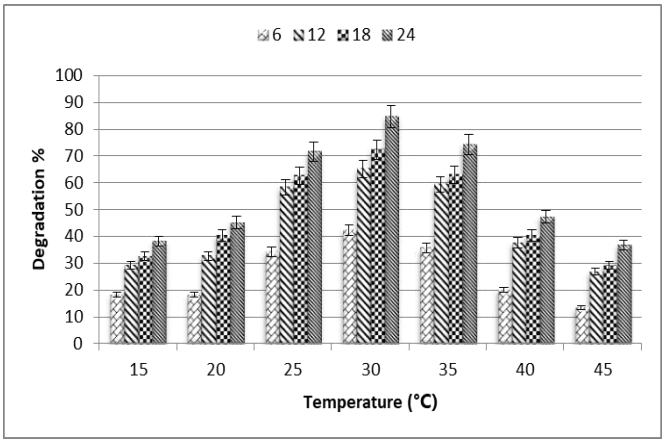


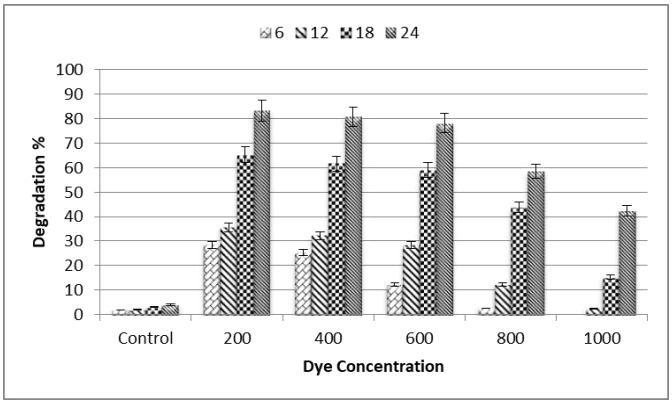
(d)

Fig. 3: Decolourization of Orange G by (a) control (b) AK 1 (c) AK2 and (d) AK3 at different pH values

The results indicated that the decolorization capability was not much affected at alkaline pH. Acidic pH below 6 was unfavorable to all the strains for the Orange G decolorization. At a pH range of 6-10, these strains demonstrated good decolorizing abilities.This property is most desirable as the textile wastewaters have varying pH from acidic to alkali due to addition of acids and salts during dyeing process.

3.2.3. *Effect of dye concentration on decolorization of Orange G*

Effect of initial dye concentration on decolorization efficiency of the strains was investigated by taking Orange G dye concentrations of 200, 400, 600, 800, 1000 mg/L. The results show that the strains could effectively decolorize the dye at varying initial dyeconcentrations. *Lysinibacillus sp*. AK1 decolorized over 83.1% of the dye at 200 ppm concentration, beyond which the decolorizing ability was gradually decreased (Fig.4a).



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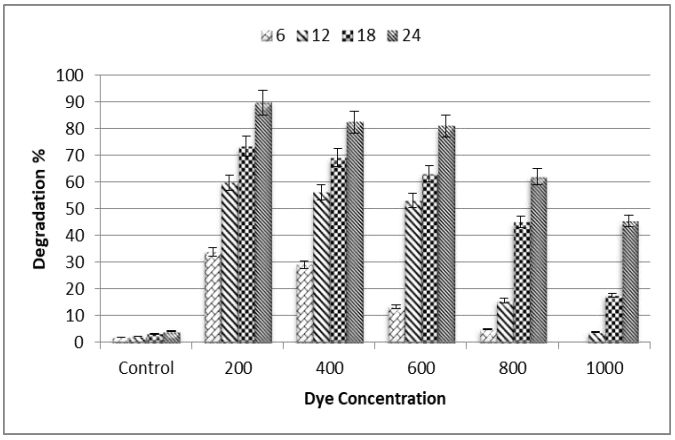

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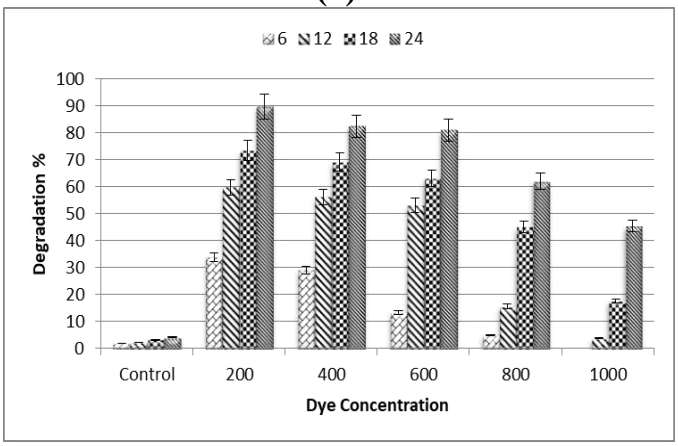

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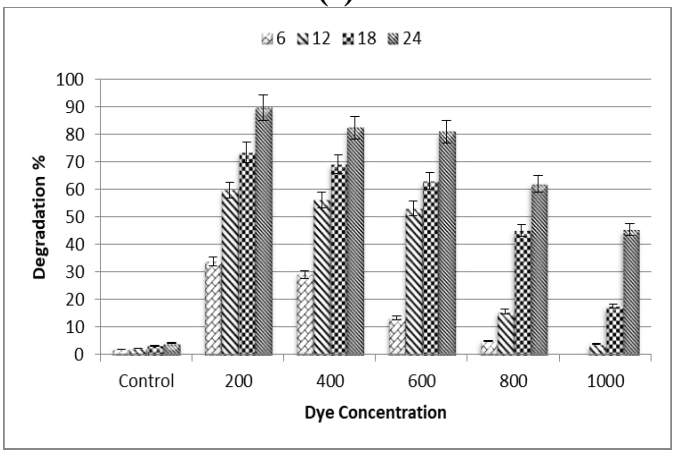


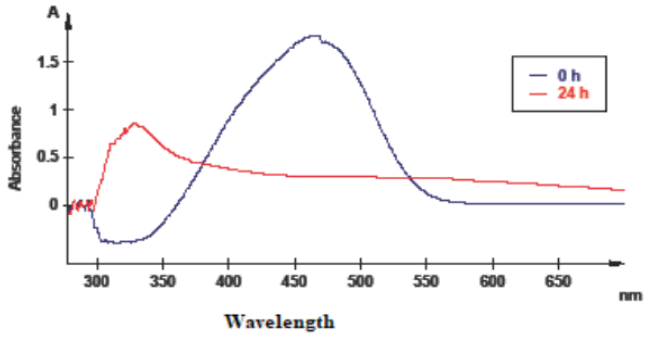
(d)

Fig. 4: Decolourization of Orange G at increasing dye concentrations by (a) AK1 (b) AK2 (c) AK3 (d) Consortium

AK2 i.e. *Bacillus muralis* AY118 decolorize 90% dye and *Bacillus thuringiensis* i.e. AK 3 decolorize upto 93%. At 1000 ppm dye concentration the decolorizing ability of these strains was reduced (Figure 4b and 4c). The tested bacteria had a higher decolorization competency than the other bacteria strains previously described.

3.3. Analysis of Orange G decolorization products

The dye decolorized sample obtained utilizing the various strains were tested for metabolite identification and decolorization pathway analysis. Orange G's UV-Vis spectra revealed only one absorption peak at 475 nm. With the passage of time, the absorption maxima of the spent medium shifted, resulting in the reduction of the peak at 475 nm and the appearance of a new peak in UV region. The peak at 475 nm dissipates, indicating that the azo link is totally broken. Peaks in the UV area suggested the formation of aromatic metabolites with a lower molecular weight. (Fig.5).



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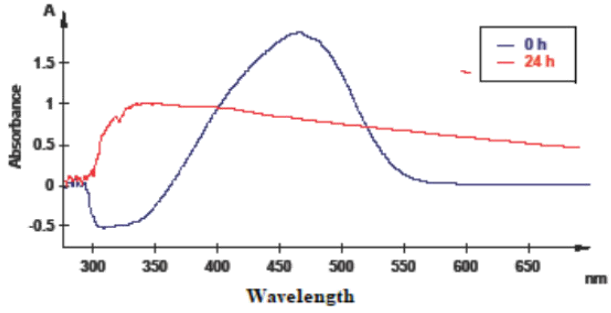

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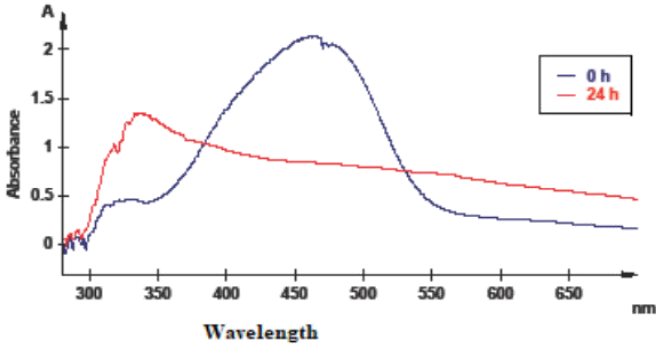
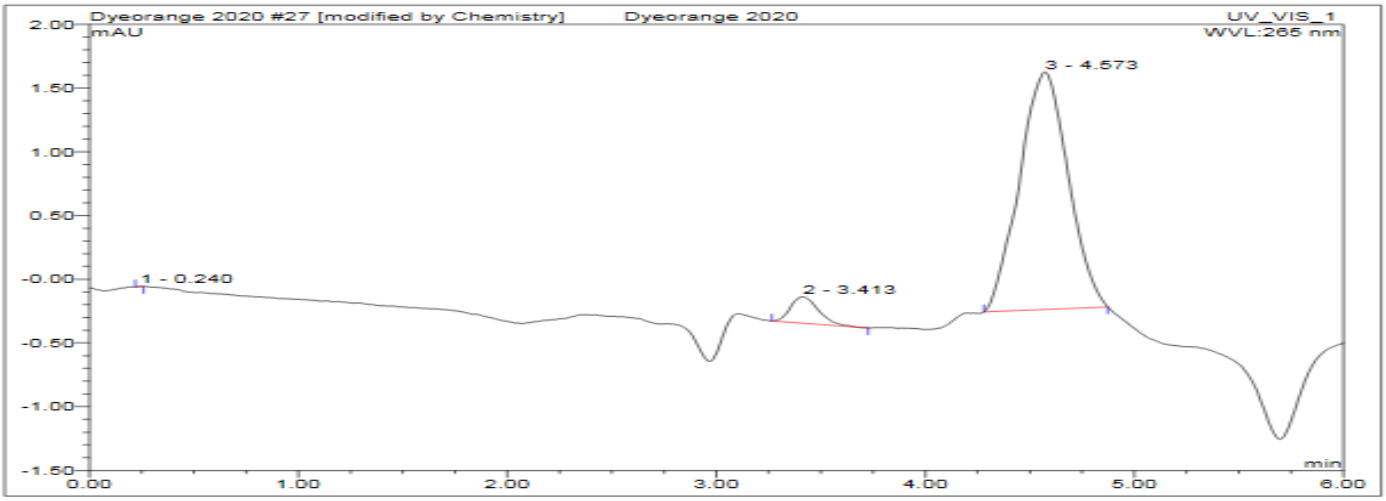


(c)

Fig.5: UV-Vis spectra of Orange G before and after decolourization by (a) AK 1 (b) AK 2 (c) AK 3

3.4. HPLC Analysis

The production of new compounds is suggested by a comparison of the HPLC chromatogram of Orange G and degradation products, as shown in (Fig.6). The consortium has two large peaks at 3.847 and 4.553 minutes, as well as two minor peaks at 4.360 and 4.853 minutes, which is substantially different from Orange G's one major peak at 4.573 minutes in Fig.6.



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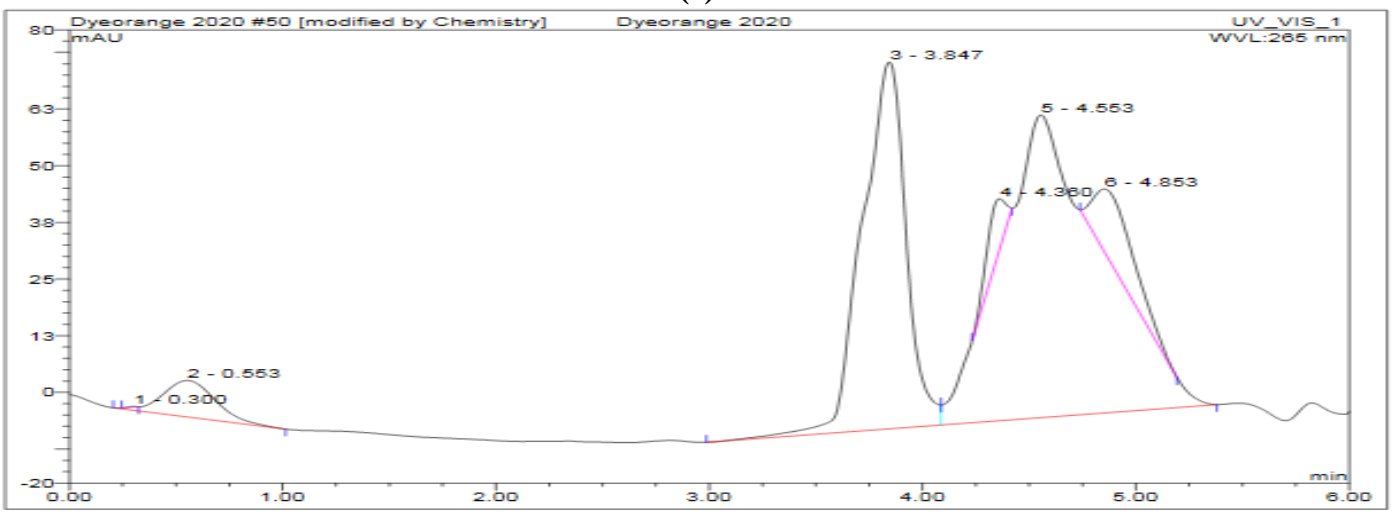

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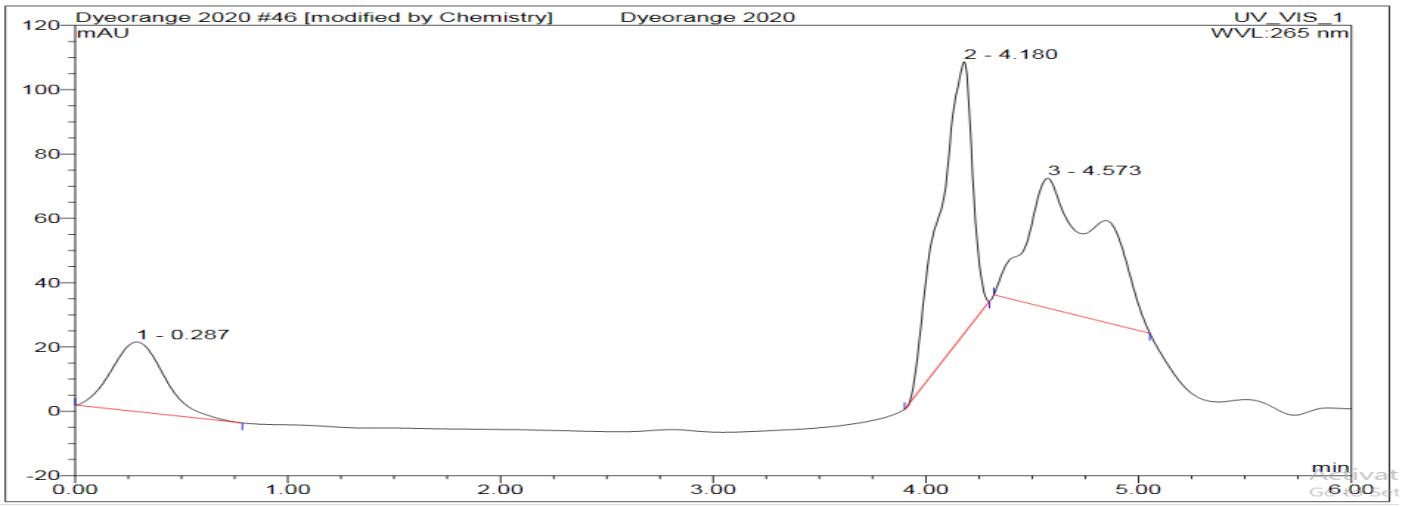

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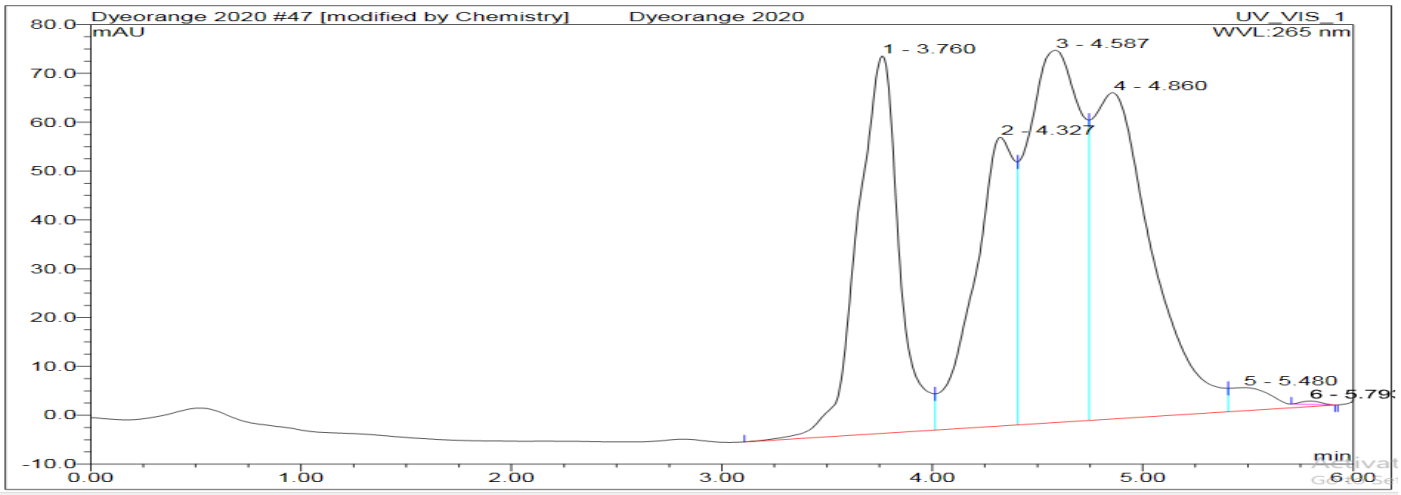


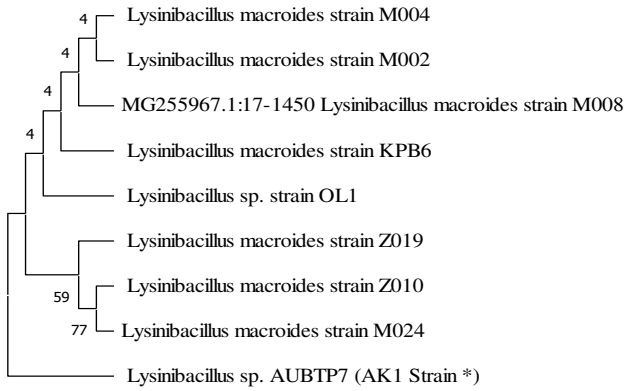
(d)

Fig.6: HPLC chromatogram of (a) standard (Orange G at 1 ppm) (b) AK 1 (c) AK 2 (d) AK 3

3.5. Phylogenetic analysis and identification of the strains

After screening, DNA was extracted from the Bacterial culture. Emerald Amp GTPCR Mastermix, catalogue number-RR310, was used to amplify the 16 rDNA region of isolated DNA. We amplified a 1500bp amplicon with the primers 27F and 1492R, and no amplicon was evident in the negative (no DNA) control, but the predicted sized amplicon (1500bp) was visible in the positive control.Gel elution/SAP was used to purify the 1500 bp test amplicon. Sanger's method of DNAsequencing was used to sequence the purified product using the methodology (bioserve- D/Seq-02 to 06). The findings of the sequencing were compiled and compared to the NCBI database.

Mega X software was used for phylogenetic analysis and tree construction. Pairwise alignment of BLAST related sequences for each AK strain was used to create a phylogenetic tree. For each phylogenetic tree, a total of ten related blast sequences are chosen at random. The Neighbor joining technique is used to create a tree from sequence distances. The NCBI website was used to calculate distances between sequences. By comparing the 16S rDNA sequences of all isolates within different subgroups to those of representatives of various genera, the phylogenetic positions of all isolates within different subgroups were studied.It is evident from the Phylogenetic tree that AK1 is closely related to Lysinibacillus sp. AUBTP7,In theevolutionary history of AK1, the percentage of replicate trees in the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. A K2 is closely related to *Bacillus muralis*strain 1Y118 in AK2analysis involved 10 nucleotide sequences.For each sequence pair, all unclear locations were deleted (pairwise deletion option). There were a total of 1454 positions in the final dataset and AK3 to Bacillus sp. Strain N3 (98.68%) and it was observed therewere a total of 501 positions in the final dataset. (Fig.7).



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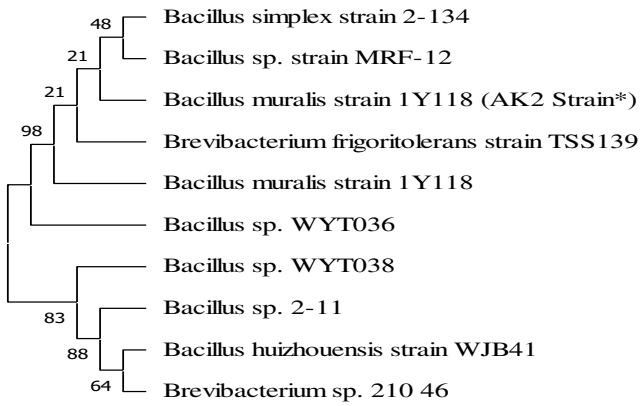

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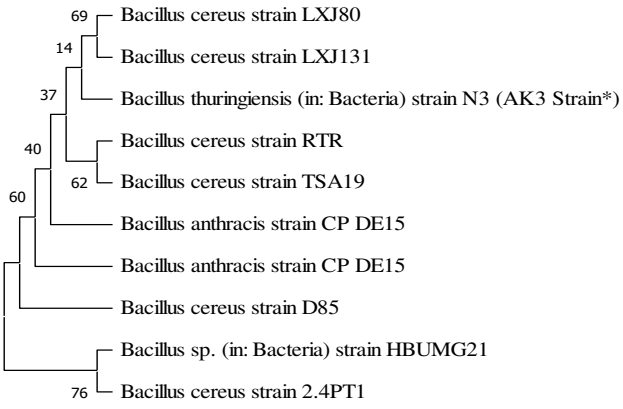

(c)

Fig.7: Phylogenetic tree for cultivated bacterial strain (a) AK1, (b) AK2, (c) AK3

4. CONCLUSION

Environmental biotechnology is continually expanding its efforts in the biological analysis of colored textile discharge, which is an environmentally friendly and low-cost alternative to the physicochemical decomposition method. The textile industries are chemical in nature, relying on a variety of dyes. Environmental contamination, as well as health and aesthetic issues, are caused by the assembly of dye materials. The use of dye has become unavoidable with the advancement of civilization, and its release in the environment poses a significant problem. As a result, there is a pressing need for a technologically feasible and cost-effective treatment method to be devised to address this issue. Due to their environmental friendliness and cost-effectiveness, microbial decolorization and degradation of various azo dyes have exceptional potential to address a variety of problems. These bacteria also have a variety of other properties. Because their growth rate is fast and their hydraulic retention is great, they are productive in the treatment of high-strength waste water.

5. ACKNOWLEDGEMENTS

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Conflicts of interest

The authors declare no conflict of interest.

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