

ISOLATION AND CHARACTERIZATION OF DYE DEGRADING BACTERIA FROM TEXTILE EFFLUENTS

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ABSTRACT

The bacteria present in dye effluents often show diverse features including different biochemical properties, ability to grow in a variety of media, tolerance towards varying physical conditions and ability to degrade dye. It plays an important role in the toxic dye remediation program and may be used as an effective organism for removing the toxic dye present in textile and other associated industrial effluent. The present investigation was carried out to isolate dye degrading bacterial species from an untreated textile mill effluent. On characterization of the yellow coloured sample, the bacterial isolate was screened for its ability to degrade azo dye (highly toxic metanil yellow dye degradation). A simple bioassay was carried out by serial diluting and pour plating the dye in nutrient medium. A single strain of bacteria was taken into account for further studies including colony

Morphology, Biochemical tests and enzyme assay. The bacterium was identified as *Bacillus cereus* on 16S rRNA identification. On performing the enzyme assay experiment, it was found that the bacteria is capable of producing mainly azo-reductase along with catechol 1,2-dioxygenase and peroxidase. The total hydrocarbon present was scanned by Reverse Phase OD S2 column.

KEYWORDS: Dye degradation, Citrate Agar, Nitrate utilization test, Azo dye, Bioremediation.

INTRODUCTION

Environmental pollution and its subsequent degradation has become rampant over the past few decades and has been increasing at an alarming rate ever since. Among the huge number of toxic and chemical substances that routinely contaminate the environment, dyes happen to be major contributors. The textile industry is one of them which uses a variety of synthetic dyes and other harmful chemicals during the production process. Pollution via dye effluents has affected large sections of soil and water bodies rendering them unfit for human use. These effluents are difficult to treat because of their high BOD, COD and also due to the presence of metal ions. Even when their effect is not immediately apparent, the threats of Bio-accumulation, relay toxicity, bio-magnification and secondary poisoning still remains. Presently the use of azo dyes have increased due to their ease and cost effectiveness in synthesis as compared to natural dyes. However use of azo dyes has many disadvantages as they are toxic, carcinogenic and mutagenic. Azo bonds are resistant to breakage, thus increasing the chances of their accumulation in the environment. There are several physico-chemical techniques available for the treatment of industrial effluents. These include adsorption on different materials, oxidation and precipitation by Fenton's reagent, bleaching with chloride, ozone photo degradation or membrane filtration. All these methods are very expensive and result in the production of wastes like sludge which ultimately results in secondary pollution in the form of land pollution. Hence the removal of these dyes from the effluent is an important issue. Dwindling natural resources and their rampant spoilage has caused the development of new disciplines and new strategies to combat their ill effects. Bio remediation is one such emerging strategy which utilizes living organisms to counter the harmful effects of pollution and reduce their impact on natural resources. It has been observed that certain microorganisms are capable of decolorizing or degrading the harmful dyes to form non-toxic products. Azo reductase catalyzes the NAD (P) H-dependent reduction of azo compounds to the corresponding amines, which involves cleavage of the azo linkages ($-N=N-$), resulting in azo dye degradation. Although these enzymes reduced certain types of azo dyes, some dyes were not degraded efficiently. To establish biological wastewater treatment system for azo dye removal, it is advantageous to screen for microorganisms that express Azo reductases with broad substrate specificities. A suitable source for such microbes is the different industrial effluents. This project work thus concentrates on isolation and characterization of bacteria from dye textile effluent. Studying and characterizing the bacteria thus provides information regarding their ability to degrade dye as well as the prospect of using them for bio-remediation.

MATERIALS AND METHODS

1. Sample collection

Industrial effluents were collected in screw capped sterilized bottles from the vicinity of Bara Nagar Textile Industries in Kasba, Kolkata and designated as sample "C". The following parameters were taken up for study.

2. Determination of physical characteristics

A. Determination of Total Dissolved Solid (TDS) of the sample

What man no. 1 filter paper disc was dried at 90°C for 10 minutes in a hot air oven and subsequently the dry weight was measured. The dried filter paper disc was used to filter out 10 ml of the effluent sample. The filter paper along with the adhered residue was dried at 90°C for 10 minutes and the dry weight was again measured. The process was repeated thrice.

B. Determination of Electrical Conductivity (EC) and Total Suspended Solid (TSS) of the sample.

50 ml of the effluent sample was filtered using What man no. 1 filter paper and the EC and TDS were determined at 25°C using a standard water analyser.

3. Determination of chemical characteristics

Determination of pH of the effluent sample

The effluent sample was filtered using Whatman no. 1 filter paper and the pH was subsequently determined using a standardized pH meter.

4. Isolation of dye degrading bacteria

For isolation of dye degrading bacteria, serial dilution of the sample was carried out and 20 µL of the 10⁻² dilution was pour plated in sterile Nutrient Agar (NA) Plates. The growth of the bacterial colonies was observed after 24 hours of incubation at 37 C. On performing Gram staining, the bacterium was found to be gram positive rods. Further the bacteria were grown in different broths having different pH as given in Table 3 and O.D. was measured to find out the optimum pH.

5. Biochemical analysis

The biochemical characterization of bacteria was done by performing the following tests:

1. Indole

2. Methyl Red
3. Voges-Praskauer
4. Citrate Utilization
5. Nitrate Reductase
6. Oxidase
7. Catalase
8. Urease
9. Starch Hydrolysis
10. Casein Hydrolysis

These tests help in the identification of the biochemical behavior of the bacteria.

6. Dye Degradation

1g and 2g of metanil yellow was measured and mixed separately in 100 ml of autoclaved distilled water. Hence two concentrations of 1% and 2% solutions of the dye were obtained. The media selected for the growth of the bacteria in this experiment was minimal media. 50ml of 1% solution was taken in one conical flask. In the next set, 10ml of 1% solution mixed with 40ml of media was taken[dye : media]. In the third set, 10ml of 1% solution was mixed with 40ml of media followed by inoculation with the isolated bacterium [dye: media (inoculum)]. The same procedure was repeated for 2% solution of the dye. The two conical flasks for 1% and 2% solutions containing dye, media and inoculum were incubated for 48 hours. After incubation, definite amount of the solutions were taken from each and centrifuged. As a result of centrifugation, bacterial pellets settle down leaving a clear soup on the top. The soup was extracted out with a micropipette and kept separately in a test tube. Finally, the O.D of all the different solutions of 1% and 2% of concentrations and the two different soups obtained after centrifugation were measured. The spectrometer was standardised by using the media.

7. Enzyme assay

Three bacterial enzymes were identified – Peroxidase, Azo-reductase and Catechol 1,2-dioxygenase. Assays for the following enzymes were carried out.

7.1 Peroxidase (EC 1.11.1.7) Assay

3ml of pyrogallol solution and 0.1ml of enzyme extract were taken in a cuvette. The optical density (O.D) of the mixture was measured at 430nm and was taken at the control. This

mixture was taken as the blank and later 0.5 ml of 1% H₂AO₂ was added and mixed thoroughly. The O.D was measured at regular time intervals at 430nm.

7.2 Azo-reductase (EC 1.7.1.6) Assay

The assay was carried out in a total reaction volume of 1 ml. The reaction mixture contained 25 mM potassium phosphate buffer (pH 7.0), 20 μ M of azo dye and enzyme extract (water source). The reaction was initiated by addition of 0.1 mM NAD (P)H. The extinction coefficient for Methyl Red at 430 nm was 23,360 M⁻¹cm⁻¹.

The enzyme activity was measured by a decrease in absorbance over a 2 min period using an Ultraviolet-visible spectrophotometer. The enzyme activity was defined as the decrease in azo dye concentration (μ M) per minute. For the control, the enzyme was denatured by boiling it for 15 minutes, followed by the addition of few drops of hydrochloric acid.

7.3 Catechol 1, 2-dioxygenase (EC 1.13.11.1) Assay

Catechol 1, 2-dioxygenase activity was assayed using a spectrophotometer. The standard assay of enzyme activity was performed by making an assay mixture containing 500 μ l crude extract as source of Catechol 1, 2-dioxygenase, 200 μ l of 10mM Catechol as a substrate and final volume adjusted to 1ml, with 50mM sodium phosphate buffer (pH 7.0). The enzyme activity was monitored by measuring the formation of cis, cis-muconic acid at 260 nm (ϵ = 16.8 mM).

8. HPLC

Visible spectrophotometric scan in liquid chromatography column revealed the total hydrocarbon present in the sample. This test was performed using Reverse Phase OD S2 Column facilitated with a rotatory pump. Retention time was maintained between 0 to 55 minutes. The eluent was scanned within 200 nm to 700 nm and the detection was done within 340 to 700 nm with flow rate 1ml/min.

9. 16S rRNA sequencing

- A. DNA was isolated from the culture provided by the scientist. Quality was evaluated on 1.2% Agarose Gel, a single band of high-molecular weight DNA has been observed.
- B. Fragment of 16S rDNA gene was amplified by PCR from the above isolated DNA. A single discrete PCR amplicon band of 1500 bp was observed (Refer to gel figure-1)
- C. The PCR amplicon was purified and further process for the sequencing.

- D. Forward and Reverse DNA sequencing reaction of PCR amplicon was carried out with **8F** and **1492R** primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.
- E. Consensus sequence of **1342bp** 16S rDNA gene was generated from forward and reverse sequence data using aligner software.
- F. The 16S rDNA gene sequence was used to carry out BLAST alignment search tool of NCBI genbank database. Based on maximum identity score first fifteen sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 5.

RESULTS

1. Characteristics of sample

i) Physical characteristics

A. Electrical Conductivity and Total Dissolved Solid of the sample:-

Table 1: Determination of EC and TSS of the effluent sample.

Sample	Electrical conductivity (μS)	Total Suspended Solid (ppt)
Effluent from textile industry in Kasba, West Bengal	0.668	0.603

B. Determination of total suspended solid present in the sample

Table 2: Total suspended solid in the effluent.

No. of Observation	Dry weight of the filter paper (gms) [A]	Dry weight of the paper and the adhered residue(gms)[B]	Amount of sample used (ml)	TSS = (B - A)×1000 /ml of sample used (mg suspended solid/ L)	Mean ± Standard deviation
1.	0.332	0.3372	10 ml	0.52	0.7830
2.	0.469	0.4765		0.75	
3.	0.483	0.4881		0.54	

ii) Chemical characteristics

pH of the sample: The pH of the effluent was found to be 6.5.

2. Isolation of dye degrading bacteria

Colonies were raised in Nutrient Agar media to obtain pure cultures. The strain was Gram-positive, short rod shaped, motile bacteria. The micrometry results revealed the bacterial size to be 3.60 μm (Table 4). The biochemical characteristic of the bacteria is reported in the later in Table 5.

Table 3: Micrometric measurements of the bacterium.

S. No.	No. of ocular divisions [O]	No. of stage divisions [S]	Value of 1 ocular division [S/O×10]	Mean (μM) ± Standard deviation
1.	3	1	3.33	3.6069
2.	6	2	3.33	
3.	8	3	3.75	

The growth of the isolated organism was checked in different pH solutions.

Table 4. Determination of optimal growth conditions.

Ph	Growth
3	No
6	Yes
7	Yes
8	Yes
9	Yes
10	No
11	No

3. Biochemical tests

The biochemical characterization of bacteria was done by performing the following tests and the results obtained are as follows.

Table 5. Results of biochemical tests.

1. IMViC Test:	
(A) Indole production	-
(B) Methyl red test	-
(C) Voges proskauer test	+
(D) Citrate utilisation test	+
2. Urease test	-
3. Nitrate reduction Test	+
4. Catalase Test	+
6. Oxidase Test	-
7. Starch hydrolysis test	+
8. Casein hydrolysis Test	+

4. Dye degradation

The dye degradation was performed using minimal media for the growth of the bacterium. Different concentrations of the metanil yellow dye was prepared and were mixed with media and water to obtain different ratios. The O.D of all the solutions were measured to check the amount of dye getting degraded. Results of dye degradation are specified in Table 6.

Table 6. Results of dye degradation.

Dye	Control 1 (Media)	Control 2 (Dye)	Control 3 (Dye: Media)	Dye: Media: Inoculum	Mean \pm Standard Deviation
1%	0.00	0.17	0.09	0.05	0.0533
		0.16	0.09	0.06	
		0.18	0.09	0.04	
2%	0.00	0.29	0.11	0.04	0.04583
		0.30	0.10	0.04	
		0.33	0.12	0.05	

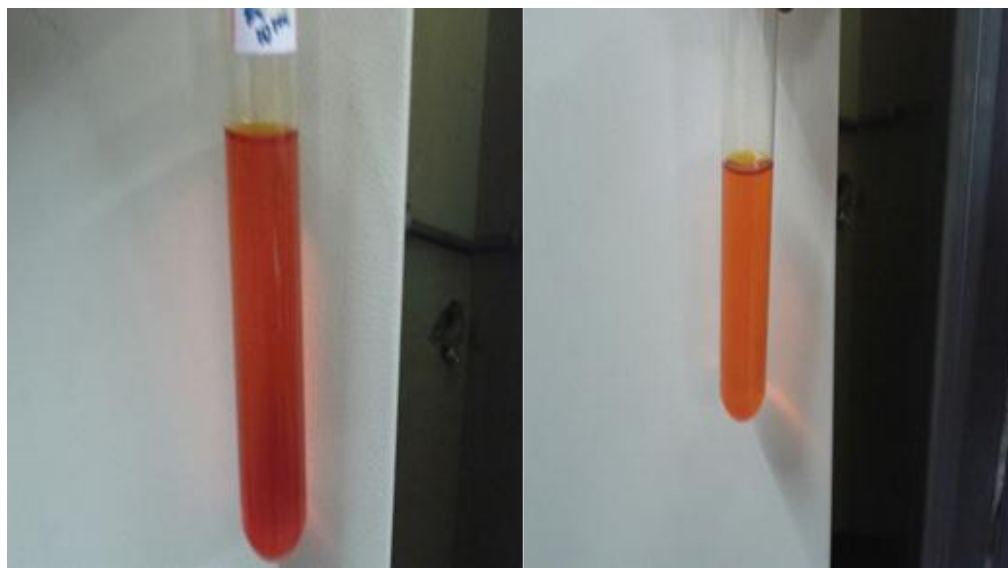
**(a) Dye before degradation****(b) Dye after degradation**

Figure 1: (a) shows the original colour of the dye and (b) shows the colour of the degraded dye.

4. Enzyme assay

Three bacterial enzymes were identified – Peroxidase, Azo-reductase and Catechol 1, 2-dioxygenase. The estimated amounts of enzyme are given as follows:

Peroxidase: $\Delta O.D/min = 0.45$

Catechol 1, 2-dioxygenase: $0.12\mu M$

Azo-reductase: $0.06\mu M$

5. Analysis of sequencing results

Comparative analysis of the sequences with already available database showed that the strain is close to the members of genus *Bacillus* species. The highest sequence similarities of the dye degrading bacterium was found to be with *Bacillus* sp.(99%).

Chromatogram data file detail

The following chromatogram files will be attached separately along with this report.

1. **ANAMICA_8F_S011564_A07_063.ab1: Data obtained with Forward primer**
2. **ANAMICA_1492R_S011564_B07_061.ab1: Data obtained with Reverse primer**

ANAMICA_8F_S011564_A07_063 (670 bp)

GCGGCGGACGGGTGAGTAACACGTGGGTAACTGCCATAAGACTGGGATAACT
CCGGGAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATT
GAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTG
GTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATC
GGCCACACTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTAGGG
AATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAG
GCTTTCGGGTCGTAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAG
CTGGCACCTTGACGGTACCTAACCAGAAAAGCCACGGCTAACTACGTGCCAGCA
GCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCG
CGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGG
GTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTTCCATGT
GTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTT
CTGGTCTGTAACTGACACTG

ANAMICA_1492R_S011564_B07_061 (970 bp)

GGTGTACAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGT
ATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCATGTAGGCG
AGTTGCAGCCTACAATCCGAACCTGAGAACGGTTTTATGAGATTAGCTCCACCTCG
CGGTCTTGACGCTCTTTGTACCGTCCATTGTAGCACGTGTGTAGCCCAGGTCATA
AGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTACCCGGCAGT
CACCTTAGAGTGCCCAACTTAATGATGGCAACTAAGATCAAGGGTTGCGCTCGTT
GCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACC
TGTCACCTCTGCTCCCGAAGGAGAAGCCCTATCTCTAGGGTTTTTCAGAGGATGTCA
AGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTT
GTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGCCTTGCGGCCGTACTCCCCAGG
CGGAGTGCTTAATGCGTTAACTTCAGCACTAAAGGGCGGAAACCCTCTAACACTT
AGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCC
ACGCTTTCGCGCCTCAGTGTACAGTACAGACCAGAAAGTCGCCTTCGCCACTGGT

GTTCTCCATATCTCTACGCATTTACCGCTACACATGGAAATTCCACTTTCCTCT
TCTGCACTCAAGTCTCCCAGTTTCCAATGACCCTCCACGGTTGAGCCGTGGGCTTT
CACATCAGACTTAAGAAACCACCTGCGCGCGCTTTACGCCCAATAATTCCGGATA
ACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTT
TCTGGTTAGGTACCGTCAAGGTGCCAGCTTATTCA

CONSENSUS SEQUENCE ANAMICA (1342 BP)

GCGGCGGACGGGTGAGTAACACGTGGGTAACTGCCATAAGACTGGGATAACT
CCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATT
GAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTG
GTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATC
GGCCACACTGGGACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTAGGG
AATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAG
GCTTTCGGGTCGTAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAG
CTGGCACCTTGACGGTACCTAACCAGAAAAGCCACGGCTAACTACGTGCCAGCA
GCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCG
CGCGCAGGTGGTTTTCTTAAGTCTGATGTGAAAGCCACGGCTCAACCGTGGAGG
GTCATTGGAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTTCCATGT
GTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTT
CTGGTCTGTAAGTACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGA
TACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGC
CCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAA
GGCTGAAACTCAAAGGAATTGACGGGGGCCCCGCACAAGCGGTGGAGCATGTGGT
TTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAAAACCC
TAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGT
CAGCTCGTGTCGTGAGATGTTGGGTAAAGTCCCGCAACGAGCGCAACCCTTGATC
TTAGTTGCCATCATTAAAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGA
GGAAGGTGGGGATGACGTCAAATCATCATGCCCTTATGACCTGGGCTACACAC
GTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTC
ATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGA
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CACACCGCCCGTCACACCACGAGAGTTTGTAACACC

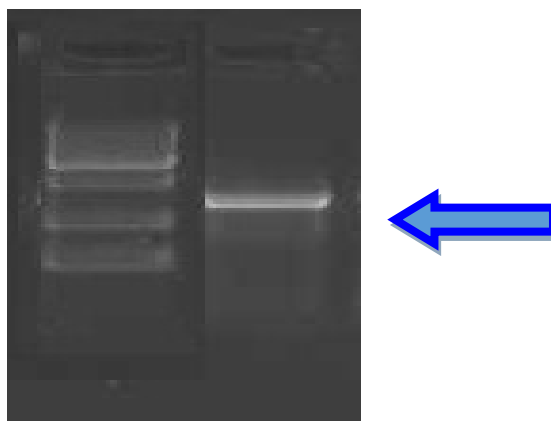


Figure1: 1.2% Agarose gel showing single 1.5 kb and 16S rDNA amplicon

Lane 1: DNA marker (1kb ladder)

Lane 2: 16S rDNA amplicon

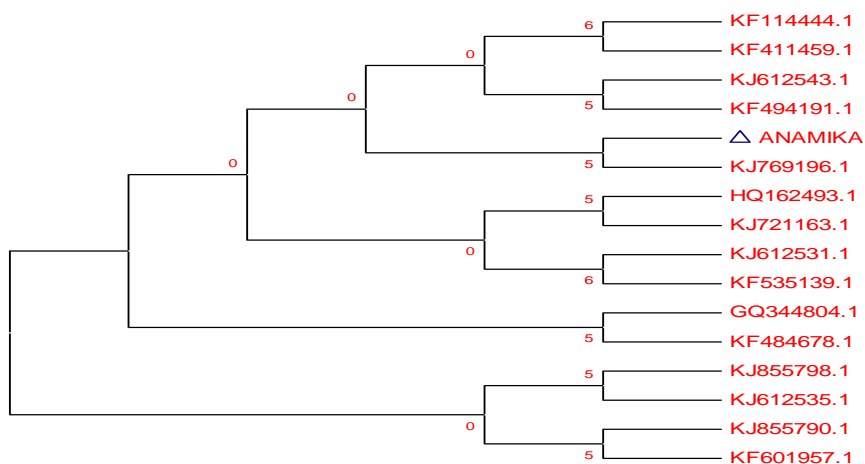


Figure 2: Phylogenetic tree showing evolutionary relationship of 11 taxa.

6. HPLC

The total hydrocarbon present was scanned by Reverse Phase OD S2 column which is represented in Figure 3.

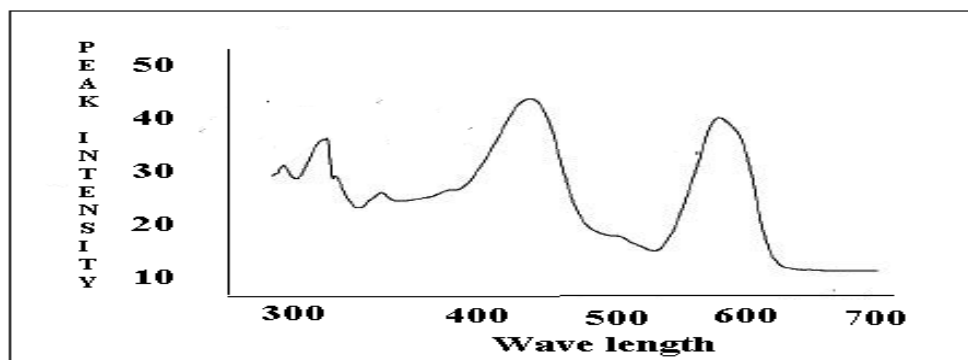


Figure 3: Visible spectrophotometric scan in liquid chromatography column.

A Scope for Bioremediation Using Such Bacteria

This bacteria is found to degrade metanil yellow and other complex azo dyes by various mechanisms. These mechanisms can be utilised for detoxification and removal of toxic forms of dyes from polluted environment. According to these results the present day study evaluates that the identified bacteria can be used to remediate textile effluents.

DISCUSSIONS

The tests performed were followed by 16S rRNA sequencing which reveals that the organism present in the textile effluents is *Bacillus*. They belong to class Bacilli and were first mentioned in the eight edition of Bergey's Manual. These bacteria are gram-positive, obligate aerobes living in textile effluents rich in complex azo dye compounds. Studies have shown that a number of members belonging to this genus are capable of degrading textile dyes and hence serve as organisms for bio-remediation. *Bacillus megaterium* shows considerable reduction (57.41%) of Red 3BN dye using peptone as nitrogen source. Certain studies also showed the influence of AzoR1 in case of *Bacillus subtilis* ORB7106 in decolourising complex azo dyes like Azobenzene, Methyl Red, Orange G and Congo Red. The azo-nitrogen of the dye substrates provide nitrogen requirement of the organism in cultures in absence of nitrogen. Decolourization of dyes is a reduction process which requires redox equivalents (electron donors) that transfer electrons to the chromophoric group dyes. Nitrate (NO_3^-) has higher oxidation character compared to the chromophoric group dyes. The result obtained was attributed to competition between (NO_3^-) and the Chromophoric group for the redox equivalents, which result in preferential reduction of (NO_3^-) relative to the chromophoric group. These bacteria thus serve as potential candidates for bio-remediation. Inoculating a given area of land contaminated with the aforementioned contaminants, with a definite dose of these bacteria, will not only help to restore land use but also serve a long way in protecting human health. Further information derived from emerging disciplines like Metagenomics, Transcriptomics can be incorporated into the studies of Ecotoxicology to develop mathematical, probabilistic models which will help in accurate selection of remediating organisms against specific contaminants.

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