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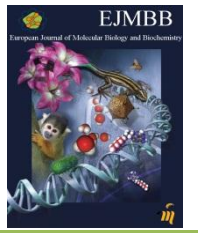

BIOREMEDIATION OF AZO DYES USING BACILLUS ARYABHATTAI ISOLATED FROM TEXTILE EFFLUENT

Arul yesudoss L[1*], Radha PV[2], Rajesh C[3]

[1]Assistant Professor & Head, Department of Biochemistry & Biotechnology, Sourashtra College, Madurai, Tamilnadu, India.
[2]Assistant Professor, Department of Biochemistry & Biotechnology, Sourashtra College, Madurai, Tamilnadu, India.
[3]Territory Manager, Neon Laboratories, Bangalore, Karnataka, India.

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ABSTRACT
Improvement and development of different industries in the world enlarge the environmental pollution. The contamination of the industrial effluent considered as a major environmental crisis. The Azo dyes from the textile industry pollute the surface water that represents a serious environmental trouble. An attempt has been made in the present study to isolate and identify suitable microorganisms which are capable of growing in the azo dye contaminated sites. The effluent samples were enriched with minimal media. Then the enriched samples were inoculated in the minimal medium agar with two different dyes and microorganisms were isolated. The isolated microorganisms were identified using morphological, biochemical and molecular characterization. The DNA from the isolates was purified, amplified sequenced and the isolates were identified as *Bacillus* sp. using BLASTn analysis. The dye degradative potential of the isolates were analyzed by LCMS method. From this study it was clear that *Bacillus aryabhattai* has high potential to degrade the dye than the other bacterial isolates.

INTRODUCTION

Rapidity of industrialization and urbanization around the world has been lead to the recognition and understanding of relationship between environmental pollution and public health [1]. While, the pollutions triggered by the human activities become the top most challenge for modern civilization [2]. Among the most concerned environmental pollutions, water pollution is the most threatening and major one of our biodiversity, where effluents from dye-based industries serve as principal sources.

The textile industry accounts for two-thirds of the total dyestuff market. During the dyeing process, approximately 10–15% of the dyes are used and it is released into the water.

Corresponding Author

Arul yesudoss L
Email: - arulyesudoss28@gmail.com

The presences of these dyes in the aqueous ecosystem are the serious cause of environmental and health concerns. Several methods are used to treat textile effluents to achieve decolorization which include such as filtration, coagulation, carbon activated and chemical flocculation [3]. These methods are effective but expensive and form of a concentrated sludge that creates a secondary disposal problem. The majority of azo dyes (food and textile) have LD_{50} values between 250-2,000 mg/kg body weight, indicating that, for a lethal dose many grams of azo dyes have to be utilized in a single dose. As azo dyes are highly water soluble, they do not accumulate in the body, but are metabolised in the liver and excreted in the urine. And also azo dyes have very strong colours and become hyperactive to cause allergies. The exact mechanism why Tatrazine increases allergic reactions or asthma is still not fully understood.

In general, the waste water from textile industry contains many dyes. Bacteria is a cheaper and improved environment friendlier, an alternative for color removal in

textile dye effluent. So that various degrading bacteria are used to exhibit degrading ability for a wide range of azo dyes. This study is aimed to isolate some bacterial strains which possessed the ability to degrade the azo dyes and the bacterium with the highest degrading ability has chosen for further studies.

MATERIALS AND METHODS

Sample collection, morphological and Biochemical characterization

The samples used for the present study were collected from effluent waters disposed from dyeing unit located in Thirupur. For the conformation of microbial presence Simple and Gram's staining was done, observed under microscope for the conformation of bacterial morphological identification. Biochemical methods such as MR-VP test, catalase test, citrate utilization and starch hydrolysis tests were performed.

Molecular identification

Isolation of genomic DNA

1.5ml of culture was taken in a micro centrifuge tube and a pellet out by centrifugation at 10,000 rpm for 5 minutes. The pellet was re-suspended in 500µl of TE buffer. 200µl of lysozyme was added and incubated at 37°C for 30 minutes. 60µl SDS and 3µl of proteinase K was added and incubated at 55°C for 2 hours. After incubation, equal volume of Tris saturated phenol (900µl) was added. The mixture was centrifuged at 10,000 rpm for 10 minutes. The supernatant was transferred to a fresh tube and the equal volume of PCI (Phenol: Chloroform: Isoamylalcohol) was added.

The extraction was repeated until to obtain without protein precipitate at interface. The aqueous phase was transferred to a fresh tube and 0.1 volume of 3mM Sodium Acetate (pH-5.5) was added. It was mixed gently and 2.5 volumes of the ice cold Ethanol was added, mixed by the inverting the tubes. The tubes were incubated at -80°C for 30 minutes.

The content was centrifuged at 10,000 rpm for 10 minutes and the supernatant was discarded. The pelleted DNA, equal volume of 70% ethanol was added and centrifuged at 10,000 rpm for 10minutes. The supernatant was discarded. The pellet was dried and dissolved in 100µl of TE buffer.

Separation of isolated genomic DNA by agarose gel electrophoresis

10µl of DNA was isolated in 1% agarose gel. The DNA was separated and visualized with UV illuminator.

PCR AMPLIFICATION OF 16S rRNA GENE

Primers Used

- Primers for 16S rRNA gene

8F 5'-AGA GTT TGA TCC TGG CTC AG-3' and 1490-5'-GAC TTA CCA GGG TAT CTA ATC C-3' (Sigma Genosys)

TEST: (Total volume 50µl)
Genomic DNA - 3µl
8F Primer - 2.5µl
1490 R - 2.5µl
Genei master mix - 25µl
Deionized water - 17µl
CONTROL: (Total volume 50µl)
Genomic DNA - 3µl
Forward Primer - 2.5µl
Reverse Primer - 2.5µl
Deionized water - 17 µl

Thermal cycler programme

Sl.No	Step	Temperature (^{0}C)	Time (min)
1	Initial denaturation	95	3.00
2	Denaturation	94	1.30
3	Annealing	55	0.40
4	Primer extension	72	1.30
5	Steps 2& 4	34 cycles	
6	Final extension	72	20.00

Purification of PCR products:

Products obtained in PCR reaction were purified using PCR purification Kit (Column Based), Bio serve, following the procedure as per the manufacture's guidelines.

- 3 volumes of solution A to 1 volume of the PCR sample were mixed. It is necessary to remove mineral oil or kerosene. For example, add 75µl of solution A to 25µl PCR sample (not including oil).
- A bio serve spin column was placed in a provided 2ml collection tube.
- The sample was applied to the Bio serve spin column and centrifuge for 1 minute at 6,000rpm for DNA binding.
- The flow was discarded. The spin column was placed back into the same tube.
- The column was washed twice by adding 500µl solution B to the Bio serve spin column and centrifuged for 1 minute at 11,000rpm.
- The flow was discarded and placed the Bio serve spin column back in the same tube.
- Bio serve spin column was placed in a clean 1.5ml micro centrifuge tube.
- To elute DNA, 20µl Solution C was added (10mM Tris-Cl, pH 8.5) or H_2O to the center of the membrane. The column was allowed to stand for 2-5minutes, and then the column was centrifuged for 1 minute at 11,000rpm.

Database searching and deposition of gene in GenBank

Nucleotide database was searched with the sequences obtained with NCBI (National Centre for

Biotechnology Information) BLAST (Blastn) tool http://www.ncbi.nlm.nih.gov/BLAST.

RESULTS

The sample was collected from effluent water disposed from dyeing unit and the organisms were isolated. The samples were serially diluted and spread on nutrient agar, the single colonies were isolated (Figure 1).

The isolated colonies are inoculated into the Selective Medium (Figure 2). The colonies isolated were subjected to differentiate their morphological characters by using a staining method (Simple and Gram stain). It was observed that a purple, rod shaped bacteria (Figure 3).

Then the biochemical characters of the isolates were done with biochemical tests shows results (Figure 4, 5 and Table 1). Isolated colonies were identified by isolate the genomic DNA. The DNA isolated was separated by agarose gel electrophoresis (Figure 6). The DNA was amplified by PCR (Polymerase Chain Reaction) (Figure 7. Then the samples were sequenced to identify the isolates (Figure 8). The identified isolate was *Bacillus aryabhattai* by using BLAST analysis (Figure 9 and Table 2).

Figure 1. Isolation bacteria from the effluent

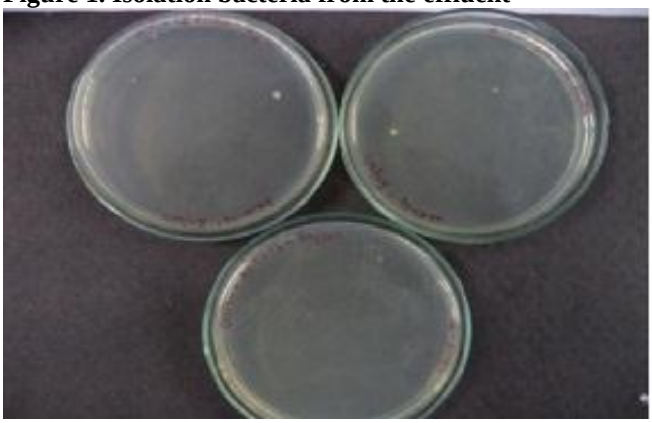


Figure 2. Dye degradation of isolted baceteria

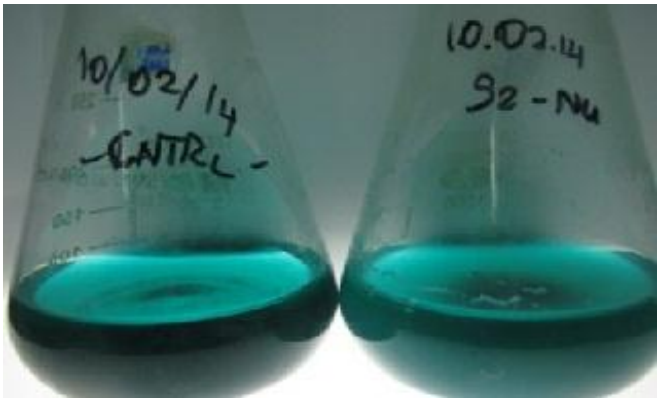


Figure 3. Microscopic observation of isolated bacteria by Grams's staining technique

Figure 4. Biochemical analysis for the conformation of bacterial characteristics

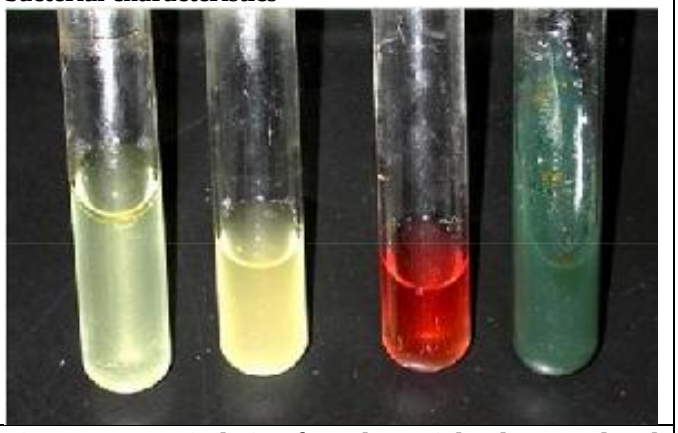


Figure 5. Starch Hydrolysis analysis for the conforamtion test

Figure 6. DNA isolation from bacterial culture and Gel documentation

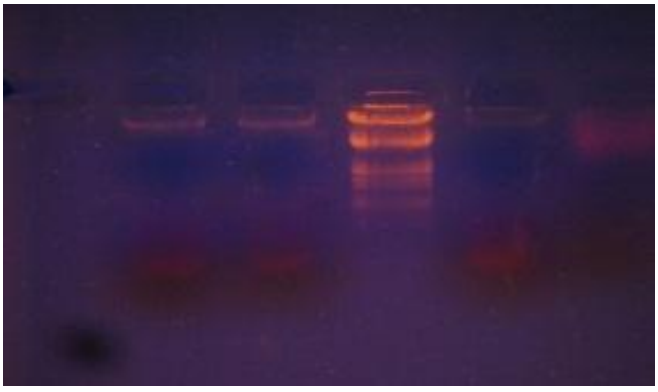


Figure 7. PCR amplification of amplified DNA

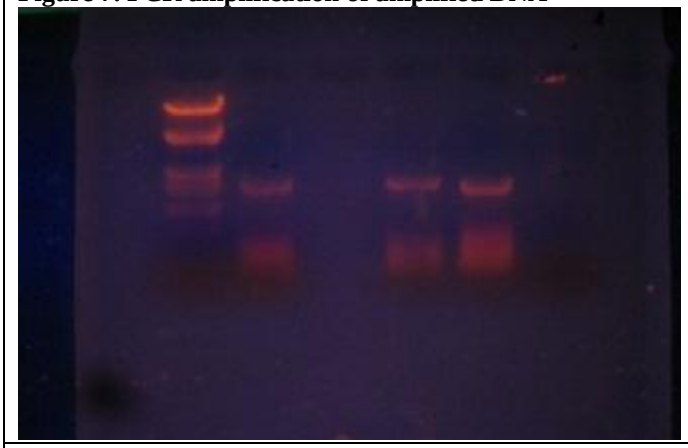


Figure 8. Blastn Analysis of DNA isolated from bacteria isolated from the effluent

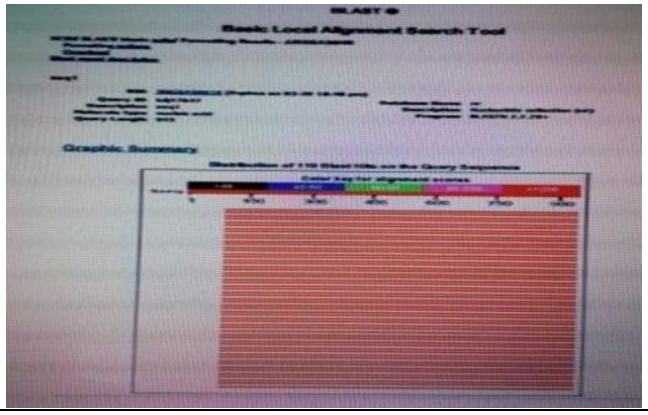
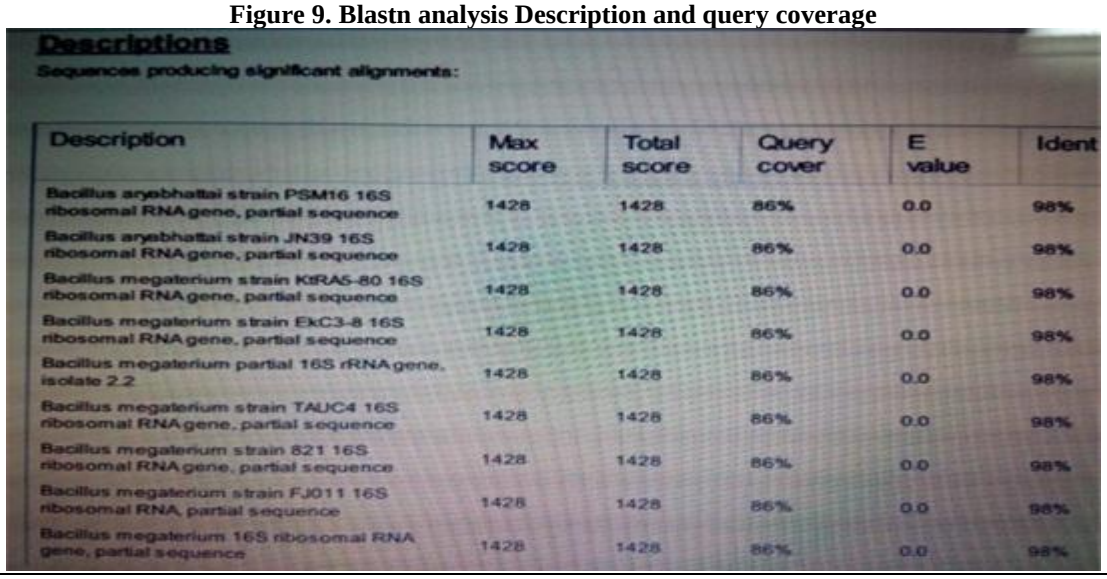


Figure 9. Blastn analysis Description and query coverage



Descriptions

Sequences producing significant alignments:

Description	Max score	Total score	Query cover	E value	Ident
Bacillus aryabhattai strain PSM16 16S ribosomal RNA gene, partial sequence	1428	1428	86%	0.0	98%
Bacillus aryabhattai strain JN39 16S ribosomal RNA gene, partial sequence	1428	1428	86%	0.0	98%
Bacillus megaterium strain KtRA5-80 16S ribosomal RNA gene, partial sequence	1428	1428	86%	0.0	98%
Bacillus megaterium strain EkC3-8 16S ribosomal RNA gene, partial sequence	1428	1428	86%	0.0	98%
Bacillus megaterium partial 16S rRNA gene, isolate 2.2	1428	1428	86%	0.0	98%
Bacillus megaterium strain TAUC4 16S ribosomal RNA gene, partial sequence	1428	1428	86%	0.0	98%
Bacillus megaterium strain 821 16S ribosomal RNA gene, partial sequence	1428	1428	86%	0.0	98%
Bacillus megaterium strain FJ011 16S ribosomal RNA, partial sequence	1428	1428	86%	0.0	98%
Bacillus megaterium 16S ribosomal RNA gene, partial sequence	1428	1428	86%	0.0	98%

Table 1. Biochemical characters of bacterial isolate

	Grams staining	Morphology	Citrate test	Methyl red test	Starch hydrolysis	Genus
Isolate I	Positive	Rod	-	+	+	*Bacillus* sp

Table 2. Bacterial species identification by BLASTn analysis

S.No	Sample	Organism
1	Isolate I	*Bacillus aryabhattai*

DISCUSSION

The potential bacterial isolates that degrade the dye was identified using the morphological, biochemical and molecular characterization. The four different isolate was identified as *Bacillus aryabhattai*.

Similar result was reported that a screening for dye decolorization alkali-thermophilic microorganisms resulted in a *Bacillus* species [6]. Strain isolated out of the wastewater drain of a textile finishing company. An NADH-dependent azoreductase of this strain, *Bacillus* sp. Strain SF, was found to be responsible for the decolorization of azo dyes. This enzyme was purified by a combination of ammonium sulfate precipitation and anion-exchange and affinity chromatography and had a molecular mass of 61.6kDa and an isoelectric point at pH 5.3. The optimum of the azoreductase dependent on the substrate and was within the range of pH 8-9, while the temperature maximum was reached 80^0C decolorization only took place in the absence of oxygen and was enhanced by FAD, which was not consumed during the reaction.

It was stated that various carbon source, nitrogen source, temperature, pH and inoculums size were optimized for decolorization of Orange 3R by using bacterial isolates [7]. *Pseudomonas* and *Bacillus* Showed maximum dye decolorization of 89% at the end of 144hr

under optimum condition. Similarly the *Bacillus* sp. was found to be more efficient in dye decolorization. It was reported that a moderately halo tolerant microbe (0.5-2.5 M NaCl sustaining) was isolated from water sample from Marina beach and was identified as *Bacillus aryabhattai* [4]. Percentage dye degradation by the isolated *Bacillus aryabhattai* was found to be 64.89%. The enzyme involved in degradation Azoreductase was assayed and purified by anion exchange chromatography. Total activity of the purified enzyme was 36.03U. The enzyme gave a single band in the SDS–PAGE with a molecular weight of 65 kDa. Decolorization of azo dye Red 3BN by two bacterial species *Bacillus cereus* and *B. megaterium* has been analyzed using mineral effluent, consisting of known concentration of the dye in ZZ medium [5]. Physico chemical parameters like carbon source, nitrogen source, temperature, pH and inoculum volume are optimized for the decolorization process by changing one parameter at a time. Optimal condition for *B. cereus* was found to be 1% sucrose 0.25% peptone, pH 7, 37°C and 8% inoculum and that for *B. megaterium* was found to be glucose 1%, 0.25% yeast extract, pH 6, 37°C and 10% inoculum. Extent of decolorization recorded by *B. cereus* under ideal conditions was 93.64% and that by *B. megaterium* was 96.88%. The study has confirmed the potential of *B. cereus* and *B. megaterium* in the decolorization of Azo dye Red 3BN and opened scope for future analysis of their performance in the treatment of textile effluent. Decolorization of azo dye by two bacterial species Bacillus aryabhattai and Bacillus cereus has been analyzed using mineral effluent, consisting of known concentration of the dye in ZZ medium [8].

Physio chemical parameters such as carbon and nitrogen source, temperature, pH and inoculum volume are optimized for the decolorization process by changing one parameter at a time. Optimal condition for Bacillus *cereus* was found to be 1% sucrose, 0.25% peptone, pH 7, temperature 37°C and 8% inoculum and that for *Bacillus aryabhattai* was found to be glucose 1%, 0.25% yeast extract, pH 6, temperature 37°C and 10% inoculum. Extent of decolorization recorded by Bacillus cereus under ideal conditions was 95% and that by *Bacillus aryabhattai* was 98%. The study has confirmed the potential of Bacillus cereus and *Bacillus aryabhattai* in the decolorization of Azo dye and opened scope for future analysis of their performance in the treatment of textile effluent.

CONCLUSION

The present study it could be concluded that the microbial community may be applied in the environment. The varieties of catabolic activities by microorganisms can be modified to degrade dye contaminated area. The continued development and application of biotechnology for biodegradation was limited primarily by physical and economic factors. The idea of recombinant strains, which can degrade variety of compounds under extreme conditions, must be developed. Thus the biodegradation prove to be an effective means of treatment of pollutants.

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CONFLICT OF INTEREST:
The authors declare that they have no conflict of interest.

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