Isolation and Identification of Azo Dye Degrading Mcroorganisms

Mohanapriya B. and C. Kanagaraj

Research Journal of Agricultural Sciences
An International Journal

P- ISSN: 0976-1675
E- ISSN: 2249-4538

Volume: 13
Issue: 04

Res. Jr. of Agril. Sci. (2022) 13: 1073–1078

CARAS

Res. Jr. of Agril. Sci. (Jul-Aug 2022)
13(4): 1073–1078

ISSN: 0976-1675 (P)
ISSN: 2249-4538 (E)

www.rjas.org

Full Length Research Article

Isolation and Identification of Azo Dye Degrading Mcroorganisms

Mohanapriya B*[1] and C. Kanagaraj[2]

Received: 14 May 2022 | Revised accepted: 16 Jul 2022 | Published online: 18 July 2022

A B S T R A C T

Industrial effluents containing unreacted azo dyes contaminates the water heavily and is one of the major sources of environmental pollution. Hence bioremediation by removal of dye effluents from water is essential. The aim of this research is to isolate bacterial strains that can efficiently degrade azo dyes and identify the strains using. Wastewater samples were collected from five different zones rich with dye Effluents across Tirupur. 9 bacterial strains were isolated out of which three strains were positive for dye decolorization activity. Biochemical Tests were done for identifying the characteristics of the positive strains. Quantitative analysis was also done for those strains using magenta dye to find out the rate of dye decolorization of each strain. Isolates Culture-5 (D-5) and Culture-6(C-6), were among the best dye degraders. D-8 was efficient enough to degrade magenta dye up to 80% in a week.

***Key words*:** Decolorization, Dye degradation, Wastewater, Bioremediation *Bacillus spp., Staphylococcus spp.*

Biodegradation is an environment friendly method used for the degradation of pollutants. In this study, Azo dyes is degraded by using microorganism, which are isolated from the textile industry effluent [1]. The biodegradation of synthetic dyes is an economic, effective, bio friendly, and environmentally benign process. Bioremediation of xenobiotic including synthetic dyes by different microbes will hopefully prove a green solution to the problem of environmental soil and water. Bacterial oxidoreductive enzymes are the key role in the degradation of synthetic dyes. This dynamic metabolism of bacteria able to utilize complex xenobiotic compounds of the dyestuff as a substrate. In the process, they are broken down to less complex metabolites [2]. Azo dyes and nitro-aromatic compounds are considered as potential xenobiotic. They are extensively used worldwide in textile, paint, printing, cosmetics, and pharmaceutical industries. A high discharge of untreated wastewater from these industries is the major source of azo dyes to enter into the ecosystem. Azo dyes containing nitro and amine moieties are toxic and mutagenic to biological systems. Synthetic nitro-aromatic compounds are also potential mutagenic and carcinogenic to biological system [3]. The rapidly increasing population and industrial development have led to the addition of an array of man-made chemicals in the environment, leading to a tremendous deterioration in environmental quality [4].

* **Mohanapriya B.**
✉ mohanapriya.bio@rathinam.in

[1-2] Department of Biotechnology, Rathinam College of Arts and Science, Coimbatore - 641 021, Tamil Nadu, India

Contamination of soil, air, water, and food is one of the major problems in the industrialized world today. Significant regulatory steps have been taken to eliminate or to reduce the production or release of these chemicals into the environment. A major contribution to these categories is azo dyes, most of which are toxic and hazardous in nature. Application of microbial processes to decontaminate environmental polluted with these compounds will require a better understanding of why and how microorganisms can degrade and utilize for their own survival as well as for cleaning the environment. Here, focuses on different anaerobic microbial degradation of azo dyes and enzymes involved therein are responsible for degradation [5].

Generally, the wastewater from dye industries contains different types of dyes. So, the bacterial strain that can efficiently degrade different types of dyes would be considered the most effective and powerful strain [6]. This project involves isolating bacterial strains around Tiruppur textile industrial areas that can decolorize 3 different colors of Azo dyes. The strains with maximum efficiency were chosen for further studies.

A number of researches and project works were carried on in the field of bioremediation by removal of dye effluents from water that is cost-effective, produces less sludge and is less benign to the environment. Siddique and Ahmed [7] reported that Bacterial strain *Brevibacillus centrosporus* had a 90% decolorizing rate for Reactive Violet 5R dye, compared to *Paenibacillus azoreducens* showed 85% decolorizing rate. Mishra and Maiti [8] reported that *Pseudomonas species* can decolorize reactive Azo, anthraquinone and triphenylmethane dyes either individually or mixed or with metal ions. Abd El-Rahim *et al.* [9] reported that some species of the fungi

Aspergillus and a single *Lichtheimia spp*. showed 70- 90% decolorization rate for violet dye. Bayoumi *et al*. [10] reported that *Pseudomonas aeruginosa*, *Pseudomonas putida* and *Bacillus cereus* showed dye degrading properties and could decolorize acid red, Sulphur black and drimarene Blue. Shah [11] reported that *Pseudomonas aeruginosa* could degrade Black B dye up to 93%, while *Bacillus subtilis* showed maximum degradation of Red RR. *Pseudomonas putida*, degraded Blue RR dye up to 95%. Ponraj *et al*. [6] reported *that Pseudomonas sp*. and *Bacillus sp*. successfully degraded orange 3R dye almost 90%.

MATERIALS AND METHODS

The materials and laboratory equipment used for this research work included Falcon tubes, test tubes, petri dishes, inoculating loop, micropipettes (200µL and 1000µL), ethanol, tissue paper, conical flasks, distilled water, shaker incubator, Incubator with temperature controller, horizontal laminar air flow system, refrigerator, microcentrifuge and microfuge tubes, laboratory autoclave, laboratory discard autoclave, sterile cotton plugs, spectrophotometer, 500mL glass jars, laboratory reagents. Different areas across the Tirupur were identified and collect untreated effluents from dye industries. Liquid samples were collected from these areas in a Falcon Tube and then safely carried to the laboratory.

Dye used

Industrial Grade Clothing dyes were purchased from a local dye shop. The dye magenta (purple color) was obtained.

Isolation of microorganisms from sample

In order to isolate the bacterial colonies from the dye effluents a series of steps were followed. At first, the liquid sample was diluted using 4-fold serial dilution method. After that, each diluted sample was spread on a petri-plate using spread plate technique. Mixed bacterial colonies were identified after 24 hours of incubation at 37 degrees Celsius. Pure colonies of each of the bacteria were obtained by quadrant Streaking method. Finally, they were inoculated in LB broth medium to obtain pure bacterial culture. The result was furnished in Table-1 and Fig-1.

Serial dilution

Five Test Tubes were thoroughly washed with detergent, wrapped and autoclaved at 121 degrees Celsius at 15 psi for 15 minutes. After autoclaving, they were brought inside laminar air flow cabinet and 4.5 ml of autoclaved distilled water was added to each of the test tubes. Now, 0.5 ml of water sample was added to the first test tube and mixed thoroughly with the distilled water to make a 5 ml diluted solution Now, 0.5 ml of solution from the first test tube was pipetted out and added to the second test tube and mixed thoroughly. Again, 0.5 ml of solution from the second test tube was added to the third one, and so on. At the end, five test tubes were obtained that contain the diluted samples with a dilution range of 10^-1 to 10^-5.

Spread plating

Four petri dishes were thoroughly washed with detergent, wrapped and autoclaved at 121 degrees Celsius and 15 psi for 15 minutes. 100 ml of LB agar media was prepared and autoclaved along with it. After autoclaving, they were brought inside laminar air flow cabinet and the liquid agar was poured on each of the petri Dishes. It was allowed to solidify. 100 µl of diluted water samples were pipetted out from each of the test tubes (10^4 and 10^5) and added to the each of the corresponding solidified agar plates. An L shaped glass spreader was sterilized by dipping into 99.9% ethanol followed by flaming it over Bunsen Burner. The sample was then evenly spread using the sterilized spreader by rotating the petri dish carefully. Finally, the petri dishes were incubated at 37 degrees Celsius for 24 hours. After 24 hours, mixed bacterial growth was observed on the agar plate and Nine variable bacterial colonies were identified.

Inoculation into LB Broth

5 ml of LB Broth was poured into Nine (9) Test Tubes each and autoclaved at 121degrees Celsius and 15 psi for 15 minutes. A discrete bacterial colony from each of the streak plates was picked up using sterilized Inoculating Loop and inoculated into each of the five autoclaved Test Tubes inside Laminar Air Flow Cabinet. The Test Tubes were incubated at 37 degrees Celsius on a shaker for 24 hours. After 24 hours, bacterial culture growth was observed on the test tubes that are identified by the turbidity of the broth.

Isolating dye degrading microorganism

5ml of LB broth was put into nine test tubes each. Then 5.5 mg (0.01%) of Manjatta Dye was added to each of the test and they were Autoclaved at 121 degrees Celsius and 15 psi for 15 minutes. 100 µl of each of the bacterial culture is added to the respective test tubes inside laminar air flow cabinet. The tubes were incubated at 37 degrees Celsius for 48 hours. After 48 hours, the Tubes were taken out and were checked for potent dye degrading bacterial colonies on the basis of decolorization of the azo dye. The samples showed high rate of decolorization were identified for further tests.

Dye decolorization assay

Dye decolorization experiments were performed to find out which bacteria is most efficient in degrading dyes. A Test Tube was washed thoroughly and labelled as ‘Control’ The samples were taken into microcentrifuge tubes each and centrifuged at 5000 rpm for 5 minutes. Now, optical density of the supernatant was measured at 520 nm using a spectrophotometer keeping the control solution as reference the OD of the control solution was also measured at 520 nm keeping distilled water as reference, to find out the initial absorbance of the control.

Quadrant streaking

Three agar plates were prepared. An inoculating loop was sterilized by flaming it over Bunsen burner until it is red hot and then allowed to cool down. Each of the three isolated bacterial strains with the best dye degrading (D-5, D-6, D-7) were picked from the liquid culture using the inoculating loop and Quadrant Streaking was done on the respective agar plates. The inoculating loop was flamed after streaking of each quadrant of the agar plate. Finally, agar plates were incubated at 37 degrees Celsius for 24 hours. After 24 hours, discrete colonies were identified from each of the Streak Plates.

Bacteria identification

Test After the positive dye degrading bacterial colonies were identified, biochemical tests were performed to identify the isolated bacterial colonies. The following biochemical test were performed.

Morphological screening

Gram staining: A glass slide was sterilized with ethanol and bacterial culture was smeared on it using inoculating loop inside the laminar air flow. It was heat fixed. Crystal Violet was

flooded over the smear for 1 minute and gently washed with water. Now, Gram's Iodine was flooded over the smear for 1 minute and again gently washed with water. Now 70% ethanol was flooded over the smear for about 15 seconds and gently washed. Finally, safranin was flooded over the smear for 1.5 minute and gently washed with water. The slide was allowed to dry and observed under microscope. Helical or cocci Shape was also observed under the Microscope (Fig 6-8).

Biochemical test

Azo reductase test: Three Conical Flask was washed and sterilize with ethanol. 150ml of nutrient broth was prepared and separated in the three conical flasks in equal volume and autoclaved at 121 degrees Celsius and 15 psi for 15 minutes. 1ml of bacterial culture was added to each flask (D-5, D-6, D-7) respectively and incubated at 37 degrees Celsius till decolorization process is confirmed. After keeping it in water bath at 60 degrees Celsius for 20 minutes. Cells from the mid log phase culture were harvested by centrifugation at 10000 rpm for 10 minutes at 4°C. Pellet were disrupted by sonication at 40% power for 6 minutes. The cell lysate (supernatant) was subjected to fractionated ammonium sulfate of 70% saturation in a second step to precipitate the azoreductase. After 24 hours, the precipitated protein is centrifuged for 10 minutes at 10000 rpm at 4°C and the pellet was dissolved in equal volume of 50mM potassium phosphate buffer (pH 7.2).

Molecular weight estimation by SDS – PAGE

The molecular weight of the azo reductase was estimated by sodium dodecyl sulphate – polyacrylamide agarose gel electrophoresis (SDS – PAGE) Technique. To be able to estimate the molecular weight of proteins on the SDS - PAGE, proteins of known molecular weight need to be run simultaneously on the gel. A mixture of these proteins is called protein standards or protein molecular weight markers. 10% SEPARATING GEL: 4 ml distilled water, 3.6ml Acrylamide, 2.5ml Tris HCL (pH 8.8), 0.1ml 10%APS, 0.1ml 10%SDS, 0.004ml TEMED; STACKING GEL:

3.4ml distilled water 0.83ml Acrylamide, 0.63ml Tris HCL (pH 6.8), 0.05ml 10% APS, 0.05ml 10% SDS, 0.004ml TEMED. Place all microcentrifuge tubes containing samples for SDS - PAGE into a water bath for 15min. Load all samples into gel lanes starting with the molecular weight standards. Sample loading volumes should be dependent on gel. Load 100µl of staining dye then all the loading samples range from 50µl.Connect both the anode and the cathode and set the voltage on the electrophoresis power supply to a constant voltage of 150 V. Allow the gel to electrophorese for 3 hours and after proceed the desired direction method. The result was captured in (Fig 11).

Quantification of proteins by Lowry's method

Different dilutions of BSA solutions are prepared by making stock BSA solution (1mg/ml) and water in the test tube as given in the table. The final volume in each of the test tubes is 5ml. The BSA range is 0.05 to 1 mg /ml. From these different dilutions, pipette out 0.2 ml protein solution to different test tubes and add 2ml of alkaline copper sulphate reagent. Mix the solutions well. This solution is incubated at room temperature for 10 mins. Then add 0.2 ml of reagent folin ciocalteau solution to each tube and incubate for 30 minutes. Zero the colorimeter with blank and take the optical density at 660 nm. Plot the absorbance against protein concentration to get a standard calibration curve. Check the absorbance of unknown sample and determine the concentration of the unknown sample using the standard curve plotted.

RESULTS AND DISCUSSION

Spread plating technique

After 24 hours, mixed bacterial growth was observed on the agar plate and Nine variable bacterial colonies were identified. Bacterial colonies were successfully isolated.

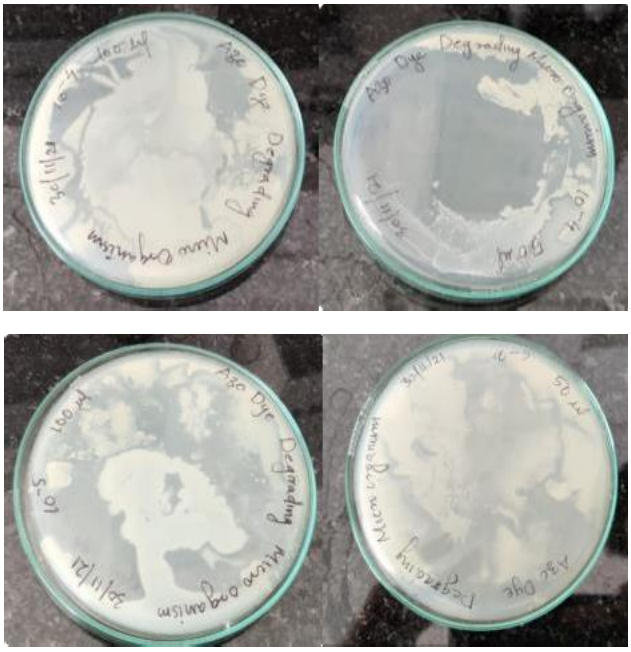


Fig 1 Isolation and enumeration of microorganisms in a mixed sample by spread plating

Dye degrading bacterial colonies

A total of nine bacterial colonies were isolated from the water samples, that were named from D-1 to D-9 respectively, out of which only six were positive samples that could degrade dyes, namely, D-3, D-4, D- 5, D-6, D-7, and D-8.

Table 1 Screening positive dye degrading bacteria strains

Colony	Dye degraded
D-1	NO
D-2	NO
D-3	YES
D-4	YES
D-5	YES
D-6	YES
D-7	YES
D-8	YES
D-9	NO

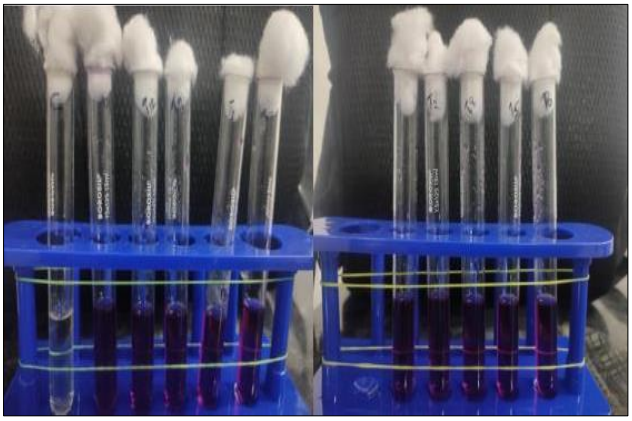


Fig 2 Before dye decolorization process incubated@ 37 degrees Celsius

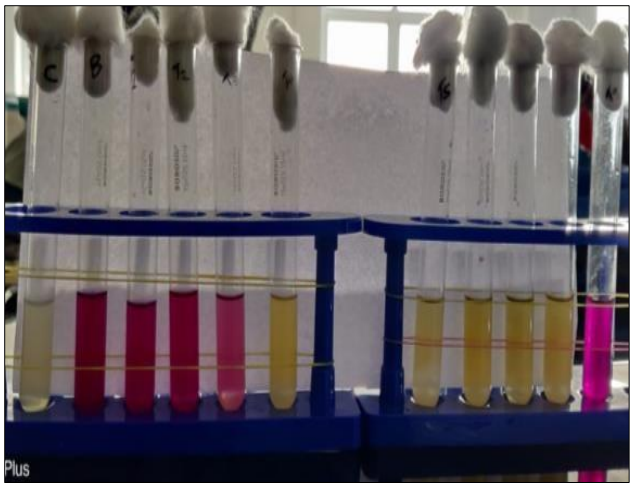


Fig 3 After decolorization period of 48 hours, D-4, D-5, D-6, D-7, D-8 Shows a visible decolorization

Quantitative assay

The optical densities for Manjatta dye are given below:

Color: Manjatta (5.5 mg / 55 ml), Absorbance at 520 nm Inoculation Date: 2nd December 2021, Control Absorbance: 1.47.

Table 2 OD and % reduction of magenta dye over 48 hours

Colony	Absorbance	Percent of reduction
D-1	1.41	4.000
D-2	1.38	6.100
D-3	0.56	62.000
D-4	0.55	62.500
D-5	0.35	76.200
D-6	0.32	78.200
D-7	0.27	80.000
D-8	0.35	76.200
D-9	1.35	8.200

Control OD = 1.47nm Blank (water) = 0.00nm

$$\text{Percentage reduction} = \frac{\text{Control OD} - \text{Test OD}}{\text{Control OD}} \times 100\% \ldots\ldots\ldots (1)$$

Top three strain (D-5, D-6, D-7) were selected for further analysis.

Decolorization graph

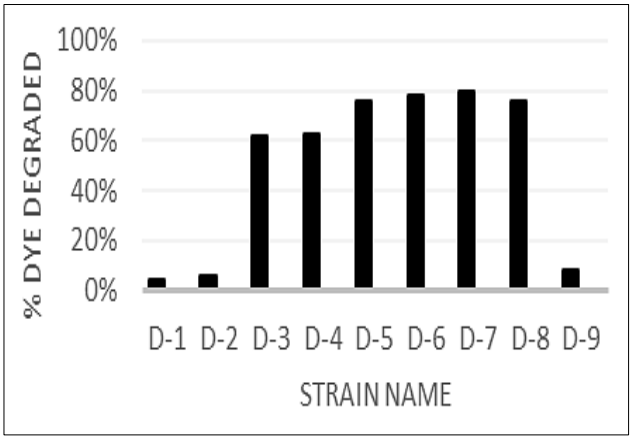


Fig 4 % decolorization of magenta dye by respective bacterial strain

Quadrant streaking

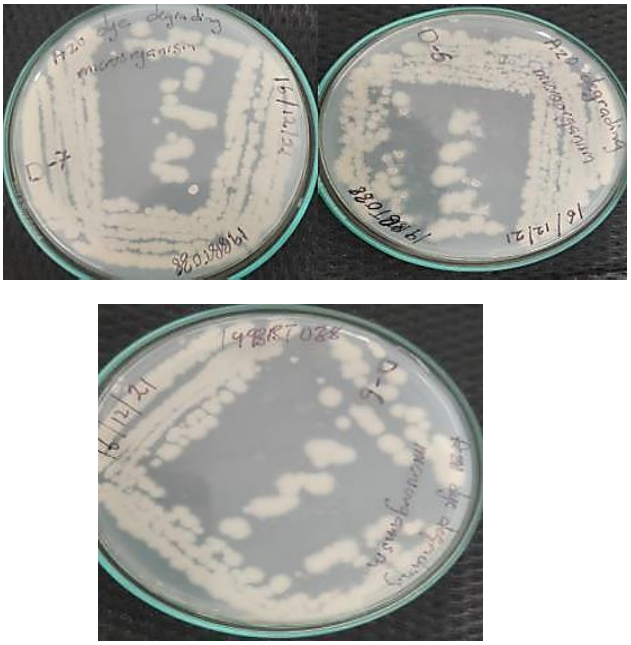


Fig 5 Quadrant streaking of the three strains to obtain pure culture

Identification of isolated bacterial sample

Morphological test

Table 3 Morphological test of the three selected strain

Test	D-5	D-6	D-7
Gram staining	-	-	-
Helical shape	+	+	-
Rod shape	-	-	-
Coccal shape	+	-	+

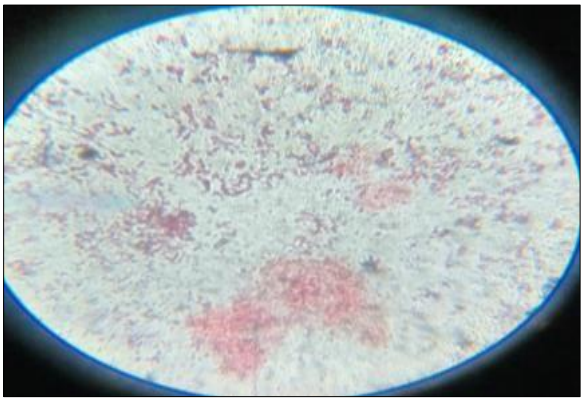


Fig 6 Gram negative (red to purple) bacterial strain (D-5), helical and cocci shape identified

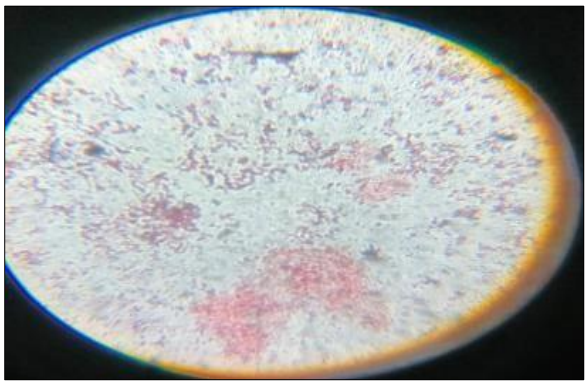


Fig 7 Gram negative (red to purple) bacterial strain (D-6), only helical shape present

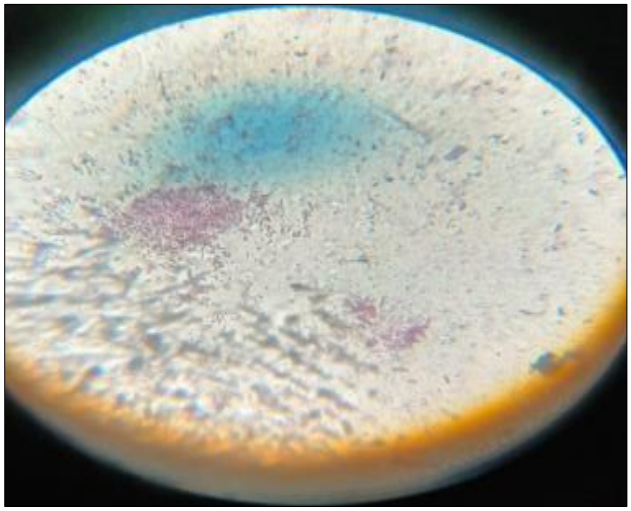


Fig 8 Gram negative (red to purple) bacterial strain (D-7), only cocci shape identified

Protein estimation

The protein concentration of Azoreductase was estimated by following the method of Lowry *et al*. [13].

Table 3 Optical densities and protein concentration of standard solution

Standard sample	Protein conc. (ug/mg)	Optical density @ 660nm
Blank	000	0.00
Standard 1	100	0.09
Standard 2	200	0.32
Standard 3	300	0.38
Standard 4	400	0.41
Standard 5	500	0.54
Standard 6	600	0.61
Standard 7	700	0.70
Standard 8	800	0.68
Standard 9	900	0.82
Standard 10	1000	0.94

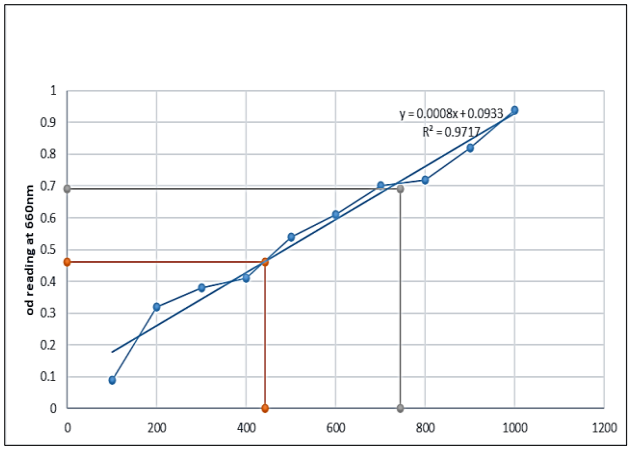


Fig 9 Standard curve of protein concentration (ug/ml)

Table 4 Optical densities and protein concentration of unknown samples

Unknown sample	OD reading at 660nm	Concentration of protein [ug/ml]
Sample 1 (supernatant)	0.46	441.63
Sample 2 (pellet)	0.69	744.13
Sample 3 (supernatant)	0.65	694.13

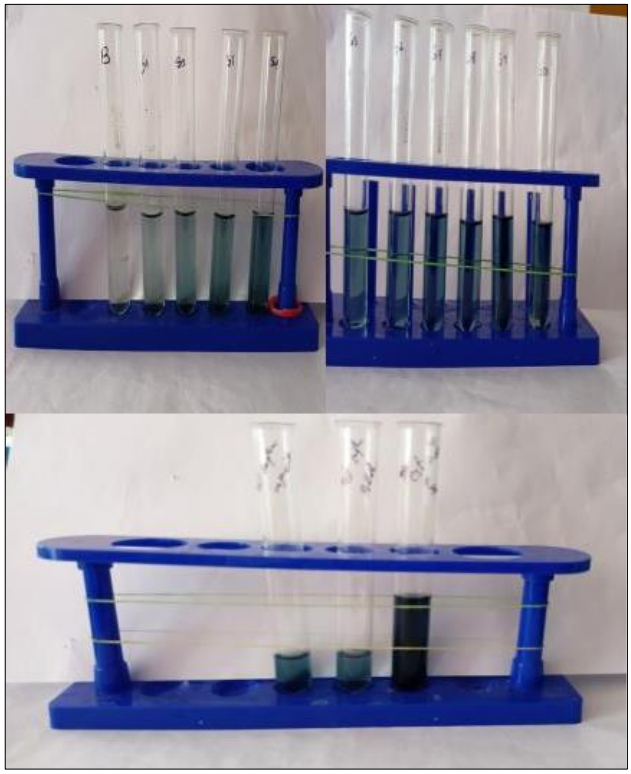
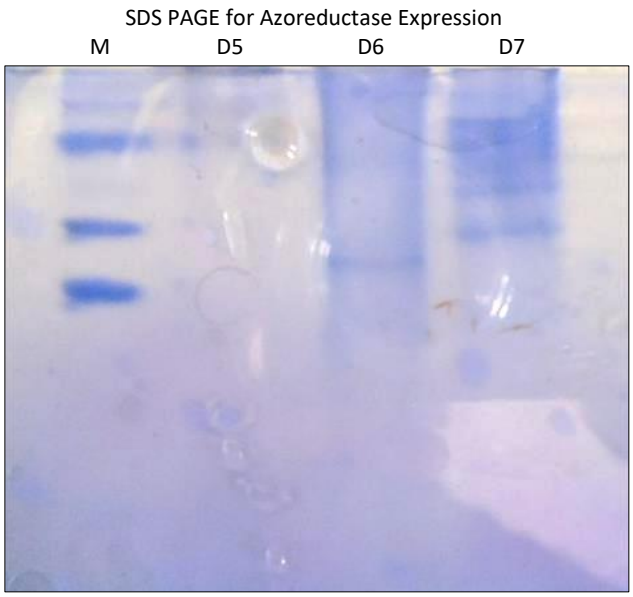


Fig 10 Quantification of protein by Lowry's method



M – Molecular weight marker; D – Purified Azoreductase enzyme
Fig 11 SDS PAGE of Azoreductase activity

Dye decolorization strain

From the 9 Bacterial Strains obtained, only three of the strains D-5, D-6 and D-7, showed maximum dye degrading properties [14-18]. Hence, only those strains were considered for the quantitative assay.

While analyzing the performance of the bacterial strains on their ability to degrade dyes, we got the following results.

Magenta dye

The Bacterial Inoculation was done on 12th December 2021 (Day 0) and the Control Absorbance was measured at 627 nm. OD was taken on 14th December (Day 2), and from the data obtained, the rate of decolorization by each strain is found out. D-5 decolorized around 57% of the dye on Day 1, and finally decolorized up to 76.2% on Day 2. D-6 was initially faster in decolorizing the dye, starting with 64% decolorization

on Day 1, and showed tremendous decolorizing potential with up to 79% decolorization on Day 2 [19-22]. D-7 was the fastest of all three strains in decolorizing the dye initially, starting with 68% on Day 1, and finally decolorized up to 80% on Day 2 [23].

CONCLUSION

From the observations of quantitative assay, we conclude that the Bacterial Strains D-5 could degrade Magenta Dye around 76% within 48 hours. The Strains D-6 degraded 78% Dye within 48 hours. D-7 degraded the most, around 80%. All of the Strains degraded dye above 70% within 48 hours. From this research, it can be concluded that industrial wastewater contaminated with dye effluents are the best source for obtaining dye degrading bacterial species. The isolated bacteria showed a dye degrading activity and can be utilized for bioremediation It can degrade the dye mixed with water before the water mixes with bigger water canals and rivers. The future prospects for this project will involve isolation of genomic DNA from dye degrading bacteria and using rDNA technologies to prepare a competent cell having dye degrading potential. It is also a challenge to build a recombinant dye degrading bacteria that can survive extreme climatic conditions, and even work with equal efficiency.

LITERATURE CITED

1. Knackmuss HJ. 1996. Basic knowledge and perspectives of bioelimination of xenobiotic compounds. *Journal of Biotechnology* 51: 287-295.
2. Bragger JL, Lloyd AW, Soozandehfar SH, Bloomfield SF, Marriott C, Martin GP. Investigations into the Azo reducing activity of a common colonic microorganism. *Int. Jr. Pharm*. 157: 61-71.
3. Sani RK, Banerjee UC. 1999. Decolorization of triphenylmethane dyes and textile and dyestuff effluent by *Kurthia sp*. *Enzyme Microb. Technology* 24: 433-437.
4. Kalyani DC, Telke AA, Dhanve RS, Jadhav JP. 2009. Ecofriendly biodegradation and detoxification of Reactive Red 2 textile dye by newly isolated *Pseudomonas sp*. SUK1. *Jr. Hazard Mater*. 163: 735-742.
5. Moosvi S, Kher X, Madamwar D. 2007. Isolation, characterization and decolorization of textile dyes by a mixed bacterial consortium JW-2. *Dyes and Pigments* 74: 723-729.
6. Alhassani HA, Rauf MA, Ashraf SS. 2007. Efficient microbial degradation of Toluidine Blue dye by *Brevibacillus sp*. *Dyes and Pigments* 75: 395-400.
7. Siddique R, Ahmed HH. 2017. Isolation, identification and characterization of azo dye reactive violet 5r degrading bacterial strains from the textile sludge. *Jr. Bangladesh Acad. Science* 41(2): 137-143.
8. Mishra S, Maiti A. 2018. The efficacy of bacterial species to decolorize reactive Azo, anthroquinone and triphenylmethane dyes from wastewater: A review. *Environ. Sci. Pollut. Res. Int*. 25: 8286-8314.
9. Abd El-Rahim WM, Moawad H, Abdel Azeiz AZ, Sadowsky MJ. 2017. Optimization of conditions for decolorization of Azo-based textile dyes by multiple fungal species. *Jr. of Biotechnology* 260: 11-17.
10. Bayoumi MN, Al-Wasify RS, Hamed SR. 2014. Bioremediation of textile wastewater dyes using local bacterial isolates. *International Journal of Current Microbiology and Applied Sciences* 3: 962-970.
11. Shah MP. 2011. Biodegradation of azo dyes by three isolated bacterial strains: An environmental bio-remedial approach. *Journal of Microbial and Biochemical Technology* S3: 007.
12. Ponraj M, Gokila K, Zambare V. 2011. Bacterial decolorization of textile dye-orange 3R. *Int. Jr. Adv. Biotechnology Research* 2: 168-177.
13. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. *Jr. Biol. Chem*. 193(1): 265-275.
14. Durai S. 2015. Isolation of dye degrading bacteria from textile effluent. *Jr. Chem. Pharm. Research* 7: 2214-2218.
15. Ren S, Guo J, Zeng G, Sun G. 2006. Decolorization of triphenylmethane, Azo, and anthraquinone dyes by a newly isolated *Aeromonas hydrophila* strain. *Appl. Microbiol. Biotechnology* 72: 1316-1321.
16. Henderson AL, Schmitt TC, Heinze TM, Cerniglia CE. 1997. Reduction of malachite green to leucomalachite green by intestinal bacteria. *Appl. Environ. Microbiology* 63: 4099-4101.
17. Donlon B, Razo-Flores E, Luijten M, Swarts H, Lettinga G, Field J. 1997. Detoxification and partial mineralization of the azo dye mordant orange 1 in a continuous up flow anaerobic sludge-blanket reactor. *Appl. Microbiology Biotechnology* 47: 83-90.
18. Gahlout M, Chauhan P, Prajapati H, Saroj S, Narale P. 2017. Isolation and screening of dye decolorizing bacteria from industrial effluent. *Jr. App. Biol. Biotech*. 5(4): 76-79.
19. Asad S, Amoozegar MA, Pourbabaee AA, Sarbolouki MN, Dastgheib SMM. 2007. Decolorization of textile azo dyes by newly isolated halophilic and halotolerant bacteria. *Bioresource Technology* 98: 2082-2088.
20. Kudlich M, Keck A, Klein J, Stolz A. 1997. Localization of the Enzyme system involved in anaerobic reduction of azo dyes by *Sphingomonas sp*. Strain BN6 and effect of artificial redox mediators on the rate of azo dye reduction. *Appl. Environ. Microbiology* 63: 3691-3694.
21. Mohamed WS. 2016. Isolation and screening of reactive dye decolorizing bacterial isolates from textile industry effluent. *Int. Jr. Microbiol. Research* 7: 01-08.
22. De-Souza SM, de-AGU, Bonilla KAS, de-Souza AAU. 2010. Removal of COD and color from hydrolyzed textile Azo dye by combined ozonation and biological treatment. *Jr. Hazard Mater* 179: 35-42.
23. Ghodake GS, Talke AA, Jadhav JP, Govindwar SP. 2009. Potential of *Brassica juncea* in order to treat textile-effluent-contaminated sites. *Int. Jr. Phytoremediation* 11: 297-312.