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Isolation, Characterization and Optimization of Bacteria for Eco-friendly Degradation of Azo-dyes

• **Maha Tarique** • **Soha Ahmad** • **Himani Bhushan**
• **Sonal Suman**

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Corresponding Author: Sonal Suman

Abstract: *Azo dyes are organic compounds bearing the functional group R"N=N"R2. They are pervasively used to treat textiles, leather articles, and some foods. In this work the bacteria with the potential to degrade azo dyes were screened. For this study, the azo dyes Methylene blue and Eosin Yellow were used as a carbon source in MSM (Minimal Salt Medium). Micrococcus sp., Bacillus sp., Streptococcus sp., Staphylococcus sp. and Pseudomonas sp. were the most potent strains among all the 17 bacterial strains isolated and subjected to degradation of the dye. The degradation of the dye was characterized by change in colour visually and by UV-Vis Spectroscopy. The decolourization activity of the bacterial isolates was also calculated. Seven of the most efficient bacterial strains (DL1, DL2, DL3, DL5, DS1, DS2, and DS3) were selected which had the highest decolourization activity of Methylene Blue (50mg/l, 75mg/l and 100mg/l), i.e., between 72-95% and Eosin yellow (100mg/l and 200mg/l). The bacterial activity was then optimized at different physical conditions (pH and temperature) and it was found that most of the bacterial isolates had optimal growth at temperature 55°C and alkaline pH of 10.5. So, the main aim of the present study was to isolate and characterize efficient bacterial strains which show the remarkable ability to decolorize/degrade various azo dyes used in various disciplines. Since the bacterial isolates originated from the dye contaminated waste water of local industry, they can easily adapt to the prevailing local environment. Therefore, such bacteria can be used to develop an effective biological treatment system for the wastewaters contaminated with azo dyes.*

Keywords: *Azo dyes, bacteria, decolourization, degradation, Methylene Blue, Eosin Yellow, UV-Vis Spectroscopy.*

Maha Tarique
B.Sc. III year, Indistrial Microbiology (Hons.),
Session: 2015-2018, Patna Women's College,
Patna University, Patna, Bihar, India

Soha Ahmad
B.Sc. III year, Indistrial Microbiology (Hons.),
Session: 2015-2018, Patna Women's College,
Patna University, Patna, Bihar, India

Himani Bhushan
B.Sc. III year, Indistrial Microbiology (Hons.),
Session: 2015-2018, Patna Women's College,
Patna University, Patna, Bihar, India

Sonal Suman
Assistant Professor, Deptt. of Indistrial Microbiology,
Patna Women's College, Bailey Road,
Patna–800 001, Bihar, India
E-mail : sonal.micro89@gmail.com

Introduction :

One of the major problems that humans are facing today is the restoration of the contaminated environment. Synthetic dyes have been used since the Bronze Age and are being released into the environment in the form of effluents by Textile, Leather, Food, Paper & Printing industries causing severe ecological damages. Among synthetic dyes, azo dyes are the largest and most versatile class of dyes which account for more than 50% of the dyes produced annually. Azo dyes are aromatic hydrocarbons and derivatives of benzene, toluene, naphthalene, phenol and aniline (Robinson*et al.*, 2001).

The effluents from textile and dyeing industries are generally coloured and contain high concentrations of dissolved solids, total solids, BOD/COD, salts and variation in pH and temperature. Such effluents also have the presence of heavy metals, such as Cr, Zn, Cu and Al. They inhibit the growth of bacteria, protozoa, algae, plants and different animals including human beings. Improper discharge of textile dyes (effluent) in aqueous ecosystems also leads to reduction in sunlight penetration, photosynthetic activity, dissolved oxygen concentration and water quality. Thus, untreated dye containing wastewater causes severe environmental and health problems worldwide (Saratale***et al.***, 2011; Solis ***et al.***, 2012).

Government has set limits for parameters for quality of water to discharge in sewage treatment plant and environment. A number of biological and physico-chemical methods have been developed for the efficient removal of industrial azo dyes (Solis ***et al.***, 2012). "Bioremediation" has become a key microbial tool to deal with different pollutants. A number of bacteria, fungi, yeasts, algae and actinomycetes have been capable of decolorizing a range of azo dyes. Particularly, bacteria are the most frequently applied microorganisms for degradation of azo dyes, as they generally multiply rapidly under aerobic, anaerobic, facultative conditions as well as in extreme environmental conditions, like high salinity and wide variations of both pH and temperature.

Two of the widely used azo dyes are Methylene Blue and Eosin Yellow. The present study was carried out to observe whether the isolated strains were capable of degrading and using these dyes as efficient carbon sources. The effectiveness of these treatment systems depends upon the survival and adaptability of microorganisms during the treatment processes. The aim of the present study was to isolate and characterize efficient bacterial strains which show the remarkable ability to decolorize/degrade various azo dyes used in various industries. Since the bacterial isolates originated from the dye contaminated effluents of local industry, they can easily adapt to the prevailing local environment. Therefore, such bacteria can be used to develop an effective biological treatment system for the wastewaters and soil contaminated with azo dyes.

Materials and Methods:

Sample collection: The effluent sample was collected from the premises of Keshav Industries (PVC pipes manufacturers), Patliputra Industrial Area, Patna. The soil sample was collected from the regular paint dumping site of Keshav Industries.

Isolation of microorganisms: The bacterial strains were isolated through spread plate technique. Serial dilutions from 10^{-1} to 10^{-6} were made from the effluent and soil sample separately. From each dilution, 0.1ml was spread over the solid plate containing Nutrient Agar medium (peptone 5g, beef extract 3g, sodium chloride 5g, D W 1 lit., pH 7.2-7.4) and incubated at 37°C for 24 hours. (Thakur ***et al.,*** 2012)

Characterization and identification of bacteria: Pour plate technique was used for the isolation of dye decolourizing bacteria. Well grown bacterial colonies were picked and further purified by streaking. The isolated strains were maintained on Nutrient Agar slants and stored at 4°C (Shah ***et al.,*** 2014).

Bacterial isolates were characterized in a four-step approach (Mehmet ***et al.,*** 2013). The four-step approach in the characterization of bacterial isolate includes the colony features, microscopic characteristics, biochemical tests and identification of bacterial isolates using Bergey's Manual of Determinative Bacteriology.

Screening of efficient azo dye decolorizing bacterial isolates: Screening was done to find out the efficient bacterial strains capable of decolorizing Methylene Blue dye using modified Mineral Salt Media (MSM- sodium chloride 1g, $CaCl_2.2H_2O$ 0.1g, $MgSO_4.7H_2O$ 0.5 g, KH_2PO_4 1g, Na_2HPO_4 1g, yeast extract 4g, distilled water 1000ml, pH- 7.0). For this purpose, isolates were first grown in MSM broth and the selected strains were grown in MSM broth supplemented with 50mg/l of methylene blue. The decolourization was determined by spectrophotometer at 670 nm. Uninoculated blanks were run to determine abiotic decolorization (Mahmood ***et al.,*** 2011).

Decolourizing activity: The decolorizing activity was expressed in terms of percentage decolourization and was determined by monitoring the decrease in absorbance at absorption maxima (Amax) of dye. The uninoculated MSM media with dye were used as control. All assays were performed at regular time intervals (1, 3, 5, 7, 9 and 11 days) and compared with control.

The decolorization efficiency was expressed using following equation:

Decolourization activity = (A-B)/A × 100

Where A= initial absorbance and B= observed absorbance (at specified time interval) or absorbance of decolorized medium. (Verma***et al.***, 2015)

Optimization of physical factors for efficient growth of bacterial strains: Factors like temperature and pH were optimized during the experimentation for identifying the best growth conditions of the isolates.

To optimize the most suitable pH for growth of isolates, MSM media with initial pH of 5.5, 7.0 and 10.5 were set, using 0.1 N HCl and 0.1 N NaOH before inoculation.

To optimize the most suitable temperature for growth of isolates, MSM media were inoculated with isolates and incubated at 26°C, 37°C and 55°C.

Physico – chemical characterization of the collected soil sample: Analysis of physical and chemical properties of soil sample was done at Central Soil Testing Laboratory as shown in Figure 1.

Physical properties include pH and electrical conductivity using pH meter and conductivity meter (KaviKarunya and D. Reetha, 2012).

Chemical properties include concentration of P_2O_4 by Olsen method, organic carbon by Walkley and Black method, Potash by ammonium acetate method and micronutrients (Zn, Cu, Mn and Fe) using atomic absorption spectrophotometer and flame spectrometer.

Figure 1a **Figure 1b**

Fig. 1. Physical tests: 1a) pH test; 1b) electrical conductivity tests

Results and Discussion:

A. Physico-chemical characterization of collected samples: The soil and effluent samples were collected in sterilized container from respective sites. Physico-chemical characteristics like color, pH, BOD, COD, etc. were measured on the same day of collection of sample as per Table 1.

Table 1. Characteristics of samples collected from Patliputra Industrial Area.

S. No.	Nature of sample	Colour	BOD (mg/L)	COD (mg/L)	pH	Organic carbon (%)	Nitrogen (kg ha^{-1})	PO_4 (kg ha^{-1})	Potash (kg ha^{-1})
1.	Liquid	Light green	1.2	58.4	8.15	–	–	–	–
2.	Solid	Dark brown	–	–	7.79	0.42	238	44	291

B. Cultural and Morphological Characterization of Bacterial Isolates: Eleven colonies were isolated from effluent sample and six colonies were isolated from soil sample. The 17 isolates were then screened in MSM with methylene blue at a concentration of 50 mg/L, and based on their decolourizationactivity, seven isolates were selected – DL1, DL2, DL3, DL5, DS1, DS2 and DS3. On microscopic observation at 100X, they were found to be rods

and ***Coccus*** in shape. On observing the plates, the colonies were found to be creamy to white in colour having opaque and transparent density with regular or irregular margin. DL1, DL2, DL3, DL5, DS2, and DS3 were gram positive while DS1 was gram negative.

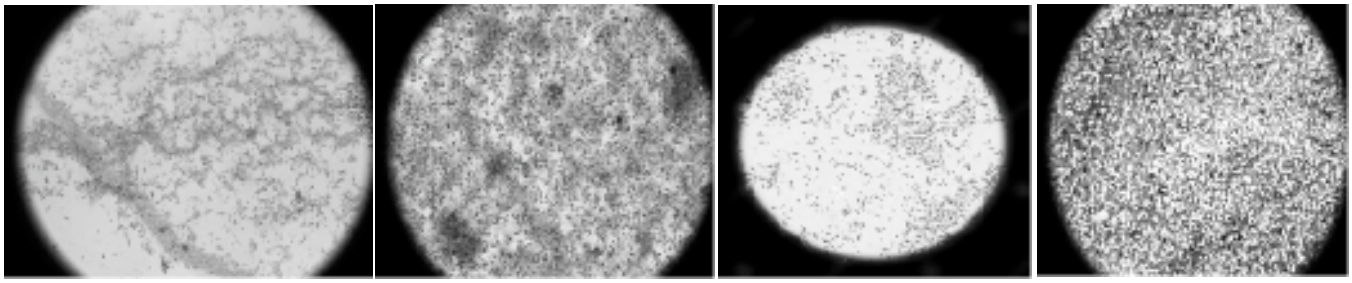

Fig. 2a **Fig. 2b** **Fig. 2c** **Fig. 2d**

Fig.2. Microscopic view of the bacterial isolates

C. Biochemical Characterization: The observation of biochemical tests performed for the selected strains is shown in Table 2.

Table 2. Observation of the biochemical tests performed for DL1, DL2, DL3, DL5, DS1, DS2 and DS3 isolates.

S. No.	Biochemical Tests	Selected Bacterial Isolates						
		DL1	DL2	DL3	DL5	DS1	DS2	DS3
1	Amylase	–	–	+	–	+	+	+
2	Casein Hydrolysis	–	–	–	–	+	+	+
3	Catalase	+	–	+	+	+	+	+
4	Gelatin Hydrolysis	+	–	+	+	+	+	+
5	Nitrate Reduction	–	–	+	-	+	-	+
6	Citrate Utilization	+	+	+	+	+	+	+
7	Indole Production	–	–	–	–	–	–	–
8	MR	–	+	+	+	-	+	+
9	VP	–	–	–	–	–	–	–
10	Urease	–	–	–	–	–	–	–
11	Dextrose	+	+	+	+	-	-	+
12	Sucrose	+	+	+	–	+	–	–
13	H_2S	+	–	+	+	+	–	–
14	Motility	–	–	–	–	+	–	–
– Negative + Positive								

Thus, on the basis of the cultural, morphological and biochemical characterization DL1 maybe ***Bacillus*** spp., DL2 maybe ***Streptococcus*** spp., DL3 maybe ***Micrococcus*** spp., DL5 maybe ***Micrococcus*** spp., DS1 maybe ***Pseudomonas*** spp., DS2 maybe ***Micrococcus*** spp., and DS3 maybe ***Staphylococcus*** spp.. However, for confirmation of the bacterial strains molecular characterization should be done. A number of dye decolourizing bacteria have been reported and their characteristics reviewed. ***Pseudomonas*** spp. are reported to decolourize triphenyl methane and azo dyes (Banat ***et al.***, 1996; Azmi***et al.***, 1998).

D. Degradation and Decolourization of Azo dye: Absorbance taken at 670 nm (for Methylene Blue) and 490 nm (for Eosin Yellow) for 192 hours to check for degradation of azo dyes by bacterial isolates as summarized in Fig. 1 and Fig. 2.

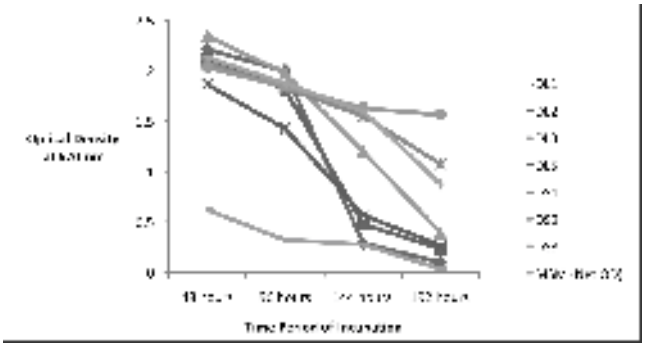

Fig. 3. Degradation trend of azo-dye (Methylene Blue) by selected bacterial strains

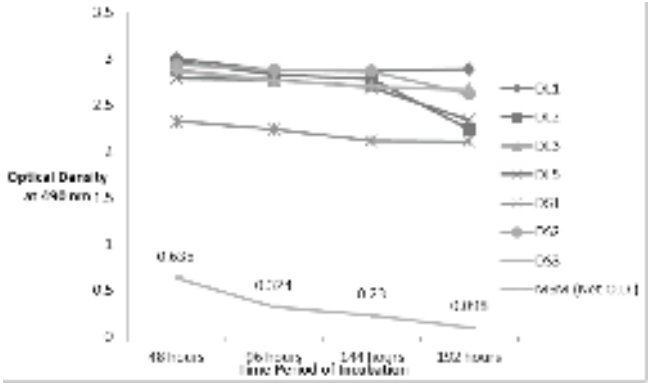

Fig 4. Degradation trend of azo-dye (Eosin Yellow) by selected bacterial strains

The decolorization activity of the selected bacterial isolates was calculated (Verma***et al.***, 2015) after every 48 hours of incubation, as mentioned in Table 3.

Table 3. Decolourization Activity of the different bacterial isolates after different incubation periods.

Strains	***Decolourization Activity (%) after different Incubation Periods***			
	48 hr	***96 hr***	***144 hr***	***192 hr***
DL1	2.21	11.4	87	95.5
DL2	6.28	17.02	78.9	89.51
DL3	3.8	14.9	47	82.97
DL5	17.2	36.79	14.81	88.2
DS1	8.18	92.4	18.1	18.7
DS2	10.03	18.35	26.8	10.2
DS3	5.48	72	29.1	17

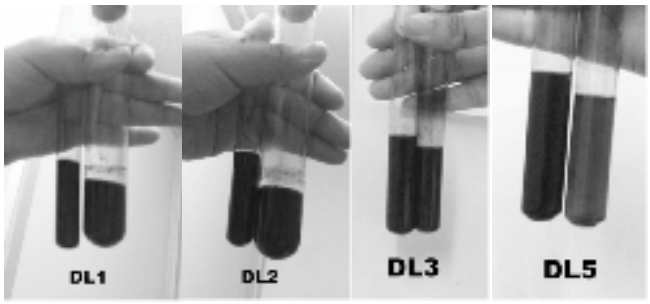

Fig.5a **Fig.5b** **Fig. 5c** **Fig.5d**

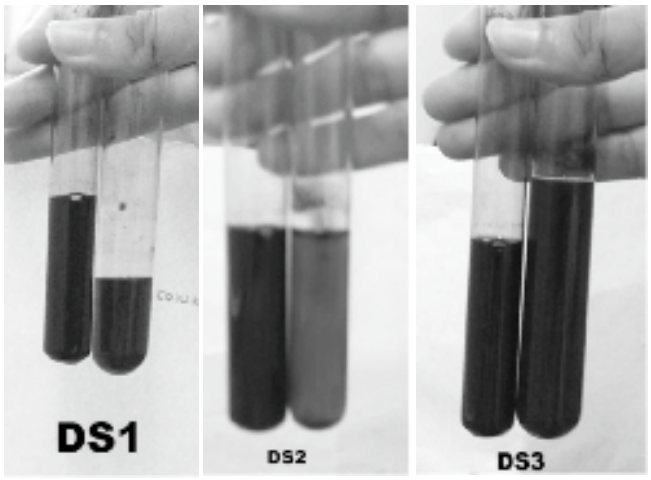

Fig. 5e **Fig. 5f** **Fig. 5g**

Fig.5. Decolourization activity observed visually in the selected bacterial isolates.

The biodegradation capability of the dyes varies from organism to organism. In our research, out of 17 isolates, seven were the most efficient in degrading the azo-dye after 192 hours of incubation. This was in accordance with the work of Barrag'an***et al.***, 2007, where it was found that out of 15 isolates, four had the maximum decolourizing capability after 72 hours of incubation.

E. Optimization: The optimum pH and temperature of the selected bacterial strains was observed, as mentioned in Table 4.

Table 4.Optimum Conditions for growth of the selected strains

Strain	**Optimum pH**	**Optimum Temperature**
DL1	7.0	55°C
DL2	10.5	55°C
DL3	10.5	55°C
DL5	7.0	55°C
DS1	7.0	26°C
DS2	7.0	26°C
DS3	10.5	55°C

Our results were in agreement with the research conducted by Guo***et al.***, 2008 in which the bacterial strains grew well and completely decolourized K-2BP. In case of pH as a variable, decolourization was on higher side at or over pH 7 whereas lower pH values (acidic conditions) decrease the decolourization efficiency of all the tested isolates. So, from this study it could be concluded that the neutral and basic pH supported bacterial activity to decolourize (Mali ***et al.***, 1999; Chang ***et al.***, 2001). Temperature is another very important parameter for treatment of waste water. Selected isolates were mesophilic and thermophilic bacteria. Most of the isolates showed better decolourization in the temperature range of 26°–55°C. This was in accordance with the work of Guo***et al.***, 2008.

Conclusion:

Relevance of traditional waste water treatment requires enormous cost and continuous input of chemicals which becomes expensive and causes further secondary pollution. Hence, economical and eco-friendly techniques using bacteria can be applied for the refining of waste water treatment. Interestingly, the bacterial species used in the decolourization of azo dye Methylene Blue and Eosin Yellow in this study was isolated from the waste sludge of dye using industry. Various strains isolated from the effluent sample were able to degrade the azo dye efficiently. Dyes being xenobiotic with complex structures are not much prone to microbe but some of the bacterial isolates have appreciable degrading potential.

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