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J. Sci. Trans. Environ. Technov. 2019, 13(1) : 10-14

Scientific Transactions in Environment and Technovation

Biodecolorization of Azo Dye by Bacteria Isolated from Cow Dung

https://doi.org/10.56343/STET.116.013.001.003
http://stetjournals.com

G. Vaijayanth[1*] G. Kalaivani[1] and R. Vijayakumar[2]

[1]Department of Microbiology, Dhanalakshmi Srinivasan College od Arts and Science for women (Autonomous), Perambalur - 621 212, Tamil Nadu, India.

[2]Department of Microbiology, Government Arts and Science College, Kurumbalur, Perambalur - 621 107, Tamil Nadu, India.

Abstract

A total of four different bacterial isolates namely *Enterobacter* sp., *Klebsiella* sp., *Serratia* sp. and *Bacillus* sp. were isolated and identified from cow dung samples. Dye degrading efficiency of bacterial isolates was evaluated against azo dyes. The dye degrading efficiency of the bacteria ranged between 23.25% and 65.11%. Among the bacterial isolates, *Bacillus* sp. was highly promising decolourizing agent, which could act as an efficient dye degrader. The degradation efficiency increased when the incubation period was increased to a greater extent. FT-IR, HPLC results of dye decolourization confirmed that, the complex, toxic azo dyes are degraded in to simple, non toxic compounds. Due to the removal or the complete degradation of azo dye, *Bacillus* sp. could be a potential candidate for the biodecolourization of synthetic dyes.

Key words: Azo dye, Bacteria, Biodecolourization, FT –IR and HPLC analysis.

Received : March 2018 Revised and Accepted : January 2019

INTRODUCTION

Synthetic dyes are colouring agents mainly used in textile industries which generate a huge amount of wastewater in the process of dyeing. Different dyes used in textile industry usually have a synthetic origin and complex aromatic molecular structures which make them more stable and more difficult to be biodegraded. Due to their ease of manufacturing methodology, azo dye constitute for almost 82% of annual production of commercial dyes all over the world. Dyes are coloring pigment that imparts colour to the substrate when they are in solution form. Dyes are derived synthetically from raw materials like hydrocarbons, benzene, toluene, naphthalene and anthracene using coal tar obtained from distillation of coal. Both organic and inorganic materials are needed to make dyes and intermediates (Patovirta *et al.*, 2003). The raw material sequence for making dye is petroleum to hydrocarbons to intermediates to dyes. Textile dyes effluents have toxic effect on the germination rates and biomass concentration of several plant species which play many important ecological functions such as providing the habitat for wildlife: protecting soil from erosion and providing bulk of organic matter that is significant to soil fertility. The toxicity of effluent is because of the presence of dye or its degraded products which are mutagenic or carcinogenic. Therefore, the treatment of industrial effluents contaminated with dye becomes necessary prior to their final discharge in to the environment (Elisangela *et al.*, 2009). Biological processes provide an alternative to existing technologies because they are more cost-effective, environmentally friendly, and do not produce large quantities of sludge. biodegradation by microorganisms is a promising approach for treating dyes contained in wastes. The effectiveness of decolorization depends on the adaptability and the activity of selected microbes. The present study investigates the degradation of azo dye by bacteria isolated from cow dung.

MATERIALS AND METHODS

Sample collection

Fresh cow dung was collected from Kuzhumani, Trichy district, Tamilnadu, South India. The sample was collected with sterilized spatula and kept in sterile polyethylene bags. The samples were transported to the laboratory in an ice box and processed within 6 h of collection.

Isolation of bacteria

Nutrient agar medium was prepared and autoclaved. Then the medium was poured into sterile Petri plates. The collected cow dung sample was diluted up to 10^{-}

*Corresponding Author :
email: *gvaijayanthimicro@gmail.com*

P - ISSN 0973 - 9157
E - ISSN 2393 - 9249

^{6} and 0.1 mL of the diluted sample was spread over the agar plates. The plate was incubated at 37°C for 24 h. After incubation, the colonies were purified by using streak plate method, then the bacterial cultures were stored at 4°C for further investigation (Teo *et al*., 2011).

Characterization of bacteria

Selected individual colonies from nutrient agar were subjected to microscopic observations by Gram staining, motility test and biochemical tests (Indole, MR-VP, citrate utilization, urea production, triple sugar iron and oxidase test) for the identification of bacterial isolates (Shrivastava and Mishra, 2014).

Collection of dye

The dye sample used was graded and supplied by the dealers of global dyes, Tiruppur, Tamilnadu, and South India. Azo dye used in this study is reactive 2B (ëm= 5m).

Bacterial incoulum preparation

All the isolated cultures were inoculated in nutrient broth and incubated for 24 hours. Fresh cultures were prepared for every time and used for dye decolourization studies (Chang and Lin, 2001).

Dye decolourization

Dye decolorization was carried out in 500 mL flask containing 250 ppm of azo dyes in 250 mL of C-limited Czapek-dox broth. The pH was adjusted to 7.3±0.2 using NaOH and Hcl solution. Then, the flasks were autoclaved at 121$^{R''}$C for 15 min. The autoclaved flasks were inoculated with 1 mL of all the bacterial isolates. The flasks were kept in a mechanical shaker at room temperature for 7-15 days. Samples were drawn at 24 h intervals for observation. 10 mL of the dye solution was filtered and centrifuged at 5000 rpm for 20 min. Decolourization was assessed by measuring absorbance of the supernatant with the help of UV spectrophotometer at wavelength maxima (580 nm) of respective dye (Waghmode *et al.,* 2011).

Assay on the dye degrading efficiency of supernatants

From 24 h bacterial cultures, supernatant was collected by the centrifugation at 10000 rpm for 30 min. The supernatant was collected separately and pellet was discarded. The supernatants of all the cultures were used for the dye degradation analysis (Waghmode *et al.,* 2011).

FT-IR Spectral analysis

The samples were dried and grinded with KBr pellets and analyzed on a Shimadzu FT-IR affinity model in the diffuse reflectance mode operating at a resolution of 4 cm$^{''1}$ (Vaijayanthi *et al.,* 2012).

HPLC Analysis

Reverse phase HPLC (Cyberlab, USA) analysis was carried out in a C-18 column (250 mm X 4.6 mm) version (Lake Forest, CA, USA) equipped with a C-18 guard column. The compounds were eluted with an isocratic elution of acetonitrile *vs* water at the flow rate of 1 mL/ min and absorbance was recorded at 680 nm (Baiocchi *et al*., 2002).

RESULTS AND DISCUSSION

In the present study, a total of four different bacterial isolates were isolated from the cow dung samples collected from Trichy district, India. They were characterized based on the morphological, biochemical and cultural properties. Morphologically, all the isolates were found as rod shaped bacteria. The isolate CDB4 alone recorded as Gram positive bacterium rest of the isolates were recorded as gram negative bacteria. Similarly, all the isolates were motile except the isolate CDB2. Biochemically, all isolates were positive for indole test except the isolate CDB3, followed by the isolates CDB1 and CDB3 which were positive for methyl red and all the isolates were positive for Voges Proskauer test except CDB1. The isolates CDB2 and CDB3 were positive for citrate utilization test, but these isolates were negative for TSI test. Oxidase was produced by CDB2 and CDB4, whereas, urease was produced by the isolates CDB1 and CDB4. Based on the phenotypic characterizations, all the isolates were identified as one each to *Enterobacter* sp. CDB1, *Klebsiella* sp. CDB2, *Serratia* sp. CDB3 and *Bacillus* sp. CDB4 (Table 1).

Similarly, six different biofouling bacteria were isolated from poultry samples and identified as *Bacillus* spp. (BFB1, BFB2 and BFB3, *Staphylococcus* sp. (BB4), *Micrococcus* sp. (BB5) and *Streptococcus* sp. (BB6) by Manikandan and Vijayakumar (2016). Hence, it is inferred that the population of bacteria is not uniform in the entire ecosystem, it could vary between sampling location.

Pollution of aquatic bodies by textile dye waste water is major environmental concerns, which also cause major health issues to biotic systems. Thus, degradation or treatment of the dye effluent using biological methods is an eco-friendly approach (Sharma *et al.,* 2009; Waghmode *et al*., 2012). In the present study, dye degrading efficiency of bacteria was evaluated against azo dye. At every 24 h intervals, sample was derived and analyzed spectrophotometrically. The dye decolourizing efficiency of the bacteria ranged between 23.25% (1st day) to 65.11% (7th day). The decolourizing efficiency increased to a greater extent when the days of incubation were increased. The bacterial isolate *Serratia* sp. (65.11%) showed maximum decolourizing ability,

Table 1. Phenotypic properties of cow dung bacteria

Name of the test	*Enterobacter* sp. C D B 1	*Klebsiella* sp. CDB2	*Serratia* sp. CDB3	*Bacillus* sp. CDB4
Gram staining	-	-	-	+
Shape	Rod	Rod	Rod	Rod
Motility	+	-	+	+
Indole	+	+	-	+
Methyl red	+	-	+	-
Voges Proskaur	-	+	+	+
Citrate utilization	-	+	+	-
TSI	acidic	-	-	acidic
Oxidase	-	+	-	+
Urease	+	-	-	+

Table 2. Biodecolourization efficiency of bacterial isolates

Days of Incubation	Degradation efficiency (%)			
	Enterobacter sp. CDB1	*Klebsiella* sp. CDB2	*Serratia* sp. CDB3	*Bacillus* sp. CDB4
1	24.03	33.09	33.09	23.25
2	30.83	33.33	35.56	33.09
3	33.09	36.43	38.75	36.43
4	40.63	43.42	43.42	65.11
5	42.1	44.18	46.31	47.28
6	48.83	47.28	53.48	52.71
7	61.52	62.9	65.11	58.9

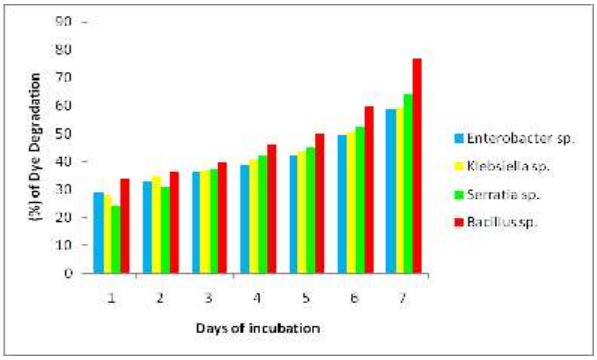

Fig. 1. Dye degradation of bacterial supernatant

followed by *Klebsiella* sp. (62.90%), *Enterobacter* sp. (61.52%) and *Bacillus* sp. (58.9%) (Table 2). In contrast to this, among the extracts of the bacterial isolates, maximum of 78.44% of decolourizing efficiency was displayed by *Bacillus* sp. at the end of 7 days followed by, 64.56%, 60.86% and 59.89% of azo dye decolourization was observed with *Serratia* sp., *Klebsiella* sp., and *Enterobacter* sp. respectively (Fig. 1). Previously, Aldoury *et al.* (2014) reported that when *Plicatilis* was cultured in malt extract medium containing 50 mg/L RB 19, the percentage decolorization increased over time, reaching a peak of 99% decolorization after 15 days of incubation. Similarly, Elisangela *et al.* (2009) studied that *Pleurotus* sp. was capable of efficient decolourization with wide range of chemically different dyes. In this study, all the bacterial supernatant extracts showed good results of dye decolourization than the whole cells

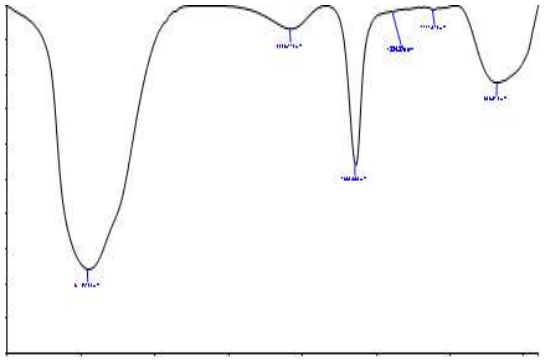

Fig. 2a. FT-IR analysis of dye (control)

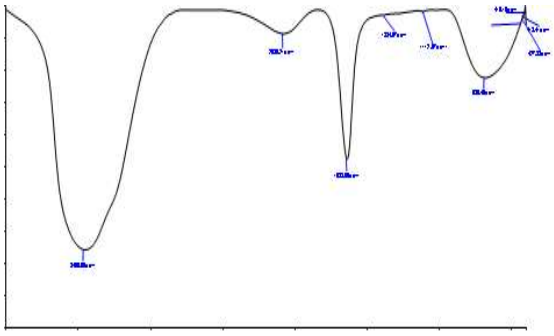

Fig. 2b. FTIR analysis of azo dye degradation by *Bacillus* sp.

Fig. 3a. HPLC chromatogram of azo dye (control)

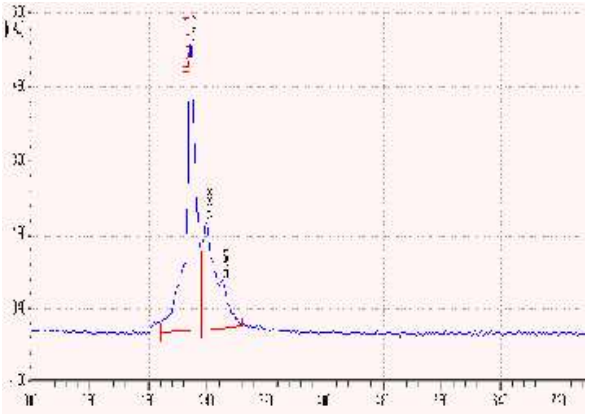

Fig. 3b. HPLC chromatogram of azo dye degraded by the *Bacillus* sp.

investigated. Especially, *Bacillus* sp. CDB4 exhibited least activity in screening test, but the biodecolourization efficiency was remarkably increased when the metabolites was extracted and introduced into the dye effluent. Therefore, *Bacillus* sp. was graded as a potential dye decolourizer when compared to other isolates, and it was selected for further FT-IR and HPLC analyses.

FT-IR spectrum test samples obtained after decolourization and control dye showed various peaks. A new peak at 1384 cm^{-1} represented –N=N- stretching vibration. The C- H deformation showed at 1112 cm^{-1}. The peak at 1384 cm^{-1} showed N-H stretching vibration. The significant change in the FT-IR spectrum of metabolites compared to control spectrum suggested that the biotransformation of complex dyes present in the mixture into simple form. The FT-IR spectrum of control dye was displayed peak at 3449 cm^{-1} for intra-molecular hydrogen bonding and O-H stretching. Peaks in the control dye spectrum represented symmetric stretching at 1384 cm^{-1} and asymmetric stretching at 1114 cm^{-1} for C–N. C-N stretching at 1637 cm^{-1} represented nature of aromatic amine group present in parent dye; 3449 cm^{-1} and 2075 cm^{-1} represented the presence of free NH group of parent dye, whereas peak at 1637 cm^{-1} represented –N=N– stretching of azo group. In degraded by extracted metabolites, a new peak at 435 cm^{-1} represented C–H deformation of alicyclic CH_2, whereas a peak at 685 cm^{-1} was observed for substituted anilines (Fig.2a& b). Likewise, azo dye Congo red sample treated with both *E. cloacae* and *H. alvei* showed a new peak in HPLC with 2.61 RT along with 2 other peaks with similar RT as observed in case of two previously mentioned treatments. Comparison between the polarities of the samples showed that the control and *H. alvei* treated samples have the peak with shortest RT 1.98. From the retention times, it can be concluded that the control has the highest polarity. Polarity refers to the number of functional groups in the dye. Highest retention time of 2.6 and thus the lowest polarity was obtained by the sample treated with the two bacterial isolates. All the treated samples showed peaks almost similar to that of control. But the peak with RT 2.94 did not appear in any of the treated samples (Selvaraj *et al.,* 2012).

In the present study, the absorption spectra of the HPLC analysis of control azo dye and *Bacillus* sp. degraded sample of dye obtained at 680 nm were depicted in fig. 3a & b. The HPLC elution profile of the azo dye (control) showed 5 peaks with retention time (RT) of 1.98, 2.18, 2.37, 2.59 and 2.94 min. The elution profile obtained for the bacteria treated samples significantly differed from the control in terms of number, height of peaks obtained and RT. The HPLC profile of azo dye treated with bacterial isolate *Bacillu* sp. showed 3 peaks with RT 1.98, 2.18 and 2.39 min. But, HPLC analysis of control dye showed the presence of one major peak at retention time of 2.702 min and three minor peaks at retention times of 2.125, 2.801, and 3.394 min (Fig. 3a). After the dye decolorization process, the disappearance of peaks as seen in case of the control and the formation of completely different three major peaks at retention times of 2.521, 3.241, and 3.564 min and two minor peaks at retention time of 3.123 and 3.910 min were observed (Fig. 3b). Bacterial enzymes such as azoreductase, riboflavin reductase, NADH–DCIP reductase, laccase, tyrosinase, lignin peroxidase and veratryl alcohol oxidase synthesized by the bacteria might have played a major role to catalyse the biodegradation of dyes in decolourization of azo dyes (Mishra and Maiti, 2019). Similar type of dye decolourization works have been reported by many workers (Chen *et al*., 2005; Kalyani *et al*., 2008; Modi *et al*., 2010; Phugare *et al*., 2011; Lade *et al*., 2015). In conclusion, the complex chemical azo dyes degraded by the whole cell and supernatant of *Bacillus* sp. isolated from cow dung could be used as a potential candidate for various dye decolourization. Instead of using hazardous, time consuming and costly chemicals, we could protect our environment from dyes by using these types of microorganisms and their metabolites.

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