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Research Article

SCREENING OF BACTERIAL ISOLATES FOR THE DECOLOURIZATION OF REACTIVE AZO DYES

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Abstract

In the present study, the bacterial isolates were screened and decolourization of Reactive azo dyes was studied. Six different bacterial isolates were isolated and identified from the textile dye effluent. The isolated bacterial isolates were identified and characterized as *Bacillus odyssey*, *Bacillus thuringiensis*, *Bacillus subtilis*, *Escherichia coli*, *Proteus mirabilis* and *Staphylococcus aureus*. The identified bacterial isolates were screened for the decolourization of reactive dyes by Plate assay. Maximum decolourization was recorded by *Bacillus odyssey* in the plate containing Reactive Orange – 16 (35 mm) followed by *Bacillus thuringiensis* (33 mm), *Bacillus subtilis* (30 mm), *Escherichia coli* (28 mm), *Proteus mirabilis* (27 mm) and *Staphylococcus aureus* (25 mm). The decolourization of textile reactive azo dyes by bacterial isolates was studied. Maximum decolourization percentage was observed in the medium inoculated with *Bacillus odyssey* (Reactive Orange – 70.66%; Reactive Black – B – 67.21%; Reactive Yellow – MR – 65.40%; Reactive Blue – MR – 63.09% and Reactive Red M5B – 61.19%).

Keywords: Textile dye, Reactive azo dyes, Bacteria, Screening and Decolourization

Introduction

Rapid industrialization and urbanization resulted in the discharge of large amount of waste to the environment, which in turn creates more pollution. Majority of the colored effluents consisting of dyes, released to the environment from textile dyestuff and dyeing industries. Color pollution in the environment is escalating problem. Such pollution is particularly associated with the reactive dyes, which accounts for a significant proportion of the total dye market. Due to the relatively low levels of dye fiber fixation, in current reactive dyeing processes, upto 50% of the dye that present in the original dye bath is lost to the wastewater (Saranraj, 2013; Saranraj and Sivasakthivelan *et al*., 2014). These highly stable reactive dyes, which are not degraded by the conventional wastewater treatment processes, enter in to environment in the form of colored wastewater (Stolz, 2001).

Azo dyes represent a major group of dyes mostly used in industry (Chen *et al*., 2004; Kumar *et al*., 2006; Jadhav *et al*., 2007), which are causing environmental concern because of their color, biorecalcitrance and potential toxicity to animals and human (Martins *et al*., 2002). It is very difficult to treat the effluents from the textile and dyeing industries by commonly used physical and chemical methods mainly because of its high BOD, COD, heat, color, pH and the presence of metal ions. Several bacterial strains that can aerobically decolorize azo dyes have been isolated during the past few years. Under aerobic conditions mono- and di-oxygenase enzymes catalyze the incorporation of oxygen from O_2 into the aromatic ring of organic compounds prior to ring fission (Saranraj *et al*., 2010; Sadeeshkumar *et al*., 2012).

Some aerobic bacteria are able to reduce azo compounds with the help of oxygen catalysed azoreductases and produce aromatic amines (Lin *et al*., 2010). It was also reportedthat the aerobic azo reductases were able to use both NAD(P)H and NADH as cofactors and reductively cleaved not only the carboxylated growth substrates of the bacteria, but also

the sulfonated structural analogues (Nachiyar and Rajkumar, 2005). This type of azoreductase activity was found in *Pseudomonas* species, and after purification and characterization it was observed that this enzyme system was flavin-free. These bacteria cannot utilize azo dye as the growth substrate, and require additional organic carbon sources. Moreover, there are few bacteria that are able to grow on azo compounds as the sole carbon source. These bacteria cleave –N55N– bonds reductively and utilize amines as the source of carbon and energy for their growth. Such organisms are specific towards their substrate. Examples of bacterial strains with this trait are *Xenophilus azovorans* and a) *Pigmentiphaga kullae*, which can grow aerobically on carboxy orange I and carboxy orange II, respectively (McMullan *et al*., 2001). Only few bacteria with specialized azo dye reducing enzymes have been found to degrade azo dyes under fully aerobic conditions (Nachiyar and Rajkumar, 2003).

Materials and Methods

Collection of Textile dye effluent

The dye house effluent was collected from a dyeing unit in Theco Silks, Thirubhuvanam region, Kumbakonam district, Tamil Nadu, India. It was refrigerated at 4°C and used without any preliminary treatment.

Dyes used

Reactive azo dyes were used in this present research. The dye samples were commercially graded and supplied by the dealers of "SIGMA Aldrich, USA". Reactive azo dyes used in this research were,

a. Reactive Orange – 16 (λm = 480 nm)
b. Reactive Black – B (λm = 600 nm)
c. Reactive Yellow – MR (λm = 600 nm)
d. Reactive Blue – MR (λm = 580 nm)
e. Reactive Red – M5B (λm = 580)

Isolation of bacterial isolates from Textile dye effluent

The bacterial isolates present in the textile dye effluent were isolated by Serial dilution **(**Pour plate) technique. In this method, 1 ml of sample was thoroughly mixed with 99 ml of sterile distilled water, and then it was serially diluted by following standard procedure upto concentration of 10^{-6}. Then, 1 ml of serially diluted samples from each concentration of samples were transferred to sterile petriplates and evenly distributed throughout the plates and sterile unsolidified Nutrient agar was poured and it was allowed to solidify. The Nutrient agar plates were incubated at 37°C for 24 hours. After incubation, the bacterial colonies were isolated from the plates.

Maintenance of bacterial isolates

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.

Identification of bacteria isolated from Textile dye effluent

Identification of the bacterial isolates was carried out by the routine bacteriological methods i.e.,

By the colony morphology

Preliminary tests like Gram staining, Capsule staining, Endospore staining, Motility, Catalase and Oxidase.
Plating on selective medias.
By performing biochemical tests.

Screening of bacterial isolates for the decolourization of reactive dyes by Plate assay

The decolourization of textile Reactive azo dyes by bacterial isolates was determined by Plate assay technique. The Plate assay was performed for the detection of decolorizing activity of bacteria isolated and identified from the textile dye effluent. The Nutrient agar and Reactive dyes (500 mg/l) was autoclaved at 121°C for 15 min. The bacterial cultures were platted on Nutrient agar plates containing Reactive azo dyes. The plates were wrapped with parafilm and were incubated in incubator at 37°C for 4 days. The plates were observed for clearance of the dye surrounding the colonies.

Decolourization of Textile reactive azo dyes by bacterial isolates

Inoculum preparation

The suspension of 2 days old cultures of bacteria were used to investigate their abilities to decolourize dyes. They were prepared in saline solution (0.85% sodium chloride). A loopful of bacterial cultures were inoculated into 50 ml of saline and incubated at 37°C for 3 hours (Benson *et al*., 1994).

Dye decolourization experiments

Dye decolourization experiments were carried out in 100 ml flasks containing 50 ml of Nutrient Broth and Reactive azo dyes (500 mg/L). The pH was adjusted to 7±0.2 using sodium hydroxide and hydrochloric acid solution. Then, the flasks were autoclaved at 121°C for 15 minutes. The autoclaved flasks were inoculated with 5 ml of bacterial inoculum of each isolates. The flasks were kept in mechanical shaker and incubated at 37°C for 4 days. Samples were drawn at 24 hours intervals for observation. Ten ml of the dye solution was filtered and

centrifuged at 5000 rpm for 20 minutes. Decolourization was assessed by measuring absorbance of the supernatant with the help of spectrophotometer at wavelength maxima (λm) of respective dye.

Decolourization assay

Decolourization assay was measured in the terms of percentage decolourization using spectrophotometer. The percentage decolourization was calculated from the following equation,

$$\% \text{ Decolourization} = \frac{\text{Initial OD - Final OD}}{\text{Initial OD}} \times 100$$

Results and Discussion

In the present study, six different bacterial isolates were isolated and identified from the textile dye effluent. The isolated bacterial isolates were identified and characterized as *Bacillus odyssey*, *Bacillus thuringiensis*, *Bacillus subtilis*, *Escherichia coli*, *Proteus mirabilis* and *Staphylococcus aureus*. All the bacterial isolates except *Escherichia coli* and *Proteus mirabilis* showed Gram positive reaction. The characteristics of the bacterial strains isolated from textile dye effluent were compared with MTCC Reference strains. Chen *et al*. (2003) reported that six isolates from different sources including lake-mud and wastewater treatment plant sludge showed various decolourization efficiencies for di-azo dyes. Khera *et al*. (2005) have reported isolation of organisms adapted to high dye concentration from sites near textile industries complex. The selected isolate is a sporulating Gram positive motile rod, occurring singly, grew as rough colony on nutrient agar. On the basis of conventional biochemical tests, it was identified as *Bacillus cereus* or *Bacillus thuringiensis*. Staining of the parasporal body showed its presence, which indicated the identity of the isolate as *Bacillus thuringiensis* (Saranraj and Stella, 2012).

Saranraj *et al*. (2010) isolated five bacterial species *viz*., *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Proteus mirabilis* and *Klebsiella pneumoniae*. Giek Far Chan *et al.* (2012) isolated and investigated the dye decolourization ability of a novel bacterial consortium, which consists of *Citrobacter freundii*, *Enterococcus casseliflavus* and *Enterobacter cloacae*. Sriram *et al*. (2013) isolated three different bacterial isolates *viz*., *Bacillus* sp., *Escherichia coli* and *Pseudomonas fluorescens* from textile dye effluent contaminated soil sample and used for the degradation study (Saranraj and Stella, 2012; Jayanthi *et al*., 2013; Saranraj and Sujitha, 3013). Recently, Saranraj *et al*. (2014) isolated and identified six different bacterial isolates *viz*., *Bacillus odyssey, Bacillus thuringiensis, Bacillus subtilis, Bacillus cereus, Alcaligenes* sp., and *Nocardiopsis alba* from the textile dye effluent sample.

The bacterial isolates (*Bacillus odyssey*, *Bacillus thuringiensis*, *Bacillus subtilis*, *Escherichia coli*, *Proteus mirabilis* and *Staphylococcus aureus*) were screened for the decolourization of reactive dyes by Plate assay. Maximum decolourization was recorded by *Bacillus odyssey* in the plate containing Reactive Orange – 16 (35 mm) followed by *Bacillus thuringiensis* (33 mm), *Bacillus subtilis* (30 mm), *Escherichia coli* (28 mm), *Proteus mirabilis* (27 mm) and *Staphylococcus aureus* (25 mm). The zone of inhibition in the plates containing the remaining reactive dyes was also recorded by the bacterial isolates in the above given order. Next to Reactive Orange – 16, the bacterial isolates showed maximum zone of inhibition in the plate containing Reactive Black – B followed by Reactive Yellow – MR, Reactive Blue – MR and Reactive Red M5B (Table – 1). Burchmore and Wilkinson (1993) studied the zone of inhibition with control dyes (Crystal violet, Phenol red, Malachite green, Methyl green and Fuchsin) with *Staphylococcus epidermidis* strains at a concentration of 100 ppm and at a concentration of 500 ppm. Whereas, degradation products did not show growth inhibition. These findings suggest the non-toxic nature of the product formed. Previous reports showed Malachite green and Crystal violet degradations into leuco-malachite and leuco-crystal violet are equally toxic to Malachite green and Crystal violet. The result of the present study was in line with the findings of Saranraj *et al*. (2014).

The decolourization of textile reactive azo dyes by six bacterial isolates *viz*., *Bacillus odyssey*, *Bacillus thuringiensis*, *Bacillus subtilis*, *Escherichia coli*, *Proteus mirabilis* and *Staphylococcus aureus* was studied. Maximum decolourization percentage was observed in the medium inoculated with *Bacillus odyssey* (Reactive Orange – 70.66%; Reactive Black – B – 67.21%; Reactive Yellow – MR – 65.40%; Reactive Blue – MR – 63.09% and Reactive Red M5B – 61.19%) followed by *Bacillus thuringiensis*, *Bacillus subtilis*, *Bacillus cereus*, *Alcaligenes* sp. and *Nocardiopsis alba*. The decolourization percentage was maximum in the medium containing Reactive Orange – 16. Next to Reactive Orange – 16, maximum decolourization was observed in Reactive Black – B followed by Reactive Yellow – MR, Reactive Blue – MR and Reactive Red M5B (Table – 2). These results provided obvious evidence of biodegradation of reactive dyes by bacterial isolates in the decolourization process, and also supported the earlier conclusion that decolourization by bacteria is mainly due to biodegradation, rather than inactive surface adsorption (Zhang *et al*., 2007; Saranraj and Stella, 2014; Jayanthi *et al*., 2014). Ayed *et al*.

Table – 1: Screening of bacterial isolates for dye degradation by plate assay

S.No	Bacterial Isolates	Zone formation (in mm)				
		Reactive Orange – 16	Reactive Black – B	Reactive Yellow – MR	Reactive Blue – MR	Reactive Red –M5B
1	*Bacillus odyssey*	35	34	33	31	29
2	*Bacillus thuringiensis*	33	31	30	28	24
3	*Bacillus subtilis*	30	29	27	25	20
4	*Escherichia coli*	28	26	24	20	16
5	*Proteus mirabilis*	27	24	21	17	13
6	*Staphylococcus aureus*	25	21	19	13	8

Table – 2: Decolourization of textile reactive azo dyes by bacterial isolates

S.No	Bacterial Isolates	% Decolourization				
		Reactive Orange – 16	Reactive Black – B	Reactive Yellow – MR	Reactive Blue – MR	Reactive Red –M5B
1	*Bacillus odyssey*	70.66%	67.21%	65.40%	63.09%	61.19%
2	*Bacillus thuringiensis*	66.97%	64.37%	62.51%	57.21%	55.46%
3	*Bacillus subtilis*	64.20%	61.58%	58.02%	52.53%	50.94%
4	*Escherichia coli*	60.93%	60.01%	52.70%	48.15%	46.09%
5	*Proteus mirabilis*	56.72%	53.05%	47.38%	42.72	36.34%
6	*Staphylococcus aureus*	53.99%	49.28%	42.48%	34.21%	26.67%

(2010) also documented similar reduction in toxicity of Congo red after consortial treatment.

The *Bacillus* species isolated have the capacity to decolourize the dye with chromophoric group. Members of the genus *Bacillus* have been reported to decolourize azo dyes. Since, dyes are extensively used in textile industries and are therefore a major source of industrial effluent contamination (Asad *et al*., 2007; Jadhav *et al*., 2007; Kim *et al*., 2008), 14 different dyes were tested. Barragan *et al*. (2007) describes *Bacillus* strains with continuous growth after 192 hrs of incubation. In the present study also, four different types of *Bacillus* sp. also isolated and used for decolourization studies.

Senan and Abraham (2004) also developed a consortium of three organisms to degrade a mixture of the dyes by co-metabolism and observed that the consortium could decolorize efficiently all the three dyes tested. Lucas *et al*. (2006) describes decolourization on plates by yeasts, with the development of cream - coloured colonies. The presence of colour in the colonies is probably due to bioaccumulation prior to biodegradation. Similar results have been described by other researchers (Yu and Wen, 2005).

Gopinath *et al*. (2009) studied the biodegradation of Congo red by *Bacillus* sp. isolated from tannery industry environment. Apart from acclimatization, many other strain development techniques are practiced that includes bio-engineering of organism focusing mainly on genetically improving required proteins.

Saranraj *et al*. (2010) investigated the decolourization and degradation of Direct azo dyes and biodegradation of textile dye effluent by using bacteria isolated from textile dye effluent. They isolated five different bacterial species from the textile dye effluent sample and the isolates were identified as *Bacillus subtilis, Pseudomonas aeruginosa, Proteus mirabilis, Klebsiella pneumoniae* and *Escherichia coli.* The bacterial inoculums were inoculated into flasks containing Direct azo dyes (500 mg/L) with trace amounts of yeast extract, glucose and sucrose and then sterilized and incubated for 4 days. In their research, *Pseudomonas aeruginosa* (97.33%) was identified as the best decolourizer of Congo Red. The best decolourizer of Direct Green-PLS was *Bacillus subtilis* (99.05%). *Klebsiella pneumoniae* (87.27%) highly decolourized the Direct Violet-BL. *Escherichia coli* (61.56%) was the best decolourizer of Direct Sky Blue-FF. The best decolourizer of Direct Black-E was *Klebsiella pneumoniae* (92.03%).

Anjaneya *et al.* (2011) isolated two different bacterial strains capable of decolorizing a highly water soluble azo dye Metanil Yellow from dye contaminated soil sample. The individual bacterial strains *Bacillus* sp. and *Lysinibacillus* sp. decolorized Metanil Yellow (200 mg L^{-1}) completely within 27 and 12 hrs, respectively. Various parameters like pH, temperature, NaCl and initial dye concentrations were optimized to develop an economically feasible decolourization process. The maximum concentration of Metanil Yellow (1000 mg L^{-1}) was decolorized by bacterial strains within 78 and 84 hrs respectively.

Kunal Jain *et al*. (2012) developed a bacterial mixed culture proficient in complete decolourization of azo dye – Reactive Violet 5R by enrichment technique. Bacterial community composition based on 16S rRNA gene analysis revealed that mixed cultures SB4 composed of six bacterial strains namely *Bacillus* sp., *Lysinibacillus* sp., *Bacillus* sp., *Ochrobacterium* sp., grew well in minimal medium containing low amount of glucose and yeast extract (YE) (1 g/L) and decolorized 200 mg/L of RV5 within 18 hrs under static condition. Decolourization efficiency was found to be unaltered under high RV5 and salt concentration where 1500 mg/L of RV5 was decolorized in presence of 20 g/L NaCl.

Sriram *et al*. (2013) carried out an experiment to degrade the textile Reactive azo dyes by using bacteria isolated from dye contaminated soil. Three different bacterial isolate such as, *Bacillus* sp*., Escherichia coli* and *Pseudomonas fluorescens* were isolated from textile dye effluent contaminated soil sample and used for the degradation study. It was noticed that there was a decrease in the OD in all the three species of all the five dyes as the incubation period increased. *Pseudomonas fluorescens* was more effective followed by *Bacillus,* and *Escherichia coli.* It was found that all the isolated bacteria were efficient decolourizers of Reactive textile azo dyes.

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