International Journal of Environmental Bioremediation & Biodegradation, 2014, Vol. 2, No. 2, 50-54
Available online at http://pubs.sciepub.com/ijebb2/2/1

DOI:10.12691/ijebb-2-2-1

An Application of Bioaugmentation Strategy to Decolorize & Degrade Reactive Black Dye by *Pseudomonas spp*

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Received April 08, 2014; Revised April 11, 2014; Accepted April 13, 2014

Abstract Pseudomonas species was isolated from the textile effluent polluted soil in Ankleshwar, Gujart, India and identified based on Biochemical and 16S RNA Sequence. The present study was carried out in an attempt to decolorize a commonly used yet tougher dye to decolorize, Reactive Black. The decolorization percentage was calculated from UV-Vis spectrophotometric analysis. Dye decolorization was probably due to the biotransformation and depended upon the biomass. Replacement of nutrient broth with minimal media did not show any decolorization property. The decolorization was confirmed by studying the spectral analysis of the dye. 16S rRNA partial gene sequencing (772 bp) of isolate *Pseudomonas* was also performed and a phylogenetic tree was prepared. Decolorization was optimized and found to be up to 95% at 7 pH, 40°C under static and non-aerated condition.

Keywords: *reactive dyes, Azo dyes, Bacterial isolates, dye decolorization*

Cite This Article: Maulin P Shah, "An Application of Bioaugmentation Strategy to Decolorize & Degrade Reactive Black Dye by *Pseudomonas spp.*" *International Journal of Environmental Bioremediation & Biodegradation*, vol. 2, no. 2 (2014): 50-54. doi: 10.12691/ijebb-2-2-1.

1. Introduction

The growth in industry and the changes in manufacturing processes have resulted in an increase in the volume and complexity of wastewater discharges to the environment. Many traditional and novel treatment processes are being modified and developed to try to eliminate the release to surface waters of the diverse chemical substances found in wastewater discharges1. Many industries use dyes and pigments to color their products. Among which, Azo dyes, constituting the largest class among the synthetic colorants, are considered as the widespread environmental pollutants associated with many important industries such as textile, food colorants, printing and cosmetic manufacturing. The textile industry is one of those industries that consume plenty of water in their manufacturing process. Synthetic dyes are extensively used in textile dyeing, paper printing, colour photography, pharmaceutical, food, cosmetic and other industries (Rafii et al., 1990; Singh et al., 2012; Sahasrabudhe and Pathade, 2012). Approximately 10,000 different dyes and pigments are used industrially, and over 0.7 million tonnes of synthetic dyes are produced annually worldwide. Major classes of synthetic dyes include azo, anthroquinone and triaryl methane dyes, and many of them are toxic or contain carcinogenic compounds with long turnover times (Hartman et al., 1978). It has been estimated that 10 to 15% of dyes are lost in the effluent during dying processes (Zollinger, 1987; Olligaard et al., 1999; Mathur, 2012). Colour is the first contaminant recognized in textile wastewater which affects aesthetics, water transparency and gas solubilities in water bodies (Faraco et al., 2009; Satyawali et al., 2009) and has to be removed before discharging the wastewater into a receiving water body (Vijaya and Sandhya, 2003). Effluent discharged from the textile industries has variable characteristics in terms of pH, dissolved oxygen, organic and inorganic chemical content, etc. Pollution caused by dye effluent is mainly due to durability of the dyes in wastewater (Jadhav et al., 2007). Existing effluent treatment procedures utilize pH neutralization, coagulation followed by biological treatment, but they are unable to remove recalcitrant dyes completely from effluents. This is because of the color fastness, stability and resistance of dyes to degradation (Anjaneyulu et al., 2005). Bioremediation is the microbial clean up approach which can transform various toxic chemicals to less harmful forms. Several reports suggest the degradation of complex organic substances, which can be brought about by bacterial enzymes like oxygenase (Ren et al., 2006), laccase (Hatvani and Mecs, 2001), lignin peroxidase (Shanmugam et al., 1999), etc. Many microorganisms capable of decolorizing the dyes include Gram- positive and negative bacteria (Sani and Banerjee, 1999) and fungi (Balan and Monteneiro, 2001). Gram-negative bacteria are *Pseudomonas*, Acinetobacter, Alkaligenes, Moraxella, Achromabacter and Flavobacterium spp. The Gram-positives include all in the actinomycete line and they are Mycobacterium, Nocardia, Rhodococcus and Arthrobacter spp. (Alexander, 1994). *Pseudomonas* is a rod-shaped,

flagellated, gram-negative bacterium that is found in most soil and water habitats where there is oxygen. It grows optimally at 25 to 30°C and can be easily isolated. This bacterium is unique because it has most genes involved in breaking down aromatic or aliphatic hydrocarbons. Thus, researchers are attracted to using *Pseudomonas* as the "laboratory 'workhorse' for research on bacteria-remediated soil processes" (Kowalski, 2002). There is great interest in sequencing the genome of *Pseudomonas* due to its strong effect in bioremediation (Marcus, 2003). The present study focuses on the use of *Pseudomonas* strain isolated from the natural habitat of textile effluent environment, to degrade one of the frequently used textile dye, that is, reactive black. The effect of different physicochemical parameters on the growth and color removal were also studied. Microbial decolorization has been proposed as a less expensive and less environmentally intrusive alternative. In the present study, we focused our attention on the isolation of dye decolorizing microorganisms from contaminated soil of an industrial estate and analyzed the ability of these isolates to degrade Reactive Black.

2. Materials & Methods

2.1. Chemicals

All chemicals and reagents used in this investigation were of Analytical grade. The common name of the dye (Reactive Black) has been used for convenience and was procured from local textile industries, Ankleshwar, Gujarat, India. The stock solutions of the dyes were filter sterilized and added to the growth medium in the concentration of 100 ppm (mg/litre).

2.2. Spectrum Study of the Dye

The dye procured from the industry was initially studied for absorption maxima in a Double Beam UV-Vis Spectrophotometer from 350 nm to 800 nm (Shimadzu, UV-Vis 1800, Japan).

2.3. Isolation of Bacterial Cultures

Soil sample taken from the dumping grounds of the sludge was used for the isolation of dye decolorizing microorganisms owing to long – term usage of the location for over 5 decades since the establishment. Bacteria from the soil sample were isolated by pour plate method and serial dilution technique using nutrient agar medium. All the plates were incubated at 37°C for 24 hours.

2.4. Study of Decolorization Activity

All decolorization experiments were performed in triplicates. A loopful of each isolated bacterial culture was inoculated into a separate 250 ml Erlenmeyer flask containing the Reactive Black (100 mg/L) in Nutrient broth and incubated for 24 h at 37°C for initial screening of the isolates for the ability to decolorize the dye. Aliquots of the culture (3 ml) was withdrawn at different time intervals, centrifuged at 5000 rpm for 15 min to separate the bacterial cell mass. Decolorization was determined by measuring the absorbance of the supernatant at 520 nm (max) and percentage of decolorization was calculated (Saratale *et al.*, 2006) as follows:

(%) Decolorization = (Initial absorbance – Observed absorbance) / Initial absorbance X 100

A loopful of culture was inoculated in 250 ml Erlenmeyer flask containing 100 ml nutrient broth. Separate study was carried out for different temperatures (20, 30, 40 and 50) and pH (3-10). Decolorization was also studied under shaking (150 rpm/min) and static (nonaerated) conditions. Decolorization was also studied in minimal media (mg/L): Glucose 1800; $MgSO_4.7H_2O$ 250; KH_2PO_4 2,310; K_2HPO_4 5,550; $(NH_4)_2SO_4$ 1,980.

2.5. Identification of the Isolate by 16SrRNA Gene Amplification and Sequencing

DNA was extracted from pure culture of the isolate that showed prospective application. A partial DNA sequence for 16SrRNA gene was amplified by using ATG GAT CCG GGG GTT TGA TCC TGG CTC AGG(forward primer) and TAT CTG CAG TGG TGT GAC GGG GGG TGG (reverse primer) (Jing et al., 2004). Amplifications performed in 50 µl reactions mixtures containing the template DNA, 40ng, 0.2 µM, for each of the primers, dNTPs 200 µM, Taq DNA polymerase 2.5 U and 10X buffers 5 µl. The mixture was subjected to the following amplification conditions; 2 min at 94 C for 1 min, and ended by a final extension step at 72 C for 7 min. The PCR products mixture was purified and sequenced at Bangalore Genei, Bangalore India. The identity of the bacterium determined by sequencing method was verified and confirmed through biochemical tests.

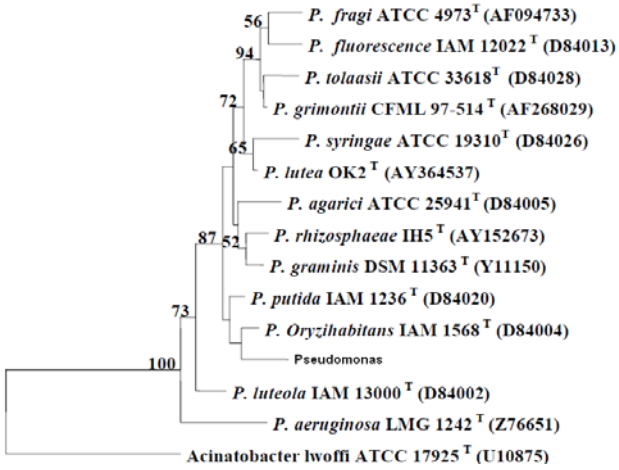

Figure 1. Phylogentiec tree of Pseudomonas

2.6. Study of Physicochemical Parameters

The isolated microbial strain *Pseudomonas* was cultivated in liquid media under different physiological conditions to study the growth kinetics and also the decolorization process. These parameters included temperature, pH, dye concentration, addition of various carbon sources (sucrose, glucose, lactose, sodium acetate and starch) in the media, etc. The spectrophotometric analysis was carried out to study the effect of various parameters on the growth and the decolorizing ability of the isolate. The mineral base medium without the dye was used as a blank.

2.7. Effect of Temperature

The effect of different incubation temperatures on growth and decolorization process was studied by keeping inoculated flasks, at different temperatures in the range of 30°C to 40°C together with the control (0.1 g/L dye) for 24 h incubation.

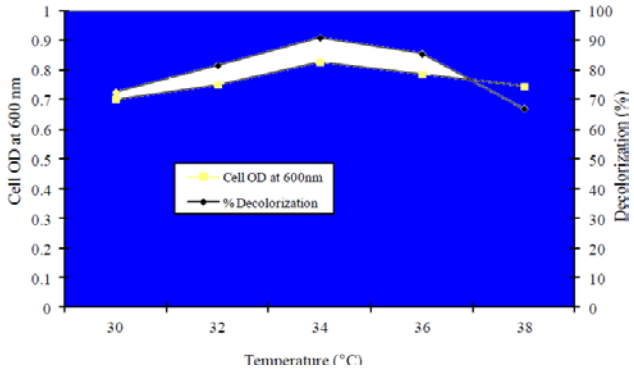

Figure 2. Effect of temperature on cell growth and decolorization of reactive black (100 mg/L)

2.8. Effect of pH

The effect of medium pH on the growth and decolorization efficiency of the isolate was investigated in the pH range of 6.5 to 7.5.

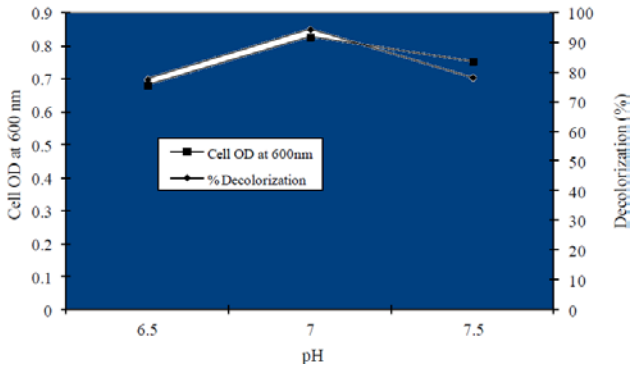

Figure 3. Effect of pH on cell growth and decolorization of reactive black (100 mg/L)

2.9. Effect of Dye Concentration

To find out the most appropriate concentration of dye that could be decolorized in a shorter duration. The concentration of the dyes used was 50, 100 and 200 mg/L, respectively. These flasks were then incubated at 37°C.

2.10. Effect of Various Carbon Sources

The decolorization efficiency of the isolate using methyl red (100 mg/L) was also evaluated in the presence of different carbon sources like glucose, lactose, starch, sodium acetate and sucrose in the mineral base medium at a concentration of 1 g/L keeping all other parameters constant.

2.11 Growth Kinetics

The isolate was also characterized in terms of their growth profile at optimal conditions in which the various parameters such as the increase in the cell biomass and the exact time of decolorization, etc, was monitored.

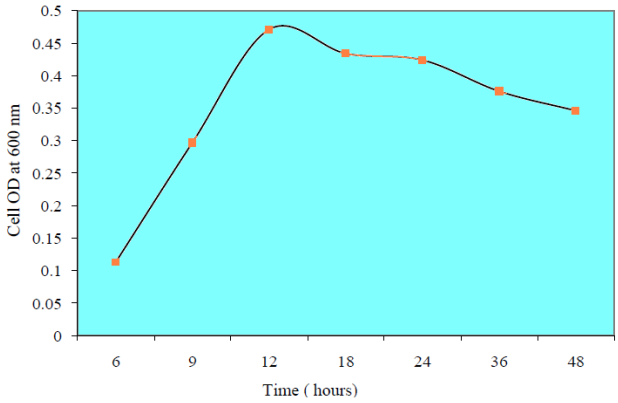

Figure 4. Growth profile of the isolate in reactive black

3. Results

3.1. Isolation and Identification

Soil and sludge samples collected from contaminated sites around various dye industries in Ankleshwar, Gujart, India were used for isolation of dye decolorizing culture in mineral base medium at pH 7.0. The isolate was Gram negative rod shaped bacteria. The partial nucleotide base sequencing (1529 base pairs) of 16S rRNA of isolate was done at Bangalore Genei, Bangalore, India. Basic Local Alignment Search Tool (BLAST) search (data not shown) for sequence homology at GenBank (www.ncbi.nlm.nih.gov) was also performed which showed that the bacteria had 100% homology with *P. putida* strain BASUP87 16S ribosomal RNA gene partial sequence, *Pseudomonas monteilii* strain SB3091 16S ribosomal RNA sequence, *Pseudomonas* sp. J4 (2008) 16S ribosomal RNA sequence and *Pseudomonas* sp. BJQ-D4 16S ribosomal RNA, *P. putida* strain LH-R1 16S ribosomal RNA gene and 99% identity with *Pseudomonas* sp. HB01 gene for 16S ribosomal gene partial sequence. A phylogenetic tree (Figure 1) suggests that the isolate shows very near evolutionary relationship with *Pseudomonas oryzihabitans* IAM 1568T (D84004). Thus, the isolate was identified as *Pseudomonas.*

3.2. Decolorization Performance

The isolate *Pseudomonas* could decolorize the dye reactive black as well as some other dyes to an appreciable extent (data not shown). The effect of various physicochemical parameters like pH, temperature, carbon source on decolorization of dye reactive black by the isolate was studied in mineral base medium with 100 mg/l methyl red.

3.3. Effect of Temperature

The incubation temperature affected the growth and activity of the *Pseudomonas*. Based on the results of Figure 2, the maximum decolorization was obtained when the isolate was incubated at 34°C followed by 37°C. A very low efficiency of decolorization was obtained under lower and higher temperature of incubation.

3.4. Effect of pH

It was observed that maximum growth and maximum decolorization was achieved at pH 7.0. Even below and

above the neutral pH, the isolate was able to grow and decolorize the methyl red (Figure 3).

3.5. Effect of Various Methyl Red Concentrations

The rate and extent of decolorization were affected by increasing concentrations of dye ranging from 50 to 200 mg/L. The spectrophotometric analysis (scanned in a spectrophotometer under the range of 200 to 600 nm) revealed that the maximum decolorization (99.65%) of the dye was seen at dye concentration of 100 mg/l within 24 h, whereas lesser decolorization of 92.72 and 63.67% was seen at dye concentration of 50 and 200 mg/l, respectively.

3.6. Effect of Various Carbon Sources

While trying to enhance decolorization performance of reactive black, extra carbon sources were added in the medium. The spectrophotometric analysis revealed that the percentage decolorization was maximum (95.39%) when no extra carbon source was added in the medium and 94.99 and 92% decolorization was seen with glucose and sucrose, respectively, while less decolorization was seen with starch (88%) and lactose (86%).

3.7. Growth Profile of Isolate

It was observed that with increase in incubation time, the decolorization efficiency of the isolate increased and the dye reactive black was completely decolorized at 24 h of incubation while the cell biomass also increased up to 12 h and then decreased (Figure 4).

4. Discussion

The isolate *Pseudomonas* could decolorize the dye reactive black (no peak) and some other dyes like Yellow FG (4.27%), Red RH (16.69%), Ponceau S (3.19%) and Brown GR (7.28%). The observed variation in percent decolorization of different dyes by the isolate was attributed to the difference in structure and complexity of each dye (Zimmermann et al., 1982; Sani and Banerjee, 1999; Khehra et al., 2004). Different physicochemical parameters like temperature, pH, dye concentration and carbon source influence decolorization of textile dyes by the isolate. In our present investigation, the optimum pH and temperature required for the efficient decolorization of dye reactive black by the isolate *Pseudomonas* in the liquid culture was 7.0 and 34°C, respectively. Normally, dye decolorizing bacteria have a narrow pH range (Chen et al., 2003). Presently, it was found that maximum growth of isolate and maximum decolorization (94.63%) was achieved at pH 7.0. This is in agreement with the studies previously conducted on degradation of reactive black by Adedayo et al. (2004). Increase in temperature proved to have a positive effect on the growth of the isolate and reactive black decolorization, which was maximum (0.828 and 91%, respectively) at 34°C. These observations could be attributed to the increase in enzyme activity and growth increase with the temperature (Asad et al., 2006). However, further temperature increase proved to be quite limiting for the growth and related decolorization of reactive black by the isolate. The isolate decolorized reactive black maximally (99.65%) at dye concentration of 100 mg/l. Further increase in dye concentration resulted in decrease in the percentage of decolorization and cell growth. This might be due to toxicity of dye through the inhibition of metabolic activities (Asad et al., 2006).

4.1. UV-Vis Spectral Analysis of Biodecolorization

The UV-Vis spectra of the media containing the dye before decolorization showed a maximum absorption at 600 nm (0 hour). In the final stage after decolorization, the absorption maxima totally disappeared from 600 nm (12 hours). Disappearance of the peak from 600 nm is a clear evidence of molecular rearrangements in the dye structure and degradation thereof (Figure 5).

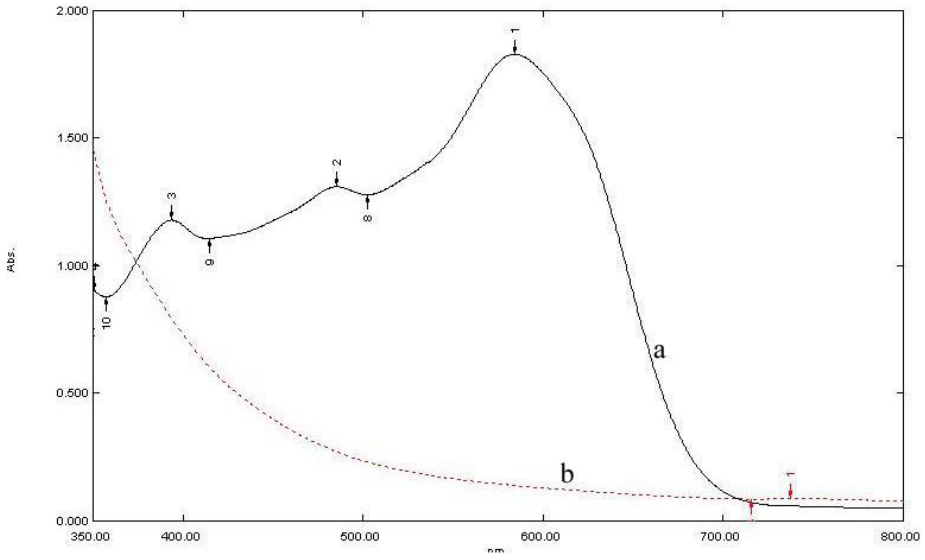

Figure 5. UV-Vis spectra of Reactive Black HFGR biodegradation at 0 hr (a) and 12 hrs (b)

As reported by Asad *et al.* (2007) decolorization of dyes by bacteria is due to adsorption by microbial cell as a surface phenomenon or to biodegradation. In case of adsorption, the UV-Vis absorption peak would tend to decrease approximately in proportion to each other, whereas, in biodegradation either the major visible light

absorbance peak disappears completely or a new peak appears. The observation of *Pseudomonas* cells mass retained their natural colour after decolorization of reactive black. Based on this, it is confirmed that the reactive black has undergone biotransformation and not due to simple adsorption over the surface. Physiological differences among the bacterial isolates may account for differences in the decolorization abilities (Reddy, 1995; Asgher et al., 2006). The complex enzymatic system responsible for the dye degradation and pattern of its expression may also vary among the isolates (Nagai et al., 2002; Boer et al., 2004); however, the relative rates of decolorization for the reactive blue dye cannot be easily explained. Degradation of dye involves aromatic ring cleavage which is dependent on the identity of the ring substituents with the presence of phenolic, amino, acetamido, 2-methoxy phenol or other easily biodegradable functional groups resulting in a greater extent of degradation (Spadaro et al., 1992; Mazmanci and Unyayar, 2005). In the present study, reactive black was found to be decolorized to different extents by the individual bacterial isolates. Different isolates have degraded the dye to different levels following a different pattern during the incubation period as is commonly observed in studies elsewhere (Knapp et al., 1997; Toh et al., 2003). However, overall complexity of structure alone is not an indicator of the difficulty of decolorization of a particular dye (Maas and Choudary 2005).

5. Conclusion

The dye reactive black was completely decolorized by a bacterium isolated from the soil samples collected from textile dyeing industrial region of Ankleshwar, Gujarat, India. The isolate was identified as *Pseudomonas*. The isolate decolorized reactive black dye within 24 h of incubation. Maximum decolorization was achieved at temperature 34°C and pH 7. The partial gene sequence of 16S rRNA, BLAST search and phylogenetic tree of the isolate were also made.

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