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BIODEGRADATION OF THE AZO DYE (REMAZOL BLACK B) BY *Pseudomonas putida* ISOLATED FROM TEXTILE WASTEWATER SAMPLE

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ABSTRACT

The synthetic aromatic compounds, Azo dyes, which have one or more –N=N– groups, are the most important and largest class of the used dyes with commercial applications especially in food and textile industries. The cleaning of textile wastewater containing dyes has attracted much attention in the recent years. The present study is an attempt to determine the ability of some bacterial isolates obtained from textile wastewater sample from Al Khumra station for decolorization of the Azo dye, Remazol black B. Three bacterial isolates were isolated from wastewater of textile effluents containing azo dye on basal nutrient medium. These isolated bacterial isolates were identified as *Bacillus cereus*, *B. pumilus* and *Pseudomonas putida* by phylogenetic analysis of their 16S rDNA. The most promising bacterial isolate was *Pseudomonas putida* and was used for further study. Results of study revealed that *P. putida* have ability of complete biodegradation (100%), if concentration is at 400 ppm while this degradation was 60 and 30 percent at 600 and 700 ppm concentration of azo dye respectively. Further, rate of biodegradation also depend on the pH and temperature of the surrounding medium, at 400 ppm concentration of azo dye, maximum degradation was obtained at pH 6.5 and temperature 40°C. This decolorization potential increased the applicability of this bacterium for the dye removal and the results suggest that the obtained isolate of *P. putida* can be used as a useful tool to treat waste water containing Azo dyes. Degradation potential of the bacterium depend on the presence of azoreductases, laccases and peroxidases enzymes.

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1 Introduction

The synthetic Azo dyes are most important class of dye, are widely used as colorants in the textile and food and pharmaceutical industries. These azo pigments are non metalized and metalized colorants; make up 50-60% of all dyes used by the world colorant industries (Anon, 1996; Aspland, 1997; Corbett, 2000, Lalnunhlmi & Krishnaswamy, 2016). Some of the Azo dyes are water soluble while rest are water insoluble but solvent soluble. Azo compounds are water-soluble compounds possessing the characteristic –N=N– (azo) bond (Figure 1). Water-soluble azo dyes are very important class of dyes for dyeing hydrophilic textiles such as cotton and viscose rayon. Both water soluble and insoluble class of azo dyes have carcinogenic properties and when these dyes are absorbed by human body can undergo decomposition and form amines by the enzymatic activity of body. Currently this includes the 24 amines classified as substances known to be human carcinogens (Waring, 1984; Gregory, 1986; EPA, 1999; Ziarani et al., 2018).

Discharge of untreated Azo dyes contaminated wastewater into

Figure 1 the chemical structure of Vinyl Sulfone dye, also known as Remazol

environment cause considerable environmental pollution. Various procedures such as filtration, carbon activated, coagulation and chemical flocculation have been widely used to treat dye containing wastewater. Many textile industries have coagulation–flocculation and advanced oxidation like azo dye containing wastewater treatment system but these procedures are complex and costly and because of these reasons these methods are not feasible for many countries (Erkurt, 2010; Sivakumar et al., 2013). Furthermore, concentrations of organic matter and particular persistent colorants (dyes) are also affecting the efficiency of treatment procedure (Olejnik & Wojciechowski, 2012). Kadlec & Wallace (2009) designed vertical-flow wetlands ecosystems to remove pollutants from wastewater; this system imitates the natural wetlands by relying on heterotrophic microorganisms, aquatic plants, and a combination of naturally occurring processes.

A wide range of microorganism including actinomycetes, bacteria, fungi and yeast have capability to degrade azo dyes in ecofriendly manner (Kirby et al., 2000; Olukanni et al., 2006). These microorganisms' possess various types of azo dyes degrading

enzymes such as laccase, azo reductase, peroxidase, and hydrogenase (Khalid et al., 2008; Sudha et al., 2014); these enzymes degrade complex azo dyes in simpler compounds under both aerobic and anaerobic conditions and provide energy to these organisms for their metabolism (Lodato et al., 2007; Sudha et al., 2014). Among the identified microorganisms, due to rapid and easy growth, survival under both aerobic and anaerobic conditions, bacteria is most commonly used for the treatment of azo dyes containing wastewater. Zhang et al. (2010) detected the presence of *Klebsiella pneumoniae*, *Aeromonas hydrophila*, *Enterobacter cancerogenus*, *Proteus hauseri* and *Acinetobacter johnsonii* as decolorizers in dye-bearing wastewater from Taiwan. Similarly, Shah et al. (2013) isolated different bacterial isolates having azo dye degrading capability from textile industry wastewater sample. Like this, Hussain et al. (2013) isolated a dye degrading bacterial strain from a textile industry wastewater of Faisalabad, Pakistan in liquid mineral salts medium. Najme et al. (2015) isolated 49 bacterial isolates from industrial waste water which can decolorize various azo dyes like Reactive Orange 16, Reactive Yellow 2 and Reactive Red 120 under the presence of different heavy metals (Zn, Cd, Cr, Pb and Cu). Among the isolated bacterial strains, *Serratia* sp. RN34 had highest ability to decolorize various reactive azo dyes (Yellow-2 dye, Red-120 and Orange-16) under static conditions. Bacterial azo dye degrading capacity also depends on the pH and temperature of wastewater or surrounding mediums. Under static conditions, highest decolorization activity was reported at pH 8 and 25°C when yeast extract used as an additional carbon source. The aim of the present study was isolation and identification of some active and local bacterial isolates capable to degrade azo dyes from wastewater treatment plants located at Jeddah, Saudi Arabia.

2 Materials and Methods

2.1 Wastewater sample

The effluent wastewater sample (50 ml) from wastewater treatment plant, WWTP at Al Khumra in the industrial area, south part of Jeddah, was collected in sterile plastic bottle and transferred directly to laboratory in ice bag for chemical and biological analysis. The pH and temperature of collected waste water were recorded by standard procedures at sampling site. Different water quality parameters such as Biological Oxidation Demand (BOD), Chemical Oxygen Demand (COD) and Total Suspended Solids (TSS) in addition to Total Dissolved Solids (TDS) were determined by standard protocol (Rainwater & Thatcher, 1960; USEPA, 1985; APHA, 1992; Rabah et al., 2007). The quantity of sodium in water is known as Sodium adsorption ratio and is measured from the equation given by Lesch & Suarez (1999).

$$\text{SAR} = \frac{\text{Na}^+}{\sqrt{0.5(\text{Ca}^{2+} + \text{Mg}^{2+})}}$$

2.2 Preparation Azo dye stock solution

Vinyl Sulfone dye (trademarked name Remazol® dye “Remazol Black B”) is a fiber reactive dye used for silk, wool and cotton coloring, obtained from local market, Jeddah, Saudi Arabia. Stock solution of dye was prepared in de-ionized water (1000 mg/l) and filter sterilized using 0.45 µm filters and used for preparations of different concentration of the dye. The calculated volume of stock dye solution was taken, filter sterilized and added to the sterilized medium.

2.3 Isolation of bacteria

About 5 ml of wastewater was added into 250 ml Erlenmeyer flask containing 50 ml of the Mineral Salt broth medium (MSM). The basic composition of MSM broth was: NaCl (1g/l), CaCl₂·2H₂O (0.1g/l), MgSO₄·7H₂O (0.5g/l), KH₂PO₄ (1g/l) and Na₂HPO₄ (1g/l). Medium was also supplemented by Remazol Black B (100 mg/l) and 0.5 (g/l) yeast extract. All flasks having medium along with wastewater were incubated at 30°C under static conditions. After 3 days, bacterial cells were collected by centrifugation at 5000 rpm and these were transferred to the fresh medium containing flasks with Remazol Black B (100 mg/l). After 48 hrs of incubation bacterial growth was estimated and this was followed by the application of 1.0 ml culture broth on Nutrient Agar containing Remazol Black (100 mg/l). The colonies exhibiting color removing ability (with clear zones around) were selected, purified and preserved on Nutrient Agar slant at 4°C for further studies. Concerning *Escherichia coli*, it was obtained from King Fahad General Hospital, Jeddah, Saudi Arabia

2.4 Preculture of the bacterial isolates

A loopfull of culture of the selected bacteria were inoculated in 250 ml Erlenmeyer flasks containing 50 ml of Nutrient broth medium for 2 days at 30°C and 120 rpm.

2.5 Selection of the most active bacterial isolates

For bacterial selection, the isolates which can tolerate higher concentration of the Azo dye, were selected and maintain on MSM liquid medium supplemented with Remazol Black B (100 mg/l) and yeast extract (0.5 g/l). Each flask was inoculated with 2 ml of bacterial preculture (4x10⁶CFU/ml). At every 12 hrs intervals, bacterial growth was measured by turbidity (A_{450 nm}) and the culture broth was centrifuged at 5,000 rpm for 15 minutes. Percent decolorization was monitored by measuring initial and final absorbance at 595 nm and calculated according to the equation given by Saratale et al. (2009).

$$\text{Percent Decolorization} = \frac{\text{Initial OD} - \text{Final OD}}{\text{Initial OD}} \times 100$$

2.6 Bacterial growth at different dye concentrations

To examine the effect of different dye concentration on bacterial growth, 2 ml (4x10⁶CFU/ml) of best isolate was inoculated in a series of parallel flasks having MSM media supplemented with increased concentrations (100-1000 ppm) of Remazol Black B, these culture flasks were incubated at 30°C under static conditions. For estimation of percent degradation and bacterial growth, samples were collected for 3 days at each 12 hrs intervals and the concentration of toxic dye and bacterial growth was estimated as mentioned earlier, uninoculated samples were used as control.

2.7 Factors affecting decolorization by bacteria

2.7.1 Effect of pH on dye decolorization

For estimating effect of pH on dye decolorization, sterile mineral salt broth medium with 400 ppm of Remazol Black B at different pH values (4, 5, 6, 6.5, 7, and 8) was prepared in 250 ml Erlenmeyer flasks and 2 ml of the preculture (4x10⁶CFU/ml) was inoculated in each flask, each treatment was replicated thrice. Uninoculated medium was used as control. All the inoculated and uninoculated flasks were incubated at 30°C under static conditions. The percentage decolorization was measured as mentioned earlier.

2.7.2 Effect of temperature on dye decolorization:

Erlenmeyer flasks containing 50 ml mineral salt broth medium at pH 6.5 were used to determine the best temperature for dye degradation. The used concentration of the dye was 400 mg/l and all flasks was inoculated with 2 ml of the preculture (4x10⁶ CFU/ml) and were incubated at 25, 30, 35, 40, 45 and 55°C for 3 days under static condition. Uninoculated medium was used as control, percentage decolorization was measured at each 12 hours intervals and obtained results were compared with controls.

2.8 Bacterial identification

The bacterial isolate which showing highest dye degradation ability was identified by using morphological, physiological, biochemical and molecular studies. Some morphological and physiological characters were determined as per the Bergey's manual of determinative bacteriology. Biochemical tests like Methyl Red, Voges-Proskauer, Indole, Citrate, Catalase, and Oxidase, Nitrate reduction and hydrolysis of Casein, Starch, Urea and Gelatin were also determined. Utilization of different carbon sources, D-glucose, D-fructose, galactose, mannitol and D-maltose and some nitrogen sources were detected at 30°C for 24-48 hrs.

Crude lysate of cells were prepared, DNA was extracted and subjected to PCR amplification by using the 16S rRNA forward

primer (5'-AGTTTGATCATGGTCAG-3') and reverse primer (5'-GGTTACCTTGTTACGACT-3'). The primers were designed based on the highly conserved region of 16S rRNA from various bacteria (Weisberg et al., 1991). The amplified PCR product was sequenced using a big dye terminator cycle sequence kit. The product of the sequencing reaction was analyzed using DNA Sequencer ABI PRISM 310 genetic analyzer (Perkin Elmer) and DNA sequence was compared with sequences of some members of the genus available through GenBank database.

2.9 Statistical analysis

All decolorization experiments were performed in three sets and mean values were calculated.

3 Results and Discussion

Jeddah Bay received massive discharges of partly-treated wastewater, Al Khumra which is a multiport diffuser pipe also contribute in enhancing waste water level of bay (Peña-García et al., 2014). Textile dyeing, leather tanning, paper and sugarcane industries produce large quantities of wastewater which cause serious water pollution not only in rivers, pond or bay but also contaminate groundwater and soil (Abrahart, 1977; Rafi & Daugherty, 1997; Senan & Abraham, 2004; Akarslana & Demiralay, 2015). In current study physico-chemical properties of the collected textile waste effluent were estimated (Table 1). The

Table 1 Chemical and biological analysis of the collected wastewater

Parameter	Wastewater	FAO
pH	6.45	6-9
Temperature	43°C	-
Color	Gray	-
EC (dS/m)	3.30	0-3
NH ₄ ⁺ (mg/l)	36.31	30
NO ₃ ⁻ (mg/l)	21.48	-
P ³⁻ (mg/l)	4.56	-
SAR	6.60	15
TDS (mg/l)	1735.40	2000
TSS (mg/l)	1134.90	-
COD (mg/l)	17.32	-
BOD (mg/l)	3.48	-

TS :Total Solids, TDS: Total Dissolved Solids, TSS: Total Suspended Solids, EC = Electrical Conductivity (dS/m), COD: Chemical Oxygen Demand (mg/l) , BOD: Biological Oxygen Demand (mg/l), FAO: Food and Agriculture Organization (1985), -: not detected

color of the collected water was dark gray, it was slightly acidic (pH 6.45) and have mild temperature. In general, the obtained pH value fall within the limit (pH 7.0 to 8.5) suggested by the World Health Organization (WHO, 1989; DWAF, 1996). Results of present study are in agreement with the findings of Tyagi & Mehra (1990) while these are contradictory to the findings of Shah (2013) those who reported alkaline nature (pH 7.45) of waste water, this difference might be because of the nature of pollutant in wastewater. The pH value of water is very important, as it affects the development of microbial community and other biochemical reactions. Contamination of water by textile pollutant also affects the solubility of gases in water which marked by higher BOD, this higher BOD value indicate the presence of pollutant and higher microbial load in tested water samples (APHA, 1992). Further, electrical conductivity of the collected water samples was reported 3.3 dS/m while mean COD value was reported 17.32 mg/l. On the other hand, biochemical oxygen demand (BOD) of the collected wastewater sample was reported 3.48 mg/l and the value of total suspended solids (TSS) were found 1134.9 mg/l. The concentrations of NH₄⁺, NO₃⁻ and P³⁻ were found 36.31, 21.48 and 4.56, mg/l, respectively (Table 1). In the present study, BOD value was reported lower than the permissible limits. Lokhande et al. (2011) reported higher BOD values (1047.3 mg/L) for the water sample collected from pharmaceutical industries while it was reported 776.2 mg/l and 535.8 mg/L for the samples collected from dyes and paint industries. In present study electrical conductivity was reported higher than that recommended concentration (0-3.0) of FAO (1985), which means collected water samples are not good for general use (Abdo et al., 2010). It was well known that wastewater contained many nutrients and ions especially nitrate (NO₃⁻), affecting the plant growth adversely (Magesan, 2001). In present study, sodium adsorption ratio (SAR) was reported 6.60 which is very higher the recommended limit and it indicate the unsuitability of this water for irrigation and cultivation purpose. Further, TDS is high for the collected samples which reduce purification of wastewater by microorganisms (Junkins, 1982; Kim, 1994).

The water samples collected from wastewater treatment plant of Al Khumra, industrial area, were spread on mineral salt broth medium containing 100 ppm of the Remazol dye. Bacterial colonies surrounded by clear zone were consider as an effective strain and were selected for further study related to the azo dye removal capability; total three bacterial isolates were selected and isolated. These isolated isolates were characterized and identified according to Bergey's Manual of

Systematic Bacteriology, physiological and biochemical tests (Holt et al., 1993; Harrigan, 1998), these isolated strains were identified as *Bacillus pumils* ABO23, *Bacillus cereus* ABO13 and *Pseudomonas putida* ABO23. Among these, *Bacillus pumils* ABO23, *B. cereus* ABO13 were Gram positive spore forming bacteria while *P. putida* ABO23 was gram negative, rod shape, non spore forming bacterium. Identification was confirmed by using 16 rRNA studies and phylogenetic trees of the selected bacteria compared with available genera database (Figures 2 and 3). Figure 4 showed the percentages of decolorization by the selected three most promising bacteria isolates, grown on broth medium supplemented with 100 ppm of Remazol B for 3 days, and these results were compared with *E. coli*. Further, the decolorization percentages after 72 hours were 100, 80 and 72 percent for *P. putida* ABO23, *Bacillus cereus* ABO13 and *B. pumils* ABO23, respectively while it was less than 20% in case of *E. coli*. Figure 5 represents the percentage decolorization by the *P. putida* ABO23 grown on broth medium containing increasing concentrations of Remazol black B for 3 days. The percentage of degradation was 100 percent up to 400 ppm concentration of Remazol black B and afterward increasing concentration of Remazol black B decreased degradation. At 700 ppm, decolorization percentage was less than 30 percent after 72 hrs of inoculation (Figure 5). Effect of pH and temperature was also evaluated in present study and maximum decolorization percentage was recorded at pH 6.5 and temperature 40°C whereas 100% decolorization was found after 60 hrs instead of 72 hr at pH 7 and 30°C (Figure 6 & 7). The pH value of the medium affect degradation of the dye and maximum

Figure 2 Phylogenetic relationship between *Bacillus pumils* ABO23, *B. cereus* ABO13 and other related reference isolates based on the 16S rRNA gene sequence analysis

decolorization percentage was reported at pH 6.5 but this dye degradation percentage reduced at pH 6 and 7 and the lowest percentages were at pH 4, 5 and 8 (Figure 6). Findings of present study are in agreement with the findings of Chang et al. (2001) and Shah et al. (2013). Similarly, Aburas (2016) isolated *P. putida* from waste water sample and reported highest ability of PHB production. Azo dye degradation was enhanced by alkaliphilic bacterial consortium (Mahmoud et al., 2017) or by special fungal degradation including *Aspergillus niger* strain isolated from textile wastewater. Bioremediations of contaminants and toxins using bacteria has been intensified and reported by many authors (Dawkar et al., 2008; Singh et al., 2014). Similarly, Roy et al. (2018) reported that bioremediation of industrial effluent containing textile dyes using bacteria is an emerging and important tool in

Figure 3 Phylogenetic relationship between *Pseudomonas putida* and other related reference isolates based on the 16S rRNA gene sequence analysis

Figure 4 Percent decolorization of the three bacteria isolates grown on broth medium supplemented with Remazol B for 3 days and results compared with *E. coli*

Figure 5 Percent decolorization by the selected *P. putida* ABO23, grown for 3 days on broth medium containing increasing concentrations of Remazol B

Figure 6 Effect of temperature on degradation of Remazol Black B by the selected *P. putida* ABO23

Figure 7 Effect of pH on degradation of Remazol Black B by the selected *P. putida* ABO23

environmental protection and pollution treatment. In conclusion, industrial effluent containing textile dyes was considered as mutagenic and mitotic poisoning water and bioremediation of these toxic materials by bacteria, is becoming an emerging and important technique. All three selected bacterial isolates from the textile effluent showed excellent decomposition and decolorization of the textile Azo dye. Among these *P. putida* reported most potent one under the best conditions for bioremediation process.

Conflict of interests

There is no conflict of interest.

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