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ANALYSIS OF BACTERIAL DEGRADATION OF AZO DYE CONGO RED USING HPLC

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ABSTRACT

The efficiency of four bacterial species namely *Enterobacter cloacae*, *Hafnia alvei*, *Klebsiella pneumoniae*, *Serratia marcescens* isolated from agricultural soil in degrading the azo dye Congo Red was analyzed. The bio-degradation was monitored using HPLC (High Performance Liquid Chromatography). Appearance of new peaks and disappearance of certain peaks were observed which showed that either there is bio-degradation or bio-transformation of the azo dye. Sample treated with *H. alvei* obtained the peak with shortest retention time (RT), 1.98. Highest retention time and thus the lowest polarity were obtained in the sample treated with *H. alvei* and *E. cloacae*. There was synergistic effect upon dye degradation since the sample treated by all the bacterial species in combination showed better degradation than that treated by individual bacteria.

INTRODUCTION

Azo dyes constitute more than fifty percent of the dyes produced annually and are the most important group of synthetic colourants that are extensively used in textile, food, pharmaceutical and printing industries. They are recalcitrant and pose toxicity to various aquatic organisms and animals (Puvaneswari *et al*., 2006). Azo dyes are the largest and most versatile class of dyes with one or more azo (N=N) bridges linking substituted aromatic structures viz. aromatic hydrocarbons, derivatives of benzene, toluene, naphthalene, phenol and aniline.

A wide variety of azo dyes with anthraquinone, polycyclic and triphenylmethane are being used in textile dyeing and printing processes. These synthetic colorants have been found to be toxic, mutagenic, carcinogenic and lethal in various test systems and linked to human bladder cancer, splenic sarcomas, hepatocarcinomas and nuclear anomalies in experimental animals and to chromosomal aberrations in mammalian cells. They are capable of producing intestinal cancers and some are teratogenic capable of causing cerebral and skeletal abnormalities in fetuses (Murugesan and Kalaichelvan, 2003). The carcinogenicity may be due to the dye itself or due to the aryl amine derivatives generated during the reductive biotransformation of the azo linkage (Zimmerman *et al*., 1982).

It is estimated that about fifteen percent of dyes

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come out along with effluent during textile processing (Wesenberg *et al*., 2003), with azo dyes being the most common one released into the environment (Bumpus, 1995). Wastewater from the textile industry is a complex mixture of many polluting substances ranging from residual dyestuff to heavy metals (Hoad, 2002). Furthermore, the colour and chemical composition of textile effluents are usually subject to daily and seasonal variations dictated by the production routine. Treatment of dye wastewater involves physical / chemical methods such as coagulation, precipitation, adsorption by activated charcoal, oxidation by ozone, ionizing radiation, ultra filtration, incineration, photocatalysis and chemical flocculation. (Lin and Peng, 1996; Lambert *et al*., 1997 and De Moraes *et al*., 2000). Commonly used waste removal treatments do not adequately eliminate many azo dyes from the effluent waters of textile mills and dye stuff factories (Shaul et al., 1991) because of its high content of BOD, COD, heat, pH, color and heavy metals (Dubrow *et al*., 1996).

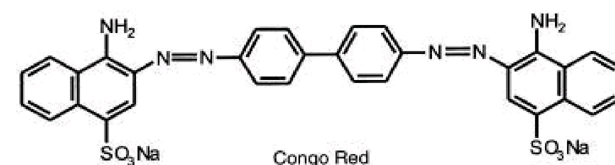
Microbial decolourization and degradation is an environment friendly and cost- competitive alternative to chemical decomposition processes (Verma and Madamwar, 2003). Microbial degradation can also detoxify the effluent effectively without leaving any residue. Among the microorganisms, bacteria are the most commonly used for various bioremediation processes. Bioremediation of azo dyes in textile effluents by bacteria or fungi is an alternative to conventional methods and a very promising area of study because of the relatively low expense involved. This biological process includes biodegradation and biotransformation, with a goal to mineralize hazardous contaminants in the environment. The method is targetted to break down the complex dye molecules to non-toxic inorganic compounds (i.e) nitrate, carbon-di-oxide and water which could be used as precursors for the synthesis of their own cell material by the micro-organisms involved.

For biotreatment purpose, the final degradation products as well as the intermediates have to be identified and quantified as these compounds may further increase the toxicity of the effluent. The true potential of bacteria in azo dye degradation cannot be evaluated without insight into the intermediates generated in the process. High performance liquid chromatography (HPLC) has been used for analysis of various dyes in waste water and metabolites from various degradation procedures (Conneely *et al*., 1999; Baiocchi *et al*., 2002; Nachiyar and Rajkumar, 2003). The purpose of the study is to use HPLC technique to examine the bacterial degradation products of selected model azo dye Congo Red.

Materials and Methods

Dyes and Chemicals

The dye Congo Red, a secondary diazo (IUPAC name sodium sodium 3,3'-([1,1'-biphenyl]-4,4'-diyl) bis(4-aminonaphthalene-1-sulfonate) dye was procured from a local dye store. Stock solution of the model azo dye was prepared by weighing 0.1 g of the dye and transferring it into a 1000 ml volumetric flask and dissolved in distilled water. pH of the solution was adjusted between 6-7 and the volume was made upto the mark. Working solutions were prepared by further dilution of the stock solution. All chemicals used in the study were of analytical grade.



Microorganisms

Four bacterial species namely *Enterobacter cloacae, Hafnia alvei, Klebsiella pneumoniae, Serratia marcescens* were isolated from agricultural top soil using standard isolation procedures. Biochemical characterization and identification of the microbes were based on Bergey's Manual of Determinate Bacteriology (Holt *et al*., 1994).

Bacterial inoculum

Standard inoculum of each bacterial isolates was prepared by sub culturing a single colony of the respective bacterium in 125 mL conical flasks containing 10 ml minimal medium (MM) composed of (gL-1) D-Glucose 0-1; Ammonium Sulphate 0.1; Potassium phosphate dibasic 1.27; Sodium Chloride 4.0; Magnesium Sulphate 0.42; Potassium Chloride 0.29; EDTA 0.5; yeast extract 0.6; Calcium Chloride 0.02; Ammonium nitrate 1.0; Sodium carbonate 0.1; Hydrochloric acid 1 mol L -1 and distilled water 100 mL. The pH was adjusted between 7- 8.5 and the culture was incubated at 30°C for 3 days.

Decolourization (Degradation) assay

The experiments were carried out in 250 mL conical flasks containing 200 mL of nutrient broth to which 5 ml of dye from the stock solution was added. 1 mL of the bacterial inocula were also inoculated and incubated at 37°C. The dye medium was treated with the four bacterial species individually and in different combinations. 3 mL of supernatant from each bacterial culture were taken for 10 days and decolourization studies were performed. The same amount of MM containing the azo dye was added to each culture after each sampling to keep a constant volume in the culture flask. Three replicate flasks with the same dye concentration and inoculum size were used for the study and the results were reported as an average of the three samples. A control was also set up with 200 mL nutrient broth and 5 mL dye solution but without any bacterial inoculum. Care was taken to reduce variations induced by photo-degradation of the due. HPLC analysis of degraded products

The samples after 10 days of treatment were filtered through a 0.45 µm membrane filter prior to HPLC analysis.

Column Specification

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 & absorbance recorded at 680 nm.

Sample preparation

One mL of the sample was centrifuged (at 3000 rpm for 15 mts) and dissolved in specific solvent of HPLC grade and filtered through 0.22 micro filter. The filtrate was collected and degassed using sonicator for 50 times at 4°C.

Solvent preparation

Solvent was prepared using Acetonitrile and water in the ratio 65:35 and degassed using sonicator for 50 times at 4°C.

Column equilibration

Column equilibration was done using 65% Acetonitrile in water until zero base line.

Sample injection

Twenty microlitre of the sample was injected in to the injection head using injection needle. Required time and wavelength were set and the purification profiles were seen on the screen that shows the degraded compounds with its retention time.

RESULT AND DISCUSSION

The absorption spectra of the samples obtained at 680 nm are presented in figures 1-5. The HPLC elution profile of the dye Congo Red (Control) showed 5 peaks with retention time (RT) of 1.98, 2.18, 2.37, 2.59 and 2.94 minutes (Fig. 1). 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 Congo Red treated with bacterial isolate *Hafnia alvei* showed 3 peaks with RT 1.98, 2.18 & 2.39 (Fig. 2). Sample treated with *Enterobacter cloacae* revealed the same number of peaks and almost similar RT as that of *H. alvei* (Fig. 3). The sample treated with both *E. cloacae* and *H. alvei* obtained a new peak with 2.61 RT along with 2 other peaks with similar RT as those observed in case of two previously mentioned treatments (Fig. 4).

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 Saleh (2005). 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. In the treatment by the bacteria *H. alvei* and *E. cloacae*, 2 peaks were not observed which were present in the control. Similarly, in the treatment by the bacterial consortia (Fig. 5), a new peak had appeared and two peaks had disappeared. From this, it can be assumed that either there is degradation of the dye in the culture medium or there is biotransformation of the dye from one form to another. It is not confirmed whether the metabolite or the breakdown product formed is a low molecular weight non toxic component or another toxic product which needs further analysis.

Bacterial strains reduce dyes both under aerobic and anaerobic conditions. The ability of microorganisms to decolorize different dyes vary depending on the structure and complexity of the dye (Cripps *et al*., 1990). Many bacterial strains possess unspecific cytoplasmic enzymes which act as azo reducates (Walker, 1970). Azo dyes can be cleaved symmetrically or asymmetrically depending on the structure of substrate and active site of an enzyme. Asymmetric

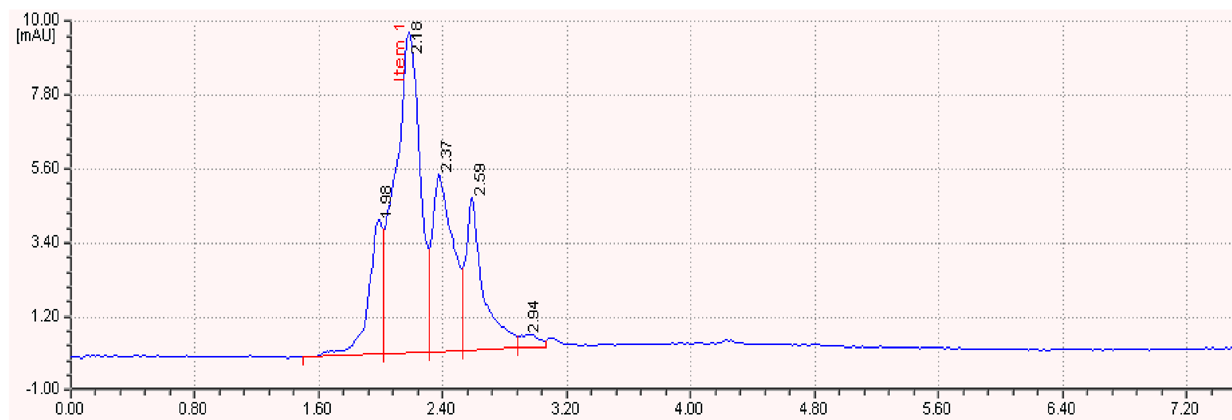


Fig. 1 HPLC chromatogram of Congo Red (Control)

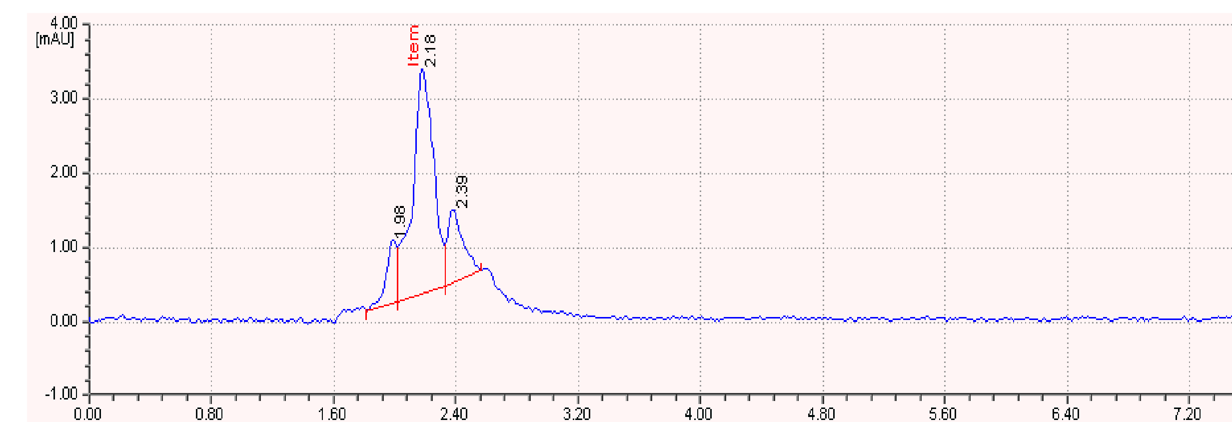


Fig. 2 HPLC chromatogram of Congo Red treated with *Hafnia alvei*

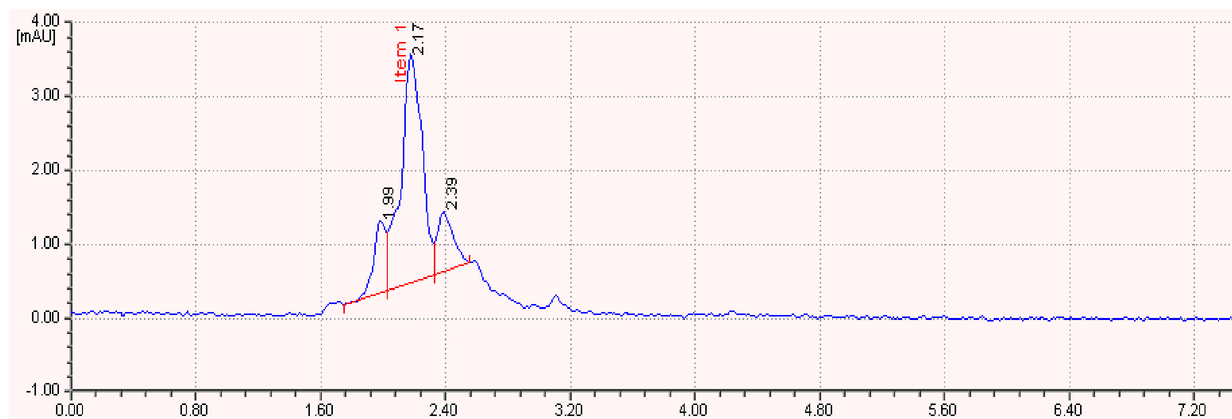


Fig. 3 HPLC chromatogram of Congo Red treated with *Enterobacter cloacae*

oxidative cleavage leads to formation of mono azo intermediates initially which can be further oxidatively and reductively cleaved into simple metabolites. There is still a gap in current knowledge between decolourization and degradation mechanisms. Due to involvement of various enzymes it is practically very difficult to define the exact role of each enzyme system involved in biodegradation (Telke *et al*., 2008). It is generally agreed that practical application of biodegradation systems using bacterial strains

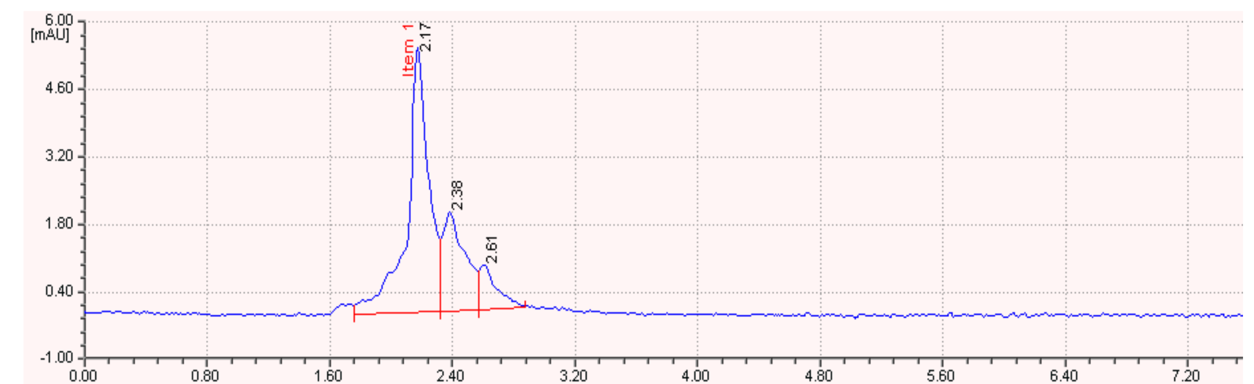


Fig. 4 HPLC chromatogram of Congo Red treated with *Hafnia alvei* and *Enterobacter cloacae*

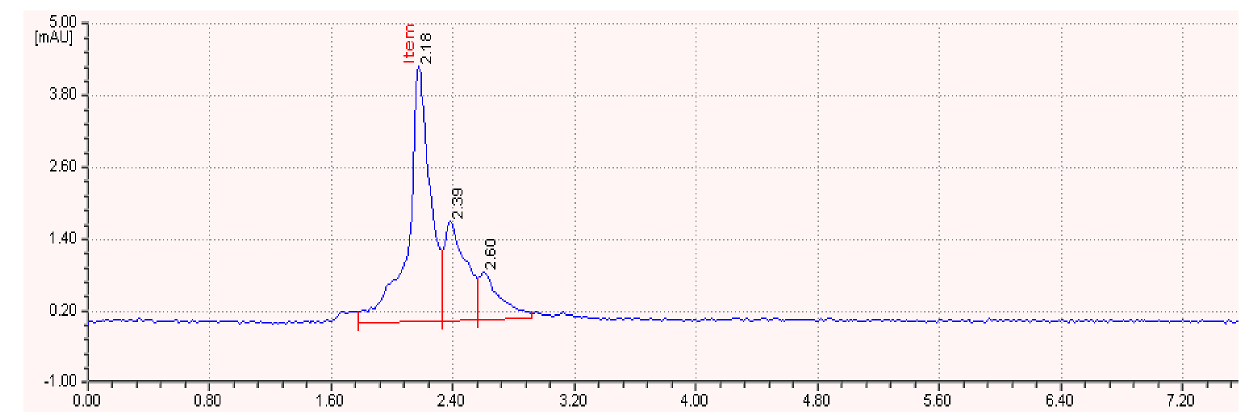


Fig. 5 HPLC chromatogram of Congo Red treated with *Hafnia alvei* and *Enterobacter cloaca, Klebsiella pneumoniae, Serratia marcescens*

must be proceeded by a better understanding of the biodegradation mechanisms involved. In the present study, the azo dye Congo Red has been effectively degraded by the chosen microbial isolates in consortia than in individual treatments which clearly demonstrates the importance of microbial synergism in the dye degradation. How ever the potential of the bacterial culture needs further study for its application in treatment of real dye-bearing wastewaters using appropriate bioreactors.

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