Journal of Environmental Science and Pollution Research 2(2) (2016) 79–82

ISSN: 2455-0272

Contents List available at JACS Directory

Journal of Environmental Science and Pollution Research

journal homepage: http://www.jacsdirectory.com/jespr

Comparative Study of Decolorisation of an Azo Dye by A Single Bacterial Isolate and Soil Consortia in Defined and Complex Media

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ARTICLE DETAILS

Article history:
Received 21 April 2016
Accepted 13 May 2016
Available online 23 May 2016

Keywords:
Azo Dyes
Decolorisation
Textile Dyes
Bioremediation

ABSTRACT

The discharge of toxic effluents from various industries adversely affects water resources, soil fertility, aquatic organisms and ecosystem integrity and chronic adverse health effects. Additionally, with diminishing water resources due to rapid population growth and industrial development, reuse of municipal and industrial waste water after treatment and elimination of potential pollutants become more critical. Conventional treatment processes have inherent problems of safe disposal of sludge and additional carbon credits. Bioremediative strategies employing pure cultures of bacteria have been successfully applied for the biodegradation of toxic dye effluents. However consortial studies on this aspect are scarce. In the present study, decolorisation capacities of a pure culture *vis-a vis* bacterial consortia in defined and complex media have been studied with the view to establish their usefulness in bioremediation. The findings point to an increase in decolorisation by an order of magnitude of ten by soil consortia as compared to bacterial isolate, in defined media.

1. Introduction

The dye industry is an important sector of the chemical industry. Synthetic dyes are used extensively in textile dyeing, photography and as additives in petroleum products. Azo dyes are the largest and most versatile class of dyes and account for more than 50% of the dyes produced annually. Approximately, 40,000 different dyes and pigments are industrially presumably more than 2,000 different azo dyes are currently used over 7×10^5 tons annually worldwide [1]. Large amount of azo dyes are used in the dyeing of textiles and it has been estimated that about 10% of the dyestuff in the dyeing processes do not bind to fibres and are therefore released to the environment [2]. Azo dyes are synthetic colors that contain an azo group -N=N- as part of the structure. Azo groups do not occur naturally. Most azo dyes contain only one azo group but some contain two (disazo), three (trisazo), or more. The different, mainly aromatic, side groups around the azo bond to stabilize the -N=N- group by making it part of an extended delocalized system. This also has the effect of making many azo compounds colored, as delocalized or conjugated systems often absorb visible frequency of light. Aromatic azo compounds are usually stable and tend to produce strong vivid colors. The general formula for making an azo dye requires two organic compounds- a coupling component and a disazo component. Since these can be altered considerably, an enormous range of possible dyes are available, especially as the starting molecules are readily available and cheap. Furthermore, the simplicity of the reactions mean that the process can be scaled up or down very easily. Energy requirements for the reaction are low, since most of the chemistry occurs at low or room temperature. The economic impact is reduced by the fact that all reactions are carried out in water, which is easy and cheap to obtain and dispose off. All of these factors make azo dyes very cheap to produce.

Increasing industrialization and urbanization has led to environmental pollution. Azo dyes cause severe contamination in the river and ground water in the vicinity of dyeing industries [3]. The discharge of toxic effluents from various industries adversely affects water resources, soil fertility, aquatic organisms and ecoysytem integrity. They are important group of xenobiotic compounds and are recalcitrant in biodegradation processes. They pose toxicity (lethal effect, genotoxicity, mutagenicity and carcinogenicity) to aquatic organisms (fish, algae, bacteria etc.) as well as animals. Chronic effects of dyestuffs, especially azo dyes, have been studied for several decades. The environment and subsequent health effects of dyes released through textile industry waste water are increasingly becoming subject to scientific scrutiny.

Some azo dyes have been linked to human bladder cancer, splenic sarcomas, hepatocarcinomas and nuclear anomalies in experimental animals and there is a higher incidence of bladder cancer and Methomoglobinemia in dye workers exposed to large quantities of azo dyes [4, 5]. In mammals, both hepatic and bacterial azoreductases, reduce the azo compound to corresponding amines [6]. Bacterial azoreductases are more active than hepatic azoreductases in reducing azo dyes and are capable of converting some azo dyes to mutagenic and carcinogenic amines.

Intensive irrigation of agricultural lands with waters polluted with various industrial effluents severely affect soil fertility and susceptibility of plants to various pathogens [7]. They reduce the rate of seed germination, Chlorophyll content and growth of plants [8-12] .

Toxic compounds from dye effluent find their way via food chains and food webs to human beings causing various physiological disorders like hypertension, sporadic fever, renal damage, cramps, decrease of protein contents of muscle, liver, gill and intestine with increasing conc. of dye effluents [13] etc.

Carbohydrates represent principal and immediate energy precursor of animals when exposed to stress conditions [14]. Reduction in lipid content may be due to the utilization of lipid for energy demand under stress [15].

Naphthalene selectively accumulates in gills, liver, gut and gall bladder of the mud-sucker, *Glichthus mirabilis* and brain of the nose kill fish, *Fundulus similes.* At lethal concentrations, naphthalene primarily affects blood component while at sublethal concentration, it causes neurosensory damage and metabolic stress [16].

Glycolysis under anaerobic conditions results in the production of lactate and the rate of reaction regulated by lactate dehydrogenase (LDH). The inhibition of succinate dehydrogenase (SDH) activity in different tissues indicates depression of cellular metabolism resulting in a shift to anaerobic metabolism, resulting marked increase in LDH activity in fish. [17, 18].

The textile industry is a major user of water, starting from washing raw wood or manmade fiber production up to garment manufacturing. Waste water from textile industry is a complex mixture of many polluting substances ranging from organochlorine based pesticides to heavy metals

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Cite this Article as: Shruti Mathur, Priyanka Jain, Comparative study of decolorisation of an azo dye by a single bacterial isolate and soil consortia in defined and complex media, J. Env. Sci. Pollut. Res. 2(2) (2016) 79–82.

associated with dyes and dyeing process. With diminishing water resources due to rapid population growth and industrial development, reuse of municipal and industrial waste water after treatment and elimination of potential pollutants become more critical. Conventional treatment processes have long been established in removing many chemical and microbial contaminants of concern to public health and the environment. Methods like chemical precipitation, coagulation, adsorption and flocculation have their own disadvantages. A huge amount of sludge is formed during effluent treatment process by chemical precipitation method. This sludge is toxic and highly problematic for safe disposal. The detoxification and disposal of sludge is a problem to the textile dye units. Environmental legislation is also being imposed to control the release of azo dyes, through waste water from dyeing factory and textile industry into the environment.

Ability of microorganisms to decolorize and metabolise dyes has long been known and the use of bioremediation based technologies for treating textile waste water has attracted interest [19]. Potential microbial processes for developing feasible remediation technology to combat environmental pollution due to dye bearing waste waters are routinely worked out by several research groups [20].

The culturable fraction of the microbial population, which accounts for nearly 1% of the total microbial diversity have been exploited for the degradation of the dye and other toxic wastes. Pure cultures of bacterial isolates have been successfully applied for the biodegradation of toxic dye effluents. However consortial studies are scarce.

In the backdrop of the above deleterious effects of azo dyes coupled with their extensive industrial uses and the bioremediative potential of microrganisms, the present study aims to study degradative capacities of a pure culture *vis-a vis* bacterial consortia in defined and complex media with the view to establish their usefulness in bioremediation.

2. Experimental Methods

2.1 Chemicals

The following list of chemicals and components were used. Brilliant Red H8B (Reactive Red 31) azo dye, yeast extract, peptone, Standard protein (Bovine Serum Albumin), KH_2PO_4, Na_2HPO_4, $MgSO_4$, $FeSO_4$, $CaCO_3$, H_3BO_3, $CuSO_4$, Alkaline sodium carbonate solution(Reagent A) [20 g/L Na_2CO_3 in 0.1 mole/L in NaOH], Copper sulphate-sodium potassium tartarate solution(Reagent B) [5 g/L $CuSO_4.5H_2O$ in 10g/L Sodium potassium tartarate], Alkaline Solution (Reagent C) prepared on the day of use by mixing 50 mL of Reagent A and 1 mL of Reagent B, Folin-Ciocalteau reagent (Commercial reagent diluted with an equal volume of water on the day of use. This is a solution of sodium tungstate and sodium molybdate in phosphoric and hydrochloric acid).

2.2 Sample Collection

Soil sample was collected from the effluent discharge site of the Sanganer dyeing industry, Jaipur. The commercially available azo dye Brilliant red H8B (Reactive Red 31) was purchased from the local market and used for the present study without any further purification.

2.3 Isolation of Pure Culture

Nutrient agar media supplemented with azo dye Brilliant Red H8B at concentration of 50 mg/L was prepared. The media were sterilized at 15 lb/in^2. , 121 °C for 15-20 minutes. Dye degrading bacterium was isolated by Gradient plate technique. Nutrient agar plates of 100 mg/L dye concentration were prepared and streaked using inoculum from the gradient plate and incubated for 24 hrs at 37 °C.

Further, nutrient agar plates of increasing dye concentration were prepared viz. 500; 1000 ; 3000; 5000; 7000; 9000 ; 10,000 ; 30,000 and 50,000 mg/L and streaked using inoculum from agar plate of dye concentration 100 mg/L and incubated at 37 °C for 24 h to see the highest dye concentration on which growth occurred. An isolated colony was picked from the highest dye concentration plate and transferred to the tubes containing 3 mL of nutrient broth supplemented with the same dye concentration and incubated at 37 °C for 24 hrs. Loopful of inoculum was streaked on the nutrient agar plate of serially higher dye concentrations after incubation at 37 °C for 48 hrs at every step. The pure colony obtained at highest dye concentration was maintained on slants of highest dye concentration. All glasswares and media were sterilized at 121 °C for 15 minutes.

2.4 Determination of the (λ_{max}) of the Dye Brilliant Red H8B

An aqueous solution of dye concentration 1000 µg/mL was used to determine the maximum absorption wavelength (λ_{max}) of the dye colourimetrically. It was found that the present dye showing the maximum absorption (λ_{max}) at 520 nm (Fig. 1). The further observations has made at this wavelength for the subsequent studies.

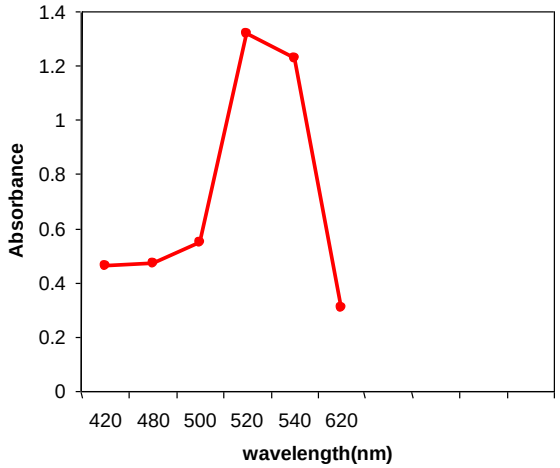

Fig. 1 Plot of absorbance v/s wavelength showing the maximum absorption wavelength (λ_{max}) of the dye at 520 nm

2.5 Determination of the Dye Concentration Present in the Soil Sample

A standard curve of varying dye concentration (1-9 µg/mL) against absorbance at λ_{max} of the dye was prepared and used to determine environmental dye concentration. About 1 g soil was suspended in 3 mL distilled water, mixed well and centrifuged. The supernatant was used to determine the concentration of dye colorimetrically from the standard curve.

2.6 Growth Studies of Isolated Bacterial Culture

The growth kinetics of the bacterial colony isolated was studied in nutrient broth media with and without dye supplementation. Growth was determined colorimetrically by observing increase in protein content. Uninoculated nutrient broth media was used as blank.

2.7 Dye Decolorisation Studies by Bacterial Isolate in Complex and Defined Media

The pure culture was grown in 50 mL nutrient broth medium at 37 °C for 24 hrs. The culture was centrifuged at 5000 g for 15 min. The culture pellet was transferred to 50 mL of broth of both complex and defined media supplemented with environmental dye concentration. This was incubated at 37 °C and 150 rpm. After every one hour interval, aliquot (5 mL) of the culture media was withdrawn, centrifuged at 5000 g and absorbance of the supernatant at different time intervals was measured at maximum absorption wavelength of the dye.

The percentage decolorisation was calculated from the difference between initial and final values per gram biomass measured as protein content. The protein content in the pellet was determined each time using Folin-Lowry's method.

2.8 Dye Decolorisation by the Soil Consortia in Nutrient Broth Medium

About 1 gram soil was added to conical flask containing 50 mL of nutrient broth medium. The dye was added at the concentration present in the effluent site of Sanganer industry. The conical flask was placed in the shaker incubator at 37 °C and 150 rpm. After every 3 h interval, aliquot (5 mL) of the culture media was withdrawn, centrifuged at 5000 g and absorbance of the supernatant at different time intervals was measured at maximum absorption wavelength of the dye. The percentage decolorisation was calculated from the difference between initial and final values. The protein content in the pellet was determined each time using Folin-Lowry's method.

3. Results and Discussion

Bacterial growth was observed upto a dye concentration of 30,000 mg/L. No colonies were seen on media supplemented with 50,000 mg/L. The bacterial colonies turned pink with the increasing dye concentration. The colonies had dark centre with light circumference and were smooth and convex in appearance (Fig. 2).

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Fig. 2 The figure shows the isolated bacterial colonies obtained on nutrient agar plate of dye concentration 30,000 mg/L

Figs. 3 and 4 reveals, the percentage decolorisation by the isolated bacterium in the nutrient broth medium without any supplementation was 1.92 % in 24 hours, whereas in the defined medium containing various mineral constituents, the dye decolourisation was 11.29 % in 24 hours. On the other hand, the dye decolorisation by the soil consortia in 24 hours was 71.69 %. It was found that dye decolorisation proceeded in concordance with growth of culture and also its looks similar in the case of defined media.

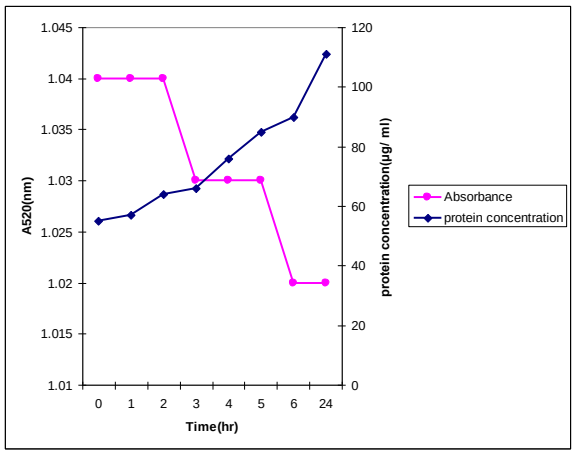

Fig. 3 Dye decolourisation and protein content of the bacterial culture at different time intervals

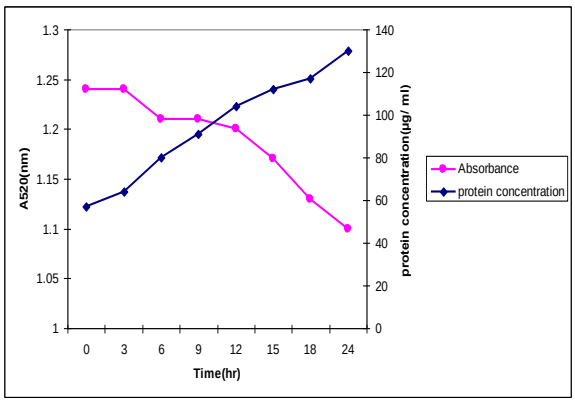

Fig. 4 Dye decolourisation and protein content of the bacterial culture at different time intervals in the defined medium

The comparative study of growth kinetics of the isolated bacterium in the presence and absence of dye revealed that the bacterium was capable of faster growth in the absence of dye than in the presence (Fig. 5).

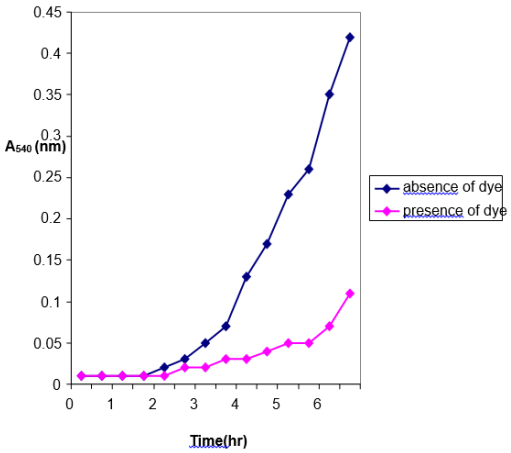

Fig. 5 A plot of A_{540} against time showing extended lag phase in case of dye supplemented media

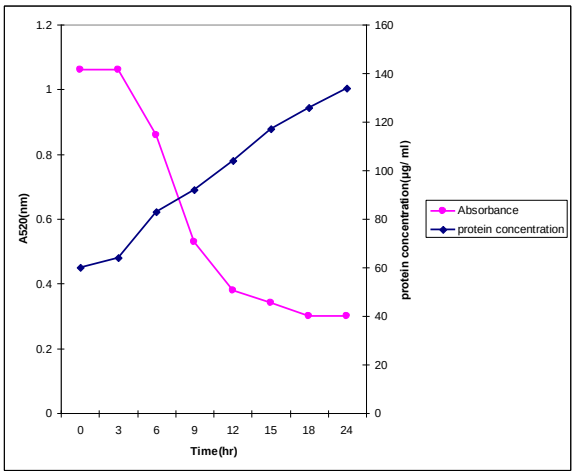

Fig. 6 Dye decolorisation and protein content of the soil consortia at different time intervals

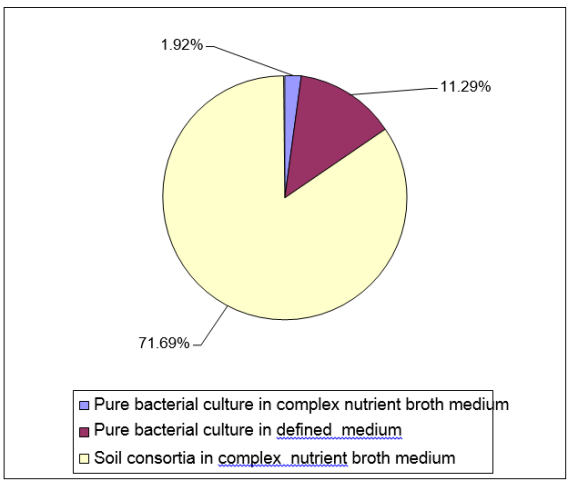

Fig. 7 Percentage dye decolorisation by the pure culture in nutrient broth medium and defined medium and by the soil consortia in complex nutrient broth medium

The study thus revealed that the dye decolorisation by the isolated bacterium was higher in the defined media as compared to complex nutrient broth medium. Also, the dye decolorisation was highest by the soil consortia than by the isolated pure culture (Figs. 6 and 7). Similar results were obtained in a study conducted by Kodam and Gawai [18].

This study reveals, for the first time, the tremendous adaptability of bacteria as seen by growth of isolate on sequentially increasing concentration of dye upto as high as 30,000 mg/L. The bacterium, having been isolated from field sample containing dye at concentrations of 182mg/L. This corresponds to a tolerance of dye of $2X10^2$ – fold higher concentrations.

The study also corroborates the fact that consortia or mixed population is far more efficient at decolorisation of the dye as shown by several

studies on mixed cultures [19]. A mixed culture of bacteria grown aerobically with 6-aminonaphthalene-2-sulhonic acid reduces the sulphonated azo dye (mordant yellow-3) [20].

White rot edible mushrooms grow efficiently on sludge and produce high level laccase and reduced the toxicity of the sludge [21]. The microbial degradation of sulphonated aromatic compounds is often accomplished by mixed cultures. A mixed culture of *Pseudomonas aeruginosa* 3MT and *Pseudomonas* sp. CP4 simultaneously and efficiently degradaed mixtures of 3-chlorobenzoate (3-CBA) and phenol/cresols. Two isolated strains BF1, BF2 of *Pseudomonas* and *P.putida* MTCC1194 have been developed for aerobic degrdadation of a mixture of seven commercial textile azo dyes in India [22]. Similarly, a mixture of bacterial isolates from domestic sewage treatment plant has been reported to be effective in decolorisation of reactive azo dyes, red RB, blue M2B and yellow. The mixed cultures could decolorize 95% of red RB and blue M2B. Decolorisation remarkably enhances when peptone is used in the medium for growing the mixed culture [23].

4. Conclusion

The present study emphasizes the fact that microorganisms may work co-metabolically and symbiotically during the decolorisation process. Also, media components accelerate the process by a factor of ten. This fact can be utilized for bioremediation strategies.

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