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# BIODEGRADATION OF AZO DYES BY MICROBIAL POPULATION ISOLATED FROM UPPER CRETACEOUS LIMESTONE ROCKS

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## ABSTRACT

The prospects of recently isolated strains from the bacterial consortium isolated from cretaceous soil were evaluated for effective biodegradation of textile dyes. High extent of decolorization was recorded when the medium was incorporated with 10 g/l of beef extract and peptone. Decolorization was strongly inhibited at non-shaken conditions. The soil was sampled from Ariyalur in Tamil Nadu, and which is famous for limestone resources.

## INTRODUCTION

Dyes, with the greatest variety of colors, have been used extensively for textile dyeing and paper printing. Approximately 10–15% of the dyes are released into the environment during and manufacturing and usage[1]. The majority of these dyes is either toxic and/or mutagenic and carcinogenic[2] and poses a potential health hazard to all forms of life. The elimination of such dye containing effluents is mostly based on physico-chemical procedures (e.g. absorption, concentration, chemical transformation and incineration). These methods are rather costly and result in hazardous byproducts and therefore other biodegradation alternatives attract attention. In the natural environment, dyes can be transformed or degraded by a variety of microorganisms, including aerobic and anaerobic bacteria and fungi[3, 4]. In contrast, bacteria could reduce the colour intensity more satisfactorily, but individual bacterial strains cannot degrade dyes completely[5, 6] and the intermediate products are carcinogenic aromatic amines, which need to be further decomposed. About 80% of color removal in effluent sample containing mixture of azo- and diazo-reactive dyes was observed by using mixed bacterial culture[7]. An isolated bacterial consortium removed upto 84% of the colour after 44 h of cultivation[8]. The present study used Amido black, Congo red, Poly red dyes for degradation by the microbial consortium from cretaceous soil taken from Ariyalur. The site is known for limestone resources and fossils. Reports have it that, the sea waters had engulfed parts of the peninsula about 100 million years ago following which clay with thin beds of sand were rhythmically formed.

## MATERIALS AND METHODS

### Chemicals

Amido black (Naphthalene disulfonic acid, disodium salt), congo red (benzidinediazo-bis-1-naphthylamine-4-sulfonic acid) and all other chemicals were purchased from Himedia, Bombay, Delhi. Stock solution of the dyes was filter sterilized and added to growth medium in the required concentration.

### Isolation of single colony

To isolate the culturable bacteria from soil, a portion of the soil samples was overlaid with sterile distilled water in a sterile falcon tube and was left undisturbed for about a week. This procedure allows the bacterial population to get concentrated in the overlaid liquid.

To separate the constituent populations, 100µl of the overlaid liquid was serially diluted ($10^{-1}$ to $10^{-10}$). An aliquot of 50µl was spread on Luria-Bertani (LB) agar (pH 7.5). Colonies obtained were selected based on morphology and were quadrant-streaked on LB agar and single colonies were isolated and inoculated in an LB broth (pH 7.2).

**Screening and selection of microorganisms**

Preliminary selection of organisms that degrade different textile dyes was done on the solid medium medium with 100 mg/l of concentration to isolate the dye decolorizing strains.

The selected cultures were inoculated in liquid minimal medium of the following composition (g/l), NaCl (5.00), Casein enzymatic hydroxylate (5.0), C6H12O6 (1.00), K2HPO4 (7.00), KH2PO4 (2.00), Na3C6H5O7 (0.50), MnSO4 (0.10), (NH4)2 SO4 (1.00) with 100 mg/l amido black, congo red, poly red. The pH of the medium adjusted to 7.5. From that the best decolorizing organism was isolated and preserved in nutrient agar slants at 158C for further use.

**Bacterial growth kinetics**

One ml of the sample was inoculated into 50ml LB broth and incubated at 37ºC in a shaker. The cultures were allowed to grow overnight. This served as the inoculum. An aliquot of 100µl inoculum was added to 30ml LB broth and kept in a shaker at 37ºC. optical density was measured at 630 nm for every 2 hours upto 40 hours.

**Biochemical characterization**

Biochemical tests were carried out to identify various metabolic properties of the different bacterial species. The tests carried out are as follows.

Indole production test

Tryptone broth containing 1g peptone in 100ml distilled water was prepared and autoclaved. 5ml of this broth was poured into a test tube. The culture was inoculated and incubated at 37ºC for 48hrs with one tube kept as control. After incubation 1ml of Kovac's reagent was added to the tubes, and the color change was recorded.

Methyl red test

MR-VP broth was prepared and autoclaved. 5ml was poured into test tubes. The culture was inoculated and incubated at 37ºC for 48hrs. Five drops of methyl red indicator was mixed and observed for the color change after 15 minutes.

Voges-Proskauer test

Sterile MR-VP broth (5 ml) was poured into a test tube. Culture was inoculated and the tubes were incubated at 37ºC for 48hrs with one tube kept as control. 40% KOH and 5% α-napthol in absolute alcohol was added into the tubes. The tubes were shaken for 30 seconds and observed for color change.

Citrate utilization test

Simmon citrate agar slant were prepared and autoclaved. The culture was inoculated and was incubated at 37ºC for 48hrs.

Catalase test

A small aliquot of the test organism was smeared on a clean slide. A drop of 3% hydrogen peroxide was added over the smear. The colour change was recorded.

**Decolorization study**

Three dyes, Amido black, Congo red, Poly red, were selected for biodegradation. falcon tubes were each filled with 20ml of broth. The tubes were mixed with 500µl of 0.1% of each dye separately followed by 1ml of pure culture and incubated in a shaker (120 rpm at 37ºC) along with a positive control.

Dye decolorization was detected by UV–Vis spectrophotometer using the supernatant from the liquid culture medium after centrifugation at 10,000 rpm for 10 min in a refrigerated centrifuge (Eppendorf 5804 R). The removal of color was reported as % decolourization (% = A0_At\A0 · 100, where A0 and At are absorbance of the dye solution initially and at cultivation time (t), respectively). Each decolorization value is a mean of three parallel experiments. Abiotic controls (without microorganism) were always included.

**Dye degradation kinetics**

One ml of the aqueous over layer was inoculated into 20 ml LB broth containing 0.1% dye. The flasks were incubated at 37ºC in a shaker. The cultures were allowed to grow overnight. This served as the inoculum. An aliquot of 100µl of the overnight culture was inoculated into 30ml LB broth and kept in a shaker at 37ºC. Sampling was done every 2 hrs for duration of 12 hours. From the samples, the decolorizing ability of the culture was determined by reading their absorbance at 493nm, 821nm and 831nm respectively. The absorbance maxima were determined by spectral scanning. According to this, the maximum absorbance of Reactive Deep Red was found to be 280nm, Diazone Green was 483nm and reactive Deep Blue was 822nm.

**RESULTS AND DISCUSSION**

**Growth kinetics of culturable population**

Four cultivable distinct bacterial populations (species) were obtained through pure culture. They were marked as AFB1, AFB2, AFB3, AFB7 and AFB8.

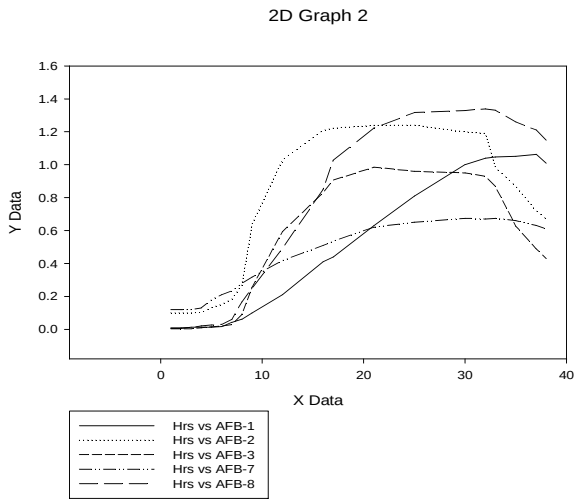


**Figure 1** Bacterial growth kinetics for AFB1, AFB2, AFB3, AFB7 and AFB8 for 40 hours.

The growth kinetics of all the four samples followed a dissimilar pattern with maximum growth at different hours which shows that AFB1 and AFB8 shows slow growth rate. (Fig. 1).

**Biochemical Characterization**

Physiological and biochemical characters of the screened bacterial strains AFB1, AFB2, AFB3,AFB5, AFB7, AFB8, were examined according to the methods described by the IMViC kit from Himedia. All the strains reduced nitrate, utilised citrate, malonate as sole carbon source, and they showed negative result for Voges Proskauer's, Methyl Red, Indole which showed no acetoin, acid production and no deamination of tryptophan respectively. In carbohydrate utilization test, AFB2, AFB3, alone hydrolysed glucose. All of the strains hydrolysed starch agar which shows the synthesis of amylase. AFB2 and AFB5 showed β-galactosidase activity. AFB3 alone produced $H_2S$. AFB2, AFB3, AFB4 were grown in MacConkey Agar and EMB agar which confirms that they are Gram negative bacteria.(Table 1)

**Table 1: Biochemical characterization**

| ***Strains*** | 1 | 2 | 3 | 7 | 8 |
|---|---|---|---|---|---|
| ONPG | - | + | - | - | - |
| Lysine utilization | - | + | - | - | - |
| Ornithine utilisation | - | + | + | - | - |
| Urease | - | - | - | - | - |
| Phenylalanine deamination | - | - | - | - | - |
| Nitrate reduction | + | + | + | + | + |
| $H_2S$ production | - | - | - | - | - |
| Citrate utilization | + | + | + | + | + |

| | | | | | |
|---|---|---|---|---|---|
| Voges Proskauer's | - | - | - | - | - |
| Methyl Red | - | - | - | - | - |
| Indole | - | - | - | - | - |
| Malonate utilization | + | + | + | + | + |
| Esculin hydrolysis | + | + | + | + | + |
| Arabinose | - | - | - | - | - |
| Xylose | - | - | + | - | - |
| Glucose | - | + | + | - | - |
| Lactose | - | - | - | - | - |
| Oxidase | + | + | + | + | + |
| MacConkey Agar | - | + | + | - | - |
| Eiosin methylene blue agar | - | + | - | - | - |

**Microbial decolorization of dyes**

Dye degradation was carried out to know if the fossil derived bacteria had the ability to utilize complex chemicals such as dyes as sole source of carbon and/or nitrogen. Three dyes were used for the investigation: Amido black, Poly red and Red M3 5. All four dyes were used at a concentration of 0.1%. Samples AFB1, AFB5 and AFB7 decolorized Red M3 5. AFB1, AFB2, AFB3 and AFB 7 performed significantly high decolorization of Poly red. Amido black was decolourised by AFB1, AFB2, AFB3, AFB7 samples. Maximum decolorization of Congo red by all the samples was observed. (Fig. 2, 3, 4) These interesting results drove us to study the kinetics of decolorization, which was carried out in a similar way as that of the growth kinetics. The decolorizing ability of each of the four samples on the dyes was noted by measuring the absorbance every 2 hours for 12 hours.

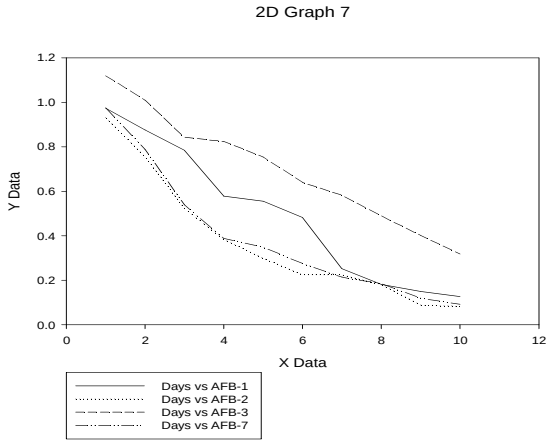


**Figure 2** Degradation kinetics of all the samples for the dye Amido black.

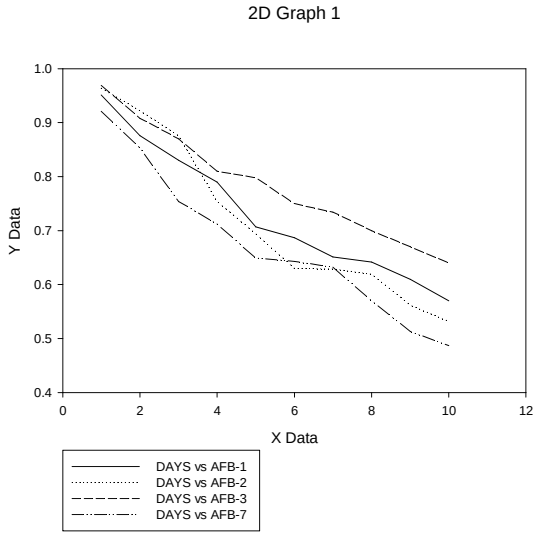


**Figure 3** Degradation kinetics of all the samples for the dye Poly red.

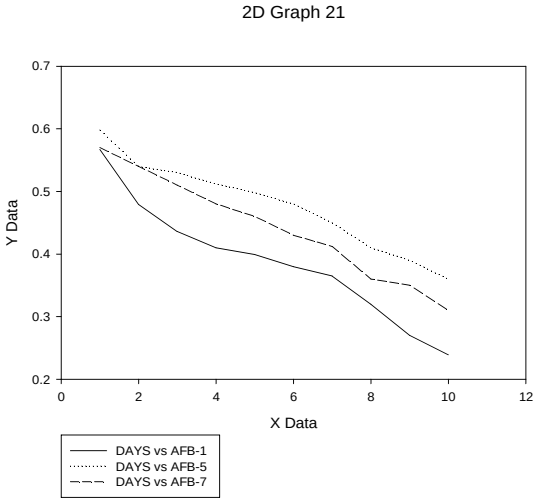


**Figure 4** Degradation kinetics of samples AFB1 AFB5 and AFB7 for the Red M5 B.

The work was started attempting to detect and bioprospect bacterial population at the site of investigation. The isolation and characterization of the cultivable bacteria was done with much ease compared to the unculturable fraction. Gram staining and biochemical tests were helped to characterize organisms. All of the four cultures gave a positive result for catalase test and are hence believed to be aerobic. Bacterial growth kinetics was performed, followed by dye degradation with pure bacterial population. This was to know if the fossil derived organisms had the ability to utilize the dye for its growth. All the four samples degraded one or the other dye. Dye degradation kinetics was also performed to understand the rate of degradation. Absorption values (OD) at respective wavelength showed the conversion of product into various by products, generally thought to be aromatic amines, along with the increasing hours.

## CONCLUSION

The soil sample was found endowed with bacterial diversity of which four populations were cultured, and subjected to biodegradation of dyes and growth kinetics. All the four populations were found to possess significant capacity for dye degradation. The future perspectives of the investigation include cloning and sequencing the amplified PCR products of metagenomic DNA and bacterial DNA so that molecular characterization of isolated colonies and evolutionary studies could be accomplished. Since the area of investigation has evolutionary significance and a vast portion of the area is yet to be investigated, intensive sequencing of the microbial genome may throw novel organisms, genes and properties. The organisms were found to degrade few dyes and the work could be extended to many other dyes. This would prove beneficial since textile effluent treatment remains a colossal problem in the state of Tamilnadu. Conventional chemical effluent treatment is highly expensive and harmful. Microbial dye degradation can be used as a safe effective alternative.

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