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Screening of Microorganisms for Azo Dye Degradation from Dye Affected Sites of Sanganer, Rajasthan, India.

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The present study deals with the screening of microorganisms for azo dye degradation. The bacteria and fungi were isolated from soil and effluent samples collected from different sites in Sanganer, Rajasthan and were screened for their degradation potential along with 14 isolates of *Aspergillus* (previously isolated from Banasthali University agricultural soil), on 3 commonly used azo dyes, viz., congo red, methyl orange and methyl red. Eleven bacterial isolates (5 Gram positive and 6 Gram negative) and 4 fungal isolates (2 *Aspergillus* sp. belonging to *niger* group, *A. ochraceous* and *A. flavus*) obtained from the dye affected soil and effluent samples and *A. niger, A. japonicus, A. aculeatus, A. foetidus* and *A. purpurogenum* from the agricultural soil showed dye decolourization activity. Bacterial isolates 2 and 4 showed 83-94% degradation, whereas, fungal strains *A. japonicus* and *A. aculeatus* showed 95-98% of decolourization of all the 3 dyes studied. The activity shown by the referred bacterial and fungal strains was more than *Pseudomonas putida*, the reference strain. The dye removal was achieved by both biodegradation as well as bisorption processes. In general, fungal isolates were more efficient dye degraders than bacterial isolates.

Key words: Azo dye, Sanganer, congo red, methyl orange, methyl red, biodegradation.

Dyes are released into the environment from two major sources, the textile and the dyeing industry effluents[1,2]. A necessary criterion for the use of these dyes is that they must be highly stable in light and during washing. They must also be resistant to microbial attack. Therefore, they are not readily degradable and are typically not removed from water by conventional wastewater treatment systems[3,4].

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Pollution of natural water bodies with waste effluents arising from various industries has become a serious problem in India. In Rajasthan, particularly in Sanganer, textile mills represent an important economic sector[5]. Sanganer is the center for 500 years old, world famous Sanganeri print, known for its exquisite hand block printed fabrics, providing employment to about 3,000 families. A survey conducted by National Productivity, New Delhi and German Technical Cooperation few years back stated that the industry uses about 17 million liters of water per day and about 75% of the effluent is discharged into the Gullar dam near the township. The effluent water released during industrial processes is untreated and discharged directly into Gullar dam. Further, the effluents

sediment in the drainage bed can percolate down and contaminate underground water. The situation further aggravates as seasonal vegetables are grown in the surrounding area using this drainage water[5]. Poor artisans who produce world-class handblock prints are being forced to move to the industrial area following a High Court directive to shift all polluting units from sanganer. Therefore, steps should be taken to protect the environment and the people.

Several methods are used in the treatment of textile effluents to achieve decolourization. These include physico-chemical methods such as filtration, specific coagulation, use of activated carbon and chemical flocculation. Some of these methods are effective but quite expensive[6,7]. Biotreatment offers a cheaper and environmentally friendlier alternative for colour removal in textile effluents. The ubiquitous nature of microbes makes them invaluable tools in effluent biotreatment[7-11]. The chemical nature of dyes varies, but azo dyes are the most widely used in textile industry[7]. Azo dyes represent a major group of dyes causing environmental concern because of their colour, biorecalcitrance and potential toxicity to animal and human[12] and their decolourization has been found to be effective under anaerobic conditions[13,14,15]. However, the anaerobic degradation yields aromatic amines which are mutagenic and toxic to humans and cannot be metabolized further under the conditions which generated them. In activated sludge treatments of dye effluents, reactive azo dyes and aromatic amine derivatives are a non-biodegradable class of compounds which can even inhibit activated sludge organism[16]. It is thus important to explore the possibilities of isolating efficient aerobic degraders for use in decolourization and biotreatment of textile effluents.

To exploit the biodegradation abilities of indigenous microbial flora, the microorganisms from dye affected area of Sanganer were isolated and screened to test their ability to decolourize the most commonly used azo dyes. In the present work, we have demonstrated the aerobic biodegradation of three commonly used azo dyes, viz., congo red, methyl orange and methyl red by bacteria and fungi isolated from the agricultural soil and Sanganer industrial soil and effluent.

MATERIAL AND METHODS

Study site and collection of samples

Sanganer is located about 60 Km from the Banasthali University Campus (latitude 25º41' N to 26º34' N and longitude 75º70' to 76º19' E). The soil and effluent samples were collected from different sites in Sanganer where generally the dyes and effluents are disposed off. Sampling was done taking all possible aseptic measures and stored at 4ºC. The samples were processed for isolation of the bacteria and fungi and were screened for decolourization potential.

Isolation and purification of bacteria and fungi

Isolation of bacteria and fungi from soil and effluent samples was done by (standard) serial dilution method. Potato Dextrose Agar (PDA) and Czapek's Dox Agar (CDA) media containing antibiotic (ampicillin @ 50 µg/ml of the media) were used for the isolation fungi, whereas, Luria Agar (LA) and Nutrient Agar (NA) media containing Nystatin were used for the isolation bacteria. All the plates were incubated at 30±2ºC. Well isolated fungal and bacterial colonies were further streaked on CDA and LA plates, respectively to obtain pure strains and were stored on the same media as slants at 4°C for further studies. Tentative identification for fungi was done with the help of literature[17], whereas; bacterial isolates were differentiated on the basis of their morphological characteristics and Gram's staining. Fourteen isolates of *Aspergillus* which were previously isolated from Banasthali University agricultural soil were also screened for their azo dye degradation potential.

Azo dyes

Three different azo dyes viz., congo red, methyl orange and methyl red, which are commonly used in various dying industry were selected for the study. Congo red and methyl orange dyes were prepared in sterile distilled water at 1 mM concentration, whereas, methyl red was dissolved in minimum amount of methanol and then volume was adjusted with the water at 1 mM concentration and were then sterilized by passing through 0.22 µ Millipore filter. The ë max of congo red, methyl orange and methyl red was determined spectrophotometrically (Systronics UV-VIS Spectrophotometer 119, Naroda, Ahmadabad, India).

Determination of decolourization on solid media

All fungal and bacterial isolates were point inoculated on CDA and LA media respectively. The media contained 100 ìM of each of the three dyes (1 mM stock was used) separately. Autoclaved, uninoculated medium (containing dye) served as a control. Plates were incubated at 30±2ºC. The dye decolourization was observed after 4-6 days of incubation.

Assay of dye decolourization by bacteria

Eleven bacterial isolates (showing decolourization on LA plates) were selected for the study. Starter cultures were prepared by inoculating single isolated bacterial colony in 10 ml of Luria Broth (LB) medium and incubating for 24 hrs at 30±2ºC and 120 rpm in an incubator-shaker (Metrex, MO-250ºC, Mercantile Engineers, New Delhi, India). One ml of starter culture was inoculated in 100 ml of the LB medium, incubated for 24 hrs at 30±2ºC and 120 rpm. Dye was added at a final concentration of 100 ìM (1 mM stock was used) and incubated under similar conditions. Autoclaved, uninoculated medium (containing 100 ìM dye) served as a control. Culture samples were harvested aseptically after every 24 hrs starting from the addition of dye till the 10^{th} day of incubation. Samples were centrifuged (Remi, Cooling Compufuge, CPR 24) at 6600 rpm (rotor no. 2, R242) for 15 min. The absorbance of supernatant was taken at the absorption maxima of each dye to determine the dye disappearance. Dye decolourization assay for the reference strain *Pseudomonas putida* (MTCC 1194, Chandigarh, India) was also carried out under similar conditions along with the other isolates.

Assay of dye decolourization by fungi

Nine fungal isolates (showing decolourization on CDA plates) were grown on CDA plates under similar incubation conditions as described previously. After the sporulation has taken place, the fungal spores were transferred to sterile distilled water and were counted by the hemocytometer. 10^4 spores/ml were inoculated in 100 ml of CD broth. It was allowed to grow for 48 hrs at 30±2ºC and 120 rpm. The dye was added to pregrown culture and was incubated again under similar conditions. Harvesting of the culture samples and determination of dye decolourization were done as described previously for the bacterial isolates.

Residual dye analysis

10 d old bacterial and fungal cultures (grown in presence of the dye) were harvested aseptically as described previously. Three ml of supernatant was extracted with equal volume of 1-butanol. The phase separation was observed. Phase containing the dye was pipetted out and absorbance was taken at respective wavelengths.

Analysis of the dye associated with bacterial pellet

After 10 days of incubation with dye, some amount of the dye remained adsorbed with bacterial cells. In an attempt to solubilize any bound dye, 1.5 ml of bacterial culture was taken and centrifuged at 6600 rpm for 15 min. Supernatant was removed and 2 ml of methanol was added to the pellet. It was centrifuged again and absorbance of the supernatant was noted.

Analysis of the dye associated with fungal mycelia

After 10 days of incubation with dye, the mycelial mat was separated from the media and homogenized in 10 ml of methanol. The homogenate was centrifuged at 2000g for 5 min, supernatant was collected in a separate tube and mycelial mat was resuspended in additional 5 ml of methanol and centrifuged. The two resulting supernatants were mixed and the absorbance was measured.

RESULTS AND DISCUSSION

Biodegradation is cost effective and environmental friendly approach. Under aerobic conditions the azo dyes are non-degradable by most of the bacteria and the isolation of microorganisms which use dye as a sole source of carbon is proved to be difficult[18]. In the present study, a total of 11 bacterial and 4 fungal isolates showing dye degradation potential were obtained from dye affected soil and effluent samples. The bacterial cultures were distinguished only on the basis of colony morphology and Gram staining and were named as isolate no. 1-11. Out of 11 bacterial isolates 5 were Gram positive and 6 Gram negative. Fungal isolates were identified with the help of the manual available and were found to belong to the genera *Aspergillus*, 2 species belonged to the *niger* group, whereas, the other 2 were *A. flavus* and *A. ochraceus*. Bacteria mediated azo dye degradation activity has been variously reported in *Pseudomonas*, *Enterobacter* and *Morganella*

sp[19]; *Escherichia coli* NO_3[20] and *Citrobacter sp*[21]. Fungi such as *Phanerochaete chrysosporium*[22,23,24] and white rot fungus *Phlebia radiate*[25] have also been found as efficient azo dye degraders. More recently, it has been shown that not only *P. chrysosporium* but also several other fungi, viz., *Geotrichum candidum*, *Trametes versicolor*, *Bjerkandera adusta, Penicillium* sp., *Pleurotus ostreatus, Pycnoporus cinnabarinus, Pyricularia oryzae*[11] and members of *Aspergillus* group such as *A. ochraceous*[26] and *A. niger*[27] are able to decolorize rather complex azo dyes.

Percent decolourization of congo red and methyl orange by the bacterial isolates is shown in Fig.1. The dye degradation started within few hours of addition of growing bacterial cells, although the pattern of decolourization was different in different isolates. The isolate number 7 showed near to maximum decolourization of congo red after 1 day of growth in contrast to the isolates 1,5,9 and 10,

Table 1. Action of bacterial isolates on various azo dyes after ten days of inoculation

Bacterial isolates	% dye decolourization*			% of dye associated with the bacterial pellet			% of dye associated with the supernatant		
	CR	MO	MR	CR	MO	MR	CR	MO	MR
1.	83	38		0.2	0.3		13.9	61.6	
2.	86	94	88	0.0	0.2	0.2	6.2	2.8	7.23
3.	78	49		0.3	0.4		13.2	43.1	
4.	83	90	91	0.2	0.2	0.3	16.4	13.1	5.12
5.	88	32		0.4	0.4		9.0	66.0	
6.	78	92		0.5	0.2		22.6	15.7	
7.	68	58		0.5	0.2		24.5	43.4	
8.	79	70		0.3	0.3		9.4	26.8	
9.	84	83		0.2	0.3		5.7	22.0	
10.	85	29		0.1	0.3		4.1	70.3	
11.	70	38		0.4	0.3		8.9	58.7	
Pseudomonas putida (MTCC 1194)	90	36	29						

*only maximum values have been given. (CR Congo red; MO Methyl orange; MR Methyl red)

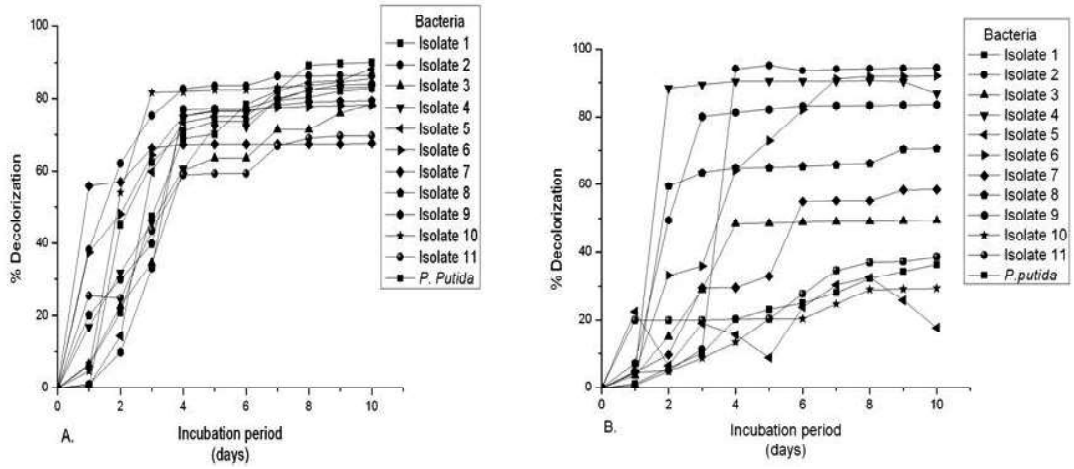

Fig. 1. Percent decolourization of Congo red (A) and Methyl orange (B) by different bacterial isolates on various days after inoculation

that could decolourize negligible amount (less than 5%) in the same time; though it increased in subsequent days (Fig.1 A). When pattern of methyl orange decolourization is compared, a different pattern is observed (Fig. 1B and Table 1). The bacterial isolates 4,5,8 and 9 carried maximum decolourization (89%, 23%, 64% and 80%, respectively) within 1-3 days of growth in contrast to the rest of the isolates that could carry only 8-30% methyl orange decolourization in the same time. Although few bacterial isolates (2 and 4) were capable of degrading methyl red dye (Table 1 and Fig. 3), but the results were quite encouraging since they showed 88-91% decolourization in comparison to the poor activity shown by the reference strain, *Pseudomonas putida* (29%). Overall, there was no fixed pattern of decolourization and such phenomenon is due to the different metabolic rates of different bacterial isolates. It is generally seen that the bacterial strains capable of degrading azo dyes show strict specificity towards the single dye to which the

Table 2. Action of fungal isolates on various azo dyes after ten days of inoculation.

Fungal isolates	% dye decolourization*			% of dye associated with the fungal mycelia			% of dye associated with the supernatant		
	CR	MO	MR	CR	MO	MR	CR	MO	MR
A. foetidus	95	84		95.1	10.3		2.6		13.2
A. purpurogenum	95	84		17.8	2.5		0.1	5.9	
A. niger	96	94		17.4	10.7		1.6	1.2	
A. japonicus	96	98	99	32.4	0.8	0.0	0.1	0.1	0.0
A. aculeatus	98	98	99	11.9	0.2	0.0	0.5	0.0	0.0
Aspergillus species (niger group) 1**	97	92		17.2	2.9		0.4	5.0	
A. ochraceus**	84	88	98	13.5	56.5	10.2	14.0	18.7	6.0
A. flavus**	85	81		14.0	10.2		14.0	15.0	
Aspergillus species (niger group) 2**	97	80		17.0	3.5		1.2	17.0	

*only maximum values have been given. ** Isolated from Sanganer soil
(CR Congo red; MO Methyl orange; MR Methyl red)

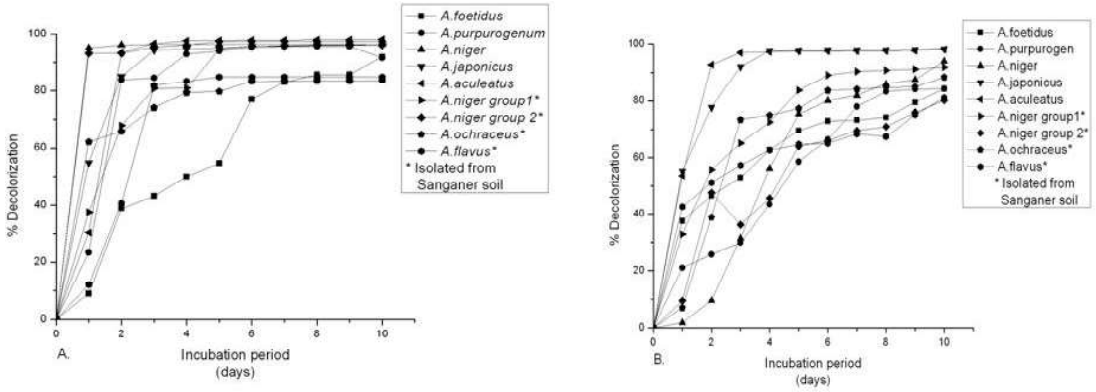

Fig. 2. Percent decolourization of Congo red (A) and Methyl orange (B) by different bacterial isolates on various days after inoculation

organism has been adapted[18,28]. In our study, although all the strains showed some amount of decolourization of all the 3 dyes, but isolates 2 and 4 were efficient among all in their action on all the 3 dyes studied (Table 1 and Fig. 3). Also, percent decolourization of congo red by these isolates are at par with the reference strain, *P. putida*, and interestingly methyl orange decolourization was also very high in the medium inoculated with isolates 2 and 4 (94% and 90% respectively) in comparison to the reference strain (36% only).

Percent decolourization of the three dyes by fungal isolates was also studied (Fig. 2,3 and table 2). On the whole fungi were better degraders than bacteria. Only a few aerobic bacteria have been found to reduce azo dyes under aerobic conditions, and little is known about the process[24]. On an average, all the dyes were efficiently decolourized (>80%) by fungal isolates in comparison to the bacterial strains which showed variable performance starting from very low value (30%) to high value (90%). Surprisingly, maximum decolourization of congo red and methyl red by

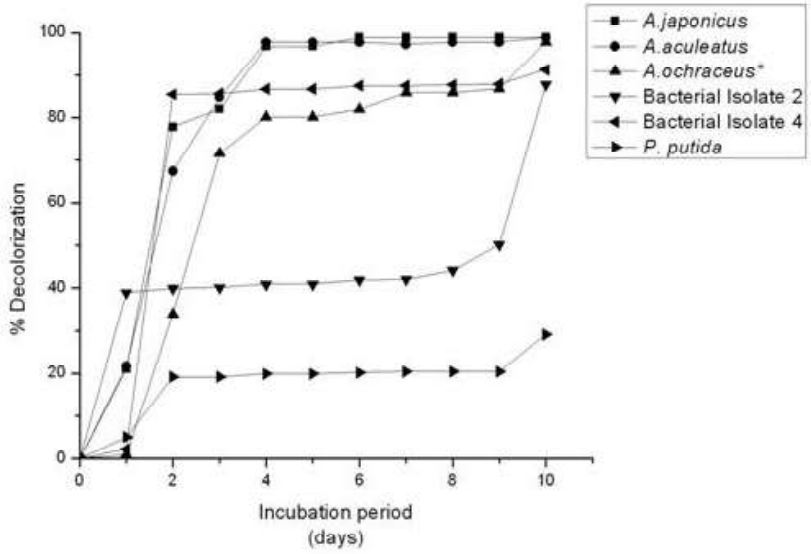

Fig. 3. Percent decolourization of Methyl red by few selelcted fungal and bacterial isolates on various days after inoculation

most of the fungal strains occurred within 4 days of incubation and this attribute is very important from commercial point of view. On the other hand, the incubation period for maximum decolourization of methyl orange varied. Out of the 14 local isolates of *Aspergilli*, 5 species showed decolourization on solid as well as in liquid media. It is evident from the results that both *A. aculeatus* and *A. japonicus* are highly efficient in decolourizing all the three forms of dyes used in the study (96-99%). 93% decolourization of congo red has been reported in 5 days by *P. chrysosporium*, a well known degrader of azo dyes[29]. Three of the fungal strains studied viz., *A. niger, Aspergillus* sp. and *A. aculeatus* showed 93-96% decolourization of the same dye on 2nd day of the study. Again this finding may be important from commercial viewpoint.

The percentage of congo red, methyl red and methyl orange associated with bacterial cells and fungal mycelia is given in Tables 1 and 2, respectively. In general, less amount of congo red was found associated with the bacterial pellet in comparison to the fungal mycelia, thus indicating that bacterial isolates removed this dye mainly by degrading it in contrast to the fungal cells (except *A. foetidus*) which carried out the dye removal by both biodegradation as well as biosorption. The

colour removal due to biodegradation has also been reported in *Kurthia sp*[30] and *Proteus mirabilis*[31]. The maximum percentage of congo red was found in the mycelial mat of *A. foetidus* which shows that the dye was removed mainly by the biosorption process. *A. japonicus* and *A. aculeatus* mycelia contained negligible amount of methyl orange and methyl red, indicating that they removed both the dyes chiefly by biodegradation process.

A few fungal (*A. japonicus, A. aculeatus* and *A. ochraceous*) and bacterial (isolates 2 and 4) strains isolated during the study are very good degraders of congo red, methyl orange and methyl red dyes. Further studies are in offing to enhance the dye degradation and biosorption process in both bacterial and fungal systems.

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