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ORANGE-G TOLERANCE, UTILIZATION AND DEGRADATION POTENTIALS OF NATIVE BACTERIAL ISOLATES

C. Mariappan*, T. V. Gayathri Devi, R. L. Yamuna, R. Palaniappan[1] and T. Selvamohan

*Post Graduate Department of Microbiology, Sivanath Aditanar College
Pillayarpuram - 629 501 (India)
[1]Post Graduate Department of Microbiology, Sri Paramakalyani College
Azhwarkurichi - 627 412 (India)

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ABSTRACT

Soil and sediment sample obtained from Orange-G dye contaminated habitat was screened for heterotrophic bacterial population. Consistently, high heterotrophic bacterial density was recorded to the tune of 10^7 CFU/g. Six isolates comprising of three *Pseudomonas* sp. two *Escherichia* sp. and one *Bacillus* sp. were checked for Orange-G tolerance and utilization. All the bacterial strains were found to resist the azo dye, Orange-G up to 75 ppm. Above this concentration, only *Pseudomonas* sp. SACO3, *Bacillus* sp. SAC01 and *Escherichia* sp. SAC01 were able to tolerate. All the test bacterial strains were found to utilize Orange-G as a sole carbon and / or nitrogen source with distinctive decoloration. While the pH optima of these strains ranged from 8 to 10, their temperature optima was 37°C except for *Escherichia* sp. (44°C). In accordance with the abundant occurrence in the soil ecosystem, *Pseudomonas* sp. were found to decolorize orange-G more effectively that other strains.

KEY WORDS: Azo dyes, Orange-G, Decoloration, *Pseudomonas* sp., *Bacillus* sp., *Eschericia* sp.

INTRODUCTION

The textile industry in India is one of the oldest and largest industry in the country. These mills require volumes of water of high purity and generate equally large volumes of wastewaters, which is highly colored and chemically complex. The textile mill effluent contains appreciable concentration of pollutants. The BOD and TSS can be controlled by biochemical treatment (Sharma *et al.,* 1999). Color removal from textile effluent is a major environmental problem because of the difficulty in treating such enormous quantity of water (Sharma *et al.,* 1999)

Triphenylmethane and azodyes are used extensively in textile industry for dying nylon, wool, silk and cotton (Mali *et al.,* 1999). Azodyes constitute the largest group of synthetic dyes with great deal of structural and colour variation (Cripps *et al.,* 1990). Azodyes are potential mutagens and carcinogens, which necessitates their proper degradation and safer disposal (Vyas and Molitoris, 1995). Microbes can degrade azodyes both aerobically and anaerobically. Decolorization of the dyes usually occur during secondary metabolism, was suppressed at high nitrogen levels, and was dependent on the concentration of atmospheric oxygen (Platt *et al.,* 1985). Azodyes' aromatic moieties is linked together by azo (-N=N) chromophores, with different auxochromes *viz.* NH_2, NR_2 OH. The release of these compounds into the environment is undesirable, not only because of the principle compound but also their breakdown products are toxic and / or mutagenic (Van der zee, 2000).

Orange-G is a monoazo dye and is sparingly in water (10.86%). This is a valuable acidic dye used in many staining methods including Papani color's 0G6 stain. It is often combined with other yellow dyes in alcoholic solution to stain erythrocytes in trichome methods and is used for demonstrating cells in the pancrease and pituitary (William *et al,* 1998).

Realizing their wide spread usage and subsequent risk involved as a constituent of the industrial effluent, this preliminary investigation was taken pu. In this attempts were made to

assess the dye contaminated soil ecosystem bacteriologically and the residual bacterial strains were examined for their dye resistant and decoloration characteristics.

MATERIALS AND METHODS

Sampling site

Soil samples were collected aseptically from five different azo dye contaminated sites in Rajapalayam and Nagercoil, Tamil Nadu.

Sample collection

Sampling was carried out a weekly interval from the dye contaminated site. Soil sample from a visibly polluted area with textile effluent was aseptically collected in a pre-sterilized 250 ml conical flask. The sample was placed in an ice box maintained at 4°C and was brought to the laboratory for bacteriological analysis within 4-6 hours.

Enumeration of Total Heterotrophic Bacterial Population (THBP) in the soil sample

One gram of dye contaminated soil sample was serially diluted using 9 ml sterile saline and the dilution was made upto 10^{-8}. From this, 1 ml of diluted soil sample was poured into sterile petridish to which 20 ml of the sterile nutrient agar was added aseptically and mixed well. After solidification, the petriplates were incubated at 37°C for 24 hours. The plates containing enumerable bacterial colonies were selected and the THBP determined.

Isolation of Orange-G tolerant bacterial isolate

The dye contaminated soil sample was enriched in Orange-G (10 ppm) incorporated nutrient broth and kept in an orbital shaker (37°C) for five days. One ml of this culture was taken, serially diluted and pour plated on air-dried nutrient agar medium. The plates were incubated at 37°C for 24 hours. Morphologically different colonies were selected from the plates purified by quadrant streaking on air-dried nutrient agar medium and stored in nutrient agar slant.

Identification of Orange-G resistant bacterial isolates

Pure cultures of Orange-G resistant soil bacterial isolates were identified upto generic level on the basis of their microscopic, biochemical and physiological characteristics (Aiso and Simudo, 1962). For this, Bergey's manual of systemic bacteriology and the Prokaryotes were also referred.

Dye resistant pattern of the native bacterial isolates

Mineral slat agar (MSA) (KH_2PO4- 2.38g; K_2HPO4- 5.65g; NH_3SO_4- 22.64g; $MgSO_4$- 1g; Glucose- 0.15g; Urea- 0.1g; Agar- 15g; pH - 7; Distilled H_2O - 1000 ml) plates were prepared with varying concentration of the chose azodyes, Orange-G (25 ppm, 50 ppm, 75 ppm and 100 ppm). Fifty three soil bacterial isolates were saline washed and the saline suspension of them was streaked on the air dried dye incorporated MSA plates.

Dye utilization assay (qualitative)

To determine the ability of the Orange-G tolerant strains to utilize the chosen dye, plate assay technique was employed. For this, MSA without carbon (C) or nitrogen (N) source was prepared and the Orange-G was incorporated into this medium at 75 ppm concentration. Also MSA was prepared with Orange-G either as the sole C and N source (50 ppm) or with C and N supplementation. On these plates, saline washed suspension of the chose bacterial isolate was single streaked and its ability to utilize the dye namely Orange-G was recorded.

Optimization studies

Determination of temperature optima-

Nine ml of sterile MS broth was inoculated with one ml of saline washed bacterial culture and incubated at different temperatures (30°C, 37°C, 44°C). The OD_{595} was taken at different intervals (24 hours, 48 hours, 72 hours, 96 hours) in a spectrophotometer (Systronics 118) with uninoculated medium as the blank.

Determination of pH optima-

The pH of the MS broth was adjusted to 6, 8, 10 with 1N HCl or 1N NaOH and one ml of saline washed bacterial culture was inoculated and incubated at their respective optimal temperature. The OD_{595} was taken at different intervals (24 hours, 48 hours, 72 hours, 96 hours) in a spectrophotometer (Systeronics 118) with uninoculated medium as the blank.

Dye decoloriztion assay (qunatitative)-

MS broth (50 ml) incorporated with 150 ppm Orange-G dye was taken aseptically in a 100 ml conical flask, inoculated with the dye utilizing

soil bacterial isolate (saline suspension) and incubated under optimized conditions. An aliquot (5 ml) of the broth was withdrawn at different intervals (24, 48 and 72 hours) and centrifuged (10,000 rpm for 10 minutes). The supernatant was taken and the OD was recorded in a spectrophotometer (Systronic 118) with uninoculated medium as the blank.

RESULTS AND DISCUSSION

Coloring agent has become the integral part of human development. Apart from contributing aesthetics sense, dyes also provide multiple applications, which includes scientific and other fields. Most of the coloring agent used in the textile industry are azodyes that have characteristic -N=N, which is stubborn molecule that resist natural degradation. Probably, this stubbornness makes these dyes as choice coloring agent in most industries. On the contrary, contamination of these dyes and their subsequent accumulation in an ecosystem pose serious threat to the environment. While the recalcitrant dye and its metabolites alter the soil porosity, and chelate with ions, they were also observed to exert negative impact on the soil micro and macro flora.

Azo dyes constitute the largest group of synthetic dyes with a great deal of structural and color variety (Cripps *et al,* 1990). They are extensively used in textile, leather, food, cosmetics, pharmaceuticals and paper industries and eventually more than 7×10^5 tons of these dyes are produced annually world wide. Realizing the importance of selecting suitable bacteria for dye degradation and also to understand the impact of the dye industry effluent on soil microflora, textile mill effluent contaminated soil was enumerated and the residual bacterial load was recorded to be in the range of 10^7 CFU/g (Table 1). Appreciable THBP in a soil ecosystem inspite of continued exposure to azodyes is suggestive either of the enrichment of dye degradable population or the dilution of the dye, which had eventually lowered the toxicity of the dye. Sudhakar *et al,* 2002 had reported similar load of THBP in sediment samples collected from a dye contaminated habitat.

From THBP, 6 strains were chosen on the basis of their morphological dissimilarity and their generic identity was determined by employing standard bacterial identical techniques. As it is well known, soil is predominantly inhabited by the gram negative *Pseudomonas* sp. and gram positive *Bacillus* sp. Bacteria belonging to these genera are known for their abilities to utilize the variety of simple and complex nutrients and hence could survive even in hostile conditions. While *Pseudomonas* sp. by virtue of the presence of a wide variety of extra chromosomal genetic material survive environmental hostilities, *Bacillus* sp. has the ability to switch to dormancy in the event of physical, chemical or biological threats. Hence, occurrence of bacteria belonging to the genera of *Pseudomonas* and *Bacillus* in the dye contaminated soil is rather an anticipated one. But isolation of an enteric organism, *Escherichia* sp. is suggestive of the probable contamination of domestic sewage in the textile effluent that might have contributed to the entry of this strain.

As a presumptive to decolorization experiments, the chose bacteria were examined for their ability to tolerate the dye namely, Orange-G. Even though all the isolates were able to resist the stain up to 75 ppm, only three strains namely, *Bacillus* sp. SAC01, *Escherichia* sp. SAC01 and *Pseudomonas* sp. SAC01 were found to tolerate Orange-G even at high concentration of 100 ppm. The inability of the native isolate to tolerate high concentration of Orange-G confirms the possible negative effect of the accumulation of the Orange-G on the native micro flora. Further, these strains were examined for their ability to utilize orange-G as sole nutrient, which could indicate their degrade potentials. Examining the data presented in Table 3 it could be inferred that the chosen soil bacterial isolates not only have ability to utilize Orange-G as the carbon or nitrogen source, but also as a sole carbon and nitrogen source. Their ability of dye utilization was evident from the appearance of zone of decoloration around the growth. Sudhakar *et al* (2002) had reported

Table-1 : Total heterotrophic bacterial load in textile mill effluent exposed soil

Sampling Area	Bacterial load (CFU/gm)
Site 1	32×10^7
Site 2	38×10^7
Site 3	20×10^7
Site 4	13.2×10^7
Site 5	20×10^7

Table-2 : Orange-G tolerance pattern of native bacterial isolates

Test isolates	Growth Dye concentration (ppm)				
	10	25	50	75	100
Pseudomonas sp. SAC01	+	+	+	+	-
Bacillus sp. SAC01	+	+	+	+	+
Escherichia sp. SAC01	+	+	+	+	+
Pseudomonas sp. SAC02	+	+	+	+	-
Pseudomonas sp. SAC03	+	+	+	+	+
Escherichia sp. SAC02	+	+	+	+	-

+ = Presence of growth; - = Absence of growth

Table-3 : Qualitative analysis of chosen bacterial isolates for Orange-G utilization (Dye concentrtion 75 ppm)

Test isolates	As a sole 'C' source	As a sole 'N' source	With 'C' and 'N' supplementation	Without 'C' and 'N' supplementation
Pseudomonas sp. SAC01	+*	*+	+*	+*
Bacillus sp. SAC01	+	+	+	+
Escherichia sp. SAC01	+*	+*	+*	+*
Pseudomonas sp. SAC02	+*	+*	+*	+*
Pseudomonas sp. SAC03	+*	+*	+*	+*
Escherichia sp. SAC02	+*	+*	+*	+*

+ = Presence of growth; + = Presence of growth with zone of utilization.

similar concentration dependent tolerance and utilization of azo dyes by the native bacterial strains such as *Pseudomonas* sp.

In order to employ these organisms for Orange-G decolourization, their growth conditions were optimized. In spite of having co-existed in the effluent contaminated habitat, a sharp difference in pH and temperature optima was found among the isolates. While *Pseudomonas* sp. SAC01 required 37°C and pH 8 at its optima, *Bacillus* sp. SAC01 required pH 10. Even though *Escherichia* strains were found to prefer pH 8 for their growth, *Escherichia* sp. SAC02 grew maximally at 44°C. In fact this observation is suggestive of its faecal origin. While *Pseudomonas* sp. SAC02 grew comparably well at both 37°C and 44°C in alkaline pH *Pseudomonas* sp. SAC03 preferred more neutral pH and 30°C for its maximal growth.

The metabolic potentials of the members of *Pseudomonas* sp. was clear in the present study. Of the three *Pseudomonas* strains examined, two strains namely, *Pseudomonas* sp. SAC03, *Pseudomonas* sp. SAC01 had exhibited extremely high potential in the decolourization (92.93% and 91.14% respectively) of orange G (Table 4). Zimmermann *et al* (1982) recorded such potentials in *Pseudomonas* KL46 with respect to Orange II. Observations of Coughlin *et al* (1997) regarding the aerobic degradation of azodye correlates with the data presented in this study with respect to the rate and percentage of dye degradation by the chosen bacterium. Even though *Escherichia* sp. is observed to be a very good dye degrading strain, its enteric origin discourages its

Table-4 : Rate of decoloration of Orange G by the native bacterial isolates (75 ppm)

Test isolates	Percentage (%) of decoloration			Overall
	48 hrs	72 hrs	96 hrs	percentage (%)
Pseudomonas sp. SAC01	34.4	44.26	76.47	91.4
Bacillus sp. SAC01	39.22	35.48	15.0	66.67
Escherichia sp. SAC01	27.63	45.45	66.66	86.84
Pseudomonas sp. SAC02	19.69	26.41	66.66	80.30
Pseudomonas sp. SAC03	35.35	50.00	78.12	92.93
Escherichia sp. SAC02	38.18	29.41	58.33	81.82

use in bio-remedial application. Surprisingly, *Bacillus* sp. despite of being popular in pollution management had very low efficiency in Orange-G decolourization in the present study.

The present investigation clearly demonstrates the relevance of native bacteria in bio-remedial applications. Further research on the analytical aspects of dye degradation, the fate of metabolites and molecular aspects would make it possible to assemble bacteria for the bio-remediation of Orange-G polluted habitat.

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REFERENCES

1. Aiso K and Simudu V, *Bull Jap Soc Sci Fish,* **28,** 1133 (1962)
2. Coughlin M F, Kinkle B K, Teeper K and Bishop P L, *J Water Sci Technol,* **36,** 215-220 (1997)
3. Cripps C, Bumpus J A and Aust S D, *J Appl Env Microbiol,* **64**, 1114-1118 (1990)
4. Mali P L, Mahajan M M, Patil D P and Kulkarni M V, *J Sci Ind Res,* **59,** 221-224 (1999)
5. Platt M W, Udar Y and Chet H, *J Appl Microbiol Biotechnol,* **21,** 394-396 (1985)
6. Sharma P, Markendey D K and Kaur A, *J. Environ. Poll. Cont.,* 5-8, (1999)
7. Sudhakar P, Palaniappan R and Gowrisnkar R, *Asian J Microbiol Biotechnol Environ Sci,* **4**(2), 203-208 (2002)
8. Van der Zee F P, *Ph.D. Thesis,* Wageningen Univesity, The Netherlands (2000)
9. Vyas B R M and Molitoris H P, *J Appl Env Microbiol,* **69**, 3919-3927 (1995)
10. William L, Wilkins R D and Baltimore M, *Classification of azodyes,* Net download (1998)
11. Zimmermann H, Kulia H G and Leisinger T, *Int J Biochem,* **129**, 197-203 (1982)