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Isolation ond Identification of A Bacterial Consortium For Biodegradation of a Textile Dye Reactive Orange M2R and Study of Phytotoxicity of the Degraded Product on *Phaseolus Mungo* and *Triticum Aestivum*

Jagwani J. S. Department of Biotechnology, KSV, Sector-23, Gandhinagar-382023

Dr. Lakshmi B Department of Biotechnology, KSV, Sector-23, Gandhinagar-382023

Dr. M. C. Sharma Department of Biotechnology, KSV, Sector-23, Gandhinagar-382023

ABSTRACT

Synthetic dyes are extensively used in textile dyeing, paper, printing, color, photography, pharmaceutics, cosmetics and other industries. Among these, azo dyes represent the largest and most versatile class of synthetic dyes. Traditional methods of treatment are found to be expensive and have operational problems. In the present studies a mixed bacterial consortium (VSS) was isolated from Textile dye contaminated soil and water from Vatva G.I.D.C., Ahmedabad, Gujarat, India, by enrichment culture technique using C.I. Reactive Orange M2R (ROM2R), an azo dye in the Bushnell and Haas Broth (BHB). All six bacterial cultures were identified by 16S rRNA gene analysis and bioinformatics tools. The consortium showed complete decolorization of the selected dye (ROM2R-100 mg/L) within 24 hrs in static conditions. The biodegradation was monitored by UV-Vis spectrophotometric analysis and High Performance Liquid Chromatography (HPLC). The presence of azo reductase suggests its role in the degradation of the dye. The non toxic nature of the degradation metabolites of ROM2R was revealed by phytotoxicity studies on germination and growth of Triticum aestivum and Phaseolus mungo. The results suggest that the isolated consortium VSS as a useful tool to treat waste water containing reactive (azo) dyes.

KEYWORDS: Biodegradation, Textile dye, Phytotoxicity, Azo reductase, HPLC

Introduction

Textile dye wastewater has become one of the main reasons of severe pollution problems due to the greater demand for textile products and increase in production and application of synthetic dyes (Dos Santos *et al*., 2007). Dyes are broadly classified into several types. Based on the chemical structure of chromophoric group synthetic dyes are classified as azo dyes, anthraquinone dyes, etc. These dyes have an adverse effect on the environment. The dyes are toxic carcinogenic and genotoxic due to their high COD (Chemical Oxygen Demand) values. Azo dyes are considered as electron deficient xenobiotic components because they possess azo (N=N) and sulphonic (-SO_3) electron withdrawing groups, generating electron deficiency and making the component less susceptible to oxidative catabolism by bacteria. Azo dyes are the largest group of dyes used in the textile industry (Ramalho *et al*., 2002).

Existing physicochemical methods viz., chemical oxidation, reverse osmosis, coagulation, flocculation, filtration, adsorption, photo degradation and membrane processes are effective for color removal but these methods are not suitable due to high cost, low efficiency and inapplicability to a wide variety of dyes. Also they use more energy and chemicals than biological processes and may cause secondary pollution problems in the form of sludge (Tamboli *et al*., 2010; Kurade *et al*., 2011; Waghmode *et al*., 2011). Several emerging technologies such as electrochemical destruction, advanced oxidation and sorption have potential for decolorization but these approaches involve complicated procedures or are not feasible economically (Sandhya *et al*., 2005).

There is a need of novel mechanism for the removal of dyes from textile wastewater. Compared with chemical/physical methods, biological processes have received much more attentions due to cost effectiveness, lower sludge production and environmental friendliness (Joe *et al*., 2008; Wang *et al*., 2008; Phugare *et al*., 2011a).

Various attempts have been made to develop biological process for treatment of textile dyes effluent, including enzyme (Phugare *et al*., 2011b), fungi viz. *Aspergillus fumigates* XC6 (Jin *et al*., 2007); Kocuria rosea MTCC 1532 (Parshetti *et al*., 2010). Recent trend is shifting towards the use of mixed microbial cultures as compared to individual strains. Thus the treatment systems composed of mixed microbial populations possess higher degree of biodegradation and mineralization due to synergistic metabolic activities of microbial community and offer considerable advantages over the use of pure cultures in the degradation of dyes and textile dyestuff (Khehra *et al*., 2005; Saratale *et al*., 2009b; Phugare *et al*., 2011a). In consortium, the individual strains may attack the dye molecule at different positions or may utilize metabolites produced by the co-existing strains for further mineralization of complex dye molecule.

In the present study, a defined consortium of six bacterial cultures, designated as Consortium-VSS was isolated from Vatva GIDC, Ahmedabad, Gujarat, India, was used for degradation of Reactive Orange M2R (ROM2R)(an azo dye) under static (microaerophilic) conditions. The dye degradation was studied by UV-Vis spectrophotometric analysis and HPLC analysis. The bacterial cultures were identified by 16S rRNA gene sequencing and bioinformatics tools. In addition, phytotoxicity study, on *Triticum aestivum* and *Phaseolus mungo,* was also used to evaluate the toxicity of degradation products of ROM2R by the consortium VSS.

Materials and Method

Sample

Soil and water samples from textile dye contaminated area were collected from Vatva GIDC, Ahmedabad (India) and kept in cold condition until used.

Dyes

The textile azo dye Reactive Orange M2R dye was obtained from Space industries, Vatva GIDC, Ahmedabad.

Method of screening dye degrading organism

Isolation of the microbial consortia was carried out by enrichment culture technique. The media used was Bushnell Haas media (Hi media) incorporated with 0.3% of yeast extract and 100mg/l azo dye as sole source of carbon. The cultures showing decolorization on every successive transfer were selected for further studies.

Identification of the bacterial consortium

The 16S rRNA gene sequences of all six unknown bacterial cultures were compared for homology with BLAST database (nucleotide) at NCBI server (http://www.ncbi.nlm.nih.org) and corresponding sequences were downloaded. Evolutionary history was inferred using the Maximum parsimony method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches (Felsenstein, 1985). The phylogenetic tree was linearized assuming equal evolutionary rates in all lineages (Takezaki *et al*, 2004). The tree was drawn to the scale, with branch lengths in the same units as those of the evolutionary distance used to infer the phylogenetic tree (Dhanve *et al*., 2009) (Figure not shown). The sequences were submitted to the NCBI database and accession numbers were obtained.

Dye decolorization experiments

Decolorization of azo dye was studied under static and shaking culture conditions at 37 ^{0}C in 250 ml Erlenmeyer flasks containing 100ml BHB +0.3%yeast extract and 100mg/l ROM2R. Aliquots of culture superna-

tant were withdrawn after regular time intervals during decolorization process. Cells were removed from the culture medium by centrifugation at 6000 rpm for 20 minutes and the cell free supernatant was used to study the absorbance by using UV-Visible spectrophotometer (Systronics, 2203) at 488nm (absorption maxima of ROM2R).

All decolorization experiments were performed in triplicates and decolorization activity was expressed in terms of percentage decolorization (Phugare *et al*., 2011a) by the following formula:

%decolorization=Ai-Af/Ai [Ai=initial absorbance; Af=final absorbance]

Preparation of cell free extract

The consortium VSS and individual organisms were grown in the BHM incorporated with 0.3% YE for 24 hr at 37^{0}C and then transferred to the same media along with dye ROM2R (100 ppm) for 24 hrs at 37^{0}C and centrifuged at 9000 rpm for 25 min. The biomass of consortium and individual organisms was separately suspended in 50mM potassium phosphate buffer (pH 7.4) and sonicated (PEI, India) (12 strokes of 9 sec each then 2 sec interval for 2 min 30 amplitude output) at 4^{0}C. The sonicated cells were centrifuged in cold condition (4^{0}C, at 18000rpm for 20min) and supernatant used as the source of intracellular enzymes.

Azo reductase enzyme assay

Azo reductase activity was assayed by modifying earlier reported method (Telke et al.,2010). The 1.2 ml reaction mixture contained 100 µl of Methyl Red (MR), 100 µl NADH, 0.9 ml of potassium phosphate buffer (50 mmol, pH 7.4). The reaction mixture was pre-incubated for 2 min followed by addition of NADH and monitored for the decrease in color absorbance (430 nm) at room temperature. The reaction was initiated by addition of 0.1 ml of the enzyme solution. The reduction of Methyl Red was calculated using molar extinction coefficient of 0.023 per µmol/cm. One unit of enzyme activity was defined as amount of enzyme required to reduce 1 µmol of substrate/min/mg of protein. All the enzyme assays were run in triplicates.

HPLC analysis

The decolorized culture medium was centrifuged at 12,000 rpm for 15 min and supernatant was collected and extracted with ethyl acetate. The extracts were allowed to evaporate till dryness and redissolved in HPLC grade methanol. The samples were filtered through 0.2 µm filter and then analyzed using column RP C18 (250 x 4.6 mm, 5 micron particle size, Phenominex). HPLC analysis was carried out (Shimadzu, LC 20AD) on C18 column by isocratic method with 12min run time (Sahasrabudhe & Pathade, 2011). The mobile phase consisted of 20mM Ammonium acetate (in water): methanol (60:40) with flow rate of 1ml/min and UV detector (SPD 20A) was kept at 488nm.

Phytotoxicity study

The effect of original dye and its degraded product on germination and early seedling growth of two plants; *Triticum aestivum* and *Phaseolus mungo* was evaluated. The degraded product of ROM2R was extracted in ethyl acetate, were dried and dissolved in water to form the final concentration of 1000ppm. The dye solutions were also prepared with concentration of 1000 ppm for phytotoxicity studies.

The seeds were germinated in small plastic containers with sterile soil. Seeds were surface sterilized with 1:1000 $HgCl_2$ and then sown. The phytotoxicity study was carried out at room temperature in relation to *Triticum aestivum* and *Phaseolus mungo* seeds by watering separately 10 ml samples of dye and its degraded product per day. Seeds germinated in water irrigated container were used as control. Length of plumule (shoot), radical (root) and germination percentage (%) were recorded after every alternate day for 8 days. The study was carried out at room temperature and germination percentage (G) was calculated by the following equation:

$G = Ng/Ns \times 100$

Statistical analysis

Statistical analysis were analyzed by one-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparisons test.

RESULTS AND DISCUSSION

Identification of bacterial consortium

The six bacteria in the consortia belonged to *Enterococcus sp.*(VSS-1), *Bacillus sp.*(VSS-2), *Enterococcus sp.*(VSS-3), *Stenotrophomonas sp.*(VSS-4), *Enterococcus sp.*(VSS-5), *Pseudomonas sp.*(VSS-6). The sequences were submitted to NCBI and the accession numbers were obtained as KF282710 to KF282715 for VSS-1 to VSS-6 respectively. Similar study was conducted by Jadhav *et al* (2008) for identification of bacterial consortium PMB11, a combination of three bacteria.

Dye decolorization experiments

Decolorization of ROM2R using consortium VSS was carried out at aerobic (shaking) and microaerophilic (static) condition, but the decolorizaton was found to be more at static condition. Complete decolorization of ROM2R (100mg/L) was obtained within 24 hrs of inoculation. Individual cultures did not decolorize ROM2R even on prolonged incubation which suggests the synergistic role of all six bacterial cultures. Khehra *et al.* (2005) showed mutualism of individual strains in consortium for better decolorization of AR-88. The individual strains may attack the dye molecule at different positions or may use degradation products produced by another strain for further degradation (Coughlin *et al*, 1997; Forgacs *et al*, 2004).

Fig.1 about here

Azo reductase enzyme assay

The protein concentration after cell lysis was estimated as 800µg/ml by Folin-Lowry protein estimation method. The enzymatic analysis of consortium VSS (after decolorization) was calculated to be 1.425 units/ml by the method reported by Salokhe and Govindwar, 1999. Similar studies were reported by Bhatt NS *et al.* (2012), Govindwar *et al.* (2011).

HPLC analysis

The metabolites obtained after 24 hrs of decolorization of ROM2R were extracted with ethyl acetate, crystallized, dissolved in HPLC grade methanol and used for the analysis. HPLC chromatogram of control ROM2R showed one major peak at retention time of 5.102 min (Fig.2a). Decolorization of ROM2R by consortium VSS showed 2 peaks at retention time of 2.595 min and 2.850min (Fig.2b) suggested the degradation of ROM2R.(Govindwar et al, 2008). Damronglerd *et al*. (2004) studied the HPLC analysis of a dye Remazol Black B and its degraded products. And reported that metabolite peak shifted towards a lower retention time compared to the control peak which suggested the formation of less aromatic and more polar compounds by biodegradation processes of bacteria. Identification of metabolites should be the objective of further investigations.

Fig.2 about here

Phytotoxicity study

Seed germination and plant growth bioassay are the most common technique used to evaluate the phytotoxicity (Kapanen and Itavaara, 2001). Germination of both seeds (*Triticum aestivum and Phaseolus mungo*) was less with ROM2R treatment as compared to its degradation metabolites and plain water (Fig.4). The length of the plumule and radical were significantly affected by ROM2R than its degradative metabolites (Table-1), indicating less toxic nature of degraded metabolites as compared to dye (Kabra *et al.,* 2011).

Fig.3 about here

Germination percentage of seeds (Triticum aestivum) in plastic containers irrigated with dye and extracted metabolites were compared with water control and found to be 10% and 70% respectively, indicating toxic nature of the dye. Phaseolus mungo also showed similar toxicity of ROM2R with severly affected plumule and radical growth (Fig.4). Toxicity of ROM2R on *Triticum aestivum and Phaseolus mungo* was summarized in the table-1.

Table-1 about here

Toxicity study of some textile dyes on germination and early seedling growth of 4 plants: clover, wheat, lettuce and tomato had been studied by Moawad and Wafaa, 2003. Similar results were reported about Reactive blue 59 toxicity on *Triticum aestivum and Phaseolus mungo* (Patil and Shedbalkar, 2008). So, phytotoxicity studies revealed biodegradation of ROM2R by bacterial consortium resulted in the detoxification of dye.

Conclusion:

In the present study, a potential azo dye degrading bacterial consortium was isolated and identified by 16S rDNA sequencing. The identified cultures belonged to *Enterococcus sp., Bacillus sp., Stenotrophomonas sp. and Pseudomonas sp.* The activities of azoreductase enzyme in a bacterial consortium VSS resulted in an increased decolorization of ROM2R as compared to the individual strains. UV-visible spectra of metabolites formed after the degradation of ROM2R clearly showed complete decolorization. HPLC analysis confirmed the biodegradation of ROM2R. Phytotoxicity analysis of the degraded product indicated that the metabolites of dye were nontoxic. Results from this studies demonstrated that the consortium VSS could be successfully applied in the safe bioremediation of textile effluent.

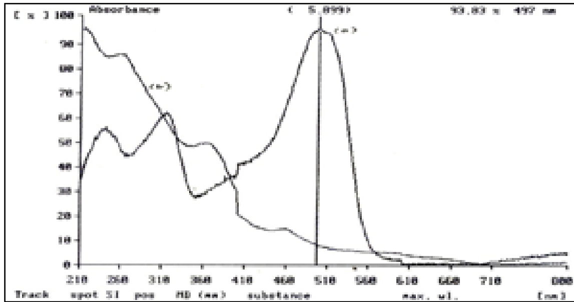

Fig.1 UV-vis spectral analysis of ROM2R 0hr (a) and after decolorization by consortium VSS (b)

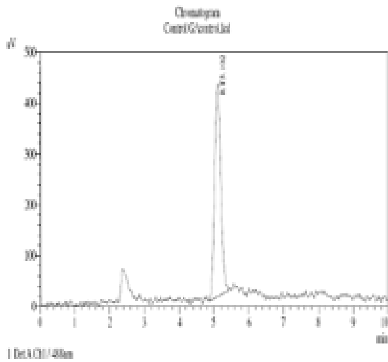

Fig.2 (a) HPLC profile recorded at 488nm of control Reactive

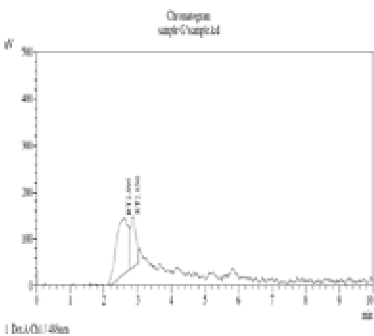

Fig.2 (b) its metaboloties obtained after 24 hrs of degradation by using consortium VSS.

Fig.3 (a) Effect of Dye (ROM2R)(ii) and its degraded product-ethyl acetate extracted(iii) on growth of *T.aestivum* compared to water control(i)

(b) Effect of Dye (ROM2R)(ii)and its degraded product-ethyl acetate extracted (iii)on growth of *P.mungo* compared to water control(i)

(c) Effect of Dye (ROM2R)(ii) and its degraded product-culture supernatant(iii) on growth of *P.mungo* compared to water control(i)

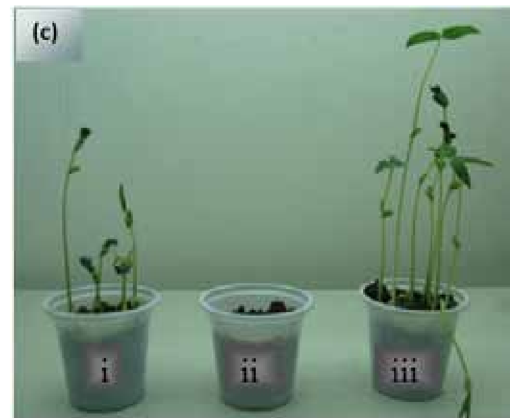

Table:1 Results of ROM2R phytotoxicity study

	Phaseolus mungo			
Parameter studied	Water	ROM2R (1000ppm)	Extracted metabolite (1000ppm)	Culture supernatant
Germination(%)	100	30	100	80
Plumule(cm)	9.63±0.3	5.62±0.2	10.3±0.4	9.2±0.9
Radicle(cm)	3.40±0.1	1.06±0.3	3.56±0.2	3.1±0.7
	Triticum aestivum			
Parameter studied	Water	ROM2R (1000ppm)	Extracted metabolite (1000ppm)	Culture supernatant
Germination(%)	60	10	70	60
Plumule(cm)	10.21±0.2	9.01±0.3	9.69±0.1	8.5±0.5
Radicle(cm)	4.8±0.1	3.73±0.2	4.86±0.2	3.4±0.3

REFERENCES

Bhatt, N.S., Vagadiya, D. R., & Junnarkar, N.S. (2012). Decolorization, degradation and azo- reductase study by bacterial transformation of reactive red HE8b. Int J of Res in Biosci, 1(1), 29-41. | Coughlin, M.F., Kinkle, B.K., Tepper, A., & Bishop, P.L. (1997). Characterization of aerobic azo dye degrading bacteria and their activity in biofilms. Water Sci Technol, 36, 215-220. | Damronglerd, S., Supaka, N., Juntongjin, K., Delia, M., & Strehaiano, P. (2004). Microbial decolorization of reactive azo dyes in a sequential anaerobic-aerobic system. Chemical Engineering Journal, 99, 169-176. | Dos Santos, A.B., Cervantes, F. J., & Van Lier, J.B. (2007). Review paper on current technologies for decolourisation of textile wastewaters: perspectives for anaerobic biotechnology. Biores. Technol., 98(12), 2369 -2385. | Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. Evol Int J Org Evol 39,783-791. | Forgacs, E., Cserhati, T., & Oros, G. (2004). Removal of synthetic dyes from wastewaters: A review. Environ Int, (30), 953-971. | Govindwar, S.P., Waghmode, T.R., Kurade, M.B., & Kagalkar, A.N. (2011). Differential fate of metabolism of a disperse dye by microorganisms Galactomyces geotrichum and Brevibacillus laterosporus and their consortium GG-BL. J of Environ Sci. DOI: 10.1016/S1001-0742(11)60899-1. | Jadhav, J.P., Kalyani, D.C., Shedbalkar, U.U., & Patil, P.S. (2008). Biodegradation of Reactive Blue 59 by isolated bacterial consortium PMB11. J Ind Microbiol Biotechnol, 35,1181-1190. | Jin, X. C., Liu, G. Q., Xu, Z. H., & Tao, W. Y. (2007). Decolorization of a dye industry effluent by Aspergillus fumigates XC6. Applied Microbiology Biotechnology, 74(1), 239-243. | Joe, M., Lim, S., Kim, D., & Lee, I. (2008). Decolorization of reactive dyes by Clostridium bifermentans SL186 isolated from contaminated soil. World Journal of Microbiology and Biotechnology, 24(10), 2221-2226. | Kapanen, A., & Itävaara, M. (2001). Ecotoxicity Tests for Compost Applications. Ecotoxicology and Environmental Safety, 49, 1-16. | Khehra, M.S., Saini, H.S., Sharma, D.K., Chadha, B.S., & Chimni, S.S. (2005). Decolorization of various azo dyes by bacterial consortium. Dyes and Pigments, 67(1), 55-61. | Kurade, M. B., Waghmode, T. R., & Govindwar, S.P. (2011). Preferential biodegradation of structurally dissimilar dyes from a mixture by Brevibacillus laterosporus. Journal of Hazardous Materials, 192(3), 1746-55. | Parshetti, G. K., Telke, A.A., Kalyani, D.C., & Govindwar, S.P. (2010). Decolorization and detoxification of sulfonated azo dye Methyl Orange by Kocuria rosea MTCC 1532. Journal of Hazardous Matter, 176(1-3), 503-509. | Phugare, S. S., Waghmare, & S. R., Jadhav, J.P. (2011b). Purification and characterization of dye degrading of veratryl alcohol oxidase from Pseudomonas aeruginosa strain BCH. World Journal of Microbiology and Biotechnology, 27(10), 2415-2423. | Phugare, S.S., Kalyani, D. C., Patil, A. V., & Jadhav, J.P. (2011a). Textile dye degradation by bacterial consortium and subsequent toxicological analysis of dye and dye metabolites using cytotoxicity, genotoxicity and oxidative stress studies. Journal of Hazardous Materials, 186(1), 713-723. | Ramalho, P.A., Scholze, H., Cardoso., Ramalho, M. H., Oliverira, M.T., & Campos, A. M. (2002). Improved conditions for the aerobic reductive decolorization of azo dyes by Candida zeylamoides, Enzyme and Microbial Technology, 7, 402-412. | Sahasrabudhe, M., & Pathade, G. (2011). Biodegradation of sulphonated azo dye C.I.reactive orange 16 by Enterococcus faecalis strain YZ 66. European Journal of Experimental Biology, 1(1),163-173. | Salokhe, M.D., & Govindwar, S.P. (1999). Effect of carbon source on the biotransformation enzymes in Serratia marcescens. World J Microbiol Biotechnol, (15), 259-263. | Sandhya, S., Padmavathy, S., Swaminathan, K., Subramanyam, Y.V., & Kaul, S. N. (2005). Microaerophilic- aerobic sequential batch reactor for treatment of azo dyes containing simulated wastewater. Process Biochemistry, 40(2), 885-890. | Saratale, R.G., Saratale, G.D., Kalyani, D.C., Chang, J.S., & Govindwar, S.P. (2009b). Enhanced decolorization and biodegradation of textile azo dye Scarlet R by using developed microbial consortium-GR. Bioresource Technology, 100(9), 2493-2500. | Tamboli, D. P., Kagalkar, A. N., Jadhav, M. U., Jadhav, J. P., & Govindwar, S. P. (2010). Production of polyhydroxyhexadecanoic acid by using waste biomass of Sphingobacterium sp. ATM generated after degradation of Direct Red 5B. Bioresource Technology, 101(7), 2421-2427. | Waghmode, T. R., Kurade, M. B., & Govindwar, S. P. (2011). Time dependent degradation of mixture of structurally different azo and non azo dyes by using Galactomyces geotrichum MTCC 1360. International Biodeterioration and Biodegradation, 65(3), 479-486. | Wang, X., Cheng, X., &Sun, D. (2008). Autocatalysis in Reactive Black 5 biodecolorization by Rhodopseudomonas palustris W1. Applied Microbiology Biotechnology, 80(5), 907-915. |