Journal of Applied & Environmental Microbiology, 2013, Vol. 1, No. 1, 1-5
Available online at http://pubs.sciepub.com/jaem/1/1/1

DOI:10.12691/jaem-1-1-1

Microbial Decolorization of the Azo Dye Methyl Red by *Enterobacter spp.* ETL-1979

Maulin P Shah*, Kavita A Patel, Sunu S Nair, A M Darji

Industrial Waste Water Research Laboratory, Applied & Environmental Microbiology Lab, Enviro Technology Limited (CETP), GIDC, Ankleshwar, Gujarat, India
*Corresponding author: shahmp@uniphos.com

Received September 26, 2013; Revised November 27, 2013; Accepted December 08, 2013

Abstract Bacterial isolates, obtained from dye-contaminated sludge, decolorized the toxic azo dye methyl red (MR). *Enterobacter spp.* ETL-1979 was selected because of its better abilities to completely decolorize MR under aerobic conditions. Effects of physicochemical parameters (temperature, stirring, concentration of glucose and pH of the synthetic medium) on the MR decolorization by the selected bacterium were studied. Under optimal conditions, *Enterobacter spp.* ETL-1979 completely decolorized 100 mg/l of MR within 6 h of incubation in synthetic medium. The high MR decolorization ability and low nutrient and environmental requirements of *Enterobacter spp.* ETL-1979 enable this bacterium to be used in the biological treatment of industrial effluent containing azo dyes.

Keywords: *azo dye, methyl red, bacterial decolorization, Enterobacter*

Cite This Article: Maulin P Shah, Kavita A Patel**,** Sunu S Nair, and A M Darji, "Microbial Decolorization of the Azo Dye Methyl Red by *Enterobacter spp.* ETL-1979." *Journal of Applied & Environmental Microbiology* 1, no. 1 (2013): 1-5. doi: 10.12691/jaem-1-1-1.

1. Introduction

The structural diversity of dyes derives from the use of different chromophoric groups (azo, anthraquinone, triarylmethane and phtalocyanine groups) and different application technologies (reactive, direct, disperse and vat dyeing) [3]. Azo dyes are the most common types of synthetic dyes and constitute the largest class of dyes used commercially [3]. Synthetic azo dyes are used extensively as dyes for textiles, food and cosmetics. More than 800,000 tons of these dyes are produced annually worldwide [3]. Most of the azo dyes, which are released into the environment, originate from the textile industry and the dyestuff manufacturing industry [2]. Azo dyes are a group of compounds characterized by the presence of one or more azo bonds (-N=N) in association with one or more aromatic systems [1]. This makes them relatively resistant to biological and chemical degradations. However, several studies have shown azo dyes to be toxic and/or carcinogenic [5,6,7,8] Most biological degradations of azo dyes are carried out by anaerobic bacteria [2,13,14,21]. Generally, azo dyes are resistant to attack by bacteria under aerobic conditions. Only some studies reported that bacteria under aerobic conditions could degrade azo dyes [3,4,9,16,18,20]. In both cases (aerobic and anaerobic conditions) the initial step in the biodegradation is the cleavage of the azo bond. Azoreductase catalyzes reductive cleavage of the azo bond to produce aromatic amines. Under aerobic conditions, the initial step of azo bond cleavage is typically followed by hydroxylation and ring opening of the aromatic intermediates [7,15]. In previous studies, *Acetobacter liquifaciens* and *Klebsiella pneumoniae* were shown to decolorize the toxic azo dye methyl red (MR) under aerobic conditions and yield two colorless compounds (Figure 1), 2-aminobenzoic acid (ABA) and N,N'-dimethyl-*p*-phenylenediamine (DMPD) [16,18]. In the present study, we report the aerobic decolorization of MR by *Enterobacter spp.* ETL-1979 growing on a synthetic medium. This bacterial strain, isolated from dye contaminated sludge, decolorizes MR faster than *A. liquefaciens* and *K. pneumoniae*. The effects of various physicochemical parameters on the decolorization of MR by *Enterobacter spp.* ETL-1979 were studied and compared.

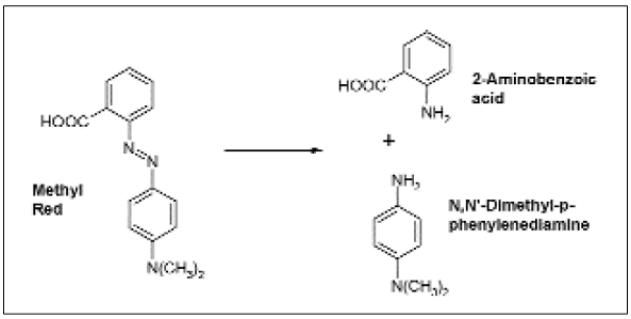

Figure 1. Reductive cleavage of the azo dye methyl red (MR) (Wong and Yuen 1996)

2. Materials and Methods

2.1. Isolation of Methyl Red Decolorizing Bacteria

Dye-contaminated sludge was collected from a wastewater outlet of an industrial area in Ankleshwar GIDC, Gujarat, India. Approximately, 1 g of sludge was suspended in 10 ml of sterile sodium chloride solution 0.85% (w/v) and mixed thoroughly. The mixture was serially diluted with sterile sodium chloride solution 0.85%. Aliquots of 0.1 ml of 10_{-1}, 10_{-2} and 10_{-3} dilutions were spread onto minimum medium (MM) plates containing: K_2HPO_4, 1.36 g/l; $MgSO_4$, 0.1 g/l; $SO_4(NH4)2$, 0.6 g/l; $CaCl_2$, 0.02 g/l; NaCl, 0.5 g/l; $MnSO_4$, 1.1 mg/l; $ZnSO_4$, 0.2 mg/l; $CuSO_4$, 0.2 mg/l; $FeSO_4$ 0.14 mg/l; MR, 100 mg/l; agar, 15 g/l. pH was adjusted to 7 with 1 M HCl. All plates were incubated at 37 °C for 3 days. Colonies surrounded by decolorized zones were picked and streaked onto MM plates containing 100 mg/l of MR. The plates were again incubated at the same conditions to confirm their abilities to decolorize MR.

2.2. Decolorization of Methyl Red by Selected Bacterium in Liquid Medium

One bacterial isolate with higher MR decolorization abilities was selected. A preculture of this bacterium was prepared by growing a single colony in 10 ml of MM containing 1% of glucose at 37 °C in a rotary shaker at 100 rpm for 18 h. An aliquot of 1 ml of precultures was inoculated into 100 ml of MM with 1% of glucose and 100 mg/l of MR. This culture was incubated under the same conditions for 24 h. After incubation, an aliquot of 10 ml of this culture was sampled and cells were pelleted by centrifugation at 12,000 × *g* at 4 °C for 15 min in a Sigma 3K15 refrigerated centrifuge. The absorbance at 430 nm (the absorption maximum of the MR) of the culture supernatant was determined using a Shimazu 1800 UV/Visible spectrophotometer.

2.3. Presumable Identification of the Selected Bacterium

The selected bacterial isolate was subject to the Gram stain and then tentatively identified by biochemical analysis according to the standardized micromethod API 20 E (bioMérieux, Inc.). All reagents and accessories were purchased from the bioMérieux Inc. and used according to the instructions of the manufacturer.

2.4. HPLC Analysis of Methyl Red and Its by-Products

The selected bacterium was grown in 100 ml of MM with 100 mg/l of MR and 1% of glucose at 37 °C. After 6 h of incubation, the culture was centrifuged to remove bacterial cells and the supernatant was concentrated to about 20 ml in a rotary evaporator at 60 °C. A concentrated sample was then extracted three times with equal volume of dichloromethane (DCM). The DCM extracts were pooled and evaporated to 5 ml at 40 °C in a rotary evaporator and then transferred to a test tube. The remaining DCM was removed by evaporation at room temperature (20 ± 0.5 °C) and placed in a hood overnight. The extracted residue was dissolved in 10 ml acetonitrile and filtered through a 0.2 µm nylon filter. Twenty µl was analyzed by a Jasco HPLC system equipped with a model 875 variable wavelength detector (detection wavelength was 254 nm) and a reverse phase C18 column (25 cm × 4 mm) packed with 5 µm particle size. The mobile phase was sodium phosphate buffer (25 mM, pH 3.0) and acetonitrile (40/60, v/v) with a flow rate of 0.2 ml/min. A control test without the bacterium and containing 100 mg/l of MR was prepared and analyzed under the same conditions. The standards of methyl red (MR) (BDH Chemicals, England), N,N'dimethyl-*p*-phenylenediamine (DMPD) (Fluka, Switzerland) and 2-aminobenzoic acid (ABA) (Fluka, Switzerland) were injected for comparison and confirmation.

2.5. Effects of Physicochemical Parameters on Methyl Red Decolorization and Bacterial Growth of Selected Bacterium

Pre cultures of the selected bacterium were prepared by growing a single colony in 10 ml of MM containing 1% of glucose for 18 h at 37 °C with shaking. Aliquots of 2 ml were inoculated in culture media containing 200 ml of MM with 1% of glucose and 100 mg/l of MR. The inoculated cultures were then incubated at different experimental conditions to determine the effects of physicochemical parameters on the MR decolorization and the bacterial growth. Effect of temperature: The inoculated cultures were incubated at various temperatures (24, 37 and 44 °C) in rotary shakers running at 100 rpm. Effect of stirring: The inoculated cultures were incubated at 37 °C without shaking (static condition) and in a rotary shaker running at 100 rpm (shaking condition).

Effect of glucose concentration: The culture medium used to determine the effect of the concentration of glucose was MM containing 100 mg/l of MR and various concentrations of glucose (0, 1, 2 and 4%). The inoculated cultures were incubated at 37 °C under shaking condition (100 rpm).

Effect of pH of culture medium: Procedures to determine the effect of pH were similar to those cited for the glucose concentration effect study, except the culture medium used was MM with 1% of glucose and 100 mg/l of MR. The pH of the culture media was adjusted to 3, 5, 7 or 9 with 1 M HCl. At several time intervals, 2 ml aliquots of each culture were sampled and their cell densities were measured spectrophotometrically at 600 nm in order to determine the bacterial growth. Cells were then pelleted by centrifugation and the supernatant was 10-fold diluted and the concentration of MR was determined spectrophotometrically at 430 nm. All experiments were carried out in triplicate.

3. Results and Discussion

3.1. Isolation of Methyl Red Decolorizing Bacteria

Preliminary selection of MR decolorizing bacteria was based on the decolorization of MR on MM plates because most azo dye decolorizing microorganisms cleave the azo bond of respective azo dye and produce colorless by-products. Nine bacterial isolates, which decolorized MR on MM plates, were isolated from dye-contaminated sludge. Among these bacteria, one bacterial isolate with higher MR decolorization ability (i.e. larger decolorized halo surrounding the colony) in MM plates was selected

for further study. We have used in the present work MM as a culture medium with low levels of carbon and nitrogen sources because previous studies have indicated that bacteria had better azo dye decolorizing abilities under such nutritional conditions (Ogawa *et al.*, 1986; Ogawa and Yatome, 1990).

3.2. Presumable Identification of the Selected Bacterium

The selected bacterial isolate was subject to the Gram stain. It was Gram negative and it was then tentatively identified by the standardized micromethod API 20 E. The results indicated the selected bacterium to be *Enterobacters* with about 88% of identification probability. This bacterial isolate belongs to the family of Enterobacteriaceae. Most members of this family are characterized by their minimum requirements for growth [10].

3.3. Identification of Methyl Red and Its by-Products by HPLC Analysis

The HPLC analysis of the control test without the bacterium exhibits one peak corresponding to MR at a retention time of 8.05 min (Figure 2A). The same retention time was obtained when MR standard was injected. Whereas, the HPLC analysis of the culture supernatant of *Enterobacter*, after 6 h of incubation in the presence of MR, indicated the disappearance of the MR peak (8.06 min) and the appearance of two major peaks X1 and X2 with the retention times of 2.12 and 4.21 min, respectively (Figure 2B). Tentative assignment of the by-products (X1 and X2) was made on the basis of the retention times of standards, supported by previous knowledge of MR degradation processes [16,17,18]. Therefore, products X1 and X2 were identified as N,N'dimethyl-*p*-phenylenediamine (DMPD) and 2-aminobenzoic acid (ABA), respectively. Similar compounds were reported as metabolites of MR degradation by other bacteria [16,17,18]. Consequently, these results suggest that *Enterobacter* completely decolorized MR and produced at least two metabolic by-products N,N'dimethyl-*p*-phenylene-diamine (DMPD) and 2-aminobenzoic acid (ABA) (Figure 1).

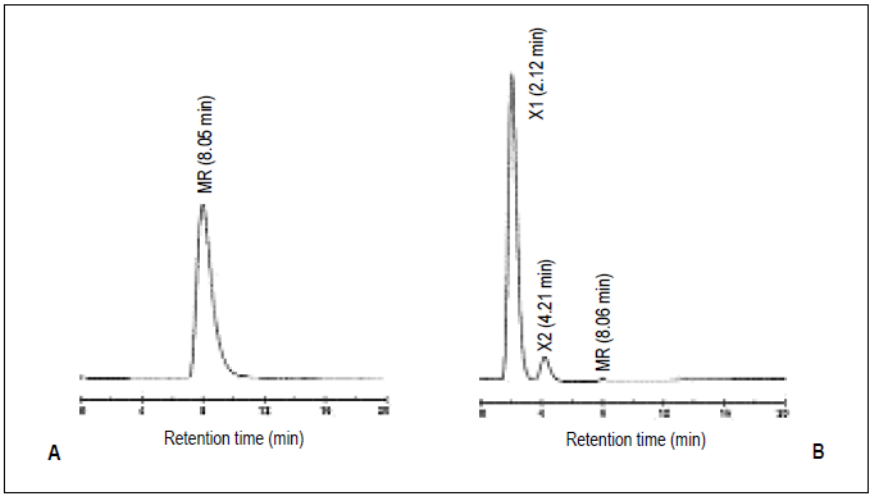

Figure 2. HPCL analysis of MR (methyl red) **(A)** and its product of decolorization (X_1 & X_2) by *Enterobacter spp.* ETL-1979 after 6 hrs of incubation **(B)** X_1 & X_2 were identified,in comparision to standards, as, N,N'dimethyl-*p*-phenylenediamine (DMPD)

3.4. Effects of Physicochemical Parameters on Methyl Red Decolorization and Bacterial Growth of Selected Bacterium

Enterobacter spp. ETL-1979 had high MR decolorization at 37 °C. However, at 24 °C and at 44 °C the MR decolorization was slower. Thus, the bacterium decolorize 92% of MR within 6 h of incubation at 37 °C whereas only 20% of MR was decolorized at this time period by the bacterium incubated at 24 °C and at 44 °C (Figure 3A). This can be explained by the variations of the bacterial growth rates of *E. agglomerans* according to the temperature of incubation (Figure 3B). Indeed, the bacterial growth is much faster and more significant at 37 °C than at 24 °C. Whereas, at 44 °C the bacterial growth was very poor. A larger population of cells could certainly decolorize more molecules of MR. (Figure 3C) shows that the stirring of the culture medium do not have an influence on the decolorization of MR by *Enterobacter* Indeed, the evolution of the rates of decolorization of MR by *Enterobacter* under shaking and static conditions is almost similar and it reaches 92% after 6 h of incubation (Figure 3C). However, the stirring of the culture medium slightly influences the kinetics of the bacterial growth of *E. agglomerans* (Figure 3D). *Enterobacter spp.* ETL-1979 decolorizes approximately 90% of MR after 6 h of incubation in the presence of 1, 2 and 4% of glucose (Figure 4A). Whereas, in absence of glucose, the rate of decolorization of MR by *Enterobacter* is only 30% and this after 10 h of incubation. This can be explained by the very weak growth of *Enterobacter* in absence of glucose (Figure 4B). Under these conditions, the bacterium uses MR as a sole source of carbon and energy for its growth. Whereas in the presence of glucose the bacterium utilized most of the energy generated from the glucose degradation for the reductive cleavage of MR. It should be noted also that in the presence of 4% of glucose the bacterial growth is slightly slower than in the presence of 1% or 2% of glucose (Figure 4B). Probably, the presence of glucose in excess in the culture medium slows down the bacterial growth of *Enterobacter*. *Enterobacter* decolorizes 90% of

MR at pH 5, 7 and 9 but with variable rates (Figure 4C). Indeed, at pH 5 the rate of decolorization of MR reaches 90% after only 4.5 h of incubation, whereas at pH values of 7 and 9, 90% of decolorization was reached after 6 h and 8 h, respectively. On the other hand, at pH 3 no decolorization of the MR was observed. This could be explained by the inhibition of the bacterial growth at this acid pH (Figure 4D). The bacterial growth of *Enterobacter* is more significant at pH 9 than at pH 5 (Figure 4D) while the decolorization of MR is faster at pH 5 than at pH 9. This can be explained by the fact that at acid pH the MR is more easily reducible than at alkaline pH.

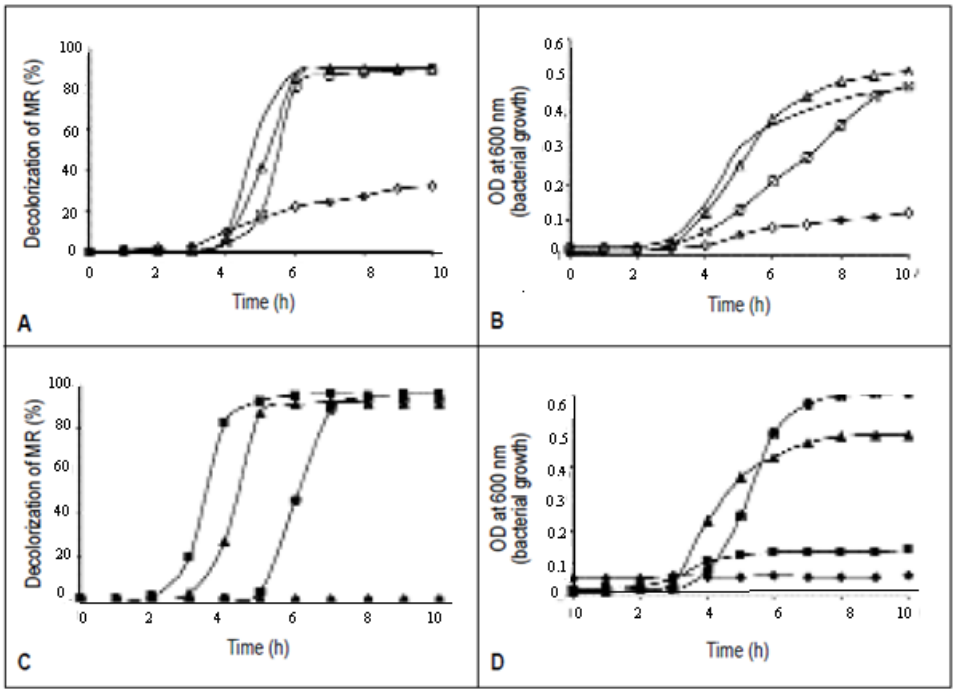

Figure 3. Effect of temperature on the MR decolorization (A) and on the bacterial growth (B) of E.spp. ETL-1979 incubated at 24 °C(-◇-), 37 °C(-) and 44 °C(-△-). Effect of stirring on the MR decolorization (C) and on the bacterial growth (D) of E. spp. ETL-1979 incubated under shaking condition 100 U/ml (-■-) and under static condition(-●-)

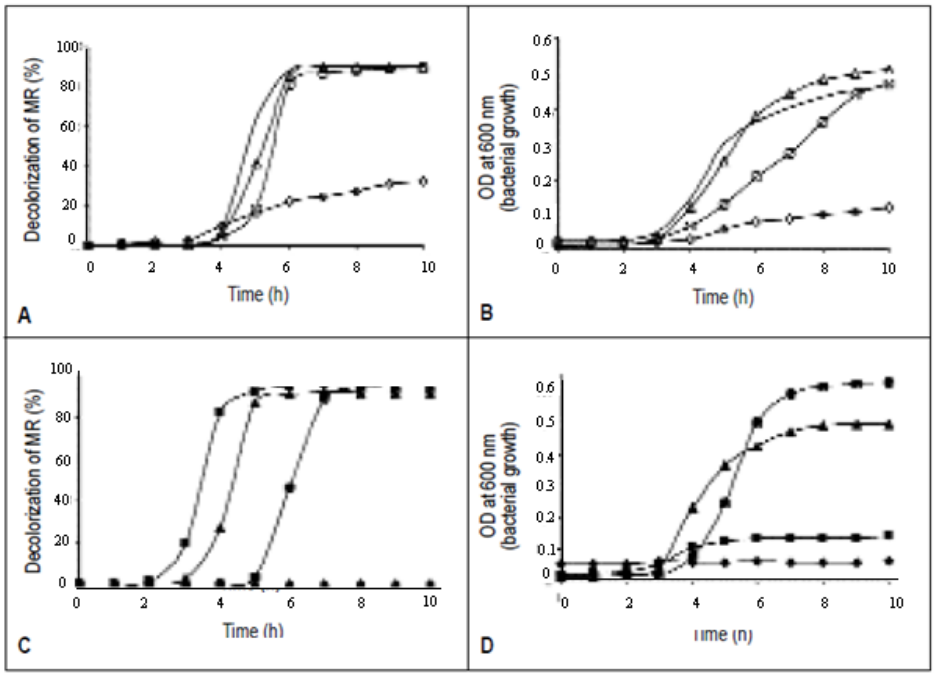

Figure 4. Effect of glucose concentration on the MR decolorization (A) and on the bacterial growth (B) of E. spp. ETL-1979 incubated in MM containing 0% (-◇-) 1% (-) 2% (-△-) et 4%(-○-) of glucose. Effect of pH on the MR decolorization (C) and on the bacterial growth (D) of E. spp. ETL-1979 incubated in MM at pH 3 (-◆-), 5 (-■-),7(-▲-) and 9 (-●-)

4. Conclusion

Enterobacter spp. ETL-1979 has a higher aerobic MR decolorization ability compared to other azo dye decolorizing bacteria incubated under similar conditions [11,16,18,19]. This bacterial strain, isolated from dye-contaminated sludge collected from an industrial area in Casablanca city, completely decolorized 100 mg/l of MR in the synthetic medium. The MR decolorization by *Enterobacter spp*. ETL-1979 was widely much faster *pneumoniae [16,18]*. Indeed as shown by the HPLC analysis, *Enterobacter* completely decolorizes the MR after only 6 h of incubation to yield the corresponding aromatic amines. This bacterial decolorization of MR by the isolated bacterium required fewer nutrients and was less sensitive to the environmental changes. These advantages allow the use of this bacterium in the treatment of azo dyes in industrial effluents with ever-changing physicochemical properties. We could then suggest the immobilization of this bacterium on various carriers to be used in a continuous process for the decolorization of azo dyes in industrial effluents.

References

[1] Anliker A. (1979). Ecotoxicology of dyestuffs - Ajoint effort by industry. Ecotoxicol. Environ. Safety, 3: 59-74.

[2] Carliell C.M., Barclay S.J., Naidoo N., Buckley C.A., Mulholland D.A., Senior E. (1995). Microbial decolourization of a reactive azo dye under anaerobic conditions. Water S.A., 21: 61-69.

[3] Chang J.S., Kuo T.S. (2000). Kinetics of bacterial decolorization of azo dye with *Escherichia coli* NO3. Bioresource Technol., 75: 107-111.

[4] Chang J.S., Chou C., Chen S.Y. (2001). Decolorization of azo dyes with immobilized *Pseudomonas luteola*. Process Biochemistry, 36: 757-763.

[5] Chung K.T., Fulk G.E., Andrews A.W. (1981). Mutagenicity testing of some commonly used dyes. Appl. Environ. Microbiol., 42: 641-648.

[6] Chung K.T., Cerniglia C.E. (1992). Mutagenicity of azo dyes: structure-activity relationships. Mutation Res., 277: 201-220.

[7] Flores E.R., Luijten M., Donlon B.A., Lettinga G., Field J.A. (1997). Complete biodegradation of the azo dye azodisalicylate under anaerobic conditions. Environ. Sci. Technol., 31: 2098-2103.

[8] Holme, I. (1984). Ecological aspects of color chemistry. In: Griffiths J., ed., Developments in Chemistry and Technology of Organic Dyes. Society of Chemistry Industry, Oxford, pp. 111-128.

[9] Hu T.L. (1998). Degradation of azo dye RP2B by *Pseudomonas luteola*. Wat. Sci. Tech., 38:299-306.

[10] Krieg N.R., Holt J.G., eds (1984). Bergey's Manual of Systemic Bacteriology. Vol.1, William and Wilkins, Baltimore.

[11] Ogawa T., Yatome C., Idaka E., Kamya H. (1986). Biodegradation of azo acid dyes by continuous cultivation of *Pseudomonas cepacia* 13NA. J. Soc. Dyers Colorists, 102: 12-14.

[12] Ogawa T., Yatome C. (1990). Biodegradation of azo dyes in multistage rotating contactor immobilized by assimilating bacteria. Bull. Environ. Contam. Toxicol., 44: 561-566.

[13] Rafii F., Franklin W., Cerniglia C.E. (1990). Azoreductases activity of anaerobic bacteria isolated from human intestinal microflora. Appl. Environ. Microbiol., 56: 2146-2151.

[14] Rafii F., Cerniglia C.E. (1993). Comparison of the azoreductase and nitroreductase from *Clostridium perfringens*. Appl. Environ. Microbiol., 59: 1731-1734.

[15] Seshardi S., Bishop P.L., Agha A.M. (1994). Anaerobic/aerobic treatment of selected azo dyes in wastewater. Waste Manage, 14: 127-137.

[16] So K.O., Wong P.K., Chang K.Y. (1990). Decolorization and biodegradation of methyl red by *Acetobacter liquifaciens*. Tox. Assess., 5: 221-235.

[17] Suzuki Y., Yoda T., Ruhul A., Sugiura W. (2001). Molecular cloning and characterization of the gene coding for azoreductase from *Bacillus* sp. OY1-2 isolated from soil. J. Biol. Chem., 276: 9059-9065.

[18] Wong P.K., Yuen P.Y. (1996). Decolorization and biodegradation of methyl red by *Klebsiella pneumoniae* RS-13. Wat. Sci. Tech., 30: 1736-1744.

[19] Yatome C., Ogawa T., Hishida H., Taguchi T. (1990). Degradation of azo dyes by cell-free extract from *Pseudomonas stutzeri*. J. Soc. Dyers Colorists, 106: 280-283.

[20] Zimmermann T., Kulla H.G., Leisinger T. (1982). Properties of purified orange II azoreductase, the enzyme initiating azo dye degradation by *Pseudomonas* KF46. Eur. J. Biochem., 129: 197-203.

[21] Zissi U., Lyberatos G. (1996). Azo dye biodegradation under anoxic conditions. Wat. Sci. Tech., 34: 495-500.

[22] Zollinger H. (1991). Color chemistry - syntheses, properties and applications of organic dyes and pigments, VCH Publications, New York. Ann.