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Influence of the chemical structure on the biodegradability of acids yellow 17, violet 7 and orange 52 by *Pseudomonas putida*

Hedi BEN MANSOUR[1,2], David CORROLER[1], Daniel BARILLIER[1], Kamel GHEDIRA[2], Leila CHEKIR[2], Ridha MOSRATI[1]*

[1]Equipe de Recherche en Physico-Chimie et Biotechnologie (ERPCB – EA3914), IUT-UFR Sciences, Université de Caen Basse-Normandie, France
[2]Unité de Recherche «Pharmacognosie/Biologie Moléculaire» (99/UR/07-03), Faculté de Pharmacie, Université de Monastir, Tunisie

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Abstract - In the present study, the textiles azo dyes acids orange 52 (AO52), yellow 17 (AY17) and violet 7 (AV7) were degraded by *Pseudomonas putida* mt-2 in mineral medium at concentration up to 100 mg/l. The culture media were completely (case of AO52) or partially (case of AY17 and AV7) decolourised under static incubation, this faster than under continuous shaking incubation. Decolourisation kinetic by intact cells indicated that AO52 disappeared completely and faster than the two other azo dyes. To understand the differentiated action of this bacterium on the three dyes, cell-free extracts activity was assessed and compared for each couple of dyes "inductor-substrate". Results showed the same half-saturation constant Km for a given dye as a substrate whatever the dye inductor, from where the assumption of a same protein with nonspecific azoreductase activity. The enzyme showed slightly more affinity for AO52 than for AY17 and AV7, but especially weaker specific activities against these two last dyes independently of the inductor. Finally, AO52 would be the best azoreductase inductor among the three tested dyes. These results indicated that the level as well the induction as the specificity of the azoreductase activity seems to be strongly dependent on the chemical structure of the dyes.

Key words: azoreductase; biodegradation; *Pseudomonas putida* mt-2; sulfonated azo dyes.

INTRODUCTION

Azo dyes are widely used for industrial, printing, and clinical purposes as well as textile dyeing because of their chemical stability, ease of synthesis, and versatility. Their durability, however, causes pollution once the dyes are released into the environment as effluent. In addition, some azo dyes are toxic and mutagenic (Medvedev *et al*., 1988; Rafii *et al*., 1997).

It has been found that some microorganisms can transform azo dyes into colourless products. Bacterial degradation of azo dyes is often initiated by an enzymatic biotransformation step that involves cleavage of azo linkages with the aid of an azoreductase and an electron donor (Zimmermann *et al*., 1982). As the azoreductase in some microorganisms can catalyze the reductive cleavage of azo groups, they have potential advantages in developing bio-treatment methods of wastewater containing azo compounds.

The rate of azoreduction was shown to be mainly affected by the chemical structure of dyes, varying with the substitution on the aromatic ring (Zimmermann *et al*., 1982; Suzuki *et al*., 2001).

Furthermore, reduction of azo dyes may produce compounds that are either more or less toxic than the parent molecules so that azoreduction may decrease or increase any indirect toxic effect of the dyes (Yahagi *et al*., 1975; Nakayama *et al*., 1983; Rafii *et al*., 1997; Ben Mansour *et al*., 2007). Among azo dyes, acids orange 52 (AO52), violet 7 (AV7) and yellow 17 (AY17) are very important commercial azo dyes used in the textiles, food, paper and cosmetic industries. In a previous study (Ben Mansour *et al*., 2007), *Pseudomonas putida* mt-2 was found to be able to decolourise these dyes, in yeast extract glucose medium, under weak or high oxygenation conditions. It has been established that the oxygen limits azo dyes reduction but in the same time that oxygen is needed for metabolization of the azo-products. On the other hand these azo dyes, except AV7, showed no toxic (genotoxic and pro-oxidant) effects. However, their metabolites obtained in static incubation were more toxic than those obtained in shaking conditions. An azoreduction product seems to contribute to the genotoxicity and to the pro-oxidant effects observed in the biodegradation extracts (Ben Mansour *et al*., 2007).

As far as acid orange 52 degradation by physical and chemical methods was well described in previous works (Liao *et al*., 2008; Okitsu *et al*., 2008; Parida *et al*., 2008), very few information on biodegradation of AV 7 (Yu *et al*., 2001) and AY 17 (Guo

* Corresponding Author. Address: ERPCB, Campus 2, Sciences 2, Université de Caen Basse-Normandie 2, Boulevard Maréchal Juin, Fr 14 032 Caen cedex, France. Phone: +33 231 567 149; Fax: +33 231 567 303; E-mail: ridha.mosrati@unicaen.fr

et al., 2006) are available. Likewise, the choice of the simple and complying structure of AO52, we have opted here for the alternate structures of AV7 and AY17 which were more complex with the presence of various substituents (mono or disulfonate, amino, hydroxyl, chloro, acetamido, etc.), various aromatic (benzenic or naphtalenic) or heteroaromatic (pyrazolic) rings and then various hydrophilies/hydrophobies of the potential metabolites. It was then important to look for the effect of variation of chemical structure on the biodegradation.

In the present study, decolourisation kinetics of acids orange 52, violet 7 and yellow 17 was compared by intact cells and cell-free extracts. The azoreductase activity was investigated as well on the level of the induction as on that of its specificity vs. dyes. Kinetic of azo dyes decolourisation, by intact or cell-free extracts, are discussed in relation to their chemical structure.

MATERIALS AND METHODS

Chemicals. The sulfonated azo dyes (Table 1) acid orange 52 (AO52), acid violet 7 (AV7), acid yellow 17 (AY17), and N,N'-dimethyl-p-phenylenediamine (DMPD), 4-aminobenzenesulfonic acid (4-ABS), sodium tetraborate, acetonitrile (HPLC grade), methanol (HPLC grade), trimethylamine, catechol, ß-nicotinamide adenine dinucleotide (NADH) as reduced dipotassium salt, proteases inhibitor cocktail, 2-mercaptoethanol, *o*-phthaldialdehyde (OPA), N-cetyl-N,N,N-trimethylammonium hydrogenosulfate (CTMA) were obtained from Aldrich (St Louis, MO, USA).

Dyes decolourisation assay.

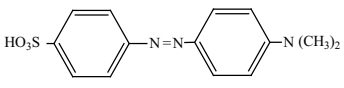
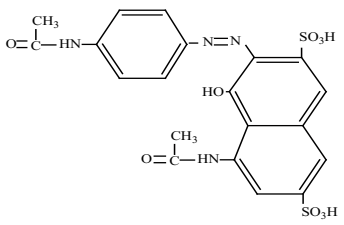
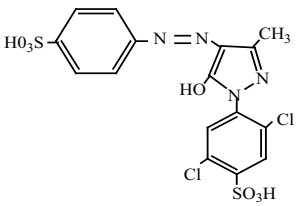
Purification procedures. Commercial azo dyes were systematically recrystallised in hot water before use. Commercial DMPD needed to be purified by a silica gel column chromatography as follows: 1 g of crude commercial DMPD was dissolved in 10 ml of diethyl ether, deposited on a 40 g silica gel bed in a glass column and then eluted with fractions of 200 ml of petroleum ether/diethyl ether mixtures corresponding to the following gradient: 7:3; 6:4; 5:5; 4:6, and 3:7. Fractions of 50 ml were collected and each one was analyzed by thin layer chromatography on a silica gel 60 F_{254} from Merck (Darmstadt, Germany; thickness 0.25 mm; petroleum ether/diethyl ether 1:1 as developing eluent). The fractions containing pure DMPD were gathered and the solvent was eliminated using a rotary evaporator. Purified DMPD was kept in a vial under a stream of gaseous nitrogen.

Culturing of Pseudomonas putida *mt-2.* Non-pathogenic *Pseudomonas putida* mt-2 (DSM 3931) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The strain harbours the plasmid pWWO encoding oxygenases for the degradation of toluene and related aromatic hydrocarbons (Franklin *et al*., 1981; Hugo *et al*., 1998). Cells from stock cultures were used for biodegradation studies after preculturing in mineral medium.

Pseudomonas putida mt-2 was grown at 30 °C in 250 ml flasks containing 100 ml of medium under a rotary shaking incubation at 200 rpm. The growth medium contained: 3.4 g/l KH_2PO_4, 8.95 g/l Na_2HPO_4, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 3 g/l NH_4NO_3, 0.15 g/l NaCl, 0.5 g/l $CaCl_2$ and 0.03 g/l $FeSO_4 \cdot 7H_2O$. The pH of the medium was adjusted to 7.2. Glucose, 10 g/l and 1 ml/l of mineral solution (1 g/l $MnSO_4 \cdot H_2O$, 0.5 g/l $CoCl_2 \cdot 6H_2O$, 0.2 g/l $CuCl_2 \cdot 2H_2O$, 0.1 g/l $ZnCl_2$ and 0.4 g/l $Na_2MoO_4 \cdot 2H_2O$) were autoclaved separately and added to the medium under sterile conditions. Azo-dye (100 mg/l) used for inducing azoreductase expression by the bacterium was sterilized using a 0.45 µm filter (Nalgene™ Labware Neerijse, Belgium).

Decolourisation assay. After cultivation of *Pseudomonas putida* mt-2 in mineral medium supplemented with 100 mg/l of azo dye (this step was necessary for the induction

TABLE 1 - Chemical structures, toxicity and commercial or technical use of the tested azo dyes

Azo dyes (Color Index)	CAS No.	Chemical structure	Genotoxicity and mutagenicity*	Commercial and/or technical use
Acid orange 52 (CI: 13025)	547-58-0		Toxic	Textiles and paper
Acid violet 7 (CI: 18055)	4321-69-1		Toxic	Textiles, food and cosmetics
Acid yellow 17 (CI: 18965)	6359-98-4		No toxic	Textiles, paper and cosmetics

* Ben Mansour *et al*. (2007).

of azoreductase expression), the exponential phase culture (~ 1.45 g/l of dry cells) was centrifuged (1700 *x g*, 10 min) to harvest cells that were transferred into a second flask (100 ml in 250 ml-flask) containing the same medium. At this time, azo dye was added to the culture medium with the aim of following its removal. We tried to investigate the influence of O_2 on azo dye degradation system by operating in two oxygenation conditions, i.e., static and shaking incubation. Flasks are incubated at 30 °C with or without agitation (100 rpm).

A sample (5 ml) of culture medium was centrifuged (1700 *x g*, 10 min) and then the absorbance of the supernatant (filtered at 0.45 µm) was measured at 520 nm for AV7, 400 nm for AY17 and 450 nm for AO52 using Pharmacia Biotech Novaspec II spectrophotometer. Residual dye concentration was quantified with construction of a calibration curve (variation of the each dye absorbance versus its concentration). The cell dry weight was estimated by recording absorbance at 660 nm (A_{660}) and according to the relation:

$$\text{Cell dry weight} = 0.61 \times A_{660}$$

All assays of biodegradation experiments were realised in triplicate. Data are expressed as mean ± standard deviation.

Preparation of cell-free extracts. Cells grown in 500 ml of mineral medium containing 100 mg/l of azo dye were collected by centrifugation (1700 *x g*, 10 min, 4 °C) and washed three times with 10 ml of 50 mM sodium phosphate buffer (pH 7). Cells were suspended in an equal volume of sodium phosphate buffer (50 mM, pH 7) containing 0.7 mM of 2-mercaptoethanol and 1 ml of protease inhibitor cocktail. The suspension was disrupted in the cold by sonification with an ultrasonic processor (Sonics Vibra-Cell - ref. 75186, Fisher-Bioblock), the cell debris and unbroken cells were removed by centrifugation (22000 *x g*, 90 min, 4 °C). The cell-free extracts were used immediately for the azoreductase assay or stored at -80 °C.

The protein concentration was determined to be 16 mg/ml using protein BioRad assay (BioRad, Richmond, CA, USA) (Bradford, 1976).

Azoreductase activity essays. Azoreductase activity was determined according to the protocol described by Zimmermann *et al*. (1982). The reaction mixture contained in a total volume of 1 ml: 50 mM sodium phosphate buffer (pH 7), 1.6 mg of soluble proteins (cell-free extract), 0.7 mM of NADH and varying concentrations of each azo dye. The reaction (at 30 °C) was started by the addition of NADH and followed by monitoring the decrease in colour intensity at the corresponding maximal absorbance for each dye. Initial enzymatic velocities were performed by varying the concentration of the substrate: each dye from 0 to 96 µM. Apparent values of the half-saturation constant (K_m) and maximal velocity (V_{max}) for each experiment were determined from the Lineweaver-Burk double-reciprocal plot.

All assays of biodegradation experiments were realised in triplicate. Data are expressed as mean ± standard deviation.

At high concentration of AO52 (96 µM) reaction mixture was treated with oxalic acid (30%) and protein were removed by centrifugation (1700 *x g*, 10 min, 4 °C), and the supernatant containing the formed potential metabolites was analyzed by HPLC according to the appropriate methodologies.

Identification of DMPD. With the aim to identify DMPD in the supernatant of decolourisation medium, this one was analyzed by the o-phtaldialdehyde (OPA) derivatization method described by Hill *et al*. (1979). The assay mixture consisted of 100 µl of the supernatant and 1 ml of a methanolic solution containing sodium tetraborate 93 mM at pH 9.5, 2-mercaptoethanol 53 mM and OPA 6.9 mM. After 15min of incubation at room temperature, the resulting mixture was subjected to analysis on a HPLC chain using a WATERS 510 pump, a Rheodyne manual injector (20 µl), a LC-18 Supelcosil (250 x 4.6 mm, 5 µm, from Interchim) reversed phase column and equipped with a JASCO FP-920 fluorescence detector. The following settings were used for detection: 5 µl flow cell, excitation monochromator set at 330 nm, emission measured with a 475 nm cut off-filter. The mobile phase was isocratic and composed of a mixture of 12.5 mM phosphate buffer (pH 7.2)/acetonitrile/methanol (1:1:1) with a flow rate of 1 ml/min. The DMPD peak at t_R=14.47 min was unambiguously identified by comparison with runs of a pure standard and of a mixed sample.

Identification of 4-ABS. The supernatant (100 µl) of decolourisation enzymatic reaction was analyzed by a HPLC chain equipped with a dual WATERS 510 pump system monitored by a WATERS automated gradient controller, a Rheodyne manual injector (20 µl) and a WATERS 486 UV-VIS detector. The sample was eluted isocratically using a Kromasil C18 reversed phase column (250 x 4.6 mm, 5 µm, from AIT) and a 20 x 4.6 mm home-made precolumn with the same phase. The mobile phase was fixed as a 60/40 mixture of eluants A and B. Eluant A: 50 mM phosphate buffer pH 5.5 and 5 mM CTMA as ion-pairing reagent in water/methanol (2:8). Eluent B: 50 mM phosphate buffer pH 5.5 and 5 mM CTMA in pure water. The flow rate of the mobile phase was 0.8 ml/min, and the UV/VIS detector was set at 245 nm. The 4-ABS peak at t_R=26.55 min was unambiguously identified by comparison with runs of a pure standard and of a mixed sample.

RESULTS

Decolourisation

Cells harvested from the incubation step were grown in flasks on the mineral medium supplemented by 10 g/l of glucose and 100 mg/l of dye. Two oxygenation conditions were compared: weak oxygenation obtained in static incubation and better oxygenation by shaking flasks at 100 rpm. The kinetics of decolourisation plotted as decreasing concentration of the three dyes vs. time is pictured in Fig. 1. It showed that *P. putida* mt-2 was able to decolourise azo dyes on both culture conditions, but the rate of decolourisation was significantly lower in the case of shaken cultures showing that excess of oxygen limited the azo dyes biodegradation. In addition, whatever the level of oxygenation, AO52 disappeared more rapidly (totally after 48 h) than AV7 and AY17 for which the rates of decolourisation were rather similar. In the same time the biomass grew and reached a plateau after 12 h for the both culture conditions with a final biomass slightly larger when flasks were shaken (respectively 2.3 and 3 g/l of cell dry weight), however, cells remained active and able to pursue the degradation of azo dyes.

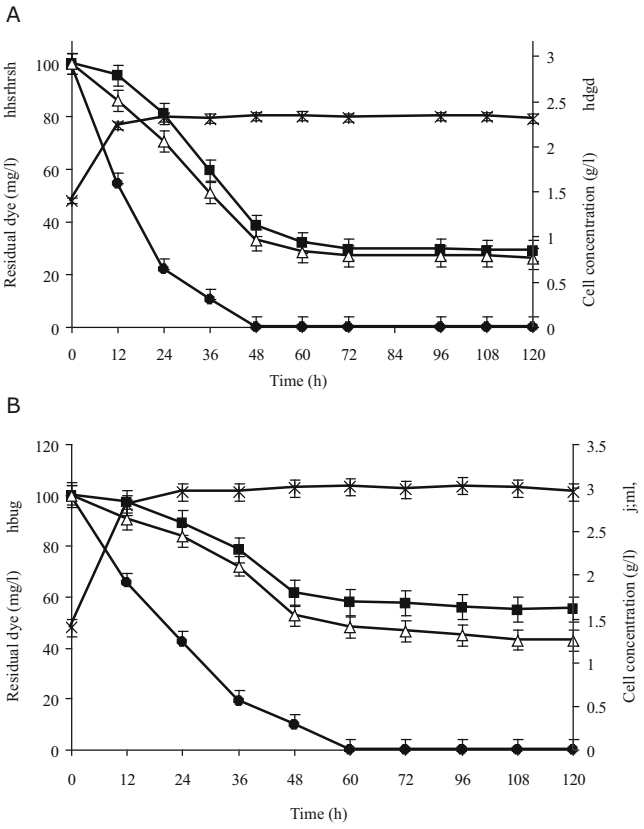


FIG. 1 - Effect of static (A) and shaken at 100 rpm (B) culture conditions on degradation of azo dyes (100 mg/l) by *Pseudomonas putida* mt-2. Symbols of residual dye concentration: (●) AO52, (△) AY17, (■) AV7 and (∗) cell dry weight.

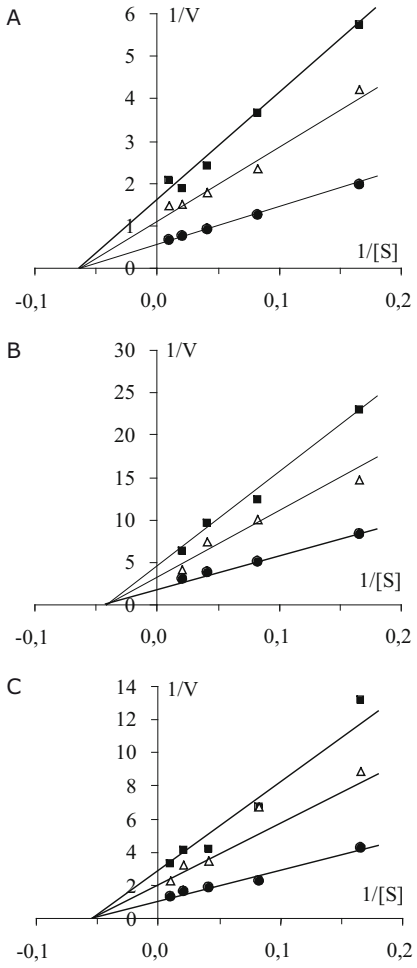


FIG. 2 - Kinetics of the azoreductase activity of cell-free extracts from *Pseudomonas putida* mt-2 inducted by AO52 (●), AV7 (■), AY17 (Δ) evaluated on the substrates: AO52 (A), AV7 (B) and AY17 (C). The substrate concentrations [S] are expressed in μM and the velocities V in μmole of substrate per mg of proteins per minute.

Cell free extracts activity

To understand the differentiated action of the bacterium on these three azo dyes, a kinetic study of the azoreductase activity was carried out. It consisted of induction of the enzyme expression by a given dye and thereafter evaluation of the enzymatic activities in cell-free extracts vs. the three dyes. Initial velocities of azoreduction were evaluated at different concentrations for each dye (from 0 to 96 μM). No inhibition by the substrates was observed in this improved concentration range. Values of apparent Michaelis-Menten parameters, K_m and V_{max}, were determined for each series from the Lineweaver-Burk double-reciprocal plot (Fig. 2). The same K_m was estimated for each dye whatever the dye inductor (Table 2). This result is in favour of the hypothesis of a same nonspecific azoreductase protein. Although the K_m values were quite similar, the enzyme showed slightly more affinity for AO52 than for AV7 and for AY17.

In Table 3 are reported data of the maximal velocity (V_{max}) of the azoreductase activity for the three azo dyes as 'substrates-inductors' in 3 x 3 full grid. On one hand, for a given inductor, the V_{max} was always highest against AO52 than AV7 and AY17, all as substrates. Compared to AO52 (as 100%) and whatever the inductor dye, the relative V_{max} were 34.6 ± 0.9% for AV7 and 55.9 ± 1.2% for AY17. On the other hand, the inductor effect can be appreciated by fixing the substrate and varying the inductor dye. The induction potential of AO52 was highest independently on the substrate. The inductor effect

TABLE 2 - Half-saturation constant (K_m in μM) of the azoreductase activity in *Pseudomonas putida* mt-2 cell-free extracts evaluated for various combinations "substrate-inductor"

Substrate	Inductor			Mean ± SD
	AO52	AV7	AY17	
AO52	14.52	16.45	16.12	15.70 ± 1.03
AV7	24.25	23.20	25.10	24.30 ± 0.95
AY17	17.26	18.58	19.89	18.58 ± 1.31

TABLE 3 - Maximal velocities (V_{max}), in µmole (of substrate)/mg (of protein)/min, of the azoreductase activity in *Pseudomonas putida* mt-2 cell-free extracts evaluated for various combinations "substrate-inductor"

Substrate	Inductor		
	AO52	AV7	AY17
AO52	1.76 ± 0.05	0.62 ± 0.03	0.90 ± 0.02
AV7	0.59 ± 0.01	0.22 ± 0.01	0.31 ± 0.02
AY17	0.99 ± 0.04	0.34 ± 0.01	0.51 ± 0.01

of AV7 and AY17 compared to AO52 were respectively 35.5 ± 1.3% and 51.7 ± 0.8%. AO52 would be the best azoreductase inductor among the three tested dyes (Fig. 3).

On the other hand, HPLC analysis of the enzymatic degradation mixture of AO52 tested at high concentration of AO52 (96 µM) let us the identification of the two expected metabolites DMPD and 4-ABS.

DISCUSSION

Pseudomonas putida mt-2 was able to degrade tested azo dyes prepared at 100 mg/l in mineral medium in two oxygenation conditions. Azoreduction products deriving from these dyes were identified in static degradation media (results not shown) which attests the expression of an azoreductase by this bacterium. This activity was similar to those reported for other *Pseudomonas* species (Chang *et al*., 2001; Nachiyar and Rajakumar, 2005). Hu (1994) and Chang *et al*. (2001) showed that oxygen had an inhibitive effect on these enzymatic reactions. This is in accordance with our results indicating that lowers was the oxygenation better was the rate of decolourisation. According to Chang *et al*. (2001), this behaviour can be attributed to a predominated competition for NADH utilization by the aerobic respiration. Moreover, the aromatic amines deriving from azoreduction could be further metabolized in several steps involving oxygenases encoded by the plasmid pWWO and by the chromosome of *P. putida* mt-2 (Results not shown).

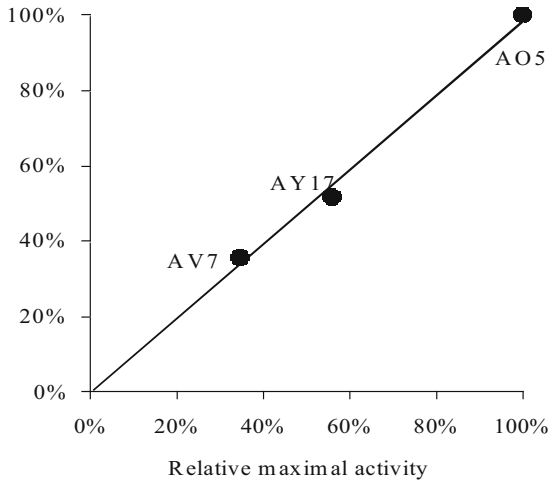


FIG. 3 - Correlation between the relative inductor potential and the relative azoreductase maximal activity of AV7, AY17 and AO52. The both are expressed in percentage (%) based on the ratio to AO52 taken as 100%.

The decolourisation kinetic of AO52 by *P. putida* mt-2 compared to those of AV7 and AY17 showed that the degradation rate of AO52 was consistently faster. With the aim of understanding the differentiated action of the bacterium on these three azo dyes, a kinetic study of the azoreductase activity was carried out on cell-free extracts. The suggestion of a degradation pathway implying enzymatic azoreduction (Ben Mansour *et al*., 2007) led us to attempt the identification of the two expected metabolites DMPD and 4-ABS in the reaction enzymatic mixture at high AO52 concentration (96 µM).

Results showed the same half saturation constant (K_m) for a given dye as a substrate whatever the dye inductor, from where the assumption of a same protein with nonspecific azoreductase activity. On the other hand, cell-free extracts activities, in several "inductor-substrate" crossing configurations, allowed concluding that AO52 was in the same time the best inductor and the best substrate of the azoreductase in comparison with the two other dyes. AV7 was the more resistant to the action of intact cells or cell-free extracts. The difference between dyes from the enzymatic attack point of view can be correlated to their chemical structures. According to Suzuki *et al*. (2001), the presence of a methyl group located on the benzene ring in para position to the azo group was identified as the most significant structural factor that improved the biodegradation of a diazoic compound. Based on this, the better biodegradability of AO52 could be explained by the presence of the hydrophobic dimethylamino group, similar to a methyl one, and also located in para position to the azo linkage. However, the presence on the naphtol ring of the polar (hydroxyl) and charged (sulfonic) groups close to the azo bond would hinder the azoreduction of AV7, as reported by Zimmermann *et al*. (1982). In the case of AY17, its weak biodegradation rate could be imputed to the presence of the sulphonic and chloro substituents as suggested by Guo *et al*. (2006). The chemical structure seems again decisive in the induction process.

Iner-classification of the dyes with respect to azoreductase (Fig. 3) is the same according to their relative inductor potential or to their relative V_{max}, and this agree well with the behaviour of intact cells whose action was marked more on AO52 than on AV7 or AY17. The azoreduction via *P. putida* mt-2 cells may be limited by the transport of azo dyes molecules across the cell membrane. Mechsner and Wuhrmann (1982) reported the importance of the transport of sulfonated azo dyes as prior step to decolourisation. These authors have compared the removal rate of sulfonated azo dyes by intact cells vs. permeabilised cells of *Bacillus cereus* and have found a better rate of decolourisation with these last. The slight

induction potential of AV7 might be a consequence of a transport limitation of this dye across the cell membrane. Indeed, two sulfonic (a strongly charged anionic moiety), and zwitterionic hydroxyl groups increase the polarity of AV7 and could prevent its penetration into the cell, which limits its induction potential.

CONCLUSION

Acids yellow 17, violet 7 and orange 52, are a very important commercial azo dyes used in the textile, food, paper and cosmetic industries. *Pseudomonas putida* mt-2 was found able to decolourise these dyes at concentrations up to 100 mg/l, level usually found in industrial effluents (Zollinger *et al*., 1987). The culture media was completely or partially decolourised under static incubation, this faster than under continuous shaking incubation, showing that excess of oxygen limited the azo dyes biodegradation. This activity is due the expression of an azoreduction system by this bacterium. Results showed the same half saturation constant K_m for a given dye as a substrate whatever the dye inductor, from where the assumption of a same protein with nonspecific azoreductase activity. We have also noticed that the level as well the induction as the specificity of the azoreductase activity seems to be strongly dependent on the chemical structure of the tested dyes. In fact, AO52 was in the same time the best inductor and the best substrate of the azoreductase in comparison with the two other dyes.

Azoreduction was followed by an oxygen-dependent metabolization of amines formed in previous step (results not shown) which may be ascribed the genotoxic effects (Ben Mansour *et al*., 2007). Azoreduction process seems to be a key step for the complete degradation of azo dyes. However, mass balances based on the parent dye and the products are needed to exclude other mechanisms of biodegradation.

The physicochemical properties of the azoreductase may open new possibilities for its biotechnological applications and allow the use of *P. putida* mt-2 in the treatment of azo dyes in industrial effluents.

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