

Bacterial Decolorization of Acid Orange 7 in the Presence of Ionic and Non-Ionic Surfactants

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The effects of the non-ionic surfactant Triton® X-100, the cationic surfactant cetyltrimethylammonium bromide (CTAB) and the anionic surfactant sodium *N*-lauroyl sarcosinate (SLS) on the decolorization of the reaction medium containing the monoazo dye Acid Orange 7 (AO7) by *Alcaligenes faecalis* and *Rhodococcus erythropolis* were studied. It was found that the surfactants influenced in different ways the rate of decolorization. At all concentrations tested the non-ionic surfactant Triton X-100 decreased the decolorization rate of *R. erythropolis*. At concentrations above the critical micelle concentration (CMC) Triton X-100 upset the usually observed exponential decay of the dye with *A. faecalis* due probably to the existence of an outer membrane in this organism. In concentrations above the CMC the anionic surfactant SLS inhibited the decolorization and, at prolonged incubation, caused partial release of the bound dye. The cationic surfactant CTAB in concentrations above and below the CMC accelerated drastically the binding of AO7 to the cells causing a rapid staining of the biomass and complete decolorization of the reaction medium. An attempt was made for explanation of the observed differences by the negative electrostatic charge of the living bacterial cell.

Key words: Azo Dyes, Biodegradation, Decolorization

Introduction

The pollution of water basins with a great number of different azo dyes wasting from various branches of the industry is regarded nowadays as one of the most serious environmental problems (Ølgaard *et al.*, 1998; O'Neil *et al.*, 1999; Robinson *et al.*, 2001). Azo dyes are synthetic organic compounds having in their structure one or more azo bonds ($-N=N-$). The azo bond is not typical for the natural products and is very resistant to biological attack (Stolz, 2001). For this reason the decolorization of azo-dye-containing effluents by the conventional wastewater treatment techniques is usually rather ineffective. Wastewater contains various compounds, which additionally complicate the problem.

The elimination of the recalcitrant azo dyes is due to the metabolic activity of many microorganisms, predominantly bacteria, belonging to various genera (Banat *et al.*, 1996). As a compulsory step the process includes binding of the dye to the microbial cell followed or accompanied by a coinciding reductive split of the azo bond in conditions of reduced partial pressure of oxygen (Keck *et al.*,

1997; Kudlich *et al.*, 1996; Li and Bishop, 2004; Stolz, 2001; Walker, 1970). It could be expected therefore that the microbial degradation of the azo dyes will depend in certain extent on the cell/medium interfacial tension, *i.e.* on the presence of surface-active agents, which are common accompanying pollutants in both the municipal and the industrial wastewater (Delée *et al.*, 1988). To elucidate their effect on this process we studied the decolorization of a reaction medium containing the monoazo dye Acid Orange 7 (AO7) by pre-cultivated cells of two bacterial species of opposite Gram-staining type in the presence of surfactants with different ionic characteristics.

Materials and Methods

Microorganisms

Two bacterial strains of opposite tinctorial type, the Gram-negative (Gm–) *Alcaligenes faecalis* 6132 and the Gram-positive (Gm+) *Rhodococcus erythropolis* 24, were chosen for the study after a preliminary screening for decolorization of AO7 by the strains maintained in the microbial collec-

tion of the Department of Microbial Biochemistry of the Institute of Microbiology of the Bulgarian Academy of Sciences, Sofia, Bulgaria.

Reagents

The surfactants Triton X-100 [polyoxyethylene-(10)octylphenyl ether, Fig. 1], CTAB (cetyltrimethylammonium bromide, Fig. 1) and SLS (sodium *N*-lauroyl sarcosinate, Fig. 1) were from Sigma (St. Louis, MO). The azo dye AO7 (Acid Orange 7, 4-[(2-hydroxy-1-naphthalenyl)-azo]-benzenesulfonic acid sodium salt, Tropaeolin 000 No.2 for microscopy, Fig. 1) was from Fluka Chemie AG (Buchs, Switzerland). All other reagents were of analytical grade.

Media

The complex nutrient broth Standard I was from Merck AG (Darmstadt, Germany). A mineral salt solution, pH 7.2, based on prescription No. 465 of DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) with omitted sources of N and S was used as the reaction medium.

Preparation of microbial suspension

The microorganisms were cultivated for 24 h at 28 °C on a rotary shaker in 200-ml cotton-plugged Erlenmeyer flasks, each containing 50 ml nutrient broth Standard I. The cells were harvested by centrifugation, washed and resuspended in the reaction medium to measure the optical density of a 10-fold diluted sample 0.5 at 540 nm. The thus prepared suspension contained 27 g l^{-1} cells (calculated as dry mass).

Permeabilization

20% v/v toluene was added to the cell suspension. The mixture was vortexed vigorously for 3 min. The cells were harvested by centrifugation, washed and resuspended in the reaction medium to the initial concentration.

Decolorization

The decolorization was carried out in 100-ml iodine flasks, each containing 30 ml cell suspension. The surfactants were added in concentrations below and above their critical micelle concentration (CMC). AO7 was added to the final concen-

tration 10 mg l^{-1} . The flasks were plugged with plastic stoppers and incubated statically at 28 °C.

Comparative experiments with non-treated cells were carried out throughout the experiment.

AO7 assay

A sample of 1 ml was clarified by centrifugation. The residual AO7 was evaluated by the optical density of the clear supernatant at 483 nm. The time of the first sample was indicated as zero time.

Fitting of the experimental data

The experimental data were fitted and evaluated statistically by means of the program Table Curve 1.10 (Jandel Scientific, AISN Software Inc., San Rafael, CA).

Results and Discussion

Time course of decolorization

The experimental results obtained in this study reveal considerable differences in the dynamics of decolorization of the reaction medium in dependence on both the concentration and the ionic characteristic of the surfactants tested.

In all control experiments, as well as in the majority of the experiments carried out with treated cells, the concentration of the residual dye was found to decrease with the time following the pattern of first-order exponential decay,

$$C = \alpha + Ae^{-\lambda t}, \quad (1)$$

where C (g l^{-1}) stands for the residual AO7 concentration at time t (h), A (mg l^{-1}) is the amplitude of the function, α (mg l^{-1}) the value of the horizontal asymptote to which the function tends to approach, and λ (h^{-1}) a time constant describing the rate of decolorization.

The model of the exponential decay (1) is widely used for the formal description of a great variety of processes occurring with decrease of quantity. Its application for the description of microbial decolorization of AO7 is also known (Méndez-Paz *et al.*, 2003). Here we have to add only that this plain model hardly could give a satisfying idea of the physiological aspect of the studied process because of the extreme complexity of the microbial cell, its life functions and interactions with the environment. However, this model gives the useful option for an exact numeric comparison of the decolorization rates observed in different conditions and

this opportunity will be used in the following discussion where possible.

Zero time concentration of the dye

In all experiments we found a considerable difference between the estimated zero time concentration of the dye, C_0 (g l^{-1}),

$$C_0 = \alpha + A, \quad (2)$$

and the amount of the dye added initially into the reaction mixture, C_i (mg l^{-1}).

The fact that the time between the addition of the dye and the first analysis was not more than 8 min suggests that the decolorization of the reaction medium includes a step of fast binding of the dye to the bacterial cell. The amount of bound dye, $C_i - C_0$, is an important index of the process, which was found to depend drastically on the surfactant properties.

Effect of the non-ionic surfactant Triton X-100

With both test organisms the rise of Triton X-100 (Fig. 1) concentration in the reaction medium leads to a corresponding rise in the amount of bound dye with a simultaneous decrease of the decolorization rate, λ (Fig. 2). With the Gm+ *R. erythropolis* the decolorization dynamics keeps the pattern of the first-order exponential decay at all concentrations of the surfactant while with the Gm- *A. faecalis* the presence of Triton X-100 in concentrations above the CMC leads to an upset of the usual time course of the process. We suppose that the observed difference relates to certain

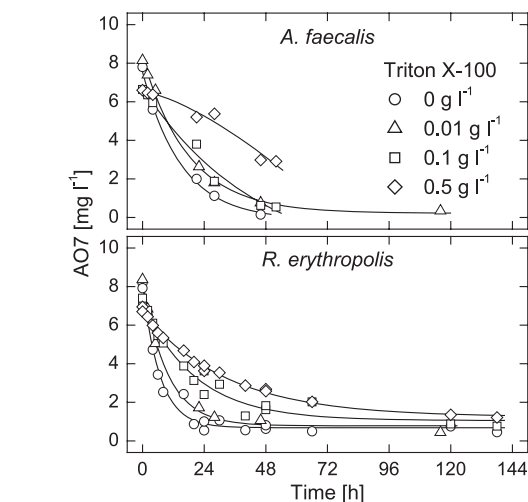


Fig. 2. Effect of different concentrations of Triton X-100 on the dynamics of decolorization of the reaction medium by cells of *A. faecalis* and *R. erythropolis*.

extent with the fundamental differences between cell wall structures of Gm+ and Gm- bacteria and, in particular, with the existence of an outer membrane in Gm- bacteria. From this point of view we do not exclude the reason of the observed disorder to be partial deterioration of the thin outer membrane of *A. faecalis* at increased concentrations of Triton X-100.

It is important to note that the inhibiting effect of Triton X-100 on the decolorization rate observed with *R. erythropolis* is not proportional to its concentration in the reaction medium. The plot

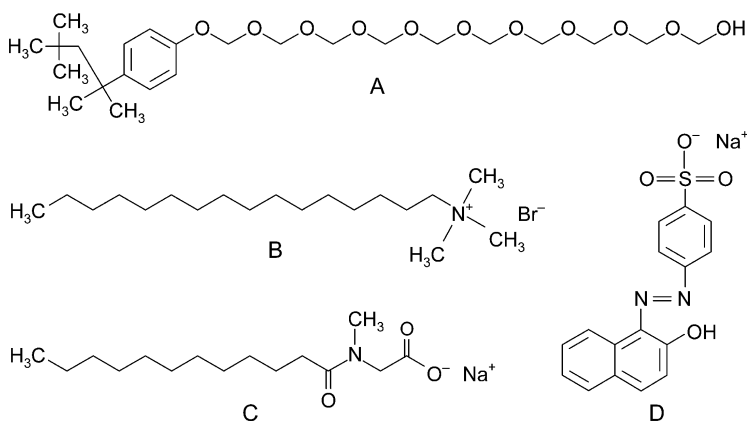


Fig. 1. Molecular structures of the organic compounds used in the study. A, Triton X-100 [polyoxyethylene(10)octyl-phenyl ether]; B, CTAB (cetyltrimethylammonium bromide); C, SLS (sodium *N*-lauroyl sarcosinate); D, AO7 (Acid Orange 7, 4-[(2-hydroxy-1-naphthalenyl)-azo]-benzenesulfonic acid sodium salt).

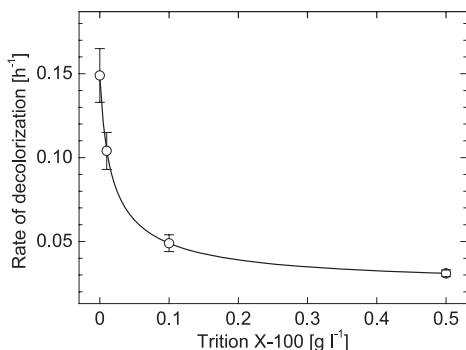


Fig. 3. Effect of different concentrations of Triton X-100 on the decolorization rate of the reaction medium by cells of *R. erythropolis*.

of estimated decolorization rates, λ , against respective surfactant concentrations (Fig. 3) reveals a fading strength of surfactant influence. Phenomenologically such an effect could be expected if the dye and the surfactant compete for the cell wall surface or for a certain definite structure.

Effect of the anionic surfactant SLS

It is notable that, unlike Triton X-100, the anionic surfactant SLS does not affect the dye-binding abilities of both *A. faecalis* and *R. erythropolis* at the first minutes after preparation of the reaction mixture (Fig. 4). At concentrations below the CMC the effect of SLS on the decolorization rate is similar to the effect observed at low concentrations of Triton X-100: with *A. faecalis* SLS at

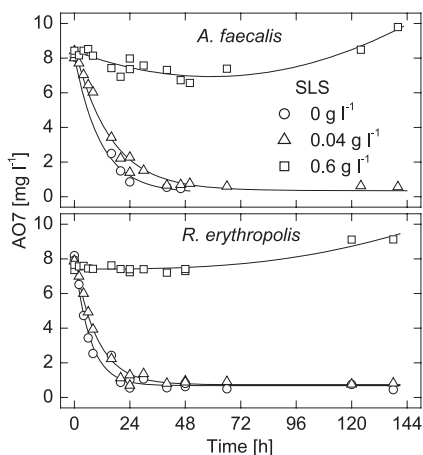


Fig. 4. Effect of different concentrations of SLS on the dynamics of decolorization of the reaction medium by cells of *A. faecalis* and *R. erythropolis*.

0.04 mg l⁻¹ causes a decrease of the decolorization rate, λ , from (0.087 ± 0.007) to (0.064 ± 0.005) h⁻¹; with *R. erythropolis* the decolorization rate at the same concentration of SLS decreases from (0.149 ± 0.013) to (0.103 ± 0.007) h⁻¹.

At concentrations above the CMC the anionic surfactant SLS stops completely the AO7 decolorization. It is known that some ionic surfactants, including SLS, have a moderate bactericidal effect. It can be assumed that the observed decolorization halt is due to cell death. The comparative experiment carried out using the strong non-specific inhibitor HgCl₂ confirmed our suggestion that both the dye-binding and its further reductive degradation are features of the living microbial cell (Fig. 5). If this assumption is true then the specific rise of the AO7 concentration at incubation for more than 120 h in the presence of SLS (Fig. 4) could be explained with a possible wash out of the dye from the cells which had already lost their dye-binding abilities.

An alternative explanation may conceive the fact that surfactants can affect the cell wall permeability and the integrity of the underlying membrane structures. This assumption seems quite reasonable and the possibility of increasing the azo dye degradation by permeabilization of the cell was already studied (Mechsner and Wuhrmann, 1982). The comparative experiments carried out with toluene-permeabilized cells (Fig. 6) show that it is not likely the effect of the surfactant to be associated with the changed permeability. As can

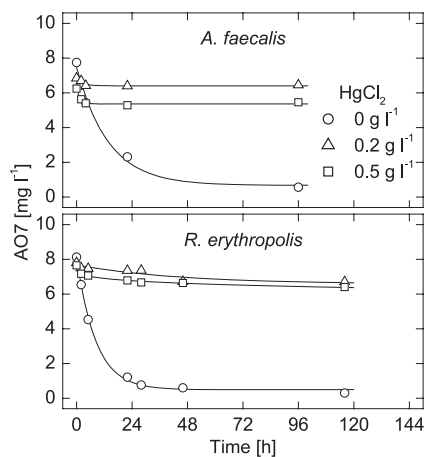


Fig. 5. Time course of decolorization of the reaction medium by cells of *A. faecalis* and *R. erythropolis* in the presence of HgCl₂.

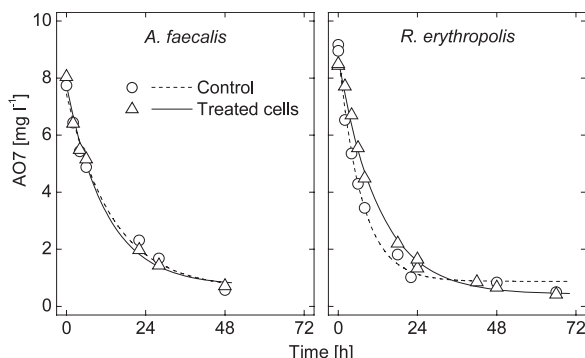


Fig. 6. Dynamics of decolorization of the reaction medium by toluene-permeabilized cells of *A. faecalis* and *R. erythropolis*. The decolorization rates are indicated in the text.

be seen by the graph the time courses of AO7 decolorization with permeabilized and non-treated *A. faecalis* cells are practically indistinguishable yielding values for the decolorization rates of (0.084 ± 0.010) and $(0.072 \pm 0.011) \text{ h}^{-1}$, respectively.

The difference between decolorization rates estimated with non-treated and with permeabilized cells of *R. erythropolis* is considerable: (0.144 ± 0.010) and $(0.081 \pm 0.005) \text{ h}^{-1}$, respectively. However, in this case we have all reasons to suppose that the observed difference is influenced in some extent by the specific high content of toluene-soluble fatty acids in the cell wall of this organism (Collins *et al.*, 1982).

In our opinion the effect of ionic surfactants is most probably associated with the electrostatic charge of the cell surface (a.k.a. zeta potential). The cell surface can be regarded as a multivalent ion containing negatively and positively charged groups, which ionize as a function of pH value, temperature and ionic strength of the suspending medium. At neutral pH most microbial cell surfaces are negatively charged (Smith *et al.*, 1998).

The fact that the change of the anionic surfactant concentration does not affect the dye-binding abilities of both *A. faecalis* and *R. erythropolis* can be interpreted as demonstration of the repellent properties of negatively charged cells towards a negatively charged surfactant.

Effect of the cationic surfactant CTAB

The effect of the cationic surfactant CTAB on the decolorization of AO7 was found to differ drastically from the effects of the non-ionic Triton

X-100 and the anionic SLS. Its influence on the process stands in good agreement with the assumption made above.

The presence of CTAB in the reaction medium causes rapid and strong binding of the dye to the cell. The reaction is so fast that at the time of the first measurement *ca.* 90% of the added dye turns out to be joined to the cells. The reaction medium becomes practically colourless and the cells become stained. The colour of the Gm– *A. faecalis* was found to fade faster compared to the decolorization of the Gm+ *R. erythropolis*.

The effect of CTAB can also be appointed to the negative electrostatic potential inducing the formation of an oriented layer of positively charged surfactant molecules around the cell. In this case the surfactant will act as a carrier of the dye molecule. This bilayer electrostatic mechanism is well established and justified for explanation of increased adsorptive properties of CTAB-modified zeolites for several reactive azo dyes (Armağan *et al.*, 2003). For a living cell this obvious mechanism is rather disputable. It could be expected that the hypothetical layer between the cell wall and the dye molecule will interfere with underlying enzyme structures, and will result in inhibition of the reductive decolorization of the bound dye: an assumption contradicting to the experimental observations. This apparent contradiction is not excluded in order to find a possible answer in the observed coadsorption of dyes and CTAB on negatively charged silica surface (Furst *et al.*, 1996). There are experimental data demonstrating that the electrostatic attraction results in the solubilization of the negatively charged dye Indigo Carmine in CTAB solution with the formation of a complex dye-surfactant monolayer on the micellar microsurface (Wang *et al.*, 2003). It should be noted however that the mechanism of coadsorption with the formation of a monolayer is still not completely elucidated.

Conclusions

The following conclusions can be drawn from the experimental results obtained in this study:

- The outer cell membrane of Gm– bacteria does not play a decisive role in the formation of their azo dye decolorization abilities. However, high concentrations of the non-ionic surfactant Triton X-100 obviously affect this outmost structure and an upset of the ordinarily ob-

served exponential decay pattern of the dye decolorization process takes place.

- b) The differences in the decolorization rate observed in the presence of surfactants with various ionic characteristics can not be explained by the expected permeabilizing effect of these compounds on the cell wall and membrane structures laying beyond the cell wall.
- c) The natural negative electrostatic charge of the living cell along with the ionic characteristic of the surfactant tested are the most important factors forming the specificity of the surfactant action.

As demonstrated, the total inhibition of cell metabolism leads inevitably to a strong decrease of the adsorptive properties of the cell and to complete loss of the azo dye decolorization abilities. Hence, the above conclusions are valid for surfactants in concentrations having no prominent antibacterial effect.

Acknowledgements

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