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Biodegradation of azo dyes by bacteria inoculated on solid media

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Abstract

Bacteria were inoculated on different solid media to attain biodegradability of an azo dye (Acid Orange 7). Kaolin, bentonite and powdered activated carbon (PAC) were selected to be used with cultures of *Enterobacter*, *Pseudomonas* and *Morganella* sp., as bacteria would be able to degrade several textile dyes. For the solid, to be employed as media, special characteristics are needed with regards to adsorption capacity for concentrating substrate within the cell environment and an adequate particle size and surface texture for assuring bacterial colonization. Only PAC with 0.490 mm particle size shows these characteristics among the solids used and it was colonized by a high number of cells from the three cultures. Dye was degraded following a second-order kinetics. A mechanism for dye degradation is proposed in which anaerobic and aerobic microniches in the PAC particle perform cleavage of the azo bond and oxidation of the amines formed in the same biocatalytic particle.

Keywords: Acid Orange 7; Biodegradation; Dye; PAC; Solid media; Textile

1. Introduction

Azo dyes are considered as electron-deficient xenobiotic compounds because they possess the azo (N=N) and sulfonic ($-SO_3^-$) electron-withdrawing groups, generating electron deficiency in the molecule and making the compound less susceptible to oxidative catabolism by bacteria. As a consequence, azo dyes tend to persist under aerobic environmental conditions [1].

Some specialized strains of aerobic bacteria have developed the ability to use azo dyes as sole source of carbon and nitrogen [2,3]; others only reduce the azo group by special oxygen-tolerant azo reductases. However, these azo reductases have a narrow substrate range [4–6]. Recently Chen et al. [7] have described bacterial strains which display good growth in aerobic or agitation culture, but color removal was obtained with a high efficiency in anoxic or anaerobic culture.

Azo linkages are easily reduced under anaerobic conditions [8,9], yielding colorless aromatic amines which, with a few exceptions [10], are not mineralized anaerobically, but are readily degraded aerobically. Therefore, a combination of anaerobic and aerobic conditions is proposed for azo compounds' mineralization [11,12].

Microbial decolorization of reactive azo dyes has been reached by sequential anaerobic–aerobic system [13]. Integration of anaerobic and aerobic conditions in a single bioreactor has been developed with a methanogenic granular sludge exposed to oxygen [14,15]. Also a simultaneous anaerobic and aerobic degradation for an azo dye by two different cultures immobilized in calcium alginate beads was described [16]. In this case, one bacterial strain caused the reduction of the azo dye in the anaerobic zone leading to the formation of aromatic amines, and the second strain could mineralize the amines in the aerobic zone.

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Several solid support media are used to attach biofilms in bioreactors, such as sand, diatomaceous earth, activated carbon, polyurethane foam and glass. Specially, the presence of powdered activated carbon (PAC) in the biological treatment of organic toxic pollutants increases the removal rate. Several mechanisms have been suggested in these processes, for example, stimulation of biological activity and bioregeneration of activated carbon [17–23]. However, a deeper knowledge regarding the role of solid media on biodegradation of recalcitrant organic toxics is required.

The objectives of this work are to investigate the effect of solid media, kaolin, bentonite and activated carbon on a monoazo dye (Acid Orange 7) biodegradation by three different bacteria under aerobic conditions and to study the kinetics and mechanism of the decolorization process. Bacteria will be inoculated on the solid surface and dye concentration will be followed in the course of time. Special attention will be paid to the properties of the solids used for fixation of bacteria. The azo dye selected for the study was Acid Orange 7 (CI 15510), widely used in textile factories and degraded by these microorganisms in previous experiments.

2. Materials and methods

2.1. Bacterial strains

Bacteria used in this research were isolated in previous studies by Márquez and Costa [19] from a PACT process, and identified as *Enterobacter* sp. (E), *Pseudomonas* sp. (P) and *Morganella* sp. (M). These microorganisms were cultivated on plates with several textile dyes as carbon source in basal mineral medium, BMM (NaCl 7 mg/L, $CaCl_2 \cdot 2H_2O$ 4 mg/L, $MgSO_4 \cdot 7H_2O$ 2 mg/L), dye 5000 mg/L, agar 3000 mg/L (Bacteriological European Type, Panreac), and pH 7.0.

2.2. Decolorization of Acid Orange 7 in liquid medium

2.2.1. Culture media

Three different liquid media were used:

a. BMM and 20 mg/L Acid Orange 7
b. BMM with 100 mg/L glucose and 20 mg/L Acid Orange 7
c. BMM with 160 mg/L peptone, 110 mg/L meat extract, 30 mg/L urea and 20 mg/L Acid Orange 7.

Assays were performed in 500 mL Erlenmeyers containing 300 mL of liquid media. Cells of *Enterobacter* sp., *Pseudomonas* sp. and *Morganella* sp., collected from agar plates (BMM, agar and Acid Orange 7) were inoculated separately and combined into the media. The cultures were incubated at 30 °C under magnetic stirring at 150 rpm. Control was performed without bacteria inoculum.

2.2.2. Addition of solid media

Solid media (0.5 g/L bentonite or kaolin of Aldrich, or activated carbon of Panreac) were added onto the liquid medium 'c' (BMM and dye supplemented with peptone, meat extract and urea).

Three particle sizes were selected for activated carbon, 0.490 mm, 0.125 mm and smaller than 0.100 mm. Adsorption capacity for Acid Orange 7, was determined for each solid before carrying out decolorization tests with bacteria inocula.

2.2.3. Analytical methods

Growth of microorganisms in liquid media was followed in the course of time by optical density at 600 nm taking samples

Table 1
Bacterial growth on plates with different dyes as sole source of carbon

Dye	Bacteria	Growth		
		96 h	144 h	192 h
Disperse Yellow 42 (CI 10338, Nitro)	E	−	−	++
	P	+	+	++
	M	−	−	+
Acid Yellow 99 (CI 13900, Monoazo)	E	+	++	++
	P	++	++	++
	M	+	+	+
Acid Blue 158 (CI 14880, Monoazo)	E	−	−	+
	P	+	+	+
	M	−	−	+
Acid Orange 7 (CI 15510, Monoazo)	E	+++	+++	+++
	P	+++	+++	+++
	M	+++	+++	+++
Acid Black 60 (CI 18165, Monoazo)	E	−	−	+
	P	+	++	++
	M	−	−	+
Reactive Red 2 (CI 18200)	E	−	−	−
	P	+	++	++
	M	−	−	++
Reactive Orange 4 (CI 18260)	E	−	−	−
	P	+	+	+
	M	+	+	+
Acid Yellow 17 (CI 18965)	E	−	−	−
	P	+	+	++
	M	−	−	−
Direct Red 79 (CI 29065, Disazo)	E	−	−	−
	P	+	++	++
	M	−	+	+
Direct Red 80 (CI 35780, Polyazo)	E	−	−	−
	P	+	++	++
	M	−	−	−
Direct Orange 34 (CI 40215, Arylamine)	E	−	−	−
	P	+	+	++
	M	−	−	−
Acid Blue 7 (CI 42080, Triphenylmethane)	E	−	−	−
	P	+	+	+
	M	−	+	+
Reactive Blue 4 (CI 61205, Anthraquinoide)	E	−	−	−
	P	+	++	++
	M	−	+	+

E: *Enterobacter* sp.; P: *Pseudomonas* sp., M: *Morganella* sp.
(+) Growth; (−) no growth.

every 24 h (5 mL). To analyze dye concentration, samples (5 mL) were centrifuged at 4000 rpm during 30 min and the absorbance values of supernatants were determined at 484 nm (wavelength of maximum absorbance for dye).

In liquid media where solids were added for supporting bacterial growth, after 30 and 45 days of incubation, surfaces of solids were visualized by scanning electron microscope (SEM) to observe colonization of microorganisms.

3. Results and discussion

3.1. Growth on plates

In order to test the activity of the studied bacteria on different dyes, experiments are carried out on plates. *Pseudomonas* sp. (P) is the microorganism that shows the highest activity on dyes selected. It grows considerably on plates since 96 h of incubation (Table 1), using different classes and chemical subclasses of textile dyes, including azo, nitro, triphenylmethane and anthraquinoide types as only source of carbon (BMM, dye and agar). At 192 h *Pseudomonas* sp. continues growing on all the selected dyes, *Morganella* sp. (M) on 10 of them and *Enterobacter* sp. (E) only on 5. Although *Enterobacter* sp. and *Morganella* sp. do not show similar activity which the *Pseudomonas* sp. does, this is the first time in which growth on textile dyes of these microorganisms is reported.

3.2. Selection of liquid medium

The three microorganisms assayed, isolated or combined, were not able to grow in liquid medium where the carbon source was only the dye (a) or supplemented with glucose (b).

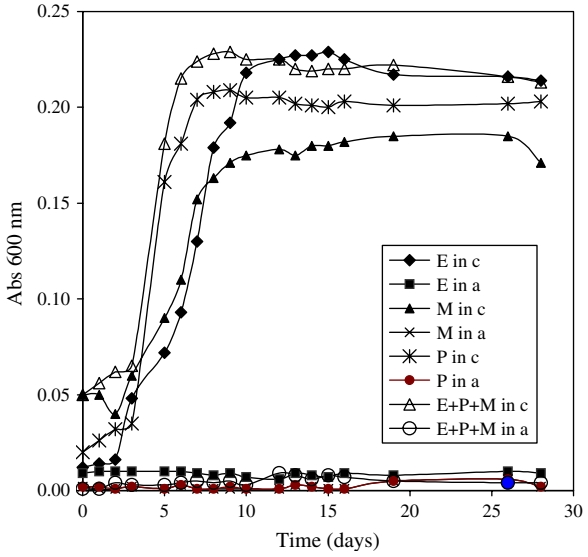

Fig. 1. Bacterial growth of *Enterobacter* sp. (E), *Pseudomonas* sp. (P) and *Morganella* sp. (M) in different media. Media a: BMM and dye and media c: BMM, dye, peptone, meat extract and urea.

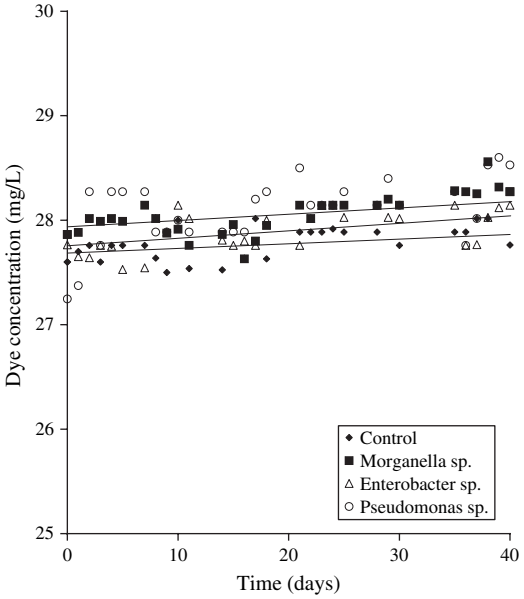

Fig. 2. Time-course profile of dye concentration for three bacteria at 30 °C in supplemented medium c (BMM, dye, peptone, meat extract and urea).

Growth was displayed only when the medium contained peptone, meat extract and urea (c) (Fig. 1). For this reason this medium was selected to continue the study.

Although the three strains showed growth in liquid medium supplemented (c), no dye degradation was observed after 45 days of incubation at 30 °C (Fig. 2). Because the strains can grow on plates in 24 h using the dye as sole source of carbon, these results suggested that a surface like agar is required for a good growth and dye degradation.

3.3. Effect of solid media: bentonite, kaolin and powdered activated carbon

Adsorption capacity for each support is presented in Table 2. In contrast to powdered activated carbon (PAC), bentonite and kaolin practically do not adsorb Acid Orange 7. Powdered activated carbon has a high adsorption capacity (120–255 mg/g) depending on the particle size and it seems to be a good solid support for microorganisms: it concentrates substrate on the surface where bacteria can degrade it. Solid supports were saturated with dye before bacteria were inoculated.

Fig. 3 reflects dye concentration profiles in the course of time for the experiments in which bacteria were inoculated on the solid surface (bentonite and kaolin). Solids concentration was

Table 2
Acid Orange 7 adsorption capacity for different supports

Support	Bentonite and kaolin	PAC		
		<0.100 mm	0.125 mm	0.490 mm
Adsorption capacity (mg dye/g support)	2	255	195	120

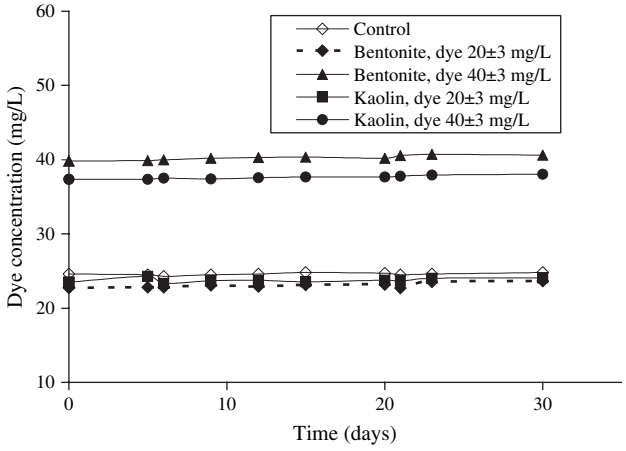

Fig. 3. Time-course profile of dye concentration in batch experiments where bacteria were added to the liquid media with bentonite and kaolin. Inoculum was the combination of the three strains (*Enterobacter* sp., *Pseudomonas* sp. and *Morganella* sp.). Control neither has support nor inoculum.

0.5 g/L and all cultures were suspended in a rotatory shaker. The concentration of Acid Orange 7 in liquid medium using bentonite and kaolin did not modify after 30 days of incubation, which means colonization of microorganisms was not produced. This behavior agrees with the one previously reported by Pala and Tokat [24], regarding the bentonite addition to an activated sludge system for the treatment of cotton textile wastewater.

Results obtained for bacteria inoculated on powdered activated carbon at different particle sizes are shown in Fig. 4. The three strains (*Enterobacter*, *Pseudomonas* and *Morganella*) were added under sterilized conditions to the liquid media in which powdered activated carbon and microorganisms were suspended by a rotatory shaker. PAC concentration in all cases was 0.5 g/L. After adsorption equilibrium (dye concentration falls to about 20% of residual dye) culture of microorganisms was maintained to attain solid colonization.

Culture with *Enterobacter* sp. (Fig. 4a) and activated carbon of particle size 0.490 mm shows clearly the dye concentration to remain constant until 16 days of incubation and a further decrease that could only be explained by the beginning of the biodegradation process. Cultures with *Pseudomonas* sp. or *Morganella* sp. and PAC 0.490 mm show activation of the biodegradation process to start at 24 and 21 days, respectively (Fig. 4b and c).

Biodegradation of Acid Orange 7 was not observed in liquid cultures with PAC particle size of 0.125 mm and <0.100 mm in all strains (Fig. 4a, b and c). Thus, particle

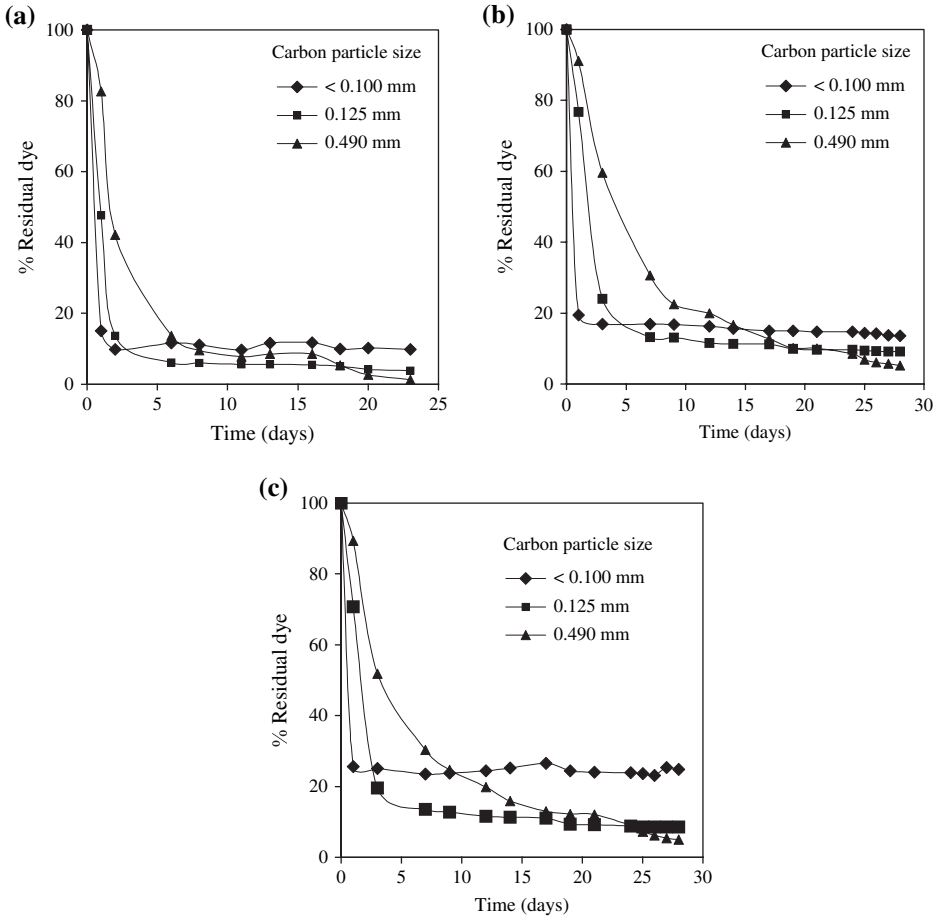

Fig. 4. Time-course profile of dye concentration in suspended cultures with PAC: (a) *Enterobacter* sp. (b) *Pseudomonas* sp. (c) *Morganella* sp.

size seems to be an important factor on surface colonization of bacteria on activated carbon.

3.4. Kinetic model of dye degradation

To study kinetic behavior of dye degradation on PAC surface, Acid Orange 7 was added to the suspensions of bacteria colonized on activated carbon and dye concentration was adjusted to 30 ± 3 mg/L for all liquid cultures. Degradation curves are shown in Fig. 5.

Kinetic model for dye degradation in liquid media can be obtained by applying a substrate mass balance to a stirred, well mixed bioreactor in these terms:

$$\text{Accumulation} = \text{Input} - \text{Output} + \text{Variation} \tag{1}$$

In mathematical form Eq. (1) becomes:

$$V\frac{\mathrm{d}S}{\mathrm{d}t} = QS_\mathrm{o} - QS + Vr_\mathrm{su} \tag{2}$$

where, S means substrate concentration in the reactor and effluent, S_o influent concentration, Q volumetric flow, V reactor volume and r_su degradation rate (substrate utilization rate).

For a batch reactor there is not incoming neither effluent flow: input and output terms are null. Eq. (2) takes the form:

$$\frac{\mathrm{d}S}{\mathrm{d}t} = r_\mathrm{su} \tag{3}$$

Several degradation kinetic models regarding substrate concentration were assayed: Monod and Contois-type equations and kinetics for different reaction orders. Results suggest second-order kinetics with regards to dye concentration:

$$r_\mathrm{su} = -kS^2 \tag{4}$$

Integrating the differential equation:

$$-\int_{S_\mathrm{o}}^{S}\frac{\mathrm{d}S}{S^2} = \int_0^t k\,\mathrm{d}t \tag{5}$$

$$\frac{1}{S}\Big]_{S_\mathrm{o}}^{S} = kt\Big]_0^t \tag{6}$$

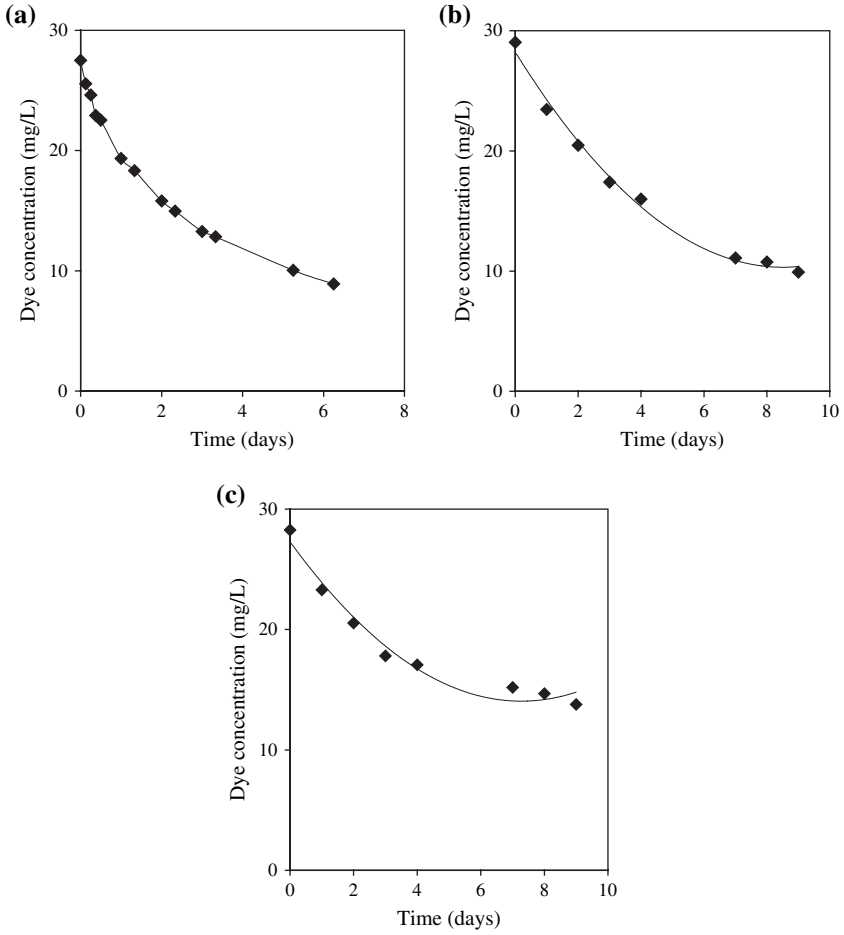

Fig. 5. Time-course profile of dye concentration in batch systems for (a) *Enterobacter* sp., (b) *Pseudomonas* sp. and (c) *Morganella* sp., with PAC of particle size 0.490 mm.

$$\frac{1}{S} - \frac{1}{S_o} = kt \tag{7}$$

$$\frac{1}{S} = \frac{1}{S_o} + kt \tag{8}$$

Correlation of experimental data shows second-order kinetics of Eq. (8) to explain the behavior of dye degradation (Fig. 6). Although in the case of Fig. 6c (*Morganella* strain) a weak deviation from the kinetic model proposed appears, dye degradation seems to follow this model.

Kinetic coefficients obtained from Fig. 6 are as follows:

For *Enterobacter* sp., $k_E = 0.0119$ L/(mg days)
For *Pseudomonas* sp., $k_P = 0.0075$ L/(mg days)
For *Morganella* sp., $k_M = 0.0037$ L/(mg days)

3.5. Mechanism of bacterial dye degradation

According to the hypothesis outlined initially in connection with the necessity of a solid surface to activate the mechanism of dye degradation, it is clear that surface should have certain characteristics. Dye decolorization by bacteria was not successful when bentonite and kaolin were used as support. This result could be explained by poor dye adsorption of these supports due to the absence of pores (Fig. 7), which means no enough concentration of substrate. On the other hand surfaces of bentonite and kaolin are not adequate for microbial growth. Kaolin (Fig. 7a) has a small particle size (less than 1 μm) and a scaly texture which is not a good environment for bacterial colonization. Particle size is in the order of bacterial size and it has to be much greater. Bentonite (Fig. 7b) is in the order of the particle size desired (more than 50 μm) which allows bacterial groups colonizing the surface, but it has a flat structure almost without pores.

However, PAC of particle size <0.100 mm and 0.125 mm with high adsorption capacity for Acid Orange 7, neither produces any effect on decolorization process (Fig. 4). Only PAC of particle size 0.490 mm with smaller adsorption capacity was able to activate strains for azo dye biodegradation. These facts are indicative of the theory defended in this article about special surface characteristics responsible for the effect on bacterial decolorization. Analysis by SEM after 30 days of incubation of the strain *Enterobacter*

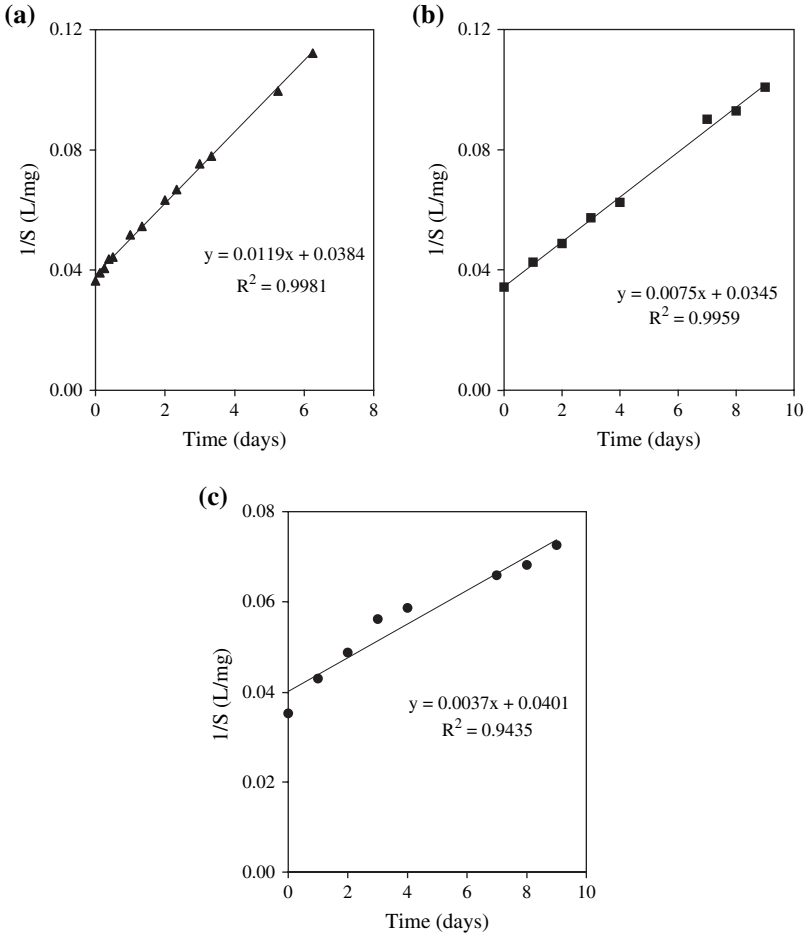

Fig. 6. Graphical correlation of dye degradation according to a second-order kinetics for (a) *Enterobacter* sp., (b) *Pseudomonas* sp. And (c) *Morganella* sp.

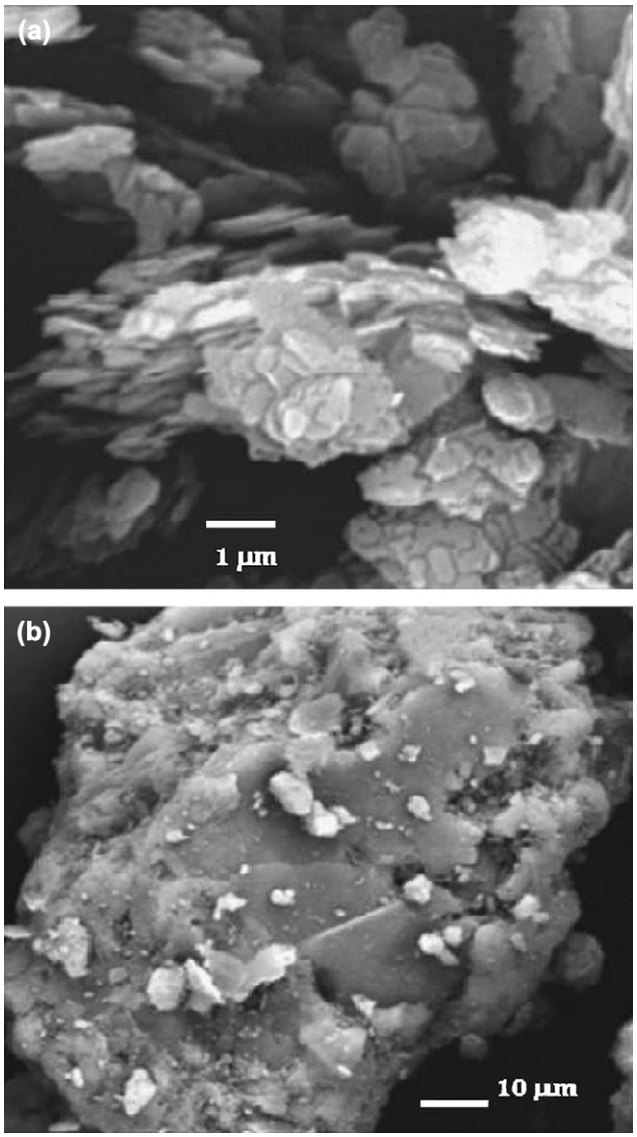

Fig. 7. SEM microphotographs of (a) kaolin and (b) bentonite.

sp. on PAC with 0.490 mm particle size (Fig. 8) confirms the microorganisms colonizing the surface, especially, inside of carbon pores. Spectacular colonization on PAC of particle size 0.490 mm after 45 days of incubation with the strain *Morganella* sp. (Fig. 9c) explains dye biodegradation in Fig. 4 and the high culture activity with this particle size. The samples from cultures of PAC with microorganisms which show no dye biodegradation in Fig. 4, did not have a high colonization after 45 days of incubation (Fig. 9a and b).

Based on these findings, we propose that mesopores of PAC with particle size 0.490 mm generate microenvironmental conditions in which anaerobic and aerobic microniches develop and coexist within a single biocatalytic particle (Fig. 10), so that oxidative and reductive activities can be accomplished simultaneously [25]. Therefore, multistep complementary biodegradation process can be

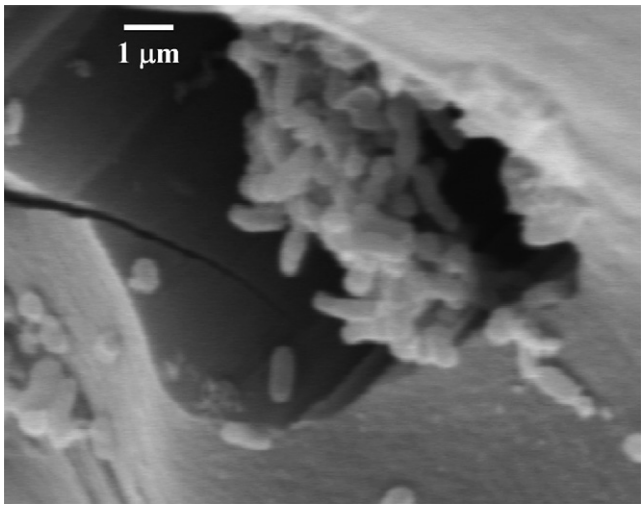

Fig. 8. SEM microphotographs of *Enterobacter* sp. with PAC of particle size 0.490 mm after 30 days of incubation.

conducted as a single stage. In this case a time of adaptation is necessary for bacteria to attach the inner surface (''anaerobic phase''), where reduced metabolites are generated and then degraded by associated microorganisms at outer surface (''aerobic phase'') which complete mineralization process.

4. Conclusions

For the removal of dyes from wastewaters, a combination of the anaerobic cleavage with an aerobic zone to degrade the amines formed was generally accepted. Two strategies have been developed: sequential and simultaneous processes. Sequential processes combine the anaerobic and the aerobic steps either in the same reaction vessel or alternatively in a continuous system in separate vessels. The simultaneous processes utilize anaerobic zones within basically aerobic bulk phases, such as observed in biofilms [25–27], granular sludge or biomass immobilized in other matrices [14–16]. In the sequential and simultaneous processes, auxiliary substrates are required, for supplying bacteria with a source of reduction equivalents for the cleavage of the azo bonds.

In this research we demonstrate that the addition of a bacterial culture on the surface of solid media (PAC) produces azo dye decolorization. Particle size and surface texture have played an important role on dye biodegradation because substrate has to be concentrated in the proximity of the cells (adsorption capacity of the solid) and an irregular solid surface and a proper particle size are required. Biodegradation on PAC follows a second-order kinetics with respect to dye concentration.

In conclusion, biodegradation of recalcitrant compounds as azo dyes can be facilitated by the presence of surfaces that have convenient pores to promote formation of microaerophilic niches and permit microbial growth. Furthermore,

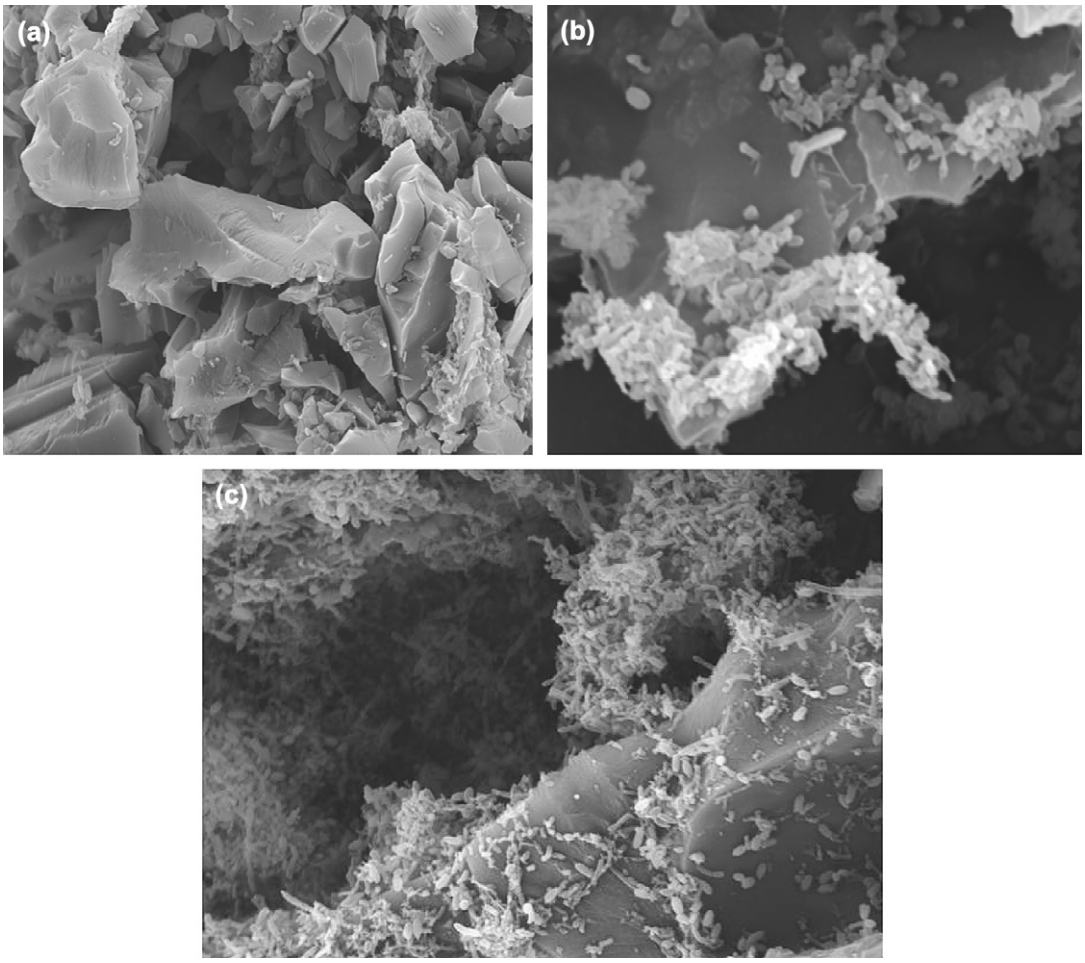

Fig. 9. SEM microphotographs of PAC with *Morganella* sp. inocula for different particle sizes after 45 days of incubation: (a) PAC < 0.100 mm, (b) PAC 0.125 mm and (c) PAC 0.490 mm.

they should have adsorptive capacity of the pollutant and nutrients to assure bacterial feeding. In this sense, some agroindustrial residues could be used as convenient supports.

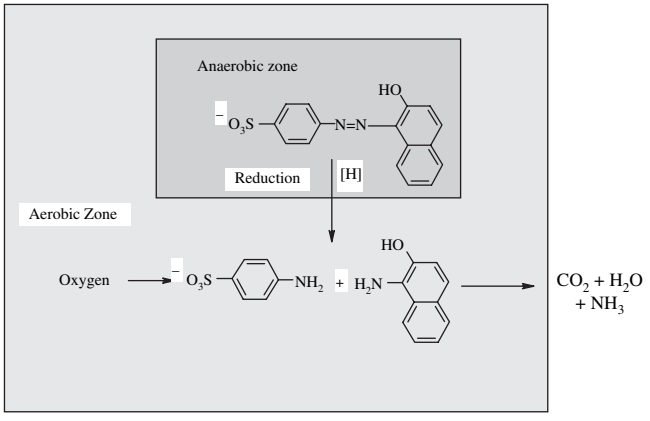

Fig. 10. Proposed mechanism for dye degradation in anaerobic and aerobic microniches of support colonized by bacteria.

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