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Degradation of immobilized azo dyes by *Klebsiella* sp. UAP-b5 isolated from maize bioadsorbent

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ABSTRACT

The degradation of two immobilized dyes by *Klebsiella* sp. UAP-b5 was studied. In batch experiments, the azo dyestuffs Basic Blue 41 and Reactive Black 5 were immobilized onto corn cobs by adsorption, and the adsorption process was characterized by a pseudo-second-order kinetic equation. *Klebsiella* sp. UAP-b5 was previously isolated from the corn waste and shown to decolorize these dyes in liquid systems. Here, we demonstrate anaerobic decolorization and reductive biodegradation of these dyes by means of spectrophotometry, HPLC, and IR spectroscopy of the solid waste and desorption solutions. We also demonstrate adsorption of compounds that resemble known degradation products.

1. Introduction

Azo dyes are significant water pollutants near textile industries. In recent years, there has been an increase in the study of microorganism-mediated azo dye degradation.

Azo dyes are the most important group of synthetic dyestuffs. Reactive Black 5 (RB5), a complex azo dye, can be degraded by continuous anaerobic/aerobic processes that yield sulfonated amines which can then be degraded aerobically [1]. Banat et al. [2,3] reviewed initial reports on microbial degradation of RB5 and other dyes. More recent studies have shown that bacteria [4–8] and different fungal strains [9] and their enzymes [10,11] can metabolize RB5. Under aerobic conditions, the enterobacteria *Enterobacter agglomerans* (reclassified as *Pantoea agglomerans*) and *Klebsiella pneumoniae* decolorized the azo dye methyl red [12,13]. In addition, several *Klebsiella* strains biodegraded *N*,*N*′-dimethyl-*p*-phenylenediamine, a hazardous metabolite produced in the bio-transformation of azo dyes [14]. Following degradation, neither RB5 nor Basic Blue 41 (BB41) releases any of the 22 amines that are restricted by a 2003 EU directive [15]. Nevertheless, 1-aminonaphthalene, which comprises part of the RB5 molecule, is a suspected carcinogen [16] and the *N*-hydroxy metabolite of 1-aminonaphthalene is carcinogenic and mutagenic [17]. In addition, 2-aminonaphthalene, which is a molecular moiety of one of the degradation products of RB5, induces bladder cancer [18]. Compounds related to RB5 degradation products, 4-(4-hydroxy-1-naphthylazo)benzenesulfonic acid salt and 4-aminobenzenesulfonic acid, have LD_{50}s of 1 g/kg and 6 g/kg (respectively) in rats [19].

Additional research on degradation of dyes has focused on joint adsorption and degradation using several different methodologies: (i) biosorption of dye from solution onto biomass [20,21]; (ii) degradation of dye by "immobilized microorganisms" that are supported on solid matter in contact with a dye that is in solution [22,23]; and (iii) dye adsorption by a moist solid substrate that is in contact with inoculated microorganisms in the absence of free water [24]. In the first two methods, efficacy is evaluated by a technique that analyzes dye concentration in solution (typically spectrophotometry). In the third method, microbial growth is evaluated by several different procedures.

Only a few agricultural waste products (wheat straw, wood chips, corn cobs) have been tested for adsorption and solid-state biodegradation [24]. This paper is one of a series of studies that reports on the adsorption of textile dyes onto adsorbents prepared from maize waste. Our aim was to adsorb the two azo dyes on corn cob waste and then evaluate the biodegradation of the immobilized dyes by the *Klebsiella* sp. UAP-b5, a species previously isolated from maize waste. We also describe a methodology that allows visualization of possible degradation products and address the hypothesis

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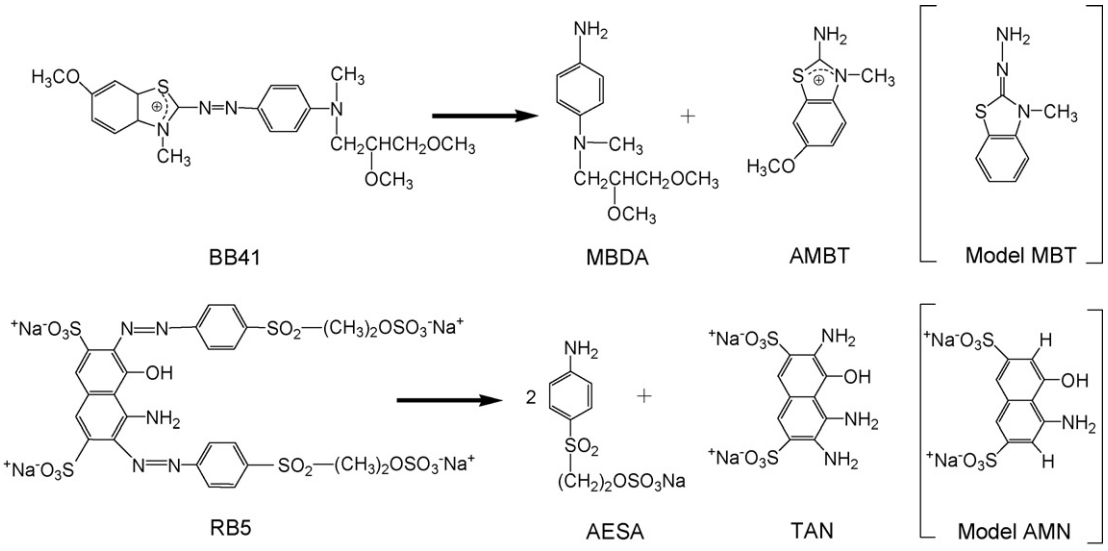

MBDA N-(2,3-dimethoxy-propyl)-N-methyl-benzene-1,4-diamine

AMBT 2-Amino-6-methoxy-3-methyl-benzothiazol-3-ium

MBT 3-Methyl-1,2-benzothiazolinone hydrazone

AESA Sulfuric acid mono-[2-(4-amino-benzenesulfonyl)-ethyl] ester, monosodium salt

TAN 3,6-Naphthalenedisulfonic acid, 2,7,8-triamino-1-hydroxy, disodium salt

AMN 3,6-Naphthalenedisulfonic acid-8-amino-1-hydroxy, disodium salt

Fig. 1. Structures of BB41, RB5, their degradation products and model molecules used in this study.

that these degradation products remain on the biosorbent in the adsorbed state.

2. Materials and methods

2.1. Bacterial isolation and identification

The adsorbent material designated as SOMAP2 was obtained from maize waste as described previously [25,26]. Particles of nonsterilized SOMAP2 were inoculated onto plates of SP medium (NaH_2PO_4, 2.44 g L^{-1}; KH_2PO_4, 1.52 g L^{-1}; $(NH_4)_2SO_4$, 1 g L^{-1}; $MgSO_4{\cdot}7H_2O$, 0.5 g L^{-1}; $CaCl_2{\cdot}2H_2O$, 0.1 g L^{-1}; NaCl, 1 g L^{-1}; fructose, 20 g L^{-1}; peptone, 10 g L^{-1}; Agar, 18 g L^{-1}) and incubated for 24 h at 30 °C. Isolates were inoculated in SP broth with RB5 (0.05 mM) or BB41 (4 mM) and incubated without shaking at 30 °C. Visual observation of the decolorization was used to select the most active isolates. The strain showing the fastest decolorization was chosen for further biodegradation experiments. This isolate was identified and inoculated into the SP broth with each of the immobilized dyes, which were previously adsorbed on SOMAP2. DNA, obtained by standard methods [27], was used as a template for the amplification of 16S rRNA with the forward primer 5′-TAGAGTTTGATCCTGGCTCAG-3′ and the reverse primer 5′-GACGGGCGGTGTGTACA-3′. PCR conditions were one cycle at 95 °C (3 min), 26 cycles at 94 °C (30 s), 55 °C (45 s), and 72 °C (2 min), plus a final extension at 72 °C (10 min). Both strands of the product were sequenced with the same primers in a capillary sequencer (PerkinElmer/Applied Biosystems 3730, Institute of Biotechnology, UNAM) and the sequence of strain UAP-b5 was deposited in GeneBank under accession number EF647622. The DNA sequence was initially compared with known sequences using BLAST [28] and these data were used for performing pairwise comparisons with the program OldDistances (Wisconsin Package 10.0). Bacterial isolates were kept at −76 °C, with 50% glycerol as cryoprotectant.

2.2. Immobilization of dyes

The two dyestuffs were: BB41 (produced by Clariant) and RB5 (produced by Ciba). BB41 (C.I. 11105) is a monoazo basic dye and benzothiazo compound. RB5 (C.I. 20505) is a diazo reactive dye and a hydroxyamino compound (Fig. 1). Dye solutions were prepared without pH adjustment in deionized water (Milli-Q reagent grade water). Adsorption isotherms were measured on SOMAP2 with a particle diameter of 40 μm at 15 °C in the concentration range 0.05–4 mM. In the batch system, resulting after 24 h adsorption of 4 mM of the dye, the solution was decanted and the solid and adsorbed dye was dried at room temperature and then at 60 °C. In this state, we henceforth denote this dye as “immobilized dye”.

2.3. Adsorption of model compounds

The model compounds 3-methyl-2-benzothiazolinonehydrazone hydrochloride (MBT) and 4-amino-5-hydroxy-2,7-naphthalendisulfonic acid monosodic salt (AMN) were purchased from Aldrich. MBT is similar to one of the degradation products of BB41 and AMN resembles the structure of the main degradation product of RB5 (Fig. 1). Batch adsorption experiments were performed at 15 °C for 24 h using an adsorbent dose of 20 mg cm^{-3}.

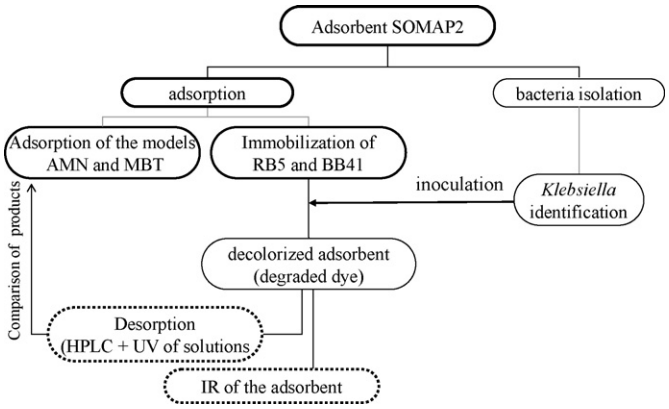

Fig. 2. Scheme of the experiments combing adsorption (thick frames), biodegradation (thin frames) and the evaluation approach (dotted frames).

2.4. Biodegradation experiments

All biodegradation experiments were also conducted using controls (without bacterial inoculum) and aliquots were measured over time. Adsorbent concentration was 1.4 g L^{-1} in the following salt medium MS: K_2HPO_4, 0.2 g L^{-1}; KH_2PO_4, 0.6 g L^{-1}; $MgSO_4{\cdot}7H_2O$, 0.2 g L^{-1}; $CaCl_2{\cdot}2H_2O$, 10 mg L^{-1}; $FeCl_3{\cdot}6H_2O$, 10 mg L^{-1}; sucrose, 10 g L^{-1}; pH 7. After inoculation with *Klebsiella* sp. UAP-b5, the tubes were incubated statically at 30 °C for 17 days.

2.5. Desorption experiments

Desorption experiments were performed by immersion of the immobilized dye (SOMAP2 + adsorbed dye) in acetone (for BB41) and in 10% NaCl aqueous solution (for RB5). The solid was extracted from the liquid medium and dried at 60 °C for 2 h before pressing the pellets for IR spectroscopy. The entire work scheme is depicted in Fig. 2. The chromatographic protocol for analysis of BB41 used column C18 AB (Macherey Nagel 150 mm × 4.6 mm), isocratic 47% methanol in phosphate buffer, pH 5.0. The chromatographic protocol for analysis of RB5 used column ODS (Beckman 250 mm × 4.6 mm), isocratic 30% methanol in phosphate buffer pH 5.0. The flow rate was 0.8 cm^3 min^{-1} and absorption at 220 nm was monitored.

2.6. Equipment

Adsorption equilibrium concentrations were measured by spectrophotometry using a DU 7500 Beckman DU 7500 spectrophotometer at the wavelength specified in each figure.

Table 1
Adsorption kinetic parameters of BB41 and RB5 on SOMAP2: pseudo-first- (k_1) and pseudo-second-order (k_2) rate constant, intra-particle diffusion constant (k_i), and adsorbed amount at equilibrium a_e for a concentration 0.1 mM L^{-1}

Model	Parameter	Dye	
		BB41	RB5
Pseudo-first order	$a_{e,exptl}$ (μmol/g)	5.2	3.5
	$k_1 \times 10^{-2}$ (h^{-1})	2.7	2.3
	$a_{e,calc(n=1)}$ (μmol/g)	2.3	2.7
	R_1^2	0.95944	0.98837
Pseudo-second order	k_2 (g $mmol^{-1}$ h^{-1})	4.4	1.4
	$a_{e,calc(n=2)}$ (μmol/g)	5.4	4.0
	R_2^2	0.99233	0.99739
Intra-particle diffusion	k_i (μmol g^{-1} $h^{0.5}$)	2.6	2.6
	R_i^2	0.97482	0.97575

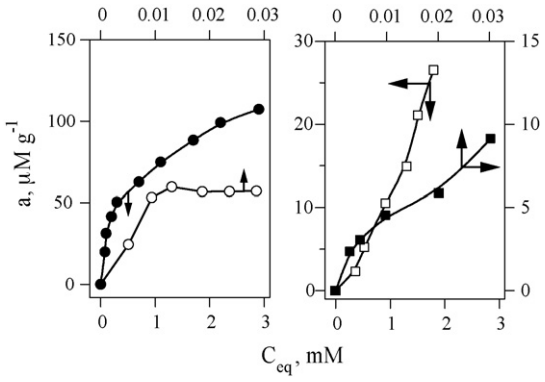

Fig. 3. Adsorption isotherms of aqueous solutions of BB41 (●), MBT (○), RB5 (■) and AMN (□) on SOMAP2 with particle diameter 250 μm at 15 °C. Detection wavelengths: 590 nm for BB41 and RB5, and 223 nm for MBT and 312 nm for AMN.

Biodegradation samples were analyzed using a Beckman Gold automated liquid chromatograph that employed a Beckman 168 diode array detector. HPLC grade methanol (Burdick & Jackson) and deionized water were used in chromatography under conditions given in each figure. The IR spectra were recorded with a Nicolet Magna FT-IR-750 spectrometer in the range of 4000–800 cm^{-1} with the same sample concentration in KBr (0.23%, w/w).

3. Results and discussion

3.1. Isolation and identification of UAP-b5

Among 38 isolates, Gram-negative UAP-b5 exhibited the fastest decolorization of immobilized BB41 [29]. BLAST analysis of the 16S rRNA sequence of UAP-b5 showed high similarity scores with the 16S genes of diverse species of the Enterobacteriaceae. Pairwise comparisons with sequences of different genera of the Enterobacteriaceae showed high similarity with species in the genus *Klebsiella* (supplementary material). The similarity scores ranged from 92.2%

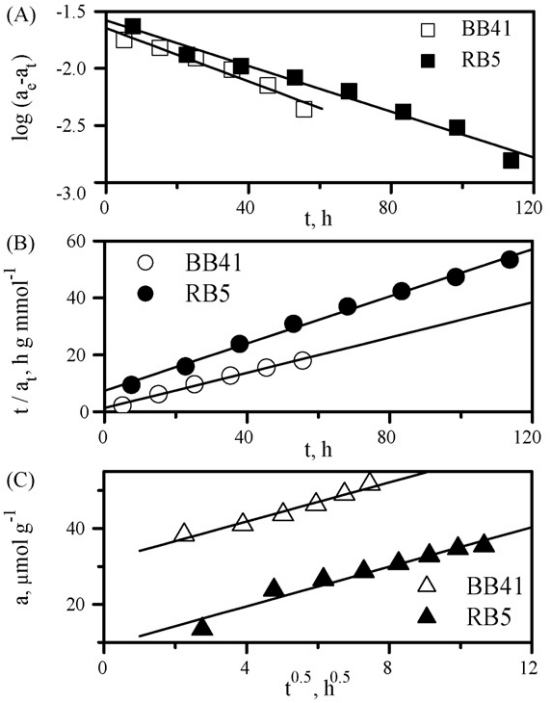

Fig. 4. Linear dependences of the kinetics evaluation according to a pseudo-first (A) and pseudo-second (B) order, and Weber–Morris (C) equation.

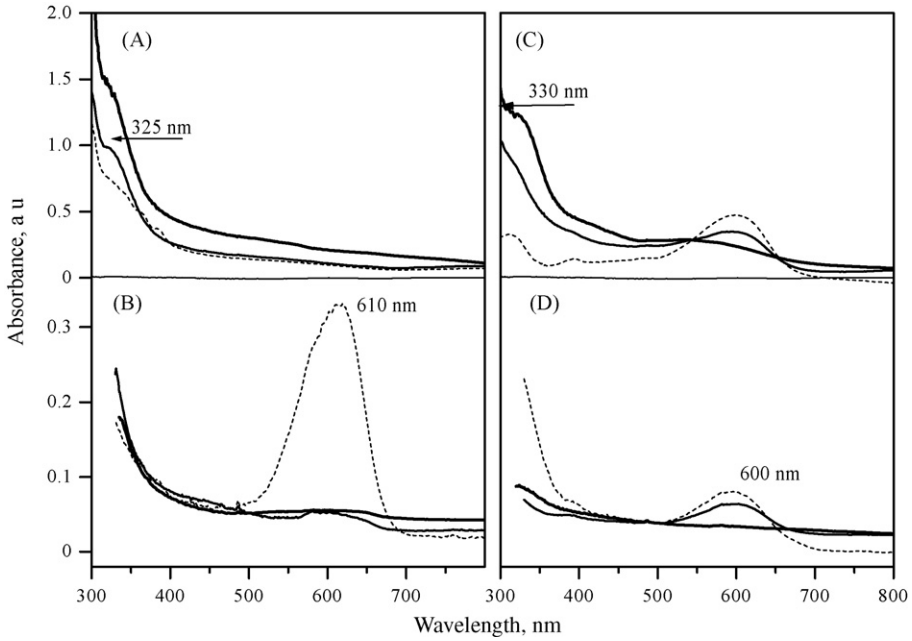

Fig. 5. UV–vis spectra of the bulk solutions (A and C) and of the desorbed solutions (B and D) for the systems containing the immobilized dyes BB41 (A and B) and RB5 (C and D) in presence of *Klebsiella* sp. UAP-b5. Medium (—), desorption solution (- - -), desorption solution after 10 (—) and after 17 days (▬▬) contact with the bacteria.

(*Klebsiella variicola*) to 97.5% (*Klebsiella granulomatis* and *Klebsiella singaporensis*).

3.2. Immobilization by adsorption

We immobilized BB41 and RB5 on the surface of the bioadsorbent by adsorption. Adsorption isotherms are shown in Fig. 3 and the associated kinetic parameters are listed in Table 1. To evaluate the solid-state biodegradation that accompanies retention of the products formed by these bacteria, we also studied the adsorption of model molecules (MBT and AMN; Figs. 2 and 3). Results showed adsorption of MBT and AMN, but less than that of the respective dyes. Except for AMN, adsorption isotherms were concave, indicating favorable interaction. The adsorption capacity of SOMAP2 toward RB5 was moderate in comparison with BB41 due to the prevailing specific interactions between the basic dye and the negatively charged surface of SOMAP2, as discussed in [26]. The kinetic parameters obtained from the plots in Fig. 4 are listed in Table 1. These data indicate that adsorption on SOMAP2 can be represented by a pseudo-second-order equation for both dyes since the value of $a_{e,exptl}$ was similar to that of $a_{e,calc(n=2)}$ and different from $a_{e,calc(n=1)}$. The biodegradation of Acid Orange 7 by different bacteria cultures on activated carbon [22] was also characterized by a second order kinetic equation. It was not completely clear whether adsorption or intra-particle diffusion is the predominant mechanism for RB5 since $a_{e,exptl}$ deviated 14% from $a_{e,calc(n=2)}$, but $R_2^2 > R_i^2$ (see Table 1). In the case of the adsorption of Acid Red 114 by pith [30], comparison of the coefficients of determination R^2 for different pith doses did not lead to a clear distinction. However, judging by the intercepts of the Weber–Morris plots (see Fig. 4C) [31], we suggest that the limiting step in the case of the adsorption of RB5 was intra-particle diffusion.

3.3. Decolorization of immobilized dyes

After 48 h of contact, *Klebsiella* sp. UAP-b5 decolorized the immobilized dye BB41 (supplementary figure). Similarly, Nigam et al. [24] previously reported fungi-mediated decolorization of RB5 after 48 h and Mohanty [1] reported a reduction of 46% in the amine metabolite concentration after 48 h following a two-stage anaerobic–aerobic treatment using sludge. Following the results of Moutaouakkil et al. [23], we assume that the anaerobic decolorization of the immobilized dye coincided with the growth of *Klebsiella* sp. UAP-b5. In contrast to the findings reported by Barragán et al. [22], who reported decolorization on small particles and decolorization + biodegradation occurring only on large particles (~500 μm) of powdered activated carbon, the particle size of SOMAP2 did not play an important role in decolorization within a reasonable period of time. This may be explained in terms of the microniches theory [22], because cob waste exhibits high porosity with meso- and macro-pores [32]. When *Klebsiella* sp. UAP-b5 was initially supported on SOMAP2, the decolorization rate of RB5 and BB41 solutions was lower than for bacteria growing in liquid medium. In contrast, 95% of a methyl red solution [23] was decolorized by supported *P. agglomerans* in 6 h.

3.4. Desorption feasibility

Because the salts contained in our MS medium might spontaneously promote desorption of the immobilized dye, we analyzed controls (without *Klebsiella* sp. UAP-b5), referred to below as "bulk solutions". Fig. 5A demonstrates that the dye BB41 remained immobilized even after 17 days, since there was no observable peak in the visible region of the spectrum. In contrast, RB5 desorbed partially and spontaneously from the solid adsorbent into solution, as indicated by the appearance of an absorption band near 600 nm (Fig. 5C). Biodegradation experiments are typically performed in liquid media and degradation evolution is monitored by spectral changes over time. In our case, the immobilized dye was present in an adsorbed state, so possible products may also have been adsorbed. Thus, we used spectrophotometry and HPLC to analyze desorption.

The dotted line in Fig. 5B shows that in the solution produced following desorption, the main component was BB41. After 10 and 17 days of contact with *Klebsiella* sp. UAP-b5, the intensity of the band decreased significantly in the desorbed solution, indicating

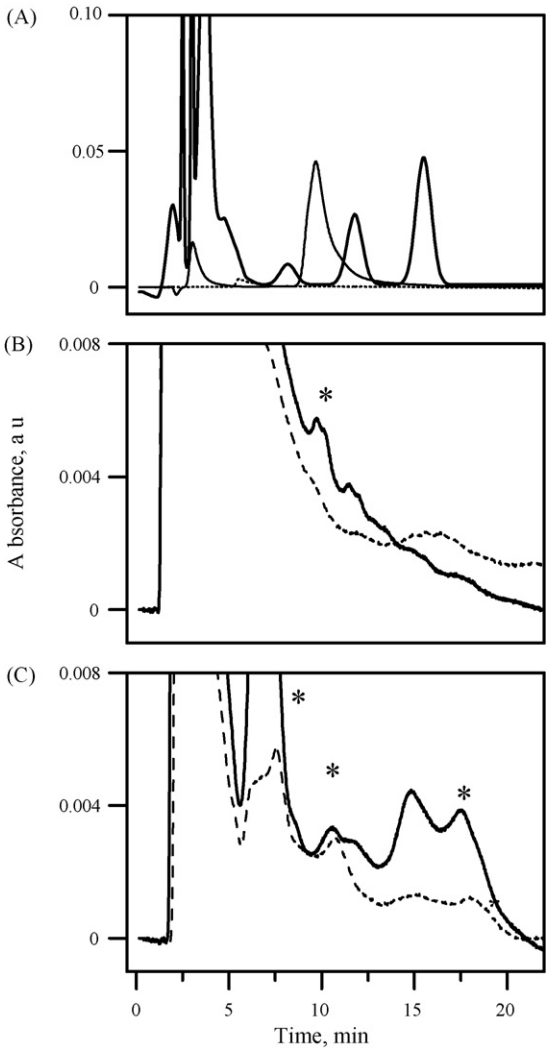

Fig. 6. HPLC chromatograms—(A) model solution: Medium ( ), BB41 (—) and the model compound MBT (—); (B) bulk solution of immobilized BB41 before (–) and after (▬▬) contact with *Klebsiella* sp. UAP-b5; (C) desorption solutions of immobilized BB41 before (–) and after (▬▬) contact with the bacteria.

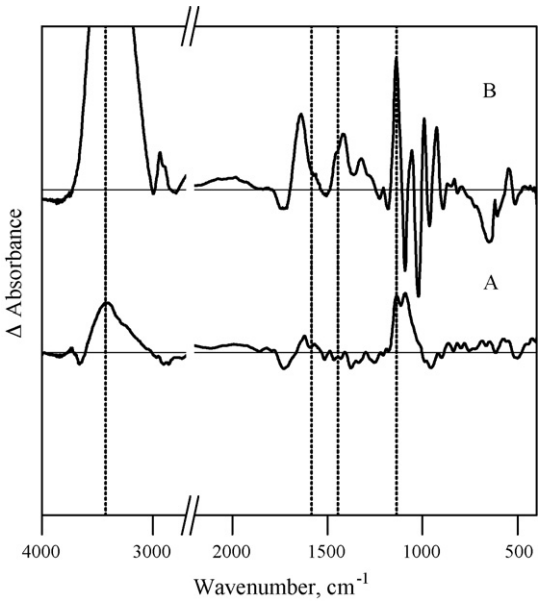

Fig. 7. FTIR difference spectrum of the immobilized dyes BB41 (A) and RB5 (B) before and after 10 days contact with *Klebsiella* sp. UAP-b5 after subtraction of the spectrum of SOMAP2.

metabolism of BB41. Desorption of RB5 was spontaneous (Fig. 5C) but not complete, since a small band still appeared in the desorption solution (Fig. 5D); this absorption band disappeared completely by 17 days. By comparing the initial spectra of bulk solutions in Fig. 5A and C with the spectra obtained after 10 and 17 days (thick lines), we infer the existence of new products in solution based on absorption bands that stretched to 350 nm.

3.5. Biodegradation assessment

We compared the composition of the bulk solution (Fig. 6B) and the desorption solution (Fig. 6C) with the chromatograms obtained for solutions of BB41 and the model compound, MBT (Fig. 6A). As a result of azo bond cleavage (Fig. 1), the products should be smaller, carry amine moieties, and be more hydrophilic and therefore, have shorter HPLC retention times than the original dye (see Fig. 6B). One of the possible degradation products of BB41, AMBT, is not commercially available but was represented by the model MBT because of its molecular similarity. After demonstrating the ability of SOMAP2 to adsorb MBT, we confirmed the presence of a similar biodegradation product in the adsorbed state. The chromatograms of the desorption solution (Fig. 6C) indicate that a mixture of products (marked with asterisks) is adsorbed after biodegradation.

In the case of degradation of RB5, Libra et al. [33] studied a two-stage anaerobic/aerobic bacterial process. They demonstrated that the fully and partially hydrolyzed forms of the dye were decolorized (65%) and the formation of degradation products (AESA, TAN, and an oxidized form of TAN; Fig. 1). AESA suffered mineralization, so the attention was centered in our work on detecting TAN in the desorption solutions. The HPLC chromatograms of the desorption solutions of the immobilized RB5 showed a peak at the retention time of the model compound, AMN (Fig. 1), whose adsorption on SOMAP2 was confirmed previously. Our results suggest then the presence of TAN in the adsorbed state. This methodological approach represents a novel application of desorption. A study of desorption of RB5 from *Corynebacterium glutamicum* biomass [22] attempted for example, only to obtain a high dye concentration.

We determined the average of three FTIR spectra collected from SOMAP2 samples and performed the same procedure for the adsorbent with immobilized dye before and after contact with bacteria. For each spectrum, the SOMAP2 averaged signals were subtracted and then the two samples' spectra were subtracted. The dotted lines in Fig. 7 identify main features of the resulting spectrum. The positive band at 3425 cm^{-1} is more intense for the degradation products of RB5 (AESA and AMN) than for BB41 and indicates the formation of amines (due to aromatic N–H stretching). This suggests that the products were TAN and AESA, which together contain four amine groups, while MBDA and AMBT, the degradation products of BB41, include only two amine groups. The positive bands at 1583 cm^{-1} indicate N–H deformation vibration; the bands at 1445 cm^{-1} indicate a change in the N=N stretching vibration coupled with ring vibrations; and the bands at 1135 cm^{-1} are indicative of C–N vibration in an azo group. These results provide indirect support for cleavage of the azo bond.

4. Conclusions

In this work we demonstrated the decolorization and cleavage of the azo bond in a heterogeneous system composed of a "contaminated solid" (dye immobilized on SOMAP2) that was maintained

in a liquid that contained bacteria. Our method differs from that of Barragán et al. [22], who explained decolorization of the azo compound in solution as being due to cleavage of the azo bond by bacteria that were supported on a solid immersed in a "contaminated" solution. In contrast to the method of Barragán et al. [22], our method demonstrated the adsorption of biodegradation products, such as amines. Thus, our technique may be particularly useful in situations where degradation products may be more toxic than the initial pollutant.

Alternative approaches may employ genetically modified bacteria or bacteria adapted to particular habitats that may exhibit higher biodegradation rates in closed environments. Efforts to isolate ecologically competitive bacteria like *Klebsiella* sp. UAP-b5 from natural environments that show proficiency in the biodegradation of pollutants could be particularly useful for restoration of contaminated environments. This problem is particularly urgent for restoration of polluted regions of developing countries, where there is often unacceptable disposal of contaminants.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhazmat.2008.04.023.

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