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Sorption isotherms and kinetics in the primary biodegradation of anionic surfactants by immobilized bacteria I. *Pseudomonas* C12B

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Abstract

The surfactant-degrading biocatalyst *Pseudomonas* C12B was immobilized by covalent linking on silanized inorganic supports and by physical entrapment of cells within reticulated polyurethane foam. Both immobilized biocatalysts have been shown to be appropriate for the effective primary biodegradation of the anionic surfactants sodium dodecyl sulphate (SDS), dodecylbenzene sulphonic acid (DBS), dioctyl sulphosuccinate (DOSS) and dihexyl sulphosuccinate (DHSS). The overall surfactant removal from water by cells entrapped in reticulated polyurethane foam exhibits a biphasic process, a rapid sorption step of the surfactant onto the cell-loaded support and the intrinsic primary biodegradation slower step, both acting cooperatively. The optimization of variables for the adsorption and the biodegradation processes (flow rate, particle size, substrate concentration) have been studied. Sorption isotherms for the surfactants on reticulated polyurethane foam have been established as type II of the Brunauer, Deming, Deming and Teller (BDDT) classification. The kinetics of the primary biodegradation of SDS by cells covalent linked on sepiolite treated with 3-aminopropyl triethoxysilane (APTS) were found to be first-order. In this case, surfactant adsorption does not exist.

Keywords: Alkyl sulphate; Linear alkyl benzene sulphonate (LAS); Dioctyl sulpho-succinate; Dihexyl sulpho-succinate; Immobilization; *Pseudomonas* C12B; Polyurethane; Silanization; Sorption; Biodegradation; Kinetics

1. Introduction

Increased use of anionic surfactants in detergent formulations means that the concentrations of these products in surface waters have increased drastically in recent years [1,2] leading to different pollution problems, although they are readily biodegraded by bacteria in sewage treatment plants and receiving lakes and streams [2]. Immobilized surfactant-degrading bacteria could be exploited for the on-site removal of excessive surfactant concentrations [3].

In relation to surfactant biodegradability, it is important to distinguish between primary and ultimate biodegradation. The breakdown of a substance as measured by a substance-specific analytical method, for example, the loss of the ester sulphate or sulphonate groups from surfactants, such as sodium dodecyl sulphate or linear alkylbenzene sulphonate, would be a primary biodegradation step that would lead to an imme-

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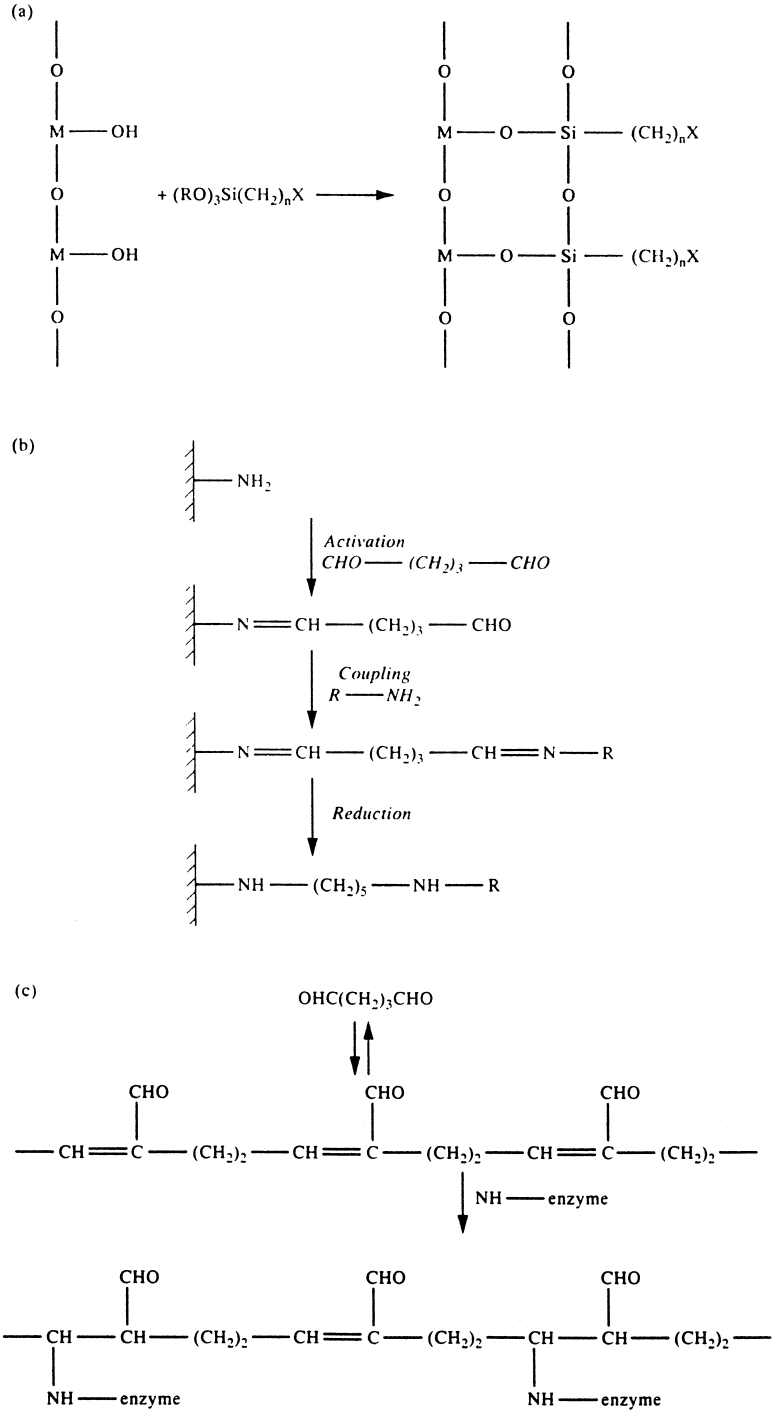


Fig. 1. Activation of silanized supports using glutaraldehyde: silanization method (A); activation of amino bearing supports by glutaraldehyde (B); conjugate addition of amino groups to ethylene double bonds of α, β-unsaturated oligomers contained in commercial aqueous glutaraldehyde solutions (C).

diate loss of surfactant properties (measured by the methylene blue anionic surfactants (MBAS) reduction test [4]). However, ultimate biodegradation implies the complete conversion of these surfactants into products such as CO_2, H_2O, inorganic salts and cellular products, and many more metabolic steps would therefore be involved. Nevertheless, confirmation of ultimate

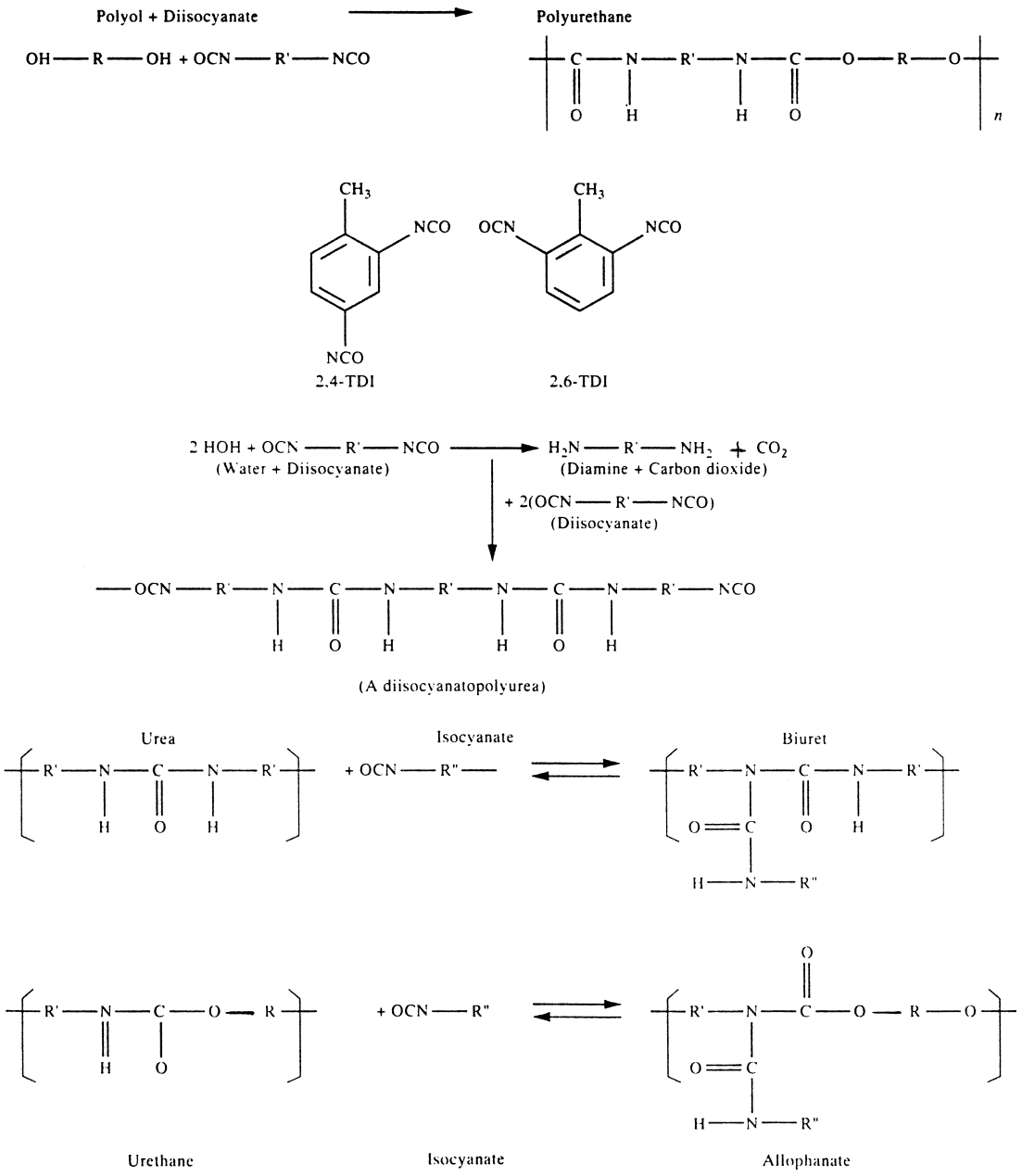


Fig. 2. Synthesis of polyurethane foams: a diisocyanate solution is added to 1000–2000 Da molecular weight prepolymer with terminal hydroxyl groups to establish the polyurethane linkages. The foaming agent is CO_2 generated in situ by controlled addition of water to the reaction. The water transforms several isocyanate groups into amine, with the release of CO_2. The amine then reacts with more isocyanate to afford urea linkages.

biodegradation (although not required by legislation) is important because primary biodegradation, although leading to losses in surfactant properties, may still yield recalcitrant and possibly toxic metabolites. Under the test criteria of CE-wide surfactant legislation, which is based on primary degradation (MBAS), alkyl sulphates and LAS have proved to be very readily degradable (99 and 95% reduction in MBAS in the EOCD (European Organization for Commerce and Development) screening) [5].

The ability to biodegrade primary and secondary alkyl sulphate surfactants is a feature of a number of bacteria common to soils and waters [6]. The bacteria involved are often capable of producing a multiplicity of alkylsulphatases hydrolyzing the sulphate esters to release long-chain fatty alcohols [7]. The biodegradation of dialkyl sulphosuccinate surfactants begins with cleavage of the hydrophobic alkyl chains from the ester linkage. The organism chosen for the present study was *Pseudomonas* C12B (NCIMB 11753), originally isolated for its ability to utilize sodium dodecyl sulphate and dodecylbenzene sulfonate surfactants as sole sources of carbon and energy [8]. This versatile microorganism can degrade a whole range of surfactants, especially alkyl sulphates and alkylethoxy sulphates [9,10].

For the primary biodegradation of surfactants, both hydrophile separation and ω-/β-oxidation destroy their amphiphilic property (specifically measured by the MBAS assay [4] carried out in this work). Considering that these activities represent a single-step biocatalytic reaction, one may immobilize either the relevant enzyme (sulphatase for hydrolysing alkyl sulphates, oxygenase for ω-/β-oxidation of alkylbenzene sulphonates, esterase for hydrolyzing dialkyl sulphosuccinate [11]), the non-viable cells exhibiting the activity in question or the viable cells (with the whole metabolic pathways, included the step of interest). Consequently, in this case, losses in viability after cell immobilization may not be that critical. A range of supports (organic and inorganic) and methods [12] have been screened for the immobilization of *Pseudomonas* cells, specifically, biofilm growth, cell entrapment and chemical binding (silanization/glutaraldehyde). Attention has focused on supports that provide high retention capacity, small or no decreases in enzymatic activity, and, particularly, satisfactory primary biodegradation activity during bioreactor performance.

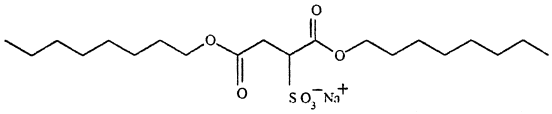
The silanization/glutaraldehyde method has proved to be efficient for immobilizing enzymes on inorganic supports [13,14], involving the use of trialkoxy silane derivatives containing an organic functional group. Coupling of these reagents to the carrier presumably takes place by displacement of the alkoxy residues on the silane, by hydroxyl groups or the oxidized surface of the inorganic support to form a metal–O–Si linkage (Fig. 1) [15].

Physical entrapment of cells inside a polymer matrix is one of the most widely used and straightforward techniques for cellular immobilisation, since it does not depend significantly on cellular properties. Polyurethanes constitute a group of polymers (Fig. 2) with highly versatile properties for immobilizing cells (high mechanical strength, inertness towards chemical and microbial attacks, good porosity, high specific surface, etc.) [16,17]. Cell viability preservation has been checked for the entrapment of plant cells in polyurethane [17–19], functioning, in some cases [18], completely active for years.

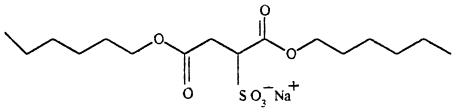
2. Materials and methods

2.1. Substrates

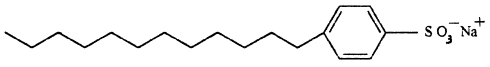
The anionic surfactants used in the present study were: sodium dodecyl sulphate (SDS), dioctyl sulphosuccinate (DOSS) (Sigma, St. Louis, MO, USA), dihexyl sulphosuccinate (DHSS) (Merck, Darmstadt, Germany) and dodecylbenzene sulphonic acid (DBS) (Sigma, USA). All products were of analytical grade. Their chemical structures are shown in Fig. 3.



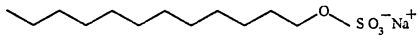
Dioctyl Sulfosuccinate (Sodium salt)



Dihexyl Sulfosuccinate (Sodium salt)



Dodecylbenzene Sulfonic Acid (Sodium Salt)



Sodium Dodecyl Sulfate

Fig. 3. Chemical structure of the anionic surfactants studied.

Most of the time, a surfactant concentration of 0.1 g l^{-1} was used in the experiments.

2.2. Microorganisms

The *Pseudomonas* C12B bacteria (aerobic/anaerobic) were kindly supplied by Dr. G.F. White (Department of Biochemistry, University of Wales College of Cardiff). These cells were batch-grown for 48 h at 30°C (150 rpm) in a medium containing 0.3% (w/v) of nutrient broth and 4 mM of SDS as enzyme-inducing agent. When the growth curve had reached steady-state ($O.D._{665\ nm} = 0.8$), the bacteria were collected by centrifugation (9000 rpm for 10 min, 4°C) and were washed with a basal salt solution (K_2HPO_4, 3.5 g l^{-1}; KH_2PO_4, 1.5 g l^{-1}; NH_4Cl, 0.15 g l^{-1}; NaCl, 0.5 g l^{-1}; Na_2SO_4, 0.15 g l^{-1}; $MgCl_2 \cdot 6H_2O$, 0.15 g l^{-1}) (MBS). Following this treatment, they were stored (0.1 g wet cells/ml of solution) in the same basal salt solution without magnesium (MBS–Mg) at 4°C.

2.3. Supports of immobilized cells

Inorganic supports were gifts from different companies: ceramic beads, Raschig rings (Lomba Camiña, La Guardia, Spain) and sepiolite (hydrous magnesium silicate) of different grain sizes (Tolsa, Madrid, Spain). The chemical composition (% w:w) of the inorganic supports was: ceramic beads Al_2O_3 (> 30), SiO_2 (65), Fe_2O_3 (< 1.6), CaO + MgO (< 0.7), $Na_2O + K_2O$ (< 3.5); ceramic Raschig rings SiO_2 (68.3), Al_2O_3 (24.5), TiO_2 (0.63), Fe_2O_3 (1.51), CaO (0.48), MgO (0.43), K_2O (2.99), Na_2O (0.42); sepiolite ($Si_{12}Mg_8O_{30}(OH_2)_4(OH)_4 \cdot 8H_2O$) SiO_2 (60.7), Al_2O_3 (3.1), MgO (21.8), CaO (1.6), Fe_2O_3 (1.0), Na_2O (0.4), K_2O (0.9). Among the physicochemical properties of the inorganic supports are: bulk density (g l^{-1}) 220 (ceramic beads and Raschig rings), 580 (sepiolite); surface area (m^2 g^{-1}), measured by Brunauer, Emmett and Teller (B.E.T.) gas–solid sorption isotherm [20], $5.4 \cdot 10^{-4}$ (ceramic beads and Raschig rings), 240 (sepiolite); mechanical strength (kg cm^{-2}) 180 (ceramic beads and Raschig rings), 5 (sepiolite). The particle sizes were between 0.1 and 3.4 cm^3.

Commercial polyurethane reticulated foams were a gift from Calther (Salamanca, Spain). For the synthesis and making up of the reticulated polyurethane foam, the prepolymer Hypol 2002 FHP supplied by Grace Service Chemicals (Heidelberg, Germany) was used. Hypol is a hydrophilic polyisocyanate, and the sample used in this work contains less than 10% of free toluene diisocyanate and 2.35 meq/g toluene isocyanate.

2.4. The bioreactor

For the removal of the surfactants, several plug flow bioreactors were made as water-jacketed metacrylate columns with the follow-

ing geometry: 3.5 cm inner diameter (d), 15 cm height (h), design ratio (d/h) 0.23. Each reactor was packed with support particles loaded with the immobilized cells and operated continuously with total recirculation (residence time equivalent to overall operational time) of the aqueous solution filling the bioreactor and tubings by means of a peristaltic pump. In the case of reticulated polyurethane foam, the column was packed with 35 cubes (1 cm^3/each) or 280 polyurethane cubes (0.125 cm^3/each). The dry weight of support within each reactor was 6.2 g and its void volume 70 cm^3 (flow rate 28.2 cm^3/min, residence time 2.5 min for one recirculating cycle). The external volume of the reactor was not taken into account because it only represents 1–3% of the total volume. The aqueous solutions loading the bioreactor were supplemented with soluble oxygen from the air after their vigorous magnetic stirring.

A suitable membrane reactor was built for measuring the internal mass transfer resistances of SDS through the polyurethane matrix. This reactor was based on a filter cartridge of polyurethane reticulated foam and encased in a polysulfone hollow cylinder (31.1 cm long, 3.2 cm outer diameter, 2.5 mm wall thickness) and equipped with suitable fittings. The lumen side of the polyurethane membrane was filled with the substrate solution consisting in 1.5 l of 0.05–0.4 g l^{-1} SDS and recirculating at 0.2 l min^{-1} flow rate within a closed loop. The shell side of the membrane was filled of ultrapure water and recirculating at 0.03 l min^{-1} flow rate within another closed loop. In such a way, these two recirculating loops only have the meeting point along the polyurethane membrane for maintaining in touch surfactant crossing through it with the solvent water.

2.5. Moisture and biomass contents

The moisture contents of the cells and supports were determined by drying the samples for 48 h, at 40°C, under vacuum, in a dichloromethane drier. The protein contents of free cells were determined according to the enhanced alkaline copper (Lowry) protein assay [21].

To measure the biomass immobilized on the support, a support particle (with immobilized cells) was treated with 5.0 ml of 3.0 M HCl for 60 min at 40°C, hydrolyzing the covalent links between the cells and the support. The Stoscheck assay for protein [21] was then carried out on samples taken from the acid solution. Standards were prepared with bovine serum albumin subjected to the same procedure. The amount of protein bound to the support was also estimated from the specific catalytic activity of free and immobilized cells, and by the difference between the amounts of protein offered and the amounts of protein found in the supernatants and washouts after immobilisation, as measured by the Lowry assay.

2.6. Surfactant biodegradative activity of free cells

The surfactant biodegradative activity of *Pseudomonas* C12B was estimated as follows: 0.25 ml of the cell suspension was mixed with 5.0 ml of surfactant solution (100 mg l^{-1}) at 30°C with stirring. At different times, 0.5 ml aliquots of mixture were withdrawn, and the remaining surfactant was analyzed by the methylene blue assay (MBAS) as modified by Hayashi [4], using a 301 Milton Roy spectrophotometer (USA).

The MBAS analysis used for the determination of the extent of degradation of anionic surfactants is specific to the class of substance. Wickbold [22] showed that the compounds detected during degradation studies of anionic surfactants (EOCD) confirmatory test) using the MBAS method were exclusively biochemically unaffected surfactant molecules, and that no alcoholic components were present. It can, therefore, be assumed that the MBAS analysis of anionic surfactants catabolites sheds light on whether alkyl chain hydroxylation has taken place on the surfactant molecule or not.

2.7. Surfactant biodegradative activity of immobilized cells

A support particle loaded with immobilized cells was incubated with 5.0 ml of surfactant solution (100 mg l^{-1}) at 30°C with stirring. At different times, 0.5 ml aliquots of mixture were withdrawn, and the remaining surfactant was analyzed by the methylene blue assay (MBAS), as modified by Hayashi [4]. Then, the dry weight of the support particle was determined and activity was measured in mg surfactant removed per minute per gram of dry support. Control assays were carried out with support particles without immobilized cells.

2.8. Kinetic assays of the sorption and biodegradation of anionic surfactants

For cells entrapped in reticulated polyurethane foam, at different operational times, a sample of the surfactant solution being recirculated was taken from the bioreactor packed with support particles without cells (sorption) or with immobilized cells (sorption + primary biodegradation), and the remaining concentration of the anionic surfactant was measured following the methylene blue assay (MBAS).

In order to distinguish between initial rates of intrinsic biodegradation and initial rates of adsorption, at least three successive operational cycles (24 h each) with the same surfactant concentration were required to saturate the foam support (without cells) with adsorbed surfactant. By subjecting the polyurethane sample (with immobilized cells) to an identical process in the fourth 24-h cycle, it was possible to measure the initial rate of biodegradation for this surfactant concentration, since the adsorption rate had been rendered null during that cycle. Even longer times would be necessary for that in the case of continuous operation.

Sorption isotherms were established in batch using different samples of support of identical weights, and incubating with different aqueous solutions of different concentrations of each of the surfactants employed. After following the adsorption kinetics of the surfactant onto the support, the final equilibrium concentration of the remaining surfactant was determined. All experiments were carried out at 25°C; in them, it was observed that the pH of the solution (not buffered) did not vary along the experimental period, a value of 7.0 (measured with a Crison digit 501 pH meter) persisted.

2.9. Scanning electron microscopy

Several scanning electron micrographs were taken of the cells immobilized on the support particles with a Zeiss Digital Scanning Microscope model 949 (Fig. 4).

2.10. Immobilization of Pseudomonas C12B cells on inorganic supports

2.10.1. Silanization of supports

The supports (ceramic beads, Raschig rings and sepiolite) were first treated with concentrated nitric acid (5 ml/g support) for 2 h, after which they were thoroughly washed with highly purified water and dried at 120°C in a furnace oven overnight. The supports were then silanized by refluxing in a boiling water-bath with 3-aminopropyl triethoxysilane (10% in dry toluene) (5 ml/g support) for 40 min, after which they were thoroughly washed with toluene and acetone, filtered and dried in a furnace oven at 120°C overnight.

2.10.2. Activation of silanized supports using glutaraldehyde

The silanized supports obtained were activated using two methods.

2.10.2.1. Method 1. To remove polymeric material, a commercial glutaraldehyde solution (25% in water) was shaken with activated carbon (33 mg/ml) and centrifuged at 7000 rpm for 15 min at 4°C. The supernatant was centrifuged again under the same conditions, and the clear supernatant was then used. The silanized supports

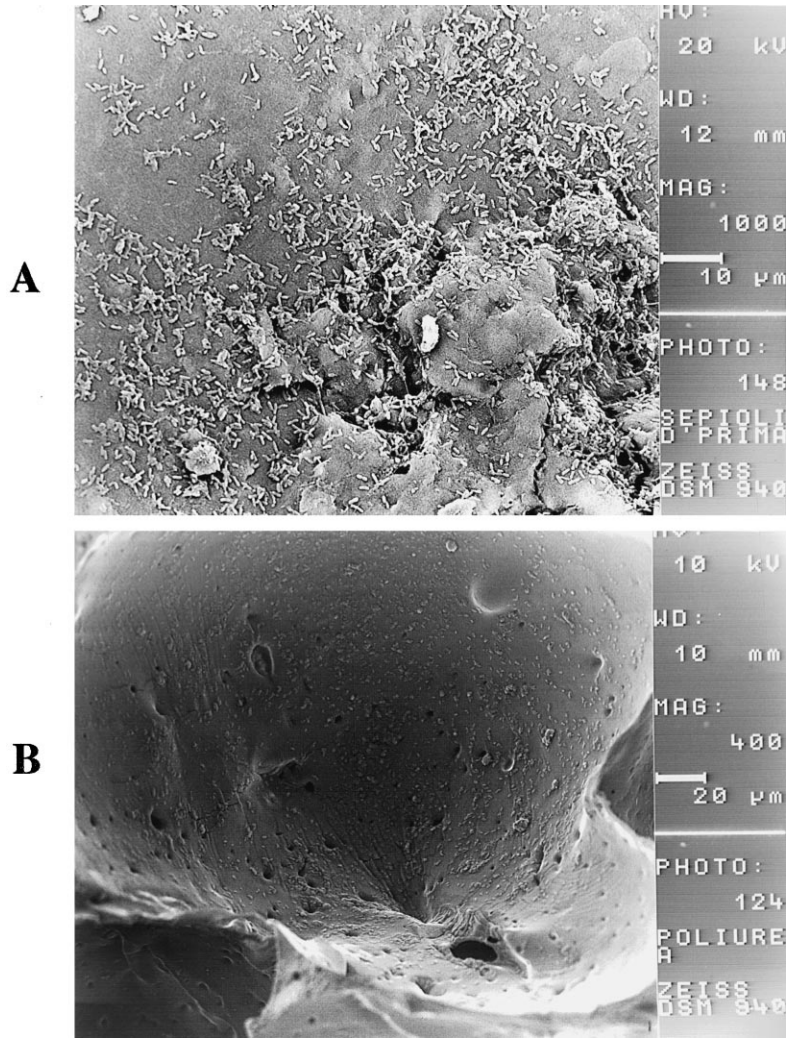


Fig. 4. Scanning electron micrographs of *Pseudomonas* C12B immobilized on sepiolite (A) and in reticulated polyurethane foam (B).

were activated at reduced pressure using a water pump at 4°C with 2.5% glutaraldehyde in phosphate buffer (0.1 M, pH 7.0) for 30 min. The Schiff bases produced were then reduced by adding sodium cyanoborohydride (7 mg/100 mg support), and the reaction was allowed to proceed for another 90 min. The activated supports were thoroughly washed with highly purified water and filtered.

2.10.2.2. Method 2. The activated supports were treated with 5% glutaraldehyde in phosphate buffer (0.1 M, pH 7.0) for 1 h at ambient temperature. Excess glutaraldehyde was filtered off, and the supports were washed several times with deionized water, and then with phosphate buffer (0.1 M, pH 7.0).

2.10.3. Immobilisation of cells onto glutaraldehyde-activated supports

2.10.3.1. Method 1. Cells were suspended in phosphate buffer (0.1 M, pH 7.0) (ca. 50 mg dry cells/ml) and kept at 4°C before being added to the aminopropyl-functionalized and glutaraldehyde-activated supports prepared by

activation method 1 (see Section 2.10.2.1) as detailed in individual experiments. Aliquots were added to the supports, resulting in a suspension of ca. 15 mg protein/100 mg support. The cells were allowed to react with the supports at 4°C under reduced pressure (using a water pump), with shaking, for 30 min. The Schiff bases produced between the cells and supports were reduced by adding sodium cyanoborohydride (7 mg/100 mg support), and the reaction was continued for 90 min. The cells-supports were washed with acetate buffer (0.1 M, pH 4.5, containing 1 M sodium chloride). The protein contents of the cell suspensions offered to the supports and of the washings after immobilisation were determined by the Lowry assay.

2.10.3.2. Method 2. Cells were suspended in phosphate buffer (0.1 M, pH 7.0) (ca. 50 mg/ml) and kept at 4°C before being added to the aminopropyl-functionalized and glutaraldehyde-activated supports prepared by activation method 2 (see Section 2.10.2.2). Aliquots were added to the supports, resulting in a suspension of ca. 10–20 mg protein/100 mg support. The cells were allowed to react with the supports for 15 h. The cells-supports were washed with acetate buffer (0.1 M, pH 4.5, containing 1 M sodium chloride). The protein contents of the cell suspensions offered to the supports and of the washings after immobilisation were determined by the Lowry assay.

2.11. Immobilization of Pseudomonas C12B cells on organic supports

2.11.1. Passive immobilization in reticulated polyurethane foam

Biofilm formation on commercial polyurethane reticulated foam was achieved by growing *Pseudomonas* sp. cells in a batch culture medium (see above) in the presence of 100 cubes (1 cm^3 each) of polyurethane foam. After 48 h of growth ($A_{665\ nm} = 0.8$), the supports were withdrawn and coated with biofilm. Preliminary assays showed that cell loading was not very high, and some washout of cells had occurred. This simple method of immobilization was discarded in favour of cell entrapment due to the higher degree of cell loading and reproducibility of immobilization resulting from this procedure.

2.11.2. Cell entrapment in reticulated polyurethane foam

Polyurethane was synthesized by quickly mixing prepolymers (polyol + diisocyanate) (Hypol FHP type 2002) with the aqueous cell solution and allowing the reticulated foam thus produced to cure as soon as possible (3 min). The polyol/diisocyanate ratio is essential for obtaining an appropriate reticulated foam with good consistency, porosity and mechanical properties [23]. The prepolymer/cells (w/v) ratio chosen for the immobilization was 15 g Hypol: 25 ml of cell suspension (0.1 g wet weight/ml). After 3 min, this fast polymerization reaction was finished, and the block of polyurethane reticulated foam entrapping the cells thus obtained was consecutively washed with basal medium without Mg^{2+} (MBS–Mg), deionized water and 200 ml of a solution 1:1 of MBS–Mg, and water to remove the excess of involved chemicals. The washings were collected, and the protein content in the total volume was determined, the loss of bacterial protein after immobilisation and washings being 15–18% of the total protein offered to the foam. Consequently, the entrapment yield was high enough (82–85%). The block was cut into cubes (0.125–1.0 cm^3 each), and the resulting particles of reticulated foam were stored in MBS–Mg solution at 4°C.

In order to increase the surfactant biodegradative specific activity of the cell-loaded supports by increasing their cell mass, two additional cell growth proliferations were carried out for a further 24–48 h, significant increases in activity and fresh weight of the cell loaded

support being detected (up to 25%). Consequently, the viability of the polyurethane-entrapped cells population was maintained thanks to the short immobilization time required for the entrapment (3 min), preventing a significant toxic and inhibitory effect of prepolymers on cells.

2.12. Curve fitting

Statistical nonlinear regression of the data on the remaining surfactant concentration vs. time was carried out by means of the EXFIT (sum of exponentials) program of the SIMFIT package, a powerful tool for the numerical analysis of experimental data in life sciences (the gift of the latest version of SIMFIT is acknowledged to the author, Dr. W.G. Bardsley of the University of Manchester, UK).

3. Results and discussion

Plug flow bioreactors packed with immobilized cells of *Pseudomonas* C12B were constructed to follow the kinetics of the primary biodegradation of anionic surfactants [sodium dodecyl sulphate (SDS), dodecylbenzene sulphonate (DBS), dioctyl sulphosuccinate (DOSS), dihexyl sulphosuccinate (DHSS)] from aqueous solutions. The biochemical process was assessed for different surfactants and different immobilized *Pseudomonas* C12B, with the observation that physical entrapment of the cells within reticulated polyurethane foam was the most efficient for removing the anionic surfactants by adsorption plus biodegradation.

3.1. Pseudomonas C12B cells immobilized on inorganic supports

3.1.1. Immobilization performance

Following the experimental procedure described, the amounts of cells immobilized by the silanization method (with and without reduction of the Schiff base) on ceramic beads, ceramic Raschig rings and sepiolite, and the activities of such immobilized cells for removing SDS were measured (Table 1). Each measurement represents the average of three replicates, and their relative standard deviations were 4–15%. Additionally, to qualitatively determine the efficiency of immobilization on each of its variants 1, 2, 4 and 5, a series of scanning electron micrographs was made. Thus, for example, in total immobilization (that is, immobilization 1 in which the reduction of the Schiff bases formed with sodium cyanoborohydride was conducted), the amount of cells present was quite significant (16.08, 50.8, >3.59 mg biomass/g support for ceramic Raschig rings, beads and sepiolite). However, unlike immobilization 2, 4 and 5 without this reduction step, the cells were clustered and surrounded by a mucilaginous material (being observed by scanning electron microscopy) and their activity was considerably decreased, mainly for sepiolite (Table 1). This fact points to a very significant effect of sodium cyanoborohydride on decreasing the viability of immobilized cells, and consequently their biodegradative activity towards SDS.

The amounts of protein bound to the inorganic supports were high enough (6.58–119 mg biomass/g support), but the corresponding SDS degrading activities were low, except in the case of sepiolite for immobilization variants 2, 4 and 5. Among the inorganic supports, the porous sepiolite particles were selected as the most appropriate for immobilizing *Pseudomonas* cells, but without the step of reduction of the Schiff bases formed between the cells and support (variant 5). The advantages of using these supports include high mechanical and chemical stability and biological inertness. However, they show a high degree of non-specific adsorption of cells due to residual charge on its high specific surface (see controls 3 and 6 in Table 1). This effect was reduced by treating the surface with the hydrophilic silane (APTS), which acts as a shield on the surface.

Table 1
Pseudomonas C12B immobilized on inorganic supports using silanization technology

	Treatment											
	Ceramic Raschig rings			Ceramic beads			Sepiolite					
	1	2	3	1	2	3	1	2	3	4	5	6
mg SDS/min/g support	0.01	8.67E-3	5.92E-4	1.55E-4	7.04E-4	1.81E-4	7.24E-4	0.21	0.051	0.236	0.322	0.025
mg *Pseudomonas*/g support[a]	9.71	8.42	0.58	56.23	26.98	6.58	3.59	105.4	24.72	87.7	119	12.44
mg *Pseudomonas*/g support[b]	16.08	7.94	0	150.8	25.5	6.58						
% Coupling yields	1.47	2.,87	0.04	14.06	6.75	1.65	0.9	26.4	6.18	21.9	29.72	3.1

1: Nitric acid + APTS + glutaraldehyde (carbon) + sodium cyanoborohydride + cells.
2: Nitric acid + APTS + cells.
3: Nitric acid + cells.
4: Nitric acid + APTS + glutaraldehyde (carbon) + cells.
5: Nitric acid + APTS + glutaraldehyde (without carbon) + cells.
6: Cells.
[a]Biomass bound to the support was estimated from the enzymatic activity retained in the immobilized preparation and the specific activity of the free cell.
[b]Protein bound to the support was estimated by the difference between the amounts of protein offered and of protein in the supernatants after immobilization measured by the Lowry assay.
Coupling yields: determined as the percentage of the total protein, offered to the support, which was bound to the support.

3.2. SDS biodegradative activity of Pseudomonas C12B immobilized on sepiolite

Kinetic experiments designed to explore the primary biodegradation of SDS were carried out both with support devoid of cells and with support loaded with immobilized cells in order to determine the effect that possible surfactant adsorption onto the support might have on the overall kinetic process. However, it was observed that surfactant adsorption onto sepiolite was null. Fig. 5 shows different kinetic curves of SDS biodegradation by *Pseudomonas* covalently immobilized on sepiolite treated with APTS. Considering that the heterogenous biocatalyst could follow Michaelis–Menten (Monod) kinetics for consumption of substrate, and that [substrate] $< K_M$, then the removal of surfactant from water was well fitted with first-order kinetics (remaining [substrate] vs. time).

$$[\mathrm{SDS}] = Ae^{-k}1^{t} + C$$

with k_1 in the range between 0.05 min^{-1} and 0.126 min^{-1}, and $t_{1/2}$ between 30 min and 40 min for $[\mathrm{SDS}] = 1 \cdot 10^{-4}$ M $- 7 \cdot 10^{4}$ M.

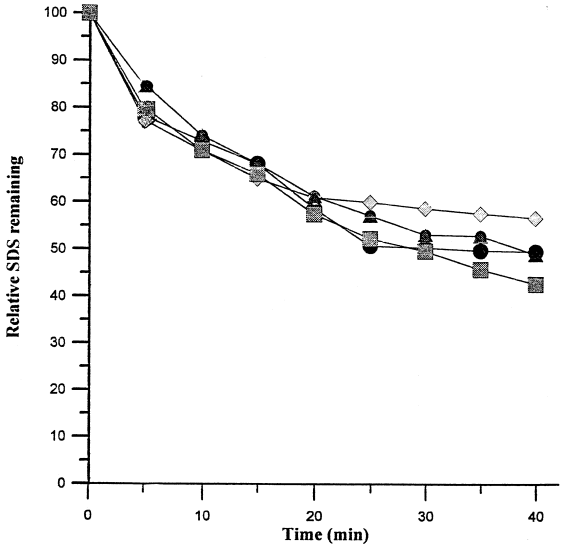


Fig. 5. Biodegradation kinetics of SDS by *Pseudomonas* covalently immobilized on sepiolite. SDS concentrations were 0.1 mM (▲), 0.2 mM (●), 0.3 mM (♦) and 0.7 mM (■), pH 7.0, 30°C.

3.3. Pseudomonas C12B cells entrapped on reticulated polyurethane foam

3.3.1. Diffusional limitations

For cells (enzymes) entrapped in polyurethane reticulated foam, diffusional limitations to substrate and/or products during surfactant conversion are expected to occur as result of cell (enzymes) within a support particle are being exposed to a microenvironment that differs from the bulk solution.

In order to check external diffusional limitations, at pH 7.0, 25°C, for 0.1 g 1^{-1} SDS, the initial SDS-removing activity of 35 cubes of 1 cm^3 with entrapped cells and packed within the reactor (see above) was measured as a function of the recirculation flow rate of the water passing through (controlled by a peristaltic pump). A 250% enhancement for such activity was found when the flow rate was increased from 18 ml min^{-1} up to 28 ml min^{-1}, decreasing the activity afterwards, meaning that the higher the flow rate, the narrower the stagnant boundary layer (Nernst–Planck unstirred layer) around the support particle, and the higher the removal of SDS activity.

The Reynolds number (Re) for the flow through the pore spaces in a packed-bed reactor is low, since both the velocity of the liquid and the width of the channels are normally small. For these cases, a modified Reynolds number (Re′) is proposed [24], considering the flow as streamline for Re′ < 2. At higher Re′, the flow starts to become turbulent in the largest channels, and subsequently, turbulence sets in successively smaller channels as Re′ increases. However, it is probable that the flow never becomes completely turbulent since the passages may be so small that streamline conditions prevail even at very high flow rates. Furthermore, higher flow rates than 28 ml min^{-1} (Re′ = 1.76) may create turbulent flow regime (at 50 ml min^{-1} Re′ = 3.15), removing some cells and disturbing the efficiency of the catalytic event. Consequently, for the following studies a flow rate of 28 ml min^{-1}, where

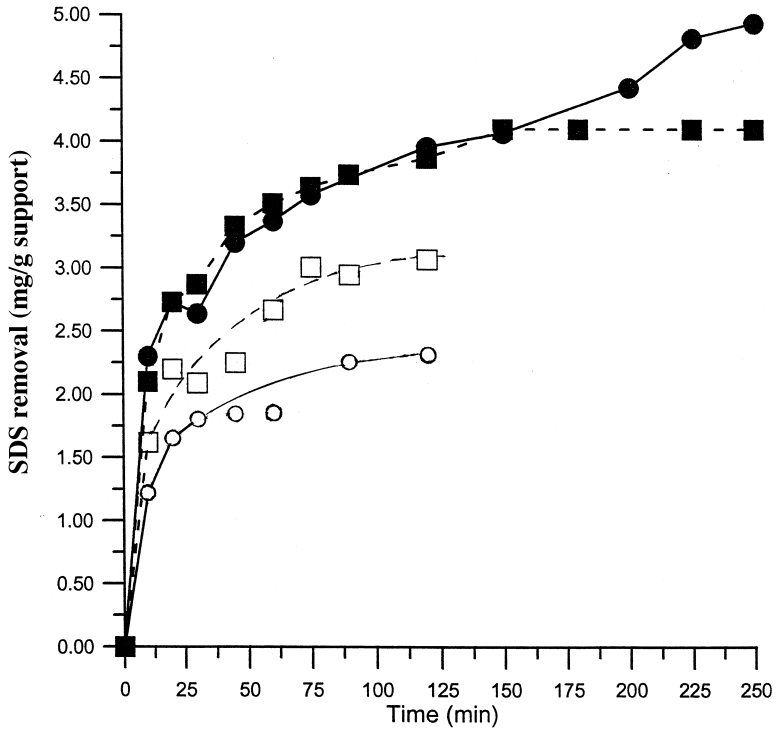


Fig. 6. SDS removal kinetics by *Pseudomonas* immobilized in polyurethane foam (●, □) and by the support without cells (○, □) at two different support particle size, 1 cm^3 (○, □) and 0.125 cm^3 (●,■). For other experimental conditions, see text.

maximum SDS removal activity was checked, and external mass transfer limitation was not the rate-limiting step of the overall removal of surfactant, was chosen.

Within the polymer matrix, diffusion takes place by passive molecular diffusion only, and is not affected by stirring speed. Internal diffusion effects will be more marked if the biocatalyst is immobilized by entrapment within the polymer matrix rather than attachment to the surface. Internal diffusional limitations were also found when a set of kinetic runs was carried out at pH 7.0, 25°C, 28 ml min^{-1} flow rate, 0.1 g l^{-1} SDS, with 35 polyurethane cubes of 1 cm^3 and with 280 cubes of 0.125 cm^3 with and without entrapped *Pseudomonas* cells (Fig. 6). The specific surface of the support particles increased threefold, and, as a consequence of increasing the mass transfer rates, the initial rate and the concentration (at 2–3 h) of removed SDS on the support with and without immobilized cells also increased up to 1.7–1.9 times. Support particles smaller than 0.125 cm^3 did not increase the kinetics of SDS removal, pointing to an optimum accessibility of the substrate to the active sites for adsorption and biodegradation throughout the plastic matrix network. Accordingly, to prevent internal diffusional limitations through the plastic matrix, 0.125 cm^3 polyurethane cubes were chosen as biocatalyst-loaded particles for subsequent experiments.

3.3.2. Diffusion coefficients for anionic surfactants

Most equations for calculating the diffusional coefficients of solutes are consequences of empirical modifications of the Stokes–Einstein equation. In this sense, Olander [25] and Hayduk and Laudie [26] have shown how the Wilke–Chang correlation [27] (see below) is very accurate for the determination of diffusion

Table 2
Diffusion coefficients of anionic surfactants

	V_c (cm^3 mol^{-1})	(V_A cm^3 mol^{-1})	D^0_{AB} (cm^2 s^{-1})
DOSS	1337.9	538.7	$3.2 \cdot 10^{-6}$
DHSS	1101.5	439.4	$3.7 \cdot 10^{-6}$
DBS	1037.7	412.3	$3.8 \cdot 10^{-6}$
SDS	817.8	321.6	$4.4 \cdot 10^{-6}$

coefficients of hundreds of solute–solvent systems, including a series of organic solutes. Following the Wilke–Chang empirical correlation, the diffusion coefficients of the anionic surfactants sodium dodecyl sulphate (SDS), dioctyl sulphosuccinate (DOSS), dihexyl sulphosuccinate (DHSS) and dodecylbenzene sulphonic acid (DBS) in water were determined (see Table 2).

The Wilke–Chang equation is:

$$D^0_{AB} = 7.14 \cdot 10^{-8} \frac{(\phi M_B)^{1/2} T}{\eta_B V_A^{0.6}}$$

where D^0_{AB} is the diffusion coefficient of solute A in solvent B in diluted solution (cm^2 s^{-1}), M_B the molecular weight of solvent B, T temperature (K), η_B viscosity of the solvent B (cP), V_A molar volume of solute A at its standard boiling temperature (cm^3 mol^{-1}) [1], ϕ association factor of solvent B (for water $\phi = 2.26$).

V_c (cm^3 mol^{-1}) is estimated by the method of chemical moities contributions following Vetere (A. Vetere, private communication to R.H. Perry (1976)):

$$V_c = 33.04 + (\Sigma M_i \Delta V_i)^{1.029}$$

where M_i is the molecular weight of the chemical group i and ΔV_i is the contribution to the volume of the chemical group i.

With regards the quantification of the internal mass transfer resistances for SDS throughout the support matrix, three controls with the reticulated polyurethane foam membrane reactor, filled its lumen loop with 0.06, 0.10 and 0.19 g l^{-1} SDS, were carried out. The another loop being filled ultrapure water. The remaining SDS concentration in the substrate loop was measured at different times. According to Tuwiner [30], the operation of this membrane reactor with these controls was considered as a batch dialysis system where the steady state is never achieved. Following Tuwiner [30], the rate equation for this transport process is:

$$kt = -m \log [(C_{so} - C_s)/C_{so}]$$

where k is the first-order rate constant for dialysis, t is the time, m is the volume of the inlet/volume of the dialysate ratio ($m = 0.5$ in this case), C_{so} is the initial solute (SDS) concentration, and C_s is the solute concentration in the dialysate. The corresponding fitting of our data to such a rate equation gave the following k values: $1.48 \cdot 10^{-7}$, $9.8 \cdot 10^{-8}$, $2.34 \cdot 10^{-7}$ s^{-1}. The average being $k = 1.5 \cdot 10^{-7}$ s^{-1} ($t_{1/2}$ = 53.5 day), i.e., very slow diffusional transport of SDS across the reticulated polyurethane foam membrane.

3.3.3. Sorption of SDS and biodegradative activity of entrapped cells

When incubating SDS with immobilized *Pseudomonas* and with empty support particles, simultaneous and synergic processes of sorption and biodegradation of SDS were found on analysing the remaining SDS concentration in water by MBAS. During the first 10 min (for 35 cubes) and 150 min (for 280 cubes), sorption of the surfactant on the support was the fastest and principal process involved in its removal from solution and, in the last case, only after sorption equilibrium had been reached did the biodegradative activity of immobilized cells become evident (Fig. 6). The relative influence of each process depends on the operational time, the sorption of the surfactant being predominant at lower operational times. As time progressed, adsorption became negligible and biodegradation became predominant.

[1] V_A was calculated following Tyn and Calus [28]: $V_A = 0.285 V_c^{1.048}$, V_c being the critical volume of the organic solute A.

3.3.4. Operational stability of the bioreactor removing different surfactants

Kinetic runs were carried out for several days, testing the remaining SDS in water vs. time of operation of the recirculation bioreactor packed with polyurethane reticulated foam particles with and without (controls) entrapped cells (flow rate 28 ml/min, pH 7.1, known weight of support, different initial SDS concentration 0.1–0.3 g l^{-1}). After each day of operation, the two bioreactors were challenged with a new cycle with the same initial SDS concentration, and after four cycles of one day of operation, when the support particles became saturated with SDS, the bioreactors were incubated in MBS–Mg solution for 24 h (for SDS being desorbed from the support) and then challenged again with successive cycles of SDS solutions. Fig. 7 shows the sorption kinetics (square points) of controls, and the sorption plus biodegradation kinetics (round points) for bioreactors challenged with consecutive cycles of SDS. The biodegradation kinetics could be obtained by subtracting the square points from the round points vs. time. Other similar experiments, with similar results, were carried out at different SDS concentrations.

From the above experiments, it may be concluded that a significant SDS-removing activity is maintained up to 5–6 cycles (days) of continuous operation, the contribution of physical sorption of SDS being significant during the first two cycles (days), while afterwards primary biodegradation of the surfactant becomes the main SDS removing step. Incubation of the packed particles in MBS–Mg solution, favouring the desorption of SDS from the support, enhances further SDS-removing activity (sorption for controls and immobilized cell bioreactor). After this, the bioreactors recover their

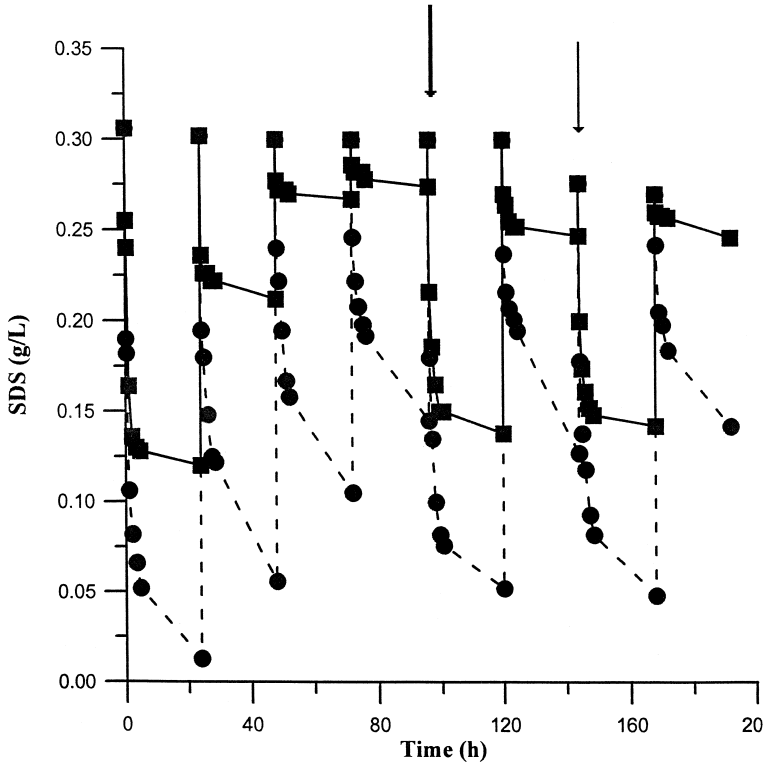


Fig. 7. Operational stability of SDS removal kinetics by *Pseudomonas* immobilized on polyurethane foam (support (■), cell loaded support (●)). For other experimental conditions, see text. Arrows mean recirculation of a high ionic strength solution (MBS–Mg) through the packed particles for 24 h.

Table 3
Kinetics of anionic surfactant removal from aqueous solutions by *Pseudomonas C12B* immobilized in reticulated polyurethane foam

Surfactant	Control k_1 (min^{-1})	Immobilized biomass in polyurethane	
		k_1 (min^{-1})	k_2 (min^{-1})
SDS	0.038	0.032	8.6 e-4
DBS	0.045	0.039	3.5 e-4
DHSS	0.089	0.05	1.1 e-3
DOSS	0.22	0.092[a]	4.9 e-4[a]

[a] For *Comamonas terrigena N3H*.
Correlation coefficient: $r = 0.96–0.99$.

surfactant removing activity after each operational cycle of 3 or 4 days by desorbing the substrate and products from the support particles by recirculating a high ionic strength solution through them for 24 h, the solution being removed afterwards.

Similar experiments, with similar results, were carried out with DHSS and DBS (Table 3).

The overall surfactant removal from water for polyurethane foam without entrapped cells was well fitted to first-order kinetics (remaining substrate concentration vs. time: [Surfactant] = $Ae^{-k1t} + C$) (see controls of Table 3). For the cells entrapped in polyurethane foam, the overall kinetics were well fitted to a biphasic process ([Surfactant] = $Ae^{-k1t} + Ae^{-k2t} + C$) (Table 3); first, a rapid sorption step of the surfactant on the cell-loaded support (k_1) followed by the slower intrinsic primary biodegradation step (k_2). Depending on time, the remaining surfactant concentration is mainly due to sorption or primary biodegradation or both cooperative processes.

It is shown in Table 3 that the k_1 for adsorption of surfactant when the support is empty of cells (control) is greater than k_1 when the support is cell-loaded, meaning that the cells on the support particles could interfere with the (specific) surfactant–support interaction by occupying some of the ligand binding sites of the support particle. Furthermore, DHSS adsorption and biodegradation by entrapped *Pseudomonas* C12B are faster than for SDS and DBS.

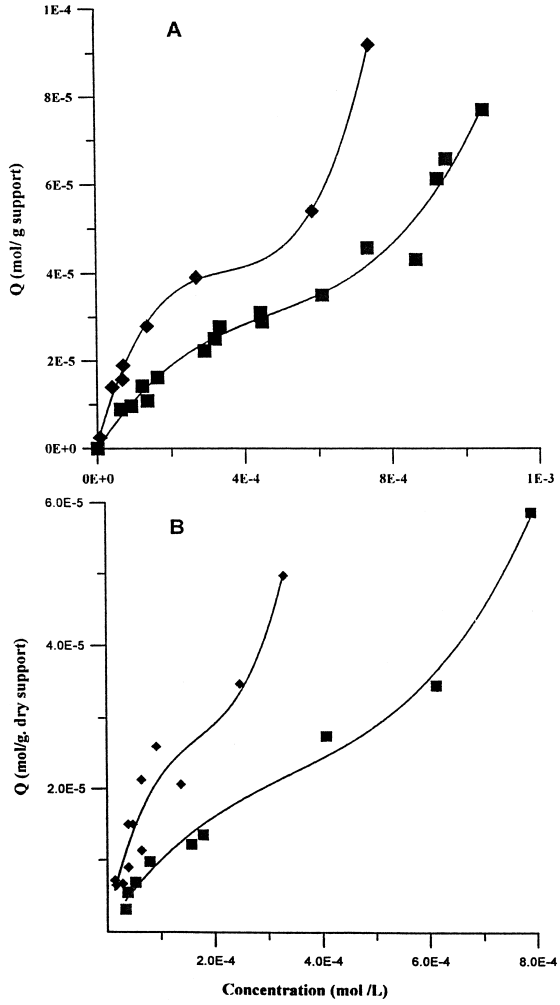


Fig. 8. Sorption isotherms for SDS (■), DOO (♦) (A); DBS (■) and DHSS (♦) (B) on polyurethane foam. For experimental conditions, see text.

Table 4
Sorption isotherms fitted to an empirical polynomial equation (see text) parameters and correlation coefficient

Surfactants	a (mol absorbed · l/ mol · g support)	b (mol absorbed · l^2/ mol^2 · g support)	c (mol absorbed · l^{-1}/ mol^{-1} · g support)	r^2
SDS	0.131	−207.8	1.46 · e5	0.981
DBS	0.111	−216.3	2.11 · e5	0.992
LAS	0.382	−738.2	5.26 · e5	0.940
DOSS	0.296	−784.8	7.41 · e5	0.998
DHSS	0.336	−1597.6	3.16 · e6	0.933

3.3.5. Sorption isotherms

The sorption isotherms for SDS, DHSS, DOSS and DBS on reticulated polyurethane foam have been established as type II of the Brunauer, Deming, Deming and Teller (BDDT) classification (Fig. 8). After being sorbed as a monolayer, the surfactant continues its sorption as a multilayer on the support particle. The data on moles of sorbed surfactant per g dried support (Q) vs. the surfactant molar concentration at equilibrium (C) are well fitted by an empirical third-order polynomial whose parameters (a, b, c) are shown in Table 4.

$$Q = aC + bC^2 + cC^3$$

As may be seen from Fig. 8 and Table 4, the shapes of the adsorption isotherms of SDS and DBS are very similar, because the values of parameters a, b, c, and consequently Q, are very close. However, DBS seems to be adsorbed less (for $C = 1 \cdot 10^{-4}$ M, $Q_{DBS} = 9.15 \cdot 10^{-6}$, $Q_{SDS} = 1.12 \cdot 10^{-5}$, a 22.4% difference) possibly because the mesomeric effect of the $-SO_3^-$ group on the benzene ring, which causes delocalization of charge, is higher than the negative inductive effect, which causes localization of charge on the $-SO_3^-$ group, consequently provokes the intermolecular electrostatic interaction (ion–dipole) ($-SO_3^-$-polar groups of the polyurethane) to decrease in intensity.

Additionally, the adsorption capacities of DOSS and DHSS are very similar (for $C = 1 \cdot 10^{-4}$ M, $Q_{DOSS} = 2.25 \cdot 10^{-5}$, $Q_{DHSS} = 2.08 \cdot 10^{-5}$), suggesting a reduced effect of the hydrocarbon side chains; that is, that the hydrophobic interaction in this adsorption process is less pronounced than the polar type. In sum, the adsorption of different surfactants onto reticulated polyurethane foam can be said to be due to a specific polar-type reversible hydrophilic interaction that becomes patent at low surfactant concentrations (up to $C = 0.2$–0.4 mM) (on the first stretch of the adsorption isotherms a Langmuir-type monolayer adsorption is seen, Fig. 8), together with a nonspecific interaction, which is also reversible, that becomes apparent at higher surfactant concentrations ($C > 0.4$ mM) (on the second stretch of the adsorption isotherms physical adsorption in multilayers is seen). These interactions are physical in nature; that is, adsorption is reversible because in the presence of high ionic strength, the spontaneous desorption of these surfactants occurs.

4. Conclusions

As a consequence of the simultaneous and synergic action of both sorption and primary biodegradation of anionic surfactants (SDS, DBS, DHSS) (DOSS) by *Pseudomonas* cells entrapped on polyurethane reticulated foam, an action that provides enhanced kinetics of removal of the surfactant, and in view of the low cost and ease of synthesizing the support, the system seems to have more advantages than other immobilized biocatalysts when attempting to remove these representative anionic surfactants.

Sorption isotherms for the surfactants on reticulated polyurethane foam have been established as type II of the Brunauer, Deming, Deming and Teller (BDDT) classification. The adsorption could be due to a specific polar-type

reversible hydrophilic interaction that becomes patent at low surfactant concentrations (Langmuir-type monolayer), together with a nonspecific interaction, which is also reversible, that becomes apparent at higher surfactant concentrations (physical adsorption in multilayers).

The kinetics for the overall surfactant removal from water for cell-loaded polyurethane foam were well fitted to a biphasic process ($[\text{Surfactant}] = A_1 e^{-k1t} + A_2 e^{-k2t} + C$), a rapid first-order sorption step of the surfactant onto the cell-loaded support ($k_1 = 0.032$–0.050 min^{-1}) and the intrinsic primary biodegradation slower step (Michaelis–Menten (Monod) kinetics under low substrate concentration regime) ($k_2 = 3.5 \cdot 10^{-4}$–$1.1 \cdot 10^{-3}$ min^{-1}), both acting synergically. Furthermore, the kinetics of the primary biodegradation of SDS by *Pseudomonas* covalently immobilized on sepiolite were found to be also first-order ($k_1 = 0.05$–0.126 min^{-1}). In this case, surfactant adsorption does not exist.

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