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Full Length Research Paper

Biodegradation of dodecylbenzene solfonate sodium by *Stenotrophomonas maltophilia* Biofilm

Hosseini Farzaneh[1]*, Malekzadeh Fereidon[1], Amirmozafari Noor[2] and Ghaemi Naser[3]

[1]Department of Biology, College of Basic Science, Islamic Azad University Research and Science Tehran branch, Tehran, Iran.
[2]Department of microbiology, Iran University of Medical Sciences, Tehran, Iran.
[3]Department of Biotechnology, College of Science, Tehran University, Tehran, Iran.

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Immobilization for microbial cultures has proved to be advantageous in municipal and industrial sewage treatment because of high degradation efficiency and good operational stability. In this survey, a bacterial strain was isolated from activated sludge that utilized branched anionic surfactants (BAS) as a sole carbon source. Identification of isolated strain was from 16S rRNA sequencing method. The immobilized cells on silanized glass beads as support and unmodified surfaces were used for removal of BAS; both types showed effective biodegrading of BAS. The removal rate in silanized surface was about 2 fold of unmodified surface. The result of biodegradation was studied by HPLC method and scanning electron microscope.

Key words: Branched anionic surfactants, biofilm, biodegradation, silanization.

INTRODUCTION

Synthetic anionic surfactants are economically important chemicals and are widely used in detergents, paints, polymers, pesticides, oil recovery, textiles and paper industries (Guang-Guo, 2005). The toxicity of a surfactant is related to its chemical structure; in general, the longer the fatty chain and branched chain anionic surfactants, the more toxic the surfactant is to aquatic organisms (Figure 1) (Andes et al., 2004; Stalmans, 2003). Anionic surfactants such as branched alkyl benzene sulfonates (BAS), which have higher sudding and weak biodegradeability potentials are less often used recently (Andes et al., 2004). Most branched alkyl sulfonates such as alkyl phenols have shown to be capable of inducing the production of vitellogenin in male fish at a low concentration (Swisher, 1963). BAS as well as linear anionic surfactants are not degraded by microorganisms in the environment. Because of its low biodegradation ability and toxic effects on environment, BAS was forbidden in advanced countries. But due to their low costs now they are widely used in detergent formulations in some countries. Water pollution by branched alkyl benzene sulfonates (BAS) is a significant environmental problem in these countries (Campos-García et al., 1999). During biodegradation, microorganisms can either utilize surfactants as sole sources of carbon and energy (Guang-Guo, 2005). It has been found in the environment as a growth-promoting agent in the rhizospheres of plants, as well as in soil, water, sediment, sewage, frozen foods and some other habitats (Whitby et al., 2000). Biofilms are structured microbial communities in which the microbial cells irreversibly attach to a surface or interface and become embedded in a matrix of extracellular polymeric substances produced by these cells (Andes et al., 2004). Bacteria that are attached to a surface express different genes and behave differently from free-floating or planktonic bacteria (Walker and Sedlacek, 2007). Banks and Bryers (1992) reported that growth rate of microorganisms plays an important role in a biofilm, with the faster growing microorganism having a competitive advantage over the slower growing microorganism (Banks and Bryers, 1992). Power et al. (1998) observed that a thick biofilm-forming bacterial species could provide a mechanism to increase the population numbers of a non-mucoid, degradative organism in a porous media environment (Power et al., 1998). Jeraaabkovaa et al. (1999) prepared bioreactors

*Corresponding author. E-mail: farzaneh953@yahoo.com. Tel: +98021-22641842.

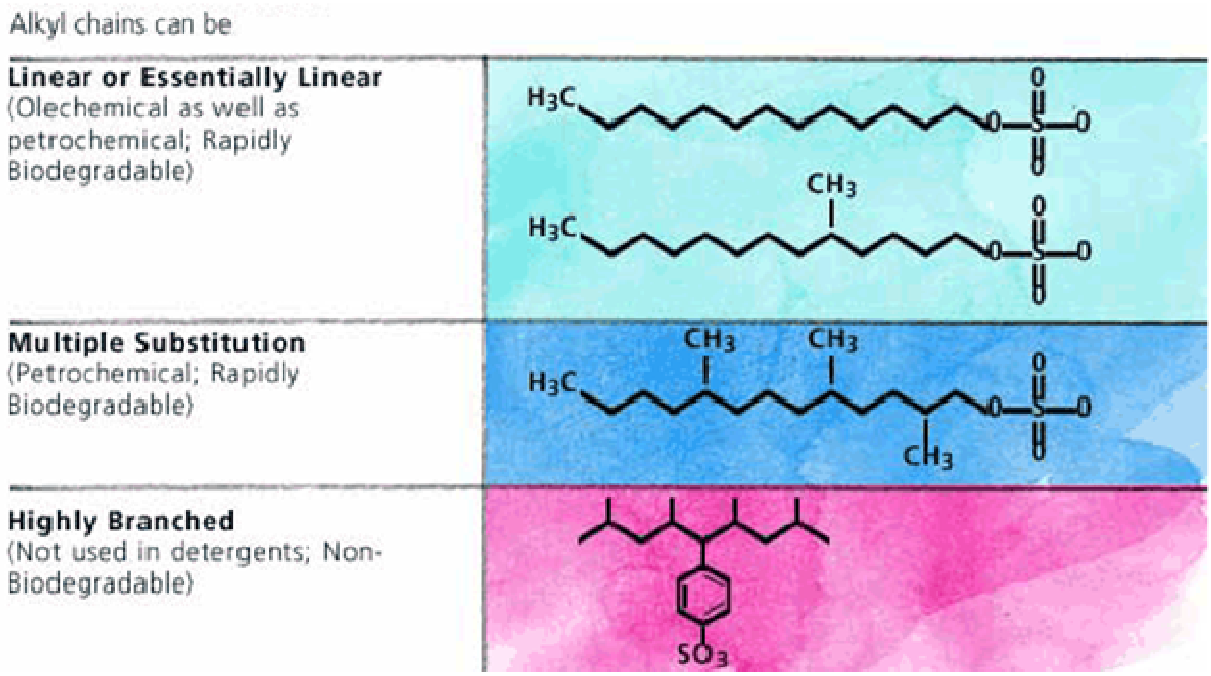


Figure1. Surfactants structures (Swisher, 1963)

Table 1: Oligonucleotide primers used for PCR (Vandamme and LiPuma, 2002)

Locus	PCR primers
I	f [0077906] CACCGCCGAGTGCGATGCCGATCTT r [0078751] ACCCGACCGTGGACATGGACGTGCG
II	f [0458122] GACGTGAAGTGGCTGCGCCTGAAGC r [0458343] CGTTCCAGCCACTGTACCGCCACCA
III	f [0897802] GTGGTGGTGATCAAGCGCGGCAAGG r [0898549] GGCAGGTCGGCTGGATGGCGGTACT

with Pseudomonas C12B cells immobilized on both types of porous glass beads for removal of SDS (Jeraaabkovaa et al., 1999).

The purpose of this work is to estimate biodegradation of BAS in immobilized biofilm of *Stenotrophomonas maltophilia* which was silanized on glass beads.

MATERIALS AND METHODS

Growth medium and enrichments cultures

In this study, commercial BAS, such as dodecylbenzene solfonate sodium with benzene sulfonic acid, acting as side chain ($C_{18}H_{29}SO_3$- Na+) containing 80.2% active matter was used (Swisher, 1963). The source of inoculate was used from activated sludge from the sewage treatment plants in Tehran, Iran. Growth medium was obtained from phosphate buffered minimal salts medium (pH adjusted to 7.2), having 5% (v/v) inoculums of BAS as the sole source of carbon and energy required for growth in 5 ml cultures in 50 ml screw- cap tubes which were aerated at 28°C with constant shaking (150 rpm) (Whitby et al., 2000). Cultures were transferred to solidified medium which was supplemented with 1% agar, 1.5 mM BAS and phosphate buffered minimal salts medium, when both growth and invisible foam occurred. After subculturing for 5 steps, a wild bacterial strain was isolated.

16 SrRNA sequencing

Identification of strains was used from 16SrRNA sequencing method. DNA preparation and 16SrDNA sequence analysis were obtained using primers as shown in Table 1. DNA was prepared as described (Vandamme and LiPuma, 2002). Isolated strain was cultured in LB medium for 24 h in 28°C. Approximately 1 ml of pure culture in LB was mixed with an equal volume of suspension buffer (50 mM trisodium citrate, 1% *N*-acetyl-L-cysteine, 2% NaOH), incubated at room temperature for 15 min and centrifuged at 15,000 × g for 5 min. The resulting pellet was suspended in 100 µl of extraction buffer (1% Triton X-100, 0.5% Tween 20, 1 mM EDTA, and 10 mM Tris-HCl [pH 8.0]) and centrifuged at 15,000 × *g* for 5 min and the pellet was resuspended in 50 µl of TBE buffer (pH 8.0). The bacterial suspension was lysed by 5 cycles of freezing in liquid

Figure 2. A deep yellow pigmentation of *S. maltophilia* in medium with phosphate buffered minimal salts medium and BAS as a sole carbon source.

nitrogen for 3 min and heating for 3 min in boiling water. After a final centrifugation step of 15,000 × g for 5 min, the supernatant, containing total DNA was used as template (5 µl) in the PCR. Each primer contained a 1 µM concentration, 10 ng of genomic DNA, a 200 µM concentration of each deoxynucleotide triphosphate, and 1.25 U of Taq DNA polymerase (Metabion) in a 3 mM $MgCl_2$ PCRbuffer (Metabion), with a total volume of 50 µl. After extraction and treatment with RNase, purified product was obtained by GFX PCR DNA and gel band purification kit. The cycle parameters consisted of annealing at 58°C for 10 s, extension at 72°C for 60 s, and dena-turation at 95°C for 10 s. For the last cycle, the extension step was 2 min. Then, 20 µl of each reaction mixture was subjected to electrophoresis in a 0.8% agarose gel in 0.5 × Tris-borate-EDTA (TBE) buffer (pH 8.0), alongside a 100 bp ladder. The PCR products were visualized and photographed after ethidium bromide staining (Whitby et al., 2000). The molecular marker (M) is a 100 bp ladder. Sequences were compared to known 16S rRNA gene sequences in the composite non-redundant database including GenBank by using the BLAST search program (Andrew et al., 2002).

Immobilized cells on glass beads

Pure culture of isolated strain cells were collected by centrifugation (5 min/5000 rpm) and rinsing with sterile minimal salts medium. Porous glass beads (pore diameter 16 ± 40 mm) as support surface were used. Half of them were exposed to 3-aminopropyltrimethoxy silane (2% v/v) for 5 min. After treatment, the beads were washed with 25:1 methanol; they were watered and dried. Suspended cells (OD 650 nm = 0.5) with treatments beads were added to an Erlenmeyer flasks, and they were shaken for 5 h in 150 rpm. Containers from Erlenmeyer were washed twice with phosphate buffered minimal salts medium for removing free cells as described. The protein concentration was determined by the method of Lowry with the bovine serum albumin as a standard (Jeraaabkovaa et al., 1999). 0.05%, w/v filter-sterilized BAS was added to immobilized cells.

Analytical methods

Biodegradation of commercial BAS was determined by HPLC (C-18 column; 18 cm length and 4 mm width using an isocratic mobile phase gradient of acetonitrile-water (80:20) at a flow rate of 1 ml/ min) in days of 1, 3, 5, 10 and 15 incubation.

Scanning electron microscopy

Glass beads were placed in fixative (2.5% (w/v) glutaraldehyde) overnight. Each sample was washed in phosphate-buffered saline 3 times for 10 min, fixed in 1% buffered sosmium tetroxide for 1 h under hooded conditions and immediately buffered washed twice for 10 min each time. Fixed samples were dehydrated in a graded ethanol series: 30, 50, 75 and 95% for 10 min each and finally with 100% ethanol for 1 h and air-dried overnight under a hood. Each was then mounted, sputter-coated with gold/palladium; the surface of glass beads was examined using a scanning electron microscopy at the Microscopy Core Laboratory of the Islamic Azad University Research and Science, Tehran branch.

RESULTS

The gram-negative bacteria were isolated for their ability to utilize dodecylbenzene solfonate sodium as the sole source of carbon and energy. The PCR amplification of the 16S rRNA genes of the bacterial isolate yielded the expected DNA amplicon of approximately 1.5 kb which falls within the size range of prokaryotic 16S rRNA gene. Analysis of the partially sequenced 16S rDNA of the isolate revealed 99% similarity to *Ralstonia* spp. and representatives of related genera from the GenBank database, using the BLAST search program (Andrew et al., 2002). Based on alignment of sequences, the results showed that the isolated strain belonged to *S. maltophilia*, previously known as *Xanthomonas maltophilia* and *Pseudomonas maltophilia* (Hsiao-Chuan, 2005).

It is an aerobic, gram-negative bacillus with deep yellow pigments widespread in a variety of environments (Figure 2). Determination of immobilized cells on glass beads was measured by optical density (OD_{650}) of the cell suspension. The OD of the cell suspension showed the lowest concentration after about 5 h. For determination of attached cell proteins, the protein concentration was determined. Comparison of the protein concentration between silanized glass and nonsilanized showed that in case of silanized glass it was about 0.894 mg/g and other was 0.452 mg/g.

The results of HPLC analysis showed that biodegradeation of DBS in medium with silanized beads was very low until 5^{th} day of incubation, whereas after 15 days it was 59.45% and in unmodified beads was 31.53% in same time (Figure 3 and 4). Micrographs from scanning electron microscope showed differences between unmo-

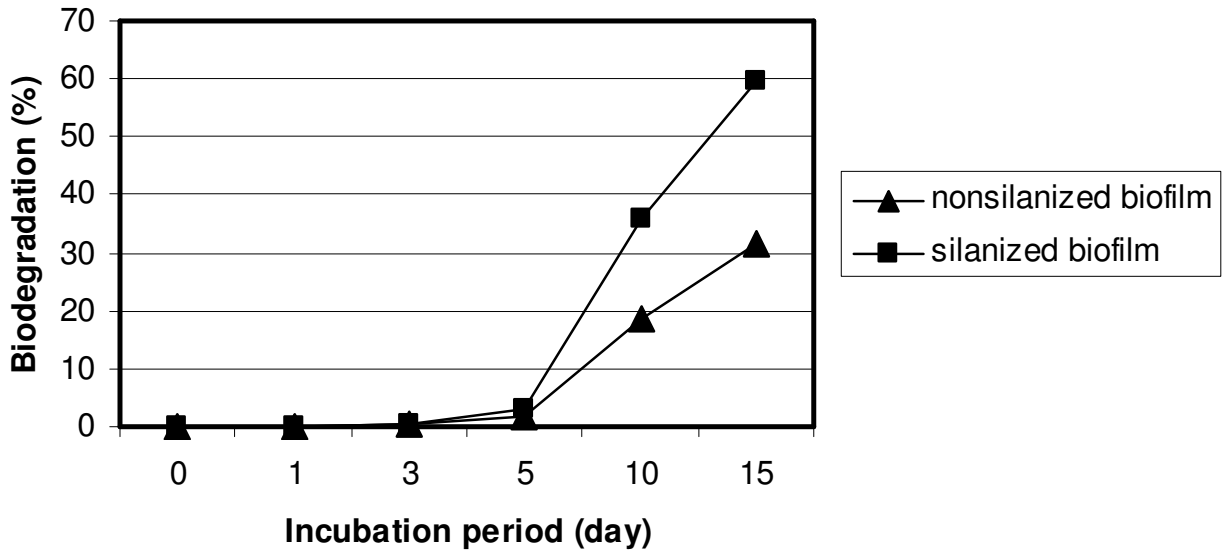
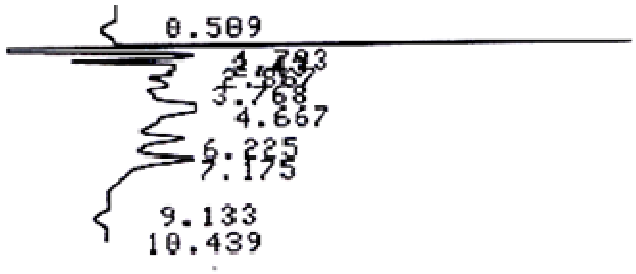


Figure 3. Biodegradation of dodecylbenzene solfonate sodium.

dified and silanized surfaces of glass beads. The size of biofilm increased for BAS concentration between 100 and 350µg/ml. On the surface of silanized particles, the high density of attached cells was seen. The distribution of cells on the surface of silanized is rather regular. On the other hand, the surface of nonsilanized glass beads was irregular and less than silanized support surface (Figure 5).

DISCUSSION

Biodegradation is the destruction of a chemical by the metabolic activity of microorganisms (Matthew et al., 2000). Many researchers used activated sludge in order to isolate bacteria that are able to degrade anionic surfactants (Schleheck et al., 2000; Schulz and Dong, 2000). Activated sludge provides elevated nutritional carbon and other factors necessary for growth of a wide variety of microorganisms; therefore it is an ideal source for isolation of specific bacteria capable of enzyme production or degradative potentials (Cain, 1981). In this study, we isolated surfactants degrading strains from activated sludge samples, indicating that such microorganisms may be widely distributed in wastewater treatment plants and discharge waters (Andrew et al., 2002). Schleheck et al. (2000) isolated α-proteobacterium strain DS-1 that could utilize commercial linear alkyl benzene solfonate (LAS) in aerobic culture (Schleheck et al., 2000). A *Pseudomonas aeruginosa* strain (W51D) was able to mineralize at least 70% of a linear alkyl benzene solfonate (LAS) commercial mixture. Branched alkenes are generally less susceptible to biodegradation than n-alkanes and certain methyl-branched alkenes (Campos-García et al., 1998). Previous studies described degradation of aminobenzenesulfonic acid by bacterial syntrophic interactions (Feigel and Knackmuss, 1993). *S. maltophilia* isolated from soil can utilize pesticides or chemicals, such as aldicarb as a source of hydrocarbon and energy through the action of esterase enzymes leading to its biodegradation (Karayilanoglu et al., 2008). *S. maltophilia* PB1, isolated from the culture, used RDX as a sole source of nitrogen for growth and degradation of hexahydro-1, 3, 5-trinitro-1, 3, 5-triazine (RDX) (Karayilanoglu et al., 2008; Binks et al., 1995). It has become accepted that biofilm-grown bacteria express different phenotypes and often exhibit totally different characteristics than do the same bacteria grown planktonically (Walker and Sedlacek, 2007). Drury et al. (1993) also observed that biofilms are significantly porous for bacterial-sized beads to become entrapped and could provide a mechanism to envelop other bacterial cells (Drury et al., 1993). In this survey, isolated strains were immobilized on silanized glass beads as a support surface. Silanization of surface of the glass support stimulated cell adsorption during immobilization. Negatively charged bacterial cells attached inerasably to positive silane groups (Jeraabkovaa et al., 1999). Continued growth and biomass accumulation of the bacteria were coincidental in the media. This indicates that the bacteria are actually utilizing BAS as their sole carbon. Although, the isolated strain had been tentatively identified based on biochemical-enzymatic characteristics, but difficulties in resolving the taxonomy of the *Ralstonia* spp. using a combination of RNA homology and phenotypic characteristics have been reported before (Palleroni, 1992). To overcome the problems associated with definitive identification, we developed species-specific PCR (SS-PCR) primers, directed to the 16S rRNA gene, and tested their utility to accurately identify *S. maltophilia* from pure



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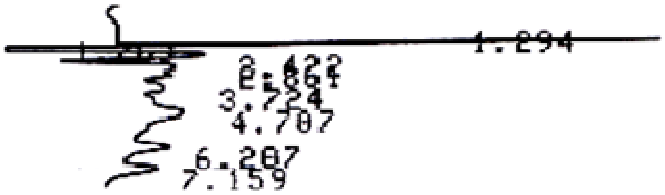
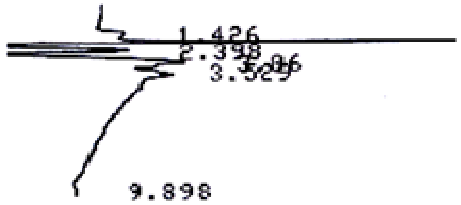
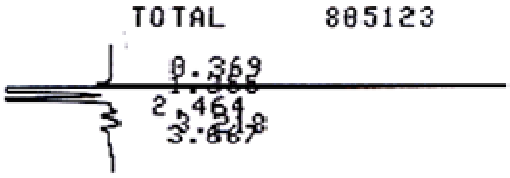
Area: 144216
Incubation day: 3



Retentio time: 2.422
Area: 138872
Incubation day: 5

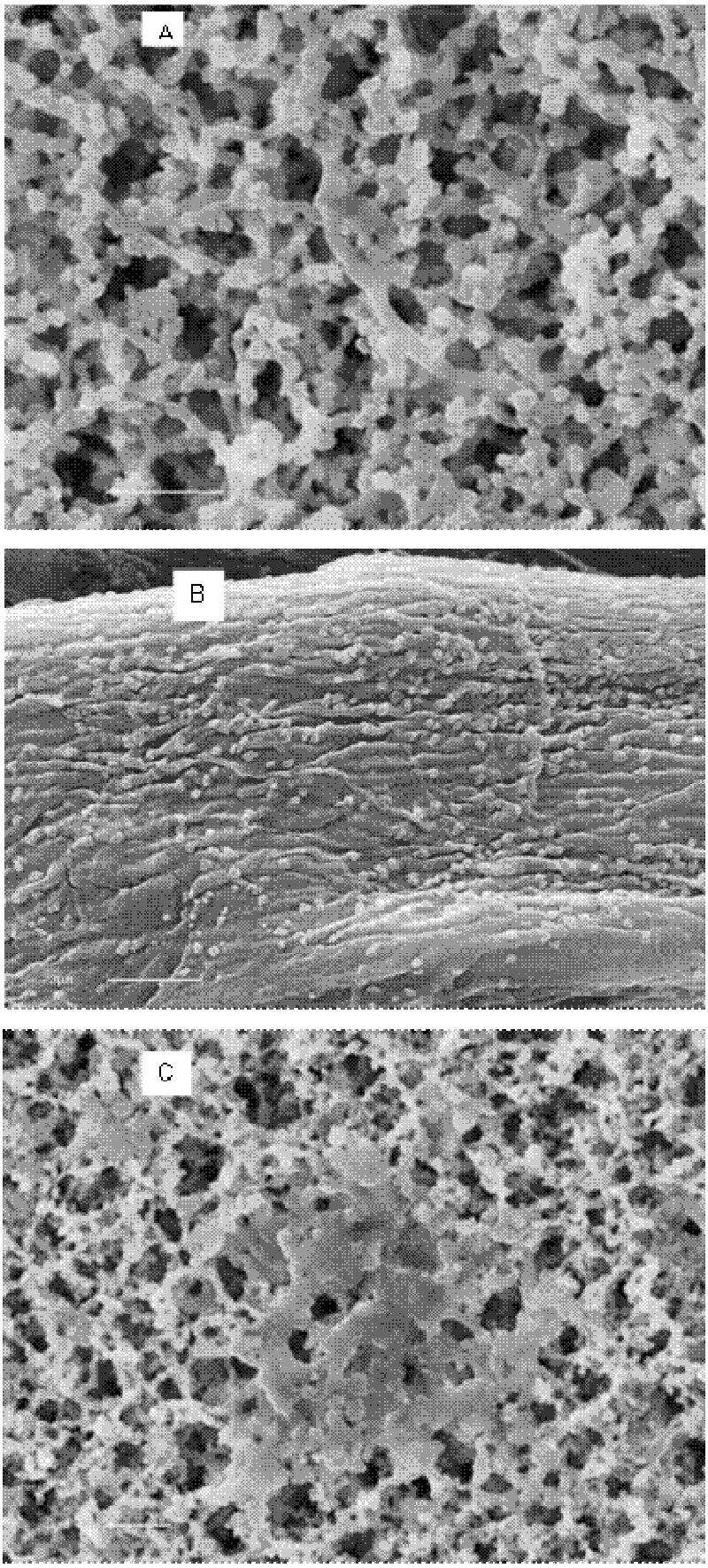


Retentio time: 2.398
Area: 91128
Incubation day: 10



Retentio time: 2.41
Area: 67312
Incubation day: 15

Figure 4. Biodegradation rate of BAS was determined by HPLC.


A
B
C

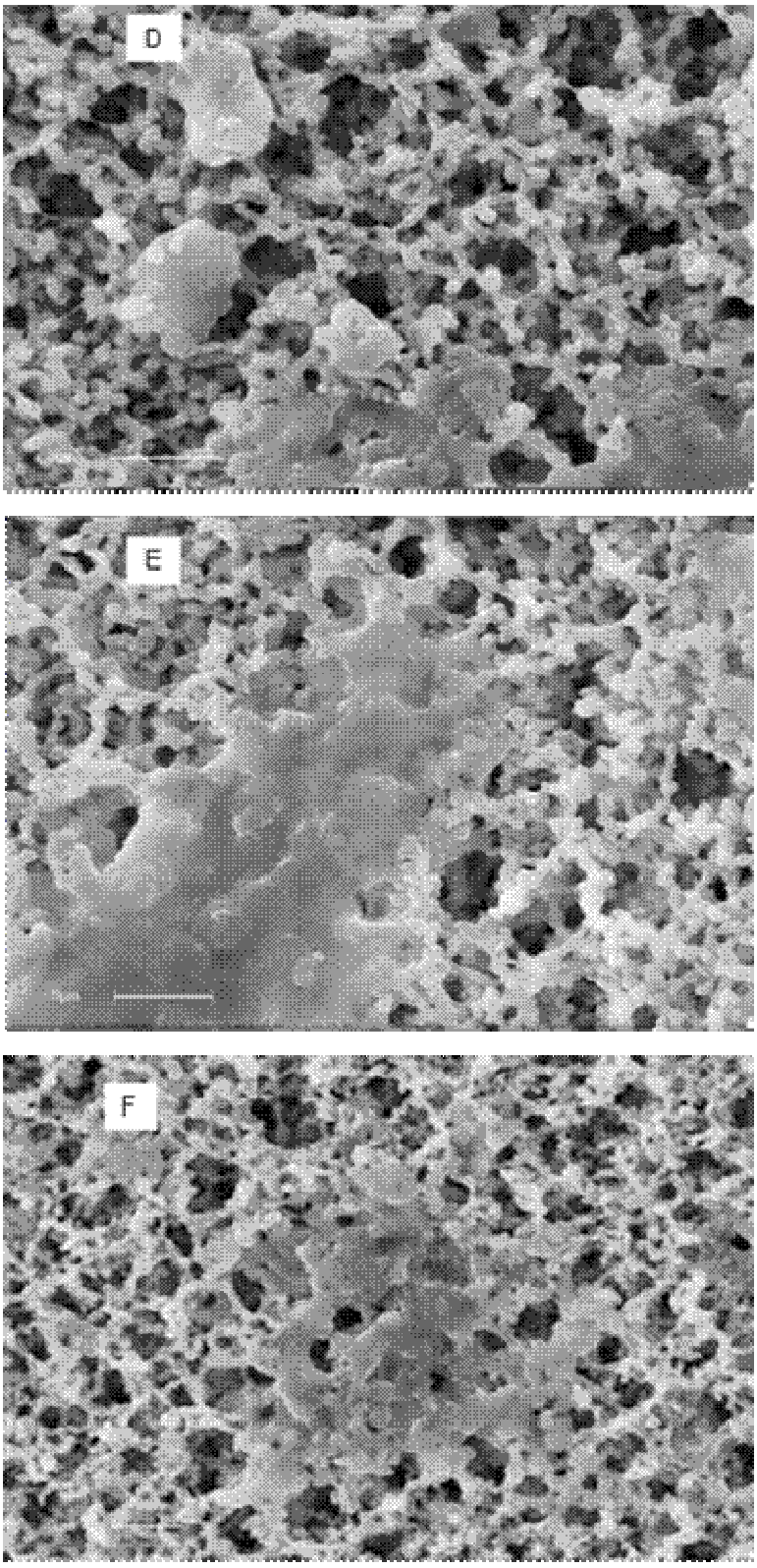


Figure 5. Scanning electron micrographs of *S. maltophilia* biofilm at 5 days of incubation; **A**, unmodified surface of glass and **B** and **C** silanized surface; **D** and **E**, at 10 days; **F** at 15 days in silanized surface. Magnification: 2500 fold, bars represent 20 mm.

culture (Withby et al., 2000). In conclusion, the results of this study showed that the main advantages of *S. maltophilia* biofilms on silanized glass beads were the simplicity of preparation, easy handling and a cost-effective method.

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