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Biodegradation of a Bioemulsificant Exopolysaccharide (EPS_{2003}) by Marine Bacteria

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Abstract The aim of the study is to analyze the biodegradation capacity of a biosurfactant exopolysaccharide (EPS_{2003}) by heterotrophic marine bacterial strains. During the initial screening performed in two sites located at the harbor of Messina for analyzing the response of marine bacterial population with the presence of biosurfactant EPS_{2003}, ten bacterial strains capable to degrade this substance were isolated. Between the bacterial strains isolated, two representative bacterial strains, isoDES-01, clustered with *Pseudoalteromonas* sp. A28 (100%), and isoDES-07, closely related to *Vibrio proteolyticus* (98.9%), were chosen for mineralization and respirometry test, performed to evaluate biodegradability potential of EPS_{2003}. Assays of bacterial growth and measure of concentration of total RNA were also performed. More than 90% of EPS_{2003} was mineralized by the isoDE01 strain for biomass formation and respiration, while EPS_{2003} mineralization by the isoDE-07 strain was less effective, reaching 60%. This approach combines the study of the microbial community with its functional aspects (i.e., mineralization and respirometry test) allowing a more precise assessment of biosurfactant degradation. These results enhance our knowledge of microbial ecology of EPS-degrading bacteria and the mechanisms by which this biodegradation occurs. This will prove helpful for predicting the environmental fate of these compounds and for developing practical EPS_{2003} bioremediation strategies from future marine hydrocarbon pollution.

Keyword *Pseudoalteromonas* · *Vibrio proteolyticus* · Biosurfactant · Bioemulsificant exopolysaccharide

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1 Introduction

One of the main reasons for the prolonged persistence of hydrophobic hydrocarbons in contaminated environments is their low water solubility, which increases their sorption to soil particles and limits their availability to biodegrading microorganisms (Desai and Banat 1997; Barkay et al. 1999). Presently, biosurfactants are mainly used in studies on enhancing oil recovery and hydrocarbon bioremediation (Banat 1995). Approaches to enhancing biodegradation often attempt to increase the apparent solubility of hydrophobic hydrocarbons by treatments such as the addition of synthetic surfactants or biosurfactants (Ron and Rosenberg 2001).

Studies employing synthetic nonionic surfactants have contributed significantly to our understanding of the mechanisms that enhance apparent solubility and the interactions among degrading bacteria, the surfactant, and the hydrocarbons (Aronstein and Alexander 1993; Volkering et al. 1995, 1997). However, the relative toxicity, low biodegradability, and limited efficiency at low concentrations reduce the potential for the applications of synthetic surfactants in contaminated sites.

The study of microbial consumption of emulsifying exopolysaccharides is relatively new. In our studies, we have used a new biosurfactant exopolysaccharide, named EPS_{2003}, produced by *Acinetobacter calcoaceticus* CBS 962.97, a hydrocarbon-degrading bacterium isolated in laboratory. As previously described (Crescenzi et al. 2003), the molecular structure of the biosurfactant corresponds to a polysaccharidic chain with hydrophobic fatty acids substitutions of 12–18 carbon atoms length (Fig. 1).

During the initial screening performed in two sites located at the harbor of Messina for analyzing the response of marine bacterial population with the presence of EPS_{2003}, ten bacterial strains capable to degrade this substance were isolated. Between the bacterial strains isolated, two representative bacterial strains, isoDES-01, clustered with *Pseudoalteromonas* sp. A28 (100%), and isoDES-07, closely related to *Vibrio proteolyticus* (98.9%), were chosen for mineralization and respirometry test, performed to evaluate biodegradability potential of EPS_{2003}. Bacterial growth and measure of concentration of total RNA were also performed.

2 Materials and Methods

2.1 Samples Collections

Seawater surface samples were collected at station "Norimberga" (38°11.46′ N–15°34.10′ E) and station "Mare Sicilia" (38°12.23′ N, 15°33.10′ E) from the harbor of Messina, Italy. The samples of water were collected in June 2008 using a 1-l Niskin bottles from the sea surface (16°C).

After collection, the water samples were immediately transported to the laboratory (5 min) in a cool box and used for further analysis. To monitor the quantitative abundance of microbial population present in the natural seawater samples, measures of bacterial density (living, dead, and total bacteria, cultivable bacteria) have been carried out.

2.2 Bacterial Community Characteristics

Total Bacterial Count Cell counts were performed by DAPI (Sigma-Aldrich S.r.L., Milan, Italy) staining on samples fixed with formaldehyde (2% final concentration). Samples were prepared as previously reported (Zampino et al. 2004; Cappello et al. 2007) and according to Porter and Feig (1980). All results were expressed as number of cells per milliliter.

Determination of Living, Dead, and Total Bacteria Living and dead bacteria were enumerated after staining with the Live/Dead BacLight Bacterial Viability Kit (Invitrogen Corp.; Molecular Probes, Inc., Eugene, OR, USA) as previously reported (Zampino et al. 2004; Cappello et al. 2007). All results were expressed as number of cells per milliliter.

Heterotrophic Cultivable Bacteria The heterotrophic cultivable bacteria were estimated by spreading 100 μl of tenfold dilutions of seawater samples on plates of Marine agar 2216 medium (Difco S.p.a, Milan, Italy), incubated at 20°C for 7 days. Results were expressed as colony-forming units (CFU) per milliliter. All measures were repeated three times.

2.3 Strains Isolation

Colonies have been isolated by spreading 100 μl of tenfold dilutions of natural seawater samples on ONR7a medium (Dyksterhouse et al. 1995) to which 1.4% of agar (Bacto Agar, Difco S.p.a., Milan, Italy) was added and 0.1% of EPS_{2003} (*w*/vol) as the sole carbon source. Plates were incubated at 20±1°C for 5 days.

2.4 Taxonomic Characterization of Isolates

Analysis of 16S rDNA was performed to the taxonomic characterization of isolated strains.

DNA Isolation and Extraction Total DNA extraction of bacterial strains was performed with the cetyltrimethylammonium bromide (CTAB) method.

PCR Protocol The 16S rDNA loci were amplified using one primer pair, the above reverse primer

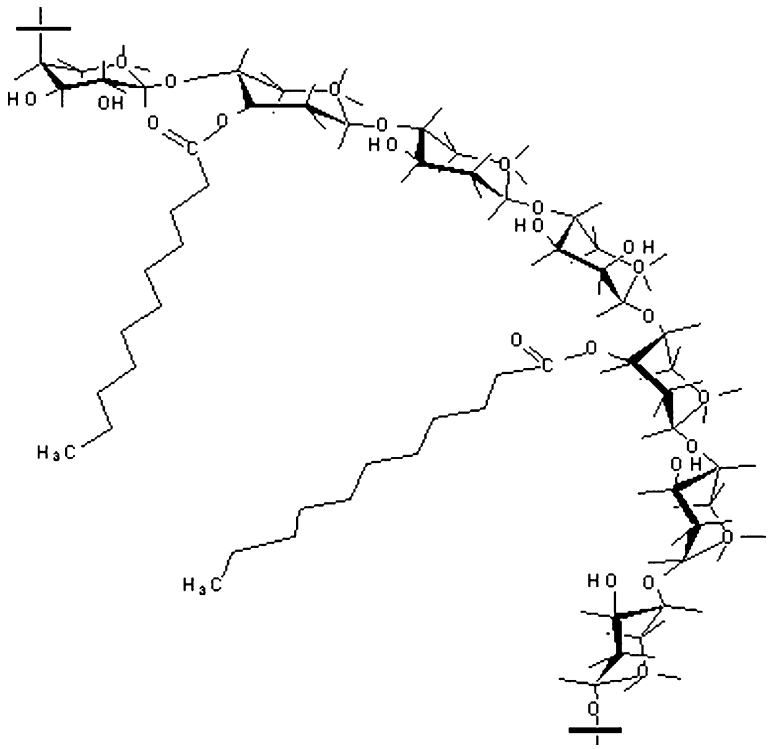

Fig. 1 Simplified structure of the biosurfactant (EPS_{2003}) used in this work

(Uni_1492R), and 16S rRNA forward domain-specific bacteria, Bac27_F (5′-AGAGTTTGATCCTGGCT CAG-3′; Lane 1991). The amplification reaction was performed in a total volume of 50 μl consisting of 50 of template, 1× solution Q (Qiagen, Hilden, Germany), 1× Qiagen reaction buffer, 1 μM of each forward and reverse primer, 10 μM dNTPs (Gibco, Invitrogen Co., Carlsbad, CA, USA), and 2.0 ml (and 2.0 U) of Qiagen Taq Polymerase (Qiagen).

Amplification for 35 cycles was performed in a thermocycler GeneAmp 5700 (PE Applied Biosystems, Foster City, CA, USA). The temperature profile for PCR was kept, 95°C for 5 min (one cycle); 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min (35 cycles); and followed by 72°C for 10 min at the end of final cycle.

Sequencing and Analysis of Amplicons The 16S rDNA amplified was sequenced with a BigDye Terminator v3.1 cycle sequencing kit on an automated capillary sequencer (model 3100 Avant Genetic Analyzer, Applied Biosystems). Analysis and phylogenetic affiliates of sequences were performed as previously described (Yakimov et al. 2004, 2005, 2006). The sequence similarity of individual inserts was analyzed with the program FASTA Nucleotide Database Query available through the EMBL-European Bioinformatics Institute.

2.5 Assays of Growth, EPS_{2003} Mineralization, and Respiration

Between the bacterial strains isolated, two representative bacterial strains were chosen for mineralization and respirometry test. Bacterial growth and measure of concentration of total RNA were also performed.

Bacterial Growth The representative strains were inoculated in a flask with 500 ml of ONR7a medium and ONR7a medium added with EPS_{2003} at 0.1% (*w*/vol). Cultures were incubated in Erlenmeyer flasks at 25°C for 12 days with shaking (Certomat IS B. Braun Biothec International, 80 rpm). Biomass variations were evaluated daily by optical density reading at 600 nm (Beckman Spectrophotometer DU-640, Beckman Coulter Inc., Fullerton, CA, USA). All measures were repeated three times.

Total RNA Rate Analysis The RNA content measurements are used as indicators of bacterial growth rate.

At regular intervals of 24 h, subvolumes (10 ml) of the bacterial culture were taken out, and bacterial cells were harvested by centrifugation at 6,000×*g* for 10 min (Eppendorf Centrifuge 5810 R). The total RNA was extracted by CTAB method (Chang et al. 1993); during the first extraction by phenol and chloroform, the concentration of total RNA was measured using a Spectrophotometer ND-1000 (NanoDrop). All measures were repeated three times.

EPS_{2003} Mineralization and Respiration The protocol which we used to measure EPS_{2003} mineralization rates has been described previously (Bruheim et al. 1997, 1999). Carbon dioxide evolution was determined by Warburg respirometry (Goksokir 1962). The cells were grown for 48 h (to the early stationary phase) in 500 ml shake flasks containing 100 ml of ONR7a medium at 25°C, centrifuged at 15,000×*g*, and washed twice in ONR7a mineral medium. The standard EPS_{2003} concentrations used was 0.01% (*w*/vol). EPS and ONR7a mineral medium (5 ml) were premixed in the central compartment during 30 min of temperature equilibration (25°C) before the cells were added. Cell concentration was determined spectroscopically with a predetermined relationship between A_{600nm} and dry biomass, and the appropriate volume was added to each flask to give a cell density of approximately 2×10^8 cells ml^{-1}. Flasks were immediately stopped with silicon rubber stoppers from which CO_2 traps consisting of glass vials with 0.5 ml of 2 M NaOH were suspended about 2 cm above the surface of the medium. Flasks were incubated for 5 days at 25°C. The content of the traps was periodically exchanged with a fresh aliquot of the base solution, and NaOH solution was later titrated to determine the amount of CO_2 evolved. CO_2 evolution reflected microbial activity. Results are presented as percent EPS_{2003} converted to CO_2. Mineralization rates were calculated during time intervals when mineralization progressed linearly with time. All experiments were carried out three times and two parallel cultures forever conditioned were analyzed for each experiment.

2.6 Statistical Analysis

Statistically significant differences between data obtained (bacterial growth, RNA rate analysis, and measures of EPS2003 mineralization and respiration) in the experiments were detected by the analysis of variance.

3 Results

3.1 Bacterial Community Characteristics

Estimations of total bacteria (DAPI count), measure of living dead cells (live/dead staining), and measure of cultivable bacteria (CFU) of microbial population present in seawater samples collected at station "Norimberga" and "Mare Sicilia" are reported in Table 1.

3.2 Strains Isolation

From the ONR7a agar plates with 0.1% of EPS_{2003} (*w*/vol), a total of ten strains were isolated. From station "Norimberga" were isolated four strains (isoDE-01, isoDE-02, isoDE-03, and isoDE-04) and six colonies (isoDE-05, isoDE-06, isoDE-07, isoDE-08, isoDE-09, and isoDE-10) from station "Mare Sicilia".

3.3 Taxonomic Characterization of Isolates

The phylogenetic position of strains isolated shows that all isolates belong to the γ-Proteobacteria are divided into two clear groups: three strains belonged to the *Vibrio* cluster (isoDE-03, isoDE-07, and isoDE-09) and seven strains (isoDE-01, isoDE-02, isoDE-04,

Table 1 Estimations of total bacteria (DAPI count), measure of living dead cells (live/dead staining), and measure of cultivable bacteria (CFU) of microbial population present in seawater samples collected

	Cellular abundance (cell ml^{-1})			
	Total cells	Live cells	Dead cells	CFU
Station "Norimberga"	9.51E+05	6.92E+05	2.59E+05	2.10E+03
Station "Mare Sicilia"	7.99E+05	6.62E+05	1.37E+05	2.40E+03

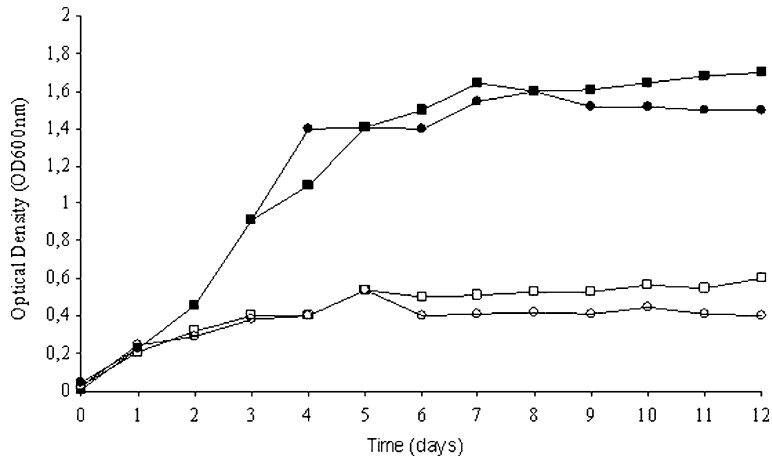

Fig. 2 Measure of bacterial growth of strains isoDE-01 and isoDE-07 during cultivation in ONR7a medium and ONR7a medium added with EPS_{2003} 0.1% (*w*/vol). Strains isoDE-01 and isoDE-07 were represented, respectively, by *square* and by *circle*. The condition growth with ONR7a is *colored in white*, with EPS_{2003} in *black*. Data presented were medium value of data obtained from experiments carried out in triplicate

isoDE-05, isoDE-06, isoDE-08, and isoDE-10) to the *Pseudoalteromonas* cluster.

3.4 Assays of Growth, EPS_{2003} Mineralization, and Respiration

Two representative bacterial strains isoDE-01 (*Pseudoalteromonas* sp. A28, 100%) and isoDE-07 (*V. proteolyticus*, 98.9%) were chosen to perform assays of growth, EPS_{2003} mineralization, and respirometry tests.

Bacterial Growth During the growth in ONR7a medium with EPS_{2003} 0.1%, the strain isoDE-01 showed an increment of biomass in the first week and then showed stable values of optical density of 1.7 (A_{600nm}). In comparison to the growth in ONR7a medium, this strain showed an increase of 2 orders of magnitude of the optical density until a final value of 0.6 (A_{600nm}).

The strain isoDE-07 showed a trend more similar than the isolate isoDE-01. During the first 4 days of cultivation on ONR7a medium with EPS_{2003} 0.1%, the biomass increased, passing from a value of 0.05 at value of 1.4 of optical density (A_{600nm}); successively stable values of optical density of 1.5 were measured during all experiment (Fig. 2).

Total RNA Rate Analysis During the growing in ONR7a medium with EPS_{2003} 0.1%, the strain isoDE-01 showed an increment of quantitative of total RNA, with values at the beginning of experimentation of 23 ng μl^{-1} that passing at values 63 ng μl^{-1} (4 days) and 85 ng μl^{-1} (12 days). During cultivation on ONR7a medium without the addition of EPS_{2003}, the quantitative total RNA remains stable at values 30 ng μl^{-1}.

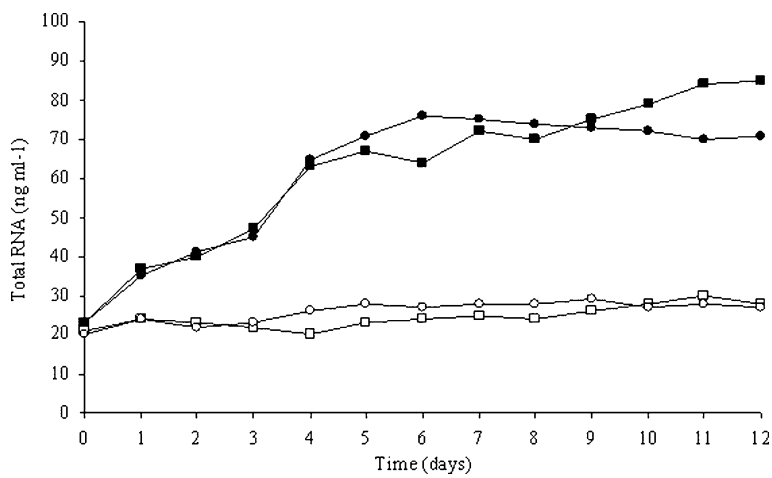

Fig. 3 Analysis of total RNA. *Square* and *circle* represented respectively strain isoDE-01 and strain isoDE-07. The *white color* represented cultivation in ONR7a medium, black cultivation in ONR7a medium with EPS_{2003} 0.1% (*w*/vol). Data presented were medium value of data obtained from experiments carried out in triplicate

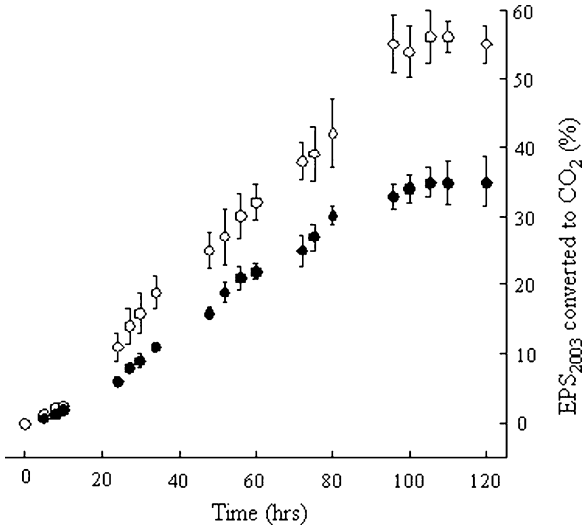

Fig. 4 Assays of EPS_{2003} mineralization by isolate isoDE-01 (*filled circles*) and isoDE-07 (*empty circles*)

Also, isoDE-07 strain showed an increment on quantitative total RNA with values 71 ng μl^{-1} at the end of the experimentation period. Constant values of 28 ng μl^{-1} were registered during cultivation in the control culture (Fig. 3).

Assays of EPS_{2003} Mineralization and Respirometry As shown on Fig. 4, mineralization of EPS_{2003} by isolate isoDE-01 proceeded faster and more complete than that of isolate isoDE-07 and was terminated almost at the same time, namely after 100–120 h of cultivation, when 35–55% of the added substrate was converted to CO_2. The calculated balance for EPS_{2003} mineralization by two strains used in this experiment is presented in Fig. 5. More than 90% of EPS_{2003} was mineralized by *Pseudoalteromonas* sp., strain isoDE-01, for biomass formation (35%) and respiration (55%), while EPS_{2003} mineralization by *V. proteolyticus*, strain isoDE-07, was less effective reaching 60% (25% and 35% for biomass formation and respiration, respectively).

Aerobic mineralization refers to conversion of exopolysaccharide EPS_{2003} to biochemical products (i.e., carbon dioxide, water, and microbial biomass). In general, respirometric devices are used to measure the total biochemical oxygen uptake and biochemical oxygen uptake rate exerted by microbial population.

4 Discussion

Almost 20 years ago, Rosenberg et al. described the microbial degradation of emulsan, polyanionic heteropolysaccharide biosurfactant produced by *A. calcoaceticus* RAG-1 (Sar and Rosenberg 1983; Shoam et al. 1983a, b). A mixed bacterial population was obtained by an enrichment culture that was capable of degrading emulsan and using it as a carbon source. From this mixed culture, an emulsan-degrading bacterium (YUV-1) was isolated. Strain YUV-1 is an aerobic, gram-negative, nonspore-forming, rod-shaped bacterium which grows best in media containing yeast extract. When placed on preformed lawns of *A. calcoaceticus* RAG-1, strain YUV-1 produced translucent plaques which grew in size until the entire plate was covered. Plaque formation was due to solubilization of the

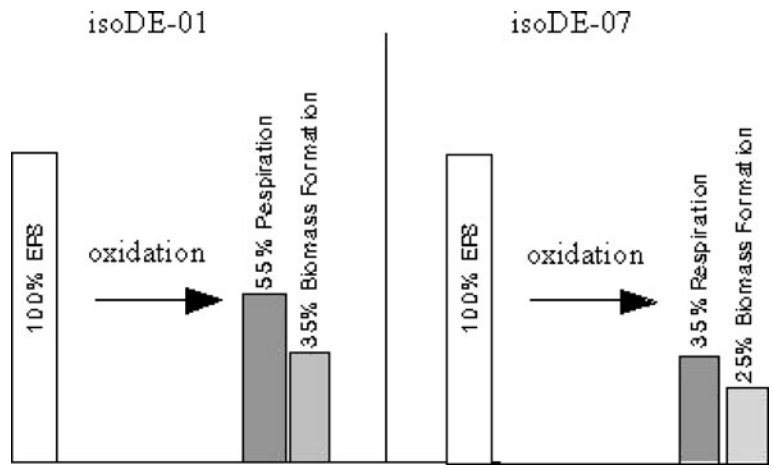

Fig. 5 Calculated balance for EPS_{2003} mineralization by strain isoDE-01 and isoDE-07 used during in this experiment

emulsan capsule of RAG-1. The enzyme responsible for the initial degradation attack was isolated and characterized as emulsan depolymerase (endoglycosidase). Exhaustive digestion of emulsan with emulsan depolymerase produced oligosaccharides with a number average molecular weight of about 3,000 Da (Shoam et al. 1983a, b). The degradation of EPS_{2003} for the strains isoDE-01 and isoDE-07 from the marine water samples confirmed EPS_{2003} biodegradation in the natural marine environment.

That study also described microbial consumption of emulsifying exopolysaccharides. In our study, it was found that EPS_{2003} was easily degraded in the marine environment, as shown by EPS_{2003} degradation by bacterial strains isoDE-01 and isoDE-07. This degradability was greater than those for similar compounds now used as emulsifying (emulsan). In fact, the microbial degradation of emulsan by bacterial strain YUV-1 has been documented as a biphasic process mere complex that often requires an additional easily degradable carbon source (for example, extract of fermentation).

The dynamic of this degradation is characterized (during the initial 24 h) by increasing cellular concentration of 10 orders of magnitude that does not correlate with visible decrease of emulsan (induction phase).

During the lag phase (from 24 at 48 h), the emulsan is inactive and depolymerized but still present. The biochemical consumption of strain YUV-1 on emulsan molecules occurs during the second growth phase (from 48 at 70 h) and consists of depolymerization and consequent decrease of its emulsification efficacy (until at 80% compared with the initial values; Shoam et al. 1983a, b). In comparison, EPS_{2003} is found to be sufficiently degradable in the natural marine environment. The bacteria used in the experimentation are autochthons of marine environment, and they can use EPS_{2003} directly as growing substrate.

The degradation dynamic follows a similar trend of other biodegradable products with an efficacy of degradation of 90% after 120 h of incubation in conditions in situ. Also, the bacterial populations that can degrade EPS_{2003} in the marine environment are well distributed in the natural communities. In fact, many isolates of bacterial genera *Pseudomonas* and *Vibrio* used EPS_{2003} as only carbon or energy source (data not presented).

The results obtained on the cultivation of sample strains isoDE-01 and isoDES-07 in a solid medium and in a liquid medium are in perfect agreement with the biomolecular results and with the mineralization and degradation tests. Both strains exhibit greater growth rates in a medium with added EPS_{2003} 0.1%, which was shown directly by increases in optical density and as total RNA rate, as well as indirectly by rates of bacterial activity and functionality.

It can be concluded that the microbial ecology of bacterial EPS_{2003} degradation and the mechanisms by which EPS_{2003} biodegradation occurs will prove helpful for predicting the environmental fate of these compounds and for developing practical EPS_{2003} bioremediation strategies from hydrocarbon pollution in the marine environment.

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