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ORIGINAL PAPER

Studies on the composition of biofilms used in the reconversion of oily greases from industrial wastewaters

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Abstract

A significant part of the research focusing on biofilms deals particularly with their (negative) effects in medicine. However, biofilms can be used in a multitude of practical scopes. Due to some characteristics of this form of supracellular organization of microorganisms, such as: functioning as a self-contained system, increased resistance to stress, ability to use limited nutritional resources, biofilms were increasingly used in different biotechnologies for wastewater treatment. Among different types of industrial wastes, oily wastewater is one of the most hazardous to the environment, as the oil molecules form a film on the water surface, thus impeding oxygenation and leading to dangerous long term - even irreversible - modifications of the aquatic biotope.

The importance of theoretical and practical knowledge on the composition of biofilms with lipolytic properties stems from their advantageous practical applications. In view of this fact, our work attempted the identification of the bacterial strains isolated from biofilms with lipolytic properties. Thus, three strains were investigated for nutritional demands and RFLP markers. The spectra of substrates required and/or metabolized by these strains, together with DNA patterns resulted from enzymatic digestions showed that one of the strains belonged to Pseudomonas putida species, one – to P. stutzeri, while the status of the remaining strain remained uncertain.

Keywords: oily wastewaters, biofilms, bio-remediation

Introduction

Among the lipolytic microorganisms, those able to adhere to glass, polymer, or stainless steel supports and to form biofilms belong to various bacterial species. [5, 17] There is an increasing number of papers describing methods to obtain and use biofilms in oily wastewater treatment, especially biofilms consisting of bacteria that belong to the *Pseudomonas* genus, also known as pseudomonads. The criteria for biological identification of *Pseudomonas* genus changed with the review of the old, artificial classification, based on phenotypic properties, to the new one based on genotypic properties (phylogenetic). [2, 4] Pseudomonads are important from both economical point of view and in nature, as they are active in the aerobe decomposition and biodegradation, and play a key role in the carbon cycle. [10, 11] They are known for their capacity to form biofilms and to participate in the decomposition of hardly degradable compounds, such as aliphatic and aromatic hydrocarbons, fatty acids, insecticides and other pollutants.

The analysis of biofilm composition and activity represents a promising scientific and practical preoccupation, which makes use of a plethora of microbiological, biochemical, and

molecular biology methods (genomic DNA isolation and purification, spectrophotometry, electrophoresis, PCR amplification, enzymatic restriction, sequencing), to allow the identification of biofixed microorganisms. The research on biofilms and their metabolic activities - which are far more diverse and efficient than those of the same isolated microbial cells in suspension -, is a reflection of the recent booming evolution of science and technology and attracts a lot of interest worldwide. [1, 14, 15].

Previously, using pseudomonad bacteria isolated from natural sources, we have investigated the aforementioned ability of biofilm formation [12] and their potential in oily wastewater treatment, with the result of a significant decrease of the chemical oxygen demand (COD) indicator [13]. The present paper aims to analyze the composition of these biofilms from the point of view of the dominant species, for further characterization and subsequent investigation of other possible applications in bio-remediation.

Materials and methods

Microorganism isolation and cultivation

The microorganisms were isolated from a biofilm spontaneously formed on glass slide supports, in a previously described oily wastewater treatment installation [12, 13]. The microorganisms were cultivated in liquid medium (7279A – Acumedia Manufacturers), at 29^0C, with stirring at 105 r.p.m., to achieve a 24 h liquid culture. Serial dilutions of this culture (10^{-5} – 10^{-7}) were made and used further for inoculation on solid medium (7329A – Acumedia Manufacturers). Isolated, well-developed colonies, at 2 to 3mm distance from one another, were sampled and transferred to test tubes containing the same slope-sided solid medium for *Pseudomonas*, for subsequent morpho-physiological examination.

Biochemical characterization of the microorganisms, using API 20 NE kit

API 20 NE kit contains 8 conventional test micro-tubes and 12 assimilation test micro-tubes. The first ones are inoculated with a saline suspension of bacterial cells (0.85% NaCl solution, up to a turbidity equivalent to 0.5 McFarland), and the last ones – with a cell suspension in minimal medium, thus reconstituting the dehydrated substrates. During incubation at 29^0C, depending on the properties of the inoculated bacterial cells, the colors of these tests change, either spontaneously or due to other added reagents. Reactions are interpreted by the help of an attached identification table. The kit also includes a test-reaction for oxidase, conducted on a microscope slide. The method and other required materials were as described by the producer (Biomérieux).

Bacterial genomic DNA isolation and purification

It was done with a Wizard® Genomic DNA Purification Kit (Promega Corporation), from a 19h liquid culture in 7279A – Acumedia Manufacturers.

DNA integrity was checked by electrophoresis in 1% agarose gel (molecular marker: BenchTop pGEM, Promega). The gels were visualized with an UV trans-illuminator (λ=302 nm), and photographed with a Polaroid GelCam.

PCR amplification of *16S RNAr* gene

For the PCR amplification reaction of *16S RNAr* gene of the *Pseudomonas* strains included in this study, a Peltier Thermal Cycler PTC-100 apparatus was used. Specific primers, GM3F - 5' AGA GTT TGA TCM TGG C 3' [8], GM4R - 5' TAC CTT GTT ACG ACT T 3' [6] and a GoTaq kit (Promega) were used as well, according to their respective

instructions. Magnesium ion concentration was adjusted to avoid non-specific amplifications. The amplification reaction protocol was adapted in the Genetics Laboratory, Faculty of Biology, Bucharest University. The initial stage of DNA denaturation/alteration was carried on at 95°C, for 1 min. There were conducted 30 amplification cycles: 1 min / 95°C, 1 min / 55°C, 1 min 45 sec / 72°C, after which the final elongation was done at 72°C, for 10 min.

Enzymatic restriction

Upon testing of a number of enzymes from the list compiled at Restriction Mapper 3 website (http://www.restrictionmapper.org/), following the analysis of the *16S RNAr* sequence, as found in the NCBI database (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=169757190&from=839694&to=841222&view=gbwithparts), *AluI*, *ApaI* (Promega) and *HphI* (New England BioLabs) were selected. Reaction mixtures were incubated for two hours, at 37°C, and the restriction fragments were visualized by electrophoresis in 1.5% agarose gel.

Results and discussions

Three strains isolated from their natural environment as described in *Materials and Methods*, which grew on the plates inoculated with the 10^{-7} dilution of the original culture were considered to be dominant and named *Strain I, Strain II* and *Strain III.* Microscopic analysis showed that the strains are Gram negative, non-sporulated, straight or slightly-curved rods with polar flagella. Obviously, these characteristics were insufficient to identify them as belonging to the *Pseudomonas* genus. Common properties of *Pseudomonas* gene were defined as being: saprophytic nutrition, aerobic metabolism, lack of fermentation, lack of photosynthesis, variable nitrogen fixing ability, and, respectively, the ability to grow and develop on a wide variety of organic substrates. [7]

Consequently, the isolated strains and *Pseudomonas putida* ICECHIM strain were biochemically analyzed and assessed for nutritional demands, using API 20 NE kit and *Pseudomonas aeruginosa* ATCC 27853 as a control.

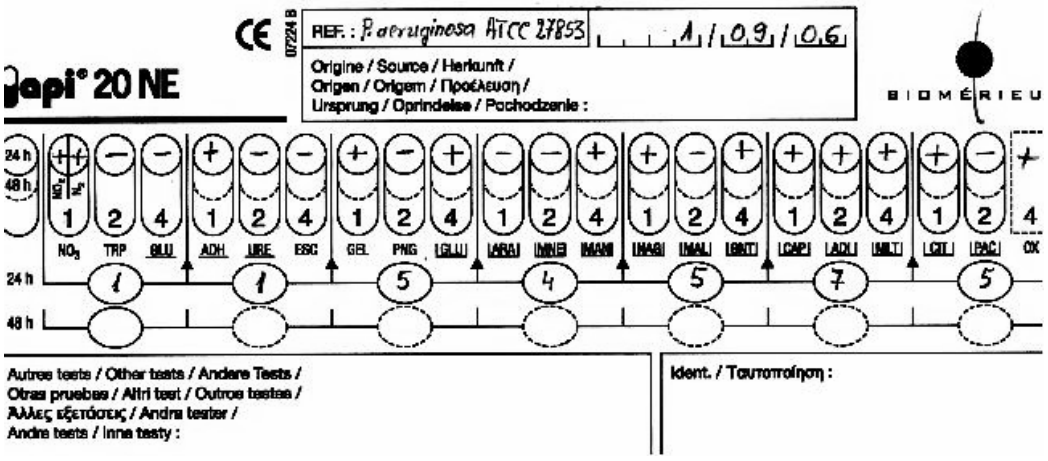
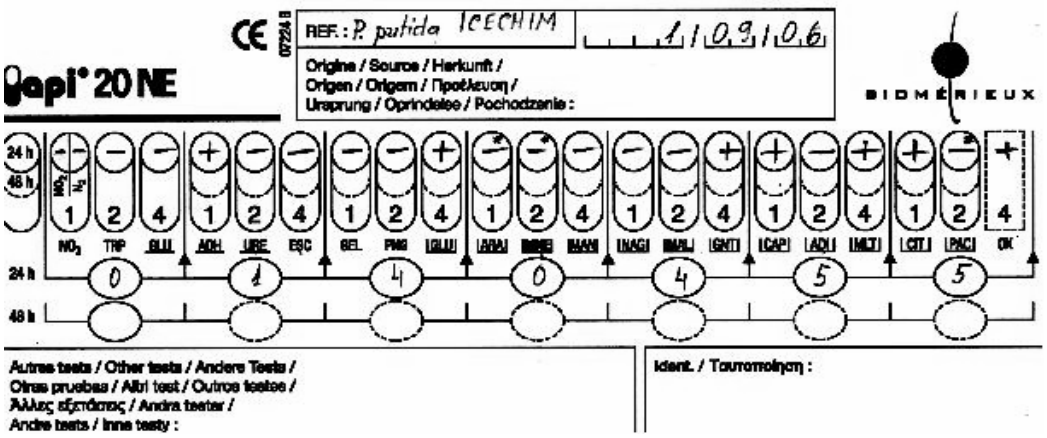


Figure 1. – Biochemical test results for *Pseudomonas aeruginosa* ATCC 27853

A pure culture of *Pseudomonas putida* ICECHIM was tested in parallel with the strains isolated from their natural environment. Reaction reading was performed according to the API 20 NE kit table, which includes the active ingredients, reactions/enzymes and a choice of expected results; similarly, microorganism identification was done according to the



attached table. The data confirmed the analyzed strain to be indeed *Pseudomonas putida* ICECHIM.

Figure 2. - Biochemical test results for *Pseudomonas putida* ICECHIM

Next, we analyzed the three dominant strains isolated from the biofilm in the oily wastewater treatment installation: more exactly colonies taken from plates inoculated from the respective 10^{-7} dilutions of the original cultures (see *Materials and Methods*). Strain I showed two slight differences when compared with *Pseudomonas putida* ICECHIM, namely: arabinose and phenylacetic acid are clearly assimilated, the test tube being opaque for Strain I and slightly opaque for *Pseudomonas putida* ICECHIM.

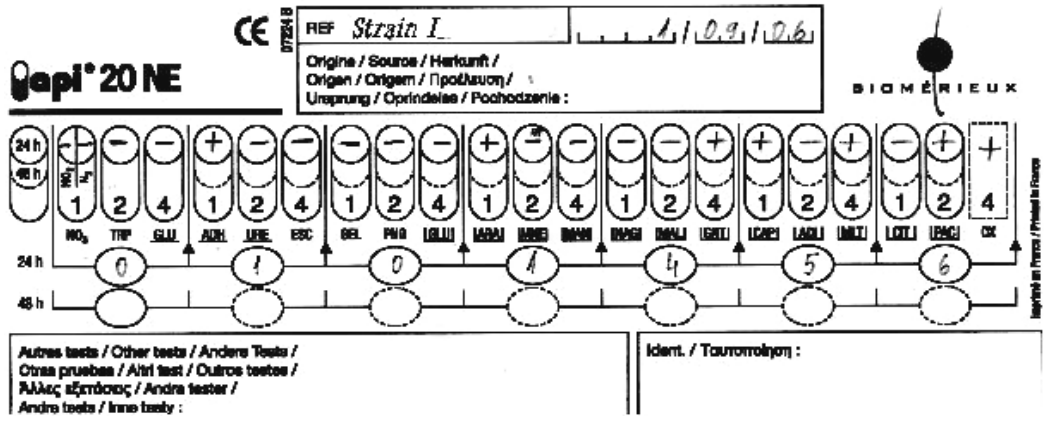


Figure 3. - Biochemical test results for *Strain I*

Strain II, in comparison to *Pseudomonas putida* ICECHIM, showed the following differentiations: the first one lacked mannase and did not assimilate D-mannose and

phenylacetic acid – the test tubes appeared transparent; the second one showed a very weak reaction, being slightly opaque, both in the case of mannose and phenylacetic acid.

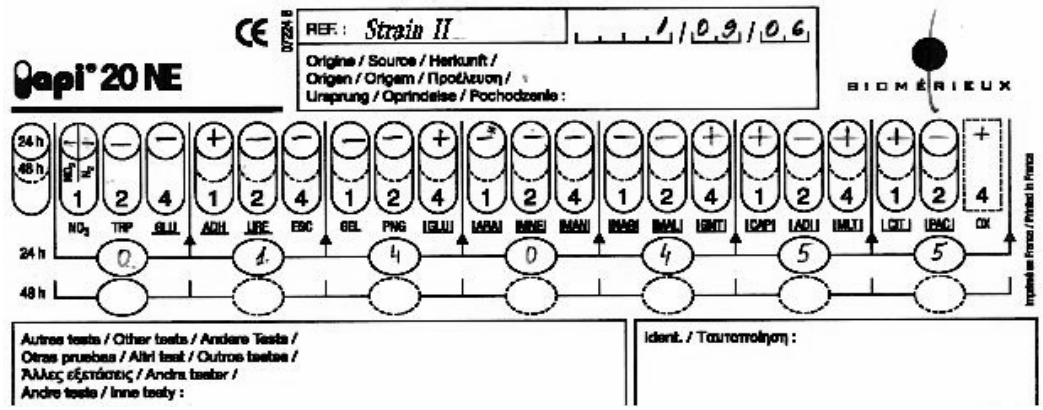


Figure 4. - Biochemical test results for *Strain II*

Strain III presented characteristics which, according to the kit identification table, indicated its belonging to *Pseudomonas stutzeri* species.

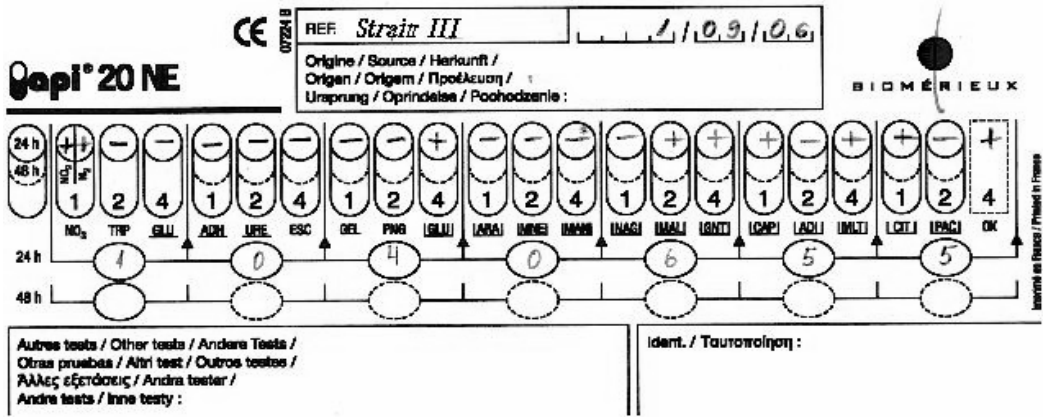


Figure 5. - Biochemical test results for *Strain III*

Table 1. The results of the biochemical tests

Tests	Active ingredients	*Pseudomonas aeruginosa* ATCC 27853	*Pseudomonas putida* ICECHIM	*Strain I*	*Strain II*	*Strain III*
NO_3	potassium nitrate	+	-	-	-	+
TRP	L-tryptophan	-	-	-	-	-
GLU	D-glucose	-	-	-	-	-
ADH	L-arginine	+	+	+	+	-
URE	urea	-	-	-	-	-
ESC	aesculin ferric citrate	-	-	-	-	-
GEL	gelatin	+	-	-	-	-
PNPG	4-nitrophenyl-β-D-galactopyranoside	-	-	-	-	-

GLU	D-glucose	+	+	-	+	+
ARA	L-arabinose	-	±	+	±	-
MNE	D-mannose	-	±	±	-	-
MAN	D-mannitol	+	-	-	-	±
NAG	N-acetyl-glucosamine	+	-	-	-	-
MAL	D-maltose	-	-	-	-	+
GNT	potassium gluconate	+	+	+	+	+
CAP	capric acid	+	+	+	+	+
ADI	adipic acid	+	-	-	-	-
MLT	malic acid	+	+	+	+	+
CIT	trisodium citrat	+	+	-	+	+
PAC	phenylacetic acid	-	±	+	-	-
OX	oxidase	+	+	+	+	+

-negative reaction; ± weak positive reaction; + positive reaction

Thus, the results of the biochemical tests included in table 1 and correlated with the table attached to the kit API 20 NE pointed to the conclusion that Strain I and Strain II were similar to *Pseudomonas putida* ICECHIM, while Strain III was similar to *Pseudomonas stutzeri*.

Furthermore, we aimed at isolating *16S RNAr* gene of *Pseudomonas putida* and of selected Strains I, II and III for the purpose of its comparative analysis by RFLP (restriction pattern length polymorphism) method [9, 16]. This approach – the isolation of *16S RNAr* gene by PCR amplification followed by RFLP analysis, eventually by DGGE (denaturing gradient gel electrophoresis) and/or sequencing – is a valuable instrument used in the last decade for the study and diagnosis of *Pseudomonas* genus, well-known for its extreme heterogeneity and for the countless taxonomic reviews. [16]

Total genomic DNA isolation was performed by the above mentioned isolation and purification technique, producing a non-fragmented DNA, concentrated enough to allow its use as a template in the PCR reaction. Next, by using GM3F and GM4R specific primers [6, 8, 9], amplicons with molecular weight of 1500 bp, specific to *16S RNAr* gene, were obtained for all strains (Fig. 6).

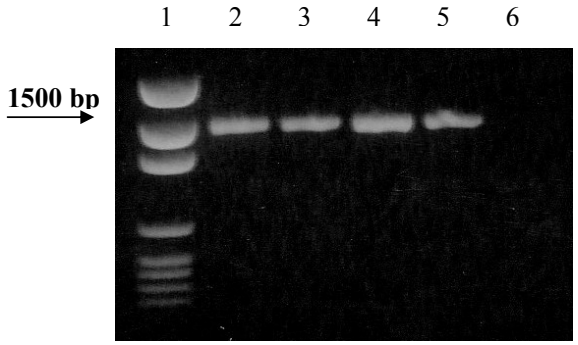


Figure 6. - Agarose gel electrophoresis of PCR products for *16S RNAr* gene (1500 bp) of strains *Pseudomonas putida,* Strain I, II and III; lanes: 1. molecular marker BenchTop pGEM; 2. amplification product for *Pseudomonas putida* ICECHIM; 3. amplification product for Strain I; 4. amplification product for Strain II; 5. amplification product for Strain III; 6. Negative control.

DNA fragments obtained by PCR were then subjected to RFLP analysis. The enzymes for the restriction reaction were selected as described in *Materials and Methods*, ultimately using *AluI, ApaI,* and *HphI*.

As shown in figure 7, following digestion with *AluI,* two restriction patterns (here indicated by *Alu*-A and *Alu*-B) were achieved; with *ApaI* – a single pattern was obtained for all the strains; with *HphI* – three patterns appeared (*Hph*-A, *Hph*-B and *Hph*-C). Thus, in the first case (restriction with *AluI*), strains *P. putida* and II showed the pattern *Alu*-A, while Strains II and III – pattern *Alu*-B. Digestion with *HphI* lead to the achievement of a pattern specific to *P. putida* (*Hph*-A), respectively for Strain II (*Hph*-C), and of a common pattern for Strains I and III (*Hph*-B).

These results partially correspond to those from biochemical analyses: Strain II was similar to *P. putida* ICECHIM; yet, Strain I seemed to be rather similar to Strain III, which, based on the culture data was, in turn considered to be similar to *Pseudomonas stutzeri*.

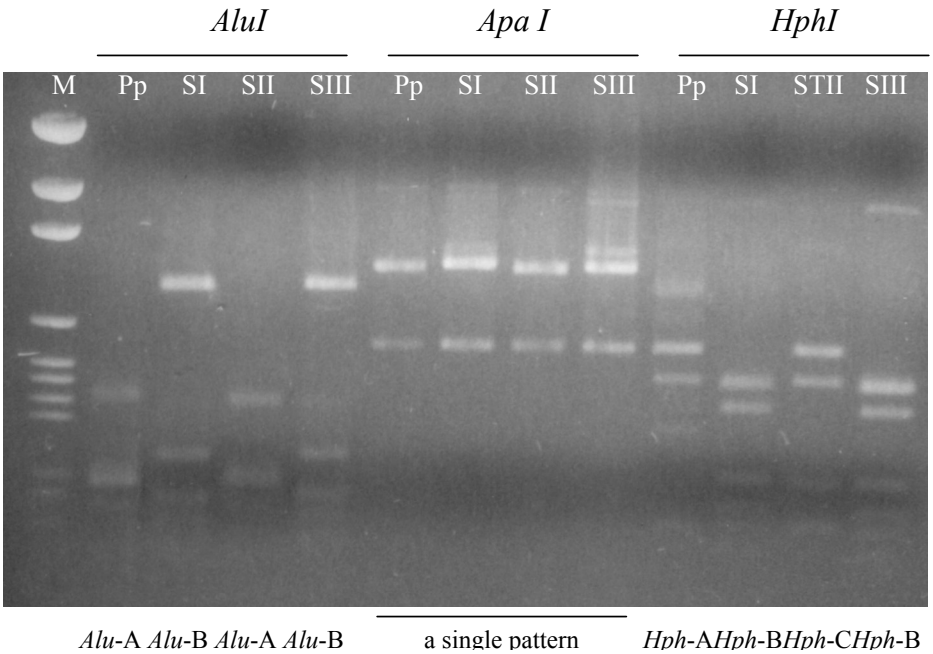


Figure 7. - Agarose gel electrophoresis of the restriction fragments of *16S RNAr* gene from strains *Pseudomonas putida* (Pp), I (SI), II (SII), and III (SIII): M - molecular weight marker BenchTop pGEM.

Conclusions

Biochemical and molecular analyses focused on systematic classification of the Strains I, II and III, isolated from their natural environment. These analyses show that Strain II was similar to *Pseudomonas putida* species, and Strain III – to *Pseudomonas stutzeri* species; in case of Strain I, results were unclear, requiring further investigations.

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