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Biodefluorination of Perfluorooctanesulphonate by *Ensifer adhaerens* M1

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ABSTRACT

A strain of bacteria M1, defluorinating perfluorooctanesulfonic acid (PFOS), was isolated from soil taken from the territory for testing fire extinguishing agents. The analysis of cultural, morphological, physiological and biochemical characters, the 16S rRNA gene sequencing allowed us to identify strain M1 as *Ensifer adhaerens*. The uniquely skill of the isolated strain to utilize PFOS in a mineral liquid medium for 5 days of cultivation was shown. It was found that *E. adhaerens* M1 transforms perfluorooctanesulfonic acid into perfluoroheptanoic acid, releasing 152 mg/L of free fluoride ions from 1,000 mg/L of PFOS. The data obtained allow us to recommend *E. adhaerens* M1 as potential treatment agent of the environment from organofluorine compounds.

1. Introduction

Distinctive features of organofluorine compounds are their complexity, wide circulation and extreme resistance to degradation. They have been found in soils, water, foods and consumer goods (Parsons *et al.* 2008). Perfluorocarboxylic acids were listed in "Annex B" of the "Stockholm Convention on Persistent Organic Pollutants", which called for actions to reduce to minimum and, if maybe, discontinue their manufacturing and exploitation (Butt *et al.* 2014; Liu *et al.* 2013).

Studies show that PFOS poses a significant threat because of its unusual resistance, persistence and accumulation in the environment and living tissues and potential toxicity (Espana *et al.* 2015; Prevedouros *et al.* 2006; Quinones *et al.* 2009; Tsuda *et al.* 2016).

PFOS was found in elevated concentrations in surface and groundwaters, in potable water, around the runways, in places of storage and frequent use of fire foam in several countries (Castiglioni *et al.* 2015; Moody and Field 1999).

Therefore, the definition of methods for the degradation of PFOS is an urgent topic.

Known methods of decomposition of PFOS-chemical processing, burning at high temperature, (Cheng *et al.* 2008; Wang *et al.* 2013) but they are high cost and inefficient.

The fluorine-carbon bond (CF) is the strongest covalent bond in organic chemistry (Ruiz-Urigüen *et al.* 2022) therefore, the biodefluorination of perfluoroalkyl substances is unlikely. It is usually characteristic only of fluorobenzene, fluoroacetate, perfluorohexylethanol and perfluorohexyl sulfonate (Carvalho *et al.* 2005; Davis *et al.* 2012; Liu *et al.* 2010; Wang *et al.* 2011; Zhang *et al.* 2013). It was previously reported about the PFOS destruction under anaerobic conditions after adding an activated sludge, but no accumulation of fluoride ion (F-) was detected (Meesters *et al.* 2004; Schroeder 2003). PFOS phytoremediation works are presented, in particular in a hydroponic experiment (Sharma *et al.* 2020).

From studies (Kwon *et al.* 2014) on the biodegradation of PFOS by individual cultures of microorganisms, one can isolate a strain of *Pseudomonas aeruginosa* HJ4, capable of its destruction within 48 hours at an initial concentration not exceeding 2 mg/L, strain 2,4-D of genus *Pseudomonas* which defluorizes it, turning it into perfluoroheptanoic acid (Chetverikov *et al.* 2017). PFOS degradation with *Acidimicrobium* sp. A6 (Huang and Jaffe 2019).

The purpose of our study was to explore taxonomic affiliation and characterization of a novel strain capable of defluorination of PFOS.

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2. Materials and Methods

2.1. Chemicals

Chemical and reagents including sodium carbonate, magnesium sulfate, iron sulfate, calcium chloride, manganese sulfate, potassium phosphate (K_2HPO_4 × 3 H_2O), sodium phosphate (NaH_2PO_4 × 3 H_2O), ammonium chloride were purchased from "Merck" (Germany). PFOS was purchased from "Sigma–Aldrich" (USA). Acetonitrile was purchased from "Panreac" (Spain) in LCMS-grade.

2.2. Microorganisms Culture Conditions

Soil samples for isolation of the bacterial strain M1 were sent from the territory for testing fire extinguishing agents (Maldives).

Accumulative and pure cultures were received using Raymond's mineral medium including PFOS as the only source of carbon (0.1 w/v %) (Raymond 1961). Cultivation of bacteria was carried out at 28°C and 160 rpm. Bacterial yield evaluated using LAMBDA-750 UV spectrophotometer (590 nm) for measuring the optical density of culture.

Morphological and physiological-biochemical indicators of pure culture were studied according to generally accepted guidelines (Gerhard 1994; Holt 1994).

2.3. DNA Isolation

Total DNA was isolated from the bacterial biomass of one colony grown on nutrient agar according (Wilson 1995). 16s rRNA gene was amplified with universal primers (Lane 1991). Using the Wizard PCR Preps kit ("Promega", USA) extraction and purification of PCR products were carried out from fusible agarose.

2.4. Sequencing and Construction of the Dendrogram of Phylogenetic Similarity

PCR products were sequenced using the ABI PRIZM 3730 automatic sequencer and the Big Dye Terminator v.3.1 kit ("Applied Biosystems Inc.", USA). Matches between the nucleotide sequences deposited in the GenBank database and those obtained experimentally were searched using the program BLAST (http://www.ncbi.nlm.nih.gov/blast) (Camacho *et al.* 2009).

The phylogenetic dendrogram was constructed in MEGA7 software (Tamura *et al.* 2011) by the neighbor-joining method with the Kimura model (Kimura 1980; Saitou and Nei 1987).

2.5. Isolation and Identification of PFOS Biotransformation Products

In order to extract, identify and quantify the bacteria-modified PFOS ultrafiltration in Vivaflow 50 ("Sartorius AG", Germany) was applied to separate bacterial cells from the environment. The obtained ultrafiltrate (≤3000 Da) was investigated using the spectrometry method on a tandem chromatographic mass LCMS-IT-TOF spectrometer ("Shimadzu", Japan) with an eluted ion injection system, a quadrupole ion trap and a time-of-flight detector.

Spectra were recorded in the negative ion mode for the mass m/z 200–800 a.e.m, column Shim–pak XR–ODS (75 × 2.0 mm) in an isocratic mode with a solvent ratio of (56:44 = 5 mM ammonium acetate in water: acetonitrile) at a flow of 0.2 ml/min was used for chromatographic separation. According to the data of general mass spectrometry, which were based on the destruction of the molecular ion and literature data, structure of the substances obtained was determined.

2.6. Biodefluorination of PFOS

PFOS biodefluorination was evaluated by the amount of fluoride ion in the culture fluid using a fluoride-selective electrode with a solid-state membrane DX219-F ("Mettler Toledo", Switzerland).

2.7. Statistics

The program MS Excel was used to calculate the average values and standard errors.

3. Results

The M1 strain studied in this work, which has the ability to utilize PFOS, was isolated using standard isolation and enrichment techniques. The strain grew noticeably on Raymond's medium, using PFOS as the only organic nutrient substrate (0.1 w/v %) at 28°C within 48 h of incubation. The results of strain's characterization are in Table 1.

Under the number MH141439, the sequence (1,319 bp) characteristic of the strain M1 was entered into GenBank. The sequence of strain M1 coincided with the sequences of strains Ensifer adhaerens LMG 20216 and *E. sesbania* LMG 26833 by 99.85 and 99.77%, respectively; they were the closest to the studied sample. Based on data on 16S rRNA sequences of typical strains of different species of the genus *Ensifer*, a phylogenetic dendrogram was

Table 1. Physiological and morphological properties of the investigated strain

Characteristic	Test result
Gram coloring	-
Shape	sticks
Mobility	+
Colony shape	convex
Type of metabolism	respiratory
Catalase	+
Oxidase	+
Hydrolyze lecithin, casein, gelatin and starch	-
Optimum growth range	26-28°C
Optimum pH	6.0-8.0
Optimum concentration of NaCl	0-2%
Growth at 4°C and 41°C	-
Denitrification	-
Gelatin liquefaction	-
Lecithinase	-
Lipase	-
Arginine dihydrolase	+
Utilization of:	
Arabinose	+
Fructose	-
Glucose	+
Galactose	+
Inositol	-
Lactose	+
Maltose	+
Mannose	+
Mannitol	+
Meso-Inositol	+
Potassium Tartrate	+
Rhamnose	+
Sorbitol	+
Starch	-
Sucrose	+
Xylose	+
2-Ketogluconate	+
Citrate	+
Ethanol	-
Alanine	+
Arginine	-
Aspartate	+
Leucine	-
Histidine	-
Lysine	+
Valine	-
Malate	+
N-Butanol	-
Propylene Glycol	+
Succinate	+

constructed, and the degree of relation of these strains with the strain M1 was determined with its help (Figure 1). As a result, the studied strain was assigned to the species *E. adhaerens*.

In periodic culture, the strain of *E. adhaerens* M1 actively uses PFOS as the source of carbon and energy (Figure 2).

The culture adapted to the substrate during the first 20–24 hours, preparatory metabolism took place (the concentration of the substrate decreases to a minimum), this was shown by the analysis of PFOS amount in the culture fluid of *E. adhaerens* M1. In this case, the optical density increases slightly. And starting to grow exponentially only after 24 hours, reaches a

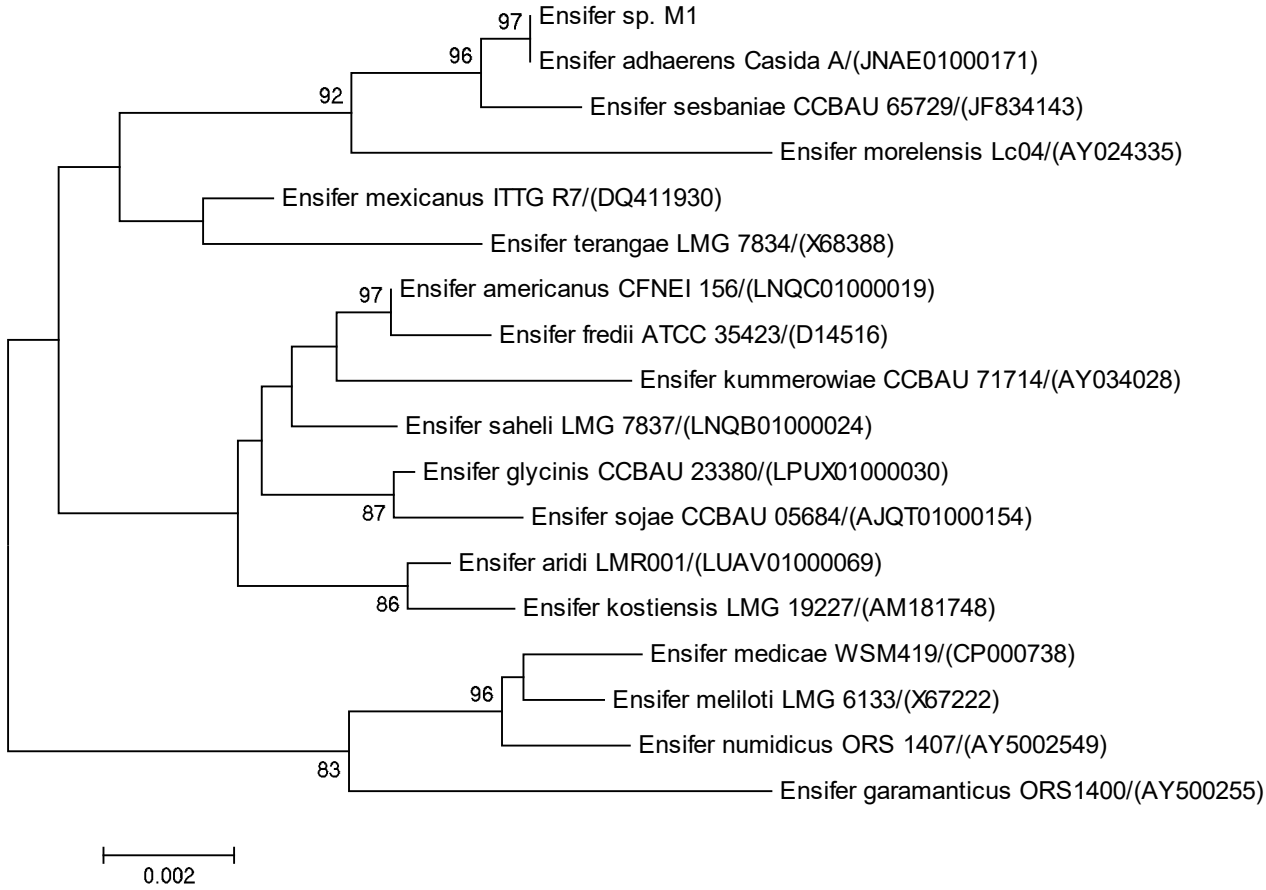

Figure 1. Phylogenetic position *E. adhaerens* M1 strain

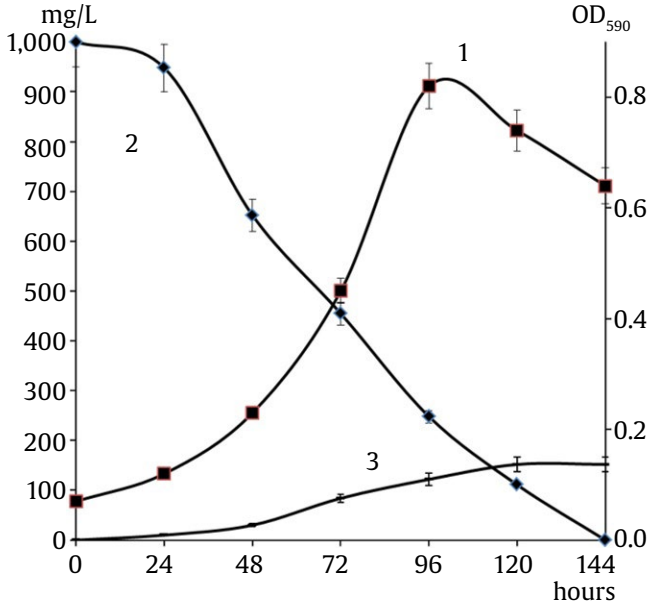

Figure 2. Relationship between OD_{590} values (1), substrate amount (2), ion F- amount (3) during *E. adhaerens* M1 cultivation in periodical culture are shown

maximum by 96–100 hours of cultivation. During this period, there was a linear increase in substrate consumption with its complete transformation in 140 hours.

Fluorine ions were transferred to the solution during the conversion of PFOS. In the culture medium (CM), their concentration reached 152 mg/L. It can be noticed that the active accumulation of fluorine ions and the rapid decrease in the concentration of PFOS in the medium began almost simultaneously and occurred in parallel.

Perfluorinated organic acids are practically not biodegradable. To evaluation the biodefluorination and biodegradation of PFOS, we used the liquid chromatography method in the variant with tandem mass spectrometry. The transformation of PFOS is presented in Figure 3 for 144 hours under conditions of periodic culture. At the starting point of the experiment, we observed a dissociated acid ion characteristic of PFOS with m/z 499 (Figure

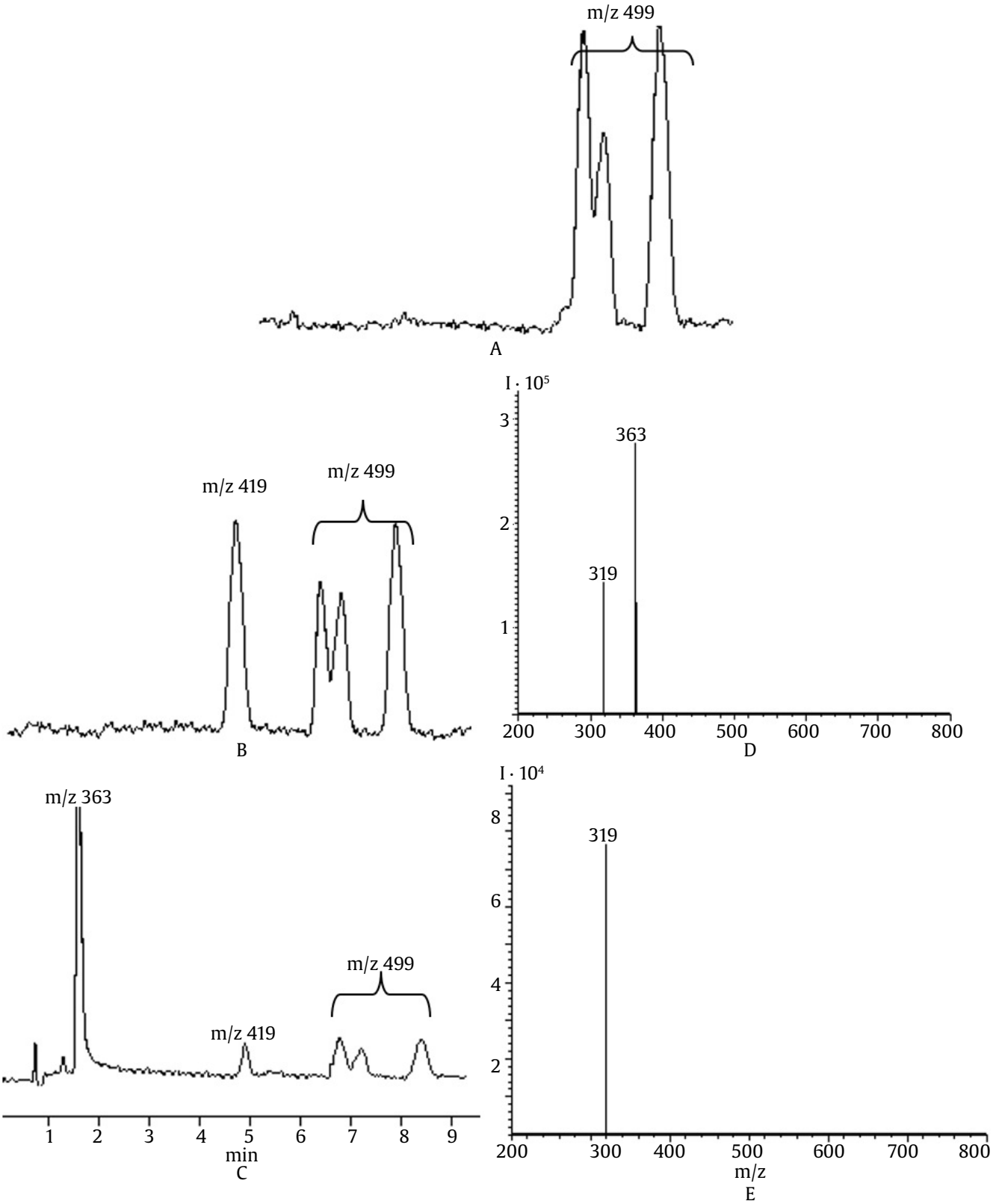

Figure 3. Mass chromatograms of ultrafiltrates of the culture fluid of *E. adhaerens* after 0 (A), 20 (B), 120 (C) hours of cultivation in a periodical culture and mass spectra of the MS1 (D), MS2 (E) component with m/z 363

3A). After 20 hours of cultivation, the existence of element with a molecular ion m/z 419 (Figure 3B) is maybe monooxygenase elimination of a sulfonate (m/z 80) radical from PFOS in shape of sulfite, which can be metabolized in starvation conditions. This conforms to the hypothesis on the bacterial preparative metabolism for future defluorination. Next transformation (20–120 hours) was follow by dynamic gain of the culture and liberation of F- into liquid. Beyond 78 hours of culturing in ultrafiltrate, a component with a molecular ion m/z 363 was found, along with elements with m/z 419 and 499 compounds (Figure 3C).

According to the spectra mass MS1, MS2 the compound with an m/z 363 was identified as perfluoroheptane acid.

Perfluoroheptanoic acid which is formed during biological defluorination was located in the culture liquid at the end of the observation period. It was identified by a decayed acidic ion. The scheme of biodefluorination of 1,000 mg/L of PFOS with the release of 152 mg/L of fluorine ions (which corresponds to the removal of four fluorine ions from one PFOS molecule) is shown in Figure 4.

Thus, as a result of the studies, a new strain M1, a representative of the species *E. adhaerens*, capable of partial mineralization of PFOS by defluorination was described. The strain *E. adhaerens* M1 is recommended for use in biotechnology transformation of organofluorine compounds to protect the environment.

4. Discussion

Perfluoroalkyl substances (PFAS) have become widespread in the environment, polluting rivers, groundwater and wastewater, soils (Eriksson *et al.* 2017; Shi *et al.* 2015; Von Der Trenck *et al.* 2018). They come there, as a rule, from various commercial products, including fire-fighting foams and non-stick coated products.

A number of technologies based on the use of physical and chemical methods have been developed to remove PFAS from the environment. Some of them have proven themselves well in laboratory testing, but their cost-effectiveness and ability to be used in the field remain questionable (Makhinrusta and Seneviratna 2020).

Bioremediation, which is the use of a biological agent to break down pollutants, can be a simple, environmentally safe and cost-effective technology for treating soils contaminated with PFAS. Commercial bioremediation has been successfully applied to eliminate various organic pollutants, such as petroleum hydrocarbons, chlorinated substances and pesticides (Adetutu *et al.* 2015; Khudur *et al.* 2019; Uqab *et al.* 2016). However, the ability of biological agents to decompose PFAS has been poorly studied (Kucharzyk *et al.* 2017).

For some PFAS, specific biodegradation pathways have been investigated (Liu and Avendano 2013), for many PFAS, the pathways are unknown. Moreover, the types of enzymes and their associated genes

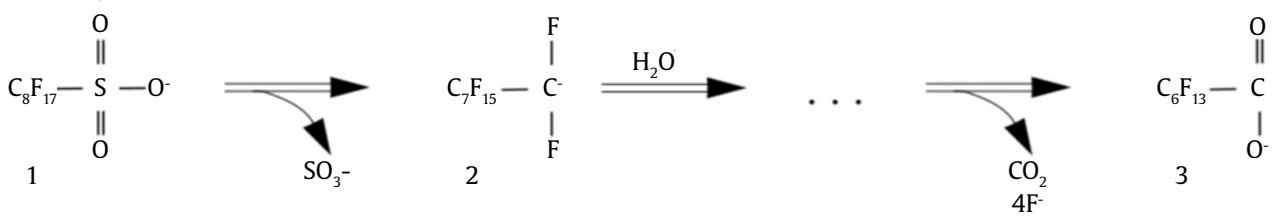

Figure 4. Biodefluorination of PFOS by strain *E. adhaerens* M1: 1 - PFOS (m/z 499), 2-perfluorooctane (m/z 419), 3-perfluoroheptanoic acid (m/z 363)

were not reported. The decomposition pathways of a particular PFAS can be investigated by evaluating intermediates, but these data are practically not found in the scientific literature.

Bacteria isolated from a PFAS-contaminated medium demonstrated the ability to decompose PFAS compounds (two *Pseudomonas* strains PS27 and PDMF10 were able to remove 32 and 28% of PFAS compounds, respectively, during 10 days of incubation under alkanotrophic conditions (Presentato *et al.* 2020). In addition, a decrease of approximately 32% in PFAS was also reported during 96-hour incubation of *Pseudomonas parafulva* (Yi *et al.* 2016), by 67% during 48-hour incubation of *Pseudomonas aeruginosa* (Kwon *et al.* 2014). It has been shown that bacteria (most of them representatives of the genus *Pseudomonas*) are able to bioaccumulate PFAS under aerobic and, to a lesser extent, anaerobic conditions; although there have been no confirmed reports of biological removal of fluorine atoms from PFAS, defluorination of monofluorinated compounds by many bacteria has been reported (Huang and Jaffé 2019).

And only one study showed that Pseudomonas plecoglossicida used PFAS as an energy source, producing perfluoroheptanoic acid and releasing fluorine ions as a result (Chetverikov *et al.* 2017).

The strain under study is also capable of such transformation and defluorination, and further destruction is presumably inhibited by fluorine ions released into the medium, and the method of biodefluorination is similar to the case with the 2.4-D strain (Chetverikov *et al.* 2017), only in a more dynamic variation. In other well-known publications (presented above) on microbial destruction and biofluorination of perfluorocarboxylic acids, intermediate metabolites are not disclosed.

In the course of our work, we isolated and characterized the strain *Ensifer adhaerens* M1, which is capable of decomposition of PFOS in a liquid medium. The unique ability of the isolated strain to utilize PFOS in a mineral liquid medium during 5 days of cultivation was demonstrated. *E. adhaerens* M1 was found to convert perfluorooctane sulfonic acid into perfluoroheptane acid, releasing 152 mg/L of free fluorine ions from 1,000 mg/L PFOS. The data obtained allow us to recommend *E. adhaerens* M1 as a potential means for cleaning the environment from organofluorine compounds.

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