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NEW *ENSIFER MORALENSIS* H16 STRAIN FOR PERFLUOROOCTANE SULPHONATE DESTRUCTION

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ABSTRACT: We studied the ability of a bacterial strain isolated from soil contaminated by chemical waste to use perfluorooctane sulfonate (PFOS) as the sole source of carbon and energy. Based on the combination of cultural, morphological, physiological and biochemical characteristics, 16S rRNA gene analysis, and phylogenetic data analysis, we identified the isolated strain as *Ensifer moralensis* H16. The strain completely utilizes perfluorooctane sulfonate in a liquid medium under the batch fermentation conditions for 120 hours. The strain biodegrades perfluorooctane sulfonate into perfluoroheptanoic acid with the release of four F-ions from one PFOS molecule into the medium (final PFOS concentration is 152 mg/L; initial PFOS concentration in the medium is 1,000 mg/L). In this study 88% of PFOS was decomposed by the new strain after treating contaminated soil in a model experiment. The isolated strain *Ensifer moralensis* H16 can be used in development of biological products for the utilization of environmental pollution caused by organofluorine compounds.

Keywords: Biodegradation; Defluorination; *Ensifer moralensis*; Perfluorooctane sulfonate; Perfluororganic compounds; Pollutants.

INTRODUCTION

Current development of human industrial activity requires the use of substances and compounds, including surfactants, which are stable in a wide range of temperatures and acidity. Many of them are halogenated compounds. Organofluorine compounds are the most stable since the C–F-bond is extremely strong.[1] Perfluorinated compounds, in particular perfluorooctane sulfonate (PFOS) and products based on it, are widely applied as surfactants due to their high stability. These compounds are used in the production of semiconductors, modern composite materials, fire fighting foams, and others.[2] However, such substances inevitably enter the environment and can pollute it for a long period of time[3] due to such characteristic properties as high toxicity, resistance to physical and biological destruction, and cumulative effect. These compounds increase the risk of cancer incidence[4] and may have a negative effect on the fetal growth and weight.[5] Perfluorooctanoic acid was reported to exhibit toxicity to enzymes.[6]

Various methods of PFOS decomposition have been proposed. Physical methods involve thermal treatment (such as incineration)[7] or ultrasound exposure.[8] However, these methods are not efficient in terms of the high cost of processing. Biological methods, e.g., phytoremediation using hydroponically grown plant cultures, are more promising.[9] But, application of this method raises the problem of plant biomass utilization. The use of bacteria as a destructor of organofluorine compounds is devoid of the disadvantages listed above, it is technologically advanced, this technology can be used directly in the pollution focus, this method is cost-effective, and it can be easily scaled up.

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There are reports on the studies in the field of microbial biodegradation of xenobiotics highlighted in the present article; however, they are mainly associated with *Pseudomonas sp.* strains.[10-12] *Pseudomonas aeruginosa* HJ4 strain degrades PFOS for 48 hours at initial concentration not exceeding 2 mg/L.[10] Huang and Jaffé observed a degradation of 60% of PFOS after 100 days of incubation with *Acidimicrobium sp.* A6 strain with the release of the fluoride-ion (F-ion).[13] It is worth mentioning that *Pseudomonas plecoglossicida* 2,4-D strain transforms PFOS to perfluoroheptanoic acid.[14]

Microbial metabolism and mechanisms of the biotransformation of organofluorine compounds, fluorinated aliphatic compounds, and others, have been described.[15] Fluoroaniline, fluoroacetate, fluorobenzene, perfluorobiphenyl, perfluorohexyl sulfonate were previously reported to undergo biodegradation.[16-21] The use of microorganisms as destructors can significantly reduce the load on the biosphere and ultimately lead to positive effects on human well-being. In this report we have studied the taxonomy and characteristics of a new strain that can degrade PFOS.

MATERIALS AND METHODS

Object: The strain was isolated from soil exposed for a long period of time to various factors of the chemical production in the industrial zone (Ufa, Russia).

Media: Cumulative and pure cultures were obtained with the use of Raymond's mineral medium[22] containing PFOS as the only source of carbon (0.1 w/v %) (g/L): Na_2CO_3 – 0.1, $MgSO_4 \cdot 7H_2O$ – 0.2, $FeSO_4 \cdot 7H_2O$ – 1.0, $CaCl_2$ – 0.01, $MnSO_4 \cdot 7H_2O$ – 0.02, $K_2HPO_4 \cdot 3H_2O$ – 1.0, $NaH_2PO_4 \cdot 3H_2O$ – 1.5, NH_4Cl – 3.0.

Methods: Bacterial cultures were incubated at 28ºC on an orbital thermostatically controlled shaker (160 rpm). The intensity of culture growth was determined with Selecta UV-2005 spectrophotometer (Selecta, Spain), (wavelength 590 nm). The object of the study was a natural strain extracted from a sample of soil contaminated by waste. The stain was characterized according to the guidelines[23-25] on the basis of cultural, morphological, physiological, and biochemical characteristics. DNA extraction was performed according to a standard procedure/method.[26] 16S rRNA gene of bacteria was amplified using universal primers.[27] Polymerase chain reaction (PCR) products were isolated and purified from low-melting agarose using the Wizard PCR Preps reagent kit (Promega, USA).

The obtained polymerase chain reaction (PCR) fragments of the 16S rRNA gene were sequenced using the Big Dye Terminator v.3.1 reagent kit (Applied Biosystems Inc., USA) on an ABI PRIZM 3730 automated sequencer (Applied Biosystems Inc., USA) following the manufacturer's recommendations. The search for nucleotide sequences of 16S rRNA genes homologous to the corresponding sequence of the studied strain in the GenBank database was performed using the BLAST software package (https://blast.ncbi.nlm.nih.gov/Blast.cgi).[28] The dendrogram of phylogenetic relationships was constructed in the MEGA version 6 software[29] using the Neighbor-Joining method[30] based on the Kimura model.[31]

For the extraction and identification of products of PFOS biotransformation and determination of its concentration in the medium, bacterial cells were separated from the medium by ultrafiltration using Vivaflow 50 (Sartorius AG, Germany). The resulting ultrafiltrate (=3 kDa) was analyzed by chromatography-mass spectrometry

using LCMS-IT-TOF liquid tandem chromatography-mass spectrometer (Shimadzu, Japan) supplied with an electrospray sample injection system, a quadruple ion trap, and a time-of-flight detector. Mass spectra were registered in the 200–800 atomic mass unit (amu) range in negative-ion mode, the detector voltage was set at 3.5 kV. A Shim-pak XR-ODS column (75 × 2.0 mm) (Shimadzu, Japan) was used for separation with an isocratic mobile phase composed of 56% 5 mM ammonium acetate in water and 44% acetonitrile delivered at 0.2 mL/min flow rate. The structure of compounds was established using combination of the mass spectra analysis data based on a molecular ion fragmentation and its comparison with published data.

The concentration of F-ions in the medium was measured using a fluoride-selective electrode with a solid-state membrane DX219-F (Mettler Toledo, Switzerland).

The ability to degrade PFOS in the soil was evaluated in the model experiment in which 100 mL of a culture suspension with OD_{590} = 0.8 (100 mL of tap water as a control) was introduced into the soil (per 1 kg) pre-mixed with 0.5% PFOS at room temperature. Soil humidity was maintained by adding 100 mL of tap water per 1 kg of soil, followed by mixing every 15 days. Soil samples (100 g each) were analyzed after 0.5, 1, 2, 3, and 6 months. The extraction was carried out with dichloromethane for 10 minutes. The extract was filtered and evaporated on a water bath at 50ºC. The residual content of PFOS was evaluated gravimetrically.

RESULTS AND DISCUSSION

The studied strain H16 was characterized as gram-negative aerobic rods (0.7–1.1 × 1.0–1.9 μm), mesophyll, with growth optimum of 28ºC, and pH optimum of 6.8–7.2, capable of denitrification. It is indole negative, does not ferment gelatin and urea, and does not assimilate gluconate and acetate, but ferments L-arabinose, D-mannose, maltose, and D-glucose. The oxidase test is positive. Strain H16 intensive growth occurs at NaCl concentration of 0–5%; higher NaCl concentrations (up to 10%) inhibit growth. It is not able to use 2-anthranilic, benzoic, coumaric, propionic, and salicylic acid as a carbon source.

The sequence of 16S rRNA gene fragments (1,351 bp, positions 33 to 1,383; *E. coli* numbering) from the strain determined in this study is available in GenBank database under the number MT267776. The closest relative of the studied strain is the bacterial species *Ensifer morelensis* Lc04 (AY024335) (level of sequence similarity, 99.93%), Comparative 16S rRNA gene sequence analysis of the relative species and the construction of a dendrogram, shown in Figure 1, allowed identification of the strain as *Ensifer moralensis* H16.

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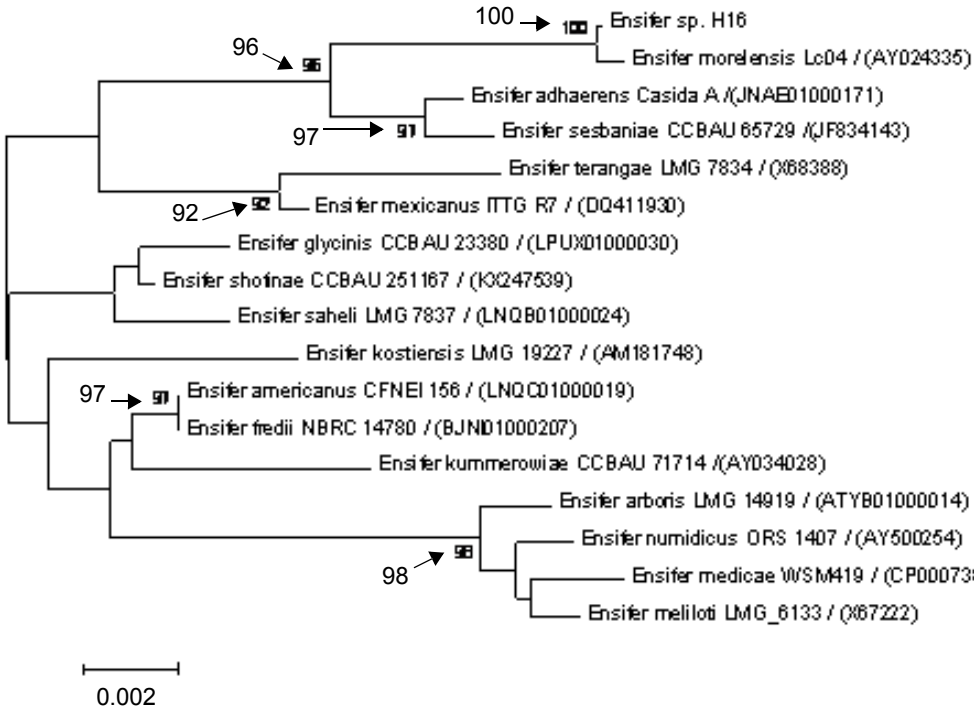

Figure 1. Phylogenetic position of the *Ensifer moralensis* H16 strain according to the 16S rRNA gene nucleotide sequence analysis. The scale shows the evolutionary distance corresponding to 2 nucleotide substitutions per 1,000 nucleotides. The numbers shows the statistical certainty of the branching order determined using the bootstrap analysis (bootstrap analysis values higher than 70% are shown). The strains shown, from top to bottom, are:

Ensifer sp. H16	/	
Ensifer morelensis Lc04	/	(AY024335)
Ensifer adhaerens Casida A	/	(JNAE01000171)
Ensifer sesbaniae CCBAU 65729	/	(JF834143)
Ensifer terangae LMG 7834	/	(X68388)
Ensifer mexicanus ITTG R7	/	(DQ411930)
Ensifer glycinis CCBAU 23380	/	(LPUX01000030)
Ensifer shofinae CCBAU 251167	/	(KX247539)
Ensifer saheli LMG 7837	/	(LNQB01000024)
Ensifer kostiensis LMG 19227	/	(AM181748)
Ensifer americanus CFNEI 156	/	(LNQC01000019
Ensifer fredii NBRC 14780	/	(BJNI01000207)
Ensifer kummerowiae CCBAU 71714	/	(AY034028)
Ensifer arboris LMG 14919	/	(ATYB01000014)
Ensifer numidicus ORS 1407	/	(AY500254)
Ensifer medicae WSM 419	/	(CP000738)
Ensifer meliloti LMG 6133	/	(X67222)

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Ensifer moralensis H16 strain used PFOS as the sole source of carbon and energy under batch fermentation conditions as shown in Figures 2A–2H. Lag phase associated with adaptation to the substrate and the accumulation of necessary enzymes was observed during first 20 hours of cultivation. There was no significant substrate concentration decrease during this period.

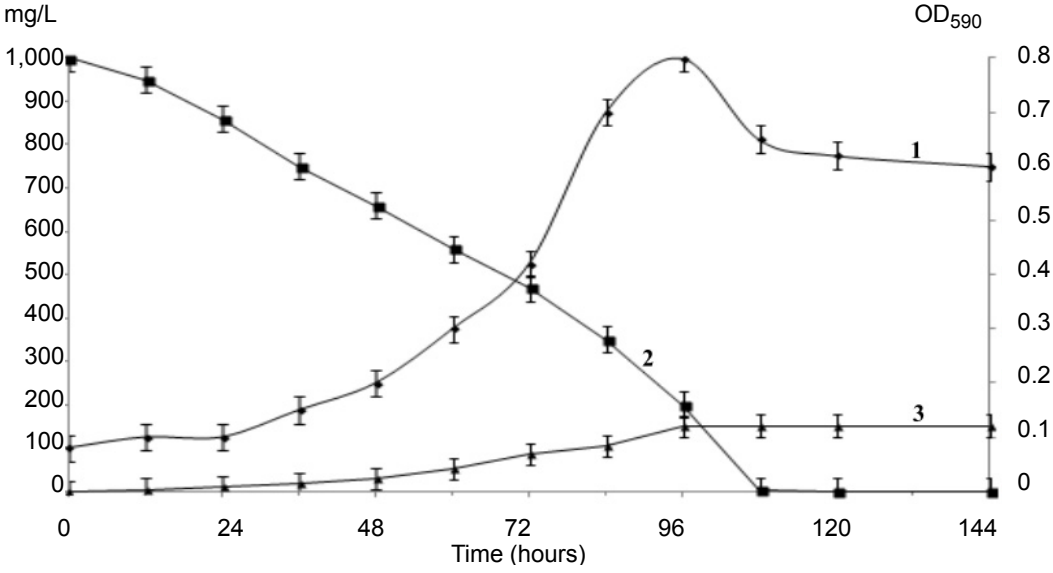

Figure 2A. Dependence of the optical density at wavelength 590 nm (OD_{590}) values of the culture medium (1) on the perfluorooctane sulfonate (PFOS) concentrations (2) and free fluoride ions (3).

1 = OD_{590} value

2 = PFOS concentration (mg/L) Initial value 1,000 mg/L.

3 = Free fluoride ion concentration (mg/L) Initial value 0 mg/L

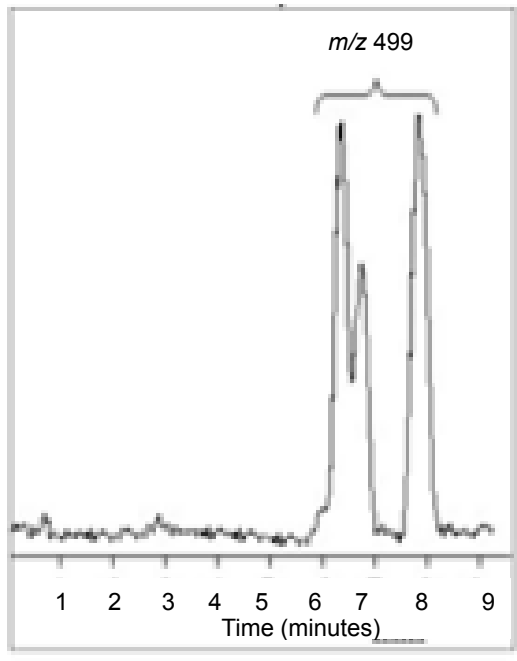

Figure 2B. Mass-chromatogram of *Ensifer morelensis* H16 culture medium ultrafiltrates (PFOS transformations) after 0 hours of batch fermentation.

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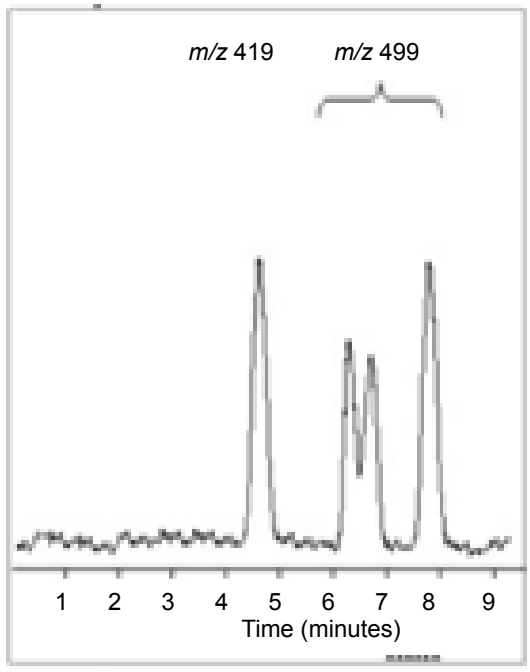

Figure 2C. Mass-chromatogram of *Ensifer morelensis* H16 culture medium ultrafiltrates (PFOS transformations) after 24 hours of batch fermentation.

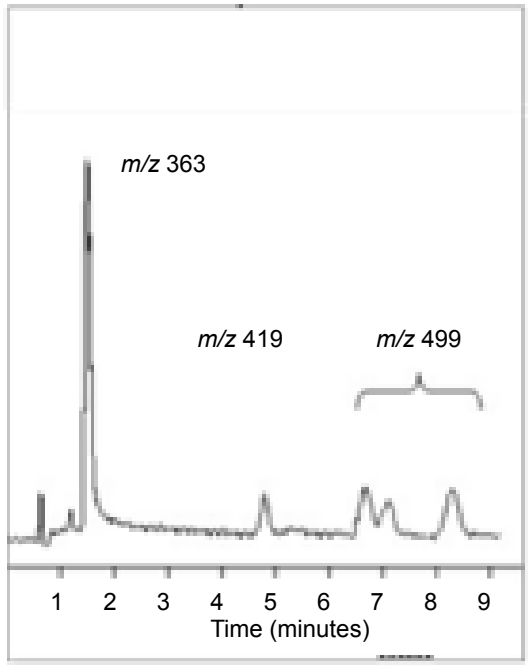

Figure 2D. Mass-chromatogram of *Ensifer morelensis* H16 culture medium ultrafiltrates (PFOS transformations) after 72 hours of batch fermentation.

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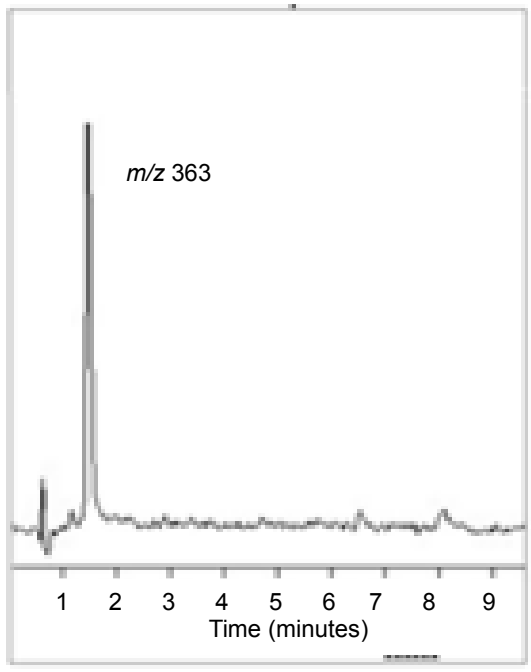

Figure 2E. Mass-chromatogram of *Ensifer morelensis* H16 culture medium ultrafiltrates (PFOS transformations) after 120 hours of batch fermentation.

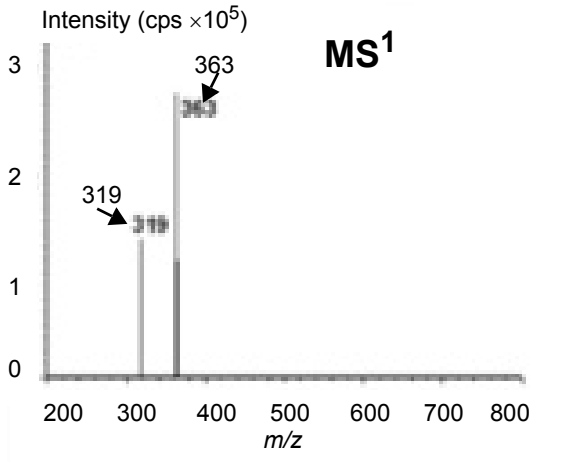

Figure 2F. MS^1 mass-spectra of the product after 120 hours of batch fermentation of *Ensifer morelensis* H16 culture medium as shown in Figure 2E.

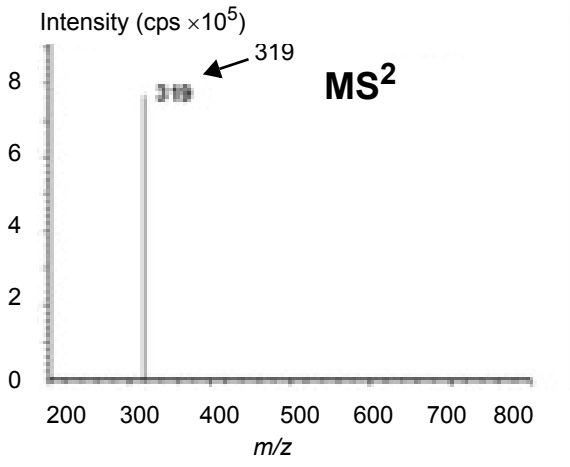

Figure 2G. MS^2 mass-spectra of the product after 120 hours of batch fermentation of *Ensifer morelensis* H16 culture medium as shown in Figure 2E.

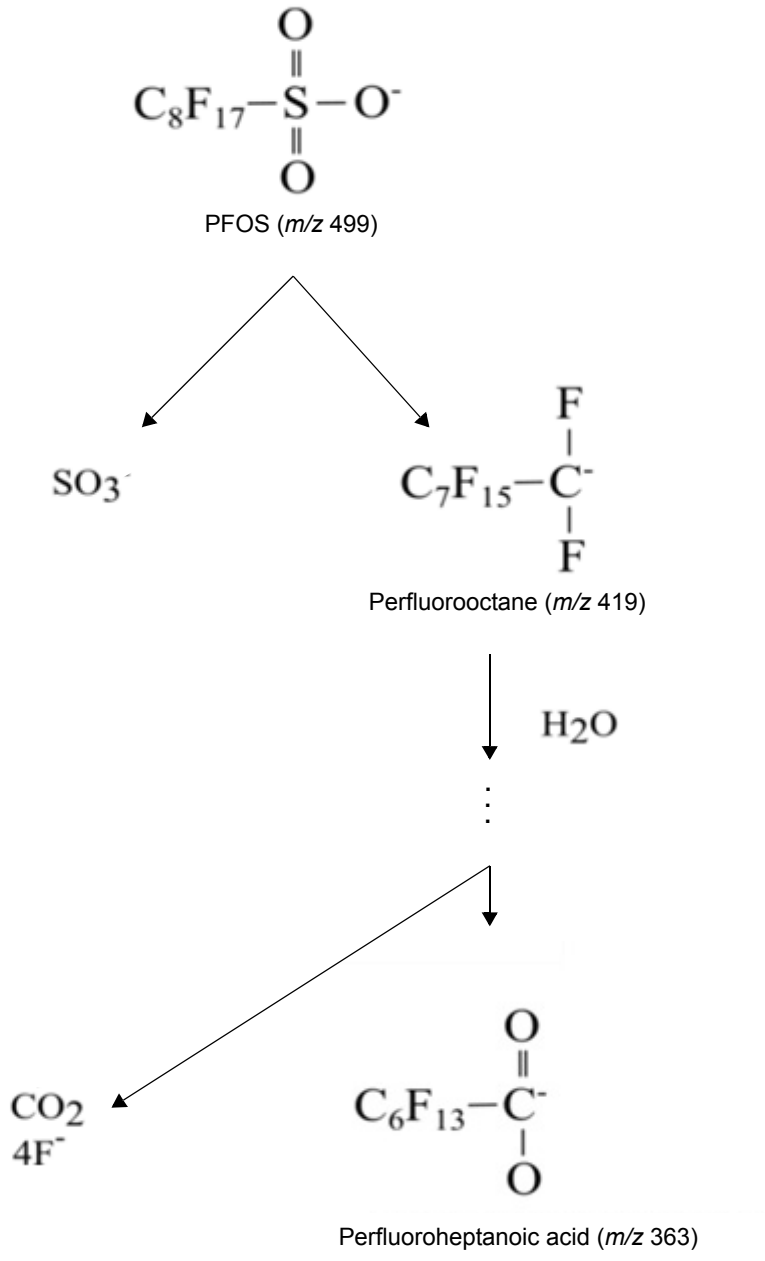

Figure 2H. Reaction scheme of batch fermentation of *Ensifer morelensis* H16 culture medium.

Biodegradation of perfluorinated acids is well known to be difficult. The most reliable method used currently in analysis of perfluorocarboxylic acids as anionic substances, including PFOS, is high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS), with detection of dissociated acid ion. At the starting point of the experiment we detected a dissociated acid ion characteristic for PFOS (*m/z* 499) in the ultrafiltrate of the culture media.

Further, after the lag phase, in 24 hours after the start of cultivation, the increase in substrate consumption is up to 20% per day, culture grows exponentially, and the optical density of the culture medium increases. A component with *m/z* 419 molecular ion is detected in the culture medium, which is probably formed as a result of monooxygenase elimination of the sulfonate group (*m/z* 80) from PFOS in the form of sulfite, which can be metabolized under starvation conditions. This is consistent with the assumption of bacterial preparative metabolism for subsequent defluorination.

Further PFOS transformation (24–120 hours) was accompanied by the active growth of the culture (the maximum optical density is reached in 96–100 hours of cultivation) and the release of fluorine ions into the medium. A linear growth of substrate consumption followed by its complete transformation due to defluorination was observed during this period (120 hours). After 72 hours of cultivation ultrafiltrate contained components with molecular ion of *m/z* 499 and *m/z* 419 and a new compound with *m/z* 363 of molecular ion, which was initially absent in the medium. By 120 hours of cultivation this compound became dominant while compounds with *m/z* 499 and *m/z* 419 were poorly detectable. The compound with *m/z* 363 of molecular ion was identified according to the MS^1 and MS^2 mass spectra as perfluoroheptanoic acid, which is characterized by the presence of a precursor ion with *m/z* 363 in the MS^1 mass spectrum. Following fragmentation of precursor ion *m/z* 363 leads to formation of a product ion with *m/z* 319 in the MS^2 mass spectrum.

Ensifer moralensis H16 strain cultivated in the presence of PFOS (1,000 mg/L) as a sole source of carbon and energy completely transformed it by defluorination into perfluoroheptanoic acid. At the same time fluoride ions accumulated in the medium to a concentration of 152 mg/L (which corresponds to the removal of four fluorine ions from one PFOS molecule). Fluoride ions released into the medium apparently inhibited strain viability, and, as a consequence, inhibited destruction of perfluorinated compounds. The presented defluorination mechanism is similar to that described for *Pseudomonas plecoglossicida* 2.4-D strain,[12] but is more dynamic. There are no reports on intermediate metabolites of microbial destruction and biodefluorination of perfluorocarboxylic acids.[8,10,11]

We observed that the bacteria *Ensifer moralensis* H16 actively assimilated PFOS, decreasing its content to the level of 12% of the initial value (according to the model experiment for remediation of the soil contaminated by PFOS) the results are shown in Figure 3. The rate of PFOS concentration decrease was linear during first 2 months of the experiment. Meanwhile, there was no significant decrease in pollutant concentration in the control experiment without introduction of *Ensifer moralensis* H16.

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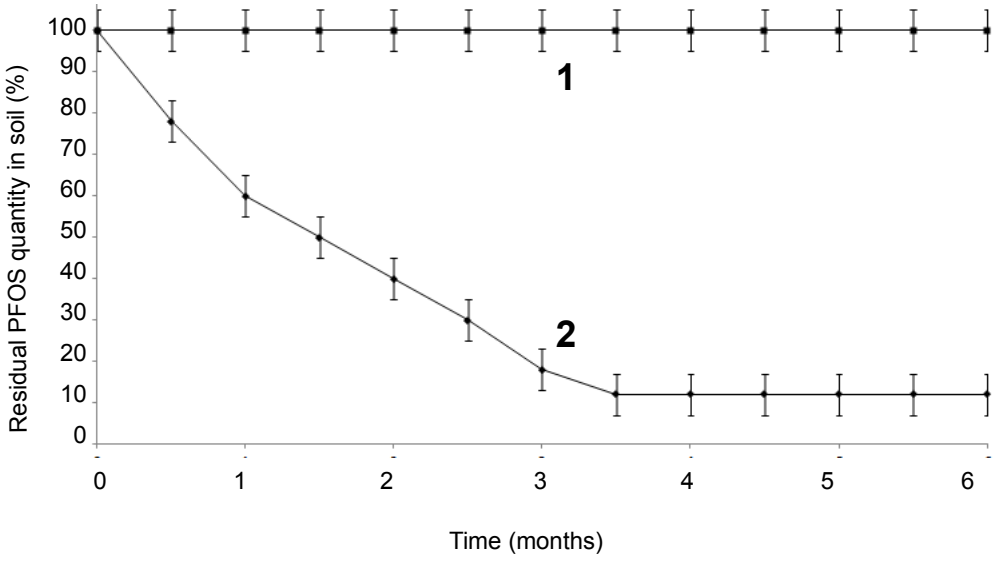

Figure 3. The residual PFOS quantity in the soil. 1: control, residual PFOS quantity in the soil without the introduction of microorganisms and 2: residual PFOS quantity in the soil in the presence of *Ensifer moralensis* H16 strain.

There are very few successful studies on microbial decomposition of organofluorine compounds. It is worth mentioning that the previous experiments showed the capacity of *Pseudomonas aeruginosa* HJ4 strain for their degradation during 48 hours at the initial concentration not exceeding 2 mg/L,[10] and a capacity of the *Pseudomonas plecoglossicida* 2,4-D strain, which defluorinates it into perfluoroheptanoic acid.[12] However, these results were obtained in experiments with liquid medium. Results for biodegradation of PFOS in soil medium are presented only for *Pseudomonas plecoglossicida* 2,4-D strain.[12] We compared the intensity and depth of PFOS biodegradation by *Ensifer moralensis* H16 and *Pseudomonas plecoglossicida* 2,4-D strains and found that the degree of PFOS decomposition by *Pseudomonas plecoglossicida 2.4-D* strain under conditions of the soil remediation experiment was only 75%, while *Ensifer moralensis* H16 strain degrades 88% of PFOS at the same exposure time.

CONCLUSION

In this study we identified *Ensifer moralensis* H16 strain according to cultural, biochemical characteristics, and phylogenetic analysis data. We proved that this strain was able to use PFOS as the sole source of carbon and energy, partially mineralizing it by defluorination. The *Ensifer moralensis* H16 strain can be used in development of biological products for the utilization of environmental pollution caused by organofluorine compounds.

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