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Biodegradation of Perfluorooctanoic Acid by *Pseudomonas Plecoglossicida* Strain DD4

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Abstract:

Organofluorines, as a pollutant, belongs to a group of substances which are very difficult to neutralize. They are part of many products of everyday use and for this reason they pollute the environment in large quantities. Perfluorinated carboxylic acids are entered into the list of the "Stockholm Convention on Persistent Organic Pollutants" in order to minimize the load on the environment by significantly reducing their use, up to their complete rejection. The DD4 strain was isolated from the soil by the enrichment method and identified using 16S rRNA method as *Pseudomonas plecoglossicida*. It is able to metabolize perfluorooctanoic acid (PFOA) as the only carbon source in Raymond nutrient medium with a concentration of 1000 mg/l with the release of 132 mg/l fluorine ions. In tests conducted on the biological decomposition of perfluorooctanoic acid, it was possible to quantify its residues using tandem LCMS-IT-TOF. The presented results characterize the *Pseudomonas plecoglossicida* DD4 strain actively utilized PFOA as the sole carbon source, which characterizes it as a candidate for the creation of biological products aimed at the utilization of organofluorine pollutants.

Keywords: Biodegradation, Defluorination, LCMS-IT-TOF, Perfluorooctanoic acid, *Pseudomonas plecoglossicida*

Introduction:

The problem of cleaning terrestrial and aquatic ecosystems contaminated with toxic substances of unnatural origin resistant to decomposition is one of the most important tasks of modern eco-biotechnology. Oil, petroleum products and pesticides are considered to be the main traditional pollutants. Bioremediation and biodegradation are successful cleaning procedures from them. But in terms of resistance, they are surpassed by halogen organic pollutants [1,2]. Halogenated pollutants are at the top of the list of persistent organic pollutants, in which perfluorocarboxylic acids (in particular, perfluorooctanoic sulfonic (PFOS) and perfluorooctanoic acids (PFOA)), which are included in "Annex B" of the "Stockholm Convention on Persistent Organic Pollutants", possess the properties of surfactants. Possible toxicological effects, coupled with resistance and a highly probable ability to accumulate in organisms, carry great risks from the point of view of ecology [3-5]. Per- and polyfluoroalkyl substances (PFASs, C_nF_{2n+1}−R) have been produced industrially for more than 80 years. Due to such properties of fluorine as large values of electronegativity, and small sizes of atoms, compounds containing fluor, in this case we are talking about the perfluoroalkyl part (C_nF_{2n+1}−), have higher consumer characteristics, which include high acidity levels, excellent surfactant properties at low dosages, high chemical resistance, high repelling capacities of oil and aqua . There is literally no such sphere left (including those related to food production) where these compounds would not be used. On the one hand, high chemical resistances with excellent consumer qualities have a certain consumer value. However, the scientific community, in particular, and the public in general, are concerned about the potential threat of numerous long-chained PFASs, which include PFCAs with more than 7

perfluorinated carbons, PFSAs with more than 6 perfluorinated carbons and their predecessor particularly PFOA and PFOS [6-8].

The ability to accumulate in nature [4] and toxicity can have enormous negative consequences. It is for this reason that regulations on the control of PFOS, PFOC (as well as other PAS) are adopted, mainly in developed countries [6]. They are also listed (PFOS) or are candidates for listing (PFOA) in the Stockholm Convention on Persistent Organic Chemicals.

The current PFOA pollution treatment usually involves expensive adsorption processes on activated carbon filters and subsequent combustion, which can only serve to recycle PFAS back into the environment [9,10]. Known methods of decomposition of perfluorinated acids are chemical processing, burning at high temperature, but they are high–cost and ineffective. These standard recovery strategies have different levels of effectiveness; in some cases they increase the risk to health [11-13].

A milder alternative to the physico-chemical variants of the decomposition of organofluorine compounds is an environmentally safe biological method, in the implementation of which microorganisms minimize the negative impact of PFOA on the environment [14,15]. The spectrum of PFOA-destructor bacteria is not so wide. Only a single number of bacterial strains capable of transforming perfluorocarboxylic acids are known. A strain of *Pseudomonas parafulva* YAB1 is known to have the ability to biodegradate PFOA. It was able to utilize 32.2% PFOA at its initial concentration of 500 mg/l [16].

It has been shown that several species of Pseudomonas can decompose perfluorochemical substances, especially perfluoroalkyl acids, under aerobic conditions. The mixed culture of *Pseudomonas* was more effective than pure cultures [17]. Strain *Acidimicrobium sp*. decomposes perfluoroalkyl acids anaerobically in the presence of electron donors [18,19]. Enzymatic pathways of PFOA degradation have been determined for the aerobic bacterium *Delftia acidovorans* isolated from a soil sample contaminated with PFOA [20]. It has also been shown that perfluorooctanoic acid undergoes olefin carbon deformation by a microbial consortium [21].

The aim of the work is to show the possibility of biological decomposition of PFOA by fluoridation using a new bacterial strain DD4 isolated from the soil of an enterprise for the production of halogen-containing herbicides.

Materials and Methods:

The studied strain was isolated from the soil of the enterprise for the production of halogen-containing herbicides (Republic of Bashkortostan, Russia).

Soil Sample Collection

Sample procedure was carried out according to the literature with slight modification [22]. Composite samples from contaminated soils at depth 0-15 cm, were collected from the point coordinates N 54°84'80.4, E 56°10'16.0''. Soil samples were well mixed, excluding stones and foreign objects. Then, they were sieved using a 2 mm sieve and kept in a cool place for analysis. The samples were taken aseptically, kept in containers, and were stored in the refrigerator until further use. The characteristics are presented in Table 1.

Table 1. The main pollutants of the soil (excerpt from [23])

The Pollutant	Maximum content in soil, mg/kg
Chlorobenzene	2670
Polycyclic Aromatic Hydrocarbons	130
Polychlorinated Biphenyls	9,72
Mineral Oils	5100
Copper	580
Lead	2,6
Zinc	206

Isolation of PFOA-degrading Microorganisms

Exemplars of soil were taken from the territory of an industrial enterprise (Republic of Bashkortostan, Russia). The sampling was carried out from contaminated areas; sterile plastic bags were used for this procedure. To enhance the bioactivity of soil microorganisms, the samples were dried in air and stored at a temperature of 4 °C. In order to isolate bacterial strains exhibiting the ability to biodegrade PFOA, culture enrichment methods were applied. Raymond's medium was used for isolation, which consists (g/l) of Na_2CO_3–0.1; $MgSO_4 \times 7H_20$–0.2; $FeSO_4 \times 7H_2O$–0.02; $CaCl_2$–0.01; $MnSO_4 \times 7H_2O$–0.02; $K_2HPO_4 \times 3H_2O$–1.0; $NaH_2PO_4 \times 3H_2O$–1.5; NH_4Cl–3.0 [24], and dissolved in 1000 ml of distilled water. Then 100 ml of the previous liquid medium was added to a 250 ml conical flask and sterilized in an autoclave. Under septic conditions, 0.1% PFOA (volume/volume) was added as the only carbon source and 1 g of soil contaminated with PFOA as the expected source of soil microorganisms decomposing PFOA, 0.1 ml was added to each flask, placed on a shaker at 30±2 °C for 10 days. Then the samples were transferred to Raymond's agarized medium, adding 0.1 ml of

PFOA. This process was repeated several times until pure colonies were obtained, and the cultures were maintained at the same previous stages.

Characterization of Bacteria

Bacteria identified according to the comparison with the characteristics contained in the Bergey's manual [25].The bacterial genera were identified. The isolates were first diagnosed based on the morphological characteristics of colonies on culture media, including size, edge, height and colour. The biochemical tests were carried out.

Detection of *Pseudomonas Plecoglossicida* Strain DD4 by 16S rRNA

Identification was done by analyzing the data of sequencing of 16S rRNA gene fragment. Copies of 16S rRNA were enlarged using a set of universal primers, 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). The polymerase chain reaction was performed in 25 ml of a mixture consisting of 10 x buffer for Taq polymerase («Silex», Russia), 0.25 mM dNTP, 1.5 mM $MgCl_2$, 0.4 microns of each primer, 5 units of act. Taq polymerase («Silex», Russia) and 10 ng of genomic DNA under the following conditions: 95 °C –5 minutes, 30 s – 94 °C, 30 s – 55 °C, 80 s – 72 °C-30 cycles, 5 min – 72 °C in the «My Cycler» amplifier («Bio-Rad», USA).

To detect PCR products, we used electrophoresis in a horizontal agarose gel (0.8%) in a TBE x 0.5 buffer (boric acid–5.5 g/l, distilled water–79.7 ml, EDTA–4 ml/l, Tris base–10.8 g/l) at room temperature, at a voltage of 5-15 V/cm for 40 minutes. Agarose gels were stained with ethidium bromide solution (0.5 mcg/ml for 5-10 minutes) then photographed in UV light using the BioDocAnalyze gel documentation system («Bio-Rad Laboratories», USA). The following molecular mass markers were used to determine the size of the fragments: O'generulertm 100 bp («Fermentas», Lithuania), O'generulertm 1 kb DNA Ladder («Fermentas», Lithuania)

Purification of PCR products and subsequent sequencing PCR was performed using a set of reagents Big Dye Terminator Cycle Sequencing Kit («Applied Biosystems», USA) according to the manufacturer's instructions.

The nucleotide sequences of functional genes and the 16S rRNA gene were determined using a set of reagents Big Dye Terminator Cycle Sequencing Kit on an automatic sequencer Genetic Analyser 3500XL («Applied Biosystems», USA).

Sequencing of the obtained PCR products of 16S rRNA gene was performed with a Big Dye Terminator v.3.1 kit («Applied Biosystems», USA) with an ABI PRIZM 3730 automated DNA Sequencer («Applied Biosystems», USA) in accordance with the instructions provided by the manufacturer. A search of the sequences homologous to the corresponding sequences of the studied strain in the GenBank database was performed by BLAST program (http://www.ncbi.nlm.nih.gov/blast) [26], for the phylogenetic tree was built with "MEGA7" program (http://www.megasoftware.net) by the neighbor-joining method [27] with the Kimura model [28].

Extraction and Identification of PFOA Biotransformation Products

Extraction and identification, as well as quantitative determination of PFOA biotransformation products in the environment, were carried out after separation of bacterial cells by ultrafiltration on "Vivaflow 50" («Sartorius AG», Germany). Then filtrate (≤3 kDa) was analyzed on tandem LCMS-IT-TOF chromatograph mass spectrometer («Shimadzu», Japan) with a system for the introduction of eluted ions, quadrupole ion trap, and time-of-flight detector. The mass spectra were recorded in the negative ion mode, in the mass range m/z 200-800 a.e.m. and 3.5 kV of the voltage in the detector. For chromatographic division a "Shim-pak XR-ODS" column (75 x 2 mm) in isocratic mode with a solvent ratio 56:44 of ammonium acetate (5 mM in water) and acetonitrile was used flow rate 0.2 mL/min has been set. The structure of the obtained substances was determined by the analysis of total mass spectrometry data based on the degradation of the molecular ion and comparison with the literature data [29].

Biodefluorination of PFOA

PFOA biodefluorination was evaluated by the magnification of concentration of fluor ion in nutrient medium using a fluoride-selective electrode with a solid-state membrane DX219-F.

Results and Discussion:

Isolation and Identification of the *Pseudomonas Plecoglossicida* DD4

The DD4 strain studied in this work, which has the ability to utilize PFOA, was isolated using standard isolation and enrichment techniques. The strain grew noticeably on Raymond's mineral medium, using PFOA as the sole carbon source (0.1 w/v %) at 28°C within 48 h of incubation. The results of its characterization (Table 2) they are in good agreement with the data [30] for *P.*

plecoglossicida bacteria in appearance, optimal growth temperature, and the profile of the substrates consumed. Thus, according to the totality of cultural-morphological and physiological-biochemical properties, the strain was initially presumably identified as *P. plecoglossicida* DD4. To confirm and accurately identify the bacteria, sequencing and comparative analysis of the nucleotide sequence of the 16S rRNA gene with known structures from GenBank were carried out (http://www.ncbi.nlm.nih.gov/genbank).

Table 2. Physiological and morphological properties of the investigated strain

Characteristic	Test result
Gram coloring	-
Shape	rods
Mobility	+
Colony shape	convex
Type of metabolism	respiratory
Catalase	+
Oxidase	+
Hydrolyze lecithin, casein, gelatin and starch	-
Optimum growth range	26-30°C
Optimum pH	6.8-7.2
Optimum concentration of NaCl	0-5 %
Fluorescent pigment	+
Growth at 4°C	-
Growth at 41°C	-
Arginine dihydrolase	+
Denitrification	-
Gelatin liquefaction	-
Lecithinase	-
Lipase	-
Utilization of:	
Arabinose	-
Fructose	-
Galactose	-
Glucose	-
Inositol	-
Lactose	-
Levan	-
Maltose	-
Mannitol	+
Mannose	-
Meso-Inositol	+
Potassium Tartrate	+
Rhamnose	-
Sorbitol	-
Starch	-
Sucrose	+
Xylose	+
2-Ketogluconate	+
Citrate	-
Ethanol	-
L-Alanine	-
L-Arginine	-
L-Aspartate	-
L-Histidine	-
L-Leucine	-
L-Lysine	+
L-Valine	-
Malate	+
N-Butanol	-
Propylene Glycol	+
Succinate	-

The genomic DNA of the isolate was used for amplification of 16S rDNA by using universal primers 27F and 1492R by PCR. The resulting bands were cut and eluted; the DNA thus obtained was subjected to sequencing. The amplified 16S rRNA of the bacterial isolate was sequenced and analyzed by BLAST search in the NCBI public database. The sequence of approximately 1562 base pairs of the 16S rRNA gene of the isolate was 99% identical to that of the 16S rRNA gene of *P. plecoglossicida*. Based on the sequence similarity, the strain was designated as *P. plecoglossicida* DD4 and its 16S rRNA sequence was deposited in the GenBank database with accession no. MZ723936.

For the isolated strain, the sequence (1413 bp) of the gene encoding 16S rRNA was determined. The bacterial species *P. plecoglossicida*, *P. juntendi*, and *P. monteilii* were the closest to the studied sample. The level of sequence similarity between strains DD4 and *P. plecoglossicida* NBRC 103162 was 99.86%, with *P. juntendi* BML3 99.83%, and with *P. monteilii* NBRC 103158 - 99.80%.

To clarify the phylogenetic position of the new strain, a comparative analysis of the nucleotide sequences of the 16S rRNA gene of species belonging to the genus *Pseudomonas* was carried out and a dendrogram was constructed (Fig. 1). From the figure it can be seen that the bacterium *P. plecoglossicida* DD4 probably belongs to the species *P. plecoglossicida* DD4.

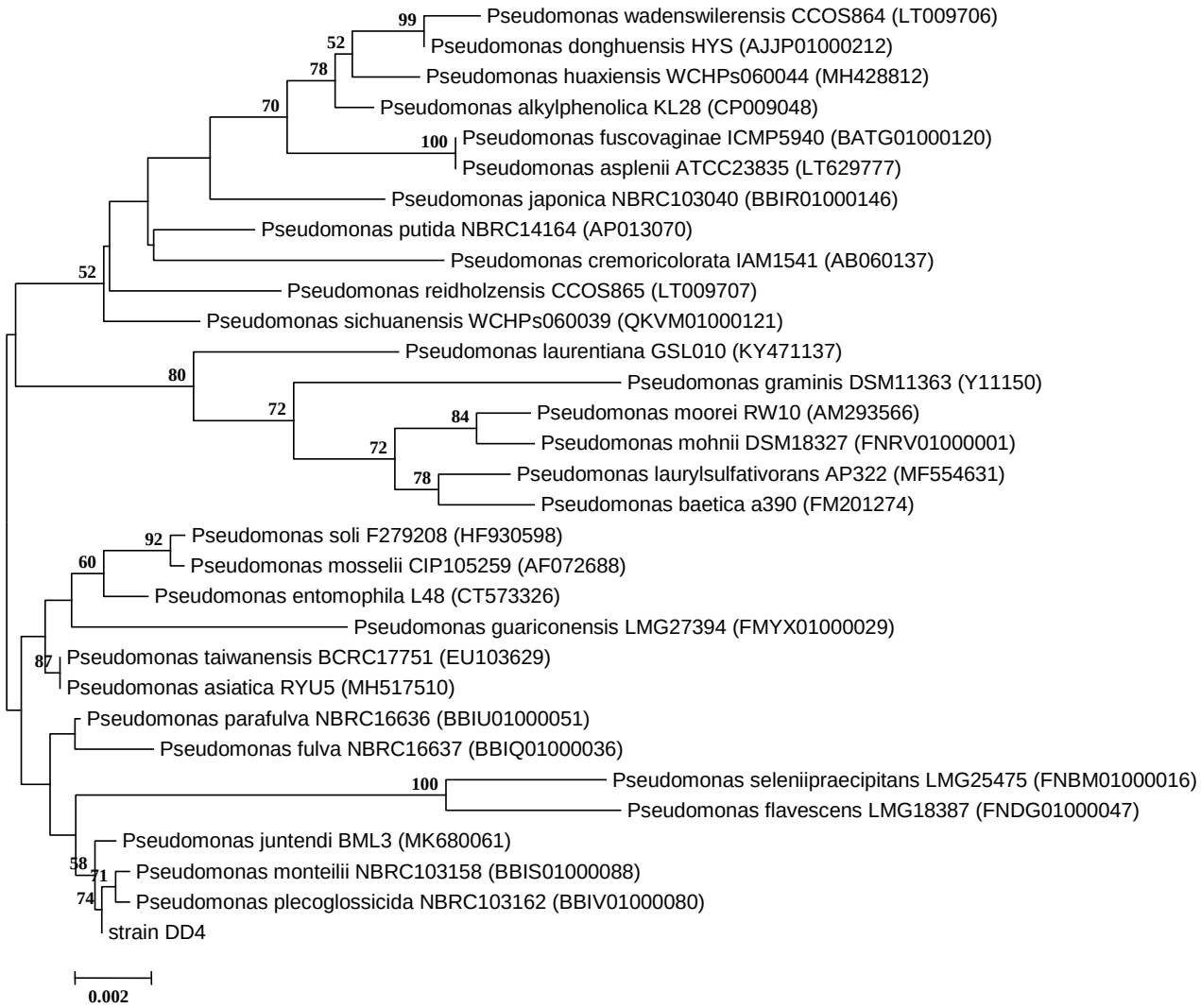

Figure 1. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences strain bacteria *P. plecoglossicida* DD4 and closely related species. Bootstrap values (expressed as percentages of 1000 replications) are shown at the branching points. Bar—two nucleotide substitutions per 1000 nucleotides

In periodical culture the *P. plecoglossicida* DD4 actively destroy PFOA as the only source of energy and carbon (Fig. 2).

The highest optical density of the culture liquid fell on the 6th day of growth when cultivated on PFOA (0.81 OD).

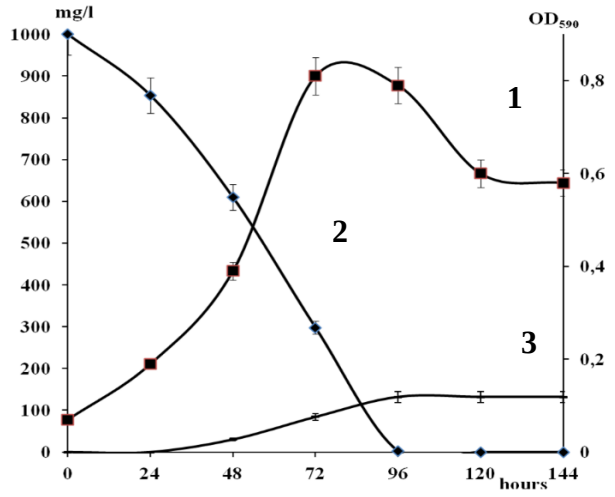

Figure 2. Dependence of the OD_{590} values of the culture fluid (1) and the concentrations of PFOA (2), free fluorine ions (3) in it, depending on the time of cultivation of *P. plecoglossicida* DD4 in a batch culture.

The *Pseudomonas plecoglossicida* DD4 strain actively destroy PFOA a sole source of carbon and energy in batch culture. Analysis of PFOA concentration decrease in the *P. plecoglossicida* DD4 culture liquid in dynamics showed that the first day was the period of adaptation of the culture to the substrate or there was a process of accumulation of the necessary enzymes. Subsequently, there was a linear growth in the consumption of the substrate. In this case, the optical density began to increase after 48 hours of cultivation, reaching a maximum value on the third day of cultivation.

The conversion of PFOA was accompanied by the extrication fluorine ions into the medium; during cultivation, their concentration reached 132 mg/L of the culture fluid, the onset of extrication was correlated the onset of a linear reduction of the PFOA concentration.

Currently, HPLC with tandem mass spectrometry is mostly applied method for analyzing perfluorocarboxylic acids as anionic substances (including PFOA). Perfluorinated organic acids are neutral and poorly biodegradable. Decomposed acid ion is usually observed during liquid chromatography with mass spectrometry of an anionic perfluorated compounds. Strain DD4 actively grew on nutrient medium with PFOA as the sole carbon source, achieving maximum OD of bacterial suspension post 70-75 hours of cultivation with its decomposition in 96 hours. During chromatographic analysis with mass spectrometry, a decomposed PFOA ion was observed in the initial culture liquid (a molecular ion with an m/z ratio of 413 a.m.u.), characteristic of anionic perfluorocompounds (Fig. 3a). After 24 hours, a compound was found whose molecular ion corresponds to m/z 369 a.m.u., which is perhaps in the issue of the ablation of carbon dioxide (m/z 44 a.m.u.) of carboxyl (Fig. 3b). After 72 hours of cultivation in the ultrafiltrate, a compound was found whose molecular ion had an m/z ratio of 363 a.m.u. (Fig. 3c). In the next day concentration of this ingredient in the culture liquid growth and the compound with m/z 369 a.m.u. after 144 hours of cultivation in the medium was not detected. 363 a.m.u., according to the mass spectra MS_1 and MS_2, it was identified as perfluoroheptanoic acid with a forerunner ion (m/z 363 a.m.u.) in the mass spectrum MS_1, which splits with the release of the product-ion with m/z 319 a.m.u. in the mass spectrum MS_2. During the conversion of the perfluorinated substrate, free fluorine ions were released into the medium, and the onset of release was noted at 22-24 hours, followed by an increase up to 96 hours to a concentration of 132 mg/L.

Perfluoroheptane acid which is formed during biological defluorination was found in nutrient medium in end of cultivating and was recognized by a decomposed acidic ion. The PFOA biodefluorination scheme at a concentration of 1000 mg/L, in which fluoride ions accumulate in the medium to a concentration of 132 mg/L (which corresponds to the removal of four fluorine ions from one PFOA molecule), is shown in Fig.4. Further destruction of perfluorinated compounds is probably inhibited by fluorine ions were emitted for the nutrient medium, and the mechanism of biodefluorination is similar to the case with the *P. plecoglossicida* 2.4-D strain [31], only in a more dynamic variation. Other well-known publications on microbial destruction and biodefluorination of perfluorocarboxylic acids do not disclose intermediate metabolites [32-34].

Thus, at the end of cultivation, perfluoroheptanoic acid identified by decomposed acid ion was detected in nutrient medium. The results obtained suggested the following scheme for the destruction of PFOA (Fig. 4). The release of the fluorine ion into the medium may have a retarding effect on further destruction of intermediary fluorinated compounds by the strain under study [33].

Assay of literature on microbial destruction of perfluorooctanesulfonic and perfluorooctanoic acids showed that the amount of strains of microorganisms capable of using them is extremely limited. The *P. aeruginosa* HJ4 strain [32] and the phylogenetically close *P. parafulva* YAB1 strain [34] have been described.

So, in the issue of research carried out in a combination of cultural-morphological, physiological-biochemical characteristics, as well as

the data of phylogenetic analysis, the DD4 strain was identified to the species. It was found that the bacteria *Pseudomonas plecoglossicida* DD4 had the unique ability to use PFOA as sole source of energy and carbon. The results obtained make it possible to recommend the strain for use in biotechnologies aimed to the decomposition of organofluorine compounds to protect the environment. They can also be a base for further research of the adaptive and destructive potential of bacteria.

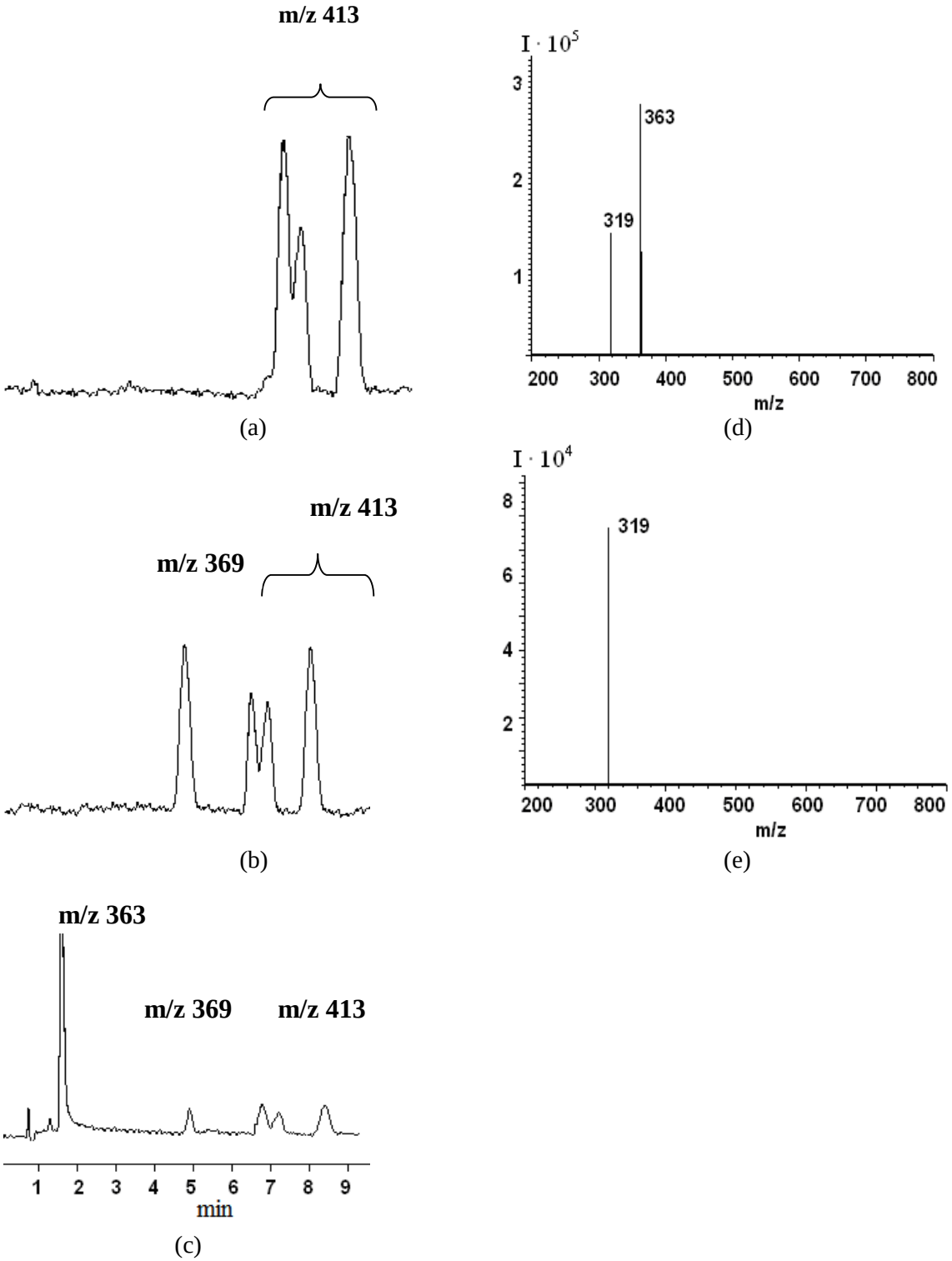

Figure 3. Mass chromatograms of ultrafiltrates of the culture fluid of *P. plecoglossicida* DD4 after 0 (a), 24 (b), 72 (c) hours of cultivation in a periodical culture and mass spectra of the MS_1 (d), MS_2 (e) component with m/z 363.

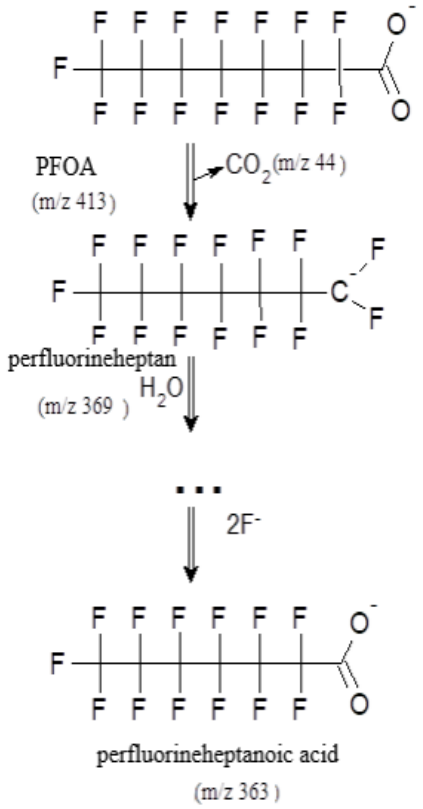

Figure 4. Biodefluorination of PFOA by strain *P. plecoglossicida* DD4 (proposed scheme).

Conclusions:

A new strain DD4, a representative of the species *P. plecoglossicida*, capable of partial mineralization of PFOA by defluorination has been described. The strain *P. plecoglossicida* DD4 is recommended for use in biotechnology transformation of organofluorine compounds to protect the environment. A new DD4 strain, a representative of the *Pseudomonas plecoglossicida* species, capable of partial mineralization of perfluorinated organic compounds (using the example of perfluorooctanoic acid (PFOA)), has been described by deftoring. The biological decomposition of PFOA at its concentration up to 1000 mg/l was confirmed by HPLC-MS/MS and potentiometry using a fluoride-selective electrode with the release of 132 mg/l fluorine ions. The strain *P. plecoglossicida* DD4 is recommended for use in biotechnology transformation of organofluorine compounds to protect the environment.

Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee in Ufa Federal Research Centre of the Russian Academy of Sciences.

Authors' contributions statement:

Sh. DA. and Ch. SP. contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript.

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التحلل البيولوجي لحمض البيرفلوروكتانويك بواسطة سلالة بليكوغلوسيسيدا الزائفة دي دي 4

تشيتفيريكوف سيرجي ب **شاريبوف دانيال أ**

معهد أوفا للبيولوجيا التابع لمركز أوفا الفيدرالي للبحوث التابع للأكاديمية الروسية للعلوم ، أوفا

الخلاصة:

تنتمي الفلورينات العضوية ، كملوث ، إلى مجموعة من المواد التي يصعب للغاية تحييدها. إنها جزء من العديد من منتجات الاستخدام اليومي ولهذا السبب تلوث البيئة بكميات كبيرة. يتم إدخال الأحماض الكربوكسيلية المشبعة بالفلور في قائمة "اتفاقية استكهولم بشأن الملوثات العضوية الثابتة" من أجل تقليل الحمل على البيئة عن طريق الحد بشكل كبير من استخدامها ، حتى رفضها الكامل. تم عزل سلالة دي دي 4 من التربة بطريقة التخصيب وتم تحديدها باستخدام طريقة الرنا الريباسي 16 ثانية على أنها بسيودوموناس بليكوغلوسيسيدا. وهي قادرة على استقلاب حمض البيرفلوروكتانويك (بفوا) كمصدر الكربون الوحيد في ريمون المغذيات المتوسطة مع تركيز 1000 ملغ/لتر مع الافراج عن 132 ملغ/لتر أيونات الفلور. في الاختبارات التي أجريت على التحلل البيولوجي لحمض البيرفلوروكتانويك ، كان من الممكن تحديد بقاياه باستخدام الترادف لممس-إيت-توف. النتائج المقدمة تميز الزائفة بليكوغلوسيسيدا دد 4 سلالة تستخدم بنشاط بفوا كمصدر الكربون الوحيد ، الذي يميزه كمرشح لخلق المنتجات البيولوجية التي تهدف إلى استخدام الملوثات العضوية الفلورية.

الكلمات المفتاحية: التحلل البيولوجي، إزالة الفلورة، مطياف الكتلة LCMS-IT-TOF , حمض البيرفلوروكتانويك، الزائفة بليكوغلوسيسيدا