MJFAS
Malaysian Journal of Fundamental and Applied Sciences

Hkudaigulov *et al.* | Malaysian Journal of Fundamental and Applied Sciences, Vol. 20 (2024) 1308-1316

RESEARCH ARTICLE

Destruction of C_7, C_9 Perfluorocarboxylic Acids by the Strain *Ensifer adhaerens* M1 Resistant to High Concentrations of Fluorine Ion

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Abstract The aim of this study is to determine the resistance to high concentrations of fluorine ions in the environment of the strain *Ensifer adhaerens* M1 isolated from soil at a fire extinguishing agent storage and testing site, to determine the growth kinetics and destruction of C_7, C_9 perfluorocarboxylic acids, perfluoroheptanoic acid and perfluorononanoic acid, respectively, as the sole sources of carbon and energy. The degradation products of perfluorinated carboxylic acids were identified using LCMS-IT-TOF. Results show that the degradation process occurs in multiple stages leading to the formation of perfluorohexanoic acid and accompanied by the release of fluoride ions. Strain *Ensifer adhaerens* M1 is resistant to F^- up to a concentration of 3.57 mM in the environment. Within 14 days of cultivation, bacteria completely utilized perfluoroheptanoic acid and perfluorononanoic acid. Based on chromatography-mass spectrometry and ion chromatography data, a scheme for the stepwise degradation of the investigated perfluorinated acids involving the enzymes coded by novR and dhlA genes is proposed.

Keywords: Perfluorocarboxylic acid, *Ensifer*, biodegradation, transformation.

Introduction

Environmental contamination with perfluorinated compounds is one of the most serious problems facing the global community. Among widely used groups of compounds are perfluoroalkyl and polyfluoroalkyl substances (PFAS). According to research, the number of known chemicals in this class exceeds 7000 different names [1]. Perfluorinated acids are the classic representatives of this group of substances, which are attracting increasing attention from researchers and ecologists and are included in Annex B of the Stockholm Convention on Persistent Organic Pollutants [2]. The intense focus on them is due to several factors: toxicity, bioaccumulation, and persistence. In other words, these compounds have the ability to migrate and accumulate, leading to prolonged environmental poisoning. Harmful substances contaminate water resources [3, 4, 5], posing a threat to human health [6]. It should be noted that industrial sites cause irreparable damage to nature even at the synthesis stage of this group of compounds [7].

One of the challenges in PFAS degradation is the negative impact of fluorine ions released, these ions have an inhibitory effect on the growth and development of bacteria by binding enzymes and/or forming complexes with metals, forming AlF_4^- and BeF_3^-*H_2O which replace phosphate ions, directly affecting metabolism and leading to disruptions and imbalances in cell metabolism [14]. Fluoride ions are known to induce oxidative stress by increasing the concentration of reactive oxygen species (ROS) in plant cells [15,16]. Additionally, fluoride ions can negatively impact not only individual bacteria but also the diversity of the microbiome [17].

Microorganisms, due to their ability to rapidly adapt, are capable of mitigating the stress caused by F^- [18]. Among microorganisms with high adaptation capabilities to negative factors, it is worth highlighting the *Pseudomonas* genus and representatives of other closely related species. For example, in the scientific literature, a strain *Acidimicrobium sp*. A6 has been described capable of utilizing up to 60% of perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) [19]. The strain *Delftia acidovorans* D4B has shown the ability to degrade perfluorinated compounds using dehalogenases [20], while

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Received: 21 June 2024
Accepted: 16 Oct. 2024

Pseudomonas parafulva YAB-1 can degrade up to 32.2% of perfluorooctane acid at an initial concentration of 500 mg/L in the environment [21]. *P. aeruginosa* HJ4 metabolized up to 67% of PFOS when present in the environment at concentrations of no more than 2 mg/L [22]. It should be noted that the number of known PFAS degraders is not very large, and there are even fewer studies identifying degradation products and potential metabolic pathways. In summary, the search for new PFAS-degrading strains resistant to high concentrations of F^- and the determination of possible detoxification methods for fluorinated compounds is a relevant issue.

The strain *E. adhaerens* M1 is capable of utilizing C_8 perfluorocarboxylic acids (perfluorooctanoic acid and perfluorooctane sulfonate) as the sole sources of carbon and energy, forming perfluoroheptanoic acid and releasing free fluoride ions into the medium [23], in addition to those described above.

If the biodegradation of PFOA has been studied by scientists, then the biodegradation of longer chain perfluorocarboxylic acids (PFCAs), which are likely more biodegradable, and more persistent shortened perfluorocarboxylic acids, has not been extensively evaluated in scientific research. The aim of this study was to assess of *E. adhaerens* M1 strains capabilities in degrading C_7 and C_9 PFCAs, perfluoroheptanoic acid (PFHpA) and perfluorononanoic acid (PFNA) as sole carbon and energy source and determination of the resistance of the studied bacterium to high concentrations of fluorine ion.

Materials and Methods

The C_7, C_9 perfluorocarboxylic acids in the following list were used: PFHpA and PFNA (all high purity > 98%). These were acquired from Sigma Aldrich (St. Louis, MO, USA). Acetonitrile (quality for HPLC) was purchased from Merck (Darmstadt, Germany). All remaining reagents were of analytical-grade grade.

Strain Source

Strain M1 was identified in previous study as *Ensifer adhaerens* and able to utilize C_8 perfluorocarboxylic acids [23].

Inoculate Preparation

To obtain the inoculate, the strain was grown on a Raymond mineral medium (gram per liter of distilled water): NH_4NO_3—2.0; $MgSO_4 \times 7H_2O$—0.2; KH_2PO_4—2.0; Na_2HPO_4—3; $CaCl_2 \times 6H_2O$—0.01; and Na_2CO_3—0.1 [24] with the addition of peptone (1 g/L) for 24 h. Prior to inoculation into experimental flasks, the biomass underwent precipitation and washing with sterile distilled water was added to experimental flasks until 0.1 OD_{600} was reached.

Fluoride Resistance Determination

The assessment of resistance to fluoride ions was conducted by cultivating the test strain on Raymond's medium in the presence of various concentrations of sodium fluoride (NaF) at concentrations 1.19, 2.38, 3.57 and 7.14 mM (50, 100, 150, and 300 mg/L, respectively). Sample selection was carried out on the ^{3rd}, ^{5th}, and ^{11th} day of the experiment.

Kinetics of Growth and C_7, C_9 Perfluorocarboxylic Acids Destruction Dynamics

To determine the kinetics of growth, decay products and dynamics of fluoride ion formation the strain M1 was cultivated on Raymond's medium with addition of PFHpA or PFNA at concentration 250 mg/L, as sole carbon and energy source in an orbital shaker-incubator ES-20/60 (SIA BIOSAN, Riga, Latvia) at 180 rpm, at a temperature of 28 °C in a liquid mineral medium containing individual PFHpA or PFNA compounds for 18 days.

A broth medium consisting of (per liter of distilled water) 15 g of peptone, 5 g of NaCl and 15 g of agar (PanReac, Barcelona, Spain), was used to isolate colonies and determine the bacterial count.

In order to evaluate the efficiency of PFCAs degradation, the fluoride balance was determined by ion chromatography using an LC-20 Prominence HPLC system with a CDD-10Avp conductometric detector (Shimadzu, Japan). Fluorine ions were separated on a Shodex column (Shodex, New York, NY, USA) at a flow rate of 1 mL/min. An aqueous solution of sodium carbonate and sodium bicarbonate was used as an eluent: 1.8 mM Na_2CO_3 + 1.7 mM $NaHCO_3$. Post-column eluent suppression was achieved by employing a Xenoic® XAMS suppressor coupled with an ASUREX-A100 (Diduco AB, Umeå, Sweden).

The content of PFCAs in the medium was evaluated, and the products of their biotransformation were identified on a liquid tandem chromatography-mass spectrometer LCMS-IT-TOF (Shimadzu, Kyoto,

Japan), (at the AGIDEL UFSC RAS Equipment Collective Use Center) in ultrafiltrates (≤3 kDa) of culture liquids obtained by ultrafiltration on Vivaflow 50 (Sartorius AG, Göttingen, Germany), as described [5].

Destruction Products Determination

The Shim-pack XR-ODS column (75 mm × 2.0 mm id, 2.2 μm) (Shimadzu, Kyoto, Japan) was used for UFLC analysis, in a gradient elution system with 5 mM ammonium acetate in acetonitrile (A) and 0.1% acetic acid (v/v) in water (B). A linear gradient elution was employed, with the following specifications: from 0 min to 10 min, the composition transitioned from 60% to 30% (B); then, from 10 min to 20 min, the composition reversed, transitioning from 30% back to 60% (B). The chromatographic separation was performed maintaining a steady flow rate of 0.25 mL/min, employing an injection volume of 5 μL. Mass spectrometry data were acquired from an electrospray ionization (ESI) source, which was operated in negative ionization mode. MS operating conditions were as follows: probe voltage, −3.5 kV; curve desolvation line (CDL) and heat block temperature, 200 °C; detector voltage, 1.57 kV; nebulizing gas (N_2) flow, 1.5 L/min; collision gas and cooling gas (argon), 50 kPa and 105 kPa, respectively. Parent ion m/z was acquired in the 150–700 range, with an ion accumulation time of 100 ms.

Structure

The structure of the obtained compounds was determined based on a set of data from the analysis of mass spectra based on the decay of a molecular ion and comparison with literature data.

Visualization

Metabolic pathways were drawn using ChemSketch 2023.1.1.

Statistics

The experimental protocols were carried out in three autonomous biological repeats.The statistical analysis was conducted utilizing Microsoft Office Excel 2021 software.

Results and Discussion

Bacteria of the genus *Ensifer* have the ability to biodegrade various xenobiotics, such as polychlorinated biphenyls [25], atrazine [26], and arsenic compounds [27]. In this study, we investigated the ability of the strain *Ensifer adhaerens* M1 to degrade PFHpA and PFNA.

Determination of Resistance of Strain to High Fluoride Concentrations

At the first stage, we established the resistance of the investigated strain M1 to high concentrations of fluoride ions in the environment, the toxicity of which is based on its exceptional electronegativity. Fluoride ions replace functional groups in enzymes responsible for binding metal ions (OH^-, PO_4^{3-}, COO^-), ultimately leading to disruptions in the configuration and function of proteins [28, 29]. Negative effects are already observed at concentrations of 10–100 μM [30]. It is for this reason that the resistance of bacteria to elevated concentrations of F^- is an important and necessary property for a potential degrader of perfluorinated compounds.

Table 1. Survival of strain M1 in the presence of fluoride ions

Concentration, NaF, mM	*Ensifer adhaerens* M1, ·10^8 CFU/mL			
	0 days	3 days	5 days	11 days
0	1.3±0.2	8.4±0.2	4.8±0.4	2±0.3
1.19	0.6±0.1	7.2±0.3	6.2±0.2	2.3±0.2
2.38	0.75±0.3	6.1±0.4	5.7±0.6	1.5±0.4
3.57	0.76±0.2	8.5±0.1	7.2±0.5	4.5±0.3
7.14	0.70±0.5	3±0.4	0.21±0.5	-

In the range of fluoride ion concentrations from 1.19 mM to 3.57 mM, differences in titers for strain M1 in the culture liquid were insignificant. At a concentration of F^- in the medium equal to 7.14 mM on the third day, the titer slightly increased, however, by the fifth day of the experiment, there was a sharp growth inhibition accompanied by a decrease in titer by an order of magnitude, and by the 11th day, there were no viable cells left in the culture liquid (Table 1). According to the results of the experiment, strain *Ensifer adhaerens* M1 shows tolerance to the presence of fluoride ions in the environment at concentrations significantly exceeding those obtained by Lesher *et al.*, indicating a substantial reduction in the amount of cell biomass of *B. subtilis* BR151 at a fluoride ion concentration of 5-10 mM [31].

One of the mechanisms of such resistance can be active transport systems for fluoride ions, and currently, two types of such proteins are known - CLC^F (CLC-Fluoride) and Fluc (Fluoride channel) proteins [32; 33]. The Fluc channel family, according to data [34], consists of primitive antiparallel one-dimensional homodimers with a bound Na^+ in the center of the protein. Sodium ion is not transported but plays a structural role in forming the fluoride-conducting state of the channel. The mechanism of CLC-Fluoride (CLC^F) differs from the operation of Fluc channels. In CLC^F, rotational movements of the side chain lead to alternating conformations changing their position between intracellular and extracellular sides for coupled antiport, in these transport systems, one fluoride ion exported from the intracellular space corresponds to one proton imported from the external environment.

The dynamics of changes in bacterial titers over time may indicate that for a certain period, bacteria are able to resist the toxic effects of fluoride ions through active transport systems that remove fluoride ions from the cells, thereby preventing their accumulation in the intracellular space and maintaining the concentration of F^- below the threshold at which negative effects become irreversible. This led to an increase in titer. However, over time, when this threshold was exceeded, the balance shifted towards the accumulation of fluoride ions entering the cell over those removed by active transport systems. This imbalance could trigger a cascade of negative effects such as oxidative stress, displacement of phosphate ions, etc., leading to a significant decrease in titer on the fifth day and complete cell death of microorganisms on the 11th day of the experiment at a fluoride ion concentration of 7.14 mM.

Growth of Strain M1 on C_7, C_9 Perfluorinated Acids

When evaluating the growth of *Ensifer adhaerens* M1 on PFHpA and PFNA perfluorinated acids, it was found that different substrates had different effects on the growth of the strain *Ensifer adhaerens* M1 (Figure 1). For PFHpA, a gradual increase in OD was observed until the end of the experiment, while for PFNA, there was a sharp decrease in optical density values after 12 days of the experiment. This phenomenon may be caused by either a suppressing metabolic effect of unidentified degradation products of PFNA acid or as a result of the fact that the breakdown of the perfluorononanoic acid unit releases a greater amount of fluoride ions, or by a combination of these two factors.

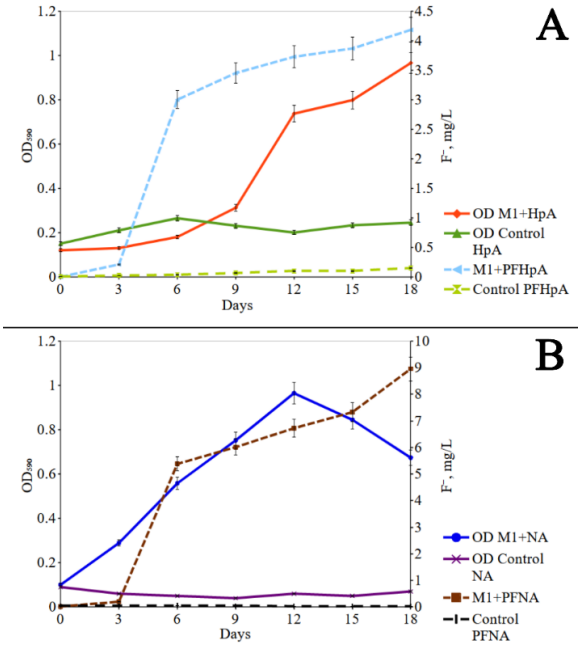

Figure 1. Dynamics of changes in optical density and fluoride ion concentration on C_7, C_9 acids: (A) - "OD M1+HpA" - optical density of the culture liquid when cultivated on heptanoic acid, "OD Control HpA" - control optical density of the nutrient medium with heptanoic acid without bacteria, "M1+PFHpA" - fluoride ion concentration in the culture liquid of strain M1 when cultivated on perfluoroheptanoic acid, "Control PFHpA" - fluoride ion concentration in the nutrient medium with perfluoroheptanoic acid without bacteria; "OD M1+NA" - optical density of the culture liquid when cultivated on nonanoic acid, "OD Control NA" - control optical density of the nutrient medium with nonanoic acid without bacteria, "M1+PFNA" - fluoride ion concentration in the culture liquid of strain M1 when cultivated on perfluorononanoic acid, "Control PFNA" - fluoride ion concentration in the nutrient medium with perfluorononanoic acid without bacteria

A recently published study showed that microbial cells experience significant stress when breaking down fluorinated substrates compared to the introduction of NaF salts (exogenous fluoride ion) into the medium, due to the formation of fluoride ions directly inside the cell. The same study reported a 22% decrease in growth rate for the investigated recombinant strain *P. putida* when introduced to 10 mM NaF in the nutrient medium, and a 41% growth inhibition when grown on 20 mM α-fluorophenylacetic acid (equivalent concentration of released F^- is 10 mM), which correlates with our results [9]. In summary, strain M1 is capable of actively growing in the presence of exogenous F^- in the nutrient medium up to 150 mg/l. We tend to attribute this to the functioning of fluoride ion active transport systems. When breaking down a perfluorinated substrate, it is likely that fluoride ions accumulate inside the cell, requiring a much smaller amount for growth inhibition. However, this issue requires more detailed and separate investigation.

It has also been noticed by us that after the growth cessation, the fluoride ion content continues to increase. This may be related to the activity of exogenous enzymes continuing to break down PFCA, accompanied by the release of fluoride ions into the medium, or endogenous enzymes that may be released into the environment after cell death. In the future, our team plans to isolate these enzymes and possibly their complexes in order to determine their contribution and functions in the degradation of perfluorinated compounds.

C_7, C_9 Perfluorinated Acids Destruction Products Determination

When cultivating the strain of bacteria *E. adhaerens* M1 on PFHpA and PFNA as the sole source of carbon and energy, products of substrate degradation were formed and analyzed using chromatography with mass spectrometric detection. The study of the dynamics of biodegradation of PFHpA (C_7-PFCA) and PFNA (C_9-PFCA) showed that the defluorination process proceeds stepwise to a substance with m/z of 313, characteristic of perfluorohexanoic acid. For perfluoroheptanoic acid, the biological degradation process takes up to 3 days, with 89% substrate decomposition by mass, accompanied by a sharp increase in released F^- ions at the same time, while OD readings change insignificantly. In the second stage, for perfluoroheptanoic acid, the intermediate product with m/z 319 is further degraded, leading to a significant increase in optical density between 3-12 days.

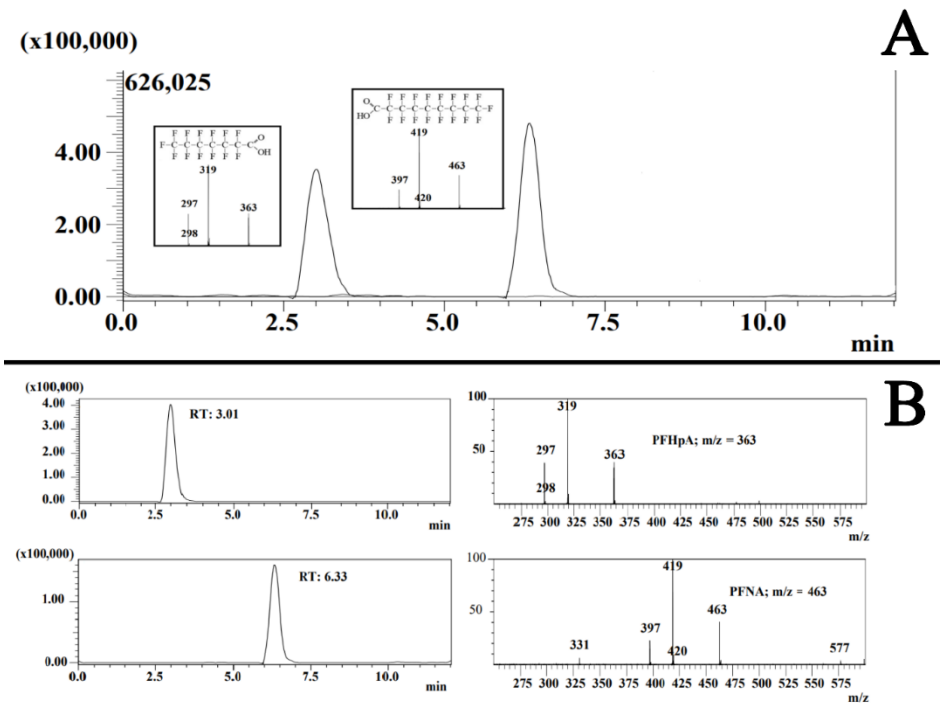

Figure 2. Temporal characteristics, chromatograms of separation (a), and chromatomass spectra of PFHpA and PFNA (b)

Table 2. Dynamics of perfluoroheptanoic acid degradation

Days	Substance concentration (%, mass)		
	m/z 363	m/z 319	m/z 313
0	100	-	-
3	54	46	-
6	11	83	6
9	2	8	90
12	-	7	93
15	-	6	94
18	-	5	95

For perfluoropelargonic acid, the picture was different, with a sharp decrease in OD_{600} observed at 12 days (Figure 2). It should be noted that in both cases, the calculated concentration of F^- does not exceed 20 mg/l, however, the amount of intermediates during perfluoropelargonic acid degradation is significantly higher (Table 3). Presumably, the inhibition of bacterial growth is caused not only by the formation of fluoride ions but also by the products of substrate degradation.

Table 3. Dynamics of perfluorononanoic acid degradation

Days	Substance concentration (%, mass)					
	m/z 463	m/z 419	m/z 413	m/z 363	m/z 319	m/z 313
0	99	1	-	-	-	-
3	47	27	17	9	-	-
6	5	12	15	16	30	22
9	1	8	12	10	21	48
12	-	-	4	4	15	77
15	-	-	-	-	8	92
18	-	-	-	-	4	96

C_7, C_9 Perfluorinated Acids Metabolism

Based on the data of biological degradation of perfluorinated C_7, C_9 acids, we have proposed a hypothetical degradation scheme (Figure 3). During the degradation of PFNA, in the first stage, the enzyme decarboxylase encoded by the novR gene [35] cleaves the COO^- group. This enzyme oxidizes the carboxyl group to form carbonic acid. It is worth noting that this enzyme is identical to the one encoded by the cloR gene - bifunctional non-heme iron oxygenase [36], which converts the substrate in two consecutive oxidative decarboxylation steps involving oxygen. After the cleavage of the carboxyl group, the enzyme encoded by the dhlA gene [37] removes two fluorine atoms.

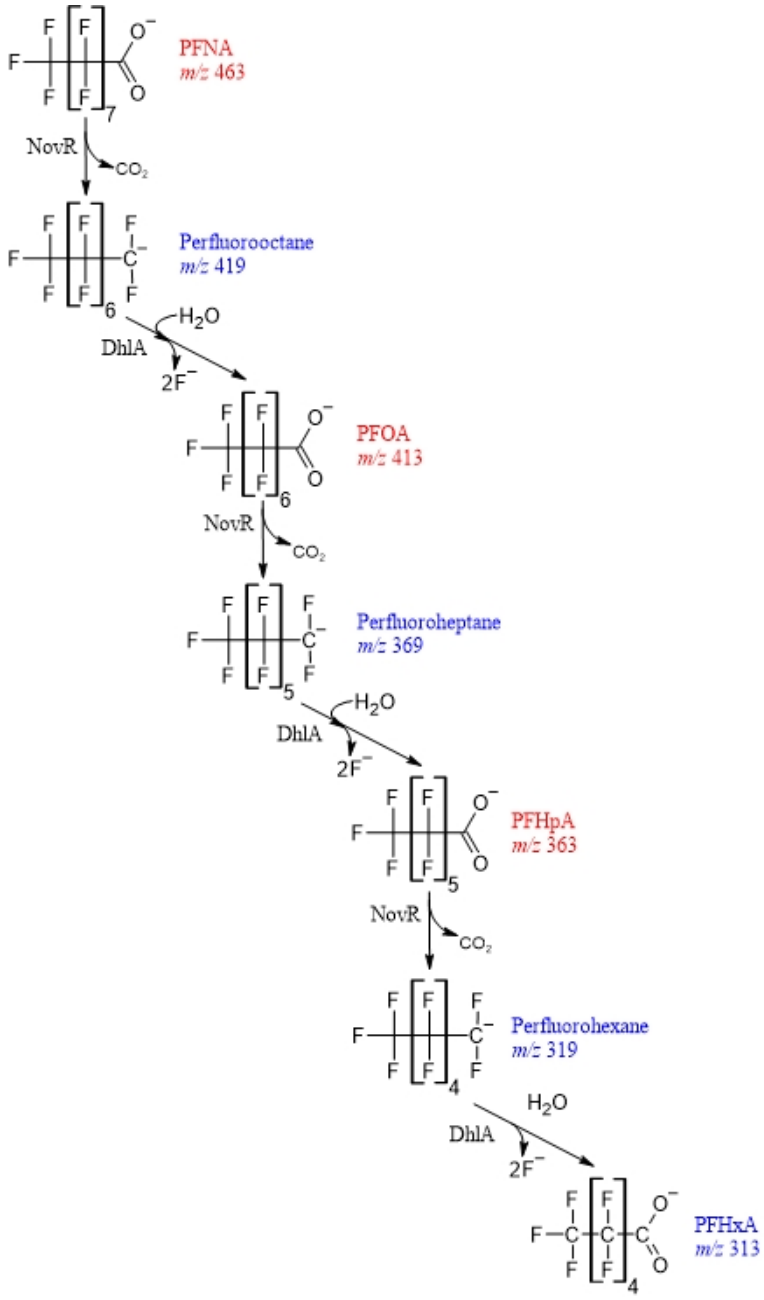

Figure 3. Hypothetical pathway of metabolism of PFHpA and PFNA perfluorinated acids involving decarboxylase enzyme (novR) and dehalogenase enzyme (dhlA)

Conclusions

Ensifer adhaerens strain M1, which was previously isolated from soil collected from a fire extinguishing agent storage and testing area (Republic of Maldives). The *E. adhaerens* M1 bacterial strain showed the ability to grow at fluoride ion concentrations in the medium up to 150 mg/L, but at a concentration of 300 mg/L, the cell titer dropped by an order of magnitude on the fifth day, and complete cell death was observed by the 11th day. Suppression and death of cells of the investigated strain occurred. The breakdown products of PFHpA and PFNA acids were identified during cultivation on Raymond medium, where PFHpA and PFNA served as the sole source of energy and carbon. Using LCMS-IT-TOF methods, the products of biological degradation of perfluorinated C_7 and C_9 acids were identified. For the degradation of PFHpA, the number of intermediates was three, whereas when cultivated on PFNA, it was six. Based on experimental and literature data, a metabolic pathway of PFCAs through stepwise

dehalogenation and decarboxylation to perfluorohexanoic acid is proposed, with the minimal m/z ratio of the intermediate detected in the bacterial culture liquid.

Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

Acknowledgement

This work is part of a research project, funded by the Russian Science Foundation, grant number 23-24-00154.

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