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A VIABLE STRATEGY FOR THE BIODEGRADATION OF HALOGENATED ORGANIC COMPOUNDS FROM THE WASTEWATER MEDIATED BY *PSEUDOMONAS PUTIDA*

DIANA IONELA STEGARUS[a], CONSTANTIN PALADI[b], ECATERINA LENGYEL[c], CORNELIU TANASE[d], ANAMARIA CĂLUGĂR[e], VIOLETA-CAROLINA NICULESCU[a,*]

ABSTRACT. Microbial degradation is to be considered a perspective solution in the elimination of microporous organic compounds in the environment. Various studies attest to the *Pseudomonas putida* species as being capable of aerial biodegradation of numerous organic compounds including chlorinated aromatic compounds. The aim of this study was to demonstrate the ability of *Pseudomonas putida* to grow on various organic substrates, being a possible candidate for dehalogenation of various chlorinated organic compounds Water samples were prepared by contamination with a solution containing 1,1,1-trichloroethane, 1,1,1,2-tetrachloroethane, trichloromethane and tetrachloromethane. The concentrations of halogenated organic compounds, before and after inoculation, were determined by gas chromatographic method. The results obtained showed the reduction, in the presence of *Pseudomonas putida*, of various halogenated organic compounds such as 1,1,1-trichloroethane, 1,1,1,2-tetrachloroethane, trichloromethane and tetrachloromethane with values ranging from 23% to 45% over a time interval of 120 days. The present study demonstrates the ability of *Pseudomonas putida* to grow on various organic substrates, being a possible candidate for dehalogenation of various types of chlorinated organic compounds.

Keywords: *biodegradation, dehalogenation, groundwater, halogenated organic compounds, Pseudomonas putida*

[a] *National Research and Development Institute for Cryogenic and Isotopic Technologies – ICSI Ramnicu Valcea, 4th Uzinei Street, 240050 Ramnicu Valcea, Romania;*
[b] *Nicolae Testemitanu University of Medicine and Pharmacy of Chisinau, 165 Ștefan cel Mare and Sfint Blvd, 2004 Chisinau, Moldova*
[c] *Lucian Blaga University of Sibiu, 9th Victoriei Street, 550024, Sibiu, Romania;*
[d] *Department of Pharmaceutical Botany, George Emil Palade University of Medicine, Pharmacy, Sciences and Technology of Târgu Mureș, 38 Gheorghe Marinescu Street, Târgu Mureș, Mureș, 540139, Romania*
[e] *Faculty of Horticulture, Advanced Horticultural Research Institute of Transylvania, Viticulture and Oenology Department, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, 3-5 Manastur Street, 400372, Cluj-Napoca Romania*
** Corresponding author: violeta.niculescu@icsi.ro*

INTRODUCTION

Remediation of wastewater has always been a major environmental and economic issue, being a complex process, which involves massive financial expenses [1], [2]. From this point of view, the use of bacteria able to biodegrade *in situ* the chlorinated compounds could potentially be an effective approach [3], [4]. This is possible as long as the availability of bacteria strains and the facility of introducing them leaves the promise of a reasonable working environment both ecologically and financial. Emission of pollutants into the environment does not only describe an ecologically fundamental problem, but it is continuously dichotomized by forming a cascade that reflects in various fields of everyday life, starting with agriculture, food industry or medicine. Water quality is a widely debated issue and its assessment is increasingly complex [5], [6].

Extensive use of halo-aromatic compounds as odorous solvents or pesticides has led to their massive release in the environment. Many of these compounds used to be considered non-degradable, but, due to the continuous evolution of microbial enzyme mechanisms, new methods for their mineralization are constantly discovered [7]. Microbial degradation is considered a perspective solution in order to eliminate the micro-porous organic compounds in the environment and it can be improved by creating genetically modified or isolated bacteria by various selection methods.

These processes may influence the soils and waters decontamination process in various industrial regions of the world [8], [9].

The hydrocarbons degradation in either soil or water is primarily conditioned by the presence or absence of strains corresponding to the chemical compound [10], [11]. The main factor in the bacteria presence becomes the accessibility of the pollutant. In order to have a positive effect on mineralization, the bacterium should come into direct contact with the pesticide / compound, either by chemotactic response or by transporting it to the contaminated area [12]. Thus, too low pollutant concentration can inoculate the soil with ineffective bacteria, as long as the amount of nutrient is inadequate [4], [13]. Various studies demonstrated that hydrocarbons from the solid tar are resistant to microbial decomposition, but, recently, the enzyme activity of aerobic and anaerobic microorganisms in the degradation of organ chlorinated compounds was investigated [7], [14]. Due to the surface/volume ratio unfavorable for the microbial growth, the substrate remains insoluble. The smaller the volume/area ratio is, the faster the microbial decomposition process of organic compounds becomes.

Another important factor in the process of the aromatic compounds microbial degradation is the temperature. Various studies confirm the hypothesis that, at low temperatures, low hydrocarbons toxicity leads to a more prolific increase of the colonies [15], [16]. In winter, however, during frost, bacterial efficiency tends to drastically decrease.

The chemical compounds such as phosphorus or nitrogen are indispensable for the aromatic hydrocarbons biodegradation [3]. Thus, at a temperature of 14°C, an absorption of 4 nmol N/mg oil was found. The maximum efficiency in decomposition of aromatics is achieved when 3.2 mg phosphate/L is introduced. In addition, the presence of phosphate and nitrogen is a limiting factor, and their addition can stimulate the biodegradation process [17], [18], [19]. Normally, bacterial metabolism also involves a certain oxygen consumption, due to the fact that both saturated and aromatic hydrocarbons involve the presence of oxygenase and molecular oxygen in the chemical decomposition process. Although aromatic compounds biodegradation in anaerobic conditions is possible, it is slower and less efficient [17].

A recent study emphasizes the enormous impact that iron oxide ions can play in the dynamics of biodegradation [20]. This can positively interact with certain *Dehalobacter* cultures in a groundwater remediation process, favoring the dichloromethane and chloroform decomposition. The same study demonstrates a three-time faster conversion of dichloromethane in the presence of iron ion [20].

Although many studies demonstrate significant biodegradation capabilities in laboratory conditions, the effectiveness of their use *in situ* (where the multitude of organic compounds present might interfere with biodegradation) is still poorly studied [21], [22], [23], [24], [25], [26], [27], [28],[29].

The aim of this study was to demonstrate the ability of *Pseudomonas putida* to grow on various organic substrates, being a possible candidate for dehalogenation of various chlorinated organic compounds (such as 1,1,1-trichloroethane, 1,1,1,2-tetrachloroethane, trichloromethane or tetrachloromethane).

RESULTS AND DISCUSSION

The results revealed that the *Pseudomonas* strain needed a fairly long period of accommodation with the organic compounds from the water, consequently, the determined values varied on a case-by-case basis. Figure 1 presents the degradation of the 1,1,1-trichloroethane over a period of 120 days, in the presence of *Pseudomonas putida* strain.

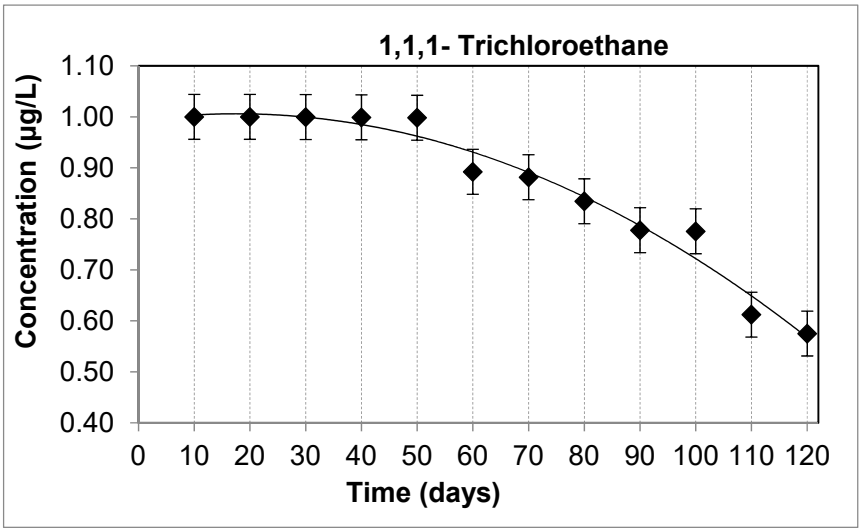

Figure 1. The graphic representation of temporal pattern of 1,1,1-trichloroethane degradation by *Pseudomonas putida* strain. Error bars indicate the ranges for triplicate samples.

The biodegradation of 1,1,1-trichloroethane was observed after 30 days, with a small decrease in concentration of only 0.0004 µg/L. The concentration of this compound decreased successively with values ranging from 0.0004 µg/L to 0.1077 µg/L, reaching a concentration of 0.8923 µg/L after the first 60 days. Between the 60 and 90 days, the 1,1,1-trichloroethane concentrations were between 0.88923 µg/L and 0.7777 µg/L, demonstrating the action of the *Pseudomonas putida* strain. Within 90 and 120 days period, the concentrations of 1,1,1-trichloroethane diminished by 23.00% to 42.49%, reaching a concentration of 0.5751 µg/L. After 120 days, the 1,1,1-trichloroethane compound has stopped the decrease in concentration.

Figure 2 reveals the degradation of the 1,1,1,2-tetrachloroethane over a period of 120 days, in the presence of *Pseudomonas* strain. As it can be seen, 1,1,1,2-tetrachloroethane undergoes a successive decomposition but the accommodation takes more than a month. Thus, after 60 days, the 1,1,1,2-tetrachloroethane concentrations were reduced with 23.7% by the action of *Pseudomonas putida* bacteria, reaching values of 0.9237 µg/L. The decrease concentration was more visible in the 60-90 days period, with values reaching 0.6572 µg/L. After 110 days, the concentration reached 0.5501 µg/L. After this period, the concentration of 1,1,1,2-tetrachloroethane remained constant, the bacteria losing its decomposition capacity.

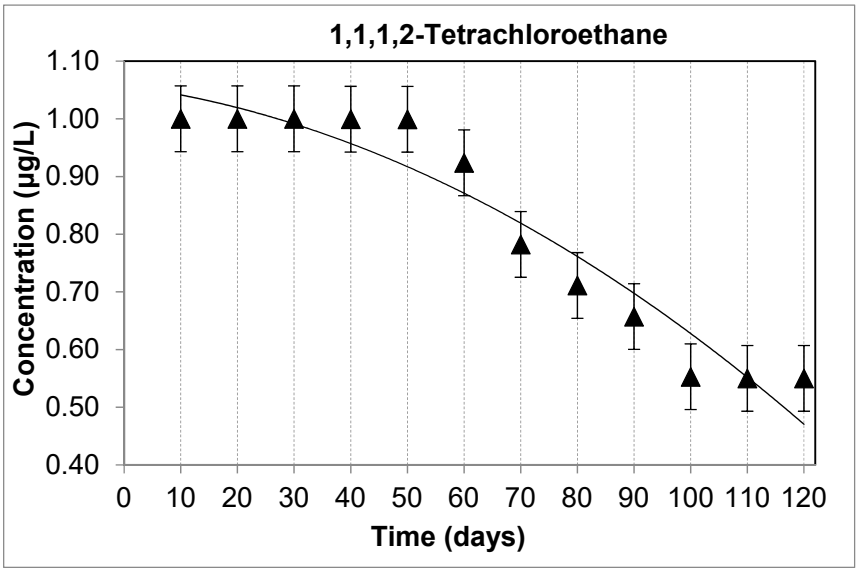

Figure 2. The graphic representation of temporal pattern of 1,1,1,2-tetrachloroethane degradation by *Pseudomonas putida* strain. Error bars indicate the ranges for triplicate samples.

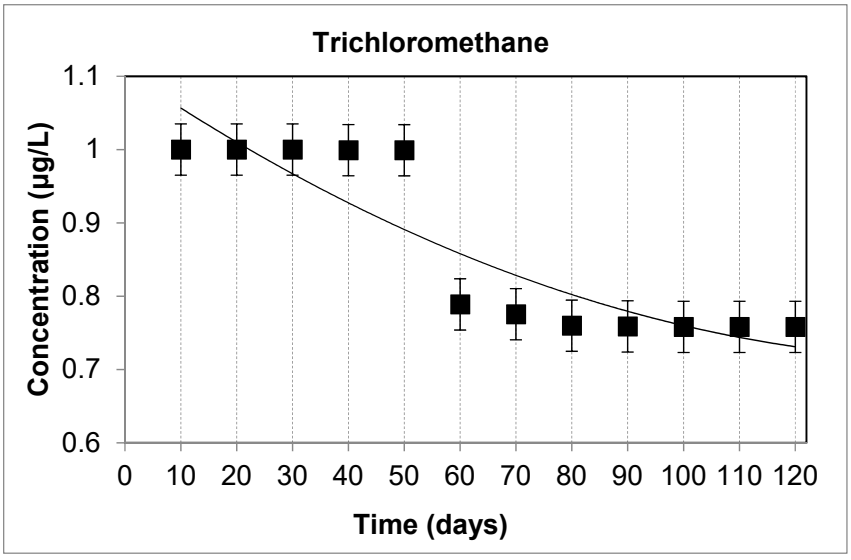

Figure 3. The graphic representation of temporal pattern of trichloromethanetime degradation by *Pseudomonas putida* strain. Error bars indicate the ranges for triplicate samples.

The other two chlorinated compounds (trichloromethane and tetrachloromethane) were also monitored, the results being displayed in Figure 3 and Figure 4. In the case of trichloromethane [30], it must be noted that the bacterial strain was accommodated with the higher contaminated environment after 40 days. Only after this period the first signs of a decrease in their concentrations appeared (0.9992 µg/L for trichloromethane and 0.9989 µg/L for tetrachloromethane). For trichloromethane, biodegradation is more significant in the 60-100 days range and the values of this compound concentration reached 0.7588 µg/L. Subsequent concentration determinations after 110 or 120 days did not show diminishes, the values remaining constant.

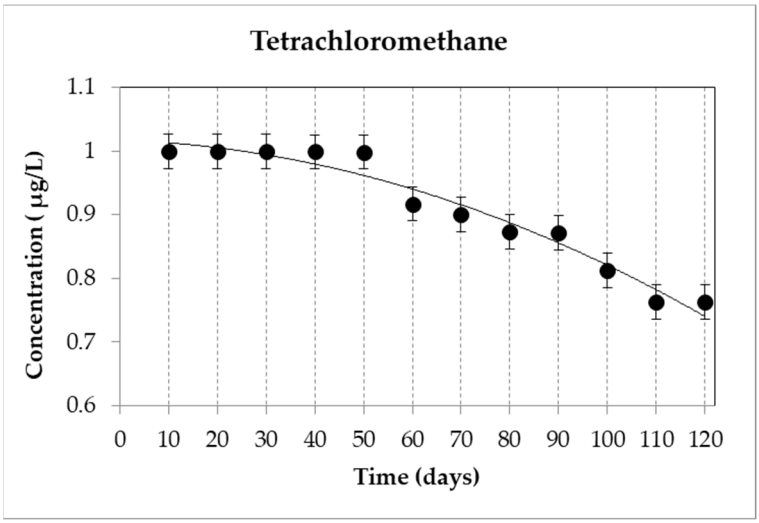

Figure 4. The graphic representation of temporal pattern of tetrachloromethane degradation by *Pseudomonas putida* strain. Error bars indicate the ranges for triplicate samples.

The biodegradation of tetrachloromethane led to the values of 0.8722 µg/L after 90 days and 0.7628 µg/L after 120 days. After 110 days there were no changes in the biodegradation of this compound in the presence of *Pseudomonas putida*.

Analysing all the results, it may be stated that, by far, the highest activity was measured for the degradation of the chlorinated ethane compounds, their concentrations being reduced at half of the initial values after the test period. Also, in their case, the accommodation of the *Pseudomonas putida* type strain was shorter than in the case of methane derivates.

An important aspect of the chlorinated compounds degradation is the formation of toxic oxidation products. For example, there were studies that reported the formation of acyl chlorides resulting from the hydroxylation of a

di-chlorinated carbon followed by elimination of one of the chlorines [31]. Acyl chlorides can act as protein modifying agents. However, during the assays used to determine the degradation rates of the different chlorinated hydrocarbons, we did not observe any decline in degradation rates. Apparently, the amounts of potentially toxic intermediates produced were too low to significantly inactivate the co-metabolic degradation.

Chlorinated aliphatics behave as unique source of carbon and energy to various bacteria. Chlorinated compounds are electron deficient, acting as electron acceptors and the energy is produced by a respiratory process in anaerobic media. In the best scenario, the chlorinated compound is degraded only if another substrate is present, due to the broad-specificity enzymes from the bacteria [32]. Future studies regarding the biodegradation of chlorinated compounds should target both basic and applied aspects of the bioremediation.

CONCLUSIONS

The use of bacteria capable of chlorinated compounds biodegradation ultimately yields convincing results under experimental conditions, and the bacterial sources facility could be an economic advantage. On the other hand, the multifactorial nature of the process (involving various environmental factor such as temperature, humidity, chemical composition of the water used as substrate) could create potential difficulties in applying the methods tested in the laboratory *in situ*. Also, irrational application of the methods in situ could lead to the accumulation of intermediary toxic compounds in microbial metabolism.

The capacity of the *Pseudomonas putida* strain to degrade 1,1,1-trichloroethane, 1,1,1,2-tetrachloroethane, trichloromethane and tetrachloromethane was reached at low concentrations, but it must be taken into consideration the variety of compounds present in polluted waters that may affect the viability of the bacterium. The biodegradation rate of the chlorinated ethane derivates was reduced to 23.72% after 60 day. The lowest biodegradation rate was for methane derivates with only 45% decrease in concentration, mainly between 60 and 100 day after inoculation with Pseudomonas putida strains.

EXPERIMENTAL SECTION

Sampling

Pseudomonas putida was isolated from a wastewater lagoon in South Carolina and deposited to the ATCC (*Pseudomonas putida* ATCC 31483) by Sybron Biochemical Corp. (Salem, VA, US) and it was activated in tryptic soy broth TSB culture media (Sigma Aldrich, Darmstadt, Germany) for 24 hours

at 30ºC. Water samples were prepared by contamination with a solution containing 1 µg/mL of 1,1,1-trichloroethane, 1,1,1,2-tetrachloroethane, trichloromethane and tetrachloromethane, using a standard mixture solution of 10 µg/mL from each compound (LGC Standards GmbH, Wesel, Germany).

Working protocol

1000 mL water were inoculated with 1 mL suspension containing 106 Pseudomonas putida cells. The cells number per mL was established by reading the suspension optical density at 660 nm with Cecil 1200 spectrophotometer (Cecil Instruments, Cambridge, UK).

The temperature was kept at 28°C and monitored for 120 days, the samples being incubated in a Memmert IPP 300 equipped with incubator Peltier system (Memmert GmbH + Co. KG, Schwabach, Germany). In order to preserve bacterial viability in water samples, $C_{12}H_{17}N_4OS^+$ (thiamine) (Sigma Aldrich, Darmstadt, Germany) was periodically introduced (once a week) at a concentration of 1 mg/L.

The identification and quantification analysis

The concentrations of 1,1,1-trichloroethane, 1,1,1,2-tetrachloroethane, trichloromethane, tetrachloromethane, before and after inoculation, were determined by a gas chromatographic method, according to ISO 10301:1997 standard, in order to establish the efficiency of the bacterial strain for the compounds degradation [33].

In order to achieve the compounds identification and quantification, the samples were prepared by taking 500 mL water which was extracted twice with 50 mL hexane (Sigma Aldrich, Darmstadt, Germany). The combined extracts were dried over sodium sulphate (Sigma Aldrich, Darmstadt, Germany) and concentrated to a volume of 2 mL with a TURBOVAP 500 concentrator (Biotage AB, Uppsala, Sweden).

The identification and quantification analysis was achieved using a 240 MS 450 GC Varian gas chromatograph coupled with a mass spectrometer (Varian, Inc., Palo Alto, CA, United States).

For the chromatographic Separation, a TG-WAXMS polar column (Thermo Fisher Scientific, Waltham, Massachusetts, United States) (dimensions: 60 m x 0.32 mm x 0.25 µm) was used. The oven temperature was maintained at 70 °C for 0.8 min, then it was increased to 110 °C, with a rate of 3°C/min, then to 140°C with 3°C/min and finally to 160°C by 1.2°C/min, where it was maintained for 2 min. Injection port and detector temperatures were maintained at 240°C and 270°C respectively, whereas the injection volume was 2 µL (splitless injection), the column being cleaned with 1.80 mL/min.

The calibration curves were designed by using 7 calibration levels: 0,01; 0,03; 0,05; 0,1; 0,3; 0,5; 0,8; 1 µg/mL from the 10 µg/mL stock standard mixture solution of 1,1,1-trichloroethane, 1,1,1,2-tetrachloroethane, trichloromethane and tetrachloromethane (LGC Standards GmbH, Wesel, Germany). Each compound was identified and quantified with a ratio of at least 3 m/z with a similarity of at least 70% compared to the spectra of similar compounds in the NIST 08 spectral library.

Analyses were conducted in triplicate and the results were expressed as average ± SD.

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