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STENOTROPHOMONAS MALTOPHILIA IMV B-7288 AS THE PROMISING DESTRUCTOR OF HEXACHLOROCYCLOHEXANE ISOMERS COMPLEX AT AEROBIC CONDITIONS

Aim *of the research was identification the "one promising" microorganism-destructor of organochlorine hexachlorocyclohexane isolated among microorganisms from places with total pesticide contamination, after studying its resistance and destruction.* ***Methods.*** *Laboratory selection of microorganisms was carried out by microbiological methods on agar plate. Identification of the isolates was realized over polyphase approach by API test method and sequence of the 16S rRNA gene fragment, followed by comparison of the results with the GenBank database using the BLASTN program. Research the ability to decompose the HCH-isomers complex (α, β, γ and δ) were studied in liquid media by gas chromatography.* ***Results.*** *On the basis of resistance to the insecticide hexachlorocyclohexane microbial isolate №6 was selected as the most promising strain and identified as Stenotrophomonas maltophilia IMV B-7288. The strain was tolerant to high (1000 mg/L) concentrations of insecticide growing on agar plate. In a liquid medium for 7 days of cultivation under aerobic conditions, the strain decomposed hexachlorocyclohexane isomers (α, β, γ and δ) by 61.6–82.1% of its initial content (20 mg/L).* ***Conclusions.*** *The selected strain of Stenotrophomonas maltophilia IMV B-7288 is an effective destructor of hexachlorocyclohexane isomers and its derivatives and can be promising for using in environmental friendly technologies.*

Key words: Stenotrophomonas maltophilia, hexaclorocyclohaxane, lindane, biodegradation, sequence of 16S rRNA.

Halogenated compounds are often considered to be relatively recalcitrant in many surface environments, such as soils, sediments, and groundwaters, due in part to their chemical stability and in other part to the lack of appropriate microbial activity for their degradation [14]. Moreover, insecticide gamma-hexachlorocyclohexane (γ-HCH or Lindane) can negatively influences the activities of microbial communities in impacted habitats [1].

Therefore, the necessity exists of finding indigenous soil microorgamisms resistant/ decomposing of chlorinated pesticides at different concentrations: low in agricultural applications, medium and high at wood treatment or spill sites. There are a sufficient number of reports about the destruction of a chlorinated cycloaliphatic compound (γ-HCH) under anaerobic conditions [8, 10]. But there are in Ukraine many areas with varying levels of pollution where it is not possible to create anaerobic conditions for microbial destruction of HCH-isomers [13]. In consequence of above it is necessary to research the autochthon (indigenous) aerobic soil microorganisms having natural resistance to HCH in heavily polluted areas, in order to obtain highly efficient destructors capable to decompose HCH-isomers and simultaneously to synthesize plant grows regulators for remediation/phytoremediation of polluted soil.

Materials and Methods

In our previous study we have isolated the natural steady to chloroorganic contaminations microbial association named Micros [15]. Micros association was selected from soil area with high organochlorines pollution level where lindane (γ-HCH) has been applied and stored over 40 years for agricultural and industrial purposes. The strain №6 resistant to the HCH-isomers (α-HCH, β-HCH, γ-HCH (lindane), δ-HCH) was isolated from this association and cultivated on liquid Menkina's mineral nutrition (MMN) medium (pH 7.2) (containing per liter: 4.0 g of glucose, 2.0 g of $NaNO_3$, 0.5 g of KCl, 200 mg of K_2HPO_4, 100 mg of $MgSO_4 \times 7H_2O$) [11]. Also nutrition medium contained chloroorganic thecnical waste with total HCH- concentration 20 mg/L as supporting selective factor. For microbial isolate cultivation at high concentration of HCH-isomers we also used peptone containing M17 medium (Oxoid, Hampshire, England) and pure analytical hexane solution of HCH-isomers (Alsi Ltd.) in concentration range from 100 to 1000 mg/L. The cultivation was performed at rotating conditions with 240 rpm/minute and 28 ± 0.1°C for 7 days. As a control has been used sterile nutrition medium with toxicant without microorganisms.

To identify the isolate №6, classical methods were used to study their physiological and biochemical characteristics. Cell morphology studies were carried out by microscopic examination of smears of daily cultures, stained according to Gramm's method. To determine the mobility of the researched microbial cells we studied the preparations of daily living cultures, which were cultivated on the nutrient medium M17. The oxidase and catalase activity of the strains was determined according to Kovacs [11].

The microbial isolate was identify using the API 20 NE system (BioMerieux, France), for non-fermenting Gram-negative rods.After 4 days of cultivation in the stationary phase of growth on MMN medium [11], colonies were recovered from the Petri plates suspended with a sufficient quantity of 0.85% (wt/vol) NaCl buffer to reach 10^9 bacteria cells per ml. This suspension was used to inoculate the API 20 NE strip (Biomerieux, Marcy l'Etoile, France) bacterial identification systems according to the manufacturer's recommendations.

The method of partial sequencing of the 16SrRNA gene were used.The isolation and purification of bacterial DNA was performed in the exponential

growth phase from 2–3 daily culture using the "Sorb-B DNA" kit according to the manufacturer's recommendations.

The amplification of 16S-rRNA gene fragments was performed using two universal primers: forward RNNF1 5′ -CGG-CCC-AGA-CTC-CTA-CGG-GAG-GCA-GCA-3 'and reverse RNNR2 5′ -GCG-TGG-ACT- ACC-AGG-GTA-TCT-AAT-CC – 3' by PCR reaction on the "2720 Thermal Cycler" amplifier.

PCR amplification was performed in a total volume of 50 µl, each reaction mixture containing: H_2O – 17 µl, mixture of dNTP – 5 µl/l, 1,0m Meach primer, 5 µl of TaqDNA polymerase (10 U/µl) (Gibco BRL, Cergy-Pontoise, France) in a buffer containing 10 mM Tris-HCl (pH 8.3), 125 mM KCl, 1.5 mM MgCl2, 0.5 µl and 7.5 µl bacterial DNA samples. The amplification temperature conditions were as follows: initial denaturation –5 min at 94 °C, the next 35 cycles – denaturation 30 sec at 94 °C, hybridization of primers 30 sec at 55 °C, polymerization 30 sec at 72 °C, final cooling to 4 °C. Analysis of PCR products was performed by electrophoresis for a period of 20 min on a 1% (wt/vol) agarose gel with ethidium bromide (1µl/ml), at a voltage of 10 V/cm. To determine the molecular mass (weight) and amount of DNA marker SM0403 (Fermentas Ltd.) was used.

The sequence was implemented according to the standard protocol using genetic analyzer "3130 Genetic Analyzer" with a set of sequence reagents "BigDye Terminator v 3.1 Cycle Sequencing Kit".

The analysis of obtained nucleotide sequence 476 n.p was performed using the BLASTN program, comparing them with the homologous nucleotide sequences of the 16S-rRNA gene detected in GenBank.

To study the ability to decompose the HCH, the isolated strain №6 have been cultivated on a MMN medium containing 20 mg/L (pure analytical substances) HCH isomer complex. Microorganisms have been cultivated in Erlenmeyer's flasks with rotating 240 rpm/h at 28 °C for 7 days. Microbial biomass was separated by centrifugation at 12000. The determination of HCH-isomers amounts was carried out in the microbial supernatants by gas chromatography according to the recommendations of the Environmental protection association (EPA) [12].

The analysis of HCH-isomers was performed applying an HP-5 column (length 30 m, internal diameter 0.32 mm, phase thickness 0.25 µm (HP cat. No. 19091J-413).

Destruction activity have been calculating in % for every HCH-isomer, according to initial content at nutrition medium.

Statistical analyses. The study was conducted in triplicates. Statistical analysis of the results were performed using MS Excel 2013. The percentage were calculated using data n=3 [3, 9].

Results and discussion

The HCH-resistant bacterium isolate №6 was found to form beige opaque flat colonies with clean edges on M17 medium agar Petri plates. It was gram-negative aerobic, non-fermentative bacterium; motile due to polar flagella, catalase-positive, oxidase-negative non-spore-forming rods, slightly smaller 0.5×2.0 and 0.4–0.6 µm in size with rounded ends.

According to the data used a special software and according to the API identification system (with ID 99.9%) the isolate №6 belongs to the species *Stenotrophomonas maltophilia.*

Since the polyphase method isused to determine the taxonomic position of microorganisms, based on the study of both physiological, biochemical and molecular genetic characteristics of investigated microorganism [6], we used molecular biological method for studying isolate №6. The nucleotide sequence of 16S rRNA gene fragment (476 bp) was determined. The results of the amplification with universal primers RNNF1 (direct) RNNR2 (reverse) are presented by electrophoresis data in 1.5% agarose gel of the obtained PCR products (Fig. 1).

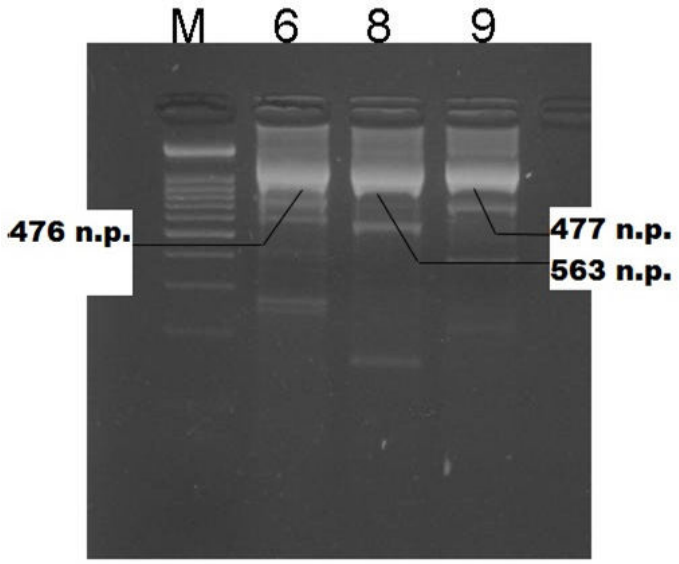

Fig. 1. PCR products16S rRNA : M – marker SM0403; 6* – S. maltophilia *6; 8* – S. maltophilia *8(the one experimental strain); 9* – P. putida *9

The amplifiers of the 16S rRNA gene were sequenced and their nucleotide sequences were obtained. Using the BLASTN program, in the GenBank database, homologous nucleotide sequences were compared.

The strain №6 have 99% homology of the nucleotide sequence with a fragment of the16SrRNA gene *S. maltophilia* 0450 (EU604758.1) and *S. maltophilia* LQB22 (GQ861457.1), which confirms belonging of isolate №6 to species *S. maltophilia*.

This microbial strain was included into Ukrainian collection of microorganisms and in depository as *Stenotrophomonas maltophilia* IMV B-7288.

The ability to destroy chloroorganic pollutions by fluorescent Pseudomonads was previously described [4], but there are a little data about similar properties of the *Stenotrophomonas* strains. It is known that *S. maltophilia* KB2 was used to metabolize broad range of aromatic compounds including phenol, some chloro- and methylphenols, benzoicacids, catechols, and others [5]. It is known that representa-

tives of *S. maltophilia* are found ubiquitously distributed in soil and often associated with roots of many plant species.

The result is very interesting for natural selected strain isolated from polluted area without any genomic manipulations. This was to be expected, because such a huge potential for biodegradation is consistent with the well-known literature on the high functional flexibility and ubiquity of *Stenotrophomonas*.

S. maltophilia IMV B-7288 demonstrates the strong ability to grow at presence high=concentration of four HCH-isomers (hexachlorocyclohexane) (Fig. 2).

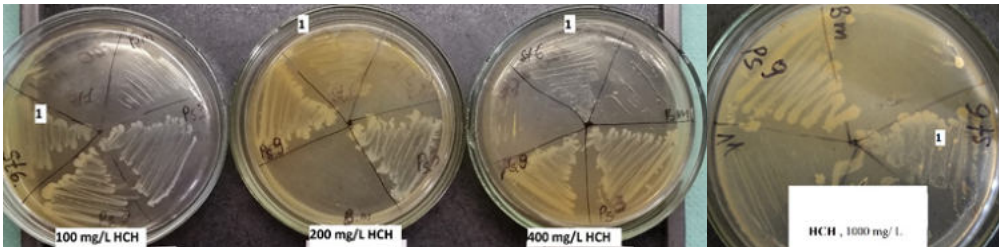

Fig. 2. The microbial growth on Petri plates (M17 nutrition medium) with concentration 100, 200, 400 and 1000 mg\L of HCH-isomers complex: 1- *S. maltophilia* IMV B-7288

The HCH-isomers degradation activity of *S. maltophilia* IMV B-7288 have been revealed under laboratory conditions (Fig. 3). We can see that *S. maltophilia* IMV B-7288 demonstrates the strong ability to decompose HCH-isomers complex. During 7 days the decomposition level of α-isomer of HCH was 73.4%, β-HCH - 61.6%, γ-HCH - 82.1%, δ-HCH - 74.5% of the initial content. The highest stability to microbial degradation had demonstrated β-HCH having the most symmetric molecule [2].

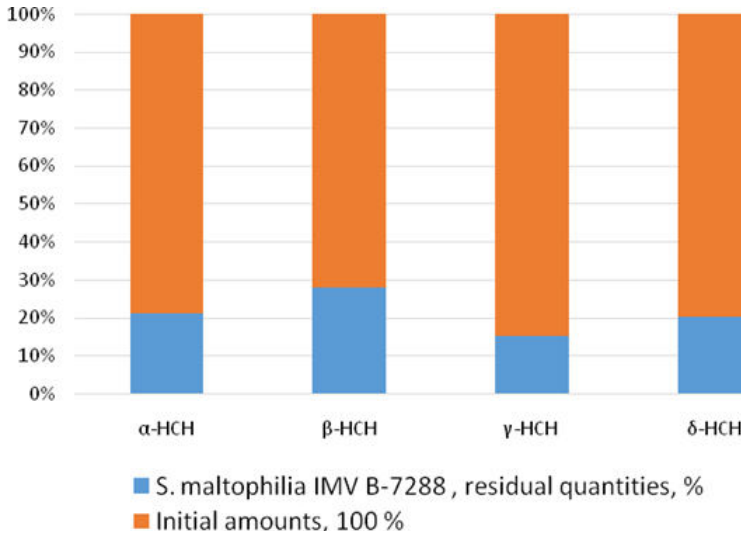

Fig. 3. Destruction of HCH-isomers by *Stenotrophomonas maltophilia* IMV B-7288, %from initial content

Notes: the percentage were calculated using data n=3

The great genetic and metabolic diversity within *S. maltophilia* makes it a "Wonder-bug" in the environment [7].

Conclusion. Our experimental data indicate that the selected strain S. maltophilia IMV B-7288 is a promising destructor of organochlorine pollutions and their derivates and can be promising for using in environmental friendly technologies. In a liquid medium for 7 days of cultivation under aerobic conditions, the strain decomposed hexachlorocyclohexane isomers (α, β, γ and δ) by 61.6–82.1% of its initial content (20 mg/L).

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STENOTROPHOMONAS MALTOPHILIA ІМВ В-7288 ЯК ПЕРСПЕКТИВНИЙ ДЕСТРУКТОР КОМПЛЕКСУ ІЗОМЕРІВ ГЕКСАХЛОРЦИКЛОГЕКСАНУ В АЕРОБНИХ УМОВАХ

Реферат

Метою *дослідження був пошук та ідентифікація перспективного мікроорганізму-деструктора хлорорганічного гексахлорциклогексану серед мікробних ізолятів, виділених із місць тотального пестицидного забруднення.* ***Методи.*** *Лабораторну селекцію мікроорганізмів та дослідження здатності розкладати комплекс ізомерів гексахлорциклогексану (α, β, γ і δ) проводили мікробіологічними методами на твердих і рідких живильних середовищах з визначенням кількості розкладеного пестициду методом газової хроматографії, ідентифікацію відібраного ізоляту проводили із застосуванням поліфазного підходу методом API тестування та за допомогою сиквенсу фрагменту гену 16Sp РНК, з подальшим порівнянням одержаних результатів з базою даних GenBank за допомогою програми BLASTN.* ***Результати.*** *За ознакою стійкості до інсектициду гексахлорциклогексану виділено ізолят №6, ідентифікований як Stenotrophomonas maltophilia IMB B-7288, який на агаризованому середовищі проявляв стійкість до високої (1000 мг/л) концентрації пестициду. У рідкому середовищі за 7 діб культивування в аеробних умовах штам розкладав ізомери гексахлорциклогексану (α, β, γ і δ) на 61,6-82,1% від його вихідного вмісту (20 мг/л).* ***Висновки.*** *Селекціонований штам Stenotrophomonas smaltophilia IMB B-7288 є ефективним деструктором ізомерів гексахлорциклогексану та його похідних і перспективним до застосування у природоохоронних технологіях.*

Ключові слова: Stenotrophomonas maltophilia, гексахлорциклогексан, ліндан, біодеградація, сиквенс16S рРНК-аналіз.

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STENOTROPHOMONAS MALTOPHILIA IMV B-7288 КАК ПЕРСПЕКТИВНЫЙ ДЕСТРУКТОР КОМПЛЕКСА ИЗОМЕРОВ ГЕКСАХЛОРЦИКЛОГЕКСАНА В АЭРОБНЫХ УСЛОВИЯХ

Реферат

Целью *исследования был поиск и идентификация перспективного микроорганизма-деструктора хлорорганического инсектицида гексахлорциклогексана (ГХЦГ) среди микробных изолятов, выделенных из мест тотального загрязнения пестицидами.* ***Методы.*** *Лабораторную селекцию микроорганизмов и исследование способности разлагать комплекс изомеров ГХЦГ (α, β, γ и δ) проводили микробиологическими методами на твердых и жидких питательных средах с определением количества разложенного пестицида методом газовой хроматографии, идентификацию отобранного изолята проводили с применением полифазного анализа методом АРЕ тестирования и с последующим сиквенсом фрагмента гена 16Sp РНК, с дальнейшим сравнением полученных результатов с базой данных GenBank с помощью программы BLASTN.* ***Результаты.*** *По признаку устойчивости к инсектициду ГХЦГ выделен изолят №6, идентифицированный как Stenotrophomona smaltophilia ИМВ В-7288, который в агаризованной среде проявлял устойчивость к высокой (1000 мг/л) концентрации пестицида. В жидкой среде за 7 суток культивирования в аэробных условиях штамм разлагал изомеры ГХЦГ (α, β, γ и δ) на 61,6-82,1% от исходного содержания (20 мг/л).* ***Выводы.*** *Селекционированный штамм Stenotrophomonas maltophilia ИМВ-7288 является эффективным деструктором изомеров гексахлорциклогексана и его производных и перспективен для использования в природоохранных технологиях.*

Ключевые слова: Stenotrophomonas maltophilia, гексахлорциклогексан, линдан, биодеградация, сиквенс16S рРНК-анализ

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