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Possible Processes and Mechanisms of Hexachlorobenzene Decomposition by the Selected *Comamonas testosteroni* Bacterial Strains

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Abstract: ***Background***: The bacterial destructing activity toward pesticides has been the focus of research in the last few decades. Hexachlorobenzene is included in the organochlorine pesticides group that are prohibited for use. However, large hexachlorobenzene amounts are still concentrated in the soil, stressing the relevance of research on hexachlorobenzene-destroying bacteria. ***Methods***: The ability to destroy hexachlorobenzene by *Comamonas testosteroni* UCM B-400, B-401, B-213 strains was investigated and established. Bacteria were cultivated (7 days at 28 °C) in mineral Luria-Bertrani (LB) medium with three hexachlorobenzene doses: 10, 20, 50 mg/L. The hexachlorobenzene concentrations were recorded by a gas chromatography method. ***Results***: The results showed that *C. testosteroni* UCM B-400, B-401 have high destructive activity toward hexachlorobenzene. The highest (50 mg/L) initial concentration decreased to 41.5 and 43.8%, respectively, for *C. testosteroni* UCM B-400, B-401. The unadapted *C. testosteroni* UCM B-213 was tolerant to hexachlorobenzene (cell titers after cultivating with 10.0, 20.0, 50.0 mg/mL were higher compared to initial titer), but had a low-destructing activity level (two times less than B-400 and B-401). ***Conclusions***: Bacterial strains *C. testosteroni* UCM B-400, B-401 can be seen as a potential soil bioremediation from hexachlorobenzene pollution.

Keywords: hexachlorobenzene; toxicity; microbial metabolism; destroying activity; bacterial decomposition; destruction potential

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1. Introduction

The last century is defined as a period of active use of pesticides against pests to increase agricultural crop yield. Organochlorines are a group of pesticides accumulating in the environment as a result of their widespread use in previous years, starting from the 40s to the end of the 20th century [1,2]. Due to the particular safety hazards, these pesticides were prohibited for use by Stockholm Convention [3]. Organochlorine pesticides are the most persistent in the environment containing five or more chlorine atoms per molecule, so they have a long half-life. Depending on the half-life, pesticides are assigned different levels of persistence in soil, ranging from low persistence (half-life < 30 days) to very high (half-life > 100 days) [4,5].

According to the EPA (Environmental Protection Association) classification, many organochlorine pesticides, including hexachlorodimethanonaphthalene (aldrin or HCDN), hexachloro-octachlorohexahydromethanoindene (chlordane), p,p-dichloro-diphenyltrichlor oethane (DDT), hexachlorobenzene (HCB), hexachlorocyclohexane (HCCH), heptachlor (tetrohydromethane heptachloroindene) are classified as persistent bioaccumulative and toxic (PBT) chemicals that persist in the environment and bioaccumulate in food chains, and

thus, pose a risk to human health and ecosystems [4]. These pesticides tend to bind strongly to soil particles and can remain on the soil surface for months to years. Hexachlorobenzene is one of the widely used organochlorine pesticides in the past, due to fungicidal activity [6].

HCB is formed as a byproduct of chemical processes due to incomplete combustion, and is also used as an additive in pesticide preparations for agriculture. Thus, large HCB amounts are still concentrated in the ecosystem [7,8]. Currently, HCB is banned in the USA and Europe due to its proven negative impact on human health and the environment [9,10]. Thus, HCB was detected in soils and animal remains from the most remote Arctic and Antarctic regions, namely in the tissues of polar bears, penguins, foxes, etc., [11–14]. Getting into the human body, orhanochlorine pesticides (OCP) can cause the disease series. Hexachlorobenzene is able to cause cysts on the hands, itching, psoriasis, eczema, leukoderma and skin rashes in humans [15]. The occurrence of liver and kidney cancer and immune and reproductive systems diseases in the human body were also reported [16]. The OCP, including HCB, have been identified in the breast milk of women in Mexico [17].

The prenatal exposure to DDT, β-BHC, and HCB leads to a decrease in the infant's body weight, i.e., OCPs are able to overcome the blood–brain barrier shown by a study conducted in China [18]. It has been reported that β-HCG, HCB and DDT residues can accumulate in maternal and umbilical cord serum, and from maternal blood can be transferred through the placenta and affect the thyroid hormone level in newborns [19].

Despite the ban on HCB production in many countries, it is a by-product or intermediate in the manufacturing process of some pesticides [10]. A significant HCB amount is concentrated in soils [20] and due to evaporation from them enters the atmosphere [14].

Organochlorine pesticides influencing studies on the microbial community structure make it possible to note that microbial communities have a unique reaction to various toxic compounds [1,21–26]. OCP were reported to cause a quantity decrease in bacteria, micromycetes, actinomycetes, and are also capable of inhibiting N_2 fixation by soil microorganisms [27–29].

Hexachlorobenzene is a persistent organic pollutant; nevertheless, in the last two decades, there have been published research reports about microbial HCB biodegradation (in anaerobic conditions) as a mechanism to reduce HCB toxicity [30,31]. For example, *Dehalococcoides* sp. CBDB1 [32] was able to reduce HCB under anaerobic conditions by dechlorination to less chlorinated benzenes such as 1,3,5-trichlorobenzene and 1,2-, 1,3- and 1,4-dichlorobenzene. However, these products can not be decomposed further and, in addition, they can lead to serious secondary pollution of the environment. Hexachlorobenzene limits are also regulated in the regulation of the European Union (Regulation (EU) 2019/1021 of the European Parliament and of the Council of 20 June 2019) [33]. Studying the microbial ability to decompose toxic compounds is also necessary. Aerobic degradation of pollutants is characteristic of the surface layers in the soil, into which chlorobenzenes and other toxic compounds enter with groundwater. In this regard, the results of a comparative study of the effectiveness of trichlorobenzene decomposition in aerobic and anaerobic conditions have been published [34]. Microbial degradation of HCB in soils was studied mainly under anaerobic conditions [6,35]. HCB-degrading aerobes obtained from natural sources are still needed for HCB-contaminated areas remediation. According to the literature, HCB is decomposed by microorganisms under aerobic conditions due to the formation of pentachlorophenol [36]. The ability of Comamonas to decompose pentachlorophenol was previously reported. Therefore, it is important that HCB destruction is studied under aerobic conditions. There are particular data concerning *Nocardioides* sp. PD653's ability to mineralize HCB [37]. The microbial metabolism of HCB under aerobic conditions is insufficiently described in the literature. The search for strains to be able to destroy HCB in aerobic conditions is promising, and consequently, the aim of the study was to determine the possible ability of *Comamonas testosteroni* bacterial strains to degrade hexachlorobenzene.

2. Materials and Methods

The study of the ability to decompose hexachlorobenzene in *C. testosteroni* UCM B-400 and B-401, B-213 was carried out. *C. testosteroni* UCM B-400 and B-401 were isolated from Kalush's organochlorine pesticides landfill (Ivano-Frankivsk region, Ukraine). In previous studies, these strains are established to be resistant to high HCB doses (50–100 mg/L) [38]. *C. testosteroni* UCM B-213 from the Ukrainian Collection of Microorganisms, which was not adapted to high HCB doses, was included in the experiment as the comparison. The laboratory experiment was conducted according to Table 1.

Table 1. Cultivation conditions of Comamonas testosteroni strains in a mineral medium LB with the HCB addition.

Conditions	Cultivations	Acetone Volume (mL)	Hexachlorobenzene (mg/L)
(a) control	cultivating the strain in LB medium contained acetone volume required to dissolve	no	Pure control
(b) control (10)		20	10
(c) control (20)		40	20
(d) control (50)		100	50
(e) cultivating	in LB medium contained	20	10
(f) cultivating		40	20
(g) cultivating		100	50

Cultivation of bacteria was carried out by the deep method in a liquid-mineral medium LB by the following composition in g/L: $(NH_4)_2SO_4$—5; KH_2PO_4—2.93; K_2HPO_4—5.87; $MgSO_4 \times 7H_2O$—0.3; NaCl—2; $CaCl_2$—0.01; $FeSO_4$—0.01 [39]. Sodium succinate (4 g/L), as a carbon source for *C. testosteroni*, was recommended [40]. Cultivation conditions: shaking (121 rpm), 7 days at 28 °C. The ability to grow *C. testosteroni* UCM B-400, B-401, B-213 in a mineral medium containing HCB was studied by cultivating the noted strains in a liquid LB medium, and three HCB concentrations were added: 10, 20, 50 mg/L. The initial titer was 1×10^6 CFU/mL. The destruction level was determined by the residual HCB concentration in the culture liquid after cultivation, the cell biomass from the culture liquid was separated by centrifugation (5000 rpm, 30 min).

Chemical analysis of the hexachlorobenzene content in the samples was performed by gas chromatography (Agilent 6890 N chromatograph in combination with HP Chemstation software (Santa Clara, CA, USA), version 4.03.016). The experiment used hexachlorobenzene (0.100 mg/mL in hexane) from the analytical standard DSZU 042.32-97.

Statistical analysis of the data was performed by GraphPad Prism 8.0.1 software using one-way ANOVA to determine reliable differences between mean values of samplings; the post-test comparison was made using Tukey's multiple comparisons test with alpha = 0.05. All values were shown as Mean ± SD (standard deviation).

3. Results

The bacterial strains of *C. testosteroni* UCM B-400, B-401, B-213 titers were the highest in samples with pure cultures of the strains since they were cultivated in the medium without the addition of solvent and HCB. After the cultivation completion, the initial titer was 1×10^6 CFU/mL (Table 2). Compared to the pure culture control under cultivation conditions with 10 mg/L HCB, the *C. testosteroni* B-400 and B-401 cells titer decreased to 1 magnitude order, with 20 mg/L HCB to 2 magnitude orders. Relative to the initial titer (1×10^6 CFU/mL), an increase was noted. The *C. testosteroni* B-213 cells titer also increased in all cultivation options, but under 50 mg/L HCB presence, its indicator was lowered to 2 magnitude orders compared to the pure culture control. Thus, according to the cell titer, tolerance to HCB of all studied strains was noted. We can assume the probability to use this pesticide as a source of carbon.

Table 2. *C. testosteroni* cells titer after adding HCB to the culture medium (CFU/mL).

Variants	*C. testosteroni* Strains		
	UCM B-400	**UCM B-401**	**UCM B-213**
Control	$1.50 \pm 0.30 \times 10^{9\ a}$	$1.77 \pm 0.21 \times 10^{9\ a}$	$9.03 \pm 0.35 \times 10^{8\ a}$
Control (10)	$7.67 \pm 1.50 \times 10^{8\ ab}$	$1.00 \pm 0.10 \times 10^{9\ a}$	$7.93 \pm 0.15 \times 10^{8\ ab}$
Control (20)	$4.53 \pm 0.59 \times 10^{8\ ab}$	$4.93 \pm 0.61 \times 10^{8\ ab}$	$3.80 \pm 0.25 \times 10^{8\ b}$
Control (50)	$2.67 \pm 0.35 \times 10^{8\ b}$	$2.70 \pm 0.36 \times 10^{8\ b}$	$8.40 \pm 0.30 \times 10^{7\ bc}$
10 mg/L	$1.23 \pm 0.32 \times 10^{8\ b}$	$1.70 \pm 0.20 \times 10^{8\ b}$	$6.87 \pm 0.35 \times 10^{7\ bc}$
20 mg/L	$7.43 \pm 0.45 \times 10^{7\ bc}$	$7.43 \pm 0.36 \times 10^{7\ bc}$	$4.07 \pm 0.47 \times 10^{7\ bc}$
50 mg/L	$1.73 \pm 0.25 \times 10^{7\ c}$	$1.80 \pm 0.30 \times 10^{7\ c}$	$1.10 \pm 0.33 \times 10^{7\ c}$

Note: various letters of upper indices a, b, c in Table indicate values that significantly ($p < 0.05$) differ one from another within one column, which indicates growing activity of studied strains as a result of comparison using the Tukey's test ($p < 0.05$). M ± the standard deviation (SD).

As a research result, the *C. testosteroni* UCM B-400, B-401, B-213 abilities to destruct HCB were revealed, as evidenced by the residual concentrations of this pesticide in the culture liquid. After the cultivation for 7 days in LB medium containing 10, 20, 50 mg/L HCB concentrations, the residual HCB concentration in the culture liquid for the first two strains was significantly ($p < 0.05$) lower (Figure 1). Thus, as a result of the metabolic activity of *C. testosteroni* UCM B-400 in the culture liquid with initial concentrations of 10, 20, 50 mg/L HCB, the decrease was monitored, respectively, by 70%, 64% and 59%.

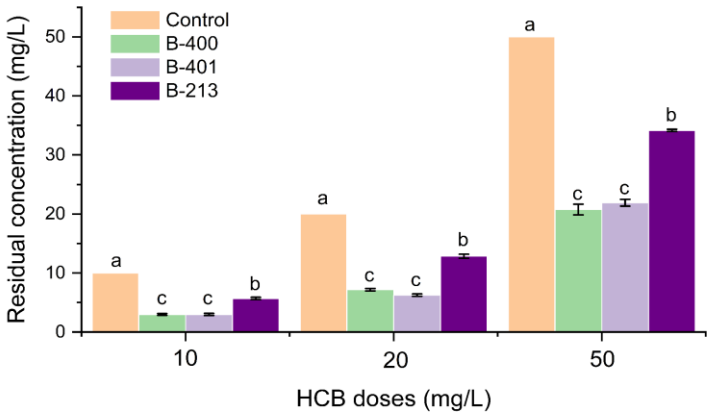

Figure 1. HCB destruction by *C. testosteroni* bacterial strains: UCM B-400, B-401, B-213. M ± the standard deviation (SD), a, b, c indicated in the graph means the values that significantly differ one from another within one column, which indicates growing activity of studied strains as a result of comparison using the Tukey's test ($p < 0.05$).

Approximately, the same HCB destruction level was established for *C. testosteroni* UCM B-401; however, at 20 mg/L HCB the initial concentration decreased by 69%, which was the highest HCB destruction level at the noted dose. The collected strain of *C. testosteroni* UCM B-213 showed much lower activity towards the HCB destruction. Under 10, 20, 50 mg/L HCB in the medium LB, the concentrations in the culture liquid decreased by 43, 36, 32%, respectively.

The confirmation of the study is also achieved by the principal component analysis (PCA) since, from Figure 2, is possible to see that, according to the eigenvalue, two groups are formed: group number 1, consisting out of B-400 and B-401 strains and group number 2, consisting out of B-213 strain. These statistical analysis results are in accordance with the obtained results since no statistically significant ($p < 0.05$) differences were obtained between B-400 and B-401 strains.

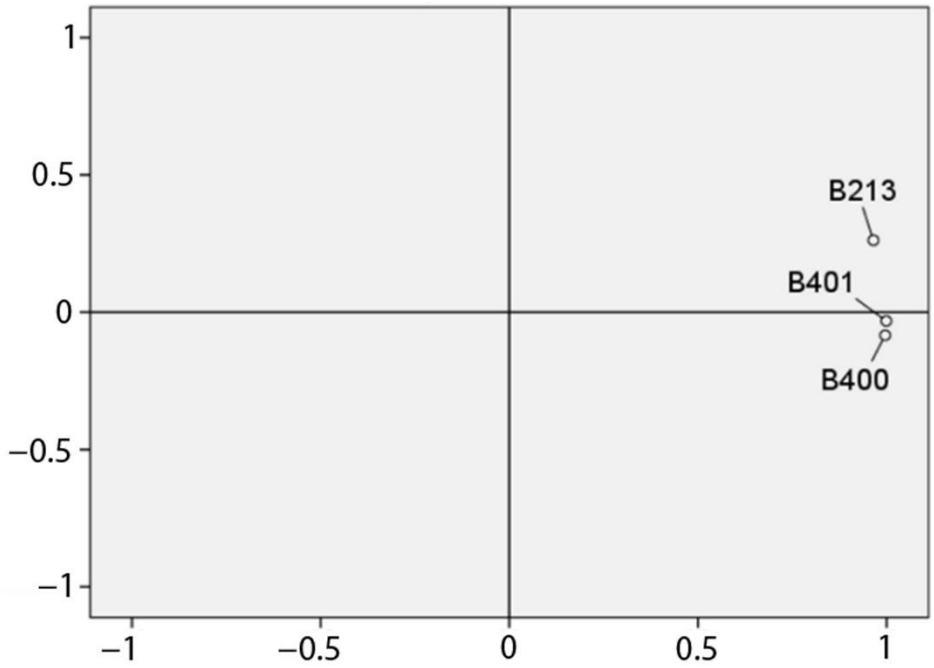

Figure 2. Principal component analysis of *C. testosteroni* cells titer after adding HCB to the culture medium (CFU/mL).

Thus, the ability of the researched *C. testosteroni* strains to destruct HCB was experimentally confirmed, while the destructing activities of B-400 and B-401 were higher compared to the collection strain B-213.

4. Discussion

In the literature, there is no data about *Comamonas* bacteria's capacity to destroy HCB. The study results showed that the strains are able to grow in the medium-contained HCB. This fact gives reason to believe that the studied strains are capable of using HCB as a carbon source. *Nocardioides* sp. PD653 bacteria are reported to have a HCB-destructing ability [37]. During aerobic cultivating of *Nocardioides* sp. PD 653 in a mineral medium-containing HCB with an initial 8 μM HCB content, after 9 days its concentration decreased to 1.5 M and the chloride ions accumulation was observed up to 34 M.

It should be noted that this is a very high HCB concentration, while the destruction was higher than 80%. In our study, we used much lower doses (less than 1 M), but also *C. testosteroni* B-400 and B-401 demonstrated a highly destructive activity. A possible (hypothetical) scheme (done according to the present literature and obtained results) of the hexachlorobenzene decomposition by *Comamonas testosteroni* bacterial strains is shown in Figure 3.

Moreover, the ability to destroy HCB in aerobic conditions by a microbial group isolated from a polluted ecotope was revealed, where *Azospirillum* and *Alcaligenes* were dominant bacteria. The HCB degradation efficiency occurred in the following pH order: 7.0 > pH 8.0 > pH 9.0 > pH 6.0. Degradation efficiency was influenced by the incubation temperature that varied in the following order: 37 °C > 30 °C > 20 °C > 4 °C. Depending on the initial substrate concentrations, the degradation efficiency was 55%, 51%, 51% at initial concentrations: 5 mg/L, 10 mg/L, and 25 mg/L, respectively [41]. In our study, the initial concentrations were 10 mg/L, 20 mg/L, 50 mg/L, and the corresponding degradation efficiency was 70.2%, 64.0–69.4%, 56.2–58.5% for *C. testosteroni* B-400 and B-401, isolated from HCB-polluted soil. The ability to utilize HCB in aerobic conditions by *Methylobacterium* and *Pseudomonas bacteria*, as the only source of carbon, was shown by adding HCB with labeled carbon (13C) to the culture medium. The confirmation of metabolic activity was the detection of bacterial DNA with 13C [42]. In addition to the study of the ability to degrade HCB by strains isolated from natural ecosystems, genetic engineering methods

are also used to create bacterial mutants potentially capable of destroying HCB by introducing a gene encoding the heme monooxygenase CYP101 (cytochrome P450cam) from *P. putida*, which is able to oxidize HCB to PCP [43,44]. Using this approach, the mutant F87W/Y96F/L244A/V247L was created, which is capable of oxidizing pentachlorobenzene and hexachlorobenzene to pentachlorophenol, which is decomposed by microorganisms. The authors also recommended the F87W/Y96F/L244A/V247L mutant for use in the bioremediation of polychlorinated benzenes [44].

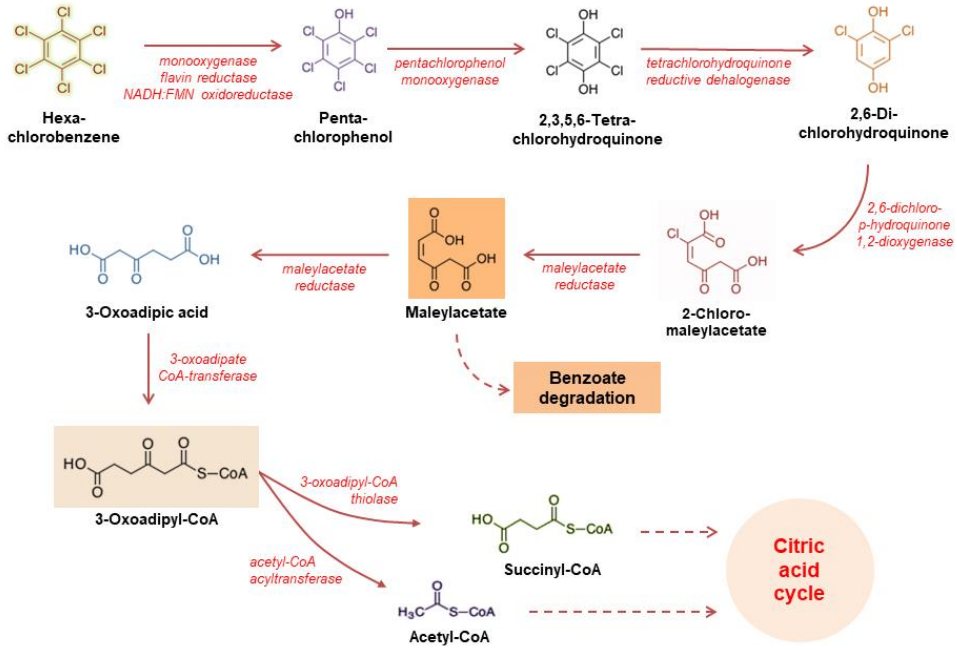

Figure 3. A hypothetical scheme of the hexachlorobenzene decomposition by *Comamonas testosteroni* bacterial strains (it was generated according to the following literature data [37,45]).

According to the literature, *Comamonas* bacteria are known to have degrading ability to some chlorobenzene compounds [46–48], such as 4-chloronitrobenzene [49]. The product of the partially reductive biodegradation pathway of 4-CHNB is 5-chloro-4-hydroxy-2-oxoacetate, which participates in the tricarboxylic acid cycle or other metabolic processes [50].

Nocardioides sp. PD653 was the first strain reported in the literature capable of degrading HCB under aerobic conditions through the intermediates pentachlorophenol, 2,3,5,6-tetrachloro-p-hydroquinone and 2,6-dichloro-p-hydroquinone [37]. *C. testosteroni* and other representatives of *Betaproteobacteria* are considered microorganisms with a high destructive potential to xenobiotics. There are reports in the literature about the ability of *Alcaligenes* sp. strain K to mineralize pentachlorophenol. *Alcaligenes* sp. and *Comamonas* sp. belong to the same order of *Burkholderiales*, class *Betaproteobacteria* [51]. Therefore, it is expected that *C.testosteroni* bacteria are capable of HCB degradation [52]. Regarding the literature, *C. testosteroni* strains are known to decompose a wide range of aromatic compounds, but the pathways of metabolism are insufficiently studied (see Figure 3).

C. testosteroni is known to be able to decompose pentachlorophenol, but the metabolic pathway has not been investigated [53]. However, in the literature are data about intermediates of pentachlorophenol metabolized by other Gram-negative bacteria, namely, *Escherichia coli* PCP1 and *Acinetobacter* sp. PCP3 (intermediates: 2,3,5,6-tetrachlorohydroquinone (TCHQ) and 2-chloro-1,4-benzenediol (DCBE)). Pentachlorophenol metabolized by Gram-negative bacteria *Sphingobium chlorophenolicum* ATCC 39723 was studied in detail, the end decomposition products are acetyl-CoA and succinyl-CoA, which are substrates of the citric acid cycle [45]. The complete 4-chlorophenol decomposition mechanism by *C. testosteroni* bacteria is known [54], where one of the intermediate products is maleylacetate (one of the benzoate degradation intermediates), which is common to the complete pentachlorophenol

decomposition mechanism. An interesting fact, *C. testosteroni* is characterized by the uniqueness of benzene acid (model aromatic compound) metabolism. Metabolizing benzoate by *C. testosteroni* bacteria is carried out by a mechanism peculiar only to this species that is hydroxylation with the m-oxybenzoate formation, which then passes through the stage of protocatechuic acid formation. In the end, two molecules of pyruvate and formate are formed to be substrates for the Krebs cycle enzymes [55–57].

According to our obtained results and the literature data analysis, it should be noted that *Comamonas testosteroni* have a high destruction potential to a wide toxic compounds range. At the same time, they are characterized by individual metabolism features.

5. Conclusions

The ability of *C. testosteroni* UCM B-400, B-401, B-213 to decompose hexachlorobenzene was investigated and their destructive activity was confirmed biochemically. It has to be emphasized that this kind of study was conducted for the first time, according to the present literature. Hypothetically, the HCB metabolism pathway by *C. testosteroni* UCM B-400, B-401, B-213 strains was explained. The initial concentration from 50 mg/L HCB decreased up to two times due to metabolism by strains UCM B-400, B-401. The destructive capacity of the strains isolated from contaminated soils was higher compared to the non-adapted collection strain. *C. testosteroni* UCM B-400 UCM B-401, at the initial 20 mg/L HCB concentration, decomposes it by 64 and 69%, respectively. These strains are promising for further studying the possibility of its use in bioremediation measures.

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Conflicts of Interest: The authors declare no conflict of interest.

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