XENOBIOTIC BIOTRANSFORMATION POTENTIAL OF *PSEUDOMONAS RHODESIAE* KCM-R_5 AND *BACILLUS SUBTILIS* KCM-RG_5, TOLERANT TO HEAVY METALS AND PHENOL DERIVATIVES

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ABSTRACT

Two environmental bacterial isolates KCM-R_5 and KCM-RG_5 were selected from xenobiotic-polluted environment. KCM-R_5 was identified as Pseudomonas rhodesiae and KCM-RG_5 as Bacillus subtilis. KCM-R_5 demonstrated tolerance to heavy metals and KCM-RG_5 – to heavy metals and phenol derivatives. Both strains were studied for xenobiotic biotransformation in order to contribute towards bioremediation of polluted environments. Pseudomonas rhodesiae KCM-R_5 and Bacillus subtilis. KCM-RG_5 possess unusual ability to utilize ortho-nitrophenol (o-NP) and 2,4- dichlorophenoxyacetic acid (2,4-D). o-NP and 2,4-D were added at concentration 30 mg/l. The possible indictive/inhibiting effect of Pb cations (40mg/l) was also studied. Pseudomonas rhodesiae KCM-R_5 removed 86 % of o-NP and below 1% of 2,4-D. Bacillus subtilis KCM-RG_5 eliminated 83% of o-NP and under 1% of 2,4-D. Biotransformation effectiveness of o-NP reached 95-100% in contrast to 2,4-D where the effectiveness was just 15-20%. Cell morphological changes were registered during the biotransformation processes. The obtained results could contribute to manage bioremediation processes in polluted with heavy metals and phenol derivatives environments.

Introduction

During last decades enormous amount industrial pollutants was released in the environment. The remediation technologies using bacteria appeared to be one of the most effective and environment-friendly techniques. Many investigations on bacteria converting metals (1,2) and phenol derivatives (5, 6, 7, 8, 12) were performed. But little is known about the bacterial activity in the environments, simultaneously contaminated with above mentioned pollutants. Possible inductive/inhibiting effect of metal cations on the biotransformation of aromatic compounds were reported in limited number of publications (3, 9). The investigation of bacteria isolated from polycontaminated environments is of special interest. Such autochthonic bacteria showed to be effective in the bioindication of environment contamination, biotransformation of xenobiotics as well as in the optimisation the bioremediation processes.

Present paper describes biotransformation abilities to nitro-and chlorophenols by two bacterial isolates collected in the region of the Pb-Zn metallurgical factory-"KCM" and the factory for producing of pesticides "AGRIA", Bulgaria. Additionally was investigated the probable inductive/inhibiting effect of Pb cations on the biotransformation processes. The study was performed in order to contribute, using above mentioned bacterial strains, for

bioremiation of similar environmental pollutants.

Materials and Methods

Microorganisms

KCM-R_5 was isolated from metal-contaminated industrial soil and KCM-RG_5- from a polluted groundwater, collected near the KCM and AGRIA factories. Both isolates were characterized using general and molecular microbiology methods (not published data). Isolate KCM-R_5 was affiliated to *Pseudomonas rhodesie* and isolate KCM-RG_5- to *Bacillus subtilis.* Isolation procedure was performed on selective media according to standard method (4). Both strains were maintained at 4 ºC and stored at -20 ºC.

2,4-D and *o*-nitrophenol biotransformation

Two aromatic compounds: 2,4-dichlorophenoxiacetic acid (2,4-D) and *o*-nitrophenol (*o*-NP) were studied for their biotransformation by *Pseudomonas rhodesiae* KCM-R_5 and *Bacillus subtilis* KCM-RG_5. Strains were grown for 144h/28 ºC/220 rpm on 2,4-D and *o*-NP (30 mg/l) as a sole carbon and energy source. The biomass accumulation, residual amount of xenobiotics and cell morphology were studied at initial time, 24h, 48h, 72h and 96h.

The effectiveness of biotransformation (Eff.) and specific rate of biodegradation (SRB) were calculated according to the following formulas:

$$\text{Eff.} = K_0 - K_t / [\text{x } 100]$$

where K_0 - quantity of xenobiotic in the initial moment;

K_t -quantity of xenobiotic in the moment t.

$$\text{SRB} = K_0 - K_t / (\text{t x biomass})$$

[мMol/h.mg Protein].

Modulative effect of Pb cations

Pb cations (40 mg/l) as lead acetate were added in additional parallel experiment in order to investigate their probable inductive/inhibiting effect on the biotransformation.

Analytical methods

Biomass accumulation was analyzed in the supernatant as protein dry weight. Residual amount of o-NP and 2,4-D was examined by HPLC (Waters, U.K.) on a reverse phase C18 column with methanol-water liquid phase.

Study of cell morphology

Variation of cell morphology was studied using light microscopy at initial time, 24h, 48h, 72h and 96h.

Results and Discussion

Environmental isolates *Pseudomonas rhodesiae* KCM-R_5 and *Bacillus subtilis* KCM-RG_5 were studied for their abilities to utilize nitro- and chlorophenols as a sole carbon and energy source. These products are hazardous pollutants released by chemical and plant-protection industry (1, 6). They demonstrate toxic, mutagenic and terratogenic effect in humans (13). Bacterial strains were isolated from polluted soil and water near the Pb-Zn smelter and factory for producing pesticides in South Bulgaria. As it was published earlier the area was strongly polluted by heavy metals. A moderate pesticides contamination was detected (10, 11).

The isolate KCM-R_5 is determined as Gram(-), motile and ovalshaped bacteria while the isolate KCM-RG_5 is a Gram(+), motile and rod-shaped one. 16S rDNA of both strains was sequenced and aligned using BLAST program to the sequences available in EMBL database. Sequences were deposited in the Gene Bank under accession numbers AJ830707 and AJ830708. As it was published earlier (10, 11) *Pseudomonas rhodesiae* KCM-R_5 demonstrated tolerance to 2,4- D and *Bacillus subtilis* KCM-RG_5- tolerance to 2,4-D as well as Pb, Cu, Zn.

Biotransformation of *o*-NP and 2,4-D at concentration 30 mg/l was studied using batch method. To evaluate the probable indictive/inhibiting effect of metal cations an addition of Pb cations at concentration 40 mg/l were added in parallel experiment. (see **Table**, in bold). The reason to choose

TABLE

Experimental scheme

	Microorganisms, phenol derivatives and metal in the experiment
1.	*Pseudomonas rhodesiae* KCM-R5+ 30mg/l *o*-NP
2.	***Pseudomonas rhodesiae* KCM-R5+ 30mg/l *o*-NP + 40 mg/l Pb^{2+}**
3.	*Bacillus subtilis* KCM-RG5 + 30mg/l *o*-NP
4.	***Bacillus subtilis* KCM-RG5 + 30mg/l *o*-NP + 40 mg/l Pb^{2+}**
5.	*Pseudomonas rhodesiae* KCM-R5 + 30mg/l 2,4-D
6.	***Pseudomonas rhodesiae* KCM-R5 + 30mg/l 2,4-D + 40 mg/l Pb^{2+}**
7.	*Bacillus subtilis* KCM-RG5 + 30mg/l 2,4-D
8.	***Bacillus subtilis* KCM-RG5 + 30mg/l 2,4-D + 40 mg/l Pb^{2+}**

in bold- parallel experiment with addition of Pb cations.

Pb as metal modulator of biotransformation is that Pb was the strongest pollutant in the area (1.5 - 2560 ppm) and exceed in times the maximum permission standard (MPL) (10, 11).

The dynamic of biomass accumulation and residual amount of xenobiotic is shown in **Fig. 1**.

Growing on *o*-NP *Pseudomonas* produced more biomass (0.3g dry weight/l) compared то *Bacillus* (0.8 g dry weight/l) (see **Fig.1-a**) Slight stimulating effect of Pb cations was found in the parallel experiment with Pb (see **Fig.1–b**). When grew on 2,4-D *Pseudomonas* again demonstrated higher biomass accumulation (0.7 g dry weight/l) while *Bacillus* biomass was reduced to 0.2 g dry weight/l. As seen from the figure Pb cations did not influence the biomass accumulation during the 2,4-D biotransformation (see Fig. 1).

In the Fig. 1 is also presented the residual amount of xenobiotics in the water phase. The reduced xenobiotic concentration (0.1 - 0.7 mg/l) in the initial time of experiment possibly resulted from its fast cell uptake. It might be due to mutagenic effect of the aromatic compounds on the cell wall permeability. Both bacterial strains removed significant amount of *o*-NP within 96^{th} hour (between 25 and 26 mg/l) (see Fig. 1). In contrast 2,4-D was weakly eliminated (between 0 and 3 mg/l) by both bacteria (see Fig. 1). The possible reason for the slight 2,4-D removal is the difficulty of chlorine atom removal and consecutive break of the aromatic nucleus.

In **Fig. 2** is shown the effectiveness of the biotransformation process. For *o*-NP it reached 95 to 100 % (see **Fig. 2-a**) while for 2,4-D it was only 15 to 20 % (see **Fig. 2-b**). As seen from the figure the SRB is significantly high: 5 to 12 мMol/h.mg protein during biotransformation of o-NP in contrast to 2,4-D biotransformation where SRB is just 0.3 to 0.6 мMol/h.mg protein (see Fig. 2).

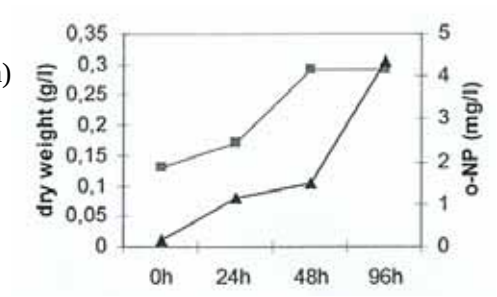
Morphological changes in bacteria was studied at initial time, 24 h, 48 h, and 96 h. Regarding

Pseudomonas rhodesiae KCM-R_5 cells became smaller and roundshaped. For *Bacillus subtilis* KCM-RG_5 a fragmentation and sporeforming was detected.

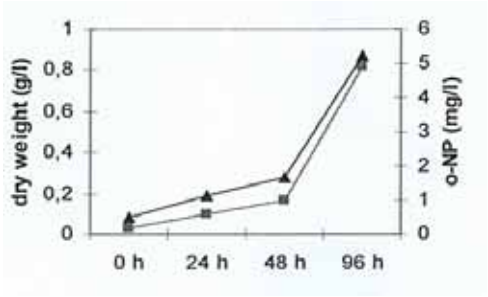
Conclusions

Pseudomonas rhodesiae KCM-R_5 и *Bacillus subtilis* KCM-RG_5 are able to utilize *o*-nitrophenol and 2,4-dichlorophenoxyacetic acid as a sole carbon and energy source. They eliminated between 76 and 83 % of *o*- NP and just 1 % and lower of 2,4-D. The effectiveness of *o*-NP biotransformation reached 90 %. Pb cations did not influence significantly the biotransformation processes. This fact proposes the development of a stability of the biodegradation apparatus towards the Pb-inhibiting

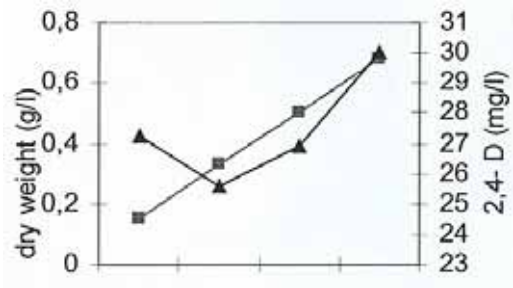
a)



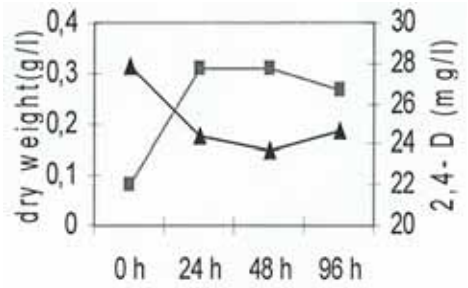
Pseudomonas + *o*-NP 86 % eliminated xenobiotic



Bacillus + *o*-NP 83 % eliminated xenobiotic

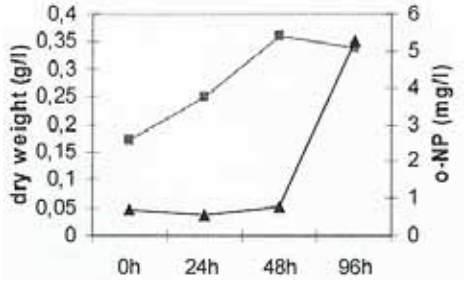


Pseudomonas + 2,4-D 0 % eliminated xenobiotic

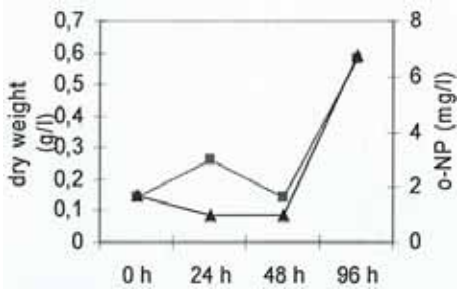


Bacillus + 2,4-D 0,1 % eliminated xenobiotic

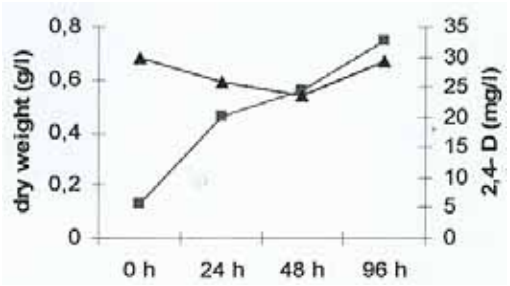
b)



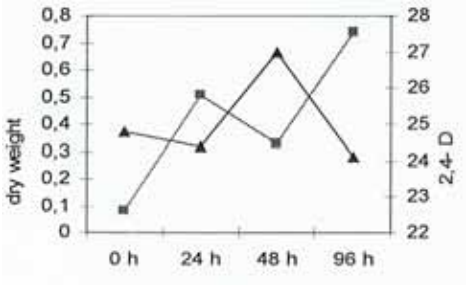
Pseudomonas + *o*-NP+Pb 83 % eliminated xenobiotic



Bacillus + *o*-NP+Pb 76 % eliminated xenobiotic



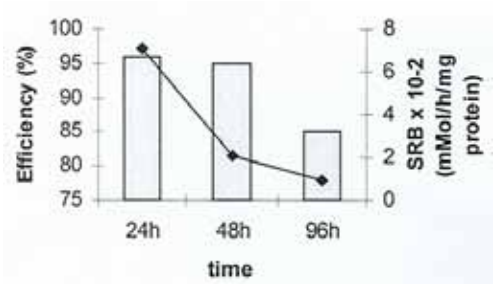
Pseudomonas + 2,4-D+Pb 0,03 % eliminated xenobiotic



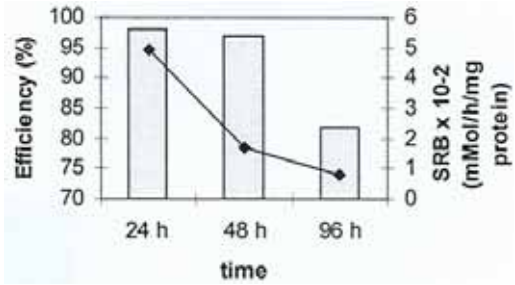
Bacillus + 2,4-D+Pb 0,2 % eliminated xenobiotic

—■— Residual amount of xenobiotic —▲— Dry weight

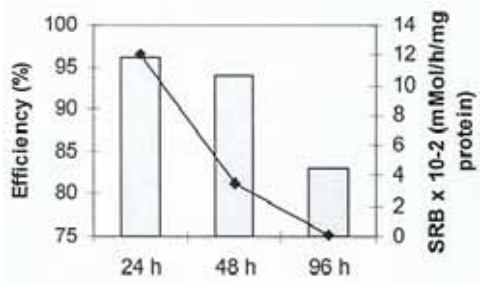
Fig. 1. Biomass accumulation and elimination of **a).** *o*-NP and 2,4-D with Pb as modulator- **b)**. Experiment was performed in triplicate.



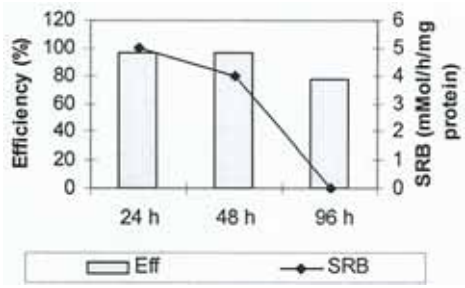
Pseudomonas + **o-NP**



Pseudomonas + **o-NP** + Pb

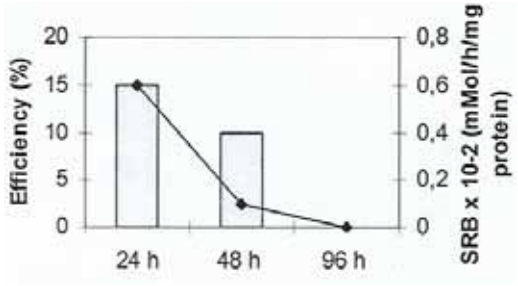


Bacillus + **o-NP**

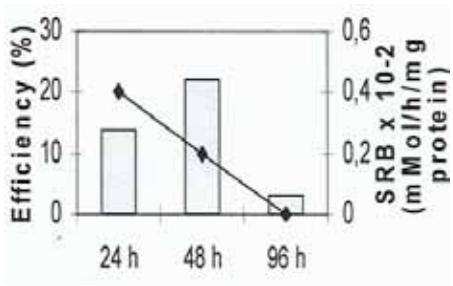


Bacillus + **o-NP** + Pb

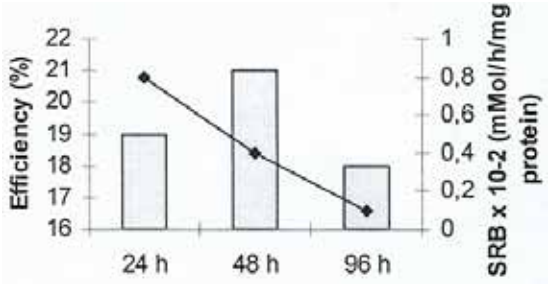
a) Effectiveness (Eff): 95-100 %, Specific rate of biodegradation (SRB): 5-12 [mMol/h.mg Protein]/24 h



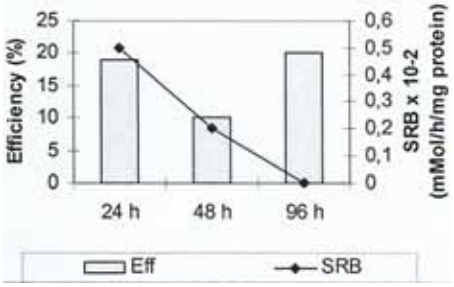
Pseudomonas + **2,4-D**



Pseudomonas + **2,4-D** + Pb



Bacillus + **2,4-D**



Bacillus + **2,4-D** + Pb

b) Effectiveness (Eff): 15-20 %, Specific rate of biodegradation (SRB): 0.3 - 0.6 [mMol/h.mg Protein]/24 h

Fig. 2. Effectiveness and specific rate of biotransformation processes of **a).** *o*-NP and **b).** 2,4-D. Experiment was performed in triplicate.

effect. Cell morphology changes as size-reducing, roundshaping, fragmentation and sporeforming of bacterial cells were registered during biotransformation. The obtained resultes showed that above mentioned bacterial strains could be applied succesfully in the bioindication of environmental pollution. Additionally they could contribute for bioremediation of environments, simultaneously contaminated with heavy metals and phenol derivatives.

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