

## ISOLATION OF MICROBES FOR THE BIOAUGMENTATION OF POLLUTANTS FROM RIVER WATER SAMPLES

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### ABSTRACT

A wide range of chemical pollutants occur in rivers, several of which may impair human health severely. The objectives of this study were the isolation, identification and characterization of xenobiotic-degrading microbes from ten different locations of the Romanian and Hungarian parts of River Maros in an international collaboration. High bacterial and fungal diversity was revealed by RISA (ribosomal intergenic spacer analysis) studies. Microbes were isolated from the water samples on media containing 1 mg/ml acetanilide, aniline-HCl, 2,6-dimethylaniline, 4-isopropylaniline, chlorpropham, diuron, Na-benzoate, 3,4-dihydroxybenzoate, 4-hydroxybenzoate, methylparaben, phenol, m-cresol, p-cresol, resorcinol, phenoxiacetic acid and 2,4-dichlorophenoxyacetic acid separately. The isolates were deposited in the Pollutant-Degrading Microorganism Collection (PDMC) of the University of Szeged. The degradation of acetanilide was monitored by spectrophotometry and the three best degraders were all identified as *Rhodococcus erythropolis*. The xenobiotic-degrading microbes isolated in this study might be used for bioaugmentation purposes.

**Keywords:** water pollutants, acetanilide, xenobiotic-degrading bacteria, *Rhodococcus erythropolis*, bioaugmentation

### INTRODUCTION

Pollutants frequently occurring in waters, such as heavy metals, polycyclic aromatic hydrocarbons (PAH) or pesticides may have various detrimental effects on human health. WANG (2012) reported about the high metal carcinogenic risk in drinking water sources in China. Cr was found to represent the highest risk reservoir water. Eleven different PAHs were detected in water and sediment in Mexico, with phenantrene being the predominant. The results of toxicity tests suggest that the determined PAH concentrations might harm aquatic organisms (JAWARD, 2012). A total of 16 PAHs with human origin were identified in water bodies in China. The findings indicate that human activities affect water resources negatively and drinking water poses potential risk to human health (LIU, 2012). The knowledge available about the human exposure to endocrine disrupting chemicals (EDC), including pesticides, was summarized by MEEKER (2012). The presence of EDCs in water may lead to far-reaching effects on human health and development.

The consumption of phenantrene by *Vibrio parahaemolyticus* was reported by SMITH (2012), while ELLENGAARD-JENSEN (2013) documented the biodegradation of the phenylurea herbicide diuron by *Mortierella* sp.

The microbial degradation of xenobiotics is suggested as a potential way of removing pollutants from rivers, thus the purposes of this study were the isolation, identification and characterization of xenobiotic-degrading microbes from River Maros. A total of 123 microbes, being able to degrade 16 different xenobiotics were isolated and deposited in the Pollutant-Degrading Microorganism Collection (PDMC) of the University of Szeged.

These isolates represent a potential source for further pollutant-bioaugmentation purposes. The degradation of acetanilide was examined by spectrophotometry and the 3 best degraders were identified as *Rhodococcus erythropolis*.

## MATERIAL AND METHOD

### Collecting river water samples

Water samples were collected at 10 different locations of the Romanian (Arad, Bodrog, Munar, Periam Port, Igris) and Hungarian parts (Nagylak, Magyarcsanád, Apátfalva, Makó, Deszk) of River Maros in April, July and October 2012, as well as in January 2013.

### Isolation of xenobiotic-degrading microbes

Fifty  $\mu$ l of the water samples were spread onto the surface of solid minimal media containing 1 mg/ml of the certain xenobiotics separately and the plates were incubated at 25 °C for 7 days.

### *Xenobiotics containing nitrogen*

Minimal medium (MM: 1 g/l  $\text{KH}_2\text{PO}_4$ , 3 g/l  $\text{Na}_2\text{HPO}_4$ , 1 g/l  $\text{MgSO}_4$ , 20 g/l agarose in distilled water) was supplemented with acetanilide, aniline-HCl, 1,2-phenylenediamine, 2,6-dimethylaniline, 3-chloroaniline, 4-chloroaniline, 3,4-dichloroaniline, 3-chloro-4-methylaniline, 4-isopropylaniline, chlorpropham, diuron o-nitrophenol, m-nitrophenol, p-nitrophenol or 2,4-dinitrophenol as sole carbon and nitrogen sources.

### *Xenobiotics not containing nitrogen*

Na-benzoate, 3,4-dihydroxy benzoate, 4-hydroxy benzoate, methylparaben, phenol, hydroquinone, o-cresol, m-cresol, p-cresol, resorcinol, phenoxiacetic acid or 2,4-dichlorophenoxiacetic was added a sole carbon sources to MM supplemented with 1 g/l  $(\text{NH}_4)_2\text{SO}_4$ .

### Monitoring acetanilide-degradation

#### *Sample preparation*

Isolates AAN1, 2, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14 and 15 were inoculated into liquid MM (1 g/l  $\text{KH}_2\text{PO}_4$ , 3 g/l  $\text{Na}_2\text{HPO}_4$ , 1 g/l  $\text{MgSO}_4$  in distilled water) supplemented with acetanilide as a sole carbon and nitrogen source in a final concentration of  $10^7$  cells/ml. The shaken (100 rpm) cultures were incubated at 25 °C for 7 days. After the incubation period 1 ml of each culture was centrifuged (10000 g, 5 min) and the supernatant was analyzed by spectrophotometry.

#### *Spectrophotometry*

Aniline - the degradation product of acetanilide - was determined in the samples with the aid of aniline reagent (1 mg/ml 4-(dimethylamino)cinnamaldehyde in 2 m/V% citric acid solution, prepared in distilled water). 100  $\mu$ l reagent was mixed with 100  $\mu$ l culture supernatant and the absorbance was measured immediately at 520 nm.

## Species identification

### Sample preparation

The bacterial strains were grown overnight in 20 ml liquid yeast extract-glucose medium (YEG: 2 g/l yeast extract, 5 g/l glucose in distilled water) at 25 °C on a rotary shaker (100 rpm). One µl of each culture was diluted with 50 µl double distilled water and used as DNA template for the subsequent PCR-amplification

### Polymerase chain reaction, sequencing and sequence analysis

A fragment of the rDNA region was amplified using primers Eub 8F (5'-AGAGTTTGATCMTGGCTCAG-3') and 534R (5'-ATTACCGCGGCTGCTGG-3'). The mixture for each reaction (50 µl) contained 5 µl 10x Taq Buffer with KCl and 15 mM MgCl<sub>2</sub>, 5 µl 25 mM MgCl<sub>2</sub>, 5 µl 2 mM dNTP Mix, 1-1 µl 10 µM primers, 33 µl double distilled water, 0.2 µl 5U/µl Taq DNA Polymerase and 5 µl template DNA. Amplification was performed in a Biometra T3 Thermocycler as follows: 1 cycle of 94 °C 2 m, 30 cycles of 94 °C 30 s, 51 °C 45 s and 68 °C 1 m, and 1 cycle of 68 °C 10 m. The amplicons were sequenced with the aid of an external service. The sequences were subjected to NCBI BLAST analysis (<http://blast.ncbi.nlm.nih.gov/>).

## RESULTS

### Isolation of xenobiotic-degrading microbes

The results of RISA (ribosomal intergenic spacer analysis) studies revealed high species diversity of bacteria as well as fungi in the water samples (data not shown). The numbers of isolates capable of degrading the certain pollutants are shown in Table 1. All strains were deposited and maintained in the Pollutant-Degrading Microorganism Collection (PDMC) of the University of Szeged. The degradation of the herbicide diuron (by *Mortierella* sp.) was reported also by ELLENGAARD-JENSEN (2013).

### Monitoring acetanilide-degradation

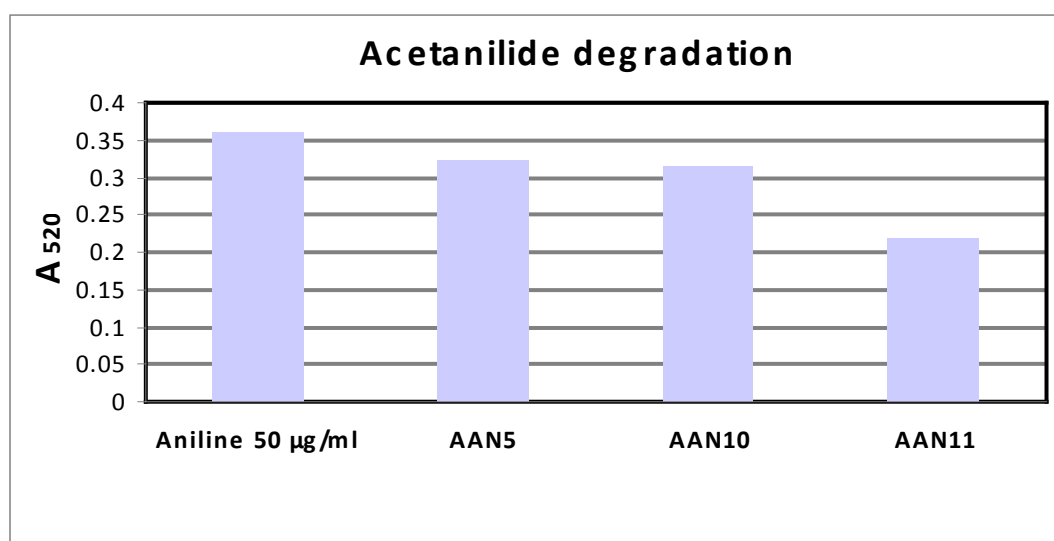
Various pesticides (alachlor, metolachlor, propachlor) are acetanilide-derivatives (STAMPER, 1998; SANYAL, 2002; MUNOZ, 2011). The highest amounts of aniline formed due to the degradation of acetanilide was detected by spectrophotometry in the case of isolates AAN5, 10 and 11 (Figure 1). The degradation of metolachlor by a mixed fungal culture was reported by SANYAL (2002), while MUNOZ (2011) documented the decomposition of metolachlor and alachlor by *Candida xestobii*.

### Species identification

Isolates AAN5, 10 and 11, showing the best acetanilide-degrading properties, were all identified as *Rhodococcus erythropolis*.

**Table 1. Numbers of xenobiotic-degrading microbes isolated in the study**

Compound	Number of isolates
Acetanilide	13
Aniline-HCl	11
2,6-Dimethylaniline	7
4-Isopropylaniline	10
Chlorpropham	7
Diuron	6
Na-benzoate	8
3,4-Dihydroxybenzoate	9
4-Hydroxybenzoate	5
Methylparaben	10
Phenol	3
m-Cresol	4
p-Cresol	5
Resorcinol	7
Phenoxyacetic acid	10
2,4-Dichlorophenoxyacetic acid	8
<b>Total: 16</b>	<b>123</b>

**Figure 1. Spectrophotometric determination (A<sub>520</sub>) of aniline formed during the degradation of acetanilide**

## CONCLUSIONS

The aims of this study were the isolation, identification and characterization of microbes with xenobiotic-degrading ability from River Maros. Bacteria and fungi capable of degrading acetanilide, aniline-HCl, 2,6-dimethylaniline, 4-isopropylaniline, chlorpropham, diuron, Na-benzoate, 3,4-dihydroxybenzoate, 4-hydroxybenzoate, methylparaben, phenol, m-cresol, p-cresol, resorcinol, phenoxyacetic acid and 2,4-dichlorophenoxyacetic acid separately were isolated. Based on the isolates a Pollutant-Degrading Microorganism Collection was established at the University of Szeged. The degradation of acetanilide was monitored by spectrophotometry and the 3 isolates possessing the best degrading properties were all identified as *Rhodococcus erythropolis*. In the future the strains capable

of degrading the other pollutants will also be identified and characterized. The established culture collection will provide a sufficient basis for future bioaugmentation purposes.

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