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ARTICLE

The complete degradation of acetanilide by a consortium of microbes isolated from River Maros

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ABSTRACT Chemical pollutants occurring in rivers may have severe effects on human health along with being harmful to the environment. Bioaugmentation is a potential tool for the removal of xenobiotics from soil and water therefore the objectives of this study were the isolation, identification and characterization of microbes with acetanilide- and aniline-degrading properties from the River Maros. Microbes isolated on minimal media containing acetanilide or aniline-HCl as a sole carbon and nitrogen source were considered as acetanilide- or aniline-degraders. The decomposition of acetanilide and aniline were followed by High Pressure Liquid Chromatography (HPLC). An acetanilide-degrading bacterium, identified as *Rhodococcus erythropolis*, was able to convert acetanilide to aniline, which was further decomposed by the fungal isolate *Aspergillus ustus* when the two microbes were co-cultivated in a minimal medium containing acetanilide as a sole carbon and nitrogen source. The strains isolated in this study might be used in approaches addressing the biodegradation of acetanilide and aniline in the environment.
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KEY WORDS

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Numerous chemical pollutants frequently occurring in rivers, such as heavy metals, polycyclic aromatic hydrocarbons (PAHs) or pesticides and their degradation products may have serious effects on environment and human health. Wang et al. (2012) observed great metal carcinogenic risk in drinking water sources in China, where the co-occurrence of PAHs with Cr represented the highest risk factor. Eleven different PAHs were detected in water and sediment in Mexico with the dominance of phenantrene. Toxicity tests revealed the potential harmful effect of the observed PAHs on aquatic organisms (Jaward et al. 2012). Liu et al. (2012) identified sixteen human-originated PAHs in water bodies in China. These examples indicate that human activities seriously affect water resources and contaminated drinking water represents a potential risk factor for human health due to the mutagenic and carcinogenic effect of PAHs. Numerous widely-used pesticides are acetanilide- and aniline-derivatives (Stamper and Tuovinen 1998; Sanyal and Kulshrestha 2002; Munoz et al. 2011; Milan et al. 2012; Sondhia 2012; Chatterjee et al. 2013), which may affect human health seriously, including for instance methemoglobinemia (Kusin et al. 2012) and various types of cancer (Hanley et al. 2012).

Biodegradation of xenobiotics by microorganisms represents an alternative way for the removal of chemical pollutants from surface and groundwater. Decomposition of phenantrene by *Vibrio parahaemolyticus* was reported by Smith et al. (2012), whereas Ellegaard-Jensen et al. (2013) documented the biodegradation of the phenylurea herbicide diuron by *Mortierella* sp.

Based on the above statements, the aim of this study was the isolation, identification and characterization of acetanilide- and aniline-degrading microbes from River Maros.

Materials and Methods

Materials

For isolating acetanilide- and aniline-degrading microorganisms solid minimal medium (SMM: 1 g KH_2PO_4, 3 g Na_2HPO_4, 1 g $MgSO_4$, 20 g agarose per liter) supplemented with 1 g acetanilide or aniline-HCl per liter as a sole carbon and nitrogen source, was used. The isolated acetanilide-degrading bacteria and fungi were examined throughout the study.

For polymerase chain reaction (PCR), bacterial strains were grown in liquid yeast extract-glucose medium (YEGM: 2 g yeast extract, 5 g glucose per liter).

The aniline- and acetanilide-degrading ability of the isolates was tested following cultivation in liquid minimal medium (LMM: 1 g KH_2PO_4, 3 g Na_2HPO_4, 1 g $MgSO_4$ per liter) supplemented with 50 mg acetanilide per liter as a sole carbon and nitrogen source.

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Unless specified, all chemicals used in the study were purchased from Sigma-Aldrich.

Methods

Water sampling and isolation of acetanilide- or aniline-degrader microorganisms

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 (Mures) in April, July and October 2012, as well as in January 2013. Fifty µl of the water samples were spread onto the surface of SMM and the plates were incubated at 25°C for 7 days. The appearing colonies were considered as potential acetanilide- or aniline-degraders. These were reinforced with sub-culturing to the same medium.

Degradation of acetanilide- or aniline by mixed microbial cultures

Each acetanilide-degrading bacterial isolate were inoculated together with the aniline-degrading fungal isolate SZMC 20913 into LMM in a final concentration of 10^7 bacterial cells/ml and the same concentration of fungal conidia. The shaken (100 rpm) cultures were incubated at 25°C for 7 days. After the incubation period, 5 ml of each culture was centrifuged (3000 g, 15 min) and the supernatants were subjected to HPLC analysis.

Sequence-based species identification

For species identification, the bacterial strains were grown overnight in 20 ml YEGM medium at 25°C on a rotary shaker (100 rpm). One µl of each culture was diluted with 50 µl distilled water and used as DNA template for the subsequent PCR-amplification. A fragment of the rDNA region was amplified by PCR using the primers Eub-8F (5`-agagtttgatCCtggctcag-3`) (Baker et al. 2003) and Eub-534R (5`-ATTACCGCGGCTGCTGG-3`) (Muyzer et al. 1993). For each reaction, the mixture (50 µl) contained 5 µl 10x Taq Buffer with KCl and 15 mmol/L $MgCl_2$, 5 µl 25 mmol/L $MgCl_2$, 5 µl 2 mmol/L dNTP Mix, 0.2 µl 5U/µl Taq DNA Polymerase (Fermentas), 1-1 µl 10 µmol/L primers, 33 µl double distilled water and 5 µl template DNA. Amplification was performed in a Biometra T3 Thermocycler as follows: 1 cycle of 94°C 2 min, 30 cycles of 94°C 30 s, 51°C 45 s and 68°C 1 min, and 1 cycle of 68°C 10 min. The amplicons were sequenced using an external service (LGC Genomics). The sequences were subjected to NCBI BLAST analysis (http://blast.ncbi.nlm.nih.gov/).

In the case of the fungal isolate, DNA extraction, PCR-amplification and sequencing of the internal transcribed spacer (ITS) region of the ribosomal DNA was carried out as described previously (Kredics et al. 2009).

Table 1. Acetanilide- and aniline-degrader microorganisms isolated in the study.

Code	Degraded compound (1 g/L)	Species
SZMC[a] 20918	acetanilide	*Pseudomonas mendocina*
SZMC 21052	acetanilide	*Rhodococcus erythropolis*
SZMC 21053	acetanilide	*Rhodococcus erythropolis*
SZMC 21056	acetanilide	*Rhodococcus erythropolis*
SZMC 20913	aniline	*Aspergillus ustus*

[a]SZMC: Szeged Microbiological Collection (WDCM 987)

High pressure liquid chromatography (HPLC) analysis

The degradation of acetanilide and aniline was quantified by high pressure liquid chromatography (HPLC) analysis, using a modular HPLC system (Merck-Hitachi) including system controller (D-7000), degasser (L-7612), HPLC pump (L-7100), autosampler (L-7200), and equipped with a reversed phase Prodigy C_{18} column (250 x 4.6 mm 5 µm; Phenomenex); a Prodigy guard (Phenomenex) with 5 µm pore size was also applied.

Detection of the selected compounds was carried out by a diode array detector (L-7455, Merck-Hitachi) in the range of 220 nm to 400 nm. Fixed wavelength was set to 233 nm. The optimal mobile phase proved to be the mixture of water and methanol (55:45) at a flow rate of 1 ml/min. Ambient column temperature was used during the analysis and the injection volume was 10 µl. Aniline and acetanilide standards (50 mg/L) were used to determine the degradation efficiency of the selected isolates. The retention time of aniline and acetanilide were 5.63 min and 6.95 min, respectively. For quantification, 9-step calibration curves were used in the range of 2 - 500 mg/L. The concentration-signal functions were $f(x) = -7.947*10^{-1} + 8.25*10^{-5}X$ for aniline and $f(x) = -4.787*10^{-1} + 7.232*10^{-5}X$ for acetanilide. The R^2 values proved to be 1.0000 for both aniline and acetanilide.

Results and Discussion

The aims of this study were the isolation, identification and characterization of microbes with acetanilide- and aniline-degrading ability from the River Maros. Four bacteria and a single fungal strain were isolated with the ability of utilizing acetanilide and aniline-HCl, respectively, as sole carbon and nitrogen source. The isolates were deposited in the Pollutant-Degrading Microorganism Collection (PDMC) of the SZMC (Szeged Microbiological Collection, University of Szeged, Szeged, Hungary) (Table 1).

The bacterial isolates SZMC 20918, 21052, 21053 and 21056, showing high acetanilide-decomposing ability, were identified as *Pseudomonas mendocina* and *Rhodococcus erythropolis* (1 and 3 strains, respectively), while the aniline-

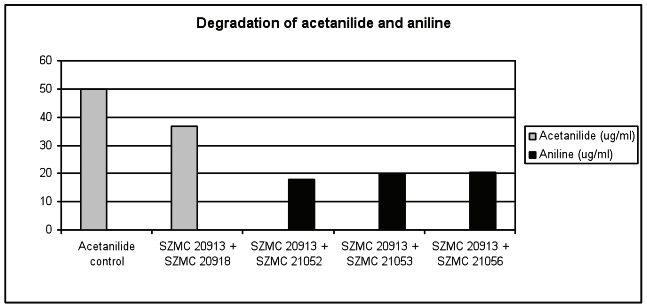

Figure 1. Concentrations of acetanilide and aniline (mg/L) in the culture supernatants of the consortia of acetanilide- (*Pseudomonas mendocina* SZMC 20918, *Rhodococcus erythropolis* SZMC 21052, 21053 and 21056) and aniline-degrading (*Aspergillus ustus* SZMC 20913) isolates. The initial acetanilide concentration was 50 mg/L.

degrader fungus SZMC 20913 proved to be *Aspergillus ustus* (Table 1). The degradation of aniline-derivatives has been examined by numerous authors. The decomposition of alachlor by a bacterial consortium was reported by Dehghani et al. (2013), whereas Sanyal and Kulshrestha (2002) documented the degradation of metolachlor by a mixed fungal culture. Munoz et al. (2011) published the degradation of both compounds by *Candida xestobii*, whereas in the study of Ellegaard-Jensen et al. (2013) a diuron-degrading strain was identified as *Mortierella* sp. These data suggest that the ability to degrade PAHs is present in a wide taxonomical range of microscopic fungi. Among acetanilide degrading bacteria, *Bacillus cereus* and *B. thuringiensis* showed the highest alachlor, propachlor and metolachlor degrading potential in previosu studies (Wang et al. 2008). The biodegradation of 2-chloro-4 nitroaniline by *Rhodococcus* sp. was examined by Khan et al. (2013), while Liu et al. (2013) documented the decomposition of the mixture of nitrobenzene and aniline by a consortium of cold tolerant microbes.

Degradation of acetanilide and aniline was examined by HPLC. When the isolated bacteria were grown in the presence of 50 mg/L acetanilide as the sole carbon and nitrogen source, no acetanilide could be detected in the samples by HPLC, indicating that the entire amount was degraded during the incubation period. The *A. ustus* strain with aniline-degrading ability was able to reduce the concentration of aniline in the culture medium from the initial 50 mg/L to 24.3 mg/L during the 7-day period examined. Combinations of the isolated acetanilide- and aniline-degrading microbial strains were cultivated in the presence of acetanilide as a sole carbon and nitrogen source. Substantial inhibition of the acetanilide-degrading potential of *P. mendocina* SZMC 20918 by *A. ustus* SZMC 20913 was observed, while a synergistic effect was detected between *A. ustus* SZMC 20913 and the *R. erythropolis* isolates tested (Fig. 1), resulting in the complete conversion of acetanilide to aniline and the further degradation of the latter compound.

The microbial strains isolated in this study are expected to provide a good basis for future approaches addressing the bioaugmentation of acetanilide, aniline and their derivatives in environmental samples.

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