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Regular paper

Identification of selected microorganisms from activated sludge capable of benzothiazole and benzotriazole transformation*

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Benzothiazole (BT) and benzotriazole (BTA) are present in the environment — especially in urban and industrial areas, usually as anthropogenic micropollutants. BT and BTA have been found in the municipal and industrial wastewater, rivers, soil, groundwater, sediments and sludge. The origins of those substances' presence in the environment are various industry branches (food, chemical, metallurgical, electrical), households and surface runoff from industrial areas. Increasingly strict regulations on water quality and the fact that the discussed compounds are poorly biodegradable, make them a serious problem in the environment. Considering this, it is important to look for environmentally friendly and socially acceptable ways to remove BT and BTA. The aim of this study was to identify microorganisms capable of BT and BTA transformation or/and degradation in aquatic environment. Selected microorganisms were isolated from activated sludge. The identification of microorganisms capable of BT and BTA removal was possible using molecular biology techniques (PCR, DNA sequencing). Among isolated microorganisms of activated sludge are bacteria potentially capable of BT and BTA biotransformation and/or removal. The most common bacteria capable of BT and BTA transformation were *Rhodococcus* sp., *Enterobacter* sp., *Arthrobacter* sp. They can grow in a medium with BT and BTA as the only carbon source. Microorganisms previously adapted to the presence of the studied substances at a concentration of 10 mg/l, showed a greater rate of growth of colonies on media than microorganisms unconditioned to the presence of such compounds. Results of the biodegradation test suggest that BT was degraded to a greater extent than BTA, 98–100% and 11–19%, respectively.

Key words: biotransformation; benzothiazole; benzotriazole, DNA sequencing, PCR

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INTRODUCTION

Benzothiazole (BT) and benzotriazole (BTA) are present in the environment — especially in urban and industrial areas, usually as anthropogenic micropollutants. BT was present in the municipal (1.7–2.2 μg/L) and industrial (5.5–687 μg/L) wastewater and rivers (0.6–12.8 μg/L) (Céspedes *et al*., 2006; Fiehn *et al*., 1994; Kloepfer *et al*., 2005; Voutsa *et al*., 2006). BTA was detected in soil (330 μg/L) and groundwater near an airport (126 mg/L), wastewater of urban area (1.2–1200 μg/L), rivers (5.0–6.3 μg/L) and in the sediments and sludge (up to 198 ng/g) (Breedveld *et al*., 2003; Cancilla *et al*., 1998; Giger *et al*., 2006; Weiss *et al*., 2006; Zhang *et al*., 2011). Moreover, BTA was proposed as an indicator of wastewater contamination in the environment (Kahle *et al*., 2009).

The origins of those substances' presence in the environment are various industry branches (e.g. food, chemical, metallurgical or electrical industry), households and surface runoff from industrial areas.

BTs were used in food industry for improvement of the overall taste, in organic synthesis for cyan dye production, in rubber industry as chemical activators of the vulcanization process, and in galvanic industry and industrial cooling systems as corrosion inhibitors (Zapór, 2005; Catallo & Junk, 2005; De Wever *et al*., 2001; Chen *et al*., 2012; Finsgar *et al*., 2010).

BTAs are present in detergents, corrosion inhibitors, UV absorbers, photography, biocides, dyes (Pillard *et al*., 2001; Castro *et al*., 2004; Voutsa *et al*., 2006; Reemtsma *et al*., 2010; Harris *et al*., 2007).

Because BT and BTA are quite well soluble in water, stable and resistant to biodegradation, a significant quantity of these substances reaches to the environment and may stay there for a long time (Wu *et al*., 1998; Giger *et al*., 2006; Vousta *et al*., 2006).

Considering this, it is important to look for environmentally friendly and socially acceptable ways to remove BT and BTA. The aim of this study is to identify microorganisms capable of benzothiazole (BT) and benzotriazole (BTA) transformation and/or degradation in aquatic environment.

MATERIALS AND METHODS

Bacterial culture medium. For the growth of bacterial strains from activated sludge, the Kojim mineral medium (Table 1) was prepared.

To each medium, 10 ppm BT and BTA was added as a carbon and energy source for the bacteria, to study degradation of those substances. In the experiment, two variations of the Kojim mineral medium were used, with (KM 1) and without (KM 2) the yeast extract. The use of KM 2 allowed to exclude the impact of yeast extract as additional carbon source.

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Abbreviations: BT, benzothiazole; BTA, benzotriazole; $BTSO_3$, benzothiazole sulfonate; Cac, concentration of BT/BTA in appropriate abiotic control; Cs, concentration of BT/BTA in the sample; diOBT, 2,6-dihydroxybenzothiazole; KM, Kojim medium; MBR, mebrane biological reactor; MBT, 2-mercaptobenzothiazole; OBT, 2-hydroxybenzothiazole; $OD600_{nm}$, optical density

Table 1. Composition of standard (KM 1) and modified (KM 2) Kojim medium

Composition	Concentration, g/l	
	KM 1	KM 2
KH_2PO_4	0.50	0.50
NH_4Cl	5.00	5.00
$MgSO_4 \times 7\ H_2O$	0.20	0.20
Yeast extract	0.01	–
Agar[a]	20.00	20.00

[a]Agar was used in the solid medium

Table 2. Characteristics of wastewater dosed to reactors

Composition	MBR 1	MBR 2	MBR 3
Synthetic municipal wastewater	+	+	+
Benzothiazole	–	+	–
Benzotriazole	–	–	+

Table 3. Conditions of PCR reaction

Step	Temperature (°C)	Time (min)	Cycle
Predenaturation	94	5:00	1
Denaturation	95	0:30	29
Annaealing	57	0:45	29
Elongation	72	1:30	29
Final elongation	72	7:00	1

Activated sludge. Activated sludge was obtained from membrane biological reactors (MBRs) treated, synthetic municipal wastewater. MBR 1 was considered as a control sample, while two other (MBR 2 and MBR 3) were sampling reactors, fed with sewage with addition of BT (96%, Sigma-Aldrich) and BTA (97%, Sigma-Aldrich) standards, respectively. Composition of wastewater dosed to MBRs is shown in the Table 2.

Screening and isolation of BT and BTA degrading bacteria. For isolation of bacterial strains capable of BT and BTA degradation, activated sludge from MBR 1, MBR 2 and MBR 3 was diluted in 0.85% NaCl (10^{-1} to 10^{-10}), placed on the Kojim solid mineral medium, and incubated for 72 hours at 37°C. After 1 week, the fastest growing colonies of bacteria were streaked on nutrient agar plates and incubated for 24 hours at 37°C.

Identification of BT and BTA degrading bacteria. Total bacterial DNA obtained from pure cultures was isolated using Genomic Mini Kit (A&A Biotechnology). PCR amplification with 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' TACGGYTACCTTGTTACGACTT 3') primers was performed (Lane, 1991). Reaction mixtures contained 1 × buffer, 2 mM $MgCl_2$, 5 pM/μL of 27F and 1492R primers, 20 pM/μL dNTPs and 1.5 U GoTAQ Flexi (Promega) in total reaction volume of 30 μL. Isolated DNA at a concentration of 0.15–0.2 μg/μL was added to the PCR mixture. Reactions underwent the cycling parameters presented in Table 3.

The presence of amplicons was confirmed by gel electrophoresis on a 1% agarose (w/v) according to standard procedure. Using Clean Up Kit (A&A Biotechnology) PCR products were purified. Then, they were reamplified and sequenced with the BigDye® Terminator v3.1 kit (Applied Biosystems). Sequences of DNA were compared with GenBank NCBI (National Center for Biotechnology Information).

Biodegradation of BT and BTA. For biodegradation study, two strains showing the fastest growth on KM 2 with addition of BT and BTA, respectively, were used. Tested strains were placed in 100 ml Erlenmeyer flask containing 50 ml Kojim liquid mineral medium with addition of BT (10 mg/L) and BTA (10 mg/L) standards, incubated for two weeks in an orbital shaker set at 25°C and 150 rpm. Abiotic control consisted of sterile Kojim liquid mineral medium with addition of the tested substances. Composition of the studied samples, analyzed in triplicate, is presented in Table 4.

Growth of bacteria was measured at 600 nm by UV–Vis spectrophotometer (Spectronic® Genesys™5). Concentration of BT and BTA was analyzed with Reverse Phase High Performance Liquid Chromatography (Chromatograph UMate 3000, Dionex) coupled with UV-VIS detector at 210 nm, 220 nm, 262 nm, 278 nm for BTA and 218 nm, 254 nm, 284 nm, 294 nm for BT. As a solid phase, Hypersil GOLD (RP-C18) chromatography column (TermoElectron Corporation) was used. Mobile phase consisted of acetonitrile and water (60:40, v/v). The efficiency of biodegradation was calculated using the formula:

$$\%B = (Cac–Cs)/Cac \times 100\%$$

where Cs is concertation of BT or BTA in the sample, Cac is concertation of BT or BTA in the appropriate abiotic control.

RESULTS AND DISCUSSION

Identification of BT and BTA degrading bacteria

In the experiment, two variants of the Kojim mineral medium were used, with (KM 1) and without (KM 2)

Table 4. Composition of samples in the BT and BTA biodegradation test

	Biotic samples						Abiotic control			
	BT_1	BT_2	BT_3	BTA_1	BTA_2	BTA_3	BT/BTA	AC_BT	AC_BTA	AC_BT/BTA
Kojim medium	+	+	+	+	+	+	+	+	+	+
BT	+	+	+	–	–	–	+	+	–	+
BTA	–	–	–	+	+	+	+	–	+	+
Strain 6_O2	+	–	+	–	–	–	+	–	–	–
Strain 7_O2	–	+	+	–	–	–	+	–	–	–
Strain 9_O3	–	–	–	+	–	+	+	–	–	–
Strain 10_O3	–	–	–	–	+	+	+	–	–	–

Table 5. Total bacterial number isolated from activated sludge on KM 1 and KM 2

Medium	Sample	Origin of activated sludge		
		MBR 1, CFU/mL	MBR 2, CFU/mL	MBR 3, CFU/mL
KM 1	Control	$2.4 \cdot 10^1$	$3.1 \cdot 10^1$	$2.2 \cdot 10^1$
	BT addition	$2.0 \cdot 10^4$	$3.5 \cdot 10^4$	$3.3 \cdot 10^4$
	BTA addition	$6.3 \cdot 10^3$	$2.4 \cdot 10^3$	$1.5 \cdot 10^4$
KM 2	Control	–	–	–
	BT addition	$1.6 \cdot 10^2$	$2.9 \cdot 10^4$	$7.0 \cdot 10^3$
	BTA addition	$6.6 \cdot 10^3$	$1.0 \cdot 10^3$	$1.6 \cdot 10^4$

yeast extract. For exclusion of the impact of yeast extract as additional carbon source, KM 2 was used. Comparison of bacterial cell number obtained with both media is presented in Table 5.

Results presented in Table 5 confirmed that in the activated sludge, a microorganism potentially capable of BT and BTA transformation was present. Moreover, yeast extract may be used by bacteria as a carbon and energy source (control of KM 1). To exclude the effect of the extract on the estimate of the BT and BTA biodegradation, in another test the KM 2 medium (without yeast extract) was used. Microorganisms previously adapted to the presence of studied substances at a concentration of 10 mg/l showed a greater rate of growth of colonies on media than microorganisms unconditioned to the presence of such compounds. The most resistant to BT and BTA bacteria were isolated from activated sludge from MBR 2 and MBR 3, which were previously adapted to the presence of those substances. However, in the activated sludge from MBR 1 which was not adapted to BT and BTA, there were bacteria resistant to both compounds. Morphological characteristics of isolated strains are presented in Table 6. Among the isolated bacteria the largest morphological group were *Coccobacilli*.

Results of genetic identification of isolated bacterial strains according to GenBank NCBI (National Center for Biotechnology Information) are presented in Table 7.

Among the identified bacterial strains capable of BT and BTA biotransformation, the most common bacteria were *Rhodococcus* sp., *Enterobacter* sp., and *Arthrobacter* sp. In other studies, *Rhodococcus* strain PA, *Rhodococcus* OBT18, *Rhodococcus erythropolis* strain $BTSO_3$1, *Rhodococcus rhodochrous* and *Pseudomonas putida* strain HKT 554 were tested for BT biodegradation (Gaja & Knapp, 1997; De Wever *et al.*, 1997, El-Bassi *et al.*, 2010; Chorao *et al.*, 2009). *Rhodococcus* strains PA and OBT18 were capable of BT and 2-hydroxybenzothiazole (OBT) degradation, but they did not remove 2-mercaptobenzothiazole (MBT). Other strain, *Rhodococcus erythropolis* $BTSO_3$1, degraded benzothiazole sulfonate ($BTSO_3$) (De Wever *et al.*, 1997). Pathways of BT, OBT and $BTSO_3$ transformation were supposed

Table 6. Morphological characteristic of isolated bacterial strains

Strain	Origin of activated slugde	Degradable subtance	Gram stain	Microscopic morphology
1_O1	MBR 1	BT	Gram –	*Coccobacilli*
2_O1	MBR 1	BT	Gram –	*Coccobacilli*
3_O1	MBR 1	BTA	Gram +	*Coccobacilli*
4_O1	MBR 1	BTA	Gram +	*Cocci*
1_O2	MBR 2	BT	Gram +	*Cocci*
2_O2	MBR 2	BT	Gram +	*Coccobacilli*
3_O2	MBR 2	BT	Gram +	*Mycobacterium*
4_O2	MBR 2	BT	Gram +	*Coccobacilli*
5_O2	MBR 2	BT	Gram +	*Corynebacterium*
6_O2	MBR 2	BT	Gram +	*Coccobacilli*
7_O2	MBR 2	BT	Gram +	*Coccobacilli*
8_O2	MBR 2	BT	Gram +	*Bacilli*
9_O2	MBR 2	BTA	Gram +	*Coccobacilli*
10_O2	MBR 2	BTA	Gram +	*Coccobacilli*
1_O3	MBR 3	BTA	Gram –	*Coccobacilli*
2_O3	MBR 3	BTA	Gram +	*Bacilli*
3_O3	MBR 3	BTA	Gram –	*Coccobacilli*
4_O3	MBR 3	BTA	Gram –	*Coccobacilli*
5_O3	MBR 3	BTA	Gram –	*Coccobacilli*
6_O3	MBR 3	BTA	Gram –	*Coccobacilli*
7_O3	MBR 3	BTA	Gram –	*Coccobacilli*
8_O3	MBR 3	BT	Gram +	*Coccobacilli*
9_O3	MBR 3	BT	Gram –	*Coccobacilli*
10_O3	MBR 3	BT	Gram+	*Coccobacilli*

Table 7. Genetic identification of isolated bacterial strains

Strain	Identification	Similarity, %	NCBI accestion number
1_O1	*Methylobacterium extorquens*	99	NC_012988.1
2_O1	*Enterobacter* sp.	97	NC_021500.1
3_O1	*Arthrobacter* sp.	99	NC_008541.1
4_O1	*Micrococcus luteus*	99	NC_012803.1
2_O2	*Rhodococcus erythropolis*	98	NC_022115.1
3_O2	*Mycobacterium sp.*	97	NC_008705.1
4_O2	*Rhodococcus opacus*	99	NC_012522.1
5_O2	*Corynebacterium variabile*	99	NC_015859.1
6_O2	*Rhodococcus opacus*	99	NC_012522.1
7_O2	*Rhodococcus pyridinivorans*	98	NC_023150.1
8_O2	*Gordonia polyisoprenivorans*	98	NC_016906.1
9_O2	*Cellulomonas flavigena*	99	NC_014151.1
10_O2	*Rhodococcus erythropolis*	94	NC_012490.1
1_O3	*Enterobacter* sp.	98	NC_009436.1
2_O3	*Bacillus* sp.	99	NC_021171.1
3_O3	*Enterobacter cloacae*	97	NC_014618.1
4_O3	*Raoultella ornithinolytica*	99	NC_021066.1
5_O3	*Enterobacter* sp.	99	NC_009436.1
6_O3	*Pseudomonas putida*	98	NC_002947.3
7_O3	*Raoultella ornithinolytica*	98	NC_021066.1
8_O3	*Arthrobacter* sp.	99	NC_008541.1
9_O3	*Enterobacter* sp.	99	NC_009436.1
10_O3	*Arthrobacter aurescens*	99	NC_008711.1

to be connected. Results of De Wever *et al.* (1998) suggest that BT and $BTSO_3$ go through an intermediate product (OBT), which is again hydroxylated to 2,6-dihydroxybenzothiazole (diOBT). Haroune *et al.* (2002) proposed that the formation of diOBT may be catalyze by monooxygenase and then it could be transformed into catechol and dicarboxylic acid by catechol 1,2-dioxygenase.

Results of Liu *et al.* (2011) show BTA transformation using activated sludge under the aerobic and anaerobic conditions yields different intermediate products: 1-methylbenzotriazol, phthalic acid, 4-methoxybenzotriazol, 5-methoxybenzotriazol and 1-methylbenzotriazole, *N,N*-dimethylaniline, carbazole, respectively.

Biodegradation of BT and BTA

For the biodegradation test, the fastest growing strains were selected, 6_O2 (*Rhodococcus opacus*) and 7_O2 (*Rhodococcus pyridinivorans*) for BT biodegradation and 9_O3 (*Enterobacter* sp.) and 10_O3 (*Arthrobacter aurescens*). Results of optical density of tested strains cultured in the Kojim mineral liquid medium are presented on Fig. 1.

The increase of optical density (OD_{600nm}) suggests that all strains of tested bacteria grow in Kojim liquid mineral medium with addition of BT and BTA standards. The results may suggest that BT and BTA may be a source of carbon and energy. The fastest growth was observed in a sample with consortium of all tested bacterial strains. Results of BT and BTA biodegradation are presented in Fig. 2.

The results of biodegradation test suggest that more degradable of the tested substances was BT. In all samples, the biodegradation rate was higher than 98%. This substance was probably used by bacteria as the source of carbon and energy. BTA was resistant to biodegradation by tested bacteria (biodegradation rate was lower than 14%). The removal of BT and BTA in a sample with consortium of all tested strains was 99% and 19%, respectively. The removal of BTA was ostensibly higher which may suggest that biodegradation of this substance is possible in consortium of various types of bacteria, but it requires further studies. The lower values of optical density (slower growth) of tested bacteria in a medium where BTA was added, were probably due to the negative impact of BTA on the studied microorganisms.

CONCLUSIONS

In all tested activated sludge, bacteria capable of BT and BTA biodegradation were present. The most bacteria resistant of BT and BTA were isolated from activated sludge from MBR 2 and MBR 3, which were previously

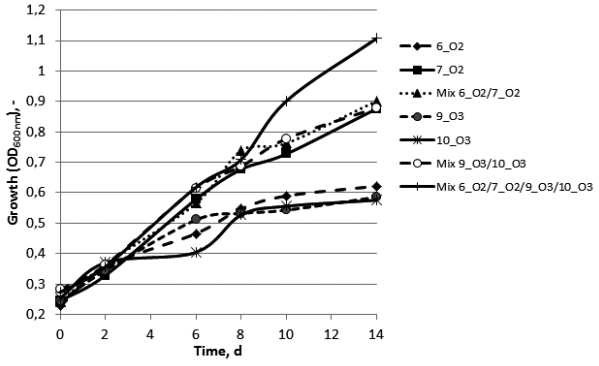

Figure 1. Optical density of tested strains cultured in Kojim mineral liquid medium

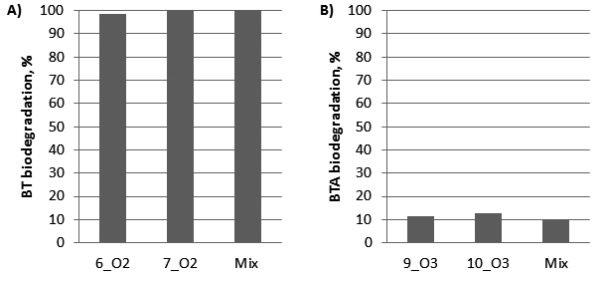

Figure 2. Biodegradation rate of: A) BT and B) BTA

adapted to the presence of those substances. However, in the activated sludge from MBR 1 which was not adapted to BT and BTA, there were bacteria resistant to both compounds. Among the identified bacterial strains capable of BT and BTA biotransformation, the most common bacteria were *Rhodococcus* sp., *Enterobacter* sp., *Arthrobacter* sp. The results of biodegradation test suggest that BT is more degradable than BTA.

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