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Isolation of *Streptomyces* sp. strain capable of butyltin compounds degradation with high efficiency

Przemysław Bernat, Jerzy Długoński*

Department of Industrial Microbiology and Biotechnology, University of Lodz, Banacha 12/16, 90-237 Łódź, Poland

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ABSTRACT

Dibutyltin (DBT), a widely used plastic stabilizer, has been detected in the environment as well as in human tissues. DBT is considered to be highly neurotoxic and immunotoxic. Hence, DBT needs to be considered as a potential toxic chemical. Degradation of butyltin compounds by *Streptomyces* sp. isolated from plant waste composting heaps was studied. Glucose grown cells degraded organotin from 10 to 40 mg l^{-1}. After 1 day of incubation 90% of DBT (added at 20 mg l^{-1}) was converted to less toxic derivative – monobutyltin (MBT). DBT metabolism was inhibited by metyrapone addition, a known cytochrome P-450 inhibitor. It could provide evidence that cytochrome P-450 system is involved in DBT metabolism in *Streptomyces* sp. IM P102. Moreover, according to our knowledge, the degradation of DBT by actinobacterium has not been previously described.

1. Introduction

Butyltin compounds have been widely used in a variety of industrial and agricultural applications and significant amounts of butyltins have entered the environment [1]. Among them, tributyltin (TBT) has been paid the most attention because of its toxicity. Due to its use as an antifouling agent in boat paints, TBT is a common contaminant of marine and freshwater ecosystems [2]. It is responsible for the imposex phenomenon, when male sex characteristics are superimposed on normal female gastropods. Moreover, low concentrations of TBT (0.1–0.5 μg l^{-1}) are immunotoxic, neurotoxic and hepatotoxic. Therefore, due to the toxic effects of TBT on marine organisms, efforts have been undertaken in order to impose a total ban on the application of TBT. International Maritime Organization (IMO) prohibited the application of TBT in antifouling paints from 17th September 2008 [3]. Another butyltin – dibutyltin (DBT) is commonly used as heat and light stabilizer for PCV materials, to speed up the production of plastics, and as a deworming agent in poultry [4]. DBT is also known as a degradation product from tributyltin (TBT). There is evidence suggesting, that DBT might target the immune system. It inhibits natural killer cells *in vitro*, induces thymic cell apoptosis and causes a decreased thymus weight in the exposed animals [5]. Dialkyltins also appeared to act by binding to dithiol groups on enzymes and cofactors [6]. Though monobutyltin (MBT), which is used as a plastic stabilizer, is generally less toxic, it is also of environmental interest [7]. Moreover, several studies showed that MBT could be as toxic as, or even more toxic than TBT for some microorganisms in soil [8]. According to the literature, DBT and MBT are present in sewage sludge, sediment or soil more often than TBT [9]. In soil, both butyltins may come from atmosphere deposition, the spread of contaminated sludge or pulverization of biocide products. Unfortunately, the terrestrial environment has not received as much attention as aquatic media [10].

There are papers concerning TBT degradation by marine microorganisms [11,12]. On the other hand, although DBT is commonly present in the environment, little is known about its biodegradation by bacteria which do not come from the marine environment. There have been some reports regarding degradation kinetics of butyl- and phenyltins in soils [13]. However, there is no information available on the ability of butyltins biotransformation by actinobacterium species, the main group of bacteria present in soils and sediments [14,15]. On the other hand, many studies demonstrated that these microorganisms, especially those of the genus *Streptomyces*, have been able to transform different toxic compounds, e.g. aldrin, DDT, metolachlor, atrazine, lindane, bisphenol A [16,17].

The use of *Streptomyces* strains for bioremediation of soils is an attractive approach due to their mycelial growth habit, relatively rapid rates of growth and colonization of semi selective substrates [18]. Moreover, their ability to produce spores contributes to streptomycete survival over long periods of drought, frost or hydrostatic pressure [19].

* Corresponding author. Tel.: +48 42 6354465; fax: +48 42 6655818.
E-mail address: jdlugo@biol.uni.lodz.pl (J. Długoński).

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Much evidence suggests that biotic degradation is the major pathway for the removal of butyltins from the environment [1,9]. There are reports on the biodegradation of TBT mediated by bacteria, fungi or microalgae [11]. Some microorganisms use TBT as a carbon source such as *Aeromonas veroni*i Av27 and *Pseudomonas* sp., which assimilates TBT up to 3 or 2 mM, respectively [12,20]. The mechanism(s) of butyltin dealkylation by bacteria is not known. The stepwise debutylation of TBT to DBT and MBT in fungi (*Cunninghamellela elegans*) or algae (*Chlorella* sp.) is believed to involve isozymes of cytochrome P-450 [21,22].

In this report, the isolation and characterization of *Streptomyces* sp. isolate capable of utilizing DBT are presented. To the best of our knowledge, this is the first paper reporting on an actinobacterium culture metabolizing butyltins. We also describe cytochrome P-450 engagement in DBT biodegradation by the isolate.

2. Material and methods

2.1. Chemicals

Organotins:dibutyltin dichloride (DBT) and monobutyltin trichloride (MBT) were purchased from Aldrich. Tetrabutyltin (TTBT) was obtained from Supelco. Other chemicals were from JT. Baker, Fluka and POCH (Gliwice, Poland). All the chemicals were high purity grade reagents. Stock solution of DBT was prepared at 5 mg ml^{-1} ethanol.

2.2. Microorganism and culture conditions

For this study, actinobacterium strains isolated from disqualificated composting heaps were used. Due to the contamination of compost raw material by pesticides and heavy metals microorganisms with intensive xenobiotic degradation ability were expected to be found. Five-day-old bacterial cultures on malt extract agar slants were used to inoculate 20 ml universal medium (per liter: 1.3 g yeast extract, 15 g peptone, 5 g glucose) (in 100 ml Erlenmeyer flasks). The cultivation was carried out on rotary shaker (180 rpm) for 48 h at 28 °C. Two ml of the homogenous preculture was introduced into 18 ml of medium with DBT (at the concentrations of 10, 20, 40, 60 and 80 mg l^{-1}) or without the xenobiotic in the control cultures (in 100 ml flasks). The cultures were incubated for 5 or 7 (DBT degradation curve) days at 28 °C on rotary shaker (180 rpm).

2.3. DBT utilisation in presence of cytochrome P-450 inhibitor

The homogenous preculture (2 ml) prepared as presented above was transferred into 18 ml of universal medium (in 100 ml Erlenmayer flasks) supplemented with cytochrome P-450 inhibitor metyrapone (1.5 mM) and DBT (20 mg l^{-1}). Uninoculated media with appropriate amounts of DBT added in ethanol served as controls. Then the flasks were incubated for 5 days in the same conditions.

2.4. Biomass analysis

Bacterial biomass was separated from culture media by filtrating through Sartourius filter (0.25 μm) and then dried at 105 °C to reach a constant weight.

2.5. Organotin analysis

The organotin analysis of cultures was carried out according to the procedure of Bernat and Długoński [23] with slight modifications. The sample was homogenised (MISONIX) with 20 ml acetone after the addition of concentrated hydrochloric acid (pH 2–3) and then centrifuged at 500 × *g* for 10 min. The supernatant was extracted twice with 25 ml of tropolone (2-hydroxy-2,4,6-cycloheptatrienone)/hexane (3:10,000, w/v). The extracts were dried over anhydrous sodium sulphate and the solvents were evaporated under reduced pressure at 40 °C. Two ml of hexane and internal standard TTBT (100 μg) were added, followed by the addition of a solution of methyl magnesium bromide (500 μl, 3 M in anhydrous diethyl ether, reaction time: 20 min at room temperature). The reaction was slowly quenched with aqueous ammonium chloride 20% (2 ml). After 20 min centrifugation (2000 × *g*), 2 μl of the upper organic layer was injected in GC/MS for an analysis.

2.6. Gas chromatography analysis of organotins

A Hewlett-Packard Model 6890 gas chromatograph equipped with 5973 Mass Detector was used. The separation was carried out in a capillary column HP 5 MS methyl polysiloxane (30 m × 0.25 mm id × 0.25 μm ft). The column temperature was maintained at 60 °C for 4.5 min, then increased to 280 °C at 20 °C min^{-1}. Finally, the column temperature was maintained at 280 °C for 3 min. Helium was used as a carrier gas at a flow rate of 1.2 ml min^{-1}. The injection port temperature was 250 °C. A split injection (2 μl) was employed. The individual butyltin species were distinctly separated and identified on the basis of their retention times and characteristic ions. Quantitative analyses were performed on single ions: 165 for MBT, 151 for DBT and 179 for TTBT (int. std.) [23].

2.7. Fatty acid analysis

The content of each flask was filtered through a Sartorious (0.2 μm) filter, dried with ethanol and stored at −20 °C. Next, fatty acids were methanolised – cells were transferred to Pyrex glass tubes and 5 ml of methanol:toluene:sulphur acid (30:15:1, respectively) was added. After 18 h at 50 °C fatty acid methyl esters (FAMEs) were extracted twice with hexane (4 ml). The extracts were dried with Na_2SO_4 and evaporated. FAMEs were analyzed by gas chromatography according to the procedure described by Bernat and Długoński [24].

2.8. Statistical analysis

Experiments were carried out with triplicate samples. One-way analysis of variance was used to determine the significance of the difference between the samples. All statistical analyses were performed using Excel 2000 (Microsoft Corporation, USA).

3. Results and discussion

3.1. Screening of actinobacterium strains for the ability to degrade DBT

One initial concentration (10 mg l^{-1}) of the compound was utilized in universal medium. The results obtained for DBT and expressed as a percentage of depletion after 5 days of incubation are presented in Fig. 1. Three bacteria strains were found to leave residual DBT concentrations lower than 50%. Among them, strain IM P102 showed the best degradation efficiency and was selected for further study.

3.2. Taxonomic characterization of DBT degrading strain IM P102

Strain IM P102 was assigned as the genus *Streptomyces* based on its morphological and chemotaxonomic characteristics, such as the presence of LL-diaminopimelic acid in whole cell hydrolysates [25]. To confirm taxonomic identifications FAME analyses of the cell lipids were done (Table 1). The cellular fatty acids of the actinobacterium included saturated straight-chain, iso- and anteisobranched

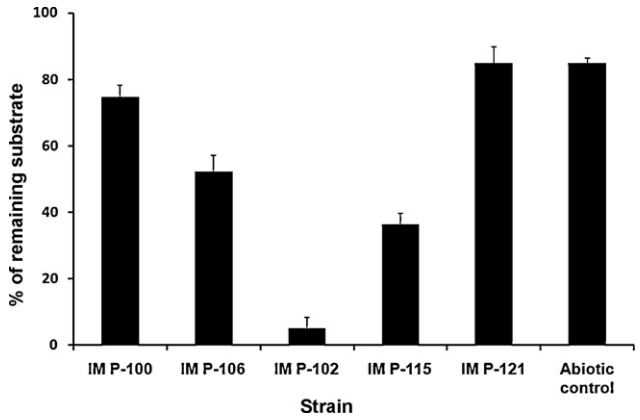

Fig. 1. DBT removal by isolated strains in universal medium after 7 days of culturing (DBT added at 10 mg l^{-1}).

Table 1
FAME composition (%) of *Streptomyces* sp. IM P102 at stationary growth phase.

	Fatty acid						
	i14:0	i15:0	ai15:0	i16:0	16:0	i17:0	ai17:0
%	32.33	20.45	23.99	5.71	6.31	2.71	8.49

i, *iso* acids; ai, *anteiso* acids.

components. The major fatty acids characteristic for the genus *Streptomyces* were iso tetradecanoic (*iso*-C14), iso pentadecanoic (*iso*-C15), anteiso pentadecanoic (*anteiso*-C15) [26,27].

3.3. Effect of initial DBT concentration on Streptomyces sp. IM P102 growth and butyltin degradation

Five initial concentrations (10, 20, 40, 60 and 80 mg l^{-1}) of DBT were utilized in universal medium supplemented with *Streptomyces* sp. IM P102. The results obtained for each concentration expressed as a percentage of remaining substrate after five days of incubation were presented in Fig. 2. The xenobiotic degradation in the concentrations of 10, 20 and 40 mg l^{-1} was the most significant. Starting from 60 mg l^{-1} the degradation efficiency dramatically decreased. Because at 60 or 80 mg l^{-1} of DBT a stepwise decrease in actinobacterium biomass concentrations was observed (Fig. 3), it seemed that *Streptomyces* sp. IM P102 better withstood

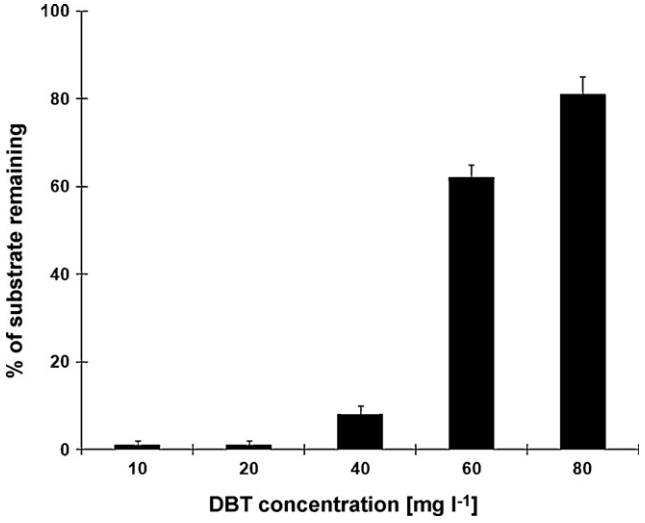

Fig. 2. Percentage of DBT degradation by *Streptomyces* sp. IM P102 in the synthetic medium after 5 days of incubation.

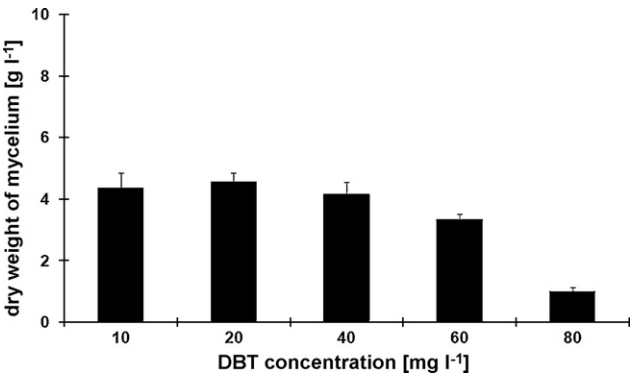

Fig. 3. Biomass of *Streptomyces* sp. IM P102 after 5 days of incubation in growth medium supplemented with different amounts of DBT.

the toxicity of DBT at 10–40 mg l^{-1} than at 60 or 80 mg l^{-1}. However, the mode of diorganotins action on bacteria has not been explored. According to other researchers they could act on dithiol-containing enzymes [6]. Miller and Cooney [28] observed inhibition of NH_4^+ uptake and accumulation of NH_2OH and NO_2^- by DBT in two *Bacillus* sp. and *Pseudomonas* sp. However, the applied concentrations of DBT did not inhibit the growth of bacteria. Probably, these effects were not merely secondary effects of general toxicity [28].

Taking into account the obtained results in the next part of the investigation the time course of DBT (20 mg l^{-1}) transformation was studied (Fig. 4). A rapid degradation of the xenobiotic (91%) and MBT formation were observed during the initial 24 h of incubation. At the same time, in the first 24 h of the exponential growth phase of *Streptomyces* sp. IM P102 was noticed. However, the intensity of xenobiotic removal was not correlated with bacterial growth, because DBT (20 mg l^{-1}) added at the stationary phase of growth was degraded in a similar manner (data not shown). Unfortunately, the selected strain was not able to transform TBT (data not shown).

Monosubstituted organotin compounds were the most abundant species in the investigated soils [29,30]. MBT was also present in bulk precipitation, throughfall, fog and litterfall (Bavaria, Germany), with the range of its concentrations observed by Huang and Matzner [30] quite similar to those reported for aquatic ecosystems. Moreover, according to the results obtained by Heroult et al. [8],who studied the degradation of butyltin compounds in agricultural and forest soils, the following corresponding scale of persistence can be proposed: TBT < DBT < MBT. Therefore, the ability of the selected strain to degrade MBT, which is the most popular and persistent butyltin in soil, could be promising (Fig. 4).

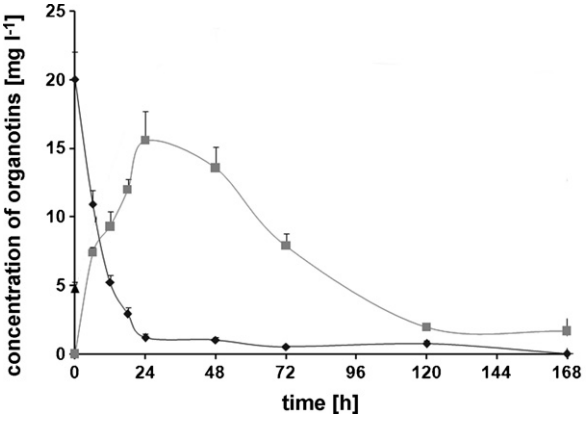

Fig. 4. DBT biodegradation (♦) and MBT formation (■) by *Streptomyces* sp. IM P102 in culture medium during 7 days of culturing (initial DBT concentration 20 mg l^{-1}).

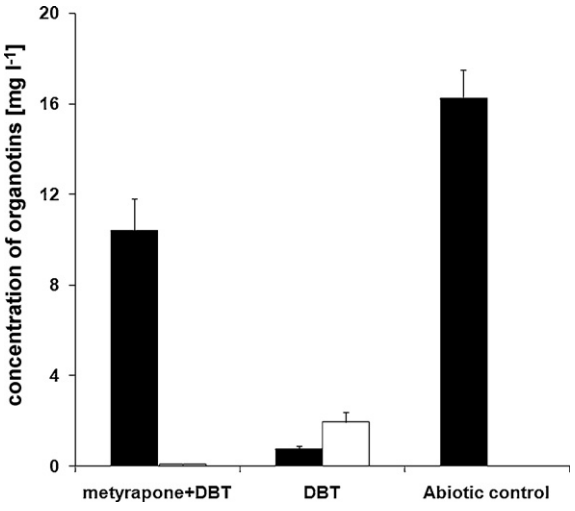

Fig. 5. DBT removal by *Streptomyces* sp. IM P102 after 5 days incubation with metyrapone (1.5 mM) (DBT added at 20 mg l^{-1}). DBT (■); MBT (□).

In the previous studies, only four Gram-negative bacteria: *Pseudomonas aeuruginosa*, *Pseudomonas chlororaphis*, *Alcaligenes fecalis* and *A. veronii* [11,12,31] were described as bacteria degrading TBT, DBT and/or MBT. For the first time, the degradation of DBT by Gram-positive microorganism – *Streptomyces* sp. IM P102 has been reported.

3.4. Cytochrome P-450 involvement in DBT degradation by *Streptomyces sp. IM P102*

Streptomyces are a rich source of soluble P450 enzymes, which play critical roles in the bioactivation and detoxification of a wide variety of compounds in biosynthetic and xenobiotic transformation reactions [32,33]. It has been shown for several actinobacteria, that cytochrome P-450 enzymes are involved in degradation of chemicals such as biphenyl, phenol, benzo(a)pyrene, aniline, pyridine [32,33]. In order to find out whether cytochrome P-450 is involved in butyltin metabolism by *Streptomyces* sp. IM P102, an experiment with cytochrome P-450 inhibitor metyrapone was carried out. Metyrapone (1.5 mM) did not affect the viability of bacteria. Moreover, its presence led to a significant inhibition of DBT degradation reactions ($p > 0.01$). After a 5-day incubation of the bacterium with the inhibitor and DBT, the xenobiotic removal efficiency was significantly lower (Fig. 5). Therefore, the strong inhibition of hydroxylation reactions in the presence of the cytochrome P-450 inhibiting substance – metyrapone points to an involvement of these enzymes in DBT transformation reactions. However, the metyrapone did not totally inhibit DBT degradation. Probably, if the inhibitor concentration had been higher, the organotin transformation have been less efficient. On the other hand, higher concentrations of metyrapone influenced microorganism growth. Interestingly, in metyrapone presence MBT was not determined (only trace amounts were found). It seems, that the inhibitor more strongly influenced DBT than MBT transformations.

Until now, there is not data concerning the involvement of cytochrome systems in DBT degradation by Procaryota. Yamaoka et al. [31] described organotins transformation by *P. chlororaphis*. DBT in sea water was degraded to MBT by pyoverdins isolated from these bacteria. However, TBT or MBT in sea water was not degraded by pyoverdins.

On the other hand, DBT degradation to beta-hydroxybutyltributyltin with DBT dioxygenase involvement by microalgae was described by Lee et al. [34]. Also, when dibutyltin dichloride was administered to male rats, butyl(3-hydroxybutyl)tin dichloride, butyl(4-hydroxybutyl)tin dichloride and butyltin trichloride were detected in their tissues [35]. In our research the hydroxyalated derivatives were not found. This may be due to the acid instability of the oxidized metabolites at positions 1 and 2 of one of the alkyl moieties [35].

4. Conclusion

In this study *Streptomyces* sp. IM P102 presented a high level of DBT and its metabolite MBT degradation. These results also confirm that the enzyme responsible for DBT degradation by this actinobacterium includes cytochrome P-450. According to our knowledge, it is the first report on a successful actinobacterium application for butyltins removal in a liquid culture. The obtained results are a very good starting point for further investigation on DBT or MBT soil polluted bioremediation with *Streptomyces* sp. IM P102.

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