Chemical Engineering Journal 249 (2014) 167–173

Contents lists available at ScienceDirect

Chemical Engineering Journal

journal homepage: www.elsevier.com/locate/cej

Triphenyltin biosorption, dephenylation pathway and cellular responses during triphenyltin biodegradation by *Bacillus thuringiensis* and tea saponin

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HIGHLIGHTS

- Triphenyltin biodegradation and cellular metabolic responses were revealed.
- Triphenyltin dephenylation occurred individually and synchronously.
- Tea saponin (TS) significantly improved phenyltin biosorption and biodegradation.
- TS significantly reduced Na^+, NH_4^+, K^+ and Mg^{2+} release by *Bacillus thuringiensis*.
- TS enhanced cellular activity, protein expression, and Cl^- and PO_4^{3-} use.

ARTICLE INFO

Article history:
Received 6 January 2014
Received in revised form 15 March 2014
Accepted 27 March 2014
Available online 2 April 2014

Keywords:
Triphenyltin
Biodegradation
Surfactant
Organotin
Bacillus thuringiensis

ABSTRACT

Triphenyltin is an endocrine disruptor that has polluted the global environment, and thus far, information regarding the mechanisms of triphenyltin biodegradation and cellular metabolic responses is severely limited. The question of whether dephenylation during degradation occurs successively or synchronously is also not clear. Here, these processes were illuminated through experiments involving surfactant-enhanced biodegradation of triphenyltin and its metabolites by *Bacillus thuringiensis*. Tea saponin significantly enhanced phenyltin solubility, biosorption, membrane permeability, protein expression, cell density and Cl^- and PO_4^{3-} use, reduced intracellular Na^+, NH_4^+, K^+ and Mg^{2+} release, and accordingly increased phenylation biodegradation. Phenyltin biodegradation was initially triggered by benzene ring cleavage, which occurred individually and synchronously, producing diphenyltin, monophenyltin, and tin. After degradation by cells in the presence of 60 mg L^{-1} tea saponin for 7 d, residual concentrations of triphenyltin, diphenyltin, and monophenyltin were decreased to 283, 270, and 235 μg L^{-1}, respectively.

1. Introduction

The global use of organotins as a component of herbicides, paints, disinfectants, biocides, wood preservatives, and plastic catalysts [1] has consequently induced their widespread exposure and resulting pollution in various ecosystems [2,3]. As one of the endocrine disruptors among thousands of organotins, triphenyltin (TPT) is highly toxic to a broad range of species [4] and its extensive use has resulted in environmental contamination worldwide [5,6].

TPT can be degraded to less toxic derivatives, including diphenyltin (DPT), monophenyltin (MPT) and tin, by biological activity [7]. Elimination of TPT and its metabolites in natural ecosystems not exposed to ultraviolet light is primarily dependent on biodegradation [8]. However, structural complexity and low water solubility limit TPT's availability to microorganisms, resulting in its persistence in natural environments. Surfactants are capable of encouraging TPT emulsification by lowering TPT surface tension and enhancing TPT affinity in effective microbes, and thereby resulting in higher TPT bioavailability. Thus far, accelerated bioremediation of polluted environments by surfactants has been investigated in a number of studies [9,10], but studies concerning surfactant enhancement of TPT biodegradation are rare. Tea saponin (TS) is a natural surfactant with low toxicity and high biodegradability in comparison with the synthetic surfactants. The potential of TS to enhance uptake of polychlorinated biphenyls and cadmium was investigated [11]. However, the application of TS as a surfactant in organotin bio-treatment has not been reported yet.

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http://dx.doi.org/10.1016/j.cej.2014.03.110
1385-8947/

Several investigations involving organotin biosorption and biodegradation have identified some enhancing factors and supplements [12,13]. Nevertheless, the severely limited information regarding biodegradation mechanisms and the influence of enhancement factors, such as surfactants, on the TPT biodegradation pathway and kinetics has limited further application of organotin biodegradation strategies. To date, the presence of successive or synchronous dearylation during TPT degradation has not been clearly revealed because all TPT benzene rings bond individually with tin atom.

As TPT biodegradation leads to the production of metabolites with their inherent ecological impacts, the resulting metabolites should be identified and their further degradation should also be evaluated. Moreover, the process of TPT catalysis is related to cellular physiological responses, which not only influence the biodegradation pathway but also control the metabolite production rate and their further degradation.

Bacillus thuringiensis widely used as a biopesticide also effectively degraded some toxic pollutants, including dimethyl phthalate [14] and fipronil [15]. Therefore, its potential to degrade phenyltins (PTs) in the presence of TS was conducted in the present work. Nutrient use, ion release, membrane permeability, cell viability, and protein secretion and expression by cells during the TPT biodegradation process with or without TS were also analyzed to illuminate the mechanisms of TPT biodegradation.

2. Materials and methods

2.1. Strain and chemicals

B. thuringiensis was isolated from organotins contaminated sediment samples collected at an e-waste processing and recycling town called Guiyu in Guangdong Province, China. TPT was purchased from Sigma–Aldrich (St. Louis, MO, USA). Mineral salt medium (MSM) contained (in mg L^{-1}) 150 $Na_2HPO_4{\cdot}12H_2O$, 50 KH_2PO_4, 30 NH_4Cl, 5 $Zn(NO_3)_2$, and 5 $MgSO_4$.

2.2. Phenyltin treatment

Biodegradation and removal of PTs at 1 mg L^{-1} by 0.3g L^{-1} *B. thuringiensis* in the presence of 5–80 mg L^{-1} TS were performed in 20 mL MSM in the dark at 30 °C on a rotary shaker at 100 r min^{-1} for 1–7 d. After treatment, cells were separated by centrifugation at 3500*g* for 5 min. Residual PTs in resultant supernatant and total residual PTs were detected to determine the removal and biodegradation efficacies, respectively. The concentrations of Cl^-, PO_4^{3-}, Na^+, NH_4^+, K^+, Mg^{2+}, and extracellular protein in MSM as well as intracellular protein content were evaluated to elaborate the correlation between TPT degradation and cellular metabolism. The metabolites and kinetic models of PT biodegradation were examined by monitoring how TPT, DPT, and MPT at 1 mg L^{-1} were degraded by *B. thuringiensis* and 60 mg L^{-1} TS.

To reveal the contribution of TPT binding by the cell surface to TPT removal, TPT biosorption by dead cells was conducted in the same condition. After inactivation by 2.5% glutaraldehyde for 24 h [16], cells were cultured in beef extract medium containing (in g L^{-1}) 3 beef extract, 10 peptone and 5 NaCl at 37 °C in Petri dish for 24 h to determine whether they were killed. These successfully inactivated cells were then applied to adsorb TPT. After biosorption, cells were separated by centrifugation at 3500 g for 5 min. Residual PTs in resultant supernatant was detected. All of the experiments were performed in triplicate and the mean values were used in calculations.

2.3. Analytical methods for ion assessments

After biodegradation, the solution was centrifuged at 3500 g for 10 min and the resultant supernatant filtered using a 0.22-μm polyether sulfone filter. Subsequently, the concentrations of Cl^-, PO_4^{3-}, Na^+, NH_4^+, K^+, and Mg^{2+} were assessed using an ICS-900 ion chromatography system (Dionex Corp., Sunnyvale, CA, USA) [16].

2.4. Analytical methods for TPT and its metabolites

After biodegradation, 10 mL hexane was added into 20 mL MSM. The PTs in the mixture were sonicated for 20 min in an ultrasonic bath and allowed to set until phase separation. After the organic phase was removed, 10 mL hexane was added into the aqueous phase, and then the operation was repeated again. The organic part was collected, followed by concentrating using a rotary evaporator at 30 °C. The residues that represented the extracellular PTs were dissolved by 5 mL methanol and derivatised in pH 4.5 acetate buffer with 2 mL of 2% sodium diethyl dithiocarbonate.

PTs were analyzed according to previously published methods [13] by gas chromatography-mass spectrometry (GC-MS, 7890/5975C, Agilent Technologies, Santa Clara, CA, USA) equipped with an Rxi–5MS GC column (30 m × 0.25 mm × 0.25 μm). Briefly, helium at 1.1 mL min^{-1} was used as the carrier gas. The column temperature program started at 50 °C for 1.5 min. Subsequently, the oven was heated to 300 °C at an efficiency of 10 °C min^{-1} for 4 min. The solvent cut time was set to 2.6 min. Mass spectra were recorded at 1 scan s^{-1} under electronic impact with electron energy of 70 eV, and mass ranged 50–650 atoms to mass unit. The recoveries of TPT, DPT and MPT were 96%, 93% and 91%, and the detection limits were 250, 110 and 110 ng L^{-1}, respectively.

2.5. Analytical methods for assessment of intra- and extracellular protein

After TPT degradation, cells were concentrated by centrifugation at 3500*g* for 10 min and the supernatant was used to determine the extracellular protein concentration. Meanwhile, the cells were washed three times in cold phosphate-buffered saline (pH 7.4), resuspended in cold lysis buffer (30 mM Tris–HCl, 7 M urea, 2 M thiourea, and 4% w/v CHAPS at pH 8.5), and lysed by sonication in an ice bath for 15 min. The cellular debris was removed from the suspension at 16,000*g* for 5 min at 4 °C and the supernatant protein concentration quantified by the Bradford method.

2.6. Cellular activities in carbon nutrient use after TPT degradation

After TPT degradation, 1 mL MSM was mixed with 99 mL 0.85% sterilized saline solution. Then samples of 150 μL was inoculated into each well of the Biolog microplate, and incubated at 25 °C in the dark. The optical density at 590 nm of each well was determined every 12 h. The Biolog data were analyzed by an average well color development (AWCD) method.

3. Results and discussion

3.1. TPT biosorption and biodegradation

An increasing trend of TPT biodegradation with ascending concentration of TS from 5 to 80 mg L^{-1} was observed (Fig. 1A). Owing to in part the increased TPT solubility induced by TS (Fig. 1B), dissolved TPT uptake into cytoplasm through active transport and cell membrane interactions was accelerated, which accordingly enhanced TPT degradation as this process mainly takes place intracellularly [13,17]. For example, tributyltin (TBT) solubility increases 13% in the presence of Tween 80 at 1 critical micelle concentration [10]. The biosurfactant rhamnolipid at 5–25 mg L^{-1} improves TPT biodegradation by 15–23% [13]. Moreover, the surfactant can regulate cellular hydrophobicity and increase membrane permeability

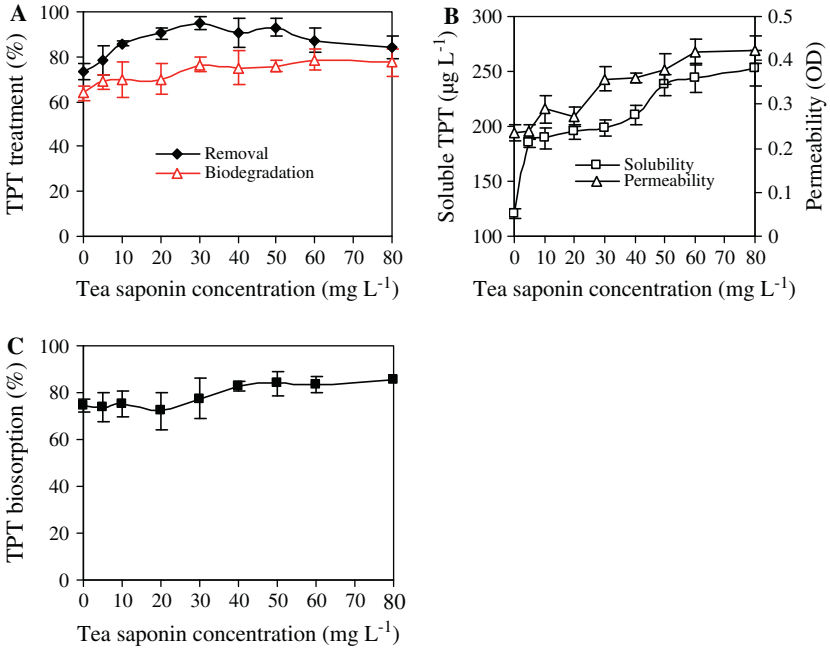

Fig. 1. Effect of TS at 5–80 mg L^{-1} on TPT treatment and solubility of 1 mg L^{-1} TPT, and cellular membrane permeability: (A) TPT degradation and removal by 0.3 g L^{-1} *B. thuringiensis* with TS for 7 d, (B) TPT solubility and cellular membrane permeability, and (C) TPT biosorption by 0.3 g L^{-1} dead cells with TS for 7 d.

(Fig. 1B), such that TS can encourage *B. thuringiensis* affinity for TPT, leading to higher TPT bioavailability. TBT degradation by *Aeromonas veronii* also has shown that TBT and its metabolites are bound to cell membrane lipids because of their hydrophobicity [18].

However, the removal exhibited a decreasing trend when the concentration of TS went up to 40 mg L^{-1} (Fig. 1A), but still higher than that of the control. This declining trend may be caused by TPT desorption or intracellular TPT release. To verify this inference, TPT biosorption by inactivated *B. thuringiensis* was conducted. The ascending biosorption efficiency exhibited in Fig. 1C confirmed that the release of intracellular TPT that made the removal efficiency decline in the addition of high level of TS. The above outcomes revealed that TS increased TPT solubility and biosorption, and membrane permeability, leading to a higher bioavailability of TPT. Similar to the effect of rhamnolipid, TS might also serve as a nutrient for cellular metabolism owing to its bioavailability and thus could be considered partially responsible for the promotion of TPT biodegradation [13]. This inference was verified by measurement of changes in protein content, cell density and nutrient use after degradation, as a reflection of *B. thuringiensis* growth.

3.2. Protein concentration and cell density after TPT biodegradation

The intracellular protein concentration increased with increasing TS at 5–40 mg L^{-1} (Fig. 2). This was consistent with TBT degradation studies in which suitable nutrients enhanced microbial activity, resulting in higher removal of this compound [10]. However, TS at high concentrations resulted in significant cell modifications that consequently govern cellular growth [19]. Thus, when the concentration was more than 40 mg L^{-1}, TS depressed the protein content increase to some extent in the current experiments but did remain enhanced in comparison with the control. The similar trend of cell density further confirmed that TS served as a nutrient that encouraged cell metabolism because of its bioavailability and was partially responsible for TPT biodegradation promotion. According to studies of cellular saturated fatty acids, surfactant rhamnolipids in MSM have also been shown as an effective carbon and energy source for *Pseudomonas stutzeri* [19].

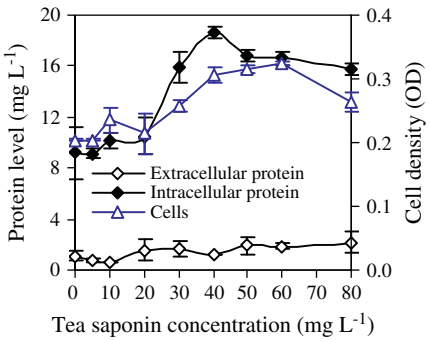

Fig. 2. Effect of TS on protein concentration and cell density after TPT degradation.

Besides the influence on intracellular protein, increasing TS concentrations also increased cellular protein efflux, further indicating that high surfactant concentrations triggered cellular membrane modification, which in turn encouraged the efflux of cytosolic materials. This finding is also consistent with the ascending trend of membrane permeability exhibited in Fig. 1B.

3.3. Ion use and release by cells during TPT biodegradation

In these experiments, ion concentration changes were measured as the experimental minus the control concentrations, such that a positive value indicated that *B. thuringiensis* released ions to the medium and a negative value implied bacterial ion absorption from the medium (Fig. 3).

In the absence of TS, considerable amounts of Cl^-, PO_4^{3-}, Na^+, NH_4^+, K^+, and Mg^{2+} ions were released to the medium, which indicated that TPT exposure led to increased membrane permeability to these ions. The high solubility of TPT in lipid, which ensures cell penetration [20], supports the present, observed ion releases. Studies using lipid and erythrocyte membranes as models for biological membranes have revealed that TPT is incorporated into these membranes and alters the molecular organization because of TPT

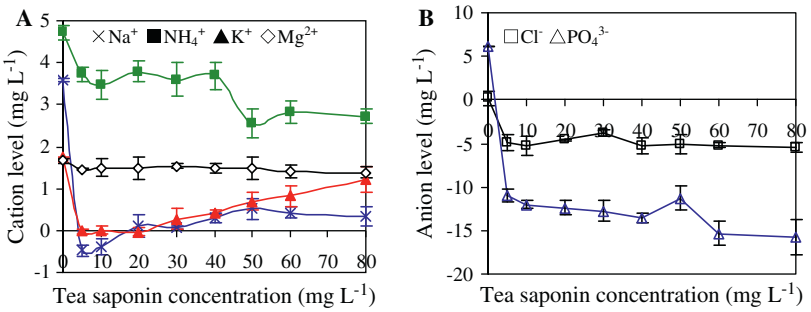

Fig. 3. Effect of TS on ion use and release during TPT degradation: (A) concentration change of cations, and (B) concentration change of anions.

interactions with membrane macromolecules of lipids and proteins [21,22].

Similar to heavy metal biosorption, the increased cation releases during TPT exposure might be related to ion exchange processes [23]. Cells could have released light metals to balance the uptake of TPT and its tin-containing catabolites. Moreover, TPT biodegradation is a metabolism-dependent process, coupled with nutrient use, that might also be partially related to the release of these ions.

These experiments showed that TS significantly decreased the efflux of cytosolic Na^+, NH_4^+, K^+, and Mg^{2+} and increased the influx of extracellular Cl^- and PO_4^{3-}. Although these released cations might still have functioned as osmolytes to balance the intracellular osmotic pressure with the environment, the significant use of Cl^- and PO_4^{3-} confirmed that TS exhibited a positive effect on cellular metabolism during the TPT degradation process, which was consistent with the present results (Figs. 1 and 2).

Na^+ and K^+ play an essential role in bacterial energy metabolism as well as in ionic homeostasis [24]. Their release is usually regarded as an objective verification of membrane permeability changes [24]. Based on the toxicity of organotins, TPT interactions with *Candida maltosa* have been assessed by the release of K^+ and alterations in membrane fluidity [25]. Similar to the present protein release, Na^+ and K^+ clearly increased with increasing TS, which further illustrated that this surfactant enhanced membrane permeability. However, the NH_4^+ efflux exhibited an opposite trend. As a component of proteins, more NH_4^+ was used to construct the N-terminal domain of proteins, considering the enhanced expression of intracellular protein in the presence of TS, which was confirmed in Fig. 2.

Mg^{2+} is an essential cofactor for many biological processes and indispensable for enzymatic activity, metabolic regulation, and nucleotide structural stability [26,27]. The Mg^{2+} release observed here was likely due to apoptosis in some cells or degradation of some Mg^{2+}-binding biological molecules. The addition of TS reduced this ion's efflux because some of the ion was used to increase cellular production.

PO_4^{3-} decreased the most significantly among all of the measured ions, reduced 17 mg L^{-1} in the presence of 5 mg L^{-1} TS, effectively shifting its cellular release to absorption. PO_4^{3-} uptake increased with increased TS concentration. PO_4^{3-} is a central component of energy-carrying molecules as well as cell membrane phospholipids. Living cells can absorb PO_4^{3-} across cell membranes during intracellular phosphorylation processes. The observably elevated PO_4^{3-} use by *B. thuringiensis* during concurrent exposure to TPT and TS demonstrated that these cells consumed more high-energy phosphate in degrading TPT and encouraged cellular reproduction. The observed changes in Cl^- concentration with increased TS showed a similar trend, compared with that of PO_4^{3-}. It is known that triorganotins mediate exchange diffusion of halides and other inorganic anions across biological and model membranes [28] and transport organic anions across the lipid bilayer by a mechanism involving exchange diffusion with Cl^- [17].

The correlation between surfactant concentration and the release of the measured ions was significant at a p value of 0.05 or 0.01 (Table 1). Moreover, the correlation among most of these ions was also significant, implying that absorption of some ions might have been coupled with the flow of others.

3.4. Cellular activities in organic carbon use after TPT degradation

The Biolog system was further used to evaluate the effect of TPT and TS on the cellular metabolic activities after TPT degradation. Although TPT has been reported to act as an inhibitor of some key enzymes [29], AWCD values shown in Fig. 4 revealed that the inhibitory effect of TPT exposure on cellular activities was lower than that of the prolonged nutrient starvation in the control, where there existed no exogenous organic carbon. To avoid the negative consequences upon exposure to toxic pollutants, some microorganisms possess resistance mechanisms, such as superoxide dismutases and metallothioneins [30]. These cellular reactions can be activated or enhanced by nutrients. It has been reported that TBT degradation by TBT-degrading bacteria was enhanced by the supplement of organic nutrient broth into the experimental medium [31]. TS in the current study also exhibited the function as nutrient, ascending the cellular metabolic activities in comparison with the control and TPT treatment.

3.5. PT dephenylation pathway

Examination of TPT, DPT, and MPT removal by *B. thuringiensis* with or without 60 mg L^{-1} TS, respectively, allowed clarification of whether dearylation during TPT degradation occurred successively or synchronously. TPT was indeed transformed into DPT and MPT, but no benzene was detected (Fig. 5A), which suggested that TPT elimination might initially proceeded by cleaving the aromatic ring, not by breaking tin-carbon bonds. The degradation rates per day during 3–7-d incubations were clearly lower than during the first 2 d, which implied that the TPT degradation potential of *B. thuringiensis* was depressed over time. As residual TPT declined with time, the collision between residual TPT and cells was reduced, thus decreasing biosorption of residual TPT. Furthermore, with longer degradation times, the secretion of intracellular material and the reduction of residual nutrients in the media (Figs. 2 and 3) exerted suppressive effects on cellular metabolic activity, which accordingly depressed the metabolism-dependent TPT biodegradation.

The observed linear, decreasing trend of DPT production illustrated that TPT was transformed to DPT, which was then further degraded (Fig. 5A). However, the MPT produced in the first day of incubation was relatively low, in the range of 51–60 μg L^{-1}, which was attributed to rapid transformation of MPT to inorganic tin or slow MPT production during the initial degradation of TPT

Table 1
Correlation between TS and ion concentrations.

Index	TS	Residual TPT	Na^+	NH_4^+	K^+	Mg^{2+}	Cl^-	PO_4^{3-}
TS	1							
Residual TPT	−0.960**	1						
Na^+	−0.224	0.254	1					
NH_4^+	−0.826**	0.832**	0.542	1				
K^+	0.288	−0.213	0.837**	0.082	1			
Mg^{2+}	−0.716*	0.670*	0.729*	0.808**	0.289	1		
Cl^-	−0.535	0.573	0.904**	0.774*	0.595	0.901**	1	
PO_4^{3-}	−0.615	0.632	0.892**	0.755*	0.563	0.887**	0.951**	1

* Correlation significant at $p = 0.05$ (2-tailed).
** Correlation significant at $p = 0.01$ (2-tailed).

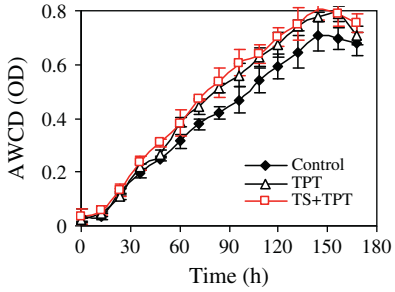

Fig. 4. Cellular metabolic activities in organic carbon use after TPT degradation for 7 d.

and DPT. After degradation for 4 d, the MPT content was higher than DPT, which suggested that TPT could be transformed into DPT and MPT synchronously, with further degradation of generated DPT to MPT, thus resulting in increased MPT production. Therefore, the ring cleavage of benzenes in TPT occurred both respectively and synchronously as these benzene rings are bonded individually with tin atom.

To further clarify the above inferences, degradation experiments with DPT or MPT at 1 mg L^{-1} were performed and it was found that the added surfactant clearly elevated DPT and MPT biodegradation. The residual concentration of MPT (Fig. 5B) was lower than that of DPT (Fig. 5C) at all time intervals regardless of whether surfactant was added or not. This indicated that MPT biotransformation to tin was not the rate-limiting reaction for TPT degradation. Thereby, MPT accumulation on the fourth day (Fig. 5A) demonstrated that a portion of MPT was formed directly from TPT transformation, in addition to being produced by DPT biodegradation. TPT contains three benzenes and one Cl^- that are connected with the tin atom through separate covalent bonds, such that in TPT's molecular structure, each benzene ring has the same chemical properties. Therefore, it was concluded that the ring cleavage of benzene could have occurred individually as well as synchronously.

3.6. Kinetics studies of PT biodegradation

In consideration of the complexity of the molecular structures of these PTs, TPT is the most complex and thus more difficult to be used by *B. thuringiensis*, while MPT has the simplest structure.

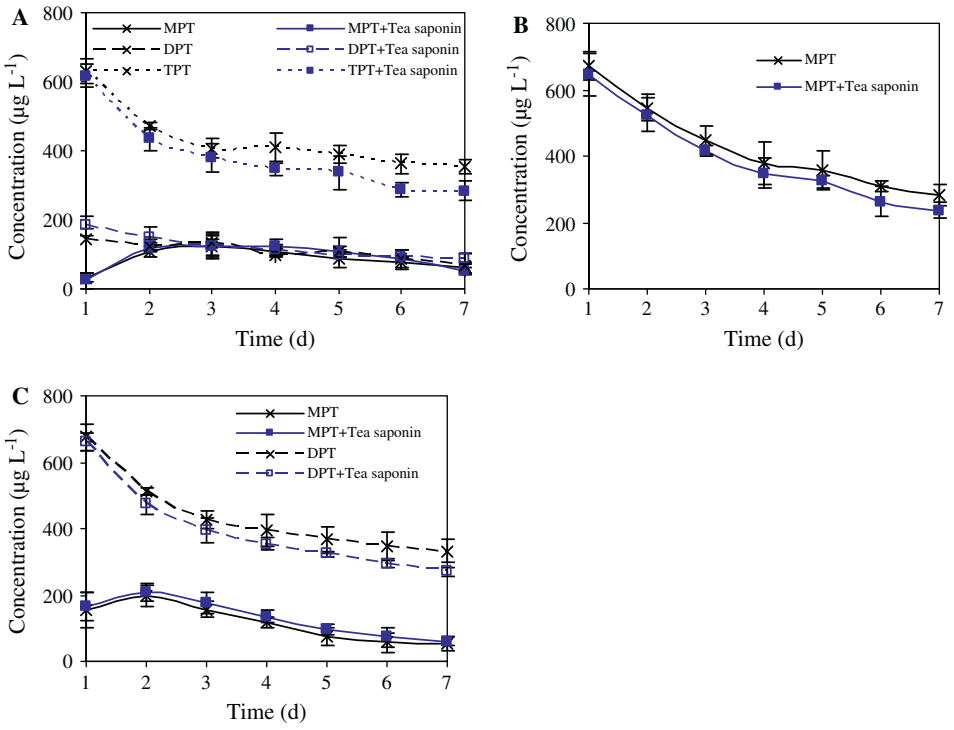

Fig. 5. PT dephenylation by 0.3 g L^{-1} *B. thuringiensis* and 60 mg L^{-1} TS for 1–7 d: (A) TPT degradation and its metabolites, (B) MPT degradation, and (C) DPT degradation and its metabolite.

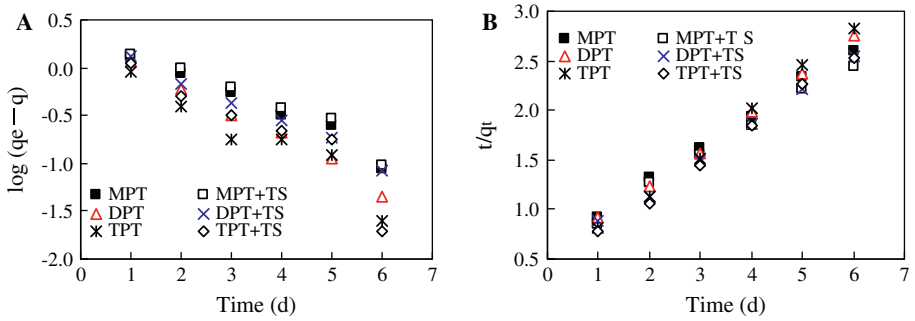

Fig. 6. Kinetic models of PTs biodegradation: (A) first-order kinetic model, and (B) second-order kinetic model.

Table 2
First- and second-order kinetic model constants for PT biodegradation.

Treatments	Q_e (exp) (mg g^{-1})	First-order rate constant K_1 (d^{-1})	First-order Q_e (theo) (mg g^{-1})	R^2	Second-order rate constant K_2 (g(mg d) $^{-1}$)	Second-order Q_e (theo) (mg g^{-1})	R^2
MPT	2.391	0.873	0.601	0.9675	0.187	2.965	0.9975
MPT + TS	2.550	0.943	0.610	0.9524	0.174	3.146	0.9951
DPT	2.227	0.539	0.767	0.9890	0.267	2.706	0.9977
DPT + TS	2.368	0.596	0.786	0.9882	0.254	2.930	0.9973
TPT	2.152	0.437	0.541	0.9039	0.503	2.408	0.9960
TPT + TS	2.389	0.889	0.508	0.8570	0.347	2.748	0.9959

The degradation kinetics of these compounds was determined by applying Lagergren's first- and second-order kinetic models to the data shown in Fig 5.

The equation for Lagergren's first–order kinetics is expressed as:

$$\log(q_e - q) = \log q_e - K_1 \cdot t/2.303,$$

where K_1 is the rate constant of first-order biodegradation (d^{-1}), q_e is the degraded quantity of PTs in terms of per gram biomass at equilibrium (mg g^{-1}), q is the amount of PTs degraded by *B. thuringiensis* at any given time (mg g^{-1}), and t any given time (min).

The second-order kinetic model is written as:

$$t/q = 1/(K_2 \cdot q_e^2) + t/q_e,$$

where K_2 is the rate constant of second-order biodegradation (g(mg d)$^{-1}$).

The resulting linear plots from this data treatment indicated that PT biodegradation fitted both the first- and second-order kinetic models (Fig. 6). The resulting rate constants illustrated that the PT degradation rate was consistent with their toxicity and molecular complexity. The more complicated the compound was, the slower it was degraded (Table 2). The slow degradation of complex PTs was also attributed to the production of benzene-containing metabolites that inhibited oxidation of their parent compounds. The high correlation coefficient of the second-order model confirmed that PT biodegradation followed this model better than the first-order version, which suggested that the biodegradation process consisted of extra- and intracellular transfer. Extracellular transfer inferred that biosorption was achieved through PT attraction by the cellular surface or via interactions of hydrophobic TPT with cell membranes [17], enhancing the efflux of intracellular materials during this process (Figs. 2 and 3). Intracellular transfer implied subsequent biotransformation as TPT degradation mainly takes place intracellularly [13].

4. Conclusions

PT biodegradation was initially triggered by cleaving a benzene ring and not by splitting covalent bonds between the aromatic rings and tin atoms. Benzene ring cleavage occurred individually and synchronously, producing DPT, MPT and tin accordingly. TS significantly enhanced PT solubility, biosorption, membrane permeability, protein expression, cell density, and Cl^- and PO_4^{3-} use, and reduced intracellular Na^+, NH_4^+, K^+ and Mg^{2+} release, accordingly increased PT biodegradation. After degradation by 0.3 g L^{-1} *B. thuringiensis* and 60 mg L^{-1} TS for 7 d, residual concentrations of TPT, DPT, and MPT decreased from 1000 to 283, 270, and 235 μg L^{-1}, respectively.

Acknowledgements

The authors would like to thank the National Natural Science Foundation (Nos. 21377047 and 21007020), the Natural Science Foundation of Guangdong Province (No. S2013010012662), and the Science and Technology Star Foundation of Pearl River (No. 2012J2200056) for their financial support.

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