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Isolation of Bacterial Culture Capable of Degrading Triphenyltin Pesticides

P. Visoottiviseth,* K. Kruawan,† A. Bhumiratana‡ and P. Wilairat§
* Department of Biology, ‡ Department of Biotechnology, § Department of Chemistry, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, and † Department of Microbiology, Faculty of Science, Burapha University, Cholburi, Thailand

A bacterial culture capable of degrading triphenyltin hydroxide (TPTOH) was successfully isolated from soil samples taken at a dockyard area in Samutprakarn province, Thailand. It was purified, identified and designated as *Pseudomonas putida no. C*. The bacterium isolated was found to have the capability of degrading TPTOH at levels of 7.0 ppm in 24 h. The addition of glucose enhanced the extent of degradation of TPTOH. Experiments were also conducted to immobilize *P. putida no. C* on various supports such as sand, cotton fibre and alginate. It was found that alginate was the best support material. Immobilized *P. putida no. C* on alginate was found to possess suitable characteristics and potential for future development in the removal of TPTOH from water and waste water systems.

Keywords: triphenyltin; degradation; bacteria; pseudomonas

INTRODUCTION

Triorganotin compounds have well known biocidal properties and have been used in many applications, including fugicides, miticides, molluscicides, nematocides, ovicides, rodent repellants, wood preservatives and antifouling paint biocides.[1] At the present time Thailand has a large and ready market for the application of these compounds, especially in the agricultural sector as pesticides for rice and other crops. However, the use of these compounds is not so widespread as in some other countries due to their high importation price. The successful development of a simple, efficient process for the manufacture of anhydrous stannic chloride ($SnCl_4$) from cassiterite ore concentrates could significantly reduce the total production costs of a range of inorganic tin and organotin chemicals.[2] The establishment of an organotin-synthesis industry in Thailand, could therefore be a possibility, and the product compounds could perhaps contaminate the aquatic system. Organotin compounds are known to be highly toxic to a variety of organisms,[3] and numerous aquatic organisms such as fish and oysters have been found to accumulate them.[4] This accumulation can result in movement up the food chain to higher trophic levels.[5]

Organotin compounds are composed of various derivatives such as tetraorganotins, triorganotins, diorganotins and monoorganotins. Each class of compounds has different degrees of toxicity towards organisms. Triorganotins are more toxic than diorganotins, which are much more toxic than monoorganotin derivatives.[6] Inorganic tin compounds ($SnCl_4$) are of low or minimal toxicity.[7] There is evidence to show that soil microorganisms can degrade triphenyltins slowly to inorganic tin via di- and monophenyltins.[8] In a study on the degradation of bis(tributyltin) oxide in soil, Barug and Vonk[9] found the products of degradation to be dibutyltin derivatives. However, attempts to isolate microorganisms capable of degrading the compounds were unsuccessful.

Triorganotins are strongly adsorbed to soil particles from which they leach out only slowly. The compounds can be concentrated by as much as 10 000-fold in the surface microlayer and up to 4000 times in sediment.[10] Thus, soils and aquatic sediments may serve as traps for toxic tin compounds, and soil and benthic organisms might be strongly involved in ecosystems which receive organotins.[11]

This research was performed in order to obtain information which will be needed if organotin-synthesizing industries are established in Thailand, or if the use of triorganotin pesticides becomes more widespread in the agricultural sector. The objectives of the research are as follows.

1. To isolate bacteria which are capable of

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degrading triphenyltin pesticides from the natural environment.

2. To investigate immobilization techniques for the isolated bacteria with the aim of removing the compounds from the waste water system of the organotin-synthesizing industries.

EXPERIMENTAL

Materials and methods

Preparation of culture medium

The minimal medium had the following composition ($g\,l^{-1}$): Na_2HPO_4, 15 g; KH_2PO_4, 3.0 g; NaCl, 0.5 g; NH_4Cl 1.0 g; trace elements* 1.0 ml; 1.0 M $MgSO_4 \cdot 7H_2O$, 2.0 ml; 1.0 M $CaCl_2$, 0.1 ml. The pH was adjusted to 7.4 with 5.0 M NaOH. If a solid medium was used, 15.0 g of agar was added.

Triphenyltin hydroxide (TPTOH) (F.W. = 367.03, lab. grade) was purchased from Ventron Co., USA and was not further purified before use.

Isolation of bacterial cultures capable of degrading TPTOH

Soil samples were collected from various locations in Thailand where there was a history of contamination from organotin biocides, e.g. sewage works, farmland, dockyards. The isolation method can be summarized as follows: each soil sample (10 g) was suspended in 100 ml of minimal medium. The suspension was filtered three times through Whatman filter papers (no. 4). Each 10 ml of suspension was added to 100 ml minimal medium containing 7–8 ppm TPTOH in a 250 ml flask and incubated at 30 °C on a shaker set at 200 rpm for seven days. The cultures were subcultured into a fresh medium and incubated under the same conditions. After three subsequent transfers of the culture to a fresh medium containing the same triphenyltin pesticide, the samples from flasks which showed high turbidity were subsequently streaked on a minimal medium agar plate containing 7–8 ppm TPTOH for isolation of single colonies. The isolated colonies were purified by restreaking on the same medium to confirm their ability to grow in the minimal medium containing TPTOH.

Identification of bacterial isolates capable of utilizing TPTOH

The selected bacterial isolates to be identified were examined for morphological appearance and biochemical properties. The tests included gram staining, catalase, citrate utilization, denitrification, gelatin hydrolysis, growth at 41 °C, indole production, Methyl Red, motility, oxidase, starch hydrolysis, triple sugar iron (TSI), unrease and Voges-Proskauer. The scheme used for identification followed the methods outlined in *Bergey's Manual of Systematic Bacteriology*.[12]

Biodegradation of TPTOH

Biodegradation tests were performed by inoculating 1% (v/v) of 24 h culture of the bacteria isolated into 250 ml Erlenmeyer flasks containing 100 ml minimal medium and 7–8 ppm TPTOH. Glucose (1% w/v) was added on one of the two flasks. Control of substrate stability was performed by preparing the medium in the same manner except no inoculum was added. Flasks were incubated at 30 °C on a shaker set at 200 rpm for 24 h. At the beginning and end of the incubation period, 10 ml of sample was withdrawn from each flask and centrifuged at 10 000 *g* for 10 min to remove bacterial cells and cell debris. The supernatant was collected and analysed for the concentration of TPTOH by the spectrofluorometric method as described by Blunden and Chapman.[13]

Kinetics of TPTOH degradation

Cultures of the isolated bacteria were grown in 500 ml Erlenmeyer flasks containing 100 ml minimal medium, 1% (w/v) glucose and 7–8 ppm TPTOH. Flasks were incubated at 30 °C on a rotatory shaker set at 200 rpm. Samples were withdrawn from each flask at 0, 2, 4, 6, 8, 10, 12, 18 and 24 h after inoculation. Each sample was centrifuged at 10 000 *g* and then the supernatant analysed for TPTOH by the spectrofluorometric method.[13] During the experiment, growth of the culture was also monitored by measuring absorption at 600 nm using a Spectronic 20 (Milton Roy Company).

The substrate control flask received similar treatment but without any inoculum. Each experiment was repeated three times.

Determination of adsorbed TPTOH by various cell components of the isolated bacteria

The isolated bacterial cultures were grown in

* Composition of trace elements: H_3BO_3, 11.4 g; $ZnSO_4 \cdot 6H_2O$, 2.2 g; $MnCl_2 \cdot 4H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.5 g; $CoCl_2 \cdot 6H_2O$, 0.16 g; $CuSO_4 \cdot 5H_2O$, 0.16 g; $NaMnO_4 \cdot 2H_2O$, 0.11 g; and EDTA, 5.0 g. All ingredients were dissolved in 100 ml of distilled water, and the pH was adjusted to 6.5–6.8 with 5.0 M NaOH.

500 ml Erlenmeyer flasks containing 100 ml minimal medium, 1% (w/v) glucose, and 7–8 ppm TPTOH. Cultures were incubated at 30 °C on a rotary shaker set at 200 rpm. Samples were withdrawn from the culture flasks at 0 and 24 h of incubation. Each sample was centrifuged at 10 000 *g* for 10 min to remove bacterial cells. The supernatant was analysed to determine the concentration of TPTOH. The pellets containing bacterial cells were washed four times with minimal medium to remove the remaining TPTOH which might be adsorbed on the cell surface. After each washing, supernatant was separated by centrifugation and analysed for the adsorbed TPTOH. To determine whether TPTOH was bound to the cell components, the cell pellet was suspended in 0.1 ml of minimal medium and suspensions were disrupted by sonicating for 7.5 min using 30 s periods at 15 s intervals by Soniprep (Soniprep 150 Ultrasonic Disintegrator, MSE sonicator Instrument Manor Royal, UK). After sonication, suspensions were centrifuged (10 000 *g*, 10 min), and the supernatant was used for the determination of TPTOH. In addition, 10 ml of toluene was added to the particulate fraction to remove TPTOH from the cell debris, and the supernatant obtained was used to determine the amount of TPTOH.

The substrate control flask received similar treatment but was not inoculated with *P. putida no. C.*

Experiment on immobilization of the isolated bacteria

Three types of media were used in the immobilization experiment, i.e. calcium alginate, cotton fibre (Central Department Store, Thailand) and Berkshire sand (Fluka Co., Germany).

Immobilization by entrapment in calcium alginate was performed according to the method of Bettman and Rehm[14] with some modifications. The isolated bacteria were grown in a minimal medium containing 1% (w/v) glucose for 24 h and centrifuged at 10 000 *g* for 10 min. The pellet was resuspended in water to obtain various cell concentrations (30, 40, 50, 60 and 70%) in equal volumes. The cell suspensions were mixed with the 2% (w/v) aqueous solution of calcium alginate. These mixtures were extruded through a needle gauge no. 24 into a 2% $CaCl_2$ solution. The alginate beads were about 4–5 mm in diameter. These immobilized cells were maintained in 2% $CaCl_2$ at 4 °C until used.

For immobilization on cotton fibre, the isolated bacterial culture was harvested by centrifugation (10 000 *g*, 10 min) and mixed with 10% gelatin (1 g cell/0.5 ml gelatin) at 45–50 °C. Cotton fibres (10 cm) were soaked in this mixture. After cooling at 4 °C for 1 h, the fibres were cross-linked using 20% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for 15 min. The fibres were subsequently washed with distilled water and maintained in 0.1 M phosphate buffer (pH 7.0). This procedure was modified from the method of Gianfreda.[15]

The procedure used for immobilization on sand was that of Ehrhardt and Rehm.[16] The cell suspension was mixed with Berkshire sand (1 g cell/5 g Berkshire sand). The sand was then washed twice with 0.01 M phosphate buffer (pH 7.0).

The ability of free and immobilized cells to degrade TPTOH

For a determination of the ability to degrade the triphenyltin pesticide, free cells or immobilized cell preparations were put into 10 ml of minimal medium containing 7–8 ppm of the pesticide. The method used in this experiment was that of Keweloh *et al.*[17] The mixture was incubated at 30 °C for 24 h. At various time intervals, samples were collected, centrifuged and the supernatants analysed for the remaining concentration of the pesticide by the spectrofluorometric method described previously.

RESULTS

Isolation and identification of bacterial culture capable of degrading TPTOH

Of 14 soil and water samples collected from various locations in Thailand, only one sample showed growth of microorganisms in minimal medium containing TPTOH after several successive transfers. This was the soil sample collected at the dockyard area. The microorganism from this culture was isolated, purified and identified.

The bacteria was a gram negative, aerobic, short rod. On nutrient agar, the isolate formed circular, smooth, entire, colourless and opaque colonies 1–2 mm in diameter when grown at 30 °C for 24 h. Further biochemical tests were performed according to the classification scheme outlined in *Bergey's Manual of Systematic Bacteriology*.[12] This isolate was identified as a *Pseudomonas putida*. Hence, it was designated as

Table 1 Degradation of TPTOH by *P. putida no. C*[a]

Carbon sources	Concentration of TPTOH (ppm)			
	Time of incubation (h)		Amount of removal	Removal (%)
	0	24		
Minimal media + TPTOH	7.45	7.35	0.10	1.34
Minimal media + 1% glucose + TPTOH	7.45	0.20	7.25	97.32
TPTOH control (no inoculum)	7.45	7.40	0.05	0.67

[a] All values are the average of three replicates.

Pseudomonas putida no. C,and this designation was used throughout the study.

Necessity for glucose in the biodegradation of TPTOH by *P. putida no. C*

The ability of the isolated bacteria to degrade TPTOH was assessed by growing the organism in the medium containing TPTOH, and the remaining concentration of TPTOH was determined by spectrofluorometer after 24 h of incubation. The results (Table 1) show that the isolate could only degrade TPTOH when glucose was present in the medium. Without glucose, the organism could neither remove TPTOH nor grow. In the presence of 1% (w/v) glucose, the organism could remove approximately 7.25 ppm of TPTOH from the total of 7.45 ppm in the medium within 24 h.

Kinetics of degradation of TPTOH by *P. putida no. C.*

The growth pattern of *P. putida no. C.* and its ability to remove TPTOH are shown in Fig. 1. The concentration of TPTOH in the medium was found to gradually decrease with concurrent increase in the turbidity of the culture of *P. putida no. C.* As shown in Fig. 1, the experiment was repeated three times,and there was not much variation in the pattern of growth nor in the pattern of decrease in the level of TPTOH. In the early incubation period, the removal rate of TPTOH was low. However, between 8 and 12 h the removal rate was higher. The removal rate was lower again after 12 h. *P. putida no. C* could remove approximately 7.25 ppm of TPTOH from the medium within 24 h. The rates of removal of TPTOH in early, middle and late incubation periods are 0.24, 1.23 and 0.05 ppm h^{-1}, respectively.

The presence of TPTOH in various cell fractions of *P. putida no. C.*

The decrease of TPTOH in the culture caused by *P. putida no. C.* in the presence of glucose raised the possibility that TPTOH might be metabolized and/or adsorbed to various cell fractions. Thus, experiments were carried out in order to determine the presence of TPTOH in various cell fractions. The cell culture was fractioned into four fractions namely, supernatant, the washing liquids (first, second, third and fourth), broken cells (cell free extract) and cell debris. The procedures employed were as described in Materials and Methods. The results in Table 2 showed that there was some TPTOH bound to the cells of the bacteria; however, more than 74% of the TPTOH could not be recovered in any of the fractions. After extractive washing there was only about 7 μg (9%) TPTOH which could be recovered. Thus, it appeared there was very little TPTOH bound to cell surfaces. Also, after the cells were broken, the amount of bound TPTOH in the cell

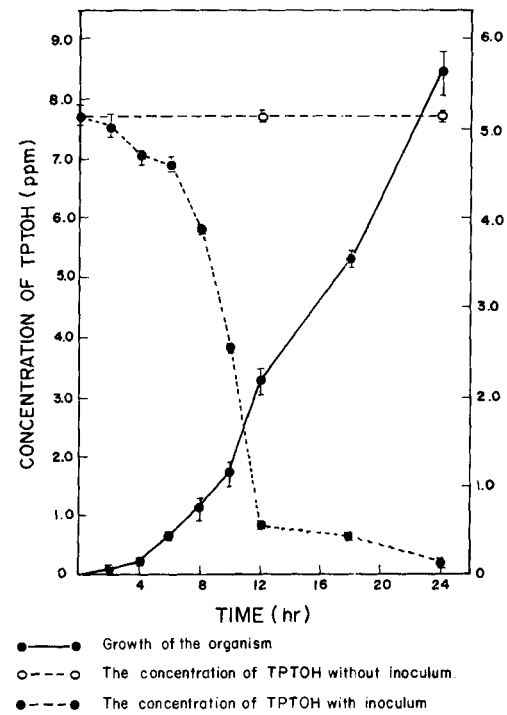

Figure 1 Kinetics for the growth and removal of TPTOH by *P. putida no. C.*

Table 2 The presence of TPTOH in supernatant and various cell fractions of *P. putida no. C*

Cell fractions	Amount of TPTOH[a,c] (μg)	TPTOH (%)[c]
Supernatant	2.5	3.36
First washing	2.0	2.68
Second washing	2.0	2.68
Third washing	2.0	2.68
Fourth washing	1.0	1.34
Broken cell	4.5	6.04
Cell debris	5.5	7.38
TPTOH control[b]	74.5	100.00

[a] Total amount of TPTOH in each fraction.
[b] Total amount of TPTOH added to the original cell suspension.
[c] All values are the average of three replicates.

fragments and the TPTOH accumulated in cells was determined. The amount of TPTOH which could be recovered from cell fragments was very low, i.e. 4.5 μg (6%). Furthermore, only 5.5 μg TPTOH was found to be attached to the cell debris. Hence, out of 74.5 μg TPTOH removed by *P. putida no. C.* from the medium, only 19.5 μg (26%) could be detected as TPTOH in these seven fractions. The quantity of 55 μg (74%), which could not be detected, might either be degraded or transformed into some other metabolites by the bacterial suspension.

Degradation of TPTOH by immobilized *P. putida*

In view of the possible application of immobilized *P. putida no. C* to remove TPTOH, various immobilization techniques, namely entrapment in calcium alginate and absorption on cotton fibre and sand were examined. The immobilized *P. putida no. C* in calcium alginate showed the highest activity in removing TPTOH, whereas immobilization on cotton fibre and on sand possessed reduced ability to remove TPTOH. The result, as shown in Table 3, indicates that the activity of the immobilized *P. putida no. C* in calcium alginate, cotton fibre and sand was 60%, 0.5% and 4%, respectively, in comparison to the free cell suspension. During the experiment, many bacterial cells were found to be released from the supporting materials into the medium when cells were immobilized on cotton fibre and on sand. However, a very small leakage of cells appeared when the cells were immobilized in calcium-alginate. Thus, the lowered efficiency in the removal of TPTOH by immobilization on cotton fibre and on sand is due to the reduced number of cells.

Table 3 The effect of immobilization techniques on the removal of TPTOH by *P. putida no. C*[a]

Method of immobilization	Activity (%)
Whole cell (non immobilized)	100
Calcium alginate	60.2
Cotton fibres	0.45
Sand	3.81

[a] All values are the average of three replicates.

Degradation of TPTOH by free and calcium-alginate immobilized *P. putida no. C.*

Earlier investigation in this study indicated that cell immobilization using calcium alginate retained considerable activity in removing TPTOH; thus, this system was investigated further. The results are summarized in Table 4. In the early period, the ability of free cells (95%) was higher than immobilized cells (57%) in removing TPTOH. However, after the third day of incubation, the ability of the immobilized cells was found to be better than the free cell suspensions. It is interesting to note that the free cell suspension changed from an orange to a brown colour by the end of the experiment. However, the immobilized cell did not change colour. The bacterial cells were not released from the immobilized medium, even after an extended period.

The free cell suspension, immobilized *P. putida no. C* and calcium alginate beads alone (no bacterial cells) were determined for TPTOH which might have remained. As shown in Table 5, there were small amounts of TPTOH which had accumulated inside the cell and which were bound to cell surface and/or cell debris. The amount of TPTOH detected from immobilized cells did not differ much from those of the free cells. Furthermore, the level of TPTOH absorbed by the alginate bead containing no *P. putida no. C* was minimal.

The effect of inoculum size on removal of TPTOH by immobilized *P. putida no. C.*

Experiments were conducted in order to investigate the effect of increasing levels of cell concentration in the alginate beads on the efficiency of

Table 4 The removal of TPTOH in free and immobilized cells of *P. putida no. C*[a]

	Free cell		Immobilized cell	
Incubation (days)	Concentration of TPTOH removed (ppm)	Removal (%)	Amount of TPTOH removed (ppm)	Removal (%)
1	2.85	100	1.6	56.14
2	2.70	94.74	1.62	56.84
3	2.25	78.95	1.53	53.68
4	1.30	45.61	1.47	51.58
5	0.93	32.46	1.55	54.39
TPTOH control	0	0	0	0

[a] All values are the average of three replicates.

removal of TPTOH. Before storage, the activity of each cell concentration in removing TPTOH was almost the same (55%). Each concentration of immobilized cells was then subjected to various storage intervals and was tested for activity every week for five weeks. After one month the immobilized cells were assayed for ability to remove TPTOH. As shown in Fig. 2, the preparation containing 30% cells gave the highest activity, i.e. about 50% TPTOH removed as compared to free cells, but its ability decreased after two months storage (to 34%). For 40% cells and 50% cells, their abilities were about 30%, except for the first treatment. Although the immobilized cells were stored for two months, their activity was not decreased. During the experiment, there was no evidence of leakage of bacterial cells from the beads, except from the beads containing 60% cells. For 70% cells, the gel beads containing cells broke at the first treatment, so they were not tested further.

Table 5 The remaining concentration of TPTOH in various portions

	Remaining concentration of TPTOH (ppm)			
Treatment	Medium	Washing liquid	Broken particle	Debris
Free cell	0.2	0.06	0.14	0.01
Immobilized cell	0.18	0.08	0.15	0.1
Gel bead	2.73	0.08	0.05	0.01
TPTOH Control	3.87	3.87	3.87	3.87

[a] All values are the average of three replicates.

DISCUSSION

Only one bacterial isolate, out of 14 potential isolates, showed persistent turbidity after several transfers in media containing a triphenyltin pesticide. This result suggests that the ability to degrade the pesticide was not widespread amongst the isolated microorganisms. some cultures grew very well in the early stages but did not persist in growth later in the presence of the pesticide. These organisms might be able to utilize nutrients in soil samples, so they died after these nutrients were diluted out upon several transfers. The isolated bacteria were identified as *Pseudomonas putida* and designated as strain no. C. It is not surprising to find that the ability to degrade triphenyltin occurs in bacteria belonging to the genus *Pseudomonas*. Bacteria in this genera are found in sewage, soil and water and are responsible for the degradation of many xenobiotic compounds in the environment. Pseudomonads were found to be involved in the bioconversion of carbofuran, methyl parathion, 3-chlorobenzoic acid and others.[18]

The isolated *P. putida no. C* could remove triphenyltin and TPTOH only in the presence of glucose (Table 1). Attempts to isolate microorganisms which could utilize TPTOH as the sole carbon source were not successful. An extensive review of the literature indicated that there were no reports on the ability of any organism to use TPTOH as the sole carbon source. Nor were there any reports on the isolation of microorganisms which can degrade TPTOH in the presence of glucose. There are two possible explanations for this. First, TPTOH was absorbed by *P. putida no. C* when the cell numbers were increased, which subsequently led to a decrease in the con-

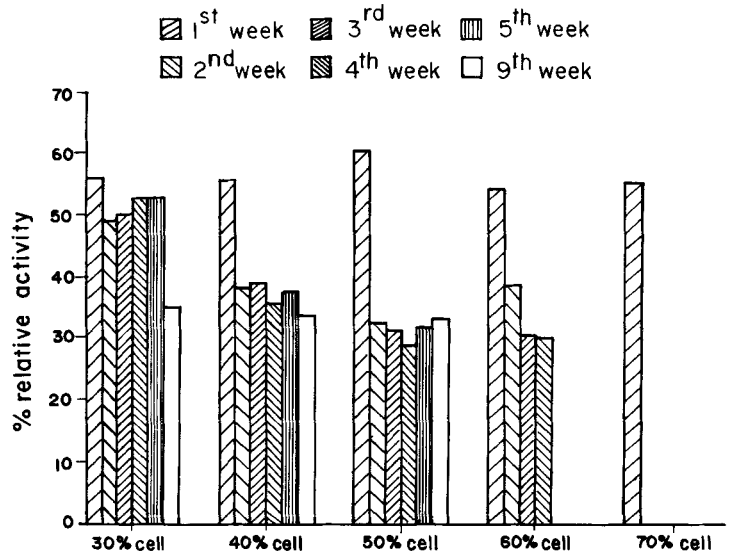

Figure 2 The effect of various concentrations of *P. putida no. C* in alginate beads and the length of each preparation on the removal of TPTOH.

centration of TPTOH in the media. Blair[19] found that there was an accumulation of the tri-n-butyltin cation by tin-resistant estuarine bacteria. Secondly, *P. putida no. C* might co-metabolize TPTOH with glucose. TPTOH could not serve as the sole source of carbon and energy for *P. putida no. C*, nor would removal of TPTOH occur only in the obligate presence of glucose. Pfaender[20] reported that the addition of glucose to raw sewage enhanced the rate of 1,1,1-trichloro-2,2-bis (*p*-chlorophenyl)ethane (DDT) decomposition. The rate of DDT decomposition and rate of its metabolite formation are diminished in the absence of glucose.

The results in this study indicate that the concentration of the pesticide decreased with time while the bacteria grew in number (Fig. 1). There was a lag period of about 4 h in the early incubation period in which the rate of growth of the bacteria and the rate of removal of the organotin compounds were low. This may be due to a need for the bacteria to adapt themselves to the new environment and consequently the reproduction rate is slow in this phase. Between 6 and 12 h there was a rapid increase in bacterial growth as well as a marked decrease in pesticide concentration. Almost 80% of the organotin compounds had now been removed from the incubation medium. After 24 h essentially all the compounds had been degraded. The results of this study agree with that of Barug[9] who reported the degradation of bis(tributyltin) oxides by *P. aeruginosa*.

This experiment also showed that the triorganotin pesticides that was removed from the solution was not absorbed to any great extent into the various cellular compartments of the bacteria. It is expected that the pesticide was degraded into di- or monoorganotin compounds, or other metabolites, which are not sensitive to the fluorescence technique used to monitor the triorganotin compounds (data not shown). This result corresponds with that of Barnes *et al.*,[8] who studied the degradation of triphenyltin fungicides. Degradation rather than absorption is proposed as the agent for removal also on the grounds that any microorganism producing cell growth would remove TPTOH from the solutions if the latter (absorption) was the case.

Studies of the presence of TPTOH in various cell components (Table 4) indicated that there were 19.5 μg (26%) TPTOH ml^{-1} present in various cell fractions of *P. putida no. C*. Cooney[11] reported that organotin compounds were more soluble in lipid, thus they could penetrate biological membranes and were more likely to accumulate in lipid-rich tissues or organelles. In addition, Blair *et al.*,[19] found that tin-resistant esturarine bacteria isolated from Chesapeake Bay could accumulate tributyltin by a process requiring no energy, probably by adsorption to the cell envelope. However, this study showed that as much as 55 μg (74%) of TPTOH ml^{-1} could not be detected. Some fractions of TPTOH might be so tightly adsorbed to the bacterial cells that the

extracting solvent could not extract them from the cells. On the other hand, they might be degraded or transformed into other metabolites. There is some evidence to show that organotin compounds can be degraded by microorganisms. Barug[21] found that two bacteria and three fungi could debutylate tributyltinoxide (TBTO). Fungal cultures isolated from wood apparently degraded TBTO to dibutyltin and monobutyltin.[22] Barnes *et al.*[8] showed that soil microorganisms degraded triphenyltin acetate (TPTOAc) slowly ($t_{0.5} = 140$ days) to inorganic tin via di- and monophenyltins. Similar degradation pathways have been found in rats and on the leaves of sugar beets.[23] Studies on the metabolism of triphenyltin compounds in rats have found that di- and monophenyltin compounds were formed along with inorganic tin.[24] Hence it can be concluded from this study that the isolate *P. putida no. C* can degrade TPTOH (7.0 ppm in 24 h) only in the presence of glucose as a co-metabolite of TPTOH.

The immobilization technique which entrapped *P. putida no. C* in calcium-alginate was more efficient in removing TPTOH than adsorption onto cotton fibre or Berkshire sand. Adsorbed cells leaked from the carrier during use in the latter two techniques. Thus, their removal efficiencies were low. Bettmann and Rehm[11] found that *Pseudomonas sp.*, immobilized in alginate, was able to degrade 2 g l^{-1} of phenol in 35 h. However, they later showed that *Pseudomonas sp.*, which were absorbed on the activated carbon, could degrade 1 g l^{-1} of phenol in 50 h.[25]

When the activities of the free and immobilized cells were compared (Table 4), it was found that the activity of free cells in removing TPTOH decreased whilst that of the immobilized cells was constant, even when the immobilized cells were reused five times. This is the advantage of immobilized cells, i.e. they may be reused repeatedly. Bettmann and Rehm[14] observed that immobilized cells could tolerate higher phenol concentrations than free cells. Moreover, in this experiment, the TPTOH was removed without the use of glucose addition.

The effect of various concentrations of *P. putida no. C* in alginate beads versus length of storage was also studied. It was found that beads containing 0.43 g wet wt. (30% cells) were best and that the beads could be used for five weeks without losing their efficiency. This might be explained by the fact that for free cells, which grow at low cell densities (typically 2.0×10^6 cells ml^{-1}), immobilization offers the potential to increase unit cell density about 20–50 fold.

Results indicate that the immobilization of *P. putida no. C.* by entrapment in calcium alginate could be a suitable method of removing TPTOH because the activity of the immobilized cells was stable and could be used repeatedly and continuously. Thus, the method of using immobilized *P. putida no. C* in calcium alginate might offer the potential to increase effectively the removal of TPTOH from water and waste water treatment systems. However, more experimental work will still be required to search for the most effective means of removal of TPTOH from waste materials using immobilized *Pseudomonas putida no. C* in calcium alginate beads.

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REFERENCES

1. Anon., *Environmental Health Criteria, 15: Tin and Organotin Compounds—A Preliminary Review*, World Health Organization, Genevaa (1980).
2. Anon., *International Tin Research Institute—Annual Report* (1987).
3. L. W. Hall, Jr. and A. E. Pinkney, *Crit. Rev. Toxicol.* **14**, 159 (1985).
4. J. W. Tas, J. L. M. Hermens, M. Van den Berg and W. Seinen, *Mar. Environ. Res.* **28**, 215 (1989).
5. L. McDonald and J. Trevors, *Water, Air and Soil Pollution* **40**, 215 (1988).
6. S. J. Blunden, L. A. Hobbs and P. J. Smith, The Environmental Chemistry of Organotin Compounds. In: *Environmental Chemistry*, 1978, International Tin Research Institute.
7. S. J. Blunden, P. A. Cusack and R. Hill, *The Industrial Uses of Tin Chemicals*, The Royal Society of Chemistry, London (1985).
8. R. D. Barnes, A. T. Bull and R. C. Poller, *Pesticide Sci.* **4**, 305 (1973).
9. D. Barug, and J. W. Vonk, *Pesticide Sci.* **11**, 77 (1980).
10. R. J. Maguire, *Environmental Sci.* **18**, 291 (1984).
11. J. J. Cooney and S. J. Wuertz, *Ind. Microbiol.* **4**, 375 (1989).
12. N. R. Krieg and J. G. Holt, *Bergey's Manual of Determinative Bacteriology*, The William & Wilkin Co., Baltimore (1984).
13. S. J. Blunden and A. H. Chapman, *Analyst.* **103**, 1266 (1978).
14. H. Bettman and H. J. Rehm, *Appl. Microbial. Biotechnol.* **20**, 285 (1984).

15. L. Gienfreda, P. Palascandola and V. Scardi, *Eur. J. Microbiol.* **11**, 6 (1980).
16. H. M. Ehrhardt and H. Rehm, *J. Appl. Microbiol. Biotechnol.* **21**, 32 (1985).
17. H. Keweloh, H. J. Heipieper and H. J. Rehm, *J. Appl. Microbiol. Biotechnol.* **31**, 383 (1989).
18. E. Dorn, M. Hellwig, W. Reineke and H. J. Kunckmuss, *Arch. Microbiol.* (submitted).
19. W. R. Blair, G. J. Olsm, F. E. Brinckman and W. P. Iverson, *Microbiol. Ecol.* **8**, 241 (1982).
20. F. K. Pfaender and M. Alexander, *J. Agric. Food Chem.* **21**, 397 (1973).
21. D. Barug, *Chemosphere*, 1981, 1145.
22. R. J. Osler and G. E. Holland, *Int. Biodeterior. Bull.* **18**, 95 (1982).
23. J. J. Cooney, *J. Ind. Microbiol.* **3**, 195 (1988).
24. E. C. Kimmel, R. H. Fish and J. E. Casida, *J. Agar. Food Chem.* **25**, 1 (1977).
25. H. Bettman and H. Rehm, *J. Appl. Microbiol. Biotechnol.* **22**, 389 (1985).