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Tributyltin (TBT) Tolerance of Indigenous and Non-indigenous Bacterial Species

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Abstract Tributyltin (TBT) is a species of organotin compound (OTC), used as antifouling biocide in boat and ship paints to prevents the attachment of marine organism on their hull surfaces. Tributyltin was found to be very toxic to a variety of targeted and non-targeted organisms and has high persistence in sediments even after the total global ban by the International Maritime Organization (IMO) in 2008. Therefore, there is an urgent need to clean up TBT-polluted environments after the global banning due to the significant risks it poses to the human and aquatic organisms for its slow degradation rate. In selecting bioremediation agents, indigenous bacteria were documented to be of great potentials compared to non-indigenous. In this study, comparison was made between a bacterial isolate *Klebsiella* sp. FIRD 2, isolated from TBT-contaminated surface sediment and *Pseudomonas* specie isolated from non-TBT-contaminated soil. Previously, we isolated, screened, and identified *Klebsiella* sp. FIRD 2 as a TBT-resistant bacterium from TBT-contaminated surface sediment of Kong Kong Laut, Johor, Malaysia. The isolate was able to resist TBT up to 1500 μg/L without addition of carbon source in minimal salt medium (MSM). *Pseudomonas* sp., isolated from non-TBT-contaminated soil was tested in MSM treated with different concentration of TBT. The bacterium did not endure to survive in TBT-treated media without addition of carbon source; thus, the strain has no ability to utilize TBT as source carbon. Growth of *Pseudomonas* sp. was observed in MSM treated with TBT at concentration of 500 μg/L and 1000 μg/L along with addition of glucose as carbon source. No growth of *Pseudomonas* sp. was observed in MSM with higher TBT concentration even with additional of carbon source. This study equally endorses the potentials of indigenous bacteria in bioremediation of TBT contamination.

Keywords Degradation · Microorganism · Organotin · Pollution · Tributyltin · Bacteria

Highlights This work was a continuation of previous study titled "*Klebsiella sp.* FIRD 2, a TBT-resistant bacterium isolated from contaminated surface sediment along the Strait of Johor Malaysia". The study is anticipated to make significant impact in conducting future research on TBT bioremediation in the tropical areas. TBT tolerances of indigenous and non-indigenous bacterial species were successfully assessed, and the study ultimately endorses the potentials of indigenous bacteria in bioremediation of TBT contamination.

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1 Introduction

Tributyltin was generally used as an antifouling biocide in boat paints to prevent the attachment of marine organism on the hull which makes it slimy (Antizar-Ladislao 2008a, b). TBT has been classified to be a very toxic compound and poses significant danger to a broad diversity of organisms at levels recounted to be present in the polluted environments. TBT usage as constituent of antifouling paints has been totally forbidden by the International Maritime Organization (IMO) since 2008 (Sakultantimetha et al. 2011). Yet, higher concentrations have been recounted in sediments from marine and fresh water in many places across the globe (Harino et al. 2008). In demands to conserve the marine habitats and prevent the danger it poses to ecosystems, there is a glaring need to contain TBT occurrence. TBT has been discovered to affect non-targeted organisms, and the most commonly reported case was imposex in marine gastropods, the superimposition of male features (notably a penis and vas deferens) onto female snails (Bettin et al. 1996; Mohamat-Yusuff et al. 2010; Santillo and Langston 2001; Terlizzi et al. 2004).

Malaysia is one of the countries with the busiest maritime activities and well documented with high level of TBT in the marine environments. Serious organotin (OT) contamination, especially from TBT, was reported in the busy Strait of Malacca and the Strait of Johor (Sudaryanto et al. 2004). Later study conducted by Harino et al. (2008) found that concentration of monobutyltin (MBT), dibutyltin (DBT), and TBT in sediments from the coastal areas of Peninsular Malaysia within the ranged from 4 to 242 μg/kg dry weight, from 1 to 186 μg/kg dry weight, and from 0.7 to 228 μg/kg dry weight, respectively (Harino et al. 2008). The author reported that the highest concentration of MBT, DBT, and TBT were found in samples collected from the Strait of Johor (MBT, DBT, and TBT ranging from 83 to 542 μg/kg dry weight, from 30 to 232 μg/kg dry weight, and from 41 to 492 μg/kg dry weight, respectively) (Harino et al. 2008). The level of OT in marine mollusk (*Thais gradata*) from the Strait of Johor and the southern part of the Strait of Malacca has been also reported at a high level (Mohamat-Yusuff et al. 2010). Its presence above the threshold limit of a particular species could cause some adverse effects such as imposex among female neogastropods (Mohamat-Yusuff et al. 2010, 2013) and even up to carcinogenic effects with no significant evidence of immunotoxicity. Among all sites reported in the literature, Kong Kong Laut area in Johor could be labeled as the hot-spot of TBT contamination since the marine mollusk collected in this site experience a high level of imposex incidence and accumulated a high level of TBT (Mohamat-Yusuff et al. 2013). TBT contaminations in sediments at Kong Kong Laut were as high as 790 μg/L even after the total global banning on the year 2008 (Mohamat-Yusuff et al. 2013). TBT was found to have deleterious effects on both prokaryotic and eukaryotic organisms (Antizar-Ladislao 2008a, b) while in humans, it was found to inhibit the immune system and cause endocrine disruption (Dubey et al. 2006). Tributyltin has been found to accumulate in the body of several marine organisms, and these organisms are very important sources of food for human as well as other organisms.

Organotin compounds are group of chemicals that have at least one tin–carbon bond, joined covalently (Rüdel 2003). A maximum of four (4) carbon-based groups can be fused to the tin (Sn) atom. The compound has the general formula $R_{(4-n)}SnX_n$ with $n = 0–3$, R is the organic group which could be alkyl or aryl that are covalently bonded with the Sn atom (Gianguzza 1997). X represents anions (–OH, –SH, $–OSnR_3$). Organotins are hydrophobic because of the hydrocarbon substituent, which depends on the degree of the alkylation/arylation. Its chemicals are designated as mono-, di-, tri-, or tetra-substituted butyltin and pentyltin compounds (MBT, DBT, TBT, MPT, DPT, TPT) (Roy et al. 2004; Thoonen et al. 2004). The properties of OTCs depend on their molecular structure and may differ significantly. It is remarkable to know that triorganotins whether aliphatic or aromatic have the peak toxicity, followed by diorganotins, then monoorganotins.

Although organotin degradation has been shown to be mediated by microorganisms, information is still limited in relation to the mechanism of degradation, tolerance of the microbes, and their relative significance. Adelaja and Keenan (2012) isolated TBT-resistant bacteria, tested it for growth in the presence of methylmercury, and found out that the isolates have potential to detoxify not only TBT but also MeHg. These bacteria were *Pseudomonas fluorescens*, *Enterobacter cloacae*, *Citrobacter braakii*, and *Alcaligenes faecalis*. They also reported that the isolates utilize carbon sources from the compound. They recommend that *E. cloacae* was the most preferable isolate resistant to TBT and MeHg and

also stated that TBT-degrading bacteria must be a TBT-resistant bacteria, however, it may not necessarily degrade TBT (Wuertz et al. 1991). Similarly, Khanolkar et al. (2014) isolated bacterial strain (*Pseudomonas stutzeri* DN2) from estuarine sediment of the west coast of India and found out that the isolate was capable of utilizing Tributyltin chloride (TBTCl) as a sole carbon source. The isolate resisted up to 3 mM of TBT level and showed maximum growth at 2 mM TBTCl in MSM.

In recent studies conducted, we reported *Klebsiella* sp. FIRD 2 isolated from contaminated surface sediment along the Strait of Johor, Malaysia, as the most preferable bacterial isolate suitable as TBT remediation agent among other TBT-resistant isolates (Abubakar et al. 2015). The isolate was found to be Gram negative and resisted up to 1500 μg/L of TBT in MSM without addition of carbon source (Abubakar et al. 2015).

This work was aimed at the comparison between bacterial isolate *Klebsiella* sp. FIRD 2 isolated from TBT-contaminated surface sediment and *Psuedomonas* sp. isolated from non-TBT-polluted soil based on their tolerance to TBT concentrations. The study is expected to validate previous studies on the potentials of indigenous bacteria in bioremediation and to contribute on the existing knowledge in order to boost the remediation of TBT and other xenobiotic compounds into a less toxic forms.

2 Materials and Methods

All chemicals were used without additional purification and purchased from Sigma-Aldrich Co., USA. TBTCl 96 %, methanol HPLC grade, minimal salt media (KH_2PO_4, $MgSO_4{\cdot}7H_2O$, NH_4Cl, $FeSO_4{\cdot}7H_2O$, $CaCl_2$, NaCl, and yeast extract) were all of analytical grade. TBT stock was prepared from 96 % TBTCl, 21,000 μg/L (1 μL in 20 mL methanol) and kept in the dark at 4 °C (Wuertz et al. 1991). All gradient concentrations were prepared using the dilution formulae $M_1V_1 = M_2V_2$ from the stock.

2.1 Isolation

Bacterium isolated from TBT-contaminated area (*Klebsiella* sp. FIRD 2) was sub-cultured from the stock of the previous studies (Abubakar et al. 2015) while the bacterium isolated from a non-TBT contaminated soil was sub-cultured from the stock collected from the Faculty of Environmental Studies, Universiti Putra Malaysia. The culture was incubated at 27 °C for 24 h.

2.2 Growth of *Klebsiella sp.* FIRD 2 in TBT Concentrations

Study on the growth behavior and TBT-resistance of the selected bacterial isolate was conducted in minimal salt broth (MSB) supplemented with varying TBT concentrations without addition of carbon source for 6 days on a rotary shaker at 150 rpm at 27 °C (Gokulakrishnan and Gummadi 2006; Khanolkar et al. 2015). The TBT concentrations were 0, 500, 1000, 1500, 2000, and 2500 μg/L. Concentration 0 μg/L was set as a negative control, cultured in MSM and inoculated with the selected isolate without carbon source and TBT.

Readings were taken as change of absorbance (OD_{600}) using a spectrophotometer at a day interval, and graph was plotted between absorbance and time interval. All steps were conducted in triplicate.

2.3 Growth of *Pseudomonas* Species in TBT Concentrations

The isolate (*Pseudomonas* sp.) was cultured in a MSM supplemented with varying TBT concentrations (0, 500, 1000, 1500, 2000, and 2500 μg/L) in MSM with TBT, and 0 μg/L TBT concentration was considered as the control. To find out the activity of the cells grown in different concentrations of TBT, the culture was incubated at 27 °C, with constant shaking at 150 rpm (Khanolkar et al. 2015). Absorbance OD_{600} of the culture was recorded using a spectrophotometer at day intervals, and the graph was plotted between time and absorbance.

3 Results and Discussion

3.1 Effects of TBT Concentrations Against *Klebsiella* sp. FIRD 2

Determination of the extreme tolerable concentration of TBT by *Klebsiella* sp. FIRD 2 bacterium was achieved by culturing the isolate in MSB for 6 days at different TBT concentrations without addition of carbon source. This was done on a rotary shaker at 150 rpm 27 °C in the dark to protect the compound from photolysis

(Gokulakrishnan and Gummadi 2006). The concentrations were 500, 1000, 1500, 2000, and 2500 μg/L. Results were taken as change in bacterial population density (i.e., change in absorbance, OD_{600}) using a spectrophotometer.

Figure 1 presents the growth pattern of the isolate at different TBT concentrations. At 500 μg/L TBT concentration (Fig. 1), the isolate grew immediately and smoothly. Also, the growth of the bacteria was exponential until day 1. The growth of the bacterium had a stationary phase between day 1 and day 2 or until day 4, afterwards a sudden decline phase of the population density that was predicted due to increase accumulation of new compounds (TBT by-products, DBT and MBT) which could be toxic to the bacteria (Berto et al. 2007; Du et al. 2014).

In contrast, at 1000 μg/L TBT concentration (Fig. 2), the growth of the bacterium was exponential until day 2, and there was no stationary phase with bit less population density measurement compared to the bacterium growth at 500 μg/L concentrations and a sudden decline at day 2.

From the growth curve in Fig. 3, there was no stationary phase at 1500 μg/L and the bacterium fails to regenerate at the initial day 1. It took the isolate some time to acclimatize with the dosage effect of the TBT before it started to have an exponential growth that lasted up to day 2 followed by a swift decline phase like the previous concentrations. Cruz et al. (2007) proposed that the delay in the regeneration was due to inducible "memory response" mechanism to TBT exposure. Like other xenobiotic compounds (e.g., heavy metals), TBT is found to be toxic to the bacteria at a given concentration. In previous studies, it was documented that TBT has some of the following effects on the bacterial cells: inhibition of growth and metabolism, toxic for bacteria (Gram negative are more resistant), decrease bacterial productivity, inhibition of solute transport and biosynthesis of molecules, and distortion of respiration (Cruz et al. 2013; Gadd 2000; Wuertz et al. 1991). At 2000 and 2500 μg/L TBT concentrations, there was a high dosage effect of the compound against the bacterial growth. The population density recorded was very low at the 2000 and 2500 μg/L TBT concentrations.

It was clearly shown that the first three concentrations (500, 1000, and 1500 μg/L) supported the growth of the bacterium until day 2, however with an abrupt death of the cells which was predicted due to the effect of by-products as a result of TBT breakdown (Cruz et al. 2007, 2015).

It was reported that the regeneration of bacterial cells is proportionally delayed because of the increase in concentrations of TBT (Adelaja and Keenan 2012; Cruz et al. 2014). Statistically, it was shown that all alternate groups were significantly different from each other in terms of mean absorbance (population density) with a tendency for the mean for the measurement to decrease with increasing TBT concentrations; however, no significant difference was detected between the most adjacent concentration groups (except between 1500 and 2000 μg/L). The highest difference was demonstrated between the 500 and 2500 μg/L concentration groups

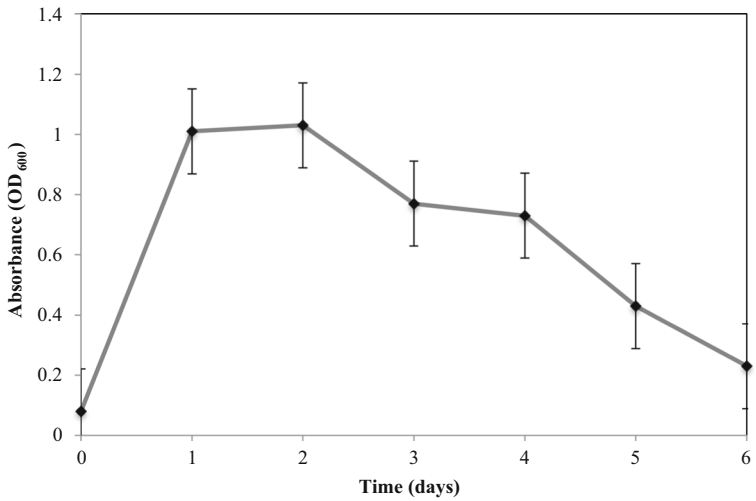

Fig. 1 Growth performance of *Klebsiella* sp. FIRD 2 in MSM in the presence of TBT concentration, 500 μg/L

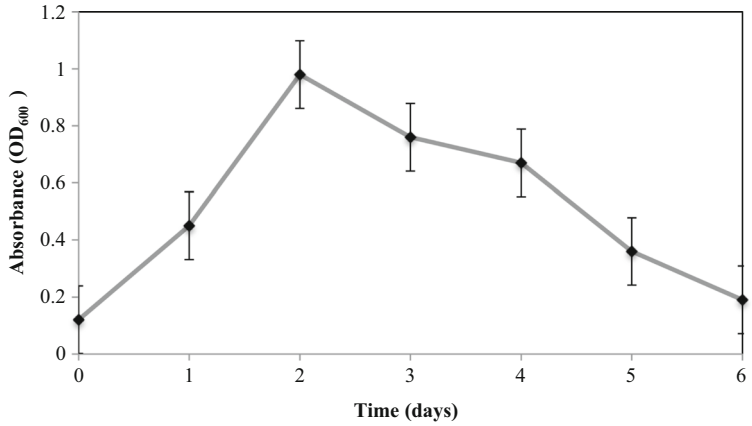

Fig. 2 Growth performance of *Klebsiella* sp. FIRD 2 in MSM in the presence of TBT concentrations, 1000 μg/L

(mean difference in absorbance = 0.1808, $p = 0.0001$). This was suggested due to the effect of higher concentrations of the compound (Jude et al. 1996).

The Brown-Forsythe test was done due to non-homogeneity of variance. The test showed the existence of a significant difference in the mean population density (absorbance) across the TBT concentration groups. The pairwise comparison also showed importance between the group differences.

In the control setup, there was no carbon source added to the media thereby the bacterial growth was not supported throughout the culture period as hypothesized earlier. This explains that the bacteria could be utilizing the TBT compound as a source of carbon.

Morphologically when observed under the microscope, the bacterial cells in MSM with lower TBT concentrations appeared to be bigger and dispersed in the growth medium; however, the bacterial cells in the higher TBT concentrations were smaller and appeared to aggregate in the culture media overtime. It is possible to suggest that at lower concentrations, TBT supports increase in the number of cells.

The maximum TBT reported environmental level in sediment of the Malaysian Peninsula, precisely Kong Kong Laut area, was 790 μg/L (Mohamat-Yusuff et al. 2013). There, these exposures of TBT to this isolate (*Klebsiella* sp. FIRD 2) showed growth at concentrations twice those reported from the location. According to the previous studies, a TBT-degrading bacteria must be a TBT-resisting bacteria, it is therefore expected that *Klebsiella* sp. FIRD 2 isolated locally from contaminated surface sediment may have the ability to degrade the TBT. However, further study of TBT remediation by bacterial isolates in other tropical environments is recommended, based upon this present study.

It is also important to note that this isolate (*Klebsiella* sp. FIRD 2) is highly resistant to TBT compared to many TBT-resisted isolates (Cruz et al. 2015). *Klebsiella* species can be found in water, soil, insects, plants, animals, and humans. They are associated with biochemical adaptations that makes them suitable to a

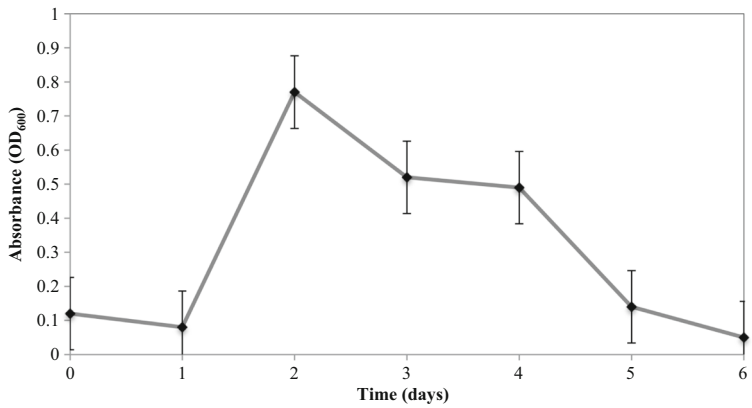

Fig. 3 Growth performance of *Klebsiella* sp. FIRD 2 in MSM in the presence of TBT concentration, 1500 μg/L

specific environment. They are found in human's flora and discovered to be pathogenic to humans.

3.2 Effects of TBT on *Pseudomonas* species

To prove an earlier hypothesis that isolated indigenous bacteria are the best candidate for remediation purpose when compared to non-indigenous bacteria, a study was done by observing the growth pattern of a commercial strain (*Pseudomonas aeruginosa*) in MSM treated with different TBT concentrations without addition of carbon source. Interestingly, there was no growth observed because of the inability of the strain to utilize TBT as carbon source; however, in another trail with supplemented carbon source in the media, growth was observed and monitored as chance in population density using a spectrophotometer. This was inconsistent with the study done by earlier works that *Pseudomonas* species resisted high concentrations of TBT (Bernat et al. 2014; Khanolkar et al. 2015; Fukushima et al. 2009). In the works done by Bernat et al. (2014), a TBT-resistant strain of *Pseudomonas* sp. was isolated from an overworked car filter. The strain was tested for tolerance to tributyltin, as well its degradation ability (Bernat et al. 2014). Another reported study on TBT-resistant *Pseudomonas* sp. was *P. aeruginosa* 25W isolated from the coastal seawater of Arabian Area (Fukushima et al. 2009). Khanolkar et al. (2014) studied the growth behavior and TBTCl resistance limit of a bacterial strain *P. stutzeri* strain DN2.

From Fig. 4, it was shown that there was a significant impact of TBT on the growth of the *Pseudomonas* sp. even at the lowest concentration. The highest absorbance measurement at the lowest TBT concentration (i.e., 500 μg/L) was 0.13 as against 1.03 compared with that of the *Klebsiella* sp. isolated from a TBT-contaminated area. This was believed to be as a result of the memory response between a bacterium isolated from a TBT-contaminated area and a bacterium isolated from a non-TBT-contaminated area as described by Cruz et al. (2007). Cruz et al. (2007) stated that bacterial isolates that have already been exposed to toxic compounds would generally have more tolerance than those that have not been exposed. It has also been found out that increase in TBT concentration completely inhibits the growth of the strain (*Pseudomonas* sp.) with the supplemented carbon source in the medium.

In the control MSM + 0 μg/L (Fig. 4), the setup was not treated with TBT and hence there was smooth growth of the strain as against that of the TBT-treated mixture.

Pseudomonas aeruginosa is a common Gram negative bacterium found in soil, water, skin flora, and most of the man-made environments. It can be pathogenic, and it uses a wide range of organic materials as source of food (Roy and Nair 2007).

4 Conclusion

The *Klebsiella* sp. isolate was found to resist TBT up to 1500 μg/L without addition of carbon source in MSM, whereas *Pseudomonas* sp. isolated from non-TBT contaminated soil could not resist TBT without addition of carbon source in MSM. However, *Pseudomonas* sp. growth was only observed in MSM with glucose as supplemented carbon source at 500 μg/L TBT concentration. Like many other reports, this study equally

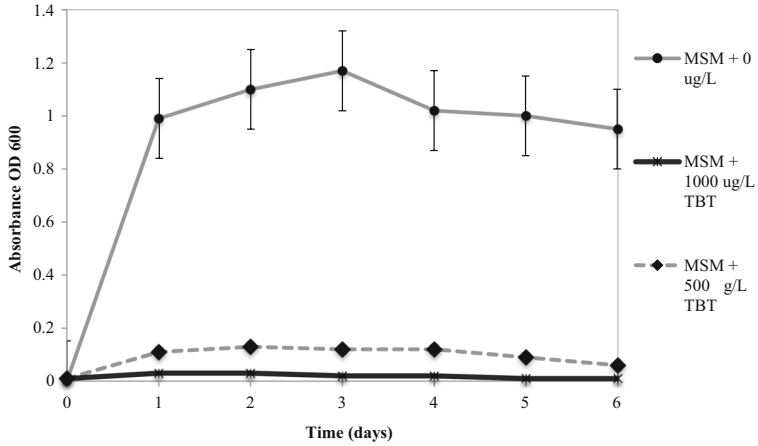

Fig. 4 Mean of growth of *Pseudomonas* sp. in MSM with glucose in the presence of TBT; A: MSM + 0 μg/L, B: MSM + 500 μg/L and C: MSM + 1000 μg/L

endorses the potentials of an indigenous bacterium in bioremediation application.

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