



UNIVERSITI PUTRA MALAYSIA

***ISOLATION AND CHARACTERIZATION OF TRIBUTYLTIN-RESISTANT
BACTERIA FROM CONTAMINATED SURFACE SEDIMENT AT KONG
KONG LAUT, JOHOR, MALAYSIA***

ABUSSAMAD ABUBAKAR

FPAS 2016 5



**ISOLATION AND CHARACTERIZATION OF TRIBUTYL TIN-RESISTANT
BACTERIA FROM CONTAMINATED SURFACE SEDIMENTS AT KONG
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By

ABUBAKAR ABDUSSAMAD

**Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in
Fulfillment of the Requirements for Degree of Master of Science**

April 2016

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DEDICATION

This thesis is dedicated to my late father Abubakar Bala Adamu for encouraging me to embark on this MSc. program whom could not live long to witness its end. May Allah (SWT) forgive his sins and grant him Jannatul Firdaus.



Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the Degree of Master of Science

ISOLATION AND CHARACTERIZATION OF TRIBUTYL TIN-RESISTANT BACTERIA FROM CONTAMINATED SURFACE SEDIMENTS AT KONG KONG LAUT, JOHORE MALAYSIA

By

ABUBAKAR ABDUSSAMAD

April 2016

Chairman : Ferdius @ Ferdaus Binti Mohamat Yusuff, PhD

Faculty : Environmental Studies

Tributyltin (TBT) is a species of organotin compound (OTC) under butyltin (BT) group, used as antifouling biocide in boat and ships paints to prevent the attachments of marine organisms on their hull surface. Tributyltin is very toxic to a variety of targeted and non-targeted organisms and has high persistence in sediments (can stay for up to thirty (30) years). Despite its global ban in 2008 by the International Maritime Organization (IMO), high concentrations of TBT in sediments from fresh and marine waters in many places across the world including the northwest coast of Portugal, Strait of Johore Malaysia, South Africa, Australia among others were reported years after the total global ban. Malaysia is one of the countries with the busiest maritime activities and well documented with high levels of TBT in the marine environments, thus there is an urgent need to remediate TBT contamination. This study aimed at isolating the best indigenous bacterial species(s) from TBT and heavy metals contaminated sediment as a prospect in bioremediation of TBT contamination based on the TBT and heavy metals tolerance ability. Sediment samples were collected from TBT contaminated surface sediments of the Kong Kong Laut area. Bacteria were isolated in Minimal Salt Media (MSM) and screened in different concentrations of TBT treated media (1000, 1200 and 1500 µg/L). Selected TBT-resistant isolates were identified using morphological, biochemical (Gram staining) and polymerase chain reaction (16S rRNA) techniques. Growth tolerance of the best isolate was ascertained in MSM treated with different TBT (500, 1000, 1500, 2000 and 2500 µg/L) and heavy metals (0, 1, 5, 15, 25, 40 and 100 mg/L) concentrations. The metals were copper, zinc, lead and cadmium. Thirty-five (35) bacterial colonies were isolated on MSM from the sediment samples. Fourteen (14) isolates (KK1, KK2, KK3, KK4, KK5, KK6, KK7, KK8, KK12, KK14, KK15, KK16, KK17 and KK18) were found to resist 1000 µg/L of TBT, a concentration level above the reported contaminated level (790 µg/L) at the sampling area. All the 14 isolates were Gram negative and three (3) isolates (KK1, KK3 and KK7) amongst were found to resist TBT up to 1200 µg/L. The 3 isolates were identified as *Citrobacter* sp. FIRD 1, *Klebsiella* sp. FIRD 2 and *Acinetobacter* sp. FIRD 3 with accession numbers KR027917, KR005669 and KR013742, respectively. This study managed to isolate, screen and identify. The best TBT-resistant bacterium from the Kong Kong Laut area, named *Klebsiella* sp. FIRD 2, a new species with high growth tolerance against TBT (1500 µg/L) and heavy metals (Cu 25, Pb 40, Zn 100 and Cd 40 mg/L). This study established

that *Klebsiella* sp. FIRD 2 isolated from Kong Kong Laut Johore has great potentials to be used as a bioremediation agent of TBT because of its high growth tolerance to TBT and heavy metals contaminated media.



Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

**PENGASINGAN DAN PENCIRIAN BAKTERIA TAHAN-TRIBUTILTIMAH
DARI PERMUKAAN SEDIMEN YANG TERCEMAR DI KONG KONG LAUT,
JOHORE MALAYSIA**

Oleh

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Tributiltimah (TBT) adalah spesies kompaun organotimah (OTC) di bawah kumpulan butiltimah (BT), yang digunakan sebagai biosida cegah-kebusukan dalam cat bot dan kapal-kapal untuk menghalang lekatan organisma marin di permukaan badan kapal. Tributiltimah adalah sangat toksik kepada pelbagai organisma sasaran dan bukan sasaran serta mempunyai ketahanan yang tinggi dalam sedimen (boleh kekal sehingga tiga puluh (30) tahun). Walaupun pengharaman diperingkat global oleh Pertubuhan Maritim Antarabangsa (IMO) pada tahun 2008, namun kepekatan TBT masih sangat tinggi dalam sedimen air tawar dan laut di banyak tempat di seluruh dunia termasuk pantai barat laut Portugal, Selat Johor Malaysia, Afrika Selatan dan Australia. Malaysia adalah salah satu negara mempunyai aktiviti maritim paling sibuk dan telah didokumentasikan mempunyai kepekatan TBT dalam persekitaran marin, dengan itu terdapat keperluan segera untuk mengatasi pencemaran TBT. Kajian ini bertujuan untuk mengasingkan spesies bakteria asal terbaik daripada sedimen yang dicemari TBT dan logam berat sebagai prospek dalam pemulihan persekitaran marin dicemari TBT berdasarkan keupayaan toleransi bakteria terhadap TBT dan logam berat. Sampel sedimen dikumpulkan dari sedimen permukaan kawasan dicemari TBT di Kong Kong Laut. Bacteria telah diasingkan dalam Media Begaram Minimum (MSM) dan didedahkan dengan media yang dirawat dengan TBT pada kepekatan yang berbeza (1000, 1200 dan 1500 µg/L). Asingan tahan TBT yang dipilih telah dikenal pasti berdasarkan teknik morfologi, biokimia (pewarnaan Gram) dan tindak balas rantai polimer (16S rRNA). Toleransi pertumbuhan asingan yang terbaik telah ditentukan dengan MSM yang dirawat dengan TBT berkepekatan berbeza (500, 1000, 1500, 2000 dan 2500 µg/L) dan logam berat berkepekatan berbeza (0, 1, 5, 15, 25, 40 dan 100 mg/L). Logam yang dipilih adalah tembaga, zink, plumbum dan kadmium. Tiga puluh lima (35) koloni bakteria telah diasing daripada sampel sedimen pada MSM. Empat belas (14) asingan (KK1, KK2, KK3, KK4, KK5, KK6, KK7, KK8, KK12, KK14, KK15, KK16, KK17 dan KK18) didapati tahan 1000 µg/L TBT, tahap kepekatan di atas tahap yang dilaporkan mencemari (790 µg/L) kawasan persampelan. Kesemua 14 asingan adalah Gram negatif dan tiga (3) asingan (KK1, KK3 dan KK7) di kalangannya didapati tahan TBT sehingga 1200 µg/L. Tiga (3) penciran telah dikenal pasti sebagai *Citrobacter* sp. FIRD 1, *Klebsiella* sp. FIRD 2 dan *Acinetobacter* sp. FIRD 3 dengan nombor kesertaan masing-masing KR027917, KR005669 dan KR013742. Kajian ini berjaya mengasing, menyaring dan mengenal pasti bakteria terbaik tahan TBT dari kawasan Kong Kong

Laut, dinamakan *Klebsiella* sp. FIRD 2, iaitu satu spesies baru bertumbuh dengan toleransi yang tinggi terhadap TBT (1500 µg/L) dan logam berat (Cu 25, Pb 40, Zn 100 dan Cd 40 mg/L). Kajian ini menetapkan bahawa *Klebsiella* sp. FIRD 2 yang diasingkan daripada Kong Kong Laut, Johor mempunyai potensi yang besar untuk digunakan sebagai agen pemulihan persekitaran dicemari TBT kerana toleransi pertumbuhan yang tinggi kepada media dicemari TBT dan logam berat.



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I certify that a Thesis Examination Committee has met on 28 April 2016 to conduct the final examination of Abubakar Abdussamad on his thesis entitled "Isolation and Characterization of Tributyltin-Resistant Bacteria from Contaminated Surface Sediment at Kong Kong Laut, Johor, Malaysia" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
GC	Gas Chromatography
GC-MS	Gas Chromatography Mass Spectrometry
%	Percentage
rpm	Revolution Per Minute
g	Gram
TBT	Tributyltin
DBT	Dibutyltin
MBT	Monobutyltin
µg	Microgram
L	Liter
OT	Organotin
BT	Butyltin
rRNA	Ribosomal Nucleic Acid
sp	Species
EFSA	European Food Safety Authority
ADI	Acceptable Daily Intake
TDI	Tolerable Daily Intake
CO ₂	Carbon dioxide
ISO	The International Standard Organization
DNA	Deoxyribonucleic Acid
KK	Lab tagging for Isolates
IMO	International Maritime Organization

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Tributyltin (TBT) is an organotin (OT) compound that has been largely used as part of antifouling biocide in boat and ships paints to restrain the attachment of marine organism on the hull surface of marine structures (Blanca Antizar-Ladislao, 2008). Organotin compounds are group of chemicals that have at least one tin–carbon bond, joined covalently (Rüdel, 2003). Tributyltin was classified as a very toxic compound, which poses danger to a wide range of organisms at the polluted environments due to the high concentrations (Fent, 1996). Leachates of tributyltin (TBT) from antifouling-based paint chemically bind themselves with suspended materials and deposited in benthic sediments via accumulation process. Accumulation of TBT in sediments is the cause of ongoing pollution sources because the residues from these compounds were slowly released back into aquatic phase (Chiron *et al.*, 2000). Tributyltin can be remobilised into aquatic phase by frequent disturbance of the contaminated sediments (Hussein *et al.*, 2011). Concern arise as this disturbance activities could further recharge the sediments with TBT as re-suspension events may increase at sites that not yet widely affected by TBT (Svavarsson *et al.*, 2001) since the sediments act as ‘reservoir’ of the pollutants (Apitz *et al.*, 2005) (Zulkifli *et al.*, 2010). Therefore, site with highly contaminated with TBT requires management for remediation.

Serious organotin (OT) contamination, especially from TBT, was reported in the busy Straits of Malacca and the Strait of Johor (Sudaryanto *et al.*, 2004). Later study conducted by Harino *et al.* (2007) found that concentration of monobutyltin (MBT), dibutyltin (DBT) and TBT in sediments from 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.*, 2007). The author reported that 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.*, 2007). The level of OT in marine mollusc (*Thais gradata*) from the Straits of Johor and southern part of the Straits 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 (Ismail *et al.*, 2004; Mohamat-Yusuff *et al.*, 2010, 2011) and even up to carcinogenic effects with no significant evidence of immunotoxicity (Lee *et al.*, 2005). 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 with high level of imposex incidence and accumulated with high level of TBT. This was supported by recent study by Mohamat-Yusuff *et al.* (2013), TBT contaminations in sediments at Kong Kong Laut was as high as 790 µg/L even after the total global banning on the year 2008.

Persistent of high concentrations of TBT in sediments from marine and fresh waters in many places across the world including the Strait of Johor, Malaysia even after the first global banning of the year 2003 (Harino *et al.*, 2008; Sakultantimetha *et al.*, 2011) and total global banning on the year 2008 (Mohamat-Yusuff *et al.*, 2013) has rose concern among scientist around the globe. The longer it stays, the higher the possibilities it transport up the food chain and end up in our diet. Unlike in non-tropical developed nations, there is still limited information on TBT degradation by microorganism in tropical countries particularly in Malaysia. Over the years, France, United Kingdom, and United States, many examples of TBT contamination and its degradation have been narrated (Cassi *et al.*, 2008; Kawai *et al.*, 1998). A potential TBT-degrading bacterium isolated from sediment/water from country of Ria de Aveiro (Portugal) successfully established by Cruz *et al.* (2007). The bacterium was identified as *Aeromonas veronii* Av27 which found tolerance to TBT up to 3 mM (Cruz *et al.*, 2007). The same author also reported that Gram-negative bacteria have more tolerance ability to TBT than Gram-positive bacteria (Cruz *et al.*, 2007). Similar works has been done by Adelaja and Keenan (2012). However, the work by Adelaja and Keenan (2012) isolated TBT-resistant bacteria and tested it for growth in the presence of methylmercury (MeHg). The isolated bacteria identified as *Pseudomonas flouresens*, *Enterobacter cloacae*, *Citrobacter braakii* and *Alcaligenes faecalis* were found to have great potentials to detoxify not only the TBT but also MeHg (Adelaja and Keenan, 2012). These bacteria could utilize carbon sources from the TBT compounds and recommended *Enterobacter cloacae* as the most preferable isolate for potential TBT and MeHg degradation (Adelaja and Keenan, 2012).

Another related study utilizing microorganism as biodegrading agent of TBT was the study of fungus, *Cunninghamella elegans* grown in Sabouraud medium added with 20 mg/L of TBT (Bernat and Długo, 2002). The study found that 70% of the chemical (TBT) was transformed into two less toxic forms (DBT and MBT) after 7 days of experiment. Concentration above than 20 mg/L was reported inhibited the growth of the fungi.

Even though microorganisms particularly bacteria and fungi, are natural degraders in their respective natural environments or elsewhere (Wuertz *et al.*, 1991), the ability to degrade pollutants in the environments could be slow due to many environmental dynamics. However, this process could be enhanced by the clever remediation technology (Abalos *et al.*, 2004). Therefore, invites for the need to isolate and study wild type microorganism, in order to explore their full potential in bioremediation process. Owing to limited information on TBT degradation in Malaysia and other tropical areas, this study is expected to make a significant contribution to studies on TBT remediation in tropical environments. Thus this work was done to identify the best indigenous bacterial candidate that has high resistance to extreme concentrations of Tributyltin (TBT) and to determine its unique characteristics as a prospect in biotechnology and bioremediation.

1.2 Problem Statement

TBT contamination is an issue of international concern because of its slow degradation. Many contaminated areas around the world are still required to be remediated years after the global ban. When deposited in sediment, TBT can stay up to 30 years and serves as

secondary source of contamination (Bray, 2006; Chiron *et al.*, 2000). In Malaysia, TBT concentrations in marine environment and the occurrence of imposex is higher and more serious than in other parts of Southeast Asia (Harino *et al.*, 2008). Above all, Kong Kong Laut at Johore has highest imposex incidence and highest TBT accumulation in the snails as compared to other sites in the southern part of Peninsular Malaysia (Mohamat-Yusuff *et al.*, 2010). In recent works, concentration of TBT in collected sediments from this sites has been reported up to 790 µg/L (Mohamat-Yusuff *et al.*, 2013). TBT was found to have deleterious effects on both prokaryotic and eukaryotic organisms (B Antizar-Ladislao, 2008) while in humans it was found to inhibit the immune system and causes 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 and TBT was found to be carcinogenic to humans. Therefore, it is a need to remediate this compound in this area. Considering works on heavy metals by Zulkifli *et al.* (2010) and Wan and Ferdaus (2014) revealed that this particular site also experience heavy metal contamination. Thus, prior for the remediation works, identification and establishment of the bioremediation agent fit with the local condition especially tolerance with heavy metals must be done.

1.3 Scope of the study

Due to the fact that bioremediation using indigenous bacteria has been proposed as the most economically efficient and effective, this study focuses on the isolation of wild type TBT-resistant bacteria from Kong Kong Laut, the hotspot of TBT polluted area in Malaysia, identification of the morphology of bacteria and morphology of the colony, species identification of the best candidates using molecular technique and determination of the growth pattern in extreme concentrations of TBT along with tolerance ability with heavy metals.

1.4 General Objective

This study was aimed at isolating the best bacterial specie(s) as a prospect in bioremediation of TBT contamination based on their TBT and heavy metal tolerance ability.

1.5 Specific Objectives of the Study

This study embarks on the following specific objectives:

1. To isolate, screen and identify TBT-resistant bacteria from surface sediments of Kong Kong Laut, Johore.
2. To determine the growth performance of the best TBT-resistant bacterial species in different concentration of TBT treated media.
3. To ascertain the tolerance ability of the best TBT-resistant bacterial species in in different concentrations of heavy metals treated media.

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LIST OF PUBLICATIONS

Conference proceedings:

Abubakar, A., Mustafa, M., Johari, W. L. W., & Ferdaus Mohamat-Yusuff, S. Z. Z. and A. I. (2014). Tributyltin-resistant bacteria from contaminated surface sediment. In C. Yong, S. Yien, W. Norhamidah, & W. Ibrahim (Eds.), *MALAYSIA INTERNATIONAL BIOLOGICAL SYMPOSIUM 2014 "Sustainable Bioresources for Bioeconomy"* (p. 142). Kuala Lumpur: ©Department of Biology Faculty of Science Universiti Putra Malaysia 43400 UPM Serdang Selangor Darul Ehsan Malaysia Tel: +603 8946 6613 <http://www.science.upm.edu.my/biology>.

Abubakar, A., Mustafa, M. B., Lutfi, W., Johari, W., Zulkifli, S. Z., Ismail, A., Yusuff, M. (2015). Isolation and identification of TBT-resistant bacteria from contaminated surface sediment. In *International Conference on Environmental Forensics "from sources to solutions"* (p. 110). Putrajaya.

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