BIODEGRADATION OF 17ALPHAMETHYLTESTOSTERONE BY MICROORGANISM ISOLATED FROM MASCULINIZING PONDS OF NILE TILAPIA FRY

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การย่อยสลายสารแอลฟาเมทิลเทสโทสเตอโรนทางชีวภาพ โดยจุลชีพที่คัดแยกจากบ่อแปลงเพศปลานิล

นางสาวพิมพ์วรัชญ์ ศรีขวัญ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	BIODEGRADATION OF 17ALPHAMETHYLTESTOSTERONE BY MICROORGANISM ISOLATED FROM MASCULINIZING PONDS OF NILE TILAPIA FRY
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พิมพ์วรัชญ์ ศรีขวัญ : การย่อยสลายสารแอลฟาเมทิลเทสโทสเตอโรนทางชีวภาพโดยจุลชีพที่คัดแยกจากบ่อแปลงเพศปลานิล (BIODEGRADATION OF 17ALPHAMETHYLTESTOSTERONE BY MICROORGANISM ISOLATED FROM MASCULINIZING PONDS OF NILE TILAPIA FRY) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ดร. ภริณดา ทยานุกูล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร. ตะวัน ลิมปิยากร, 90 หน้า.

แอลฟาเมทิลเทสโทสเตอโรน หรือ MT เป็นฮอร์โมนสังเคราะห์เพศชายที่ใช้กันอย่างแพร่หลายในกระบวนการแปลงเพศเป็นเพศผู้ในอุตสาหกรรมการเพาะเลี้ยงสัตว์น้ำ เกษตรกรผู้เพาะเลี้ยงปลานิลนิยมปลาเพศผู้มากกว่าเพศเมีย เนื่องจากปลาเพศผู้มีอัตราการเจริญเติบโตรวดเร็วกว่า มีขนาดตัวใหญ่ และน้ำหนักมากกว่า แม้ว่า MT จะมีประโยชน์มากทางด้านการเพาะเลี้ยงสัตว์น้ำ แต่สารบางส่วนที่ตกค้างอยู่ในสิ่งแวดล้อมสามารถทำให้เกิดความผิดปกติที่ต่อมไร้ท่อและการทำงานของระบบสืบพันธ์ของสัตว์น้ำตามธรรมชาติ ดังนั้นเพื่อเป็นการลดปริมาณสารที่ตกค้างในบ่อและสิ่งแวดล้อม กระบวนการย่อยสลาย MT ทางชีวภาพเพื่อลดปริมาณ MT จึงถูกนำมาศึกษา วิทยานิพนธ์ฉบับนี้มีเป้าหมายในการคัดแยกเชื้อจุลินทรีย์ที่มีความสามารถในการย่อยสลาย MT และศึกษาความสามารถในการย่อยสลาย MT ของเชื้อจุลินทรย์การคัดเลือกจุลินทรีย์ดำเนินการโดยนำตัวอย่างตะกอนดินและไบโอฟิล์มจากบ่อแปลงเพศปลานิลมาเลี้ยงเชื้อที่ความเข้มข้นของ MT ที่ 0.5 – 30 มิลลิกรัมต่อลิตร ผลการคัดเลือกพบว่ามีจุลินทรีย์ 3 สายพันธุ์ ที่มีความสามารถในการย่อยสลาย MT คือ B051, B052 และ S303 ซึ่งสามารถแบ่งเป็น 3 สายพันธุ์ในจีนัส *Acinetobacter*sp., *Ochrobactrum*sp. และ *Nocardioides*sp., ตามลำดับ นำทั้ง3 สายพันธุ์มาศึกษาความสามารถในการย่อยสลาย MT จากการวิเคราะห์ทางจลน์ศาสตร์ด้วยสมการของ Michaelis-Mentenพบว่า อัตราการย่อยสลายของ MT สูงขึ้น เมื่อความเข้มข้นของ MT สูงขึ้น จนเริ่มคงที่ที่ค่าอัตราการย่อยสลายสูงสุด โดยเชื้อสายพันธุ์B051, B052 และ S303 มีค่าอัตราการย่อยสลายสูงสุดคือ 0.34 นาโนกรัมต่อลิตรต่อชั่วโมงต่อเซลล์, 0.18 นาโนกรัมต่อลิตรต่อชั่วโมงต่อเซลล์ และ 0.24 นาโนกรัมต่อลิตรต่อชั่วโมงต่อเซลล์ ตามลำดับ และมีค่าคงที่ของ Michaelis-Mentenเท่ากับ 8.23 มิลลิกรัมต่อลิตร 1.48 มิลลิกรัมต่อลิตร 1.0 มิลลิกรัมต่อลิตร ตามลำดับ โดยสายพันธุ์S303 เป็นสายพันธุ์ที่สามารถย่อยสลาย MT ได้เร็วกว่าสายพันธุ์อื่นที่ความเข้มข้นของ MT ต่ำ นอกจากนี้ทั้งสามสายพันธุ์ยังถูกนำมาทดสอบการย่อยสลายฮอร์โมนเพศชนิดต่างๆที่มีโครงสร้างคล้าย MT และพบว่ามีการปนเปื้อนในสิ่งแวดล้อมเช่นเดียวกับ MT ซึ่งประกอบด้วย เทสโทสเตอโรน, เอสโทรน, เบตาเอสทราไดออล และ แอลฟาเอทินิลเอสทราไดออล จากการทดลองนำเชื้อจุลินทรีย์มาย่อยที่ความเข้มข้น 10 มิลลิกรัมต่อลิตรเป็นเวลา 7วัน พบว่า มีเพียงฮอร์โมนเทสโทสเตอโรนเท่านั้นที่จุลินทรีย์ทั้งสามสายพันธุ์สามารถย่อยสลายได้ ซึ่งจากการทดลองนี้สรุปได้ว่า เชื้อจุลินทรีย์ที่ถูกคัดเลือกมีความสามารถในการย่อยสลายฮอร์โมนกลุ่มเพศชายอย่างเฉพาะเจาะจง

สาขาวิชา การจัดการสิ่งแวดล้อม ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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PIMVARAT SRIKWAN: BIODEGRADATION OF 17ALPHAMETHYLTESTOSTERONE BY MICROORGANISM ISOLATED FROM MASCULINIZING PONDS OF NILE TILAPIA FRY. ADVISOR: PARINDA THAYANUKUL, Ph.D., CO-ADVISOR: ASSOC. PROF. TAWAN LIMPIYAKORN, Ph.D., 90 pp.

17α-methyltestosterone (MT) is a synthetic androgenic steroid, which is widely used in masculinization of aquaculture industry. Male population is preferred in a farming of Nile tilapia because of the faster growth rate, bigger body size and larger weight than the female. Although MT is beneficial for aquaculture production, the release of residual causes adverse effects to the endocrine and reproductive systems of natural aquatic organisms. This study aims to isolate the MT degrading microorganisms and to investigate the biodegradation characteristic, in order to enhance the biological removal. The isolation was performed with enrichment technique using MT as a sole carbon source at concentrations between 0.5 to 30 mg/L. The inoculum sources were sediment and biofilm from masculinizing ponds of Nile tilapia fry. Three strains, namely B051, B052 and S303 which affiliated to *Acinetobacter* sp., *Ochrobactrum* sp. and *Nocardioides* sp., respectively, were tested for the MT degrading kinetic and substrate versatility among other sex steroidal hormones which may coexist in farm environment. Michaelis-Menten model well explained the MT degrading kinetic for the 1 – 50 mg/L. The V_{max} and K_m value of strain B051, B052 and S303 were 0.34 $ngL^{-1}h^{-1}cell^{-1}$ and 8.23 mg/L, 0.18 $ngL^{-1}h^{-1}cell^{-1}$ and 1.48 mg/L, 0.24 $ngL^{-1}h^{-1}cell^{-1}$and 1.0 mg/L, respectively. Strain S303 was distinguished MT degrader as it had higher V_{max} and it had the lowest K_m, which can imply that strain S303 had ability to degrade MT faster than the others at low concentration close to environmental condition. In addition, all isolates also removed testosterone while they had not reduced estrone (E1), 17β-estradiol (E2) or17α-ethynylestradiol (EE2) at the concentration of 10 mg/L during 7 days. The results inferred that all three isolates are specialized in androgenic hormone degradation.

Field of Study: Environmental Management Student's Signature .....
Academic Year: 2014 Advisor's Signature .....
Co-Advisor's Signature .....

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CHAPTER I
INTRODUCTION

1.1. Introduction

Masculinization is a sex reversal process that utilizes an androgenic hormone to induce an emergence of male population. The male population is preferred in Nile tilapia (*Oreochromis niloticus*) farming, a common commercial fish species in Thailand, as they have faster growth rate, larger body size, and more weight than the female.

17α-methyltestosterone (MT), a synthetic androgenic steroid hormone, is often given to the fry at approximately 60 mg per kg of feed for 28 days (Barry et al, 2007). The residual MT in wastewater is sometimes released into the environment. Megbowon et al. (2014) addressed that the contamination of MT and other sex hormones in the environment should be concerned because of their endocrine disrupting effects to fish, animals, and human. Methyltestosterone has been detected in environment including municipal wastewater and farming wastewater. MT was detected at 617.4 µg/L in the tilapia culture pond (Barbosa et al., 2013). Liu et al. (2011) observed the concentration of MT from two wastewater treatment plants in Guangdong (China). The concentrations of MT in Huiyang WWTP was 1.5 ng/L in wastewater effluent and 1.3 ng/L in effluent of Meihu WWTP.

There are very limited studies investigating the MT degradation by microorganisms. Based on the literatures, MT degrading bacteria related to *Rhodococcus* sp. and *Nocardioides* sp. have been isolated from a masculinizing pond of Nile tilapia fry (Homklin et al., 2012). Besides, *Acinetobacter* sp., *Brevundimonas* sp., *Comamonas* sp., *Sphingomonas* sp., *Stenotrophomonas* sp., and *Rhodobacter* sp. were found in a testosterone-degrading enriched culture originated from a swine manure sample (Yang et al., 2011). They might be capable of degrading MT in fish ponds. If the MT degrading isolate are obtained, it may be possible to apply to aquacultural wastewater treatment in order to reduce the loading of MT in environment.

The objectives of this study are to obtain the 17α-methyltestosterone degrading isolates from the masculinizing ponds of Nile tilapia, to characterize the biodegradation of MT by the pure isolates, and to examine their ability to degrade other steroid hormones presenting in environment. The isolation was performed with culture techniques using MT as a sole carbon source. Ampicillin, a bacterial inhibitor, has been introduced in some enrichment flasks in order to seek for the MT degrading archaea. Besides,the substrate versatilities of the MT degrading isolates with other gonadal steroid hormones including testosterone, estrone(E1), 17β-estradiol (E2), and 17α-ethynylestradiol (EE2) have been explored as they may be coexisting with MT in the aquaculture environment. This will add profit of using the seed isolates for degrading several sex hormones at the same time.

1.2. Objective

1. To obtain the 17α-methyltestosterone degrading isolates from the masculinizing ponds of a Nile tilapia fry.
2. To characterize the kinetic of MT biodegradation by the pure cultures.
3. To examine the potential of the pure cultures on degrading other steroidal hormones.

1.3. Hypothesis

1. MT degrading microorganisms live in masculinizing ponds of Nile tilapia fry. Periodically feeding MT at low concentration can promote the growth of the organisms facilitating the isolation procedure.
2. Other sex steroid hormones, that have similar chemical structure to MT, are able to be degraded by the MT degrading microorganisms.

1.4. Scope of Study

1. Sediment andbiofilm sample were collected from masculinizing ponds of Nile tilapia fry in Thailand

2. The enrichment and isolation of microorganism were performed with different initial MT concentrations of 0.5, 1 and 30 mg/L
3. The kinetic of MT biodegradation were carried out with initial MT concentrations range of 1 - 50 mg/ L
4. The steroid hormones used in biodegradation test beside from MT, are testosterone, estrone (E1), 17β-estradiol (E2), and 17α-ethynylestradiol (EE2).

CHAPTER II
BACKGROUND AND LITERATURE REVIEW

2.1. Masculinization of Nile tilapia

According to the report from the Food and Agriculture Organization of the United Nations (FAO) in 2006, Thailand is the main producer country of Nile tilapia (*Oreochromis niloticus*). Male population is preferred because of their commercial value. Moreover the male has faster growth rate, larger body size and more weight than the female (MacIntosh and Little, 1995).

All-male-population can be produced by several techniques such as manual separation of the male and female, chromosomal manipulation, and hormonal sex reversal (Megbowon et al., 2014). The most efficient, cheap and common method is hormonal sex reversal process by using an androgenic hormone (e.g., 11-ketotestosterone, androstenedione, 17α-methyltestosterone, Mibolerone, and 17α-ethylnyltestosterone) to induce the mono-sex populations. Hormonal induction of sex reversal can be accomplished in various fish species such as *Cyprinidae, Salmonidae, Poecilidae, Anabantidae,* and *Cichlidae* (Pandian and Sheela, 1995).

17α-methyltestosterone (MT) is the most preferred hormone for induction of the male fish (Pandian and Sheela, 1995). The typical MT concentration for oral administration (mixing with food) varies between 40 to 80 mg/kg, while 60 mg/kg is the most common. (El-Greisy et al, 2012).

2.2. 17α- methyltestosterone

17α- methyltestosterone (MT) is a synthetic anabolic androgenic steroidal hormone. The structure of MT is similar to the natural androgenic hormone namely testosterone which stimulates the male characteristic. The different point is at the C-17 position where hydrogen is replaced with methyl group in MT as shown in figure 1. The water solubility of MT is rather low as it is 3.39 mg/L or only 3.36 for Log K_{ow} (Table 2.1).

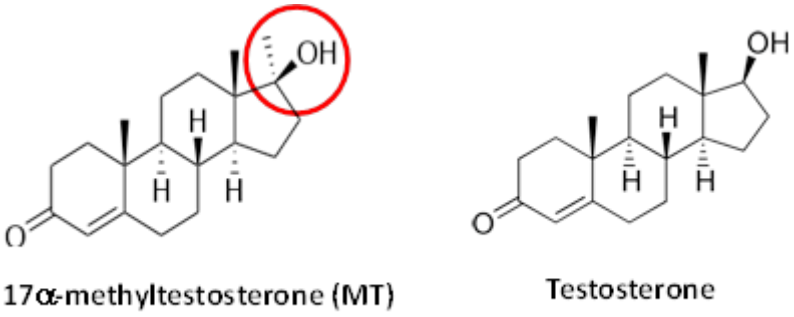


Figure 2.1[a] Structure of Methyltestosterone and testosterone (the red circle shows the C-17 position where hydrogen is replaced with methyl group)

Table 2.1 The physicochemical properties of 17α- methyltestosterone

Properties	Information
Formula[a]	$C_{20}H_{30}O_2$
CAS number[a]	58-18-4
Molecular weight[a]	302.46 g/mole
Appearance	White crystalline powder
Melting point[a]	163^{o}C
Log K_{ow}[c]	3.36
Water solubility at 25^{o}C[a]	3.39 mg/L
Vapor pressure at 25^{o}C[a]	1.85 x 10^{-8} mmHg
Henry's constant[b]	4.68 x 10^{-9} atm·m^3/mole

[a]Sigma-Aldrich material safety data sheet, [b] US EPA (2003), [c]Hansch et al. (1995)

2.3. Effect of 17α- methyltestosterone

MT is a kind of Endrocrine disrupting chemicals (EDCs) that alter functions of the endocrine system and consequently cause adverse health effect to an intact organism, or its progeny, or (sub) population (WHO and IPCS, 2002). Other male and female hormones in this group include testosterone, estrone(E1), 17β-estradiol (E2) and 17α-ethynylestradiol (EE2). They effect on the development, function, and

behavior of animals and perhaps human at low concentration in the range of few nanogramsper liter (WHO and UNEP, 2013).

For MT, many studies suggested the short-term effects on the endocrine and reproductive system of the aquatic organisms. Pawlowski et al. (2004) observed the lower egg production and fertilization rates in the adult fathead minnows (*Pimephales promelas*) exposed to 5 µg/L of MT for 3 weeks. In addition, the female fish showed territorial behavior and had darker skin color. These characteristicsusually associate with the male fish usually. Sharpe et al. (2004) found that the exposure of 100 ng/L of MT for 2 weeks inhibited the hCG-stimulating testosterone production in male fish and mummichog (*Fundulus heteroclitus*), and it interrupted the hCG-stimulating estradiol (E2) production in female fish. Korsgaard (2006) investigated the effects of MT on the circulating yolk-precursor protein vitellogenin (VTG) in female eelpouts (*Zoarces viviparous*) which were exposed to different doses of MT (10 -500 ng/L). The result showed that the VTG decreased in all MT concentrations. In adult male zebrafish (*Danio rerio*), the level of natural 11-ketotestosterone and testosterone decreased significantly up to 96.6% and 79.8%, respectively, when gradually exposed to MT concentration range from 4.5 to 62.3 ng/L in 1 week (Andersen et al., 2006). Not only the effects of MT on aquatic organisms have been reported, but the effects on other terrestrial organisms have been beenrevealed. Selzsam et al. (2005) studied the effect of MT in the Japanese quail bird (*Coturnixcoturnix japonica*) and found that the egg-laying performance was reduced by 13%, 62%, and 90% in exposure to 10 mg/L, 50 mg/L and 110 mg/L of MT, respectively.

2.4. Occurrence of MT in environment

Methylestosterone has been released to environment through municipal and farming wastewater. Fitzpatrick and Contreras-Sanchez (2000) measured the MT concentration in a masculinizing pond after the sex reversal process. For almost three months after the end of process, 2.8 – 2.9 ng/L of MT remained in the sediment. Barbosa et al. (2013) determined the MT concentration in freshwater samples of tilapia aquaculture and found it was 617.4 µg/L. Liu et al. (2011) observed the concentration of MT from two wastewater treatment plants in Guangdong (China). The concentrations of MT in Huiyang WWTP was 1.5 ng/L in wastewater effluent

while in Meihu WWTP it was 1.8 ng/L in influent and 1.3 ng/L in effluent. In 2012, Lui et al. detected 7.1 ng/L of MT in flush water of a piglet farm.

For the masculinizing ponds used in this study, Waiyaput et al. (2011) measured the MT concentration using Ultra performance liquid chromatography - tandem mass spectrometer (UPLC-MS/MS). The MT concentrations in the overflow water and suspended solid of a pond were between 0.3 to 39.5 ng/L. In addition, 0.595 ng/L of MT was detected for MT remaining in water and settle solid in the pond on the last day of hormonal sex reversal process (day 30).

In addition, the other steroid hormones were also detected in environment. Bartelt-Hunt et al. (2011) detected the testosterone, 4-androstenedione, and androsterone in wastewater impoundment at livestock facilities and found they were in range from 30 to 3600 ng/L. In groundwater samples, testosterone were found up to 390 ng/L. Ying et al. (2002) reported the concentrations of estrogenic compounds in sewage treatment plants in different countries and found up to 70 ng/L for estrone (E1), 64 ng/L for 17β-estradiol (E2), 18 ng/L for estriol (E3), and 42 ng/L for 17α-ethynylestradiol (EE2).

2.5. Microbial degradation of 17α- methyltestosterone

There are limited studies reporting on the microbial degradation of MT. Homklin et al. (2012) enriched and isolated MT degrading bacteria from water and sediment samples in a masculinizing pond of Nile tilapia fry. Two strains were isolated from the enrichment culture with 10 and 100 mg/L of initial MT concentration. These two strains were identified as *Nocardioides* sp. and *Rhodococcus* sp. The degradation rates increased with the increasing of the initial MT concentrations in the range of 1.0–10 mg/L. The degradation rate declined in the MT concentrations above this range. Among the isolated strains, *Rhodococcus* sp. was found to be most tolerant to high MT concentrations.

In addition, *Acinetobacter* sp., *Brevundimonas* sp., *Comamonas* sp., *Sphingomonas* sp., *Rhodobacter* sp. and *Stenotrophomonas* sp. were found in a testosterone-degrading enriched culture originated from a swine manure sample. Testosterone was degraded by these bacteria within 29 hours with a lag phase of approximately 22 hours. There are three degradation products of testosterone. Three

of which were identified as androstadienedione (ADD), dehydrotestosterone (DHT), and androstenedione (AD). DHT was the major testosterone degradation product within the first 48 hours (Yang et al., 2011). The biodegradation pathway of testosterone degradation is show in Figure 2.2.

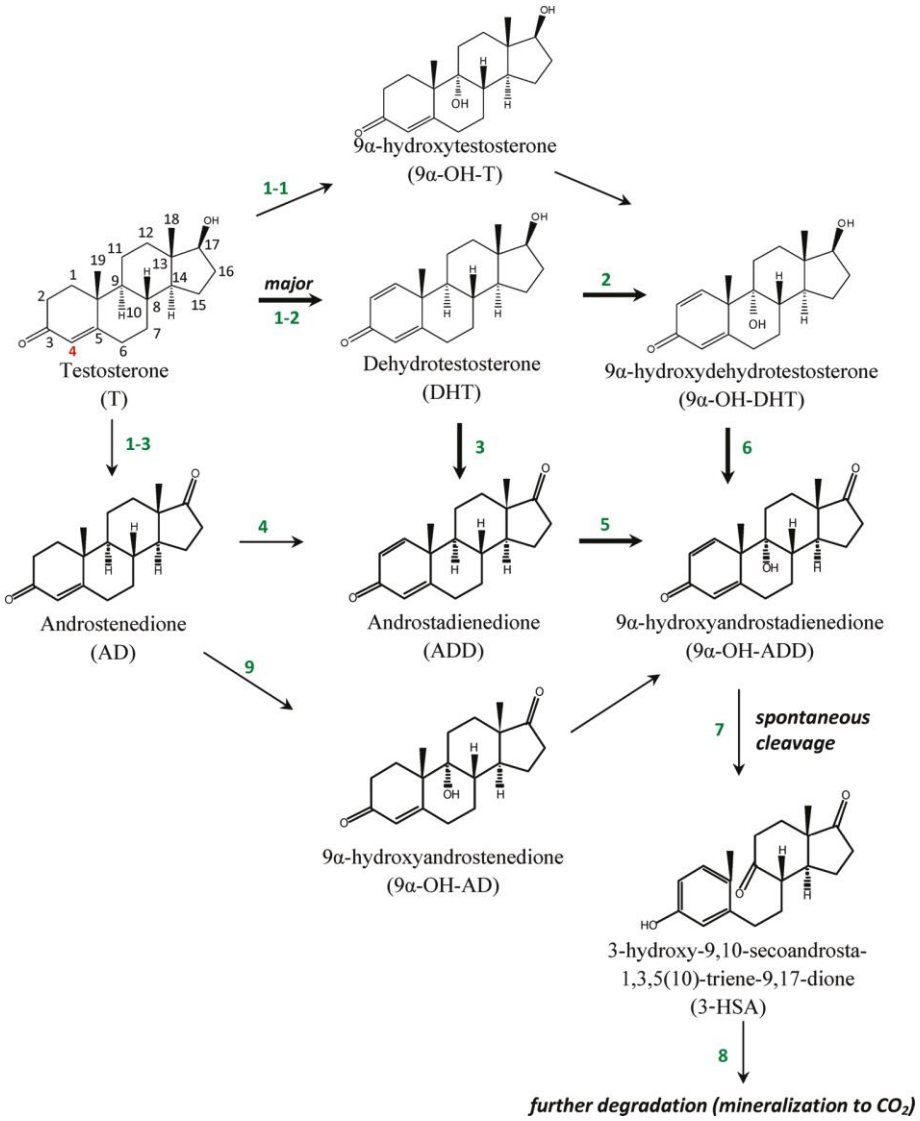


Figure 2.2 Proposed testosterone degradation pathways by bacteria enriched from swine manure (Yang et al., 2011)

MT is an environmental concerned steroidal hormones which includes testosterone, estrone (E1), 17β-estradiol (E2) and 17α-ethynylestradiol (EE2). Chemical structure of these compounds are quite similar to MT as shown in (Figure 2.3); therefore, It can hypothesize that the MT degrading microorganisms might be also capable of degrading other steroid hormones. This information is necessary when applying the MT degrading microorganisms in the biological treatment system

receiving farm wastewater. Some farms may raise various animals generating many kinds of sex hormone. It would be more beneficial if the MT degrading isolates could mineralize various steroidal hormones reducing endocrine disrupting load in environment.

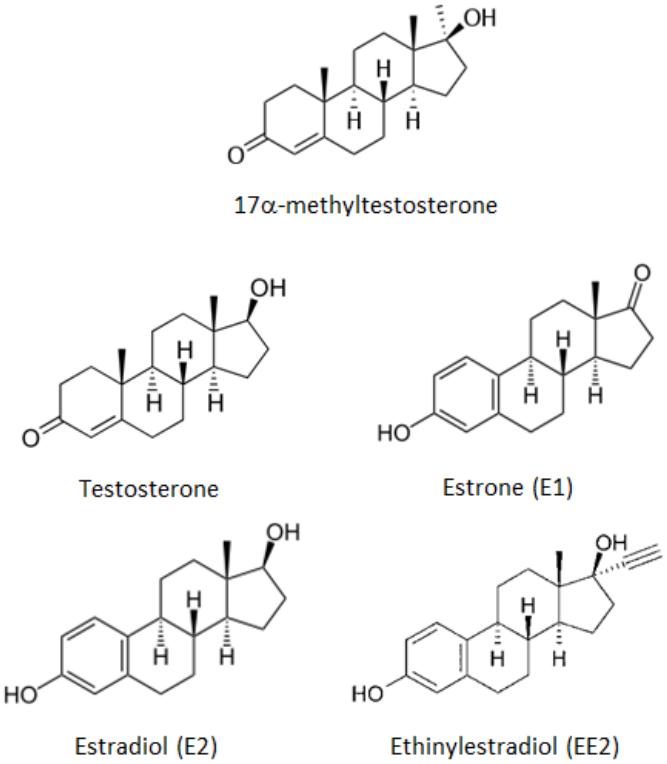


Figure 2.3 The chemical structure of other sex hormones compare with structure of MT.

Kurisu et al. (2010) isolated five strains which have ability to degrade natural estrogen from soil in agricultural field. Three strains were identified as *Rhodococcus* sp. and the others were identified as *Sphingomonas* sp.

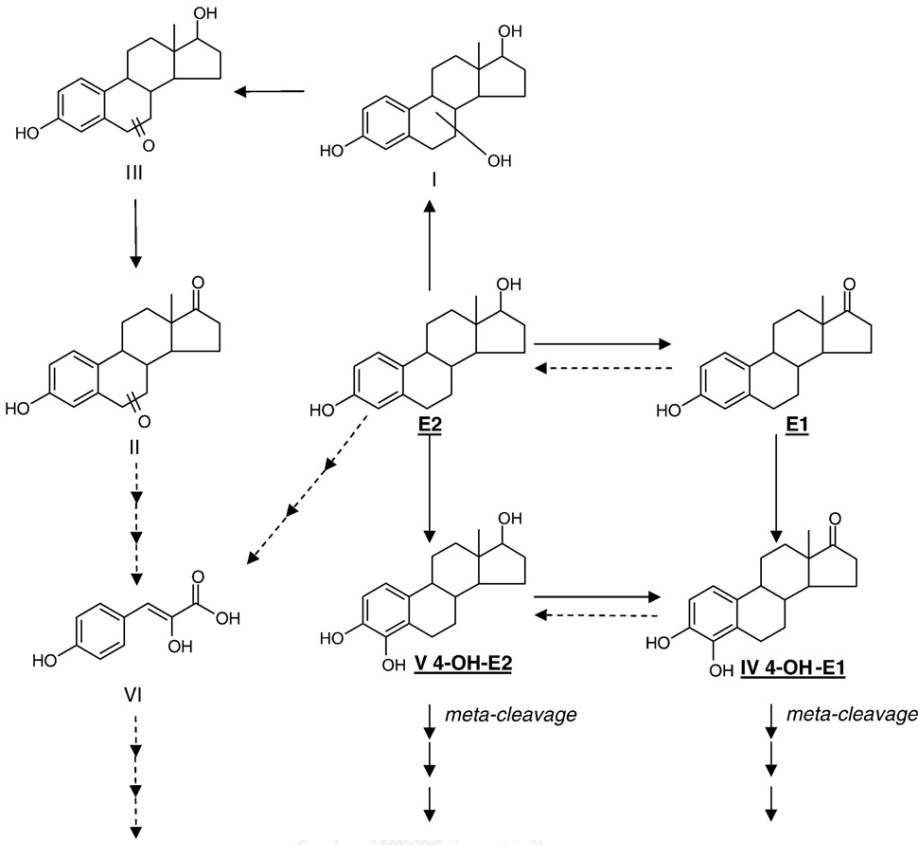


Figure 2.4 Proposed E2 degradation pathways of ED8 strain
Compounds that have been identified with authentic chemicals are shown underlined and in bold. Uncertain pathways are shown in dotted line.

Beside, five strains which related with *Bacillus* sp. were isolated from activated sludge in a wastewater treatment plant. These bacteria are capable of converting E2 to E1 within 8 days (initial concentration 1 mg/L) and only two of them were able to degrade E1 as a metabolite during E2 transformation which E2 was degraded completely and then E1 began to decline as well (Jiang et al., 2010). Haiyan et al., (2007) investigated 17a-ethynylestradiol (EE2)-degrading bacterium was isolated from the activated sludge of the wastewater treatment plant (WWTP) of an oral contraceptives producing factory in Beijing, China.

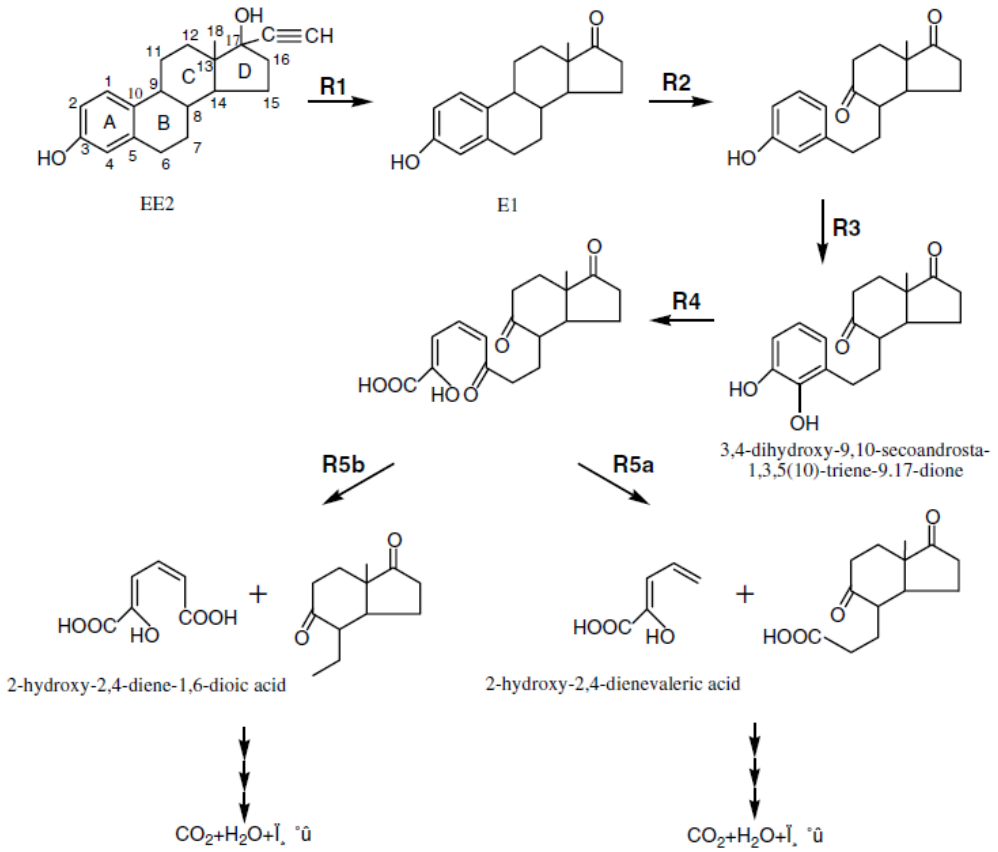


Figure 2 Proposed catabolic pathway for EE2 degradation by strain JCR5

As MT and the others can be degraded by microorganism. The numerous studies investigated the degradation of a range of steroid hormones by microorganism and proposed the degradation pathway by microorganism. For degradation pathway of MT by organisms in the environment is limit of study. However, there are many studies on the biodegradation of steroid hormones such as estrogen and testosterone (Table 2.2). It might provide information on possible biodegradation of MT.

Table 2.2 List of sex hormone degrading microorganism

Bacteria Species	Compound	Reference
*Rhodococcus*sp.	17α-Methyltestosterone	Homklin et al., 2012
	17β-estradiol (E2) estrone (E1)	Kurisu et al., 2010
Nocardioides sp.	17α-Methyltestosterone	Homklin et al., 2012
	17β-estradiol (E2)	Yu etal., 2007
*Acinetobacter*sp.	Testosterone	Yang et al., 2011
	17β-estradiol (E2)	Keet al., 2007
Brevundimonas sp.	Testosterone	Yang et al., 2011
*Comamonas*sp.	Testosterone	Yang et al., 2011
Sphingomonas sp.	Testosterone	Yang et al., 2011
	17β-estradiol(E2) estrone (E1)	Kurisu et al., 2010
Stenotrophomonas sp.	Testosterone	Yang et al., 2011
Rhodobacter sp.	Testosterone	Yang et al., 2011

CHAPTER III
METHODOLOGY

3.1 Experimental Framework

The methodology of this study were summarized in the experimental framework as shown in figure 3.1

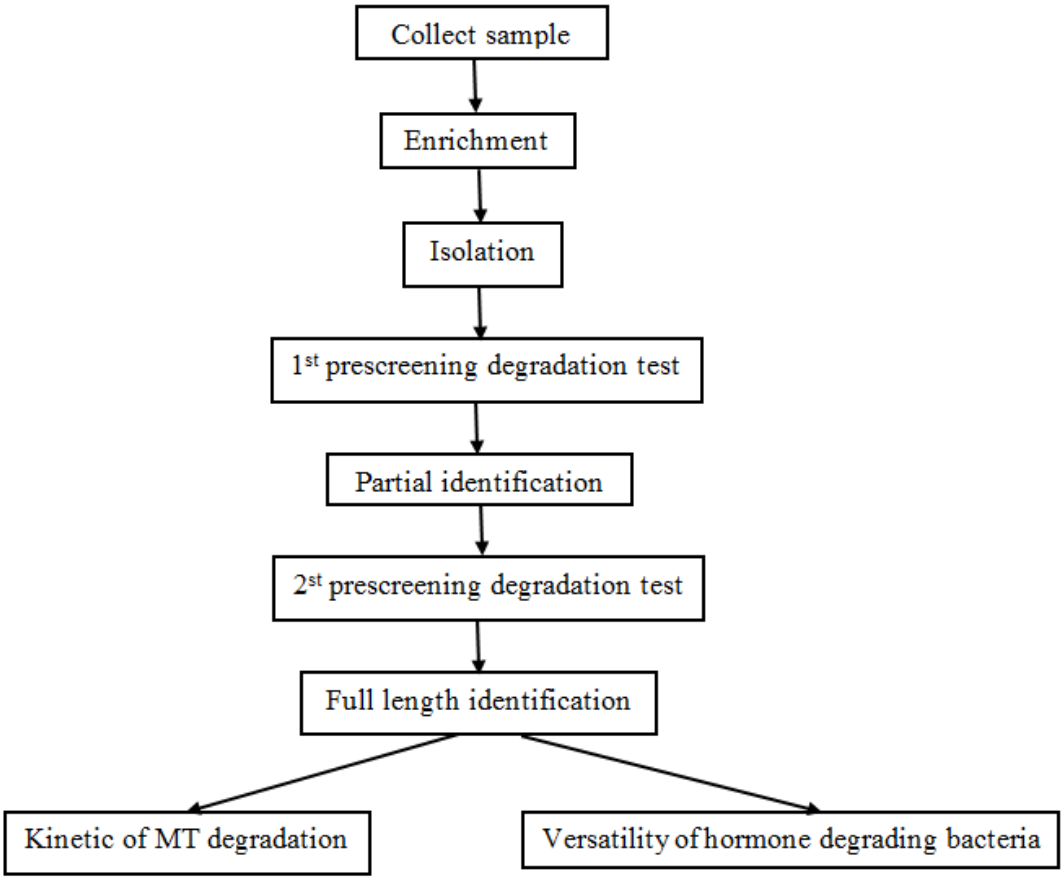


Figure 3.1 The experimental framework

3.2 Sample collection

Samples were collected from the masculinizing ponds of Nile tilapia fry located in Ang Thong province, Thailand. Two combined samples were prepared by mixing the samples from many ponds with the same proportion. For the first combined sample, the sediment was grabbed from a masculinizing earthen pond (Figure 3.2a) and a well collected dischargesfrom several masculinizing fiberglass ponds (Figure 3.2b and 3.2c). For the second combined sample, thebiofilm of slimy

settled solid was scratched from the edge of a concrete pond (Figure 3.3a) and a net of earthen pond (Figure 3.3b).

Figure 3.2 (a) an earthen pond, (b) a fiberglass ponds and (c) a discharge area of fiberglass ponds.

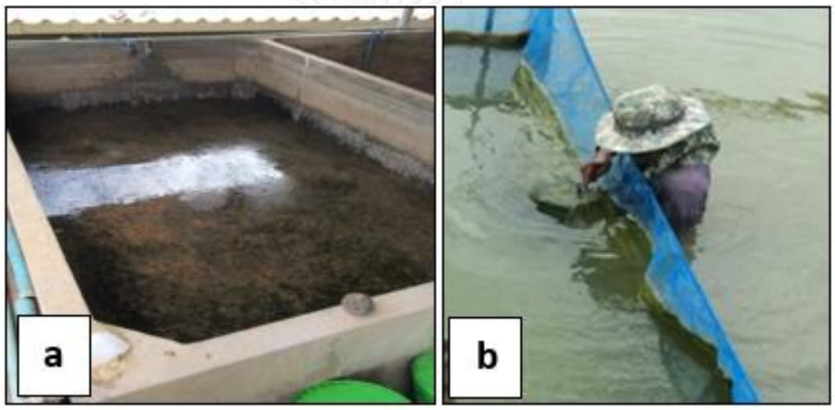


Figure 3.3 (a) a concrete pond, (b) net of earthen pond.

3.3. Fresh water medium preparation

Fresh water medium (FWM) was prepared by dissolving 1 g of NaCl, 0.2 g of KH_2PO_4, 0.5 g of KCl, 0.4 g of $MgCl_2 \cdot 6H_2O$, 0.1 g of $CaCl_2 \cdot 2H_2O$ and 5 g of HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) in one liter of water. In addition, trace element including non-chelated trace element, 1 mL; Selenite-tungstate solution, 1 mL; vitamin mixture, 1 mL; Thiamine solution, 1 mL; and vitamin B12, 1 mL were added. The composition of the trace element solution was prepared as in Table 3.1. The medium was applied from Tourna et al. (2011) who can obtain an ammonia oxidizing archaea from soil.

Table 3.1 Composition of trace element solutions

Solution	Chemicals	Unit
Non-chelated trace element	HCl (25% = 7.7M)	12.5 mL

Solution	Chemicals	Unit
(in 1 L of deionised water)	$FeSO_4 \cdot 7H_2O$	2.1 g
	H_3BO_3	30 mg
	$MnCl_2 \cdot 6H_2O$	100 mg
	$CoCl_2 \cdot 6H_2O$	190 mg
	$NiCl_2 \cdot 6H_2O$	24 mg
	$CuCl_2 \cdot 2H_2O$	2 mg
	$ZnSO_4 \cdot 7H_2O$	144 mg
	$Na_2MoO_4 \cdot 2H_2O$	36 mg
Selenite-tungstate solution (in 1 L of deionised water)	NaOH	0.4 g
	$Na_2SeO_3 \cdot 5H_2O$	6 mg
	$Na_2WO_4 \cdot 2H_2O$	8 mg
Vitamin mixture (in 100 mL of 10 mMSodium phosphate buffer at pH 7.1)	4-aminobenzoic acid	4 mg
	D(+)-biotin	1 mg
	Nicotinic acid	10 mg
	Calcium D(+)-pantothenate	5 mg
	Pyridoxine dihydrochloride	15 mg
Thiamine solution (in 100 ml of 25 mM Sodium phosphate buffer at pH 3.4)	Thiamine chloride dihydrochloride	10 mg
Vitamin B12 (in 100 mL of deionised water)	Cyanocobalamine	5 mg

3.4 Enrichment of MT-degrading bacteria

For the enrichment of MT-degrading bacteria, 10% (v/v) of either sediment sample or biofilm sample was inoculated into a flask containing 200 mL of fresh water medium (FWM). The 17α-methyltestosterone (MT: >97% HPLC grade, ALDAMEX, Switzerland) was initially supplied as a sole carbon source in the system at different concentrations (0.5, 5, 30 mg/L). For the half of culturing flasks, I aimed to investigate the role of archaea on MT degradation, 100 µg/mL of ampicillin (final concentration), which prevent the peptidoglycan formation in the bacterial cell wall, was applied. Totally, twelve culturing conditions were performed (Table 3.2).

Table 3.2 The condition of twelve culturing flasks

Source	Initial concentration	Ampicillin	Sample name
Sediment sample (S)	0.5 mg/L	-	S05
		+	SA05
	5 mg/L	-	S5
		+	SA5
	30 mg/L	-	S30
		+	SA30
Biofilm sample (B)	0.5 mg/L	-	B05
		+	BA05
	5 mg/L	-	B5
		+	BA5
	30 mg/L	-	B30
		+	BA30

Note: As the isolates name presented in the table, the alphabet S stands for Sediment sample and B stands for Biofilm sample which are the source of isolates. The alphabet A stands for the enrichment culture with ampicillin. The following numbers (05, 5 and 30) stand for the initial MT concentration of each enrichment cultures (0.5 mg/L, 5mg/L and 30 mg/L). The last numbers (1, 2, 3, ...) stands for each isolates which were selected to use in the experiment. For example, SA051 is bacteria number one which obtained from sediment sample at 0.5mg/L of MT with ampicillin

Because MT is hardly dissolved in water (3.39 mg/L, Homklin et al., 2012), the MT stock solution was prepared using methanol (HPLC grade, RCI Labscan, Thailand) at concentrations of 500 and 5,000 mg/L. The stock MT solution was added into free culturing flasks (500mL) followed by air-drying to remove methanol before filling with FWM medium. The culturing flasks were incubated at room temperature with a shaker speed of 200 rpm. The MT concentration was measured periodically using the High Performance Liquid Chromatography (HPLC 1100 series, Agilent Technologies) with diode array detector. Once the MT concentration was reduced to 90% of the original concentration, then transferred 10% of the enriched culture into a new flask containing MT medium at the designated concentration. The enrichment process was repeated for ten cycles.

3.5 Isolation of MT-degrading bacteria

The enriched culture was isolated by spreading technique with the FWM agar containing MT at their enrichment concentration. The agar was prepared by spreading the MT stock solution onto the surface and then opening the plate over-night in sterile biological hood to evaporate the methanol. The incubation was conducted for 5-7 days at room temperature. Colonies with different morphologies were selected and streaked onto the new FWM agar containing MT at their enrichment concentration. The isolation was repeated until getting the single colony of pure culture.

For the selection of MT degrading microorganisms, the isolated colonies were preliminary screened for their MT degradation by inoculating into FWM broth containing MT at their respective concentration and measured the MT reduction after 7 days (Using HPLC section 3.8).

3.6 Identification of MT-degrading bacteria

Genomic DNA was extracted using freezing and thawing technique for five cycles (Thayanukul et al., 2013). In brief, few pure colonies were selected and suspended in 30 µL of DNAse free water (Invitrogen, USA) followed by the freezing step in a -80^{o}C refrigerator for 3 min and thawing step in a dry-bath incubator at 75^{o}C for 5 min. The DNA template was amplified by PCR for sequencing in partial with the universal primers 27F (5’ AGA GTT TGA TCC TGG CTC AG 3’), 519F (5’ CAG CMG CCG CGG TAA TWC 3’) and 1492R (5’ GGT TAC CTT GTT ACG ACT T 3’) (Wang et al., 2009 and Homklin et al., 2012). The PCR mixture was prepared using Taq DNA polymerase kit from Fermentas (Thermo scientific, USA) following the company instruction. The PCR program was set as follows: 94^{o}C (10 min); 35 cycles of 94^{o}C (30 sec), 50^{o}C (30 sec), 72^{o}C (30 sec); and then 72^{o}C (5 min). The PCR product was purified and sequenced by Macrogen Inc., Korea (http://www.macrogen.com/eng/) with primers 27F (5’ AGAGTTTGATCMTGGCTCAG 3’), 518F (5’ CCAGCAGCCGCGGTAATACG 3’), 800R (5’ TACCAGGGTATCTAATCC 3’)and 1492R (5’ TACGGYTACCTTGTTACGACTT 3’). The sequence assembly was performed using DNA Baser Sequence Assembler v4.14 (Heracle BioSoft, Romania).Bacterial identification was performed by comparing the sequence similarity using BLASTn in

NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic tree was analyzed using clustal W and neighbor joining method with Mega software (Tamura et al., 2013)

3.7 MT biodegradation test

To confirm the MT degrading ability in second prescreening degradation test, the identified colonies were inoculated into 5 mL of the FWM medium containing 10 mg/L of MT in 16 mL amber glass vial. The control set was included with the same culturing condition without isolate inoculation. The cultivation was performed at room temperature for 7 days in a shaker with 200 rpm. Every 24 hours, the 5 mL of sample is taken for measuring the MT concentration.

3.8 Measurement of MT concentration

MT analysis was conducted by the method following Homklin (2012). The samples were mixed with methanol in the ratio of 1:1, filtered with 0.45 µm PTFE membrane (National scientific, USA), and filled in HPLC vials. Then, 50 µL of each sample was injected into the High Performance Liquid Chromatography (HPLC 1100 series, Agilent Technologies) installed with reverse phase C18 column (250mm×5mm×4.0µm, ODS Hypersil) and connected to the diode array detector. The detecting wave length was set at 245 nm and the column temperature was maintained at 40^{o}C. The gradient mobile phase was prepared using acetonitrile (ACN, HPLC grade, RCI Labscan, Thailand) and deionized water (18Ω, ELGA, UK). The gradient solution has started from 60% ACN, raised to 95% ACN for 6 min, reduced and hold at 60% ACN for 3 min. The flow rate was kept constant at 1 mL/min. To determine the concentration, the external standard curve was prepared in the range of 0.1 – 50 mg/L (0.1, 0.3, 0.5, 0.8, 1, 3, 5, 8, 10, 20, 30 and 50 mg/L).

Quantitative analyses of the other steroid hormones were performed using the analytical method used for MT analysis.

3.9 Biodegradation of MT by the pure isolates

The kinetics of MT degradation was studied with MT as the sole carbon source. The tests were carried out with initial MT concentrations range of 1-50 mg/ L. All experiments were performed in amber vials (16mL) with 5 mL of medium solution containing 1%v/v of 10^6 cells/mL of isolated MT degrading bacterium.

Abiotic control tests were conducted without the isolated cells. The vials were incubated at room temperature approximately 30°Cwith a rotating speed of 200 rpm. The number of cells was counted on Plate Count Agar (PCA) medium by drop plate technique which is direct dropping the bacteria culture on plate without spreading. This technique use a little amount of bacteria culture which is suitable for the experiment. Michaelis-Menten equation was used to model the kinetics of MT degradation. The equation is given below:

$$V = \frac{V_{max} S}{K_m + S} \qquad \text{(Eq 3. 1)}$$

Where; V is the specific degradation rate ($mgL^{-1}hr^{-1}cell^{-1}$)

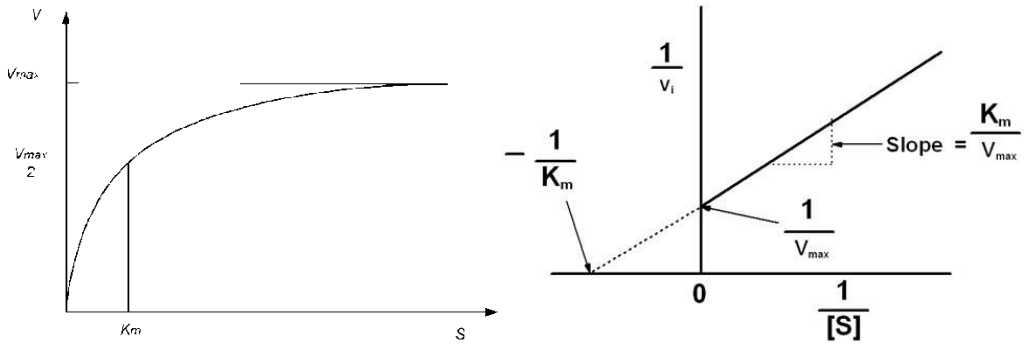
V_{max} is the maximum degradation rate ($mgL^{-1}hr^{-1}cell^{-1}$)

S is the substrate concentration (mg/L)

K_m is the substrate half saturation coefficient, the Michaelis–Menten constant (mg/L)

The value of V_{max} and K_m were determined by plotting graph between MT concentration (X axis) and specific degradation rate (Y axis) (Figure 3.4a). According to the graph, K_m is a half of V_{max}. In order to determine V_{max} from the Michaelis-Menten plot, another graph namely Lineweaver-Burke which shows linear relationship between $\frac{1}{V}$ and $\frac{1}{S}$ (Figure 3.4b) since these parameter were derived from Equation 1. The equation is given below:

$$\frac{1}{V} = \frac{Km}{Vmax} \cdot \frac{1}{S} + \frac{1}{Vmax} \qquad \text{(Eq 3. 2)}$$



(a) (b)

Figure 3.4 (a) Michaelis-Menten plot and **(b)**Lineweaver-Burke plot.

According to Lineweaver-Burke plot in Figure 3.4(b), slop is ratio between K_m and V_{max}. While $\frac{-1}{K_m}$ and $\frac{1}{V_{max}}$ are X and Y interception, respectively.

3.10 Versatile substrates degrading characteristic of isolated strains

To characterize the versatile degradation ability of isolated strains, the other hormones that are also often detected in environment and have similar chemical structures to MT were examined. Testosterone, estrone (E1), 17β-estradiol (E2), and 17α-ethynylestradiol (EE2) were selected. The degradation tests were performed following the MT experiment using hormone concentration of 10 mg/L.

Quantitative analyses of the other steroid hormones were performed using the analytical method used for MT analysis (section 3.8).

CHAPTER IV
RESULTS AND DISSCUSION

4.1 Enrichment and isolation of MT-degrading microorganisms

The enrichment was performed in twelve conditions. After ten subculturing, the pure cultures on solid FWM agar plates at the same MT concentration used in the as enrichment step. Various colony characteristics in term of color, form, elevation and margin were observed. As different microbes may have the same appearance, I collected approximately five colonies for each characteristic. In total, 130 colonies were collected for further screening the MT degrading ability (Table 4.1).

Table 4. 1 Colony morphology of MT degrading microorganism isolated from Sediment.

Source	Initial MT concentration	Colony morphology			
		Color	Form	Elevation	Margin
Sediment (S)	0.5 mg/L	White	Circular	Convex	Entire
		Transparent	Circular	Convex	Undulate
		Brown	Circular	Convex	Entire
	0.5 mg/L + Ampicillin (A)	Cream	Circular	Convex	Entire
		Brown	Irregular	Flat	Lobate
		White	Punctiform	Convex	Entire
		Brown	Circular	Convex	Entire
	5mg/L	White	Punctiform	Convex	Entire
		White	Circular	Convex	Entire
	5 mg/L + Ampicillin (A)	Cream	Circular	Convex	Undulate
		Brown	Circular	Convex	Entire
		White	Punctiform	Convex	Entire
	30 mg/L	White	Circular	Flat	Undulate
		White	Circular	Convex	Entire
		Brown	Circular	Convex	Entire
	30 mg/L + Ampicillin (A)	Yellow	Circular	Convex	Undulate
		White	Punctiform	Convex	Entire

Table 4.1(cont.) Colony morphology of MT degrading microorganism isolated from biofilm samples.

Source	Initial MT concentration	Colony morphology			
		Color	Form	Elevation	Margin
Biofilm	0.5 mg/L	White	Punctiform	Convex	Entire
		White	Circular	Convex	Entire
	0.5 mg/L + Ampicillin (A)	Brown	Circular	Convex	Entire
		White	Circular	Convex	Entire
	5 mg/L	White	Punctiform	Convex	Entire
		Brown	Punctiform	Convex	Entire
	5 mg/L + Ampicillin (A)	White	Circular	Convex	Entire
		Yellow	Circular	Convex	Undulate
	30 mg/L	Brown	Circular	Convex	Entire
		White	Punctiform	Convex	Entire
		Cream	Circular	Convex	Undulate
	30 mg/L + Ampicillin (A)	White	Punctiform	Convex	Entire
		White	Circular	Convex	Entire
		Brown	Circular	Convex	Entire

4.2 First screening of MT degradation

All 130 colonies have been subjected to the prescreening degradation test at 10 mg/L for seven days. The MT concentration was measured before and after the incubation. Among 130 tests, only 14 colonies could reduce MT greater than 80% of initial concentration of MT. Nine isolates were from a sediment sample and five isolates were from a biofilm sample. For further experiment in section 4.3, 14 colonies with high MT removal were used and also selected 6 colonies, which have unique colony morphologies though their MT degradations were poor.

Table 4. 2 Colony morphology and source of selected strains

Selected strain	Colony characteristic				Source
	Color	**Form**	**Elevation**	**Margin**	
S051	Brown	Circular	Convex	Entire	Sediment, 0.5 mgMT/L, no ampicillin
S052	Brown	Circular	Convex	Entire	
SA051	Brown	Irregular	Flat	Lobate	Sediment, 0.5 mgMT/L, with ampicillin
S51	White	Punctiform	Convex	Entire	Sediment, 5 mgMT/L, no ampicillin
S52	White	Circular	Convex	Entire	
S301	White	Circular	Flat	Undulate	Sediment, 30 mgMT/L, no ampicillin
S302	White	Circular	Flat	Undulate	
S303	White	Circular	Flat	Undulate	
S304	White	Circular	Convex	Entire	
S305	White	Circular	Flat	Undulate	
S306	White	Circular	Flat	Undulate	
SA301	Cream	Irregular	Umbonate	Undulate	Sediment, 30 mgMT/L, with ampicillin
SA302	Cream	Irregular	Umbonate	Undulate	
B051	White	Punctiform	Convex	Entire	Biofilm, 0.5 mgMT/L, no ampicillin
B052	white	Circular	Convex	Entire	
BA051	White	Circular	Convex	Entire	Biofilm, 0.5 mgMT/L, with ampicillin
BA51	White	Circular	Convex	Entire	Biofilm, 5 mgMT/L, with ampicillin
B301	Brown	Circular	Convex	Entire	Biofilm, 30 mgMT/L, no ampicillin
B302	Brown	Circular	Convex	Entire	
B303	White	Punctiform	Convex	Entire	

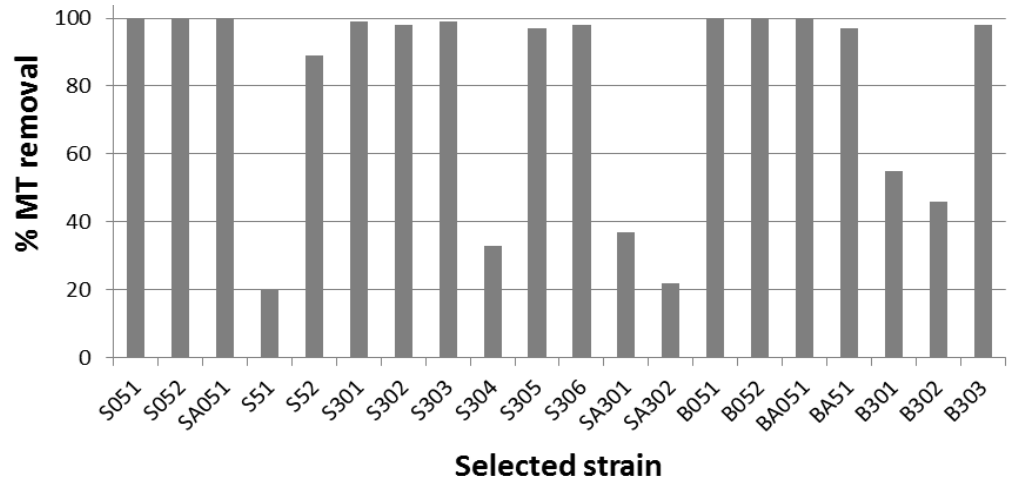


Figure 4. 1 The percentage of MT removal by selected strains within 7 days.

4.3 Preliminary Identification of isolates from MT enrichment cultures

The colony PCR was performed with the 16S rRNA gene primers. The DNA template was amplified by PCR using the universal primers 519F (5’ CAG CMG CCG CGG TAA TWC 3’) and 1492R (5’ GGT TAC CTT GTT ACG ACT T 3’). The uncommon primer 519F was selected for preliminary identification because it could bind 96.7% and 98.5% of archaea and bacterial 16S rRNA gene sequences, respectively (Wang et al., 2009). The freezing and thawing technique was used to extract DNA. The condition has been adjusted many times for the good DNA amplifying result. Figure 4.2 shows exampleof gel electrophoresisimage of the PCR product which the DNA template was extracted by freezing and thawing technique.

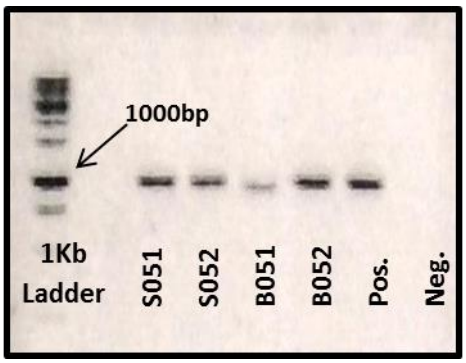


Figure 4. 2 Examples of gel electrophoresis of PCR product which the DNA template was extracted by freezing and thawing technique. The PCR product was amplified by using primer 519F and 1492R

Among twenty isolates, nineteen isolates could be amplified by PCR technique. The 16S rRNA genes of an isolate number B303 could not obtained which may be due to its unique cell wall property which was difficult to release by using freezing and thawing technique.

However, the 16S rRNA gene analysis results have been achieved for only eight strains. The rest isolates were not usable due to contamination problem. As more than one colony were collected for the DNA extraction step in order to increase DNA template density, different bacteria might be collected together.

The 16S rRNA gene sequences of eight isolates were preliminary identified using BLAST algorithm (Table 4.3). Strain S051 and S052 which came from the sediment samples were closely related to *Azotobacter chroococcum* strain YCYSat 99% similarity. In addition, both strain SA51 from biofilm sample (5 mg/L with ampicillin) and SA301 from sediment and water (30 mg/L with ampicillin) were closely related to *Pseudacidovorax* sp. NH-1 at 99% and 100%, respectively. Besides, strain B051, B052, S303, and S304 were affiliated to *Rhodobacter* sp., *Sinorhizobium* sp.,and *Nocardioides* sp.,and *Ensifer* sp., respectively.

There was no archaea obtained, but bacteria grew in the ampicillin containing enrichment culture. These bacteria strains were resist to ampicillin antibiotic. The archaea might not play the role in MT degradation in fish pond or they might be eliminated due to the bias of culturing technique.

Table 4. 3 Identification of eight isolates from MT enrichment cultures

Isolates	**Closest matched sequences**	**Accession number**	**% similarity**
B051	*Rhodobacter*sp. strain NP25b	EU580696.1	98% (505bp/1340bp)
B052	*Sinorhizobium* sp. strain SCAUS141	KF836038.1	99% (723bp/1405bp)
BA51	*Pseudacidovorax*sp. strain NH-1	HQ834240.1	99% (794bp/1493bp)
S051	*Azotobacterchroococcum* strain YCYS	JQ692178.1	99% (720bp/1429bp)
S052	*Azotobacterchroococcum* strain YCYS	JQ692178.1	99% (721bp/1429bp)
S303	*Nocardioides* sp. strain DF412	AB373748.1	100% (792bp/1463bp)
S304	*Ensifer* sp. strain A6(2012)	JX941528.1	99% (721bp/1356bp)
SA301	*Pseudacidovorax* sp. strain NH-1	HQ834240.1	100% (721bp/1493bp)

4.4. Second screening of MT degradation

In order to confirm MT degrading ability of eight isolates, the single colony of them were inoculated individually into a medium containing 10 mg/L of MT and

measured the MT reduction during 7 days. Only five colonies reduced MT considerably within 7 days (Figure 4.3). They were strain B051 and B052 isolated from biofilm sample in the enrichment of 0.5 mg/L, strain S051 and S052 isolated from sediment sample in the enrichment of 0.5 mg/L, and strain S303 isolated from the sediment in the enrichment of 30 mg/L. Three isolates, namely BA51, SA301, and S304, could not reduce MT at second screening, although they could degrade MT by 97%, 37%, and 33%, respectively, in the first screening test. Strain SA301 and S304 confirmed with the second screening that they entirely could not degrade MT. The purpose of this pre-screening was to see whether or not bacteria can degrade a particular hormone, not about how well the degradation ability of bacteria is. Therefore, the strain BA51 was discarded as it showed the disability to degrade MT. In addition, they had been isolated from the enrichment culture containing MT as a sole carbon source. Since MT was dissolved in methanol, trace amount of methanol might remain even though the solvent was removed by evaporation. These three isolates might utilize methanol that was remained on agar plate or they might need longer incubation period for growth.

According to the analysis of partial DNA, four strains including B052, S051, S052 and S303 were examined as *Sinorhizobium* sp., *Azotobacter* sp., *Azotobacter* sp. and *Nocardioides* sp. respectively. These strains could remove 100% of the given MT within two days in second screening and remove MT more than 99% within seven days in the first screening. It can be implied that these strains were absolutely the MT degrading bacteria. Whereas strain B051 affiliated to *Rhodobacter* sp. revealed 30% removal efficiency in second screening, it removed 100% of MT in the first test. This suggested that *Rhodobacter* sp. might be a bacterium capable of MT biodegradation.

Hence, these five strains exhibiting the MT degrading ability to further experiments were selected.

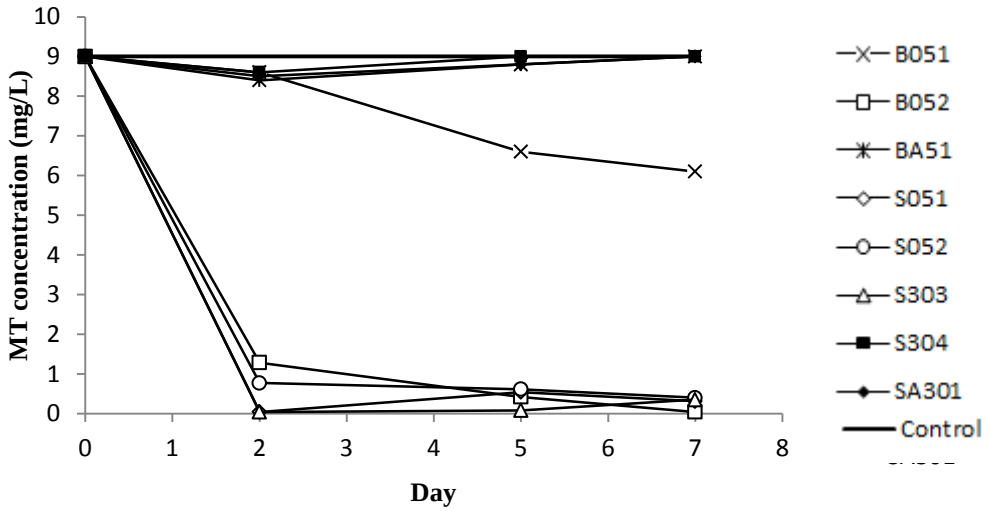


Figure 4. 3 Reduction of MT in one week at the initial concentration of 10 mg/L.

4.5 Identification of MT degrading bacteria with nearly full length16S rRNA gene sequence

The nearly full length16s rRNA gene sequences of the five MT degrading bacteria (B051, B052, B051, S052 and S303) have been reevaluated using primers 27F, 518F, 800R, and 1492R. Quality of DNA sequencing has been examined before BLAST identification. The results show in Table 4.4.

Table 4. 4 Identification of MT degrading bacteria using full length 16S rRNA gene analysis

Isolates	Closest matched sequence	Accession number	% similarity
B051	*Acinetobacter*sp. SCU-B268	KJ000864.1	99% (1301bp/1404bp)
B052	*Ochrobactrum* sp. C-1	KF479631.1	99% (1326bp/1447bp)
S051	*Acinetobacter* sp. U1369-101122-SW178-2	JQ082154.1	99% (1311bp/1443bp)
S052	*Ochrobactrum* sp. FCp1	KJ009242.1	99% (1413bp/1429bp)
S303	*Nocardioides* sp. DF412	AB373748.1	99% (1307bp/1463bp)

The analysis revealed that strain SW303 was closely related to *Nocardioides* sp. which was same with the preliminary identification result in Section 4.2 (Table 4.5). On the other hand, the rest four strains were matched with different strains in BLAST search from the preliminary identification.

Strain S051 was matched with *Azotobacter* sp. in the previous test but it changed to be *Acinetobacter* sp. in this analysis. *Acinetobacter* sp. might contaminated during the subculture on plateprocess that several colonies were picked up together. Since microorganisms in air is generally little and aseptic technique has been perform strictly with qualified instrument, the strain was likely originated from masculinizing pond samples.

In addition, strain B051 was also reexamined as *Rhodobacter* sp. instead of *Acinetobacter* sp. The culturing condition of the strain B051 seemed to be contaminated by *Acinetobacter* sp. from strain S051.

Besides, strain B052 was changed from *Sinorhizobium* sp. to *Ochrobactrum* sp. Again, the sample was likely contaminated at subculturestep. And again strain S052 is affiliated to *Ochrobactrum* sp. It might contaminated further from strain B052.

From this result, it can conclude that there is contamination during the experiment. It might be in the subculture step that many colonies have been picked up together to increase the cell density. Since the new strains likely from the masculinising ponds and they have the ability to degrade MT, thus, the next step was continued using the new identified strains; *Ochrobactrum* sp. and *Acinetobacter* sp.s.

Table 4. 5 Comparison identification result between the preliminary test and the latest of MT degrading bacteria

Isolate	Preliminary result	Current result
B051	***Rhodobacter* sp.** **strain NP25b** 98% (505bp/1340bp) Phylum: Proteobacteria **Class: Alphaproteobacteria** Order: Rhodobacterales Family: Rhodobacteraceae Genus: Rhodobacter	***Acinetobacter*sp.** **strain SCU-B268** 99% (1359bp/1404bp) Phylum: Proteobacteria **Class: Gammaproteobacteria** Order: Pseudomonadales Family: Moraxellaceae Genus: Acinetobacter
B052	***Sinorhizobium* sp.** **strain SCAUS141** 99% (723bp/1405bp) Phylum: Proteobacteria Class: Alphaproteobacteria **Order: Rhizobiales** Family: Rhizobiaceae Genus: Sinorhizobium	***Ochrobactrum* sp.** **strain C-1** 99% (1326bp/1447bp) Phylum: Proteobacteria Class: Alphaproteobacteria **Order: Rhizobiales** Family: Brucellaceae Genus: Ochrobactrum

Table 4.4 (cont.) Comparison identification results between the preliminary test and the latest of MT degrading bacteria

Isolate	Preliminary result	Current result
S051	***Azotobacterchroococcum*** **strain YCYS** 99% (720bp/1429bp) Phylum: Proteobacteria Class: Gammaproteobacteria **Order: Pseudomonadales** Family: Pseudomonadacea Genus: Azotobacter	***Acinetobacter*** **sp. strain U1369-101122-SW178-2** 99% (1369bp/1443bp) Phylum: Proteobacteria Class: Gammaproteobacteria **Order: Pseudomonadales** Family: Moraxellaceae Genus: Acinetobacter
S052	***Azotobacterchroococcum*** **strain YCYS** 99% (721bp/1429bp) Phylum: Proteobacteria **Class:Gammaproteobacteria** Order: Pseudomonadales Family: Pseudomonadacea Genus: Azotobacter	***Ochrobactrum*** **sp. strain FCp1** 99% (1413bp/1429bp) Phylum: Proteobacteria **Class: Alphaproteobacteria** Order: Rhizobiales Family: Brucellaceae Genus: Ochrobactrum
S303	***Nocardioides*** **sp. strain DF412** 100% (792bp/1463bp) Phylum: Actinobacteria Class: Actinobacteria Order: Actinobacteridae Family: Nocardioidaceae **Genus: Nocardioides**	***Nocardioides*** **sp. strain DF412** 99% (1307bp/1463bp) Phylum: Actinobacteria Class: Actinobacteria Order: Actinobacteridae Family: Nocardioidaceae **Genus: Nocardioides**

The 16s rRNA sequences of strain B051 and strain S051 were compared and found that their sequence similarity was 99.8%. The alignment was shown in figure 4.4 According to Yarza et al., (2014) that the specie cutoff is 98.7%, these two strains might be the same *Acinetobacter* sp. Other characterizing such as physiological test, DNA hybridization, and microscopic observation are required to completely identify the strains. Since these two strains are likelythe same species, only one strain for subsequent study was chosen. In the case of strain B052 and S052, 100% sequence similarity was confirmed by alignment tool of CLC Sequence Viewer 6 program (Figure 4.5). Therefore one of them was selected for further study. In summary, three species of MT degrading bacteria, including *Nocardioides* sp. (S303), *Acinetobacter* sp. (B051) and *Ochrobactrum* sp. (B052), were obtained and used in kinetic of MT degradation.

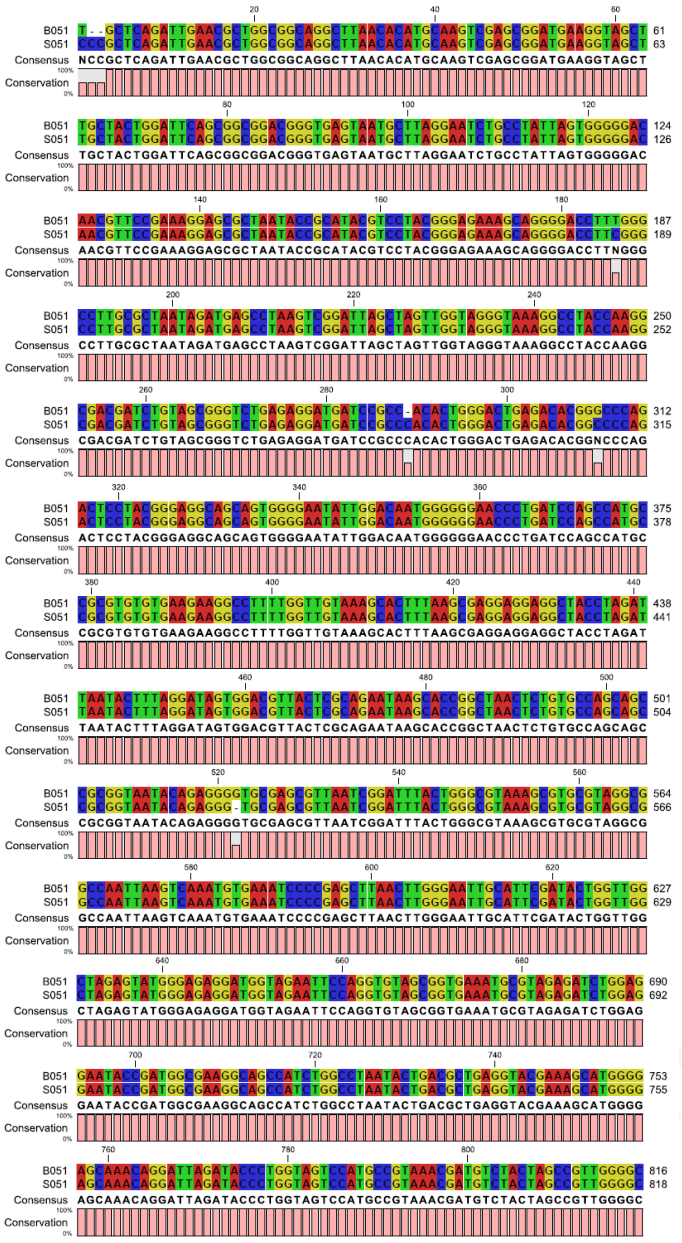


Figure 4. 4 Alignment of strain B051 and strain S051

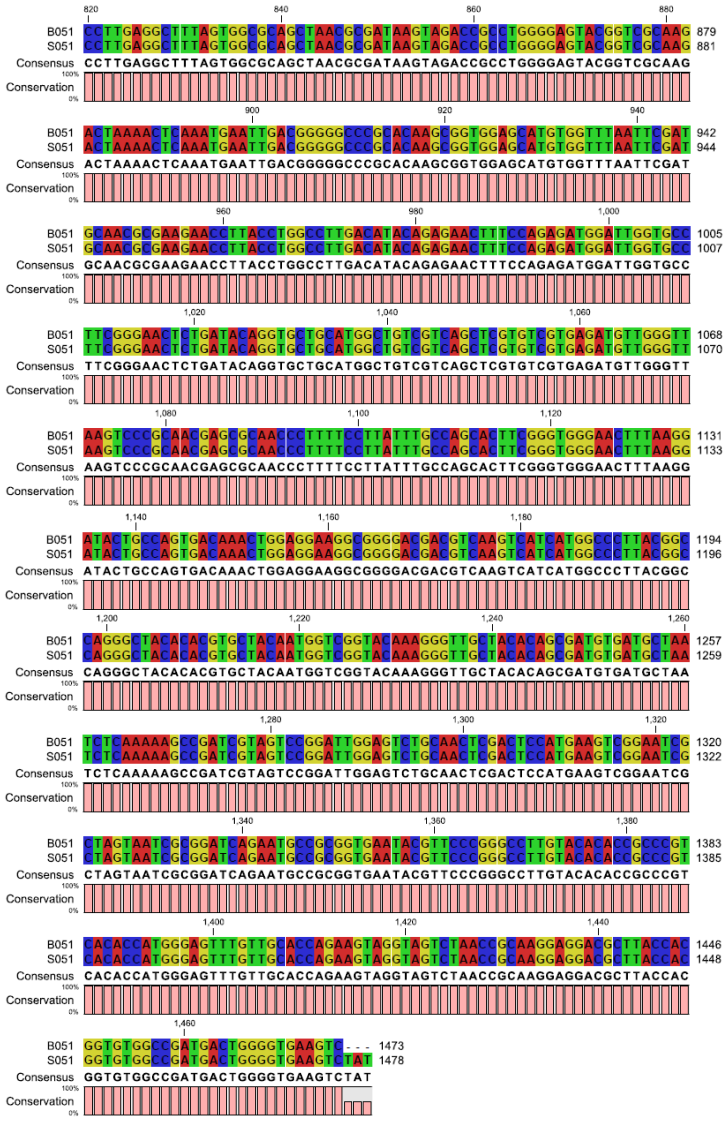


Figure 4.4 (cont.) Alignment of strain B051 and strain S051

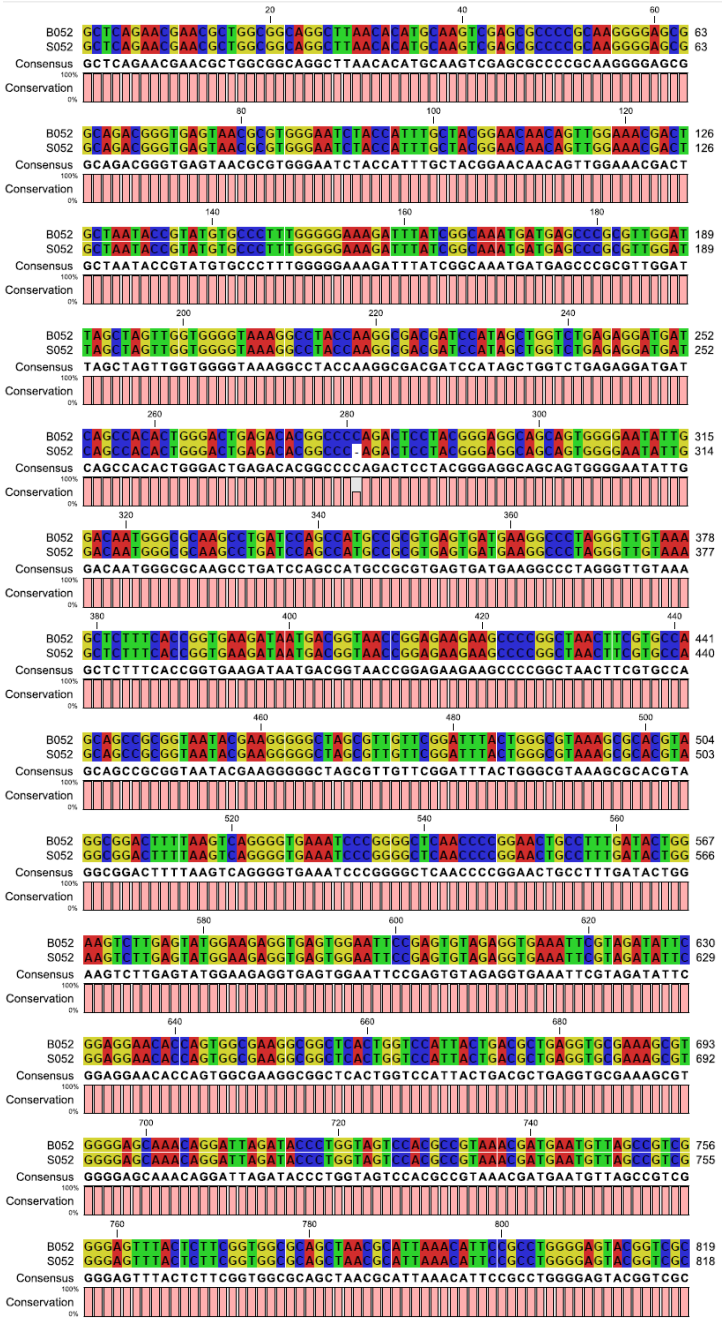


Figure 4. 5 Alignment of strain B051 and strain S051

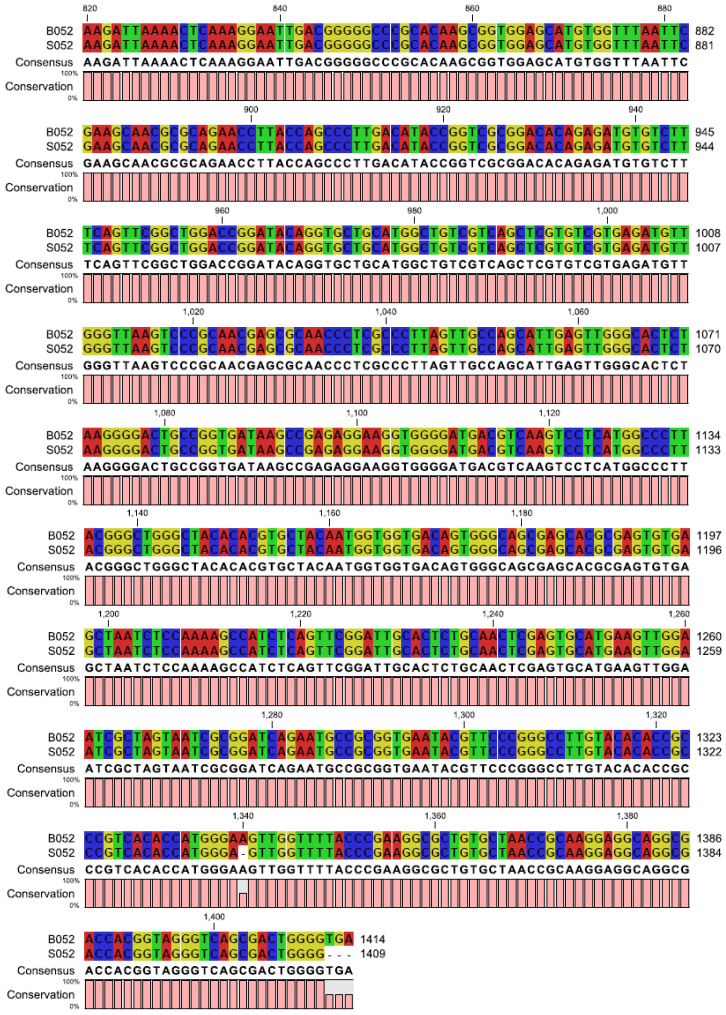


Figure 4.5 (cont.) Alignment of strain B051 and strain S051

And the phylogenetic relationships between MT degrading bacteria and related bacteria is shown in Figure 4.6 which analyzed using clustal W and neighbor joining method with almost full-length sequences of 16S rRNA of bacteria. Strain S051 and B051 were found to be closely related to *Acinetobacter* sp. Strain S052 and B052 were related closely to *Ochrobactrum* sp. and strain S303 was examined as *Nocardioides* sp.

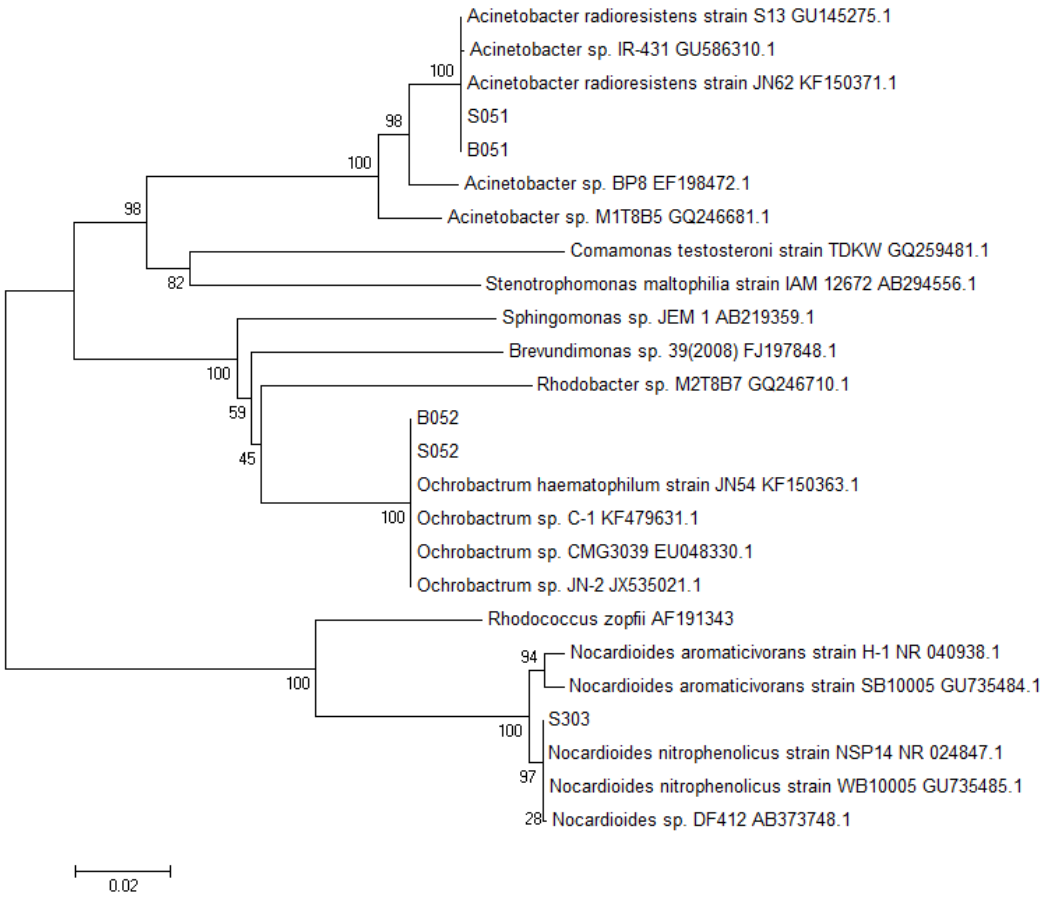


Figure 4. 6 Phylogenetic tree shows relationship among a group of bacteria analyzed using clustal W and neighbor joining method.

4.6 Kinetic of MT degradation by bacterial isolates

After classified the 16s rRNA gene sequences of all MT degrading isolates, the MT degrading kinetic of the three strains namely B051, B052 and S303 have been investigated. These strains were cultivated in medium containing MT as a sole carbon source at the different initial MT concentration ranging from 1 to 50 mg/L. This experiment was first conducted at the MT concentration of 1, 5, 10, and 50 mg/L to estimate the trend line of Michaelis-Menten model. After getting rough trend line, the researcher then added data of MT at the concentration of 3, 8, 20, 30 mg/L to delicately observe the trend.The number of cells of all strains (Figure 4.6a - 4.8a, Right) slightly changed at all initial MT concentration. These results implied that the substrate concentration used in this experiment was not high enough to support bacteria growth.

The degradation results of strain B051, B052 and S303 at initial MT concentration are presented in figure 4.7a – 4.9a (Left). All strains can degrade MT with acclimatization period ranging from 24 to 48 hours. The kinetic of MT degradations by different isolates are demonstrated. The result showed that degradation rate increased when MT concentration increased. The specific substrate degradation rate (Vmax) was calculated from substrate degradation rate divided by the number of cell at initial time. As shown in Figure 4.7b - 4.9b, the Michaelis-menten model was found to fit to the experimental data.

The strain B051 was tested using MT concentrations of 1, 3, 5, 8, 10, 20, 30, 50 mg/L. For 1 mg/L and 3 mg/L of MT, the MT was depleted within 72 hours, while it takes 36 hours to remove 5 mg/L MT. At MT concentration of 8, 10, 20, 30, 50 mg/L on the other hand, it takes more than 96 hours to complete biodegradation. The kinetic constants Vmax and Km in biodegradation of MT by strain B051 were estimated at 0.34 $ngL^{-1}h^{-1}cell^{-1}$ and 8.23 mg/L, respectively.

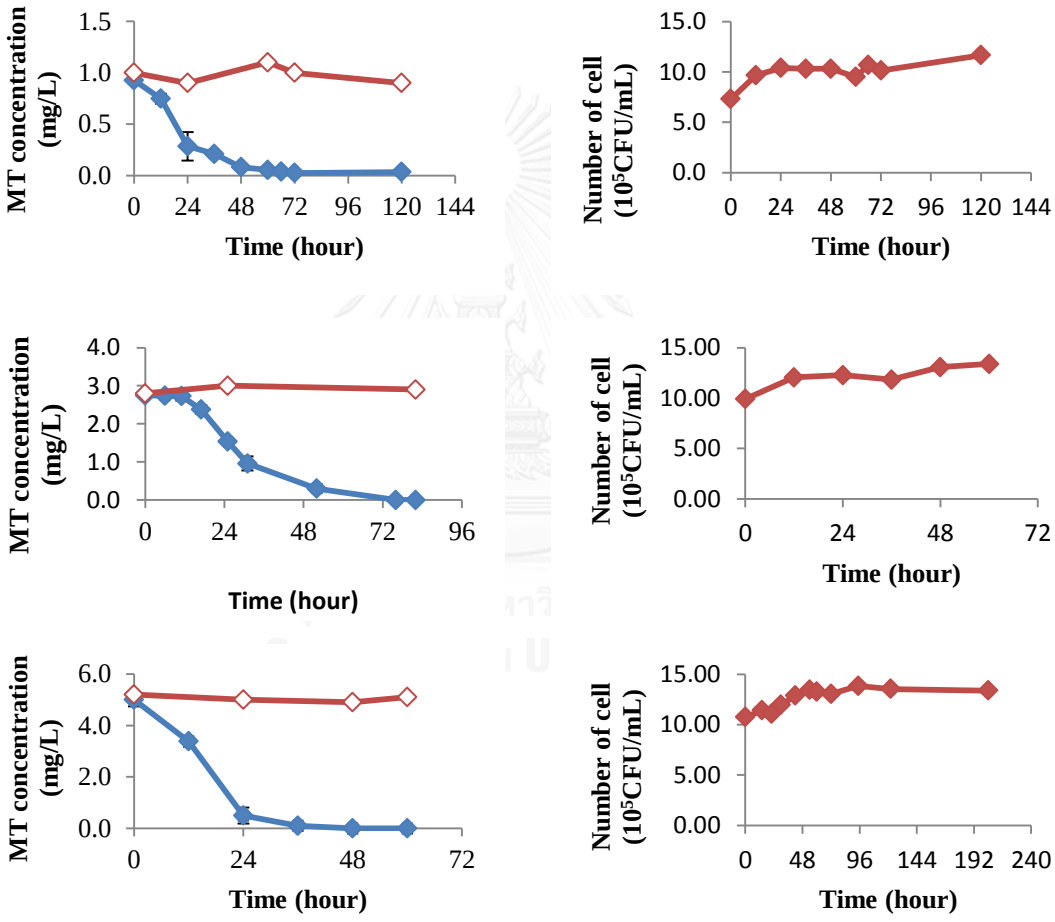


Figure 4. 7a Degradation of MT by strain B051 (Left) and number of cells at different initial MT concentration (Right).Error bars indicate standard deviation (S.D.) from triplicate data sets.

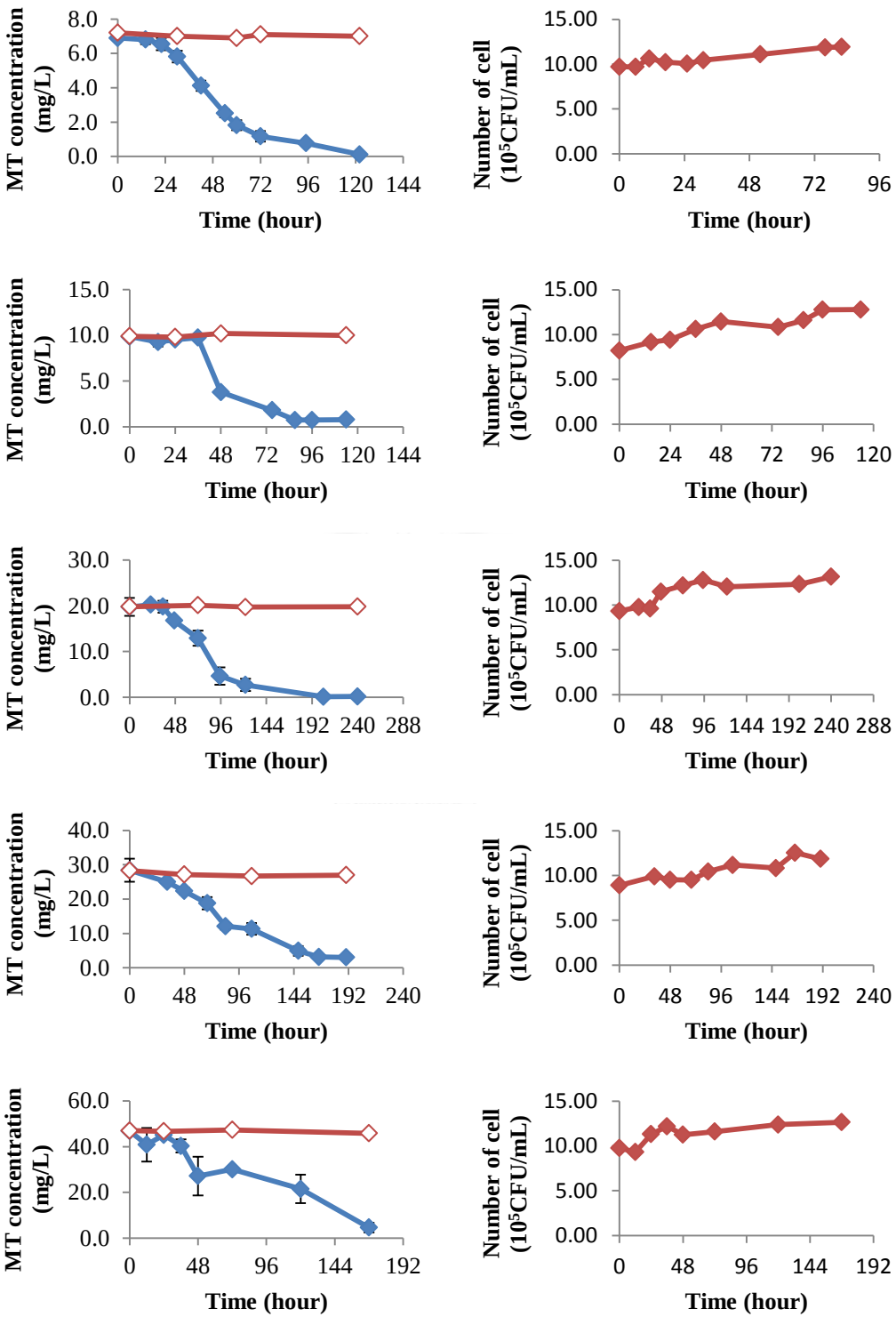


Figure 4.7a cont. Degradation of MT by strain B051 (Left) and number of cells at different initial MT concentration (Right).Error bars indicate standard deviation (S.D.) from triplicate data sets.

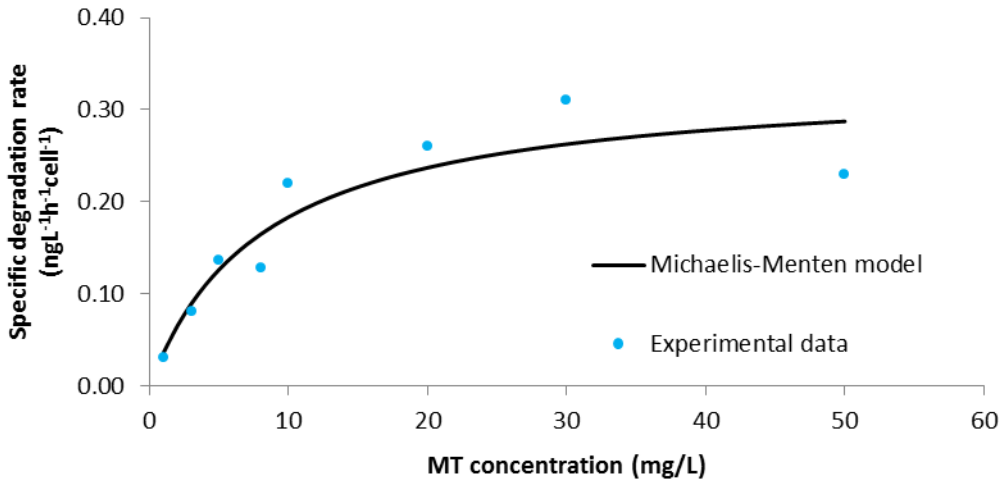


Figure 4.7b Specific degradation rate (ngL^{-1} h^{-1} $cell^{-1}$) of strain B051

The strain B502 was tested using MT at the concentrationsof 1, 3, 5, 8, 10, 20, 30, 50 mg/L. While 90% of MT was removed within 48 hours in the experiments with initial MT concentrations of 1 and 5 mg/L, it took over 150 hours to remove the same percentage of MT when using higher initial concentrations (10 and 50 mg/L). Nevertheless, in the experiments with initial MT concentration of 3, 8, 20 and 30 mg/L, no biodegradation and growth of bacteria on the culturing plate was observed. The failed result might be related to the inoculation step occurring with the second batch of the experiment, consisting of 3, 8, 20, and 30 mg/L of MT concentration used for improving detail of trend.In case of strain B052, kinetic constants Vmax and Km were 0.18$ngL^{-1}h^{-1}cell^{-1}$and 1.48 mg/L, respectively.

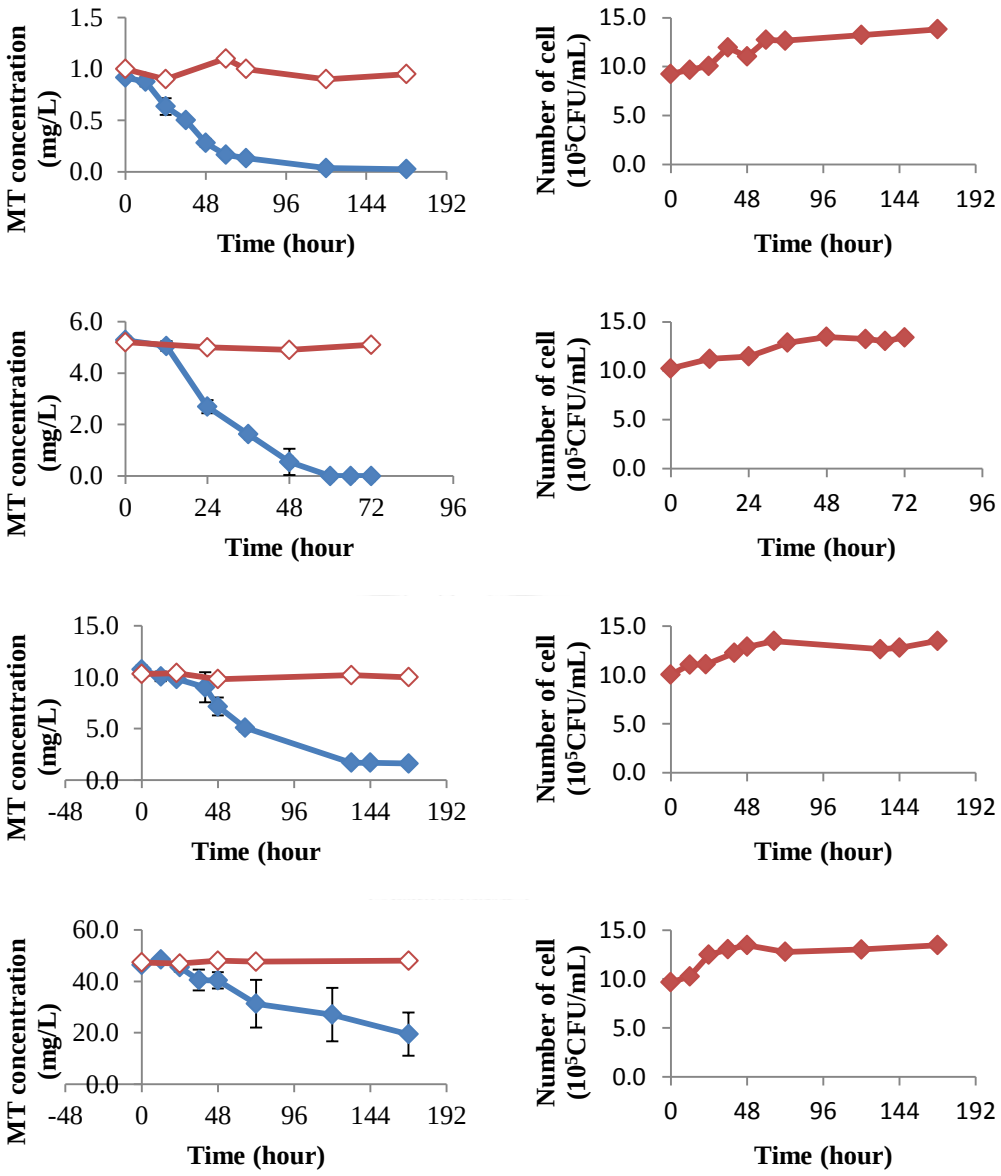


Figure 4. 8a Degradation of MT by strain B051 (Left) and number of cells at different initial MT concentration (Right). Error bars indicate standard deviation (S.D.) from triplicate data sets.

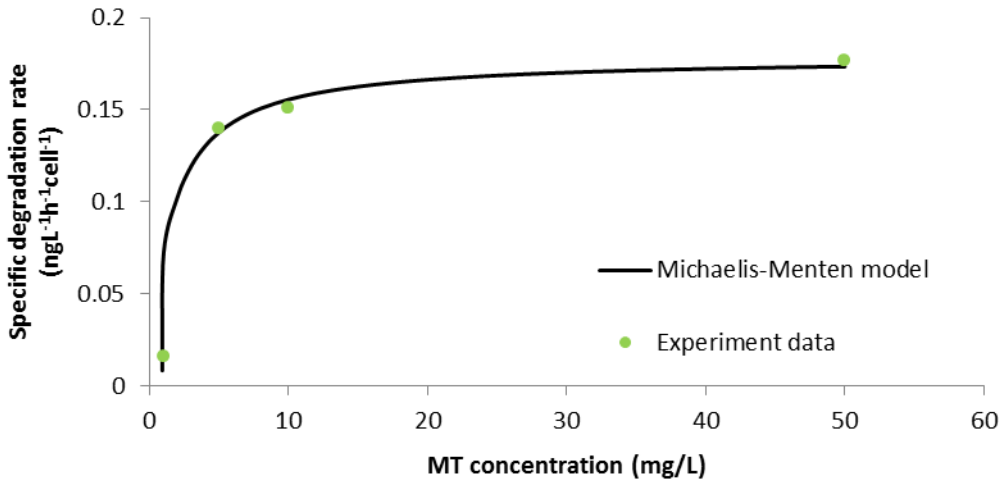


Figure 4.8b Specific degradation rate (ngL^{-1} h^{-1} $cell^{-1}$) of strain B052

The strain S303 was tested with MT which has the concentration of 1, 3, 5, 8, 10, 20, 30, 50 mg/L. At 1, 3, and 5 mg/L, the MT is used up within 24 hours. It takes 96 hours for 10, 20, and 30 mg/L of MT to be used up, while it takes way over 96 hours for 50 mg/L of MT to be completely gone. At 8 mg/L of MT, on the other hand, there was contamination in this experiment as there were two bacteria on the plate count agar. The result therefore cannot be included and had to be discarded.Biodegradation of MT by Strain S303, the kinetic constants Vmax was 0.24 $ngL^{-1}h^{-1}cell^{-1}$and Km was 1.0 mg/L.

As three MT degrading strains were affiliated to *Acinetobacter* sp., *Ochrobactrum* sp. and *Nocardioides* sp.There are a few of research which isolated MT degrading bacteria and studied their kinetic of biodegradation of MT. *Acinetobacter* sp. and *Ochrobactrum* sp. were not found that can degrade MT; however *Nocardioides* sp. were found that can degrade MT as strain S303 was identified to be the same genus. Homklin (2012) found three strain of MT degrading

bacteria. Strain SB010-03 was identified as *Rhodococcus* sp. and two strains including SB100-05 and WB100-05, which were isolated from enrichment of sediment and water at MT concentration of 100 mg/L, were closely related to *Nocardioides* sp. The kinetic of biodegradation constants V_{max} and K_m, in biodegradation of MT by strain SB100-05 were 0.22 $ngL^{-1}h^{-1}cell^{-1}$ and 14.22 mg/L, respectively. The kinetic constant of another strain WB100-05, V_{max} was 0.16 $ngL^{-1}h^{-1}cell^{-1}$ and K_m was 23.92 mg/L.V_{max} of strain SB100-05 and strain S303 were comparable, but strain S303 has lower K_m than strain SB100-05 (Table 4.5). This might be implied that strain S303 has ability to degrade MT faster than SB100-05 at low concentration of MT. In addition, the specific growth rates of these two strainsdecrease at high concentration of MT due to substrate inhibition. Strain SB100-05 and WB100-05 were decreased the MT at the initial MT concentrations above 5.0, and 10.0 mg/L, respectively.

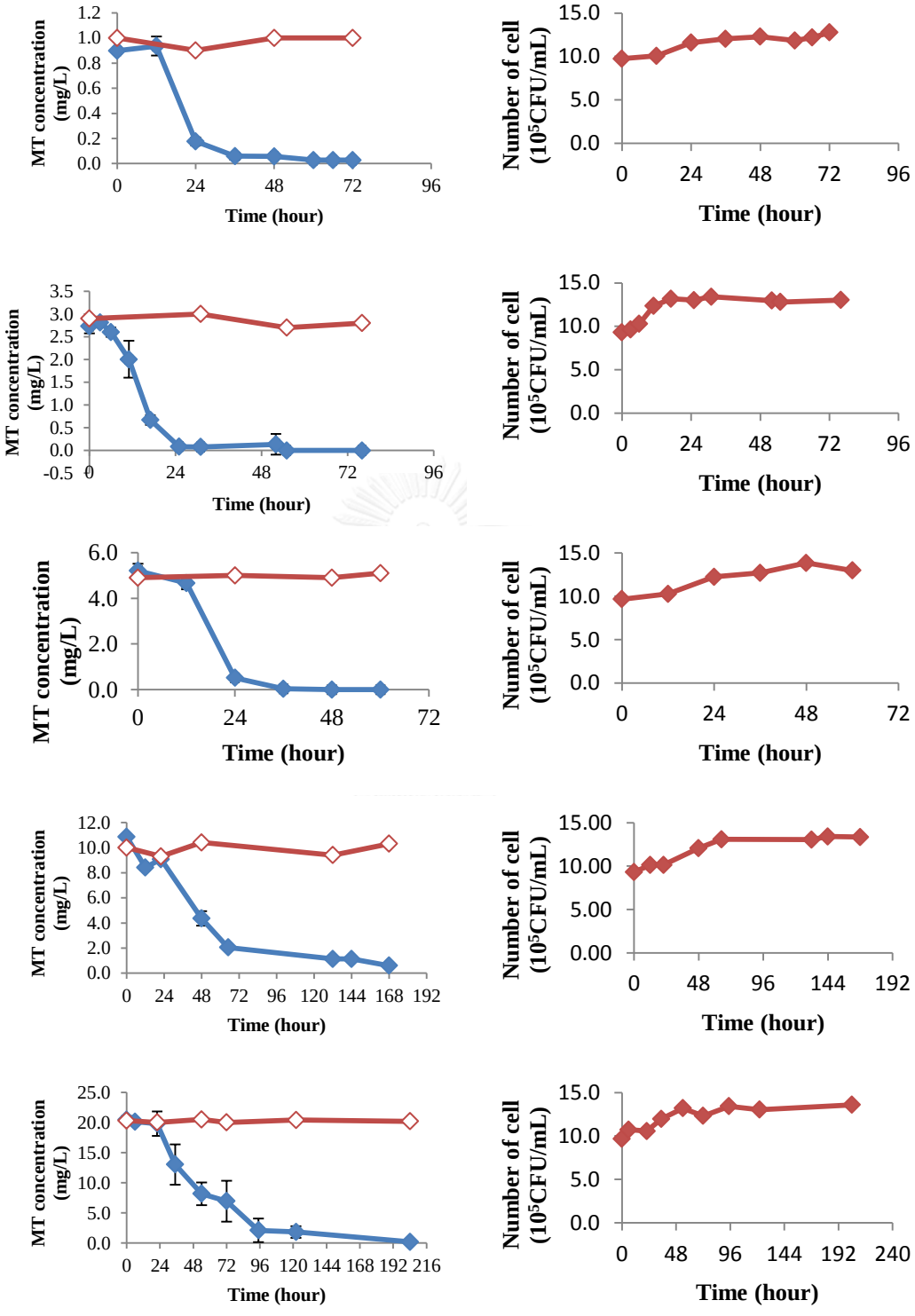


Figure 4. 9a Degradation of MT by strain B051 (Left) and number of cells at different initial MT concentration (Right).Error bars indicate standard deviation (S.D.) from triplicate data sets.

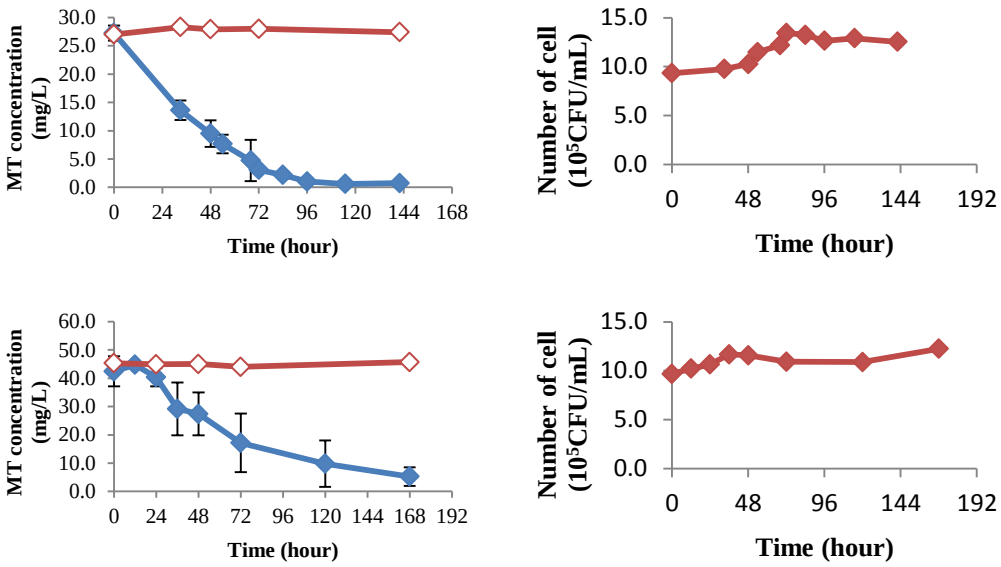


Figure 4.9a (cont.) Degradation of MT by strain B051 (Left) and number of cells at different initial MT concentration (Right).Error bars indicate standard deviation (S.D.) from triplicate data sets.

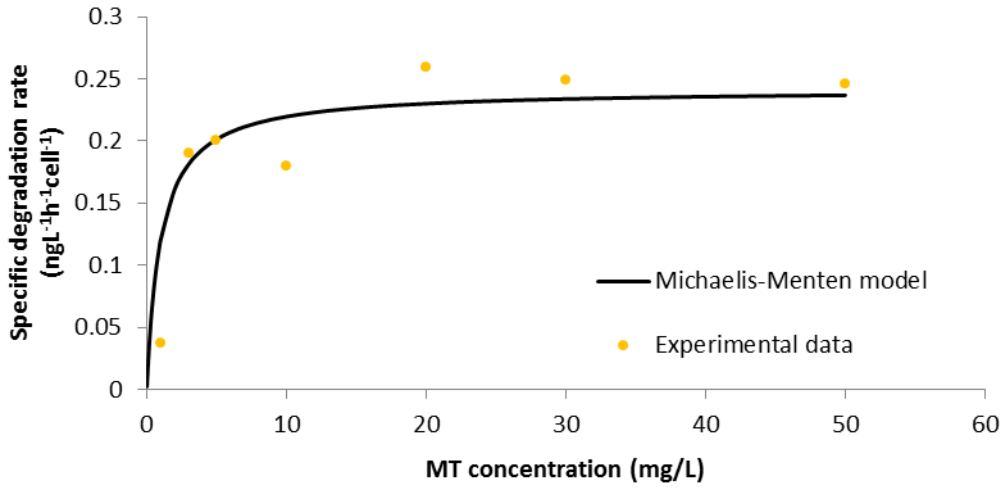


Figure 4.9b Specific degradation rate (ngL^{-1} h^{-1} $cell^{-1}$) of strain S303

The Michaelis-Menten plot of three strains is shown in Figuer 4.10. The kinetic constant of MT biodegradation in different strain is shown in Table 4.6. Higher V_{max}

value shows faster degradation rate; therefore, with consideration of K_m, Strain B051 shows the best of MT degradation ability at higher concentration which is 8.23mg/L. However, strain S303 had the lowest of K_m which is 1.0 mg/L. This implied that strain S303 had the highest capability among the tested strains to degrade MT at low concentration which is relevant to real situation. It could suggest that different genus and even the same genus of MT degrading bacteria as shows in Table 4.6 can have different efficiency of MT degradation depending on the concentration of MT and growth condition.

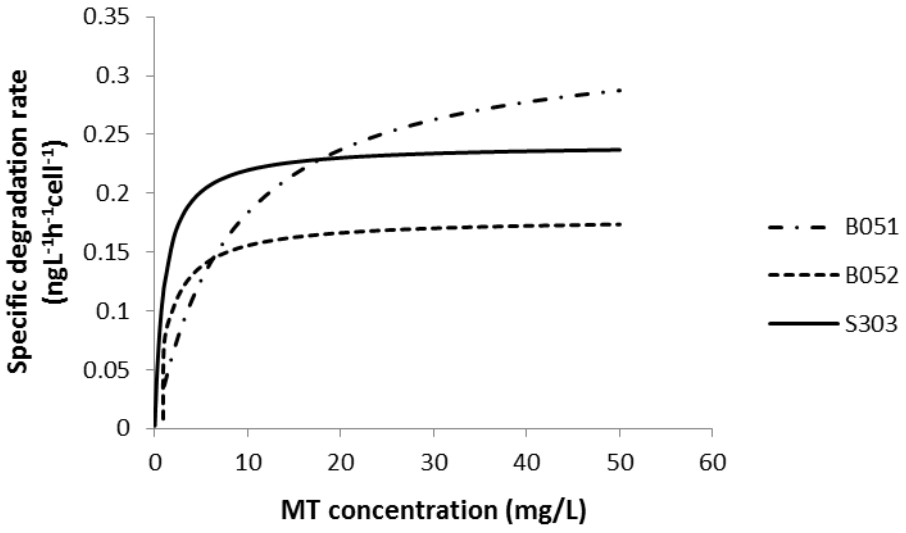


Figure 4. 10 TheMichaelis-Menten plot of three strains

Table 4. 6 The kinetic constant of MT biodegradation in different strain

Strain	Closest matched sequence	V_{max}(ngL^{-1} h^{-1} cell^{-1})	K_m (mg/L)
B051	*Acinetobacter*sp.	0.34	8.23
B052	*Ochrobactrum* sp.	0.18	1.43
S303	***Nocardioides* sp.**	**0.24**	**1.0**

SB100-05	***Nocardioides* sp.**	**0.22**	**14.22**
WB100-05	***Nocardioides* sp.**	**0.16**	**23.92**

4.7 Versatile substrates degrading characteristic of isolated strains

The capability of MT degrading strains including B051, B052 and S303 on biodegrading other hormones (testosterone, estrone (E1), 17β-estradiol (E2) and 17α-ethylestradiol (EE2); Figure 4.10) was investigated in this study. The degradation tests were performed using the initial MT concentration of 10 mg/L for 7 days.

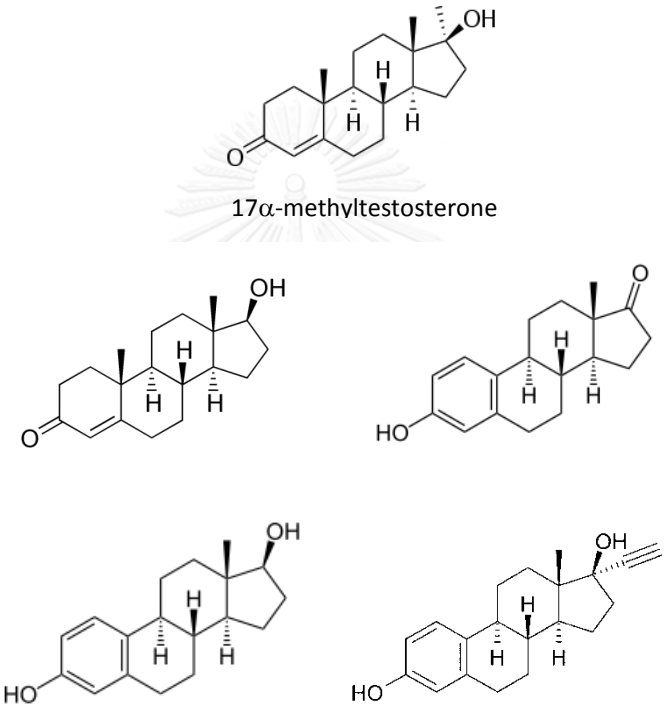


Figure 4.10 The chemical structure of other sex hormones compared with structure of MT.

Testosterone, which was the same androgenic hormone as MT, was selected to test degradation ability of isolated strains. The result showed that concentration of MT decreased by 86% within 132 hours by the strain S303, whereas strain B051 and strain B052 take 132 hours to degrade MT to reach 45% and 29% of MT removal. Testosterone has the similar chemical structure as MT, except at C-17 position of MT, where a hydrogen atom is replaced with a methyl group (Figure 4.10). This might be the reason why all strains could degrade testosterone. The performance of the three

bacteria is in agreement with those of section 4.5 where strain S303 showed the highestcapability of biodegrading MT followed by strain B051 and B052, respectively (Figure 4.11).

This study revealed that strain B051, which is classified as *Acinetobacter* sp., can biodegrade testosterone. This is in agreement with Yang et al (2011) who illustrated that *Acinetobacter* sp. could degrade testosterone. It was also found that besides *Acinetobacter* sp., *Brevundimonas* sp., *Comamonas* sp., *Sphingomonas* sp.,*Rhodobacter* sp. and *Stenotrophomonas* sp., were able to degrade testosterone. All of these bacteria can degrade testosterone at 3mg/L within 29 hours with a lag phase of approximately 22 hours.

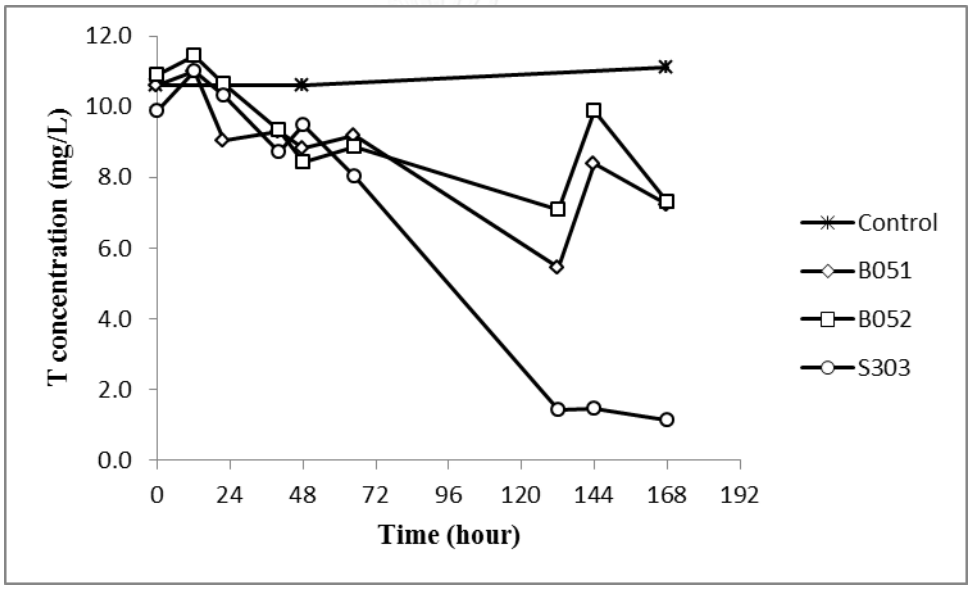


Figure 4.11 Degradation behavior of Testosterone (T) by isolated

This study also investigated the ability of isolated strains on biodegradationof estrogen hormones includingestrone (E1), 17β-estradiol (E2), and 17α-ethynylestradiol (EE2). The results showed that none of these isolated strains could degrade estrogen hormones at the concentration of 10mg/L within 7-day observation (Figure 4.12-4.14). The results appears to contradict with Pauwel et al. (2007) who reported that *Acinetobacter* sp. could degrade E2 and EE2.

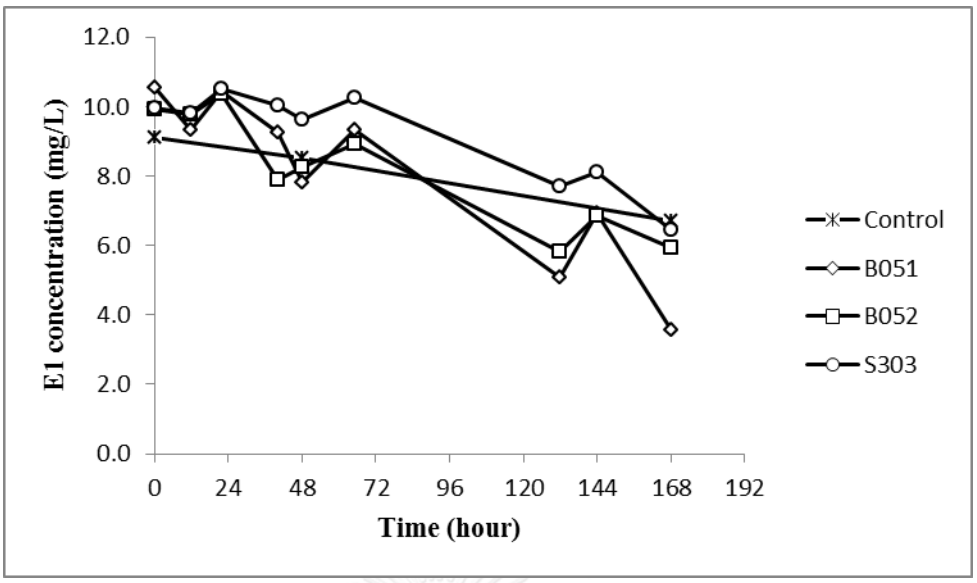


Figure 4.12 Degradation behavior of estrone (E1) by isolated

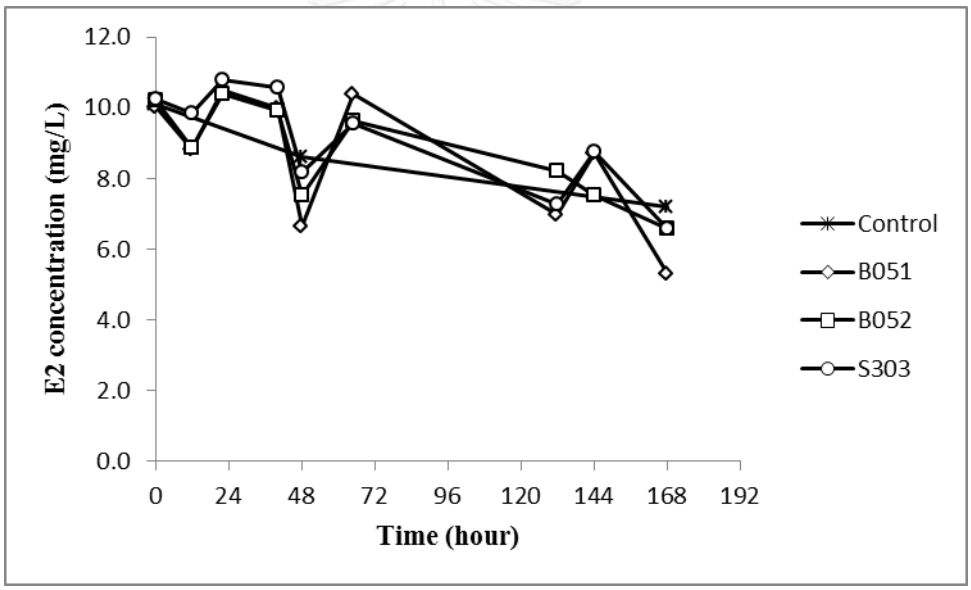


Figure 4.13 Degradation behavior of 17β-estradiol (E2) by isolated

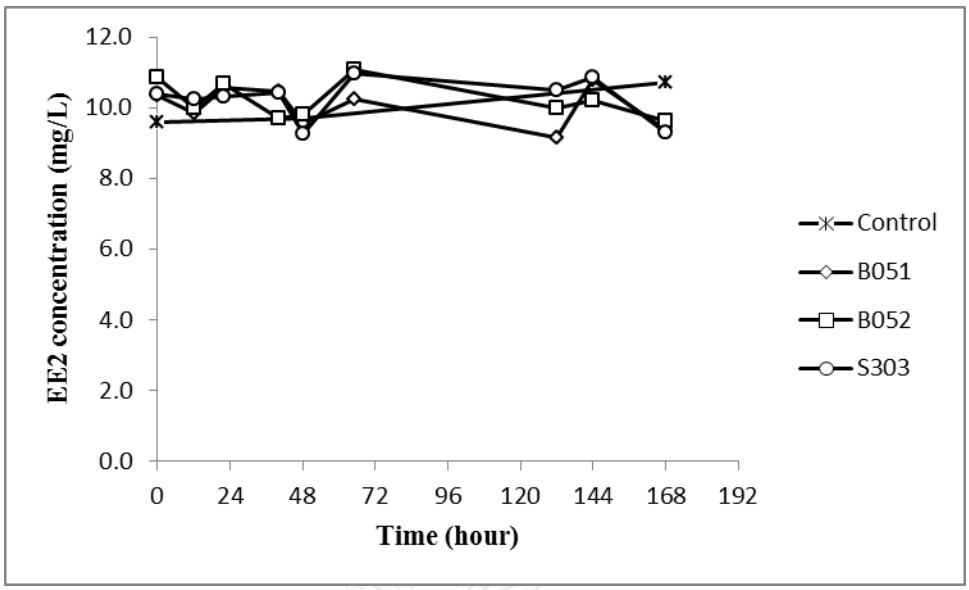


Figure 4.14 Degradation behavior of 17α-ethynylestradiol (EE2) by isolated

Yu et al. (2013) reported that many strains of*Pseudomonas* spp. were capable of degrading estrogenNote that *Acinetobacter* sp. shares the same order as *Pseudomonas* spp., but different family.

Yu et al. (2007) found that *Nocardioides*sp. was able to gradually convert E2 into E1 in 7 days at 3 mgE2/L. It is sensible to conclude that bacteria that are in the same genus should have the same trend of degrading hormones, but not all the case. It is possible that environmental factors and culturing conditions mayaffect biodegradation of estrogen hormones.

It was found earlier that bacteria in class Alphaproteobacteria such as *Shingomonas* sp. could degrade E2as well as testosterone (Roh et al, 2010). This is in contrast with the results of this study which showed that *Ochrobactrum* sp. strain B052could degrade only testosterone, but not E2. That might be because there were different strains even they were in the same class, genus or species. These results suggested that although bacteria are in different genus, bacteria in same class may be able to degrade androgenic hormones, but nor estrogen hormones.

In summary, the result shows that all strains have the potential to degrade testosterone which has similar chemical structure as MT. The strain S303 is the best when it comes to reducing testosterone. As for other sex hormones, Estrogen group including estrone (E1), 17β-estradiol (E2), and 17α-ethynylestradiol (EE2) were

examined, all stains cannot degrade E1, E2 and EE2 at the concentration of 10 mg/L during 7 days period. That might be because the enzyme which bacteria used to degrade androgen and estrogen hormone were different. This can be inferred that all three isolates are specialized in androgenic hormone degradation.

CHAPTER V
CONCLUSIONAND RECOMMENDATIONS

5.1 Conclusion

17α-methyltestosterone degrading isolates were obtained from enrichment culture of sediment and biofilm samples collected from masculinizing ponds of Nile tilapia fry. Three strains of MT degrading bacteria were isolated in this study.

Strain S303 was isolated from 30mg/L of initial MT concentration in enrichment culture. It was affiliated to *Nocardioides* sp. It can degrade MT faster than the others at low concentration. Moreover, it has ability to degrade testosterone.

Strain B051 and B052 were found in enrichment culture with initial MT concentration at 0.5 mg/L. Strain B051 and B052 were affiliated to *Acinetobacter*sp., and *Ochrobactrum*sp., respectively. These two strains were not reported in issue of MT degrading bacteria, However, *Acinetobacter* sp. was reported that it can degrade testosterone.

Testosterone can be removed by all MT degrading bacteria while they could not degrade estrone(E1), 17β-estradiol (E2) or 17α-ethynylestradiol (EE2). This implies that all three isolates are specialized in androgenic hormone degradation.

The Michaelis-Menten plot of three strains is shown in Figuer 4.1. The kinetic constant of MT biodegradation in different strain is shown in Table 4.1. Higher V_{max} value shows faster degradation rate; therefore, S303 was the best strain to degrade MT at low concentration of MT comparing among B051 and B052.

5.2 Recommendation

According to experimental result in this work, some recommendations for a further study and application are proposed as follow;

1. MT degrading strains should be confirmed their full length sequence and their hormones degradation ability because it has contamination in the experiment.

2. The intermediate substances during MT degradation process and enzyme involved should be investigated for understanding the fate and transport mechanism and to open possibility to degrade MT using enzyme technology.

3. Mixed culture experiment should be conducted to study the competitive capacity of each strain in order to enhance the degradation of MT and other hormones.

4. The application of MT degrading bacteria could be apply in practice in order to ensure that MT could be degraded in the actual situation. Bioaugmentation study with the pure isolates or mixed with other microorganism should be applied to know the role of isolates on enhancing MT degradation in wastewater.

5. For the limitation of detection of HPLC, the specific machine which had lower limit of detection and higher identification performance should to be used such as LC-MS, GC-MS, or LC-MS/MS.

6. The methods that can concentrate the MT concentration (e.g. solid phase extraction) have to be applied in order to study the degradation at lower MT concentration, more relevant to the real situation.

7. The method to optimize biodegradation efficiency should be explored; for instance, using co-substrate to induce the bacteria growth.

8. Investigation of MT concentration in the treatment ponds or contaminated areas has to be done in order to know the background MT concentration in Thai environment.

9. The treatment of sediment has to be concern because MT can be adsorbed in sediment.

10. Although MT was degraded completely, the androgen activity needs to be analyzed because intermediates and products might be still effective posing environmental risk to receiving water environment.

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APPENDIX

Table A. 1 Biodegradation of MT by MT degrading bacteria strain SS051 at initial MT concentration of 1 mg/L

Time (Hour)	MT concentration (mg/L)				
	1	2	3	AVG.	SD
0	0.9	0.9	0.9	0.9	0.0
12	0.8	0.7	0.7	0.7	0.0
24	0.1	0.4	0.4	0.3	0.1
36	0.2	0.3	0.2	0.2	0.0
48	0.1	0.1	0.1	0.1	0.0
60	0.1	0.1	0.1	0.1	0.0
66	0.0	0.0	0.0	0.0	0.0
72	0.0	0.0	0.0	0.0	0.0
120	0.0	0.0	0.1	0.0	0.0

Table A. 2 The amount of colony forming unit per ml of MT degrading bacteria strain SS051 at initial MT concentration of 1 mg/L

Time (h.)	MT concentration (mg/L)							
	1		2		3		AVG.	SD
0	830000	790000	980000	770000	530000	490000	731667	187234
12	1020000	960000	840000	930000	1130000	920000	966667	99130
24	950000	1070000	1130000	1030000	970000	1080000	1038333	68823
36	1380000	1190000	930000	1060000	840000	790000	1031667	224804
48	1170000	820000	1040000	1180000	920000	1060000	1031667	140914
60	830000	760000	1180000	960000	1040000	930000	950000	149666
66	950000	1150000	820000	1280000	960000	1250000	1068333	185409
72	1290000	830000	940000	880000	1170000	970000	1013333	178736
120	1070000	1240000	1260000	1180000	1190000	1060000	1166667	84301

Table A. 3 Biodegradation of MT by MT degrading bacteria strain SS051 at initial MT concentration of 3 mg/L

Time (Hour)	MT concentration (mg/L)				
	1	2	3	AVG.	SD
0	2.8	2.7	2.8	2.7	0.0
6	2.7	2.7	2.8	2.7	0.1
11	2.9	2.6	2.7	2.7	0.1
17	2.3	2.3	2.5	2.4	0.1
25	1.6	1.4	1.5	1.5	0.1
31	0.9	1.2	0.8	1.0	0.2
52	0.5	0.3	0.3	0.3	0.1
76	0.0	0.0	0.0	0.0	0.0
82	0.0	0.0	0.0	0.0	0.0

Table A. 4 The amount of colony forming unit per ml of MT degrading bacteria strain SS051 at initial MT concentration of 3 mg/L

Time (h.)	MT concentration (mg/L)							
	1		2		3		AVG.	SD
0	970000	1050000	960000	1120000	850000	870000	970000	103344
12	870000	930000	810000	1040000	950000	1210000	968333	141480
24	960000	1070000	930000	1180000	960000	1280000	1063333	141233
36	1130000	940000	1050000	1190000	890000	930000	1021667	121065
48	1190000	840000	930000	960000	1140000	970000	1005000	133079
60	980000	1280000	1070000	1150000	930000	850000	1043333	156418
66	970000	1040000	1290000	1120000	1300000	930000	1108333	158419
72	1090000	1260000	1150000	1170000	1290000	1160000	1186667	74476
120	1150000	1300000	1260000	1120000	1050000	1270000	1191667	99482

Table A. 5 Biodegradation of MT by MT degrading bacteria strain SS051 at initial MT concentration of 5 mg/L

Time (Hour)	MT concentration (mg/L)				
	1	2	3	AVG.	SD
0	5.0	5.0	4.5	5.0	0.3
12	3.3	3.4	3.4	3.4	0.1
24	0.0	0.6	0.4	0.5	0.3
36	0.1	0.0	0.0	0.1	0.1
48	0.0	0.0	0.0	0.0	0.0
60	0.0	0.0	0.0	0.0	0.0

Table A. 6 The amount of colony forming unit per ml of MT degrading bacteria strain SS051 at initial MT concentration of 5 mg/L

Time (h.)	MT concentration (mg/L)							
	1		2		3		AVG.	SD
0	1090000	870000	970000	1030000	930000	1060000	991667	83526
12	1160000	1040000	1280000	1130000	1350000	1270000	1205000	114673
24	1290000	1190000	1030000	1270000	1340000	1250000	1228333	108888
36	1250000	1170000	980000	1130000	1260000	1310000	1183333	118940
48	1370000	1290000	1240000	1340000	1210000	1390000	1306667	72296
60	1360000	1270000	1240000	1370000	1430000	1360000	1338333	70261

Table A. 7 Biodegradation of MT by MT degrading bacteria strain SS051 at initial MT concentration of 8 mg/L

Time (Hour)	MT concentration (mg/L)				
	1	2	3	AVG.	SD
0	6.8	6.9	7.0	6.9	0.1
14	7.1	6.7	6.6	6.8	0.3
22	6.9	6.2	6.5	6.5	0.4
30	6.0	5.4	6.0	5.8	0.3
42	3.8	4.4	4.1	4.1	0.3
54	2.7	2.3	2.6	2.5	0.2
60	2.0	1.9	1.5	1.8	0.3
72	1.2	1.4	0.8	1.2	0.3
95	0.7	0.8	0.8	0.8	0.1
122	0.2	0.1	0.2	0.1	0.1
204	0.0	0.0	0.0	0.0	0.0

Table A. 8 The amount of colony forming unit per ml of MT degrading bacteria strain SS051 at initial MT concentration of 8 mg/L

Time (Hour)	MT concentration (mg/L)							
	1		2		3		AVG.	SD
0	980000	1050000	1180000	1030000	960000	1240000	1073333	112368
14	1210000	1070000	960000	1180000	1290000	1130000	1140000	115239
22	930000	1050000	1280000	950000	1190000	1240000	1106667	150820
30	1280000	1190000	1170000	1250000	1230000	1060000	1196667	77889
42	1340000	1280000	1160000	1290000	1380000	1270000	1286667	74744
54	1350000	1270000	1360000	1290000	1380000	1420000	1345000	56125
60	1290000	1360000	1460000	1270000	1190000	1380000	1325000	94816
72	1420000	1390000	1260000	1170000	1310000	1270000	1303333	91579
95	1370000	1490000	1360000	1250000	1470000	1360000	1383333	87101
122	1280000	1340000	1420000	1290000	1370000	1420000	1353333	61210
204	1190000	1390000	1410000	1270000	1340000	1420000	1336667	90701

Table A. 9 Biodegradation of MT by MT degrading bacteria strain SS051 at initial MT concentration of 10 mg/L

Time (Hour)	MT concentration (mg/L)			
	1	2	AVG.	SD
0	9.9	9.8	9.9	0.1
15	8.9	9.6	9.3	0.5
24	9.7	9.3	9.5	0.3
36	9.8	9.7	9.7	0.1
48	3.6	4.0	3.8	0.3
75	1.7	1.9	1.8	0.1
87	0.7	0.8	0.7	0.0
96	0.7	0.8	0.7	0.0
114	0.8	0.7	0.8	0.1

Table A. 10 The amount of colony forming unit per ml of MT degrading bacteria strain SS051 at initial MT concentration of 10 mg/L

Time (Hour)	MT concentration (mg/L)							
	1		2		3		AVG.	SD
0	830000	720000	950000	840000	730000	860000	821667	86120
15	970000	910000	890000	920000	870000	940000	916667	35590
24	1060000	910000	850000	1030000	960000	840000	941667	91524
36	980000	1150000	1040000	930000	1210000	1060000	1061667	104195
48	1130000	1280000	1080000	1100000	1040000	1240000	1145000	94604
75	1070000	1140000	950000	1090000	1210000	1040000	1083333	88468
87	1260000	1040000	1270000	980000	1150000	1260000	1160000	125698
96	1340000	1240000	1190000	1250000	1360000	1280000	1276667	64083
114	1270000	1190000	1340000	1280000	1250000	1340000	1278333	57067

Table A. 11 Biodegradation of MT by MT degrading bacteria strain SS051 at initial MT concentration of 20 mg/L

Time (Hour)	MT concentration (mg/L)				
	1	2	3	AVG.	SD
0	17.5	21.2	20.5	19.7	2.0
22	20.3	20.7	19.7	20.2	0.5
35	18.6	21.2	19.7	19.8	1.3
47	16.6	17.1	16.6	16.7	0.3
72	14.5	13.0	11.3	12.9	1.6
95	3.6	3.5	6.8	4.7	1.9
122	1.4	2.7	4.1	2.7	1.3
204	0.1	0.1	0.2	0.1	0.1
240	0.2	0.2	0.1	0.2	0.1

Table A. 12 The amount of colony forming unit per ml of MT degrading bacteria strain SS051 at initial MT concentration of 20 mg/L

Time (Hour)	MT concentration (mg/L)							
	1		2		3		AVG.	SD
0	890000	960000	970000	830000	910000	1040000	933333	72847
22	1050000	930000	1020000	870000	930000	1060000	976667	77374
35	1030000	920000	940000	830000	1070000	980000	961667	85186
47	1160000	1050000	1040000	1230000	1140000	1270000	1148333	92826
72	1370000	1130000	1260000	1340000	1170000	1040000	1218333	127658
95	1340000	1280000	1190000	1260000	1240000	1360000	1278333	63377
122	1230000	1130000	1290000	1380000	1040000	1150000	1203333	121929
204	1140000	1260000	1370000	1290000	1140000	1200000	1233333	90701
240	1340000	1280000	1360000	1210000	1420000	1280000	1315000	73689

Table A. 13 Biodegradation of MT by MT degrading bacteria strain SS051 at initial MT concentration of 30 mg/L

Time (Hour)	MT concentration (mg/L)				
	1	2	3	AVG.	SD
0	32.3	26.6	26.4	28.4	3.3
33	24.1	25.3	25.6	25.0	0.8
48	23.3	21.4	22.3	22.3	0.9
54	20.4	21.4	21.5	21.1	0.6
68	20.4	19.1	16.9	18.8	1.8
75	18.3	20.1	15.9	18.1	2.1
84	11.2	13.1	12.0	12.1	0.9
96	12.1	10.1	10.4	10.8	1.1
107	9.9	13.2	10.8	11.3	1.7
142	3.7	4.1	4.7	4.2	0.5
148	4.0	6.5	4.2	4.9	1.4
166	3.2	3.1	3.1	3.1	0.0
190	3.0	3.0	3.0	3.0	0.0

Table A. 14 The amount of colony forming unit per ml of MT degrading bacteria strain SS051 at initial MT concentration of 30 mg/L

Time (Hour)	MT concentration (mg/L)							
	1		2		3		AVG.	SD
0	870000	930000	860000	950000	910000	820000	890000	48580
33	840000	940000	970000	1210000	1070000	910000	990000	131605
48	850000	1060000	1150000	830000	950000	870000	951667	128750
68	1040000	840000	930000	1080000	890000	920000	950000	91652
84	1020000	940000	1150000	1140000	960000	1050000	1043333	88242
107	1040000	1130000	1280000	980000	1060000	1210000	1116667	112546
148	890000	1050000	1070000	1320000	1170000	990000	1081667	148918
166	1220000	1380000	1130000	1320000	1280000	1190000	1253333	91141
190	1180000	1060000	1280000	1040000	1360000	1190000	1185000	123572

Table A. 15 Biodegradation of MT by MT degrading bacteria strain SS051 at initial MT concentration of 50 mg/L

Time (Hour)	MT concentration (mg/L)				
	1	2	3	AVG.	SD
0	47.8	45.8	45.7	46.8	1.2
12	32.3	45.2	45.0	40.8	7.4
24	46.6	44.6	44.1	45.1	1.3
36	37.1	42.6	41.2	40.3	2.8
48	28.2	35.0	18.2	27.2	8.4
72	30.4	29.2	30.6	30.1	0.8
120	16.0	8.1	28.2	17.4	10.1
168	2.3	5.3	6.3	4.6	2.1

Table A. 16 The amount of colony forming unit per ml of MT degrading bacteria strain SS051 at initial MT concentration of 50 mg/L

Time (Hour)	MT concentration (mg/L)							
	1		2		3		AVG.	SD
0	840000	870000	970000	1180000	1070000	930000	976667	128323
12	850000	940000	1150000	830000	950000	870000	931667	117374
24	1160000	950000	1140000	1230000	1040000	1270000	1131667	119234
36	1370000	1130000	1260000	1340000	1170000	1040000	1218333	127658
48	1080000	1140000	1140000	1090000	1260000	1040000	1125000	76354
72	1260000	1040000	1270000	980000	1150000	1260000	1160000	125698
120	1270000	1140000	1340000	1280000	1250000	1140000	1236667	80664
168	1360000	1210000	1200000	1190000	1360000	1270000	1265000	78677

Table A. 17 Biodegradation of MT by MT degrading bacteria strain **SS052** at initial MT concentration of 1 mg/L

Time (Hour)	MT concentration (mg/L)				
	1	2	3	AVG.	SD
0	0.9	0.9	0.9	0.9	0.0
12	0.9	0.9	0.9	0.9	0.0
24	0.6	0.7	0.7	0.6	0.1
36	0.5	0.5	0.5	0.5	0.0
48	0.3	0.2	0.3	0.3	0.0
60	0.2	0.1	0.1	0.2	0.0
66	0.1	0.2	0.2	0.2	0.1
72	0.1	0.1	0.2	0.1	0.0
120	0.0	0.0	0.0	0.0	0.0
168	0.0	0.0	0.0	0.0	0.0

Table A. 18 The amount of colony forming unit per ml of MT degrading bacteria strain **SS052**at initial MT concentration of 1 mg/L

Time (Hour)	MT concentration (mg/L)							
	1		2		3		AVG.	SD
0	980000	870000	890000	940000	820000	1050000	925000	82644
12	960000	1030000	840000	930000	1130000	920000	968333	100283
24	890000	930000	1170000	1040000	970000	1040000	1006667	99733
36	1280000	1310000	1080000	1140000	1090000	1270000	1195000	103296
48	1250000	1040000	1150000	980000	1030000	1170000	1103333	102697
60	1180000	1270000	1380000	1260000	1160000	1390000	1273333	96678
72	1250000	1320000	1170000	1290000	1320000	1240000	1265000	57533
120	1370000	1270000	1360000	1290000	1370000	1280000	1323333	48028
168	1360000	1290000	1460000	1390000	1380000	1410000	1381667	56362

Table A. 19 Biodegradation of MT by MT degrading bacteria strain **SS052** at initial MT concentration of 5 mg/L

Time (Hour)	MT concentration (mg/L)				
	1	2	3	AVG.	SD
0	5.4	5.1	5.3	5.3	0.1
12	5.2	4.8	5.1	5.1	0.2
24	2.4	2.7	2.9	2.7	0.3
36	1.5	1.7	1.6	1.6	0.1
48	0.2	1.1	0.3	0.5	0.5
60	0.0	0.0	0.1	0.0	0.1
66	0.0	0.0	0.0	0.0	0.1
72	0.0	0.0	1.7	0.0	1.0

Table A. 20 The amount of colony forming unit per ml of MT degrading bacteria strain **SS052**at initial MT concentration of 5 mg/L

Time (Hour)	MT concentration (mg/L)							
	1		2		3		AVG.	SD
0	1090000	870000	970000	1210000	930000	1070000	1023333	123720
12	1160000	1040000	1280000	1130000	950000	1170000	1121667	114091
24	1080000	1190000	1170000	1150000	1230000	1060000	1146667	65320
36	1340000	1280000	1160000	1290000	1380000	1270000	1286667	74744
48	1350000	1270000	1360000	1290000	1380000	1420000	1345000	56125
60	1290000	1360000	1460000	1270000	1190000	1380000	1325000	94816
66	1420000	1390000	1260000	1170000	1310000	1270000	1303333	91579
72	1380000	1240000	1420000	1370000	1280000	1350000	1340000	67231

Table A. 21 Biodegradation of MT by MT degrading bacteria strain **SS052** at initial MT concentration of 10 mg/L

Time (Hour)	MT concentration (mg/L)			
	1	2	AVG.	SD
0	11.0	10.4	10.7	0.4
12	10.4	9.7	10.1	0.5
22	9.8	9.8	9.8	0.0
40	8.0	10.0	9.0	1.5
48	6.5	7.8	7.2	0.9
65	4.8	5.3	5.1	0.4
132	1.8	1.6	1.7	0.1
144	1.7	1.7	1.7	0.0
168	1.5	1.8	1.6	0.2

Table A. 22 The amount of colony forming unit per ml of MT degrading bacteria strain **SS052**at initial MT concentration of 10 mg/L

Time (Hour)	MT concentration (mg/L)							
	1		2		3		AVG.	SD
0	980000	890000	1180000	1030000	960000	970000	1001667	98268
12	1130000	1070000	930000	1280000	1090000	1130000	1105000	113093
22	930000	1050000	1280000	950000	1190000	1240000	1106667	150820
40	1280000	1290000	1170000	1320000	1230000	1060000	1225000	96488
48	1340000	1280000	1160000	1290000	1380000	1270000	1286667	74744
65	1350000	1270000	1360000	1290000	1380000	1420000	1345000	56125
132	1090000	1380000	1250000	1380000	1290000	1180000	1261667	114091
144	1250000	1180000	1240000	1270000	1370000	1350000	1276667	71461
168	1380000	1470000	1240000	1190000	1370000	1430000	1346667	109301

Table A. 23 Biodegradation of MT by MT degrading bacteria strain **SS052** at initial MT concentration of 50 mg/L

Time (Hour)	MT concentration (mg/L)				
	1	2	3	AVG.	SD
0	45.2	47.5	45.8	46.4	1.2
12	48.7	48.1	47.9	48.4	0.4
24	45.2	45.7	42.7	45.5	1.6
36	43.3	37.6	45.5	40.5	4.1
48	37.2	43.5	40.8	40.3	3.2
72	37.5	25.0	19.5	31.3	9.2
120	17.1	37.0	21.8	27.0	10.4
168	11.4	27.5	15.2	19.4	8.4
120	0.0	0.0	0.0	0.0	0.0
168	0.0	0.0	0.0	0.0	0.0

Table A. 24 The amount of colony forming unit per ml of MT degrading bacteria strain **SS052**at initial MT concentration of 50 mg/L

Time (Hour)	MT concentration (mg/L)							
	1		2		3		AVG.	SD
0	930000	910000	1090000	850000	980000	1040000	966667	88242
12	1050000	930000	1020000	1170000	930000	1060000	1026667	90480
24	1290000	1130000	1460000	1270000	1040000	1290000	1246667	145694
36	1420000	1390000	1260000	1170000	1310000	1270000	1303333	91579
48	1350000	1270000	1360000	1290000	1380000	1420000	1345000	56125
72	1090000	1380000	1370000	1380000	1180000	1260000	1276667	122093
120	1250000	1290000	1240000	1270000	1410000	1350000	1301667	65853
168	1380000	1300000	1420000	1260000	1320000	1390000	1345000	61237

Table A. 25 Biodegradation of MT by MT degrading bacteria strain **SW051** at initial MT concentration of 1 mg/L

Time (Hour)	MT concentration (mg/L)				
	1	2	3	AVG.	SD
0	0.9	0.9	0.9	0.9	0.0
12	0.9	0.9	0.9	0.9	0.0
24	0.8	0.8	0.8	0.8	0.0
36	0.5	0.7	0.7	0.6	0.1
48	0.5	0.5	0.5	0.5	0.0
60	0.6	0.5	0.5	0.5	0.0
66	0.3	0.4	0.2	0.4	0.1
72	0.4	0.3	0.1	0.3	0.1
120	0.1	0.1	0.1	0.1	0.0
168	0.1	0.0	0.0	0.1	0.0

Table A. 26 The amount of colony forming unit per ml of MT degrading bacteria strain **SW051** at initial MT concentration of 1 mg/L

Time (Hour)	MT concentration (mg/L)							
	1		2		3		AVG.	SD
0	950000	870000	970000	980000	930000	910000	935000	40866
12	950000	1040000	920000	930000	980000	930000	958333	45350
24	970000	1040000	930000	1010000	910000	980000	973333	48442
36	1130000	1090000	1050000	1180000	1040000	1090000	1096667	52026
48	1290000	1280000	1340000	1260000	1380000	1230000	1296667	54650
66	1320000	1320000	1340000	1260000	1270000	1340000	1308333	34881
72	1130000	1360000	1190000	1290000	1320000	1300000	1265000	86891
120	1250000	1380000	1380000	1270000	1350000	1270000	1316667	59889
168	1420000	1390000	1260000	1160000	1090000	1270000	1265000	127554

Table A. 27 Biodegradation of MT by MT degrading bacteria strain **SW051** at initial MT concentration of 3 mg/L

Time (Hour)	MT concentration (mg/L)				
	1	2	3	AVG.	SD
0	2.9	2.5	3.0	2.8	0.3
3	2.9	2.9	3.0	2.9	0.0
6	2.9	2.8	2.9	2.9	0.1
11	3.0	2.8	2.6	2.8	0.2
17	3.0	2.8	2.9	2.9	0.1
25	3.1	2.9	3.2	3.1	0.1
52	3.0	3.1	2.9	3.0	0.1
76	2.8	2.6	2.5	2.6	0.1
82	2.2	2.1	2.2	2.2	0.1
106	1.8	2.0	1.8	1.9	0.1
130	0.3	1.6	1.4	1.1	0.7
146	0.4	0.0	0.7	0.4	0.3

Table A. 28 The amount of colony forming unit per ml of MT degrading bacteria strain **SW051** at initial MT concentration of 3 mg/L

Time (Hour)	MT concentration (mg/L)							
	1		2		3		AVG.	SD
0	930000	1020000	1050000	970000	1060000	920000	991667	60470
6	1160000	1030000	940000	1130000	980000	1090000	1055000	86429
25	1130000	1270000	1190000	1280000	1090000	1130000	1181667	79099
52	1090000	980000	1250000	1180000	1080000	1180000	1126667	95847
76	1190000	1290000	1360000	1250000	1170000	1260000	1253333	68896
82	1090000	980000	1130000	1100000	1060000	1110000	1078333	53448
106	1370000	1170000	1260000	1290000	1180000	1220000	1248333	75211
130	1130000	1250000	1280000	1120000	1320000	1240000	1223333	81158
146	1420000	1390000	1260000	1190000	1310000	1250000	1303333	88015

Table A. 29 Biodegradation of MT by MT degrading bacteria strain **SW051** at initial MT concentration of 5 mg/L

Time (Hour)	MT concentration (mg/L)				
	1	2	3	AVG.	SD
0	4.9	6.0	5.3	5.4	0.6
12	5.1	5.2	4.9	5.1	0.2
24	3.5	1.8	2.9	2.7	0.9
36	-0.1	2.8	1.0	1.2	1.5
48	1.3	0.7	1.2	1.1	0.3
66	0.4	0.5	0.8	0.6	0.2
72	0.0	0.0	0.0	0.0	0.0
120	0.0	0.0	0.0	0.0	0.0

Table A. 30 The amount of colony forming unit per ml of MT degrading bacteria strain **SW051** at initial MT concentration of 5 mg/L

Time (Hour)	MT concentration (mg/L)							
	1		2		3		AVG.	SD
0	960000	870000	970000	1070000	930000	980000	963333	65625
12	980000	1080000	930000	1040000	1010000	920000	993333	62503
24	1340000	1280000	1160000	1290000	1380000	1270000	1286667	74744
36	1190000	1290000	1210000	1290000	1150000	1280000	1235000	59917
48	1230000	1190000	1030000	1280000	1310000	1340000	1230000	111893
66	1280000	1290000	1170000	1320000	1230000	1060000	1225000	96488
72	1340000	1280000	1160000	1290000	1380000	1270000	1286667	74744
120	1250000	1270000	1160000	1290000	1280000	1140000	1231667	64936

Table A. 31 Biodegradation of MT by MT degrading bacteria strain **SW051** at initial MT concentration of 8 mg/L

Time (Hour)	MT concentration (mg/L)				
	1	2	3	AVG.	SD
0	6.9	7.0	5.4	6.4	0.9
6	7.3	7.4	7.1	7.3	0.1
9	7.1	7.1	6.7	7.0	0.2
14	6.6	6.5	6.4	6.5	0.1
22	6.9	7.1	6.5	6.8	0.3
30	6.8	6.6	6.9	6.8	0.1
35	6.6	6.8	7.0	6.8	0.2
42	6.3	6.8	6.4	6.5	0.3
47	6.7	6.4	6.6	6.6	0.1
54	6.3	6.4	6.7	6.4	0.2
60	6.2	5.5	6.1	5.9	0.4
66	5.4	5.4	6.3	5.7	0.5
72	5.8	5.1	5.4	5.4	0.3
95	3.1	3.3	3.8	3.2	0.4
122	1.5	1.1	1.3	1.3	0.2
204	0.5	0.1	0.2	0.3	0.2

Table A. 32 The amount of colony forming unit per ml of MT degrading bacteria strain **SW051** at initial MT concentration of 8 mg/L

Time (Hour)	MT concentration (mg/L)							
	1		2		3		AVG.	SD
0	870000	940000	1030000	980000	1090000	970000	980000	75366
14	870000	970000	1070000	930000	1020000	960000	970000	69570
30	1170000	980000	1080000	930000	1230000	1160000	1091667	117204
47	1160000	1040000	1280000	1130000	950000	1170000	1121667	114091
60	1130000	1250000	1280000	1370000	1320000	1240000	1265000	81670
72	1280000	1290000	1170000	1260000	1230000	1320000	1258333	52694
95	1230000	1190000	1030000	1280000	1310000	1340000	1230000	111893
122	1290000	1360000	1460000	1270000	1290000	1380000	1341667	72503
204	1380000	1300000	1250000	1260000	1320000	1390000	1316667	58878

Table A. 33 Biodegradation of MT by MT degrading bacteria strain **SW051** at initial MT concentration of 10 mg/L

Time (Hour)	MT concentration (mg/L)			
	1	2	AVG.	SD
0	11.0	11.0	11.0	0.0
12	11.6	11.2	11.4	0.3

22	10.2	9.8	10.0	0.3
40	10.0	10.3	10.1	0.2
48	5.2	5.2	5.2	0.1
65	4.8	5.7	5.2	0.6
132	1.1	1.2	1.1	0.1
144	0.5	0.6	0.5	0.0
168	0.5	0.5	0.5	0.0

Table A. 34 The amount of colony forming unit per ml of MT degrading bacteria strain **SW051** at initial MT concentration of 10 mg/L

Time (Hour)	MT concentration (mg/L)							
	1		2		3		AVG.	SD
0	860000	850000	890000	980000	970000	840000	898333	61779
12	950000	980000	1030000	890000	1010000	970000	971667	49160
22	1030000	970000	1190000	1080000	1180000	1210000	1110000	98184
40	1150000	1040000	1280000	1130000	1350000	1270000	1203333	115528
48	1140000	1320000	1350000	1380000	1280000	1130000	1266667	107269
65	1290000	1190000	1030000	1270000	1340000	1250000	1228333	108888
132	1140000	1290000	1240000	1270000	1360000	1350000	1275000	80685
144	1380000	1300000	1250000	1260000	1320000	1390000	1316667	58878
168	1430000	1370000	1290000	1120000	1420000	1370000	1333333	115701

Table A. 35 Biodegradation of MT by MT degrading bacteria strain **SW051** at initial MT concentration of 20 mg/L

Time (Hour)	MT concentration (mg/L)				
	1	2	3	AVG.	SD
0	20.1	20.6	20.8	20.5	0.4
6	20.6	20.1	20.4	20.4	0.2
22	20.6	21.5	21.0	21.0	0.4
35	19.6	20.0	19.3	19.7	0.3
47	18.8	19.4	19.1	19.1	0.3
60	18.6	19.2	20.6	19.5	1.0
72	17.6	18.4	19.3	18.4	0.8
95	18.2	18.1	17.3	17.9	0.5
122	16.0	14.1	16.5	15.5	1.3
204	11.4	9.0	8.0	9.5	1.8
216	11.0	10.0	7.6	9.5	1.7
240	4.8	5.8	10.6	7.1	3.1
319	4.2	8.2	4.5	5.6	2.2

Table A. 36 The amount of colony forming unit per ml of MT degrading bacteria strain **SW051** at initial MT concentration of 20 mg/L

Time (Hour)	MT concentration (mg/L)							
	1		2		3		AVG.	SD
0	940000	980000	950000	1030000	850000	870000	936667	67429
22	980000	1060000	930000	970000	1050000	980000	995000	50100
47	1050000	1270000	1160000	1290000	1180000	1140000	1181667	88412
72	1290000	1360000	1270000	1170000	1290000	1380000	1293333	74476
95	1250000	1180000	1240000	1270000	1100000	1350000	1231667	84715
122	1350000	1240000	1290000	1210000	1380000	1290000	1293333	64083
204	1370000	1490000	1360000	1250000	1470000	1360000	1383333	87101
216	1130000	1250000	1280000	1120000	1320000	1240000	1223333	81158
240	1250000	1270000	1160000	1290000	1280000	1140000	1231667	64936
319	1290000	1360000	1460000	1270000	1290000	1380000	1341667	72503

Table A. 37 Biodegradation of MT by MT degrading bacteria strain **SW051** at initial MT concentration of 50 mg/L

Time (Hour)	MT concentration (mg/L)				
	1	2	3	AVG.	SD
0	44.6	49.4	48.5	47.0	2.5
12	48.9	43.2	44.0	46.1	3.1
24	45.5	44.5	47.9	45.0	1.8

36	45.5	43.2	39.7	44.3	2.9
48	42.1	40.9	40.5	41.5	0.8
72	32.6	30.3	32.8	31.5	1.4
120	27.9	24.7	2.7	26.3	13.7
168	18.1	8.2	15.2	13.2	5.1

Table A. 38 The amount of colony forming unit per ml of MT degrading bacteria strain **SW051** at initial MT concentration of 50 mg/L

Time (Hour)	MT concentration (mg/L)							
	1		2		3		AVG.	SD
0	980000	970000	1070000	890000	1040000	970000	986667	62823
12	1030000	970000	1190000	1070000	1180000	1150000	1098333	89088
24	1180000	1160000	1030000	1080000	970000	1160000	1096667	84538
36	1350000	1270000	1360000	1290000	1380000	1420000	1345000	56125
48	1290000	1360000	1460000	1270000	1190000	1380000	1325000	94816
72	1420000	1390000	1260000	1170000	1310000	1270000	1303333	91579
120	1250000	1180000	1240000	1270000	1370000	1350000	1276667	71461
168	1380000	1470000	1240000	1190000	1370000	1430000	1346667	109301

Table A. 39 Biodegradation of MT by MT degrading bacteria strain **SW052** at initial MT concentration of 1 mg/L

Time (Hour)	MT concentration (mg/L)				
	1	2	3	AVG.	SD
0	1.0	1.0	1.0	1.0	0.0
12	0.9	0.9	0.9	0.9	0.0
24	0.6	0.5	0.7	0.5	0.1
36	0.4	0.4	0.4	0.4	0.0
48	0.4	0.3	0.3	0.4	0.1
60	0.2	0.2	0.2	0.2	0.0
66	0.1	0.1	0.2	0.1	0.0
72	0.1	0.1	0.2	0.1	0.1
120	0.1	0.0	0.0	0.1	0.0
168	0.0	0.0	0.0	0.0	0.0

Table A. 40 The amount of colony forming unit per ml of MT degrading bacteria strain **SW052** at initial MT concentration of 1 mg/L

Time (Hour)	MT concentration (mg/L)							
	1		2		3		AVG.	SD
0	1090000	870000	970000	1210000	930000	1070000	1023333	123720
12	1160000	1040000	1280000	1130000	950000	1170000	1121667	114091
24	1160000	1190000	1070000	1150000	1100000	1060000	1121667	52694
36	1340000	1280000	1160000	1290000	1380000	1270000	1286667	74744
48	1370000	1490000	1360000	1250000	1470000	1360000	1383333	87101
60	1260000	1380000	1410000	1320000	1270000	1420000	1343333	70048
72	1380000	1320000	1460000	1370000	1290000	1420000	1373333	62503
120	1430000	1370000	1290000	1120000	1420000	1370000	1333333	115701
168	1340000	1290000	1170000	1340000	1280000	1410000	1305000	80685

Table A. 41 Biodegradation of MT by MT degrading bacteria strain **SW052** at initial MT concentration of 3 mg/L

Time (Hour)	MT concentration (mg/L)				
	1	2	3	AVG.	SD
0	2.8	3.0	3.1	3.0	0.1
3	2.9	3.0	2.8	2.9	0.1
6	2.7	3.1	3.0	3.0	0.2
11	3.1	2.8	3.1	3.0	0.2
17	2.9	3.1	2.9	2.9	0.1
25	3.3	2.1	2.0	2.4	0.7
55	2.8	2.7	1.6	2.4	0.7
76	2.3	2.5	2.4	2.4	0.1
82	2.5	2.4	2.5	2.5	0.1
106	1.2	1.1	1.2	1.2	0.0
130	0.3	1.0	0.0	0.5	0.5
146	0.1	0.7	0.0	0.2	0.4
153	0.0	0.0	1.2	0.4	0.7

Table A. 42 The amount of colony forming unit per ml of MT degrading bacteria strain **SW052** at initial MT concentration of 3 mg/L

Time (Hour)	MT concentration (mg/L)							
	1		2		3		AVG.	SD
0	930000	910000	1090000	850000	980000	1040000	966667	88242
6	1050000	930000	1020000	1170000	930000	1060000	1026667	90480
17	1340000	1080000	1260000	1190000	1280000	1170000	1220000	92304
25	1230000	1190000	1030000	1280000	1310000	1320000	1226667	108197
55	1280000	1290000	1170000	1320000	1230000	1060000	1225000	96488
82	1340000	1280000	1160000	1290000	1380000	1270000	1286667	74744
106	1250000	1270000	1160000	1290000	1280000	1140000	1231667	64936
130	1290000	1360000	1460000	1270000	1290000	1380000	1341667	72503
146	1420000	1390000	1260000	1190000	1310000	1250000	1303333	88015

Table A. 43 Biodegradation of MT by MT degrading bacteria strain **SW052** at initial MT concentration of 5 mg/L

Time (Hour)	MT concentration (mg/L)				
	1	2	3	AVG.	SD
0	4.8	5.3	4.7	4.9	0.3
12	4.8	5.3	5.5	5.2	0.4
24	2.2	0.6	0.0	0.9	1.1
36	2.1	1.1	0.0	1.1	1.1
48	0.1	0.2	0.1	0.1	0.0
66	0.1	0.0	0.0	0.0	0.1
72	0.0	0.0	0.1	0.0	0.0
120	0.0	0.0	0.0	0.0	0.0

Table A. 44 The amount of colony forming unit per ml of MT degrading bacteria strain **SW052** at initial MT concentration of 5 mg/L

Time (Hour)	MT concentration (mg/L)							
	1		2		3		AVG.	SD
0	960000	870000	970000	1070000	930000	980000	963333	65625
12	980000	1080000	930000	1040000	1010000	920000	993333	62503
24	1160000	1040000	1280000	1130000	950000	1170000	1121667	114091
36	1290000	1130000	1460000	1270000	1240000	1290000	1280000	106583
48	1130000	1250000	1280000	1120000	1320000	1240000	1223333	81158
66	1260000	1190000	1030000	1180000	1240000	1270000	1195000	88713
72	1370000	1320000	1360000	1250000	1370000	1360000	1338333	47081
120	1280000	1290000	1170000	1320000	1230000	1320000	1268333	58452

Table A. 45 Biodegradation of MT by MT degrading bacteria strain **SW052** at initial MT concentration of 10 mg/L

Time (Hour)	MT concentration (mg/L)			
	1	2	AVG.	SD
0	10.6	11.2	10.9	0.4
12	11.6	11.2	11.4	0.3
22	10.5	8.1	9.3	1.7
40	8.8	9.4	9.1	0.4
48	5.7	5.6	5.6	0.1
65	5.4	6.1	5.7	0.5
132	2.6	2.5	2.5	0.1
144	2.1	1.8	2.0	0.2
168	1.7	2.1	1.9	0.3

Table A. 46 The amount of colony forming unit per ml of MT degrading bacteria strain **SW052** at initial MT concentration of 10 mg/L

Time (Hour)	MT concentration (mg/L)							
	1		2		3		AVG.	SD
0	980000	890000	1180000	1030000	960000	970000	1001667	98268
12	1040000	970000	1120000	1030000	920000	980000	1010000	69282
22	1080000	1190000	1170000	1150000	1230000	1060000	1146667	65320
40	1340000	1280000	1160000	1290000	1380000	1270000	1286667	74744
48	1350000	1170000	1260000	1290000	1180000	1220000	1245000	68920
65	1130000	1270000	1190000	1280000	1090000	1130000	1181667	79099
132	1090000	1380000	1250000	1380000	1290000	1180000	1261667	114091
144	1250000	1180000	1240000	1270000	1100000	1350000	1231667	84715
168	1420000	1390000	1260000	1160000	1090000	1270000	1265000	127554

Table A. 47 Biodegradation of MT by MT degrading bacteria strain **SW052** at initial MT concentration of 50 mg/L

Time (Hour)	MT concentration (mg/L)				
	1	2	3	AVG.	SD
0	46.4	48.0	44.4	47.2	1.8
12	45.8	47.8	47.9	46.8	1.2
24	40.1	47.3	46.9	43.7	4.0
36	38.7	33.3	35.1	36.0	2.7
48	30.2	36.4	36.5	33.3	3.6
72	32.7	35.5	32.2	34.1	1.8
120	32.8	25.6	3.1	29.2	15.5
168	12.7	11.6	4.6	12.2	4.4

Table A. 48 The amount of colony forming unit per ml of MT degrading bacteria strain **SW052** at initial MT concentration of 50 mg/L

Time (Hour)	MT concentration (mg/L)							
	1		2		3		AVG.	SD
0	860000	970000	1040000	1170000	970000	840000	975000	121450
12	1040000	970000	1130000	890000	1040000	970000	1006667	82138
24	1030000	970000	1190000	1040000	1180000	940000	1058333	104960
36	1150000	1040000	1280000	1130000	1350000	1270000	1203333	115528
48	1290000	1190000	1030000	1270000	1340000	1250000	1228333	108888
72	1140000	1290000	1240000	1270000	1360000	1350000	1275000	80685
120	1380000	1300000	1250000	1260000	1320000	1390000	1316667	58878
168	1430000	1370000	1290000	1120000	1420000	1370000	1333333	115701

Table A. 49 Biodegradation of MT by MT degrading bacteria strain **SW303** at initial MT concentration of 1 mg/L

Time (Hour)	MT concentration (mg/L)				
	1	2	3	AVG.	SD
0	0.9	0.9	0.9	0.9	0.0
12	0.9	1.0	0.8	0.9	0.1
24	0.2	0.2	0.2	0.2	0.0
36	0.1	0.1	0.1	0.1	0.0
48	0.1	0.1	0.1	0.1	0.0
60	0.0	0.0	0.0	0.0	0.0
66	0.0	0.0	0.0	0.0	0.0
72	0.0	0.0	0.0	0.0	0.0

Table A. 50 The amount of colony forming unit per ml of MT degrading bacteria strain **SW303** at initial MT concentration of 1 mg/L

Time (Hour)	MT concentration (mg/L)							
	1		2		3		AVG.	SD
0	860000	970000	1040000	1170000	970000	840000	975000	121450
12	1040000	970000	1130000	890000	1040000	970000	1006667	82138
24	1030000	970000	1190000	1260000	1180000	1320000	1158333	134077
36	1150000	1040000	1280000	1130000	1350000	1270000	1203333	115528
48	1290000	1190000	1030000	1270000	1340000	1250000	1228333	108888
60	1250000	1170000	980000	1130000	1260000	1310000	1183333	118940
66	1280000	1150000	1080000	1340000	1290000	1170000	1218333	99883
72	1410000	1340000	1290000	1120000	1320000	1180000	1276667	107455

Table A. 51 Biodegradation of MT by MT degrading bacteria strain **SW303** at initial MT concentration of 3 mg/L

Time (Hour)	MT concentration (mg/L)				
	1	2	3	AVG.	SD
0	2.8	2.8	2.5	2.7	0.2
3	2.8	2.9	2.7	2.8	0.1
6	2.6	2.7	2.5	2.6	0.1
11	1.8	1.8	2.5	2.0	0.4
17	0.7	0.6	0.8	0.7	0.1
25	0.1	0.1	0.1	0.1	0.1
31	0.0	0.1	0.1	0.1	0.1
52	0.4	0.0	0.0	0.1	0.2
55	0.0	0.0	0.0	0.0	0.0
76	0.0	0.0	0.0	0.0	0.0

Table A. 52 The amount of colony forming unit per ml of MT degrading bacteria strain **SW303** at initial MT concentration of 3 mg/L

Time (Hour)	MT concentration (mg/L)							
	1		2		3		AVG.	SD
0	930000	890000	860000	890000	960000	1060000	931667	71949
3	1090000	880000	930000	870000	970000	1050000	965000	89833
6	890000	1050000	940000	1030000	1080000	1190000	1030000	106019
11	1190000	1290000	1210000	1290000	1150000	1280000	1235000	59917
17	1280000	1320000	1290000	1360000	1290000	1370000	1318333	38687
25	1260000	1380000	1210000	1320000	1270000	1370000	1301667	66758
31	1180000	1320000	1460000	1370000	1290000	1420000	1340000	100200
52	1140000	1370000	1290000	1320000	1290000	1370000	1296667	84774
55	1290000	1360000	1190000	1270000	1190000	1380000	1280000	80994
76	1420000	1390000	1260000	1170000	1310000	1270000	1303333	91579

Table A. 53 Biodegradation of MT by MT degrading bacteria strain **SW303** at initial MT concentration of 5 mg/L

Time (Hour)	MT concentration (mg/L)				
	1	2	3	AVG.	SD
0	4.9	5.5	5.3	5.2	0.3
12	4.4	4.7	4.9	4.7	0.3
24	0.5	0.5	0.6	0.5	0.0
36	0.1	0.0	0.0	0.0	0.1
48	0.0	0.0	0.0	0.0	0.0
60	0.0	0.0	0.0	0.0	0.0

Table A. 54 The amount of colony forming unit per ml of MT degrading bacteria strain **SW303** at initial MT concentration of 5 mg/L

Time (Hour)	MT concentration (mg/L)							
	1		2		3		AVG.	SD
0	930000	910000	1090000	850000	980000	1040000	966667	88242
12	1050000	930000	1020000	1170000	930000	1060000	1026667	90480
24	1130000	1250000	1280000	1120000	1320000	1240000	1223333	81158
36	1280000	1290000	1170000	1320000	1230000	1320000	1268333	58452
48	1370000	1490000	1360000	1250000	1470000	1360000	1383333	87101
60	1190000	1290000	1380000	1290000	1350000	1280000	1296667	65625

Table A. 55 Biodegradation of MT by MT degrading bacteria strain **SW303** at initial MT concentration of 10 mg/L

Time (Hour)	MT concentration (mg/L)			
	1	2	AVG.	SD
0	10.8	10.9	10.9	0.1
12	8.6	8.2	8.4	0.2
22	9.0	9.2	9.1	0.1
48	4.0	4.8	4.4	0.6
65	2.2	1.9	2.0	0.2
132	1.2	1.1	1.1	0.0
144	1.2	1.0	1.1	0.2
168	0.6	0.6	0.6	0.0
168	0.5	0.5	0.5	0.0

Table A. 56 The amount of colony forming unit per ml of MT degrading bacteria strain **SW303** at initial MT concentration of 10 mg/L

Time (Hour)	MT concentration (mg/L)							
	1		2		3		AVG.	SD
0	930000	890000	860000	890000	960000	1060000	931667	71949
12	1090000	970000	930000	1000000	1030000	1050000	1011667	57417
22	1020000	930000	1080000	980000	1010000	1060000	1013333	54283
48	1280000	1290000	1170000	1210000	1230000	1060000	1206667	84538
65	1250000	1210000	1360000	1390000	1350000	1290000	1308333	69976
132	1260000	1380000	1310000	1320000	1270000	1290000	1305000	43243
144	1180000	1320000	1460000	1370000	1290000	1420000	1340000	100200
168	1430000	1370000	1290000	1120000	1420000	1370000	1333333	115701

Table A. 57 Biodegradation of MT by MT degrading bacteria strain **SW303** at initial MT concentration of 20 mg/L

Time (Hour)	MT concentration (mg/L)				
	1	2	3	AVG.	SD
0	21.1	19.8	20.4	20.4	0.6
6	19.9	19.9	20.6	20.1	0.4
22	18.5	18.8	22.1	19.8	2.0
35	9.2	15.1	14.8	13.0	3.3
54	10.3	6.7	7.5	8.2	1.9
72	3.1	9.4	8.4	7.0	3.4
95	0.8	1.1	4.4	2.1	2.0
122	1.8	2.9	0.9	1.8	1.0
204	0.0	0.4	0.2	0.2	0.2

Table A. 58 The amount of colony forming unit per ml of MT degrading bacteria strain **SW303** at initial MT concentration of 20 mg/L

Time (Hour)	MT concentration (mg/L)							
	1		2		3		AVG.	SD
0	980000	870000	980000	1030000	960000	990000	968333	53448
6	1210000	1070000	960000	1040000	1120000	1030000	1071667	85654
22	930000	1050000	1080000	950000	1190000	1140000	1056667	102697
35	1280000	1190000	1170000	1250000	1230000	1060000	1196667	77889
54	1340000	1280000	1360000	1290000	1380000	1270000	1320000	46043
72	1250000	1270000	1160000	1290000	1280000	1140000	1231667	64936
95	1290000	1360000	1460000	1270000	1290000	1380000	1341667	72503
122	1420000	1390000	1260000	1190000	1310000	1250000	1303333	88015
204	1370000	1410000	1310000	1260000	1420000	1380000	1358333	61779

Table A. 59 Biodegradation of MT by MT degrading bacteria strain **SW303** at initial MT concentration of 30 mg/L

Time (Hour)	MT concentration (mg/L)				
	1	2	3	AVG.	SD
0	26.8	26.2	28.7	27.2	1.3
33	15.4	12.0	13.3	13.6	1.7
48	12.2	7.9	8.3	9.5	2.4
54	8.0	9.2	5.9	7.7	1.6
68	6.5	7.1	0.6	4.7	3.6
70	3.6	1.6	4.7	3.3	1.6
72	3.3	2.6	3.4	3.1	0.5
84	1.7	2.9	2.0	2.2	0.6
96	1.3	0.8	0.9	1.0	0.3
101	1.6	1.6	1.3	1.5	0.2
115	0.6	0.6	0.6	0.6	0.0
142	0.5	1.0	0.4	0.7	0.3

Table A. 60 The amount of colony forming unit per ml of MT degrading bacteria strain **SW303** at initial MT concentration of 30 mg/L

Time (Hour)	MT concentration (mg/L)							
	1		2		3		AVG.	SD
0	890000	960000	970000	830000	910000	1040000	933333	72847
33	1050000	930000	1020000	870000	930000	1060000	976667	77374
48	1030000	920000	1040000	970000	1070000	1130000	1026667	73937
54	1160000	1050000	1040000	1230000	1140000	1270000	1148333	92826
68	1370000	1130000	1260000	1340000	1170000	1040000	1218333	127658
72	1260000	1380000	1410000	1320000	1270000	1420000	1343333	70048
84	1290000	1360000	1460000	1270000	1190000	1380000	1325000	94816
96	1140000	1370000	1290000	1120000	1290000	1370000	1263333	109484
115	1320000	1320000	1270000	1240000	1200000	1390000	1290000	67528
142	1340000	1290000	1160000	1230000	1340000	1160000	1253333	82865

Table A. 61 Biodegradation of MT by MT degrading bacteria strain **SW303** at initial MT concentration of 50 mg/L

Time (Hour)	MT concentration (mg/L)				
	1	2	3	AVG.	SD
0	47.4	36.8	43.1	42.4	5.3
12	44.2	47.7	44.0	46.0	2.1
24	39.6	41.0	45.8	40.3	3.2
36	38.2	20.1	25.3	29.2	9.3
48	33.1	21.7	36.1	27.4	7.6
72	24.5	9.8	29.7	17.2	10.3
120	1.6	18.0	11.0	9.8	8.2
168	7.9	2.7	1.8	5.3	3.3

Table A. 62 The amount of colony forming unit per ml of MT degrading bacteria strain **SW303** at initial MT concentration of 50 mg/L

Time (Hour)	MT concentration (mg/L)							
	1		2		3		AVG.	SD
0	930000	870000	970000	1040000	930000	1070000	968333	74944
12	1060000	1040000	980000	1130000	950000	970000	1021667	67946
24	1080000	970000	1170000	980000	1130000	1060000	1065000	79687
36	1140000	1080000	1160000	1140000	1210000	1270000	1166667	65625
48	1370000	1050000	1280000	1130000	1050000	1060000	1156667	136772
72	1090000	1210000	930000	1180000	1090000	1050000	1091667	99683
120	970000	1150000	1080000	950000	1140000	1240000	1088333	111967
168	1280000	1290000	1170000	1320000	1230000	1060000	1225000	96488

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