BIODEGRADATION OF TIAMULIN BY WOOD-ROT FUNGI AND ENRICHED BACTERIAL CULTURES OBTAINED FROM SWINE FARMS

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การย่อยสลายไทอะมูลินโดยราย่อยไม้และกลุ่มแบคทีเรียเอนริชจากฟาร์มสุกร

นางเหงวียน ถิ กิม ซอน

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เหงวียน ถิ กิม ซอน : การย่อยสลายไทอะมูลินโดยราย่อยไม้และกลุ่มแบคทีเรียเอนริชจากฟาร์มสุกร (BIODEGRADATION OF TIAMULIN BY WOOD-ROT FUNGI AND ENRICHED BACTERIAL CULTURES OBTAINED FROM SWINE FARMS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ดร. ภริณดา ทยานุกูล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร. อรฤทัย ภิญญาคงปริญญาเอก, 206 หน้า.

ไทอะมูลิน (Tiamulin) เป็นยาปฏิชีวนะที่มีการใช้อย่างแพร่หลายในฟาร์มสุกรของไทย และเป็นสารที่มีความคงทนในกระบวนการเก็บรักษาปุ๋ยคอกเป็นอย่างมากอันอาจก่อให้เกิดแพร่กระจายยาปฏิชีวนะและทำให้เกิดการวิวัฒนาการของจุลินทรีย์ดื้อยาได้ งานวิจัยนี้ได้นำราย่อยสลายไม้ 12 ชนิดจากฟาร์มสุกรมาทดสอบความสามารถในการย่อยสลายสารที่มีความคงทนโดยใช้สีย้อม brilliant green และ crystal violet โดยพบว่ามีเชื้อราที่คัดแยกมา 3 ชนิด และราย่อยไม้ขาว (white-rot fungi) 3 ชนิดมีชื่อว่า *Lasiodiplodia* sp. F1, *Fusarium* sp. F5, *Galactomyces* sp. F8, *Verticillium* sp., *Trametes versicolor*, และ *Trametes hirsuta* AK4 สามารถทำลายสีย้อมทั้ง 2 ชนิด และสามารถย่อยสลาย 10 mg/L ของไทอะมูลินได้ภายใน 12 วัน โดยมีประสิทธิภาพ 93.2 %, 82.4 %, 73.8 %, 89.3 %, 86.1 %, และ 66.8 % ตามลำดับ และด้วยอัตรา 27.0, 9.8, 5.8, 15.6, 12.6 และ 5.5 mL/g fungi·d ตามลำดับ *Lasiodiplodia* sp. F1, *Verticillium* sp., และ *Trametes versicolor* สามารถย่อยสลายไทอะมูลินได้มีประสิทธิภาพสูงที่สุดคือมากกว่า 85 % ยิ่งไปกว่านั้นพบว่าเอนไซม์ Manganese peroxidase ผลิตได้มากกว่าเอนไซม์ laccase และ lignin peroxidase เป็นอย่างมากในราทุกสายพันธุ์ บ่งบอกว่าเอนไซม์ชนิดนี้น่าจะเป็นเอนไซม์หลักที่ใช้ในการย่อยสลายไทอะมูลิน นอกจากนั้นงานวิจัยนี้ได้ทำการบ่มเพาะกลุ่มจุลินทรีย์ที่ย่อยสลายไทอะมูลิน 4 ประเภท ซึ่งประกอบไปด้วยกลุ่ม A (นำมาจากระบบบำบัดบ่อไร้อากาศแบบปิดคลุม), กลุ่ม AN (นำมาจากระบบบำบัดบ่อไร้อากาศแบบปิดคลุม และเลี้ยงด้วยอาหารที่มี nutrient broth), กลุ่ม S (นำมาจากระบบบำบัดแบบบ่อปรับเสถียร), และกลุ่ม SN (นำมาจากระบบบำบัดแบบบ่อปรับเสถียร และเลี้ยงด้วยอาหารที่มี nutrient broth) ที่สามารถกำจัดไทอะมูลินที่ความเข้มข้น 2.5 - 200 mg/L ภายในเวลา 16 ชั่วโมงด้วยประสิทธิภาพที่ 99.7%, 99.0%, 99.9% และ 99.6% ตามลำดับ และมีค่าตัวแปรทางจลนศาสตร์ของ Monod ค่า K_s ที่ 190.9, 469.6, 2001.0 และ 206.7 mg/L และค่า q_{max} ที่ 30.7, 63.5, 269.2 และ 48.5 mg/L·h ตามลำดับ การหาลำดับดีเอ็นเออด้วยเทคนิคยุคใหม่พบว่าจีนัส *Achromobacter*, *Delftia* และ *Pandoraea* ในคลาส Betaproteobacteria และจีนัส *Pseudomonas* และ *Stenotrophomonas* ในคลาส Gammaproteobacteria มีอยู่เป็นจำนวนมาก ซึ่งจีนัสเหล่านี้อาจจะเป็นตัวหลักในการย่อยสลายไทอะมูลิน การทดลองนี้พบว่าเอนริชแบคทีเรียมีประสิทธิภาพเหนือกว่าราที่นำมาทดสอบในด้านอัตราเร็วในการย่อยสลาย แต่หากว่าการนำจุลินทรีย์ไปใช้อาจทำได้จำกัดเนื่องจากมีความเป็นไปได้ที่จะแพร่กระจายเชื้อจุลินทรีย์ดื้อยาและยีนดื้อยาสู่สิ่งแวดล้อม เทคนิคการตรึงจุลินทรีย์ (bioencapsulation) อาจช่วยป้องการการรั่วไหลของเชื้อ และนอกจากนี้ยังอาจสกัดเอนไซม์ manganese peroxidase จากรามาใช้ในการย่อยสลายไทอะมูลินเช่นเดียวกันซึ่งต้องการการศึกษาต่อไป โดยสรุปแล้วเอนริชแบคทีเรียและราที่ได้จากในการทดลองนี้มีความเป็นไปได้สูงที่จะนำไปใช้กำจัดไทอะมูลินที่ตกค้างในปุ๋ยมูลสุกรหรือน้ำเสียที่ได้

สาขาวิชา การจัดการสิ่งแวดล้อม ลายมือชื่อนิสิต .....

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NGUYEN THI KIM XUAN: BIODEGRADATION OF TIAMULIN BY WOOD-ROT FUNGI AND ENRICHED BACTERIAL CULTURES OBTAINED FROM SWINE FARMS. ADVISOR: DR. PARINDA THAYANUKUL, CO-ADVISOR: ASSOC. PROF. ONRUTHAI PINYAKONG, Ph.D., 206 pp.

Tiamulin is a widely used antibiotic in Thai swine farms and persists in manure storage, posing risks related to the spreading of antibiotic and the evolution of resistant bacteria. Twelve strains of wood-rot fungi were collected from swine farms and preliminarily examined for their ability to degrade a recalcitrant compound using brilliant green and crystal violet dyes. Three isolated fungi and three white-rot fungal strains–namely, *Lasiodiplodia* sp. F1, *Fusarium* sp. F5, *Galactomyces* sp. F8, *Verticillium* sp., *Trametes versicolor*, and *Trametes hirsuta* AK4–capable of developing considerable decolorized zones on both dyes exhibited their abilities to degrade 10 mg/L of tiamulin over a period of 12 days: 93.2%, 82.4%, 73.8%, 89.3%, 86.1%, and 66.8%, respectively, and at the rates of 56.7, 6.5, 5.6, 58.6, 56.0, and 6.8 mL/g fungi·d, respectively. *Lasiodiplodia* sp. F1, *Verticillium* sp., and *Trametes versicolor* were able to remove TIA most efficiently (> 85%). Manganese peroxidase was predominantly produced by all strains over laccase and lignin peroxidase suggesting its main role in TIA degradation. In addition, four TIA-degrading bacterial enriched cultures including A (covered anaerobic lagoon source), AN (covered anaerobic lagoon source, nutrient broth), S (stabilization pond source), and SN (stabilization pond source, nutrient broth) were able to remove tiamulin efficiently at concentrations of 2.5 - 200 mg/L with the maximum rates of 99.7%, 99.0%, 99.9% and 99.6%, respectively within 16 h. Monod kinetic values of K_s were 190.9, 469.6, 2000.1, and 206.7 mg/L, respectively and q_{max} values were 30.7, 63.5, 269.2, and 48.5 mg/L·h. Next-generation sequencing revealed genera *Achromobacter*, *Delftia*, *Pandoraea* in class Betaproteobacteria and genera *Pseudomonas*, and *Stenotrophomonas* in class Gammaproteobacteria dominated , indicating that they might contributed to improve tiamulin elimination process. Tiamulin degrading enriched bacterial cultures had an advantage over the tested fungi in term of the degradation rate; however, its application might be limited due to the possibility of spreading antibiotic resistance microbes or genes to environment. Bioencapsulation technique might help to retard the release of microbes. Besides, the extracted fungal manganese peroxidase might be applied to degrade tiamulin. Further investigation is required. In summary, the bacterial enriched cultures and fungi obtained in this study are promising tiamulin degraders that are potentially applied for eliminating tiamulin in contaminated swine manure and wastewater.

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Student's Signature .....
Advisor's Signature .....
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LIST OF ABBREVIATIONS

Abbreviation	Full name
AK4	*Trametes hirsuta* AK4
APS	Ammonium persulfate
ARB	Antibiotic resistant bacteria
ARGs	Antibiotic resistant genes
A	Covered anaerobic lagoon
AN	Covered anaerobic lagoon + Nutrient broth
S	Stabilization pond
SN	Stabilization pond + Nutrient broth
CFU	Colony forming unit
COD	Chemical oxygen demand
d	Day

Abbreviation	Full name
DGGE	Denaturing gradient gel electrophoresis
DI	Deionized
DNA	Deoxyribonucleic acid
EC_{50}	Half maximal effective concentration
EPA	Environmental Protection Agency
Eq.	Equation
F1	*Lasiodiplodia* sp. F1
F2	*Curvularia* sp. F2
F3	*Aspergillus* sp. F3
F4	*Penicillium* sp. F4
F5	*Fusarium* sp. F5
F6	*Trichoderma* sp. F6
F7	*Hypocreales* sp. F7

Abbreviation	Full name
F8	*Galactomyces* sp. F8
F9	*Geotrichum* sp. F9
F10	*Mucor* sp. F10
F11	*Trichosporon* sp. F11
F12	*Penicillium* sp. F12
h	Hour
HPLC-DAD	High performance liquid chromatography with diode-array detection
HSM	Center of Excellence on Hazardous Substance Management
ITS	Internal transcribed spacer
K_s	Half-saturation constant
L	Litter
LB	Luria-Bertani
LC_{50}	Lethal concentration, 50%

Abbreviation	Full name
LD_{50}	Median lethal dose or lethal dose, 50%
Lac	Laccase
LiP	Lignin peroxidase
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid Chromatography - Mass Spectrometry and Liquid Chromatography - Tandem Mass Spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
μ_{max}	Maximum consumption rate
µL	Microliter
µmol	Micromole
mg	Milligram
min	Minute
mL	Milliliter

Abbreviation	Full name
mm	Millimeter
MIC	Minimum inhibitory concentration
MLS	Macrolide-lincosamide-streptogramin
MSM	Mineral salt medium
MSR	Miseq Reportern Software
MnP	Manganese peroxidase
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-susceptible *Staphylococcus aureus*
MW	Molecular weight
NB	Nutrient broth
NGS	Next-generation sequencing
NH_3-N	Ammonium nitrogen
OD	Optical density

Abbreviation	Full name
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PIS	Porcine intestinal spirochaetosis
qPCR	Quantitative polymerase chain reaction
TCS	Toxicity classification system
TCs	Total hydrocarbons
TEMED	N,N,N',N' - Tetramethylenediamine
TIA	Tiamulin
TN	Total nitrogen
TV	*Trametes versicolor*
U	Unit
U.K	United Kingdom
U.S	United State

Abbreviation	**Full name**
UV	Ultraviolet
V	*Verticillium* sp.
VA	Veratryl alcohol
v/v	Volume/volume
w/v	Weight/volume
WWTP	Wastewater treatment plant

Chapter 1 INTRODUCTION

1.1 Introduction

Antibiotics are emerging environmental issues because they are widely and intensively used in human and veterinary therapies and also used as growth promoters in animal husbandry. Over 1 million metric tons of antibiotics have been produced since 1940's [1] and from the mid of 1990's more than 500 metric tons of antimicrobials were annually produced [2]. Veterinary drugs are essentially used in swine production to prevent and treat disease, and to improve efficiency of feed and promote growth [3]. Several types of antibiotics have been introduced in swine production such as amoxicillin, chlortetracycline, colistin, doxycycline, enrofloxacin, erythromycin, oxytetracycline, sulfamethazine, tetracycline, tiamulin, and tylosin [4-6]. Usually, the concentration of antibiotics was mixed with feed ranged from 3 to 220 g antibiotic per ton of feed based on the kind and size of the animal and the types of antibiotic [7].

Antibiotics are partially metabolized after taking up and the remaining antibiotics are excreted via feces and urine as original or modified forms [8, 9]. Residues of antibiotics and their corresponding resistant genes were frequently found in swine farms and surroundings, hence swine wastes become a crucial pollution source of antibiotics and antibiotic resistant genes [10-12]. Because antibiotics are incompletely removed, it can be accumulated in the surrounding environments [13]. Antibiotics residues and antibiotic resistant genes in swine wastewater residues have a great capacity to disturb the natural ecological balance and stimulate the emerging of resistant bacteria in environment. Hence, the residues of antibiotics found in animal wastes limit its recycling value for agricultural benefit. The application of antibiotics contaminated manure to agricultural farm lands as fertilizer potentially cause the transmission of antibiotics and antibiotic resistant genes to soils and surrounding streams [14, 15]. Thus, it is necessary to select a treatment technology with both high

efficiency and reasonable operational cost to remove antibiotics residues from swine waste before releasing to the environment [16].

Among antibiotics used in swine farms, tiamulin (TIA) has been the medication commonly and widely used for swine dysentery eradication since the disease could cause major economic losses for pork producer as well as eradication rather than mere control of the disease is preferable [17]. Tiamulin is a semi-synthesized antibiotic in Pleuromutilin class that originated from a fungal *Pleurotus mutilus*. Its mode of action involves in inhibition of bacterial protein synthesis by binding to the domain V of 23S rRNA in the peptidyl transferase slot at the 50S large prokaryotic ribosomal subunit and blocking substrate binding site for peptide formation and mispositioning of the CCA ends of tRNA for peptide transferase [18]. It is effective against Gram positive bacteria and *Mycoplasma* [19]. In the U.S and U.K, there were 1,435 kg tiamulin had been used in 2000 [14]. In the investigation of 55 pig farms in Dong Nai and Binh Duong provinces of Viet Nam, the use of tiamulin accounted for 5.74% of total antibiotic uses compared to other antibiotics [4]. According to Novartis Animal Health US, tiamulin was classified as a hazardous waste for purposes of good product stewardship practices and recommended to prevent release to the environment uncontrollably. Tiamulin may be irritating to the skin, eyes, and respiratory system, so it may cause sensitization by skin contact and inhalation [20].

Tiamulin has high water solubility of 58 g/L, log K_{ow} of 5.9 and pK_a of 7.6 [21-23], so the residual of tiamulin was discovered in various environmental media. Tiamulin was found in swine manure samples at the concentration of 43 µg/kg [24], in swine wastewater at 5 µg/L [21], in two investigated soil samples at trace concentrations of 0.7 µg/kg [24], in the wastewater lagoon at a concentration of 182 ng/L [25], in all water samples from a source water collected from downstream of Dongjiang River at the average concentrations in the range of 0.77 - 18.77 ng/L [26], and in groundwater at a concentration of 29 ng/L [25]. Some studied were performed to eliminate tiamulin from livestock liquid manure in 180-day degradation, but the removal efficiency was not high since tiamulin is very persistent in manure [24, 27]. The remaining tiamulin may cause environmental problem.

In Thailand, tiamulin is one of the most often used compounds in swine farms as it applied to all stages of swine husbandry (personal communication with veterinaries and farmers). The waste from swine farms is treated by various processes such as stabilizing ponds and anaerobic digesters. These treatments are mainly aimed at removing common biodegradable organic matters but not for the hardly degraded structure of antibiotics. Residual antibiotics of tiamulin, therefore, may remain in waste which some parts release to outside of farm. The existence of tiamulin potentially disturbs natural ecological balance and stimulates the emergence of resistant bacteria in environment.

In addition, some bacteria have been found to become less susceptible to tiamulin worldwide [28-30]. *Brachyspira hyodysenteriae*, the causative agent of swine dysentery, was found to be resisted to tiamulin in the swine farms in the Czech Republic between years 1997 and 2006 [31]; tiamulin resistance in an anaerobic spirochaete *Brachyspira pilosicoli*, the etiologic agent of porcine intestinal spirochaetosis (PIS), was also reported in 2002 [32]. Wastes from livestock farming such as liquid manure and sewage sludge contain nutrients that can be used to supply for soil as a sustainable land use practice. The presence of tiamulin in liquid manure and sewage could constrain nutrient recycling potentially cause antimicrobial-resistant microorganisms. As a consequence, synthetic fertilizers are required to add into soil to make it fertile and productive for agricultural purposes. However, this practice is costly and tiamulin cannot be removed from wastes. Alternatively, tiamulin residual needs to be eliminated to open way for waste utilization.

Antibiotics in manure could be biodegraded by different treatment methods such as anaerobic digestion, composting, and constructed wetlands. These treatments could remediate several kinds of antibiotics. Chlortetracycline at the initial concentration of 6.5, 8.3, and 5.9 mg/L was reduced 7%, 80%, and 98%, respectively at 22^{o}C, 38^{o}C, and 55^{o}C slurries of swine manure anaerobic digesters, respectively for the period of 21 days [33]. Batch anaerobic digestion could reduce initial oxytetracycline from 20 mg/L to 5.4 mg/L (73%) [34]. The lagoons was able to remove tylosin efficiently in swine manure up to 75% [35]. Chlortetracycline, oxytetracycline, tetracycline,

ciprofloxacine, and sulfamethoxazole could also be nearly completely degraded by composting process [36-38]. In constructed wetland system, high removal efficiencies (69.0 - 99.9%) were achieved for oxytetracycline, tetracycline and chlortetracycline [39]. Composting could be used efficiently to remove various kinds of antibiotics such as monensin, tylosin, levofloxacine, ciprofloxacine, erythromycin, sulfamonomethoxine, sulfamethoxazole, trimethoprim, and carbamazepine with the efficiency ranged from 37% to 100% [38, 40]. Removal rate of antibiotics by composting was more efficient than by anaerobic digestion due to more aerobic bioactivity [11]. Biodegradation of antibiotics was generally depended on initial concentration because microbial activities were inhibited by antibiotic concentrations [41].

However, manure storage process and anaerobic conditions were ineffectively in biodegrading tiamulin. Indeed, results of previous studies showed that tiamulin remained unchanged during the entire experiment of livestock liquid manure 180-day degradation [24, 27] as well as it was very slow under anaerobic conditions degradation [42].

Wood rot fungi are a promising approach for bioremediation of antibiotics-contaminated sites because they produce extracellular enzymes and non-specific lignin-mineralizing enzymes including laccase, lignin peroxidase, manganese peroxidases, cellulase, and hemicellulase that can remove a diverse range of xenobiotic chemicals [43]. Synthetic dyes with complex and stable chemical structures could be degraded; for instance, *Trametes hirsuta* could degrade triarylmethane, indigoid, azo, and anthraquinoic dyes by laccase enzyme [44]. Antibiotics such as ciprofloxacin and norfloxacin could be removed more than 90% at the concentration of 2 mg/L in the period of 7 days using extracellular enzyme laccase from *Trametes versicolor* [45]. Interestingly, white rot fungi could degrade both dyes and antibiotics. For instance the white rot fungus *Phanerochaete chrysosporium* could degrade crystal violet and brilliant green [46]. It also had the capability to degrade antiviral drug oseltamivir (Tamiflu) and the antibiotics erythromycin, sulfamethoxazole, and ciprofloxacin at 10 µg/mL [47]. *Phanerochaete chrysosporium*

could degrade antibiotics due to enzyme laccase and manganese peroxidase [48, 49]. Additionally, *Galactomyces geotrichum* decolorized triphenylmethane, azo, methyl red, and reactive high exhaust textile dyes [50] while it also showed 37% the degradation efficiency in treating the lincomycin antibiotic residuals in solid wastes of fermentation drugs [51]. The example of two fungi *Phanerochaete chrysosporium* and *Galactomyces geotrichum* that could degrade both dye and antibiotics at the same time by using its non-specific enzymes leading to a research hypothesis for this study that dye degrading fungi could also degrade antibiotic tiamulin.

Moreover, swine wastes contain a variety of bacteria which are readily exposed to residues of antibiotics, so they potentially pre-adapted to antibiotics. For example, residual chlortetracycline antibiotic was completely degraded in 20 days in the batch experiments with swine wastewater after 6 months adaptation under darkness and aerobic condition [52]. Similar phenomenon may occur with tiamulin residues in swine wastes as tiamulin may act as a main substrate for growth of bacteria and its concentrations potentially reduced by bacterial communities.

From all the above mentioned points, it is important to enhance tiamulin antibiotic removal in both solid and liquid swine wastes to restrict the mitigation of tiamulin residues and their resistant genes into the surrounding environments. Biodegradation of tiamulin antibiotic has many advantages such as low operation cost, high efficiencies, and sustainable environment and development. In this study tiamulin-degrading microorganisms including both fungi and bacteria were isolated from swine farms so as to apply for further alleviating tiamulin antibiotic residue problems arisen from swine production.

1.2 Research objectives

The overall objective of this study is to enhance TIA antibiotic removal in swine farm wastewater and manure by using fungi and bacterial enrichment cultures obtained from swine farms in Thailand. The objectives were divided into three sub-objectives;

1) To obtain tiamulin-degrading fungi and investigate their ligninolytic enzymes involving in tiamulin elimination

2) To obtain TIA-degrading enriched bacterial cultures and examine the tiamulin degradation kinetics of TIA elimination

3) To study the structural communities of TIA-degrading bacterial enriched cultures in different tiamulin concentrations.

1.3 Research hypotheses

The following hypothesis were evaluated and tested during the course of this study.

1) Fungi that are capable of decolorizing both synthetic dyes including brilliant green and crystal violet can degrade a recalcitrant tiamulin antibiotic due to ligninolytic enzymes.

2) Tiamulin degrading bacterial communities in swine wastewater are captured with enrichment technique.

3) Bacteria that are capable of degrading tiamulin are various under different swine waste sources, enriched conditions and tiamulin concentrations.

1.4 Scopes of study

1) Swine wastewater, sediment, manure, decayed wood, tree trunks and leaves samples were taken from two pig farms with covered anaerobic lagoon and stabilization ponds swine waste treatment processes in Ratchaburi province and Nakhon Phathom province in Thailand.

2) Fungi were isolated from swine farm samples with potato dextrose agar (PDA) plates. The genomic DNA of all isolated fungi was extracted and their internal transcribed spacer in ribosomal rDNA genes (ITS-rDNA) were sequenced to identify all fungal isolates with genus classification.

3) Fungal abilities on producing ligninolytic enzymes were preliminary screened using PDA plates containing brilliant green and crystal violet dyes (0.5% w/v) with all isolated fungi and three previously reported white rot fungi unrelated to swine farm.

4) Positive fungi in degrading both dyes were continued to examine for tiamulin degradation in aqueous cultivation. Enzymatic activity from tiamulin degradation tests was also identified to elucidate role of enzymes involving in tiamulin removal.

5) To further enhance tiamulin removal in various conditions, TIA-degrading bacteria were enriched from swine wastewater and sediment samples in mineral salt medium (MSM) with increasing concentration of tiamulin from 4 mg/L to 20 mg/L. Nutrient broth, other carbon source, was supplied in one set of experiment with gradually decreasing from 2 mg/L to 0 mg/L.

6) The kinetics of tiamulin biodegradation and bacterial growth rates by bacterial enriched cultures were tested in aqueous cultivation at different tiamulin concentrations (2.5 - 200 mg/L). The TIA concentrations were measured by using High-Performance Liquid Chromatography with Diode-Array Detector (HPLC-DAD).

7) Tiamulin degrading bacterial communities in enriched cultures and their corresponding treatments at the lowest and highest tiamulin concentrations were identified by 16S metagenomics analysis with the next-generation sequencing technique.

1.5 Thesis organization

This thesis is organized into seven chapters. Chapter 1 mentions general introductions with research objectives, hypotheses, and scopes of this study. Chapter 2 of this document presents a literature review on the uses of antibiotics, the development of resistance, type of antibiotics, methods for detection and quantification, and previous findings on this topic. Chapter 3 provides materials and methods used in this thesis. Chapter 4 indicates the whole research results and discussions related to using wood rot fungi isolated from swine farms to decolorize the synthetic dyes and eliminate the antibiotic tiamulin. Chapter 5 of this thesis demonstrates the results and discussions correlated with using stable enriched bacterial cultures to alleviate the tiamulin antibiotic. Chapter 6 reveals structural communities of bacterial enrichment cultures from swine wastewater with the next-generation sequencing. Chapter 7 presents general conclusions for this research and reveals recommendations for future research in this field of study (Figure 1.1).

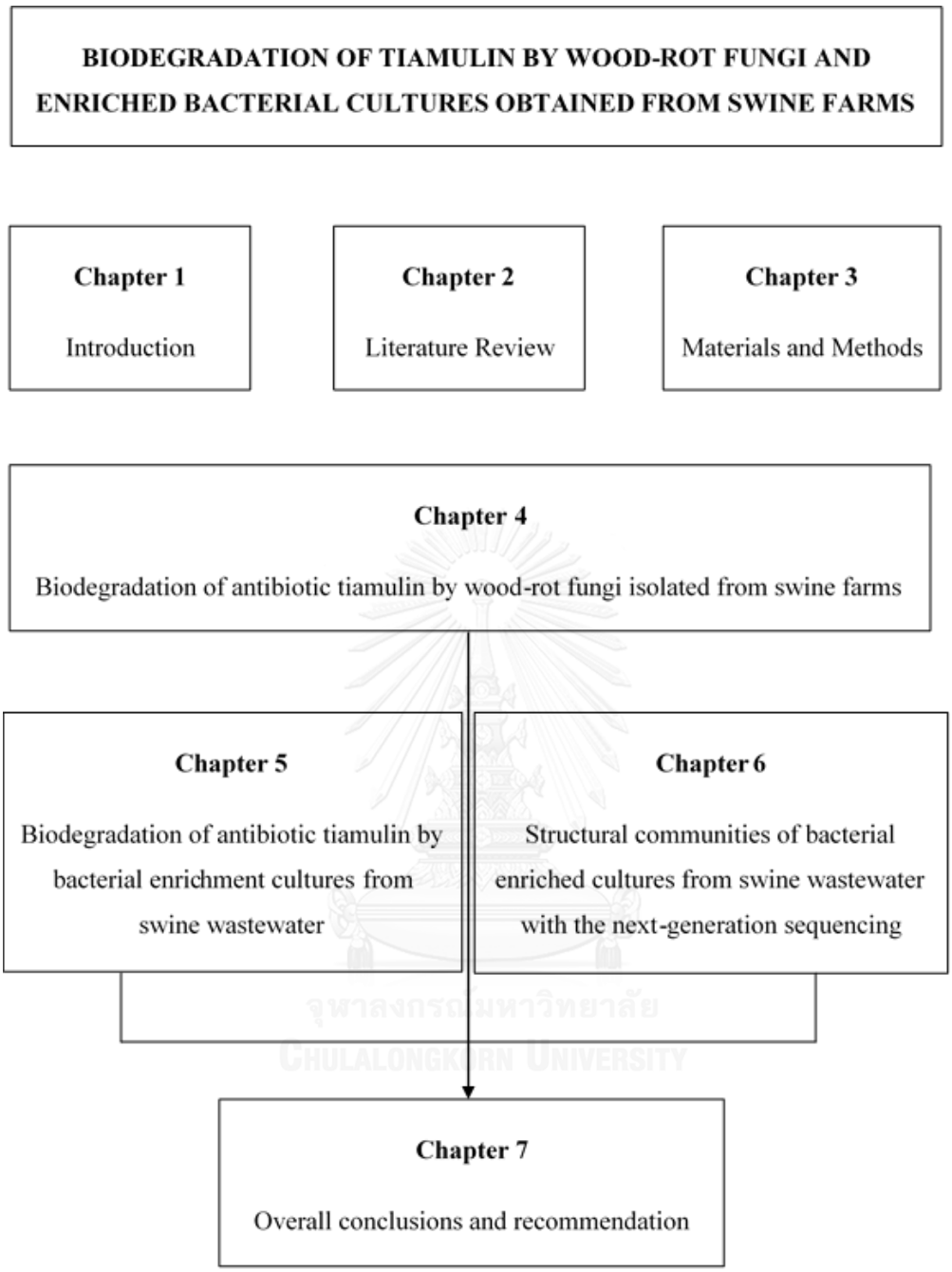


Figure 1.1 Structure of dissertation

Chapter 2 LITERATURE REVIEW

2.1 Characteristics of tiamulin antibiotic

Antibiotics were defined as “compounds produced by living organisms that impede the growth of other organisms” [53]. They are produced by nature or synthesized by human to inhibit or kill other microorganisms or viruses. Most antimicrobials are produced by the soil microorganism *Streptomyces* [54]. Antibiotics can be divided into many subgroups such as aminoglycosides, beta-lactams, quinolones, tetracyclines, macrolides, oxazolidinones, pleuromutilins, and sulfonamides [55].

Antibiotics can be further classified based on their target attacks. For example, antibiotics could inhibit cell wall synthesis, inhibit metabolism of folic acid (play essential role in multiplication of bacterial cells), alter the cytoplasmic membrane structure, and inhibit DNA gyrase, DNA-dependent RNA polymerase, and protein synthesis (Figure 2.1) [56].

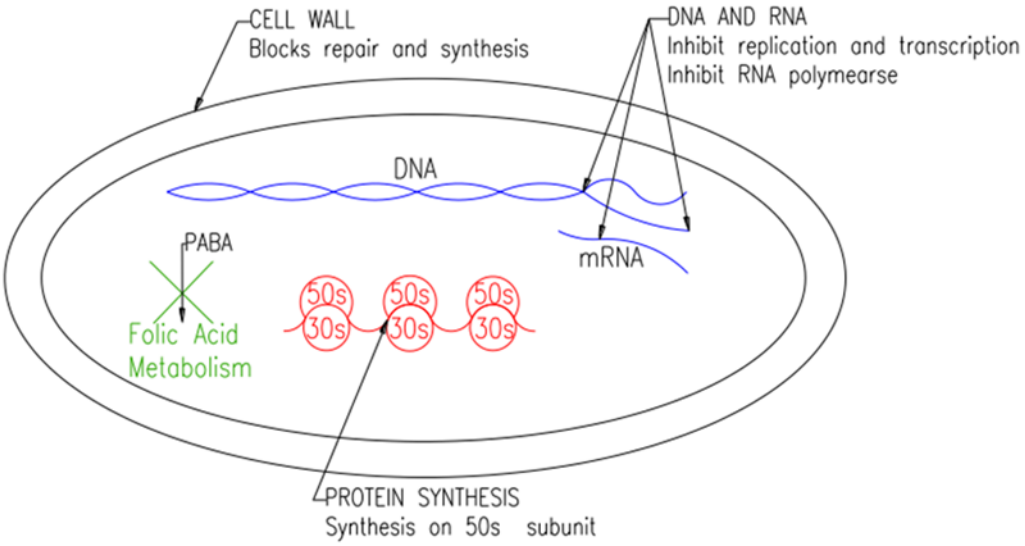


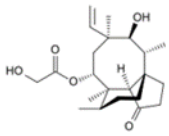
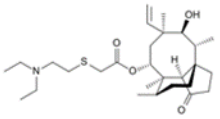
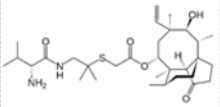
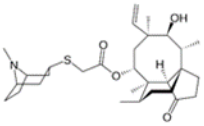
Figure 2.1 Major modes of action of antimicrobial agents

Veterinary drugs are essentially used in swine production to prevent and treat disease, and to improve efficiency of feed and promote growth [3]. Among antibiotics used in swine farms, tiamulin has been the medication commonly and widely used for swine dysentery eradication since the disease could cause major economic losses for pork producer. Eradication rather than mere control of the disease is preferable [17].

Tiamulin is a semi-synthesized antibiotic that originated from a fungal *Pleurotus mutilus*, now called *Clitopilus scyphoides*. Its mode of action involves in inhibition of bacterial protein synthesis by binding to the domain V of 23S rRNA in the peptidyl transferase slot at the 50S large prokaryotic ribosomal subunit and blocking substrate binding site for peptide formation and mispositioning of the CCA ends of tRNA for peptide transferase [18].

Tiamulin (TIA) is classified in a pleuromutilin antibiotic drug which contains other two derivatives comprising valnemulin and retapamulin. Tiamulin and valnemulin are used exclusively in veterinary medicine. Only retapamulin is authorized currently for topical use in humans from 2007 [57]. Tiamulin belongs to the class of diterpene antimicrobial and possesses a quite rigid tricyclic structure of pleuromutilin which is similar to that of valnemulin and retapamulin (Table 2.1). Tiamulin was approved for use in veterinary medicine in 1979. Tiamulin is extremely active against Gram-positive bacteria such as streptococci and staphylococci and against *Mycoplasma* and *Brachyspira*. Members of other 16 anaerobic species including *Bacteroides fragilis* and *Clostridium perfringens* with no exception are sensitive to tiamulin [19]. In pigs, tiamulin is used to treat swine dysentery, spirochaete-associated diarrhea, porcine proliferative enteropathy, enzootic pneumonia and other infections where *Mycoplasma* is involved with significantly effectiveness [57]. Additionally, tiamulin has reported that it is broad spectra but especially active against *Surpulina hyodysenteriae*, mycoplasma, gram positive bacteria, a few gram negative bacteria and certain anaerobic bacteria [23].

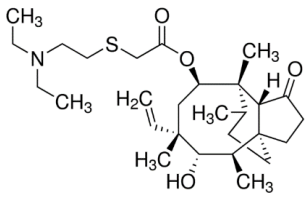
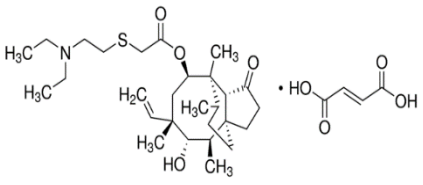
Table 2.1 Properties of tiamulin and its derivatives of pleuromutilin antibiotic group

Properties	Pleuromutilin	Tiamulin	Valnemulin	Retapamulin
Formula	$C_{22}H_{34}O_5$	$C_{28}H_{47}NO_4S$	$C_{31}H_{52}N_2O_5S$	$C_{30}H_{47}NO_4S$
Molecular weight	378.5 g/mol	493.7 g/mol	564.8 g/mol	517.8 g/mol
Structure				
No. CASRN	125-65-5	55297-95-5	101312-92-9	224452-66-8

(No. CASRN: Chemical Abstract Services Registry Number)

Source: [58]

Table 2.2 Major physico-chemical properties of tiamulin and tiamulin hydrogen fumarate

Properties	Tiamulin[a]	Tiamulin hydrogen fumarate[b]
Formula	$C_{28}H_{47}NO_4S$	$C_{32}H_{51}NO_8S$
Molecular weight	493.7 g/mol	609.8 g/mol
Structure		

Properties	Tiamulin[a]	Tiamulin hydrogen fumarate[b]
No. CASRN	55297-95-5	55297-96-6
Physical state	Soluble powder	
Color	Crystals from acetone	
Water solubility	58 g/L	
Melting point	147-148^{o}C	
$LogK_{ow}$	5.93	
pK_a	7.6	

(No. CASRN: Chemical Abstract Services Registry Number, $LogK_{ow}$: Octanol/water partition coefficient, pK_a: Acid dissociation constants)

[a] https://pubchem.ncbi.nlm.nih.gov/compound/Tiamulin#section=Related-Compounds

[b]https://pubchem.ncbi.nlm.nih.gov/compound/6434500#section=Drug-and-Medication-Information

In veterinary medicine, most commonly used drug forms of tiamulin are water-soluble powder, water-soluble granules, premix, and solution for injections [57]. There are two forms of tiamulin, including tiamulin and tiamulin hydrogen fumarate (tiamulin fumarate), in which their physical and chemical properties are not similar (Table 2.2).

Tiamulin hydrogen fumarate is more complex structure and heavier molecular weight than tiamulin because it contains fumarate in its structure.

Table 2.3 Acute toxicological data of tiamulin antibiotic on animals

LD_{50}	**Concentration (mg/kg)**	**Tested animal**
Acute oral	1,830 - 2,740	Rat
Acute oral	650 - 770	Mouse
Acute intravenous	20	Rat
Acute intravenous	50	Mouse
Acute subcutaneous	521	Mouse

According to Novartis Animal Health US, tiamulin was classified as a hazardous waste for purposes of good product stewardship practices and recommended to prevent release to the environment uncontrollably [20]. Tiamulin may be irritating to the skin, eyes, and respiratory system, so it may cause sensitization by skin contact and inhalation. However, under Resource Conservation and Recovery Act (RCRA), tiamulin was a solid (non-hazardous) waste if it is discarded in its manufactured form. It showed no sub-chronic and reproductive toxicities, no teratogenicity, mutagenicity, and carcinogenicity on toxicological data tested with rats, dogs, pigs, rabbits, and mice. Acute toxicilogical data of tiamulin showed very high concentration in median lethal dose (LD_{50}) values (Table 2.3). Moreover, it had potentially effects to ecological system when it showed low half maximal effective concentrations (EC_{50}) toward aquatic creatures (Table 2.4).

Table 2.4 Eco-toxicological data of tiamulin

Aquatic organism	**LC_{50} (mg/L)**	**EC_{50} (mg/L)**	**Tested time (h)**
Fish	5.2		96
Algae		> 0.62	96
Daphnia magna		40 - 67	48
Daphnia magna		5.4	Chronic test

Tiamulin showed large haft-lives ranging from 43 to 301 days with different soils. Under normal environmental conditions, tiamulin had a low potential to bioaccumulate, but there is a potential for tiamulin to bioaccumulate under high pH conditions. Tiamulin was readily absorbed and extensively metabolized in swine [20]. Moreover, it was reported that subtherapeutic antibiotics used on livestock and poultry caused antibiotic resistance for bacteria which were found in humans [59]. Antibiotic residues in animal wastes become human health and environmental concerns [59]. Hence, tiamulin residue from swine waste is considered as a source of antibiotic resistant pollutions.

2.2 Uses of tiamulin in veterinary medicine

The use of antibiotics for the treatment of infectious diseases has been started since the discovery of penicillin [60]. Almost 250 antibiotic compounds have been permitted to use for both human and veterinary treatment [61]. Certain amount of antibiotics have been allowed by US Food and Drug Administration [62] to add into the feedstock so as to improve meat quality, increase growth, and prevent infectious diseases in cultivating animals leading to the increase of farmer's benefit [63]. Particularly, the administration of antibiotics could improve the swine average growth

rate for 16.4% in piggy, and reduce the feed by 7%. However, the growth rate for mature male pig was only improved by 10.6% and the efficiency of feed utilization could be improved by 2%. Long-term use of antibiotics did not impact on the effective growth benefit for the swine producers because antibiotics are only effective in improving growth in young pigs [3]. Antibiotic uses bring certain benefits to the swine producers, but the intensive use may cause spreading of antibiotic residues in surrounding environments. Thus negatively influencing on human life. For example, the use of antibiotics to treat human's disease becomes less effective because the pathogens develop the resistance to the antibiotics existing in environments.

Therapeutic, metaphylactic, prophylactic, and subtherapeutic are four main types of antibiotics administration in swine production. Therapeutic is used for disease treatment, metaphylactic is for disease prevention in high risk environment, prophylactic is for disease prevention for the weak animals, and subtherapeutic is for facilitating growth or improving feeding efficiency within the herd [64]. Subtherapeutic antibiotics were the most common used. It has been estimated that nearly 4.7 million kilograms of antibiotics were used in 90 million swine per year in the United State [65].

Lists of some common antibiotics and chemotherapeutics approved for uses in swine feed by the US Food and Drug Administration [62] are presented in Table 2.5. The United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), and National Animal Health Monitoring System (NAHMS) conducted an extensive survey of swine producers in 2006. It was estimated that 55% of finishing operations administered an antimicrobial or feed additive for growth promotion with the most common products being chlortetracycline (52%), tylosin (44%), bacitracin (29%), ractopamine (28%), and tiamulin (10%) [66].

Tiamulin is authorized nationally in the member states of the European Union (EU) and is available in most EU member states. Following a recent referral, tiamulin is indicated in pigs for the treatment and prevention of swine dysentery (*Brachyspira*

hyodysenteriae), treatment of colitis (*Brachyspira pilosicoli*), treatment of ileitis (*Lawsonia intracellularis*) and treatment of enzootic pneumonia (*Mycoplasma hyopneumoniae*) [67]. Other indications might still be listed, as different products containing tiamulin are nationally approved. Tiamulin is also authorized for chickens for the treatment and prevention of chronic respiratory disease and air-sacculitis caused by *Mycoplasma gallisepticum* and *Mycoplasma synoviae*; for turkeys for the treatment and prevention of infectious sinusitis and air-sacculitis caused by *Mycoplasma gallisepticum, Mycoplasma meleagridis* and *Mycoplasma synoviae*; and for rabbits for the treatment of epizootic rabbit enterocolitis. Tiamulin is available as an oral solution, a powder for medication in drinking water, medicated feed premixes and as an injectable formulation for pigs [68].

For pigs, the dose recommendation following the EU article 34 referral for the Tiamulin premix varies between 2 and 10 mg/kg depending on indication and the duration of treatment, but can be related to clinical responses and varies between 7 and 28 days [67]. According to the outcome of the referral, preventive treatment with tiamulin should only be initiated after confirmed infection with *Brachyspira hyodysenteriae* and then as part of a program including measures aiming to eradicate or control the infection within the herd [67]. It is not known whether such recommendations are included in the Summary of Product Characteristics for all products containing tiamulin. There are limited data on the extent to which this advice on preventive use is followed; e.g. it is very important that such use is not undertaken without appropriate accompanying measures in order to minimize the emergence of resistance [57].

Table 2.5 Lists of some common antibiotics and chemotherapeutics approved for uses in swine feed by the US Food and Drug Administration

Antimicrobial	Class	Subtherapeutic rate	Trade name
Antibiotics			
Apramycin	Aminoglycoside	150 g/ton	Apramycin
Bacitracin Methylene Disalicylate	Bacitracin	45 - 90 g/ton	Coban, BMD
Baciracin Zinc	Bacitracin	10 - 50 g/ton	Albac
Bambermycin	Bambermycin	2 - 4 g/ton	Flavomycin
Chlortetracyline	Tetracycline	10 - 50 g/ton	Aureomycin
Lincomycin	Lincosamide	20 g/ton	Lincomix
Neomycin	Aminoglycoside	NA	Neomycin
Oxytetracycline	Tetracycline	10 - 50 g/ton	Terramycin
Penicillin	β-lactam	10 - 50 g/ton	Penicillin
Tiamulin	Diterpene	10 g/ton	Tiamulin
Tylosin	Macrolide	20 - 100 g/ton (starter)	Tylan
		20 - 40 g/ton (grower)	Tylan
		10 - 20 g/ton (finisher)	Tylan
Virginiamycin	Streptogrammin	5 - 10 g/ton	Stafac

Antimicrobial	Class	Subtherapeutic rate	Trade name
Chemotherapeutics			
Arsanilic acid	Arsenical	10 - 30 g/ton	Mecadox
Carbadox	Quinoxaline	10 - 25 g/ton	3-Nitro
Roxarsone	Arsenical	22.7 - 34.1 g/ton	Sulfamethazine
Sulfamethazine	Sulfonamide	100 g/ton	Sulfathiazole
Sulfathiazole	Sulfonamide	100 g/ton in combination with chlortetracycline	

Source: [56]

Tiamulin is one of the most often used compounds in swine productions for swine dysentery eradication. According to personal communication with veterinarians and farmers, tiamulin was applied to all stages of swine husbandry in Thai swine farms. In the U.S and U.K, there were 1,435 kg tiamulin had been used in 2000 [14]. In the investigation of 55 pig farms in Dong Nai and Binh Duong provinces of Viet Nam, the use of tiamulin accounted for 5.7% of total antibiotic uses, in which it was the sixth common antibiotic used in swine production compared to the five other antibiotics (tylosin - 16.4%, amoxicillin - 11.9%, gentamicin - 8.6%, enrofloxacin - 6.6%, and penicillin - 6.2%) [4].

2.3 Occurrence of antibiotics and tiamulin in environment

Antibiotic residues in swine production were found not only in pig farms but also in surrounding environments that are closed, even far from the swine farms. Several previous studies reported that the residual antibiotics were detected in swine manure

in many countries. In fact, tetracycline concentrations were found ranged from 0.1 to 4.0 mg/kg, 0.1 to 24.4 mg/kg, 0.1 to 46.0 mg/kg in swine manure in Germany, Denmark, and Austria, respectively [69]. In China, the range of 0.2 to 134.8 mg/kg of oxytetracycline and 0.3 to 121.8 mg/kg of chlortetracycline were frequently detected. The maximum concentrations of chlortetracycline and oxytetracycline ever detected in pig manure were 764.4 mg/kg and 136.0 mg/kg, respectively [69]. The other antibiotics such as sulfadimidine, sulfadiazine, sulfamethazine and sulfathiazole occurred in swine manure in European nations at the amounts of 20.0, 2.0, 8.7 and 12.4 mg/kg, respectively [69], whereas the maximum concentration of sulfamonomethoxine, sulfadimidine and sulfadimethoxine were measured in swine manure in China at 4.1, 24.8, and 26.4 mg/kg, respectively.

In the Mekong Delta of Vietnam, the sulfamethazine was detected at extremely high concentration (18.5 - 19.2 µg/L) in pig farm wastewater. It was previously reported that sulfamethazine accounted for 98% of all antibiotics detected in pig farm waste with concentration range of 18,512 - 19,512 µg/kg [70]. In Malaysia, eight sulfonamides were detected in swine wastewaters at concentrations ranging from 5.1 to 95 ng/L [21]. In Switzerland, total sulfonamide concentrations in the mixed cattle and swine manure slurry was reported to be 20 mg/kg, while in China it was found at the concentration of 49.5 µg/L in animal wastewater. The swine wastewater samples were unavoidably contaminated with antibiotics tetracycline, oxytetracycline, chlortetracycline and doxycycline, with maximum concentrations of individual contaminants reaching 13.65 µg/L [71-74]. The tylosin detected in swine wastewater showed concentrations of 56, 72 and 8.6 µg/L, in breeding-gestation, nursery pigs, and grow-finishing area in Spain, respectively [75]. Multiple classes of antimicrobial compounds (commonly at concentrations of > 100 µg/L) were detected in swine waste storage lagoons [76]. Tiamulin and tetracycline were also found at the concentration of 43 µg/kg in swine manure and 4.1 µg/L in animal wastes, respectively [24, 77].

The antibiotics also occurred in surrounding environments such as streams, aquatic environment, soil and groundwater. Concentration of overall twenty-one antibiotics were approximately 1 mg/L in 139 streams in the United States [76]. Sulfamethazine

was a major antibiotic contaminant in pig farm pond (475 - 6,662 ng/L) whereas erythromycin (154 - 2246 ng/L) and clarithromycin (2.8 - 778 ng/L) were found at lower concentrations. In addition, sulfamethazine was detected in soil and groundwater with concentrations of 2,000 µg/kg and 240 ng/L, whereas chlortetracycline was detected in soil and surface water at the concentrations of 39,000 µg/kg and 690 ng/L, respectively [15, 78]. The residues of antibiotics were detected in surface and groundwater samples collected proximal to the swine and poultry farms [76].

Table 2.6 Occurrences of antibiotics in the environment

Samples	Antibiotics	Concentration	Country
Swine manure	Tetracycline	0.1 - 4 mg/kg	Germany
		0.1 - 24.4 mg/kg	Denmark
		0.1 - 46 mg/kg	Austria
	Oxytetracycline	0.2 - 136 mg/kg	China
	Chlortetracycline	0.3 - 764.4 mg/kg	
	Sulfamonomethoxine	4.1 mg/kg	
	Sulfadimidine	24.8 mg/kg	
	Sulfadimethoxine	26.4 mg/kg	
	Sulfadimidine	20 mg/kg	European
	Sulfadiazine	2 mg/kg	
	Sulfamethazine	8.7 mg/kg	
	Sulfathiazole	12.4 mg/kg	

Samples	Antibiotics	Concentration	Country
Swine manure slurry		20 mg/kg	Switzerland
Swine liquid manure	Tiamulin	43 µg/kg	Germany
	Tetracycline	4.1 µg/L	
Swine wastewater	Sulfamethazine	18.5 - 19.2 µg/L	Vietnam
	8 antibiotics	5.1 - 95 ng/L	Malaysia
	Tylosin	8.6 - 72 µg/L	Spain
	Sulfonamide	49.5 µg/L	China
	Tetracycline		
	Oxytetracycline	13.65 µg/L	
	Chlortetracycline		
	Doxycycline		
Soil	Sulfamethazine	2,000 µg/kg	European
	Chlortetracycline	39,000 µg/kg	

Samples	Antibiotics	Concentration	Country
Streams	21 antibiotics	1 mg/L total	United State
Groundwater	Sulfamethazine	240 ng/L	European
Surface water	Chlortetracycline	690 ng/L	European
Pond	Sulfamethazine	475 - 6,662 ng/L	European
	Erythromycin	154 - 2,246 ng/L	
	Clarithromycin	2.8 - 778 ng/L	

2.4 Fates and transport of tiamulin

Tiamulin has high water solubility of 58 g/L and pK_a of 7.6 [21, 22], so the residual of tiamulin was discovered in various environmental media such as swine manure, swine wastewater, soil, river, and groundwater. Previous studies reported that tiamulin was found in liquid swine manure samples in European countries at the maximum concentration of 43 µg/kg [24], in swine wastewater at 5 µg/L [21]. In Shandong province in China, Tiamulin were detected with the lowest detection frequencies (< 4.8%), and their median concentrations were 0.1 mg/kg in swine manure samples of 21 concentrated animal feeding operations (CAFOs) [69]. It was also detected tiamulin in two investigated soil samples at trace concentrations of 0.7 µg/kg [24], in the wastewater lagoon at a concentration of 182 ng/L [25], in all water samples from a source water collected from downstream of Dongjiang River at the average concentrations in the range of 0.77 - 18.77 ng/L [26], and in groundwater at a concentration of 29 ng/L [25]. However, tiamulin has also reported that it is high log K_{ow} of 5.9 and tends to excrete with faeces [23]. Tiamulin may leach to surrounding

environments when tiamulin-contaminated manure is applied as fertilizer on fields. Some studied were performed to eliminate tiamulin from livestock liquid manure in 180-day degradation, but the removal efficiency was not high since tiamulin is very persistent in manure. Tiamulin also persisted in the soil of the field as the concentrations of tiamulin remained ranging from 0.9 µg/kg up to 1.9 µg/kg after one year fertilizing with tiamulin contaminated manure at initial concentration of 43 µg/kg. The upper surface layer (0 - 15 cm) of the field soil revealed more contaminated with tiamulin since tiamulin concentration was raising when tiamulin-containing manure was used as fertilizer several times in the soil [24, 27].

2.5 Removal of antibiotics and tiamulin

Antibiotic residues contained in manure and wastewaters of swine farms may be biotically or abiotically transformed, sorbed in soil column, reach the surface water by run-off, or leach into ground water as the swine wastes are applied to agricultural fields as fertilizers [79, 80]. However, most of the residual antibiotics are only partly degraded in the environment or in wastewater treatment processes. This makes to expose antibiotic residues to the environment and may rise the production or maintenance of bacterial resistances [55, 81].

In general, antibiotics were degraded in abiotic condition was less than biodegradation. For examples, sorption is the major factor influencing the loss of tylosin in soils of about 6% [82], but the lagoons was able to remove tylosin in swine manure up to 75% efficiently [35]. Abiotic hydrolysis and photolysis were primarily in cephalosporins degradation in lake surface water, whereas biodegradation was responsible for the elimination of the cephalosporins in the sediment [83]. Hydrolysis and photolysis phenomena were removed oxytetracycline about 20% and 70%, respectively in water [84], while it could also be nearly completely degraded by manure composting process [36]. Therefore, it is necessary to enhance antibiotic degradation in biological processes before releasing it to the surroundings in order to restrict spreads of antibiotic and antibiotic resistant pollutants.

Different biological treatment methods, including anaerobic digestion, composting and constructed wetlands were conducted to remediate several kinds of antibiotics in swine wastes. For instance, batch anaerobic digestion of swine manure could reduce approximately 80 - 98% of chlortetracycline and 73% of oxytetracycline for the period of 21 days [85]. Chlortetracycline and oxytetracycline could also be nearly completely degraded by composting process [36-38]. In constructed wetland system, high removal efficiencies (69.0 - 99.9%) were achieved for oxytetracycline, tetracycline and chlortetracycline [39]. Composting could be used efficiently to remove various kinds of antibiotics such as monensin, tylosin, levofloxacine, ciprofloxacine, erythromycin, sulfamonomethoxine, sulfamethoxazole, trimethoprim, carbamazepine with the efficiency ranged from 37% to 100% [40]. Removal rate of antibiotics by composting was more efficient than by anaerobic digestion due to more aerobic bioactivity [11]. Biodegradation of antibiotics was generally depended on initial concentration because microbial activities were inhibited by antibiotic concentrations. Furthermore, it was also depended on composition of the microbial activity, biotic and abiotic degradation of antibiotics, and sorption of antibiotics [41].

However, manure storage process and anaerobic conditions were ineffectively in biodegrading tiamulin. In investigation of tiamulin biodegradation using the storage of pig manure in liquid form [27], the results indicated that this antibiotic exhibited persistent capacity in the liquid manure with the long half-life of more than 200 days. This was in accordance with the degradation result that the concentration of tiamulin was unchanged during the experiment. In a separate study, it was found that tiamulin was very slowly degraded in anaerobic conditions [42]. Moreover, there are no report related to abiotic degradation of tiamulin. Hence, improving tiamulin remediation by biological processes using microbes from swine farms is an effective and appropriate alternative method in this study contributes to minimize the impact of swine wastes containing tiamulin antibiotic on the environments.

2.6 Uses of wood-rot fungi in environmental treatment

2.6.1 Classification, isolation and identification of wood-rot fungi

Wood-rot or wood-decay fungi are called lignicolous fungi because they are capable to digest moist wood and cause it to decay in various ways such as attacking the carbohydrates in wood and decaying lignin. Basing on the decaying types, wood-decay fungi were classified as brown-rot, soft-rot, and white-rot fungi. Each produces different enzymes to degrade different plant materials and colonize on different environmental niches. Brown-rot fungi break down hemicellulose and cellulose. Soft-rot fungi secrete cellulase from their hyphae to break down cellulose in the wood. White rots break down lignin and cellulose and commonly cause rotted wood to feel moist, soft, spongy, or stringy and appear white or yellow [86].

Wood-rot fungi are normally isolated on potato dextrose agar plates, in which fungal fruiting bodies were collected from various sources of environments such as manure, soil, tree trunks, and decayed wood samples. Fungi were molecularly identified by a complete sequence analysis of its 18S rRNA gene and the internal transcribed spacer region using primers of ITS 1 and ITS 4. The ITS region of fungal rDNA has been successfully used for species identification because it is present at a very high copy number in the genome of fungi, as part of tandem repeated nuclear rDNA which coupled with PCR amplification produces a highly sensitive assay [87, 88]. This study followed numerous previous studies that have used PCR-based methods to identify fungi using ITS 1 and ITS 4 primers.

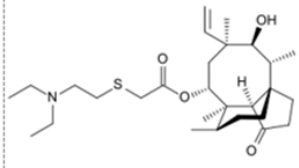
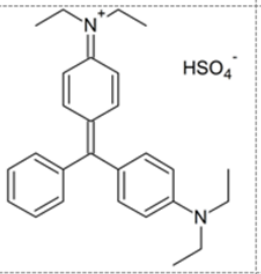
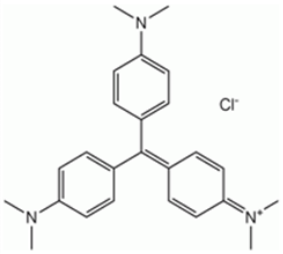
2.6.2 Degradation of recalcitrant compounds by wood-rot fungi

Fungi can produce an array of enzymes that are capable of degrading a wide range of recalcitrant compounds [89, 90] that contaminate the environments. The colorless halo around the fungal colony reflects the production of ligninolytic enzymes [91]. Similar approach was conducted for decolorization of Remazol Brilliant Blue R (RBBR) and degradation of 1,1,1-trichloro-2,2-bis(4-chlorophenyl)-ethane (DDT) by *Trametes versicolor* [92]. In addition, white rot fungus *Phanerochaete chrysosporium* was able to degrade various synthetic dyes as well as biotransformed three pharmaceutical active compounds (diclofenac, ibuprofen and naproxen) [46, 93, 94],

sulfamethoxazole, tetracycline and oxytetracycline antibiotics [48, 49, 95] owing to its lignin-degrading system. Therefore, the decolorization of dye might be used as a simple method to preliminarily select fungi with the ability to degrade other recalcitrant compounds.

Before examining tiamulin degradation, wood rot fungi was pre-screened with brilliant green and crystal violet dyes. Some major physical and chemical properties of tiamulin and these two synthetic dyes are presented in Table 2.7. Although brilliant green and crystal violet are different formulas and structures in comparison with those of tiamulin, these dyes have their molecular weight of 482.6 g/mol and 408.0 g/mol, respectively that are approximately the same with tiamulin (MW 493.7 g/mol). Moreover, they are resistant to sterilization at 121^{o}C in 20 minutes. Thus two dyes were appropriately selected for screening ligninolytic enzymatic activities in wood rot fungi for tiamulin degradation.

Table 2.7 Major physico-chemical properties of brilliant green and crystal violet dyes

Properties	Tiamulin[a]	Brilliant green[b]	Crystal violet[c]
Formula	$C_{28}H_{47}NO_4S$	$C_{27}H_{34}N_2O_4S$	$C_{25}H_{30}N_3Cl$
Molecular weight	493.7 g/mol	482.6 g/mol	408.0 g/mol
Structure			
No. CASRN	55297-95-5	663-03-4	548-62-9
Physical state	Soluble powder	Green crystalline powder	Bright blue-violet crystals
Water solubility	58 g/L	40 mg/mL	1 - 4 mg/mL
Melting point	147-148°C	210°C	215°C

(No. CASRN: Chemical Abstract Services Registry Number)

Source: [a] https://pubchem.ncbi.nlm.nih.gov/compound/Tiamulin#section=Related-Compounds;

[b, c] https://pubchem.ncbi.nlm.nih.gov/

The use of wood rot fungi is a promising approach for the bioremediation of antibiotic-contaminated sites because they produce extracellular enzymes and non-specific lignin-mineralizing enzymes, including laccase, lignin peroxidase, manganese peroxidases, cellulase, and hemicellulase, that can remove a diverse range of xenobiotic chemicals [43]. Many white rot fungi can thus degrade the complex molecular structures of synthetic dyes and antibiotics. For instance, the white rot fungus *Phanerochaete chrysosporium* can degrade crystal violet, brilliant green and

other dyes owing to its lignin-degradation system [46]. It also had the capability to degrade the antiviral drug oseltamivir (Tamiflu) and the antibiotics erythromycin, sulfamethoxazole, and ciprofloxacin at 10 µg/mL [47]. *Phanerochaete chrysosporium* is able to degrade antibiotics due to its laccase and manganese peroxidase content [48, 49]. Additionally, *Galactomyces geotrichum* decolorized triphenylmethane, azo, methyl red, and reactive high exhaust textile dyes [50] and effectively treated the antibiotic lincomycin in the solid waste of fermentation drugs [51]. *Trametes hirsuta* was shown capable of degrading various synthetic dyes including triarylmethane, indigoid, azo, and anthraquinoic by laccase [44], while *Trametes versicolor*, of the same genus, removed more than 90% of antibiotics such as ciprofloxacin and norfloxacin (2 mg/L) in 7 days using laccase [45]. The immobilized fungal cell of *Coriolus versicolor* RC3 was also able to decolorize Orange II at the concentration of 20 mg/L [96]. *Phanerochaete chrysosporium* ATCC 24725 mycelium were able to degrade phenolic compounds in palm oil mill discharge with the maximum phenol removal efficiency of 61.22 % after 6-day experimental period [97].

Moreover, non-specific enzyme activities of some fungi can also degrade antibiotics such as *Phanerochaete chrysosporium* removed up to 74% of 10 mg/L sulfamethoxazole within 10 days [48]. Laccase activity was expected to play main role in degradation. Another study extracted manganese peroxidase from *Phanerochaete chrysosporium*. The pure enzyme degraded 73% and 84% of tetracycline and oxytetracycline (at 50 mg/L), respectively [49]. *Trametes versicolor* also removed more than 90% of ciprofloxacin and norfloxacin at the concentration of 2 mg/L in the period of 7 days using extracellular enzyme laccase and the P450 system [45]. *Trametes versicolor* removed 83% of pharmaceutical active compounds (PhACs) and 53% of some endocrine disruptor compounds (EDCs) from hospital effluents [98].

There are many examples of fungi which have abilities to degrade pharmaceutical compounds, dyes, phenolic compounds, and antibiotics using non-specific enzymes. The white rot fungal strain *Verticillium* sp. could eliminate organophosphate insecticide, chlorpyrifos [99] and it also degraded 0.5 - 2.0 mg/L of ampicillin in 7

days [100]. *Trametes versicolor* also shows a high capacity for degrading diverse recalcitrant compounds as well as various antibiotics (ciprofloxacin, norfloxacin, sulfamethoxazole and tetracycline groups), pharmaceuticals, insecticides, pesticides, and hormones [45, 98, 101, 102]. *Trametes hirsuta*, additionally, can dephenolize and decolorize phenolic compounds and many synthetic dyes such as triarylmethane, indigoid, azo, and anthraquinoic [44, 103], and reduced 90% of endosulfan and endosulfan sulfate insecticides [104]. *Lasiodiplodia* sp. was highly effective in the degradation of endosulfan, a persistent organochlorine pesticide [105], phenolic compounds in olive mill wastewater [106], plastics [107], and PAHs [108]. *Fusarium* sp. has been able to dissipate PAH pollutants such as benzo[a]pyrene, phenanthrene, pristine, and pyrene [108-110]. *Galactomyces* sp. was shown capable of decolorizing wastewater effluent from the dye industry and removing lincomycin antibiotic residuals in the solid wastes of fermentation drugs [50, 51, 111].

2.6.3 Enzymatic activities of wood rot fungi in biodegradation

Fungi possess ligninolytic enzymes directly involved not only in the degradation of lignin in their natural lignocellulose substrates [112, 113] but also in the elimination of diverse recalcitrant pollutants [89, 114, 115]. Manganese peroxidase (MnP) from white rot fungus *Phanerochaete chrysosporium* was the main enzyme that could completely deplete orange G color, 60% of tartrazine dyes [93], and degrade 72.5% and 84.3% of tetracycline and oxytetracycline antibiotics, respectively [49]. Furthermore, *Phanerochaete chrysosporium* and *Trametes versicolor*, with low MnP levels (5 - 48 U/L), to be effective in the elimination of anti-inflammatory drugs [94, 116]. The *Trametes hirsuta* strain AK4 illustrated the greatest MnP activity at 3,046 U/L when eliminating phenolic compounds and color at a removal efficiency of 66.5% and 64.7%, respectively, after 8 days [103].

Besides MnP, laccase (Lac) and other enzymes are also related to recalcitrant compounds degradation. Lac from *Trametes versicolor* was mainly responsible for the removal of 30 diverse trace organic contaminants, consisting of eleven pharmaceuticals, six pesticides, five steroid hormones, three industrial precursors and

products, three UV filters, two phytoestrogens [102], and sulfamethoxazole [117]. Similarly, many synthetic dyes such as triarylmethane, indigoid, azo, and anthraquinoic as well as tetracycline antibiotics could be mainly degraded by the Lac enzyme of *Trametes hirsuta* [44, 118]. *Lasiodiplodia* sp. also exhibited a major contribution of the Lac enzymes on plastic and phenolic-compound degradations [106, 107, 119]. *Lasiodiplodia* sp. and *Fusarium* sp. can utilize PAHs owing to their lipase productions [108]. Some studies have reported that the induction of Lac and LiP activities from *Galactomyces* sp. contributes to the decolorization of textile dyes [50, 120]. Besides, *Lentinula edodes* fungi showed 65% decolorization and 75% elimination of total phenol in olive mill wastewater with the main lignolytic activity of Lac production [121]. Lac also removed approximately 66% and 77% of sulfathiazole and sulfamethoxazole antibiotics, respectively [122].

2.7 Uses of bacterial enriched cultures in antibiotic treatment

2.7.1 Enrichment of bacterial cultures under target antibiotic conditions

Bacteria with the ability to grow at the expense of target antibiotic as sole nitrogen or carbon source were enriched and isolated from various sources in mineral salts medium (MSM) under aerobic conditions. Sulfamethazine-degrading bacteria were enriched in MSM from soil with long-term treatment of sulfamethazine [123]. Sulfamethoxazole antibiotic was used as sole source of carbon and nitrogen in enriching bacterial community with activated sludge from the high-load stage of a two-stage municipal WWTP [124-126]. Three tetracycline antibiotics, comprising of tetracycline, chlortetracycline, and oxytetracycline was also added into the basal medium to enrich tetracyclines-degrading bacterial cultures with swine wastewater samples collected from the effluent of five wastewater treatment systems at five pig farms in Taiwan [52]. Following such studies, tiamulin-degrading bacterial communities were enriched by adding this target antibiotic to MSM containing swine wastewaters as microbial sources and subcultured 14-day interval during seven months.

2.7.2 Application of bacterial enriched cultures in biodegradation of antibiotics and tiamulin

Antibiotics can be partially or fully degraded by microorganism in certain environmental conditions. Tetracycline was partially degraded (46%) under methanogenic condition because some microorganisms under this condition were not significantly inhibited by tetracycline. However, tetracycline was not biodegraded under nitrate- and sulfate-reducing conditions due to the tetracycline toxicity effect on nitrate- and sulfate-microorganisms [127]. Tetracycline, oxytetracycline, and chlortetracycline have been frequently detected in swine wastewater. The biodegradation tests in batch experiments were performed using isolated bacteria from the swine farms [52]. The results showed that oxytetracycline and chlortetracycline were more biodegradable than tetracycline. Oxytetracyline and chlortetracycline were totally removed by microbial action after 18 and 20 days in wastewater, respectively. However, 7.1% of tetracycline still remained after 20-day incubation. The study also found that initial tetracyclines concentration showed effects on biodegradation rates in the manner that higher tetracyclines, slower biodegradation rates.

Similarly, degradation of fluoroquinolone antibiotics ofloxacin, norfloxacin, and ciprofloxacin also depended on its initial concentrations (0.8 to 30 μM) [128]. These antibiotics were found to be aerobically degraded by using fluoroorganic-degrading strain *Labrys portucalensis* F11. After 28-day period of experiment, *Labrys portucalensis* F11 was able to degrade 55, 22 and 16 % of norfloxacin, ofloxacin, and ciprofloxacin, respectively. It should be noted that sodium acetate as second carbon source was added to final concentration of 5.9 mM to simulate an influent of a wastewater treatment system. As can be seen from the previous studies, several kinds of bacteria could degrade various types of antibiotics. The degradation of antibiotics by bacteria depends on pH, temperature, concentration of antibiotics, and chemical structure of testing antibiotics.

Tiamulin was extensively metabolized in pigs (40%). The metabolites of tiamulin had been found from the incubation of tiamulin in liver microsomes from pigs. The

structures of the metabolites were found to be 2β-hydroxy-tiamulin, 8α-hydroxytiamulin and N-deethyl-tiamulin. In addition, the LC-MS chromatograms revealed that there are also two other minor metabolites of tiamulin, including 2β-hydroxy-N-deethyl-tiamulin and 8α-hydroxy-N-deethyl-tiamulin [129].

Although microbial enriched cultures have been used to effectively eliminate antibiotics in laboratory scale from enormous previous studies, application of bacterial enriched cultures to directly treat swine wastewater in pig farms may spread antibiotics, their metabolites and their resistant bacteria to surrounding environments. Conventionally using bacterial mixed cultures with liquid formulations might encompass several problems of the low viability of microorganisms during storage and field application. Nevertheless, bioencapsulation technique may help to overcome this drawback because immobilization of microorganisms is capable to extend bacteria's shelf-life and control microbial release from formulations. This makes to enhance field application efficacy [130]. Tetracycline, sulfathiazole and ampicillin antibiotics, for instance, were effectively biodegraded and biotransformed (approximately 70%) through immobilized bacterial cells containing the detoxifying enzyme glutathione S-transferases in bioreactor to treat animal feed contaminated wastewaters [131]. Three tetracycline antibiotics, including tetracycline, chlortetracycline and oxytetracycline were significantly eliminated in bioreactors using tetracycline-degrading enriched bacteria supplementing enzyme-extract microencapsules at great efficiencies of 98.2, 99.2, and 99.8%, respectively [52].

In this study, application of tiamulin-degrading bacterial enriched cultures in treating tiamulin-contaminated swine waste sites may be practicable as mixed microbes are encapsulated or immobilized in microencapsulation and supplemented in the sites. In order to enhance effectiveness of tiamulin degradation as well as restrict tiamulin release, immobilized bacterial cells and crude enzymes of wood-rot fungi shall be combined.

2.8 The next-generation sequencing technology for determination of bacterial community structures

Next generation sequencing (NGS), massively parallel or deep sequencing are related terms that describe a DNA sequencing technology which has revolutionised genomic research. In the genome research, NGS has mostly superseded conventional Sanger sequencing. For example, using NGS an entire human genome can be sequenced within a single day, while the previous Sanger sequencing technology, used to decipher the human genome, required over a decade to deliver the final draft [132]. The advent of next-generation sequencing has extraordinarily enhanced capabilities to deeply sequence mixed amplicon pools, wherein declining costs continue to render it increasingly feasible to incorporate NGS into ongoing research programs [133] as well as enhanced the scope of research in the life sciences [134].

The NGS technology has been recognized as a powerful tool to study microbial communities [135], and the growing power and reducing cost sparked an enormous range of applications [136], of which has been widely used to study the ecology in gut, human, marine, hospital, and food [137-139] and the application of NGS technology has been expanded in earth or environments as well. For example, NGS revealed that river sediment microbial communities are highly complex and sensitive to changes in land use practices in the Tongue River, Montana, USA [67]. NGS was used to indicate the similarities and differences of microbial communities between various eight sand beaches in the US [140]. Bacterial community structures, including tetracycline resistant bacteria and genes in activated sludge of Jiangxinzhou sewage treatment plants in Nanjing, China were greatly affected by tetracycline treatment thanks to 454 pyrosequencing and Illumina high-throughput sequencing 16S rRNA gene [141]. Several thermophilic anaerobes were detected in the Sungai Klah hot spring in Malaysia by amplicon metagenome sequencing at the V3-V4 region of 16S rRNA gene [142]. The four different pollution sources (WWTP, urban, suburban, and agriculture) exhibited dissimilar impacts on the sediment bacterial community composition in the tributary of Taihu Lake via 16S rRNA gene clone library technique [143]. NGS was potentially used for assessment of microbial water quality [144].

Metagenome studies are becoming increasingly widespread, yielding important insights into microbial communities covering diverse environments from terrestrial and aquatic ecosystems to human skin and gut [145]. Metagenomic studies are commonly performed by analyzing the prokaryotic 16S ribosomal RNA gene (16S rRNA), which is approximately 1,500 bp long and contains nine variable regions interspersed between conserved regions. Variable regions of 16S rRNA gene are frequently used in phylogenetic classifications such as genus or species in diverse microbial populations.

2.9 Summary

Antibiotics are used in the swine industry to prevent and treat disease, and to improve efficiency of feed and promote growth, as a result this introduces high levels of tiamulin contamination in the environment. This in turn has likely led to an increase in tiamulin resistance in the natural environment. The study of biodegradation of tiamulin antibiotic by wood rot fungi and bacterial enriched cultures play an important role in agriculture and in human health because it will enhance antibiotic tiamulin removal in both solid and liquid swine wastes in order to restrict the release of tiamulin residues and their resistant bacteria and genes to surroundings. This literature review has provided an overview of these broad topics and how they relate to the environmental concern at hand.

Chapter 3 MATERIALS AND METHODS

3.1 Study sites

3.1.1 Sampling locations

Fungal and bacterial samples were collected from two swine farms with covered anaerobic lagoon and stabilization ponds treatment systems in Ratchaburi and Nakhon Pathom provinces located at 13.53^{o} N and 99.81^{o} E and 13.82^{o} N and 100.06^{o} E, respectively on the Western of Bangkok (Figure 3.1). Two such farms raised large numbers of pig with all ages from breeding/gestating to finishing stages as well as used huge amount of tiamulin for infectious disease prevention and treatment. Therefore, two such swine farms with two commonly and representatively swine waste treatment processes in Thailand were chosen to collect samples for enriching bacteria and isolating wood-rot fungi using to degrade tiamulin in this study.

Water and sediment samples were collected from two pig farms in January of the year 2015. In the first farm, the waste matters on pig house floors, walls, and pig bodies were washed and flowed into swine waste collection pond that was connected with covered anaerobic lagoon for methane generation. The covered material is opened for collecting accumulated sediment a few times a year and then transferred to fertilizer companies. Overflow wastewater was further treated in stabilization pond with wetland plants surrounding. In the second farm, the fresh swine manure was gathered at swine house, sun dried, and sold to fertilizer companies. Floor cleaning swine wastewater was passed through a stabilization pond system with one opened anaerobic pond, two facultative ponds and two aerobic ponds.

Sediment (~ 200 g) and wastewater samples (~ 600 mL) were collected at five sampling points from each swine farm of covered anaerobic lagoon or stabilization ponds swine waste treatment systems (Figure 3.2 - 3.3) with separated plastic vessels. All samples were stored in ice boxes during transportation to the laboratory and

preserved in – 4^{o}C until conducting bacterial enrichment experiment. In addition, swine manure, decayed wood, tree roots, trunks and leaves that were obtained from different locations were also collected for fungal isolation (Figure 3.4). These samples were kept at room temperature and cultured on potato dextrose agar plates no more than one week storage.

Figure 3.1 Location of sample collecting farms in Thailand

Source: http://www.maps-thailand.com/

3.1.2 Experimental locations

This research was carried out in HSM research laboratory of Center of Excellence on Hazardous Substance Management and Microbiology laboratory in Department of Microbiology, Faculty of Science, Chulalongkorn University, Phayathai Road, Pathumwan, Bangkok 10330 Thailand.

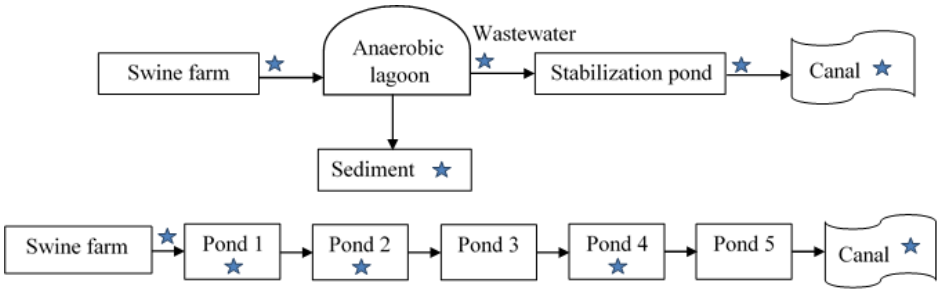


Figure 3.2 Sampling location in covered anaerobic lagoon and stabilization ponds swine wastewater treatment systems

Figure 3.3 Sample collection from swine farms

[(a), (b), and (c) - swine wastewater and sediment samples for bacterial enrichment; (d) - swine manure for fungal isolation].

Figure 3.4 Some sources of fungal sample collection

[(a) - Swine manure, (b) - Dried grass, (c) - Dried coconut fruits and leaves, (d) - Fresh tree leaves, (e) - Decayed wood].

3.2 Research materials

3.2.1 Laboratory equipment

Current research was used various types of equipment from different companies and countries that are presented in Table 3.1.

Table 3.1 Major laboratory equipment used in this study

Equipment	**Company**	**Country**
Autoclave	Hirayama	Japan
C18 column (Hypersil ODS 5µm, 4.0 × 250 mm)	Agilent Technologies	USA
Denaturing Gradient Gel Electrophoresis (DGGE)	Bio-Rad	USA
FastPrep® FP120	Bio 101 Thermo Savant	USA
Gel documentation WealTec Dolphin - DOC	Bio-Rad	USA
High Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD) Agilent 1100 series	Agilent Technologies	USA
Illumina Miseq	Illumina	USA
Labofuge 200	Heraeus	Germany
Biosafety cabinet	Telstar	Spain

Equipment	Company	Country
Micropipette	Eppendorf	Germany
MiniRun Gel Electrophoresis	Bio-Rad	USA
NanoDrop 2000/2000c Spectrophotometer	Thermo scientific	USA
Orbital shaker	Thermo scientific	USA
Oven	WTB Binder	Germany
pH meter	HACH	USA
Polymerase Chain Reaction (PCR)	Bio Rad	USA
Protector Laboratory Hood	Labconco	USA
Refrigerated Centrifuge, 5804R	Eppendorf	Germany
Ultrasonic	Bandelin electronic	Germany
UV spectrophotometer	Thermo Electron Corporation	USA
Vortex	Scientific Industries, INC	USA

Equipment	Company	Country
Water distillers 15 Ω and 18 Ω	H2o Labs	USA

3.2.2 Chemicals and culturing media

1) Chemicals

This study used many kinds of chemicals for bacterial enrichment culture, fungal isolation, enzymatic activity test in fungi, TIA degradation test with fungi and bacteria, and identification of fungal and bacterial community as shown in Table 3.2.

Table 3.2 Lists of chemicals used in this study

Chemicals	Company	Country
2X PCR Master mix solution (i-Taq^{TM})	European Biotech Network	Belgium
Agarose	Bio-Rad	USA
Absolute ethanol (99.9% purity) (CH_3CH_2OH)	Merck	Germany
Ammonium persulfate (APS) ($(NH_4)_2S_2O_8$)	Sigma-Aldrich	Germany
Acrylamide (C_3H_5NO)	Sigma-Aldrich	Germany

Chemicals	**Company**	**Country**
Bis-Acrylamide ($C_7H_{10}N_2O_2$)	Sigma-Aldrich	Germany
Brilliant green ($C_{27}H_{33}N_2.HO_4S$)	Carlo Erba	France
Crystal violet ($C_{25}N_3H_{30}Cl$)	Carlo Erba	France
Deionized formamide	Sigma-Aldrich	Germany
Acetic acid (CH_3COOH)	Sigma-Aldrich	Germany
Glycerol ($C_3H_8O_3$)	Sigma-Aldrich	Germany
Guaiacol ($C_7H_8O_2$)	Sigma-Aldrich	Germany
Hydrogen peroxide (H_2O_2)	Merk	Germany
Luria-Bertani (LB)	Titan Biotech LTD.	India
Manganese (II) Sulfate ($MnSO_4$)	Carlo Erba	France
Methanol (CH_3OH) HPLC Grade	Sigma-Aldrich	Germany
N,N,N',N' - tetramethylenediamine (TEMED) ($C_6H_{16}N_2$)	Sigma-Aldrich	Germany

Chemicals	Company	Country
Nutrient broth	HiMedia Laboratories Pvt. Ltd.	India
Plate counting agar	HiMedia Laboratories Pvt. Ltd.	India
Potato dextrose agar (PDA)	Titan Biotech LTD.	India
Succinic acid [$(CH_2)_2(CO_2H)_2$]	Carlo Erba	France
Sulphuric acid (H_2SO_4) 98%	Carlo Erba	France
Tiamulin fumarate ($C_{32}H_{51}NO_8S$)	Fluka Analytical	Germany
Tris-acetate-EDTA buffer (TAE buffer)	Sigma-Aldrich	Germany
Urea (CH_4N_2O)	Sigma-Aldrich	Germany
Veratryl alcohol ($C_9H_{12}O_3$)	Sigma-Aldrich	Germany

2) Culturing media

2.1) Mineral salt medium (MSM)

Mineral salt medium (MSM) in this study was obtained from Reis et al. 2014 that had been used in biodegradation of sulfamethoxazole and other sulfonamides antibiotics by *Achromobacter denitrificans* PR1 bacteria. MSM contained basal components without any carbon source that was appropriately for the growth of any microorganisms. The components of this basal medium comprised of 3.5 g/L K_2HPO_4, 0.5 g/L NaCl, 1.5 g/L KH_2PO_4, 0.15 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L $(NH_4)_2SO_4$

and it was adjusted the pH to 7.0. It was selected to use in the enrichment of TIA-degrading bacterial cultures from swine farm sediment and liquid wastes with tiamulin fumarate antibiotic ($C_{32}H_{51}NO_8S$, Fluka Analytical, Germany) as sole carbon source (Figure 3.5).

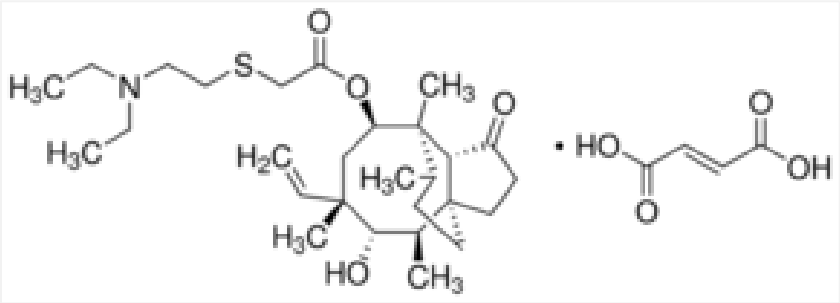


Figure 3.5 Structure of tiamulin fumarate antibiotic

(Source: /www.sigmaaldrich.com/)

2.2) Defined aqueous medium for fungal cultivation with tiamulin

The chemical components of defined aqueous medium used to examine the tiamulin degradation by fungi per liter were 10 g glucose, 1 g KH_2PO_4, 0.5 g $MgSO_4$, 0.14 g $CaCl_2$, 1 g yeast extract, and 0.0025 g Thiamine. This medium has been also used to screen for dye decolorization of white rot fungi with glucose as co-substrate in the previous study [146]. Glucose was a good carbon source for metabolism with many target xenobiotic pollutants in liquid degradation tests using fungi compared to other cosubstrates including fructose, saw dust, corn starch, and cane molasses. [147-149].

2.3) Luria-Bertani (LB)

The Luria-Bertani (LB) (Titan Biotech, India) medium includes tryptone 10 g, yeast extracts 5 g, and NaCl 5 g. The components were dissolved in 1 liter of distilled water, adjusted to pH 7.0 and autoclaved at 121ºC for 20 min.

2.4) Plate count agar

Plate count agar (HiMedia Laboratories, India) is a standard method agar for counting bacteria which are composed of casein enzymic hydrolysate (5g/L), yeast extract (2.5 g/L), dextrose (1 g/L), and agar (15 g/L) at pH of 7.0 ± 0.2.

2.5) Nutrient broth (NB)

Nutrient broth (Sigma-Aldrich, USA) comprises of meat extract (1 g/L), peptone (5 g/L), sodium chloride (5 g/L), and yeast extract (2 g/L) prepared at pH 7.4.

2.6) Potato dextrose agar (PDA)

Ingredients of PDA (Titan Biotech, India) consist of potato infusion (200g/L), dextrose (20 g/L), and agar (15 g/L) in 1 L of distilled water at pH of 5.6 ± 0.2.

3.3 Research methodology

3.3.1 Biodegradation of antibiotic tiamulin by fungi

1) Fungal source and isolation

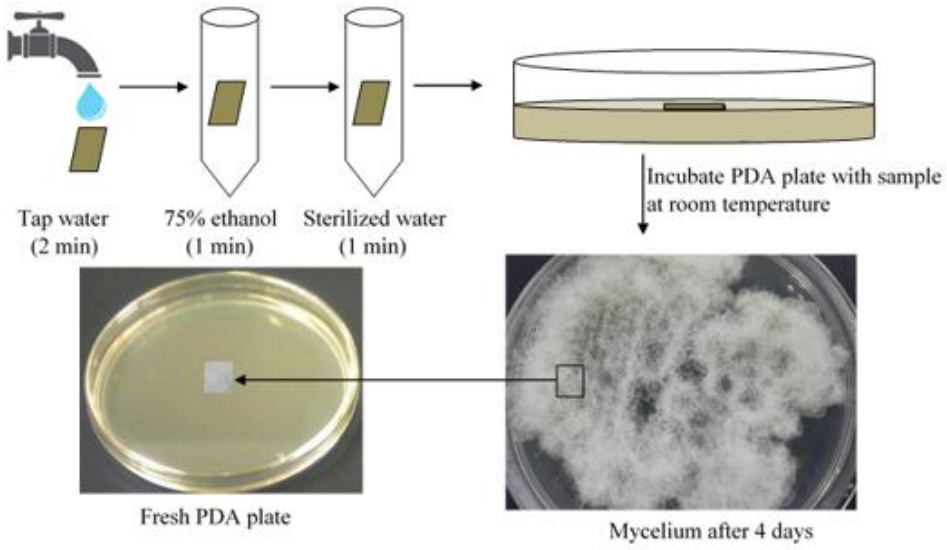


Figure 3.6 Procedure of fungal isolation

Many rotten wood samples and swine waste materials such as manure, decayed wood, tree trunks and leaves were collected as the sources for fungal isolation (Figure 3.4). Fungal isolation was performed as shown in Figure 3.6, samples were washed under running tap water about 2 min, then subjected to surface sterilization with 1.5 mL of 75% ethanol for 1 min and submerged in 1.5 mL sterilized water approximately 1 min under aseptic conditions. Samples were cut into small pieces, inoculated onto potato dextrose agar (PDA; Titan Biotech LTD., India) plates, and then incubated at room temperature. The cultures were repeatedly transferred by inoculating a new mycelium cut into a fresh PDA plate every 4-day interval until getting a pure culture. Subsequently the pure cultures of all fungi were preserved on PDA slant at 4°C.

2) Fungal identification

Genomic DNA of the isolated strains showing dye-decolorizing potential were extracted using Fast DNA Spin extraction kit for soil (QBiogene, Solon, Ohio, USA) following the manufacturer's instructions. The 2 µL product from DNA extraction was verified by loading together with 2 µL loading dye into 2% agarose gel of electrophoresis (Bio-Rad, USA) and run in MiniRun Gel Electrophoresis (Bio-Rad, USA) approximately 30 min. Finally, fungal DNA extracts were visualized on Gel documentation WealTec Dolphin - DOC (Bio-Rad, USA) and stored at - 20^{o}C until use.

The internal transcribed spacer in ribosomal rDNA (ITS-rDNA) were amplified using a Bio Rad thermocycler PCR machine (Bio-Rad, USA) with the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [150]. The PCR mixture was prepared using a Takara polymerase (Takara Bio Inc., Japan), in which components of 12.5 µL PCR reaction mixture consisted of 8.6875 µL distilled water, 1.25 µL $MgCl_2$, 1.25 µL 10× buffer, 0.25 µL dNTP, 0.25 µL primer ITS1, 0.25 µL ITS4, 0.0625 µL Fermentas taq, and 1 µL fungal template DNA. The PCR condition was 5 min at 95°C followed by 35 cycles for 40 s at 95°C, 40 s at 55°C, and 1 min at 72°C, followed by 10 min of final extension at 72°C. 5 µL of each PCR product was confirmed using electrophoresis technique at 120 V for 30

min in 2 % (w/v) agarose gel and 1× TAE buffer, then visualized and photographed by using a Gel documentation WealTec Dolphin - DOC (Bio-Rad, USA). The PCR products were then used for sequencing (Macrogen Inc., Korea). The obtained sequences were then assembled into one completed sequence using DNA Baser v4.23.0 (Heracle BioSoft SRL, Romania) and compared to the GenBank database using BLASTn. Genetic distances were analyzed based on a neighbor-joining algorithm with 500 bootstrap numbers using MEGA 6 software [151]. The DNA sequencing information of the twelve isolated fungi (F1-F12) were deposited at Genbank with the accession numbers KX224478 - 89.

3) Dyes decolorization

All fungi were cultivated on Potato Dextrose Agar plates containing dye solutions either brilliant green ($C_{27}H_{34}N_2O_4S$, MW 482.64 g/mol) or crystal violet ($C_{25}H_{30}ClN_3$, MW 407.98 g/mol) (Figure 3.7) at 0.05% (w/v) over 12-day period. These dyes were selected due to the similar molecular weight with tiamulin (MW 493.7 g/mol), and the stability to sterile treatment. Mycelial discs of approximately 4 mm were cut from actively growing region using sterile forceps, transferred onto the center of three new dye-containing PDA plates, and incubated at room temperature (~ 28^{o}C). Three of uninoculated dye-containing PDA plates were served as control (Figure 3.8). The dyes decolorizing experiment was carried out in triplicate. The decolorization of dyes was observed by the formation of clear zones under and around developing mycelia. Diameters of fungal mycelia and the size of the decolorized zones were measured in mm-unit for every two days (Figure 3.9). Pearson correlation analysis was computed using SPSS version 22.0 (IBM Corp, USA).

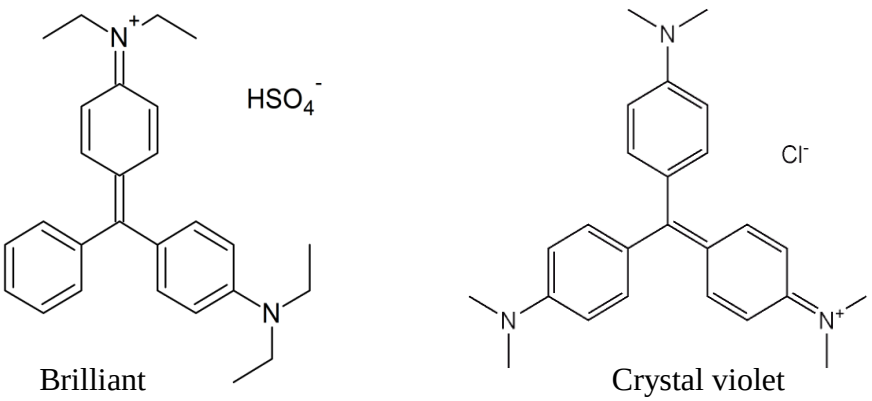


Figure 3.7 Structure of brilliant green and crystal violet dyes

(Source: Wikipedia)

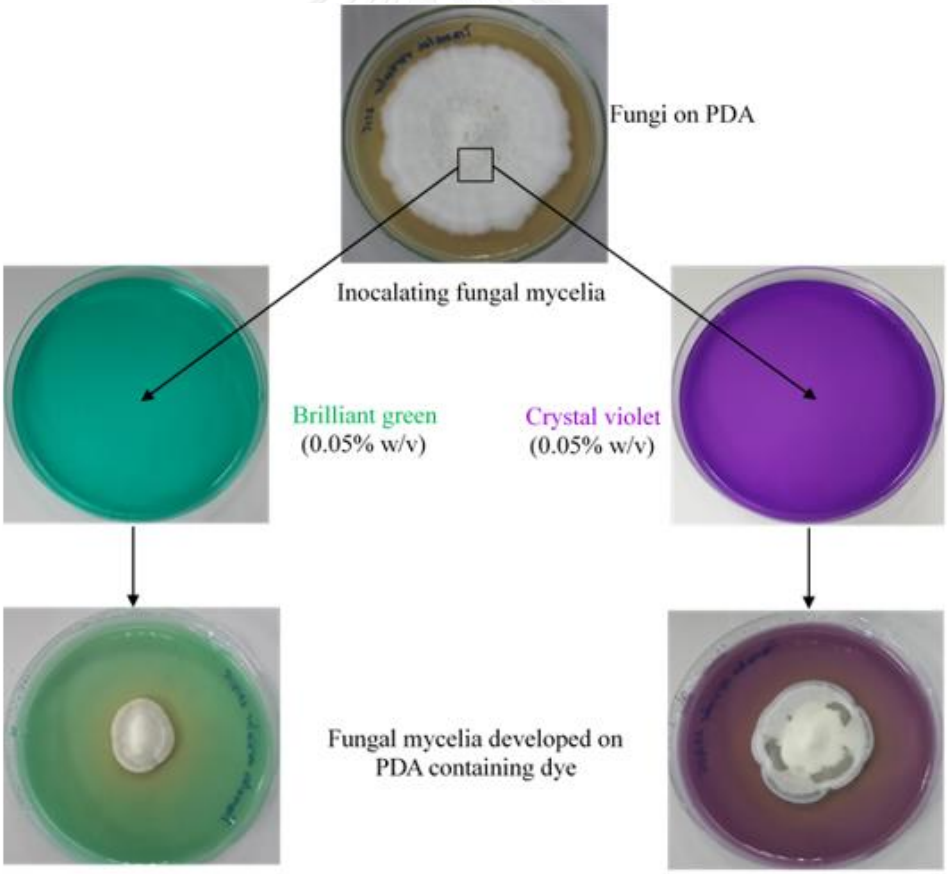


Figure 3.8 Screening fungi with brilliant green and crystal violet dyes

The decolorization of dyes was observed by the formation of clear zones under and around developing mycelia. Three diameter values were determined from different directions and estimated for the averaged value. Decolorization zone equal or larger than 80 mm was defined as +++. The zone from 60 mm to 80 mm, and from 1 mm to 59 mm were assigned as ++ and +, respectively. No clear zone formation was recorded as –.

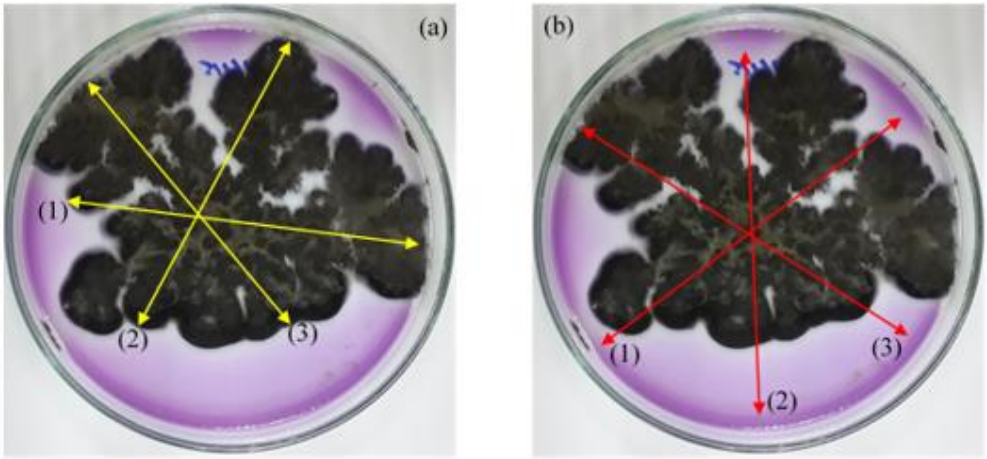


Figure 3.9 Measurement of fungal diameters and decolorization zones

[(a) - Diameter of fungal mycelia; (b) - Size of decolorized zone)]

4) Tiamulin degradation tests by fungi

The examination of tiamulin degradation by fungi was carried out in batch mode using 250-mL Erlenmeyer flasks. Each flask contained 100 mL of a chemically defined medium with 10 mg/L of tiamulin fumarate (VETRANAL Sigma, USA, MW 609.8 g/mol), the common veterinary medicinal form of tiamulin. The components of the defined medium are showed in section 3.2.2. This medium was used to screen for dye decolorization in the white rot fungi of a previous study [146]. Then, 5 g of mycelium pellets (wet weight - ww) taken from the PDA plate aseptically was added to the medium. Uninoculated flasks containing 100 ml of the defined medium and heat killed cultures were employed as the medium and sorption controls, respectively.

The experiment was conducted in triplicate. The flasks were incubated in the rotary shaker at 200 rpm at room temperature (approximately 28^{o}C) for 12 days. All flasks were covered with aluminium foil in order to avoid photolysis phenomenon. The aqueous samples were harvested at 0, 1, 2, 4, 8, and 12 days to measure the residual tiamulin and test the enzymatic activity of manganese peroxidase (MnP), lignin peroxidase (LiP), and laccase (Lac). The sampling time intervals were selected to provide an overall view of tiamulin degradation. Experimental procedure is presented in Figure 3.10.

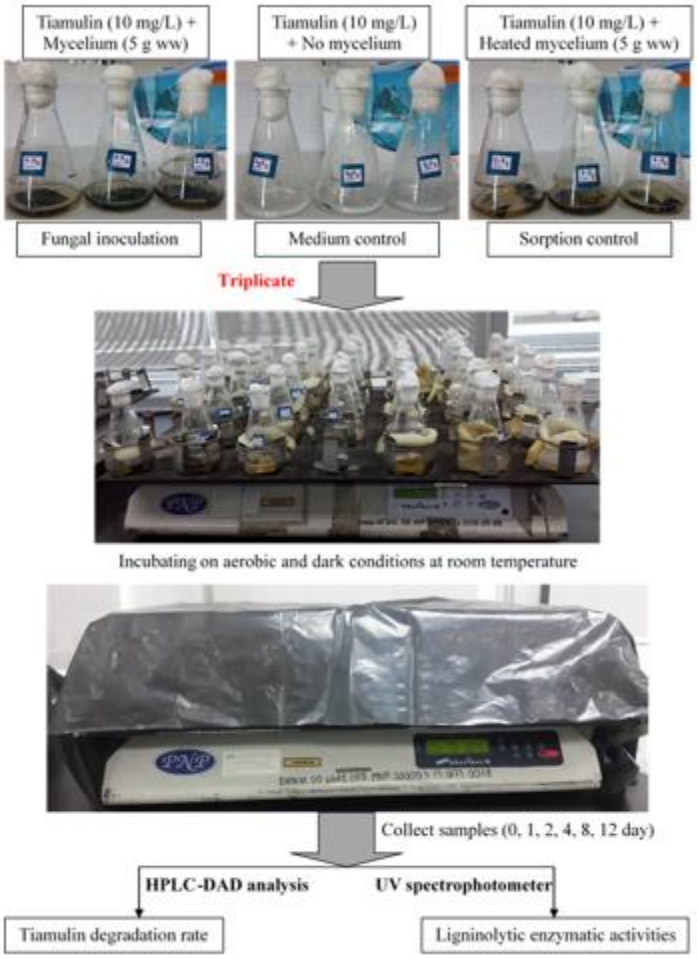


Figure 3.10 Tiamulin degradation test with dye-degrading fungi

A Tiamulin residue was detected by high performance liquid chromatography with diode-array detection (HPLC-DAD) that is presented in HPLC-DAD analysis for detecting tiamulin of section 3.3.3 for analysis methods. Student *t*-test and one-way ANOVA have been used to discuss the difference in tiamulin degrading results using SPSS version 22.0 (IBM Corp, USA).

5) Determination of the enzymatic activity of the fungi

Liquid samples for enzymatic examination were collected from the triplicated tiamulin degrading flasks at the particular times and used directly as crude enzymes in this experiment. In order to determine the role of enzymes in eliminating tiamulin substrate, three ligninolytic enzymes including lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Lac) were examined their enzymatic activities in the same experiment with tiamulin antibiotic and no tiamulin supplementation. Three ligninolytic enzymes have been studied the most due to their high potential to degrade various toxic substances in nature [152]. The activities of such three extracellular enzymes were tested following the methods described elsewhere [48, 153]. Two experimental conditions have been set with tiamulin addition and without tiamulin addition and all were done in triplicate. Statistical analysis has determined using Two-factor ANOVA with replication in Microsoft Office Excel 2013. All ligninolytic enzymatic activity was expressed as U/L, with 1 U defined as 1 µmol of the substrate oxidized per minute (Appendix B).

Lignin peroxidase was estimated based on the oxidation of veratryl alcohol (3, 4-dimethoxybenzyl alcohol) (Sigma-Aldrich, USA) to veratraldehyde in the presence of H_2O_2 at room temperature. The reaction mixture contained 2 mmol/L veratryl alcohol, 0.4 mmol/L H_2O_2, 50 mmol/L sodium succinate buffer (pH 4.5) and 660 µL crude enzyme, comprising a total volume of 1 mL. The changing absorbance at 310 nm in 2 min was recorded using a UV spectrophotometer (Thermo Electron Corporation, USA).

Manganese peroxidase was measured using spectrophotometer at the wavelength of 238 nm within 10 secs at room temperature. The reaction mixture consisted of 0.1

mmol/L $MnSO_4$, 0.1 mmol/L H_2O_2, 100 mmol/L sodium succinate buffer (pH 5.0) and 540 µL crude enzyme in total volume of 1 mL.

Laccase activity was determined by measuring the amount of oxidized guaiacol (Sigma-Aldrich, USA). One milliliter of the reaction mixture contained 50 mmol/L sodium succinate buffer (pH 4.5), 0.4 mmol/L guaiacol and 760 µL crude enzyme. After a 30 min reaction at 30°C, guaiacol oxidation was monitored by observing the absorbance change at 465 nm in 1 min by UV spectrophotometer.

3.3.2 Biodegradation of antibiotic tiamulin by bacterial enriched cultures

1) Collection and preparation of swine waste samples

Five swine wastewater and sediment samples were collected from several locations in two swine farms (Figure 3.2). To increase the chance to get bacteria that could degrade tiamulin, all collected samples of each farm were completely mixed and then used as sources for the enrichment of TIA-degrading bacteria with liquid mineral salt medium.

2) Enrichments of TIA-degrading bacteria

Enrichment of TIA-degrading bacteria was performed in two conditions with and without other carbon sources. The enrichment was conducted in 250-mL Erlenmeyer flasks containing mineral salt medium containing some components in culturing media of section 3.2.2 that had been used elsewhere to cultivate *Achromobacter denitrificans* PR1 to degrade sulfamethoxazole [126]. For the enrichment with supplementing other carbon source, the nutrient broth (Sigma-Aldrich, USA) was added to the final concentration of 2 mg/L in culturing flasks. The ingredients of nutrient broth are also showed in culturing media of section 3.2.2.

Four enrichment cultures comprised of A, AN, S, and SN were obtained. Their sources and cultivated conditions are described in Table 3.3.

Table 3.3 Bacterial mixed enrichment cultures with their sources and cultivated conditions

Culture	Source	Cultivated conditions
A	Covered anaerobic lagoon	Tiamulin + Mineral salt medium
AN	Covered anaerobic lagoon	Tiamulin + Mineral salt medium + Nutrient broth
S	Stabilization ponds	Tiamulin + Mineral salt medium
SN	Stabilization ponds	Tiamulin + Mineral salt medium + Nutrient broth

Tiamulin stock solution of 2,000 mg/L was prepared by mixing 0.5 mL of tiamulin 20% with 49.5 mL deionized water. In the enrichment experiment, 250-mL Erlenmeyer flasks were used and the working volume was 100 mL with the final tiamulin concentration of 4 mg/L. Firstly, 89.8 mL and 89.4 mL MSM were filled into the enrichment flasks with and without nutrient broth, respectively. Then, 10 mL of swine mixed liquor samples and 0.2 mL of tiamulin stock were added. Nutrient broth (0.4 mL of 500 mg/L stock solution) was added into the flasks containing other carbon source.

A control set consisted of 4 mg/L tiamulin and MSM without swine mixed liquor sample. All flasks were incubated on a shaker with the speed of 200 rpm, at room temperature, and in dark condition. After 14-d intervals, 10% (V/V) of each enrichment culture was transferred to new flasks containing fresh MSM in which

tiamulin concentration was increased up to 5 mg/L and nutrient broth was decreased down to 1.5 mg/L (Figure 3.11).

In order to check the survival of bacteria in enriched cultures, all flasks were spread in Luria-Bertani (LB) agar plates with and without adding 4 mg/L of tiamulin antibiotic. 50 µL of sample from each bacterial enriched flask was spread on LB agar plates in triplicate, incubated at room temperature on two days, and observed living cells on each plate. Living cells of all flasks were recorded as + or – if it appeared bacterial colonies or not. Bacterial colonies were numerous in all bacterial enriched samples, but they did not occur in any control flasks.

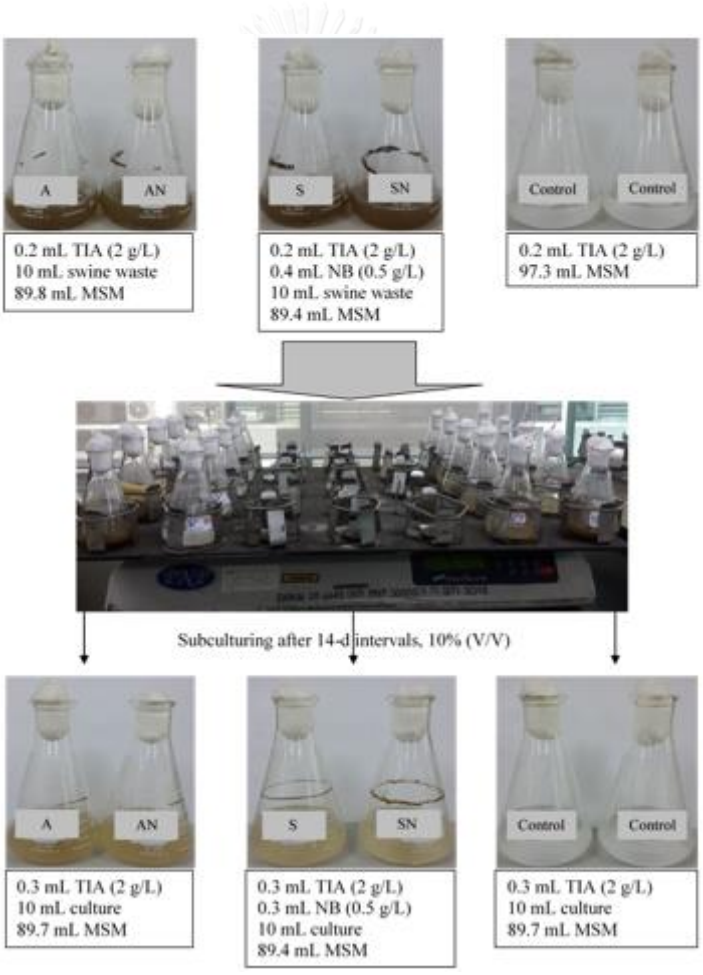


Figure 3.11 Bacterial enrichment culture setup

After the fourth subculture, the subculture process was performed when bacterial numbers reached 10^5 CFU/mL. The bacterial numbers were enumerated on plate

counting agar (HiMedia Laboratories Pvt. Ltd., India) in triplicate by using serial dilution technique. The culture plates were incubated at room temperature for two days. The subcultures (1%, V/V) were continually done in such a way that the proportion of tiamulin concentration is gradually increasing to 20 mg/L, but the nutrient broth concentration is gradually decreasing to 0 mg/L for the enrichment set that containing other carbon source.

After seven months with 14 times of subcultures, bacterial mix enriched cultures were used to set up experiment for either examining their abilities on degrading tiamulin or determinating tiamulin degradation kinetics.

3) Examination of enrichment culture ability on degrading tiamulin and determination of tiamulin degradation kinetics

The batch experiments were conducted using 250-mL Erlenmeyer flasks containing 100 mL mineral salt medium with 1% (V/V) of enrichment cultures. Ten final tiamulin concentrations of 2.5, 5, 10, 20, 30, 50, 70, 100, 150 and 200 mg/L were used in this experiment. Each concentration was carried out in triplicate. The experimenting flasks were shaken with the speed of 200 rpm, at room temperature in darkness under aerobic condition. Abiotic controls were performed with tiamulin at ten above concentration plus only mineral salt medium. Aqueous samples in the experimenting flasks were periodically collected to measure the concentration of residual tiamulin by HPLC-DAD and to enumerate number of bacteria by dropping serial dilution bacterial mixed culture on plate counting agar in triplicate well (Figure 3.12). Degradation rate of tiamulin was calculated by comparing the rate of tiamulin consumption in flasks containing bacteria with the abiotic controls. Kinetics of tiamulin degradation were established based on the correlation of tiamulin concentration and bacterial growth rate using software SigmaPlot version 11.0 (Systat Software Inc., UK)

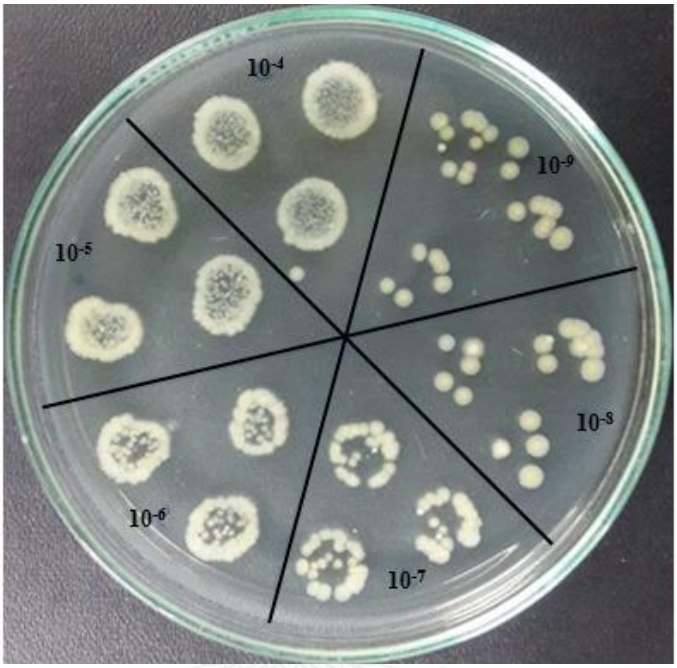


Figure 3.12 Dropping plate technique for enumerating bacterial number

4) Identification of TIA-degrading bacterial community in enrichment culture

4.1) Bacterial DNA extraction

The genomic DNA of bacteria in four original enrichment cultures and their corresponding treatment at three different tiamulin concentrations including 2.5 mg/L, 50 mg/L, and 200 mg/L was extracted with the Fast DNA SPIN kit for soil (QBiogene, USA) from 10 mL of bacterial culture volume. The protocol of bacterial DNA extraction is presented in the Appendix B of the genomic DNA extraction topic. DNA extraction quality was verified by directly loading 2 µL DNA template and 2 µL dye mixture into 2% agarose gel and running with MiniRun Gel Electrophoresis (Bio-Rad, USA) approximately 30 minutes, soaking in 10% Ethidium bromide solution

about 1 h and finally reading the results under Gel documentation WealTec Dolphin - DOC (Bio-Rad, USA).

Concentrations and purity of bacterial DNA were then quantitated and determined through NanoDrop 2000/2000c Spectrophotometer (Thermo scientific, USA) in triplicate. The protocol of measurement of nucleic acid samples includes in NanoDrop measuring DNA concentration topic of the Appendix B.

4.2) Polymerase chain reaction (PCR) amplifying 16S rRNA gene of bacterial enriched cultures

The bacterial 16S rRNA gene of all sixteen samples was amplified by PCR techniques using the bacterial universal pair of primers with their sequences: 27-F (5′ - AGAGTTTGATCCTGGCTCAG- 3′) and 1492-R (5′ - GGTTACCTTGTTACGACTT - 3′). The 20 µL of 1× PCR reaction mixture was created by mixing 7 µL distilled water, 10 µL 2× PCR Master mix solution (European Biotech Network, Belgium), 1 µL primer 27-F, 1 µL primer 1492-R, and 1 µL bacterial template DNA. The PCR condition was programmed at 94^{o}C for 5 min, followed by 35 cycles of 94^{o}C for 30 seconds, 55^{o}C for 30 seconds, and 72^{o}C for 1 min, and a final extension step of 72^{o}C for 7 min. The positive and negative controls were simultaneously set up which 1 µL of *Mycobacterium* sp. PO1 strain DNA and distilled water took the place of bacterial DNA template. The amplified products were confirmed with agarose gel 2% with positive and negative controls under Gel documentation WealTec Dolphin - DOC (Bio-Rad, USA).

4.3) PCR amplification for denaturing gradient gel electrophoresis (DGGE)

PCR approach was continuously applied to bacterial V3 region of 16S rRNA gene amplification of all sixteen samples using the bacterial V3 universal primer set 341-F-GC (5’ - CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGCC TACGGGAGGCAGCAG - 3’) and 520-R (5’ - AGCAGCCGCGGTAAT - 3’). PCR amplification was performed in a total volume of 50-µL reaction containing 25 µL 2× PCR Master mix solution (European Biotech Network, Belgium), 0.4 µM of each

primer, 22 μL distilled water, and 1 μL of DNA template. The PCR condition was 94^{o}C for 5 min, followed by 30 cycles of 94^{o}C for 1 min, 55^{o}C for 1 min, and 72^{o}C for 2 min, and a final extension step of 72^{o}C for 10 min. Subsequently, 5 μL of each PCR product was confirmed using electrophoresis technique at 120 V for 30 min in 2 % (w/v) agarose gel and 1× TAE buffer. PCR products, including positive of *Mycobacterium* sp. PO1 bacterium and negative controls, were visualized and photographed by using a Gel documentation WealTec Dolphin - DOC (Bio-Rad, USA).

4.4) Denaturing gradient gel electrophoresis (DGGE)

After electrophoresis of PCR amplification, approximately 30 μL of each unpurified PCR product (including loading dye) was loaded on a 8 % (w/v) polyacrylamide gel with a 30∼70 % linear gradient of DNA denaturant agents (urea and formamide). The DGGE gel was electrophoresed in 1× TAE buffer at 65°C and a constant voltage of 130 V for 4.5 h (Bio-Rad, USA). The DGGE gel was stained in 20 mL of 10% Ethidium bromide diluted 20 μL: 200 mL in DI water for 15 minutes and visualized using a Gel documentation WealTec Dolphin - DOC (Bio-Rad, USA) (Appendix B).

After separating PCR fragments, DGGE patterns of all samples were compared, particularly patterns of each bacterial enriched culture with its treatment at three different tiamulin concentrations (2.5, 50 and 200 mg/L), to select appropriate samples for further molecular identification. Because the numbers of bands in DGGE patterns reflected the numbers of predominant microbes in the microbial communities (Muyzer and Smalla, 1998), samples showed different DGGE patterns were obtained for identifying bacterial community structures through advanced technique of the Miseq next-generation sequencing (NGS) system using Illumina Miseq (Illumina, USA).

5) Identification of bacterial community in enriched cultures with the next-generation sequencing

5.1) Preparation of 16S rRNA gene amplicons for the Illumina MiSeq system

PCR amplification was performed using the 515F-806R bacterial/archaeal primer pair that used by the Earth Microbiome Project (Walters et al., 2016) (515-F: 5' - AATGATACGGCGACCACCGAGATCTACACTATGGTAATTGTGTGCCAGCMGCCGCGGTAA - 3' and 806-R: 5' - CAAGCAGAAGACGGCATACGAGATXXXXXXXXXXXXAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT - 3') targeting region V4 of the 16S rRNA gene in all of the twelve extracted DNA. The PCR reaction mixture of 50 µL contained 25 µL 2× PCR Master mix solution (European Biotech Network, Belgium), 22 µL distilled water, 1 µL primer 515-F, 1 µL 806-R primer, and 1 µL of sample DNA. Amplification was carried out under the following conditions: initial denaturation at 95°C for 5 min, followed by 28 cycles of denaturation at 95°C for 30 s, primer annealing at 55°C for 45 s, and extension at 68°C for 30 s, with a final elongation step of 68°C for 7 min. The PCR products were confirmed on a 2% agarose gel electrophoresis and visualized using a Gel documentation WealTec Dolphin - DOC (Bio-Rad, USA).

All amplified PCR products of 16S V4 amplicon were then purified using AMPure XP beads kit (Agencourt Bioscience, USA) following the manufacturer's instructions. Index PCR step attaching dual indices and Illumina sequencing adapters was carried by using the Nextera XT Index Kit. Before quantification, the final library was cleaned up again with AMPure XP beads kit. The purified PCR products were subsequently quantified and adjusted to the final concentration 4 nM of each library using a fluorometric quantification method and pooled equimolar for the next-generation sequencing. The library pools were submitted to Omics Science and Bioinformatics Center, Faculty of Science, Chulalongkorn University. The next-generation sequencing was performed on an Illumina MiSeq platform (Illumina, USA).

5.2) Bioinformatics by Miseq reporter metagenomics workflow

After loading the samples, the Miseq system provided on-instrument secondary analysis Miseq Reportern software (MSR). Among several options for analyzing

Miseq sequencing data provided from MSR, metagenomics workflow was selected to demonstrate 16S protocol. Using a database of 16S rRNA data and the Greengenes database (http://greengenes.lbl.gov/), microorganisms were classified at several taxonomic levels, such as kingdom, class, order, family, genus, and species. The secondary analysis data obtained from this Metagenomics workflow contained cluster graph, sample table and cluster pie chart. Cluster graph exhibited numbers of raw cluster, clusters passing filter, clusters that did not align, clusters not associated with an index, and duplicate were also obtained. Sample table summarized the results of the sequencing for each sample. Cluster pie chart showed a graphical representation of the classification breakdown for each sample.

3.3.3 Tiamulin determination by High-Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD)

1) HPLC-DAD set up for tiamulin determination

Tiamulin analysis was performed following a previous study with some modifications [154]. For preparing standard solution, a stock tiamulin solution was prepared by dissolving 100 mg of the compound in 100 mL ultrapure water 18 Ω and stored at 4^{o}C refrigerator for no longer than 3 months. Working standard solutions ranging from 0.1 to 10 mg/L and from 0.5 mg/L to 100 mg/L were diluted with ultrapure water for tiamulin degradation experiment with fungi and bacterial enriched cultures, respectively.

HPLC-DAD Agilent 1100 series diode array and multiple wavelength detectors system with autosampler was used to detect tiamulin. The chromatographic separation was performed on the Hypersil ODS C18 column (250 × 4.0 mm, particle size 5 µm) with column guard, which was maintained with controlling temperature at 25^{o}C. The optimal compositions of acidic mobile phase were 10% HPLC grade methanol (A) and 90% ultrapure water acidified to pH 2.2 with sulfuric acid (B). The volume of 20 µL standard solutions or samples was injected into HPLC-DAD. Mobile phases were run at the flow rate of 0.25 and 0.5 mL/min towards samples of tiamulin degradation experiments by fungi and bacterial mixed enriched cultures, respectively. Tiamulin

was detected at 208 nm with approximately 20 and 10-minute retention times for fungal and bacterial enrichment experiments, respectively.

2) Calculation of limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection (LOD) and the limit of quantification (LOQ) were determined relatively to the standard deviation (SD) of ten noise peaks near the tiamulin detected retention time of three runs of blank solution. The LOD of tiamulin analysis was 0.09 mg/L (LOD = 3 × standard deviation of noise/slope of standard curve) and the LOQ was 0.3 mg/L (LOQ = 10 × SD_{noise}/slope). Linearity of the standard curves was obtained in the range from 0.1 to 10 mg/L. The correlation coefficient (R^2) in general was 1.0 indicating good linearity of the calibration curve for the method in the considered concentration range.

3.3.4 Data analysis

All measurements were replicated twice. The mean values and standard error of all parameters were taken from three replicates. The data were subjected to analysis of variance by one-way ANOVA, Pearson's correlation using SPSS statistics version 22.0 (IBM Corp, USA), Student's *t*-test, two-factor with replication (ANOVA) analysis in Microsoft Office Excel 2013, and kinetics determination by software SigmaPlot version 11.0. Critical *P*-value was set at 0.05 or the least significant differences test was at the 5% significant level.

In experiment of biodegradation of tiamulin using wood rot fungi, the relationship between fungal growth and clear zone formation was analyzed Pearson's correlation coefficient (r). The TIA concentrations at the beginning and the end of experiment were compared by performing Student's *t*-test and one-way ANOVA to determine if the differences between the means were statistically significant. Two-factor with replication (ANOVA) analysis in Microsoft Excel 2013 was also performed to test the significant difference in the amounts of enzyme production at two different cultivation conditions.

For biodegradation of tiamulin by bacterial enriched cultures, kinetics plot of tiamulin degradation were established based on the correlation of tiamulin removal concentration and bacterial growth rate by software SigmaPlot version 11.0. The significant differences between treatment means of tiamulin concentrations and bacterial numbers were determined with the Student's *t*-test in Microsoft Office Excel 2013. Proportion of total reads in the next-generation sequencing results from kingdom to species levels and Shannon diversity index values at species level were also analyzed in Microsoft Office Excel 2013 to compare diversity of the four enriched cultures and their corresponding treatments at different sources, cultivation conditions, and tiamulin concentrations.

3.3.5 Calculation

1) Calculation of TIA concentration

From the standard curves of TIA degradation in the Eq. (3.1), TIA concentration was calculated as following Eq. (3.2).

$$y = ax + b \qquad \text{Eq. (3.1)}$$

$$x = \frac{(y-b)}{a} \qquad \text{Eq. (3.2)}$$

Where: x is TIA concentration (mg/L)

y is Peak area of TIA chromatogram

a and b are constants from TIA standard curves.

2) Calculation of first-order specific TIA degradation rate (k_{biol})

From the Eq. (3.3) described as a pseudo-first-order degradation rate of the degradation of pharmaceuticals in the water phase [155], the Eq. (3.4) was obtained to calculate the first-order specific TIA degradation rate in TIA degradation experiment of wood rot fungi.

$$\frac{dC_w}{dt} = -k_{biol} \times X \times C_w \qquad \text{Eq. (3.3)}$$

$$\ln\left(\frac{C_w}{C_o}\right) = -k_{biol} \times X \times t \qquad \text{Eq. (3.4)}$$

Where: C_w is the TIA concentration in water phase of degradation test (mg/L)

C_o is the initial TIA concentration (mg/L)

X is fungal concentration (g/L)

k_{biol} is the first-order biodegradation constant (L/g fungi·d)

t is the time (d).

This equation suggests that a plot of $\ln(C_w/C_o)$ versus X × t yields a straight line and first-order biodegradation constant k_{biol} is obtained from the slope of the straight line by applying the least-squares method.

3) Calculation of enzymatic activity

The following formula of Eq. (3.5) was used to calculate the enzyme activity (U/L) in TIA degradation experiment by six wood rot fungal strains.

$$U = \frac{(\Delta A_{sample} - \Delta A_{blamk}) \times V_{cuvette} \times 10^3}{\varepsilon \times \Delta_{time} \times \text{path length} \times V_{sample}} \qquad \text{Eq. (3.5)}$$

Where: 1 U = 1 μmole/min

ΔA_{sample} presents the change in absorbance of the sample containing enzyme from the beginning to the end of the measurement period.

ΔA_{blank} is the change in absorbance of a sample containing reaction mixture except the crude enzyme.

$V_{cuvette}$ is volume of total reaction mixture in cuvette (mL).

10^3 converts the mmoles of ε to μmoles.

ε is the molar extinction coefficient converts absorbance values to concentrations. The molar extinction coefficient of Lac, MnP and LiP are ε465 of guaiacol (12.1 L/mM·cm), ε238 of Mn^{3+} (6.5 L/mM·cm), and ε310 of veratraldehyde (9.3 L/mM·cm), respectively.

Δ_{time} is the time interval the absorbance was measured (min)

V_{sample} is volume of the crude enzymes adding into the total 1 mL of reaction mixture (mL).

The cuvette path length is 1 cm.

4) Calculation of Monod kinetics parameters

Tiamulin consumption rates in the degradation tests with bacterial enriched cultures were calculated by using Eq. (3.6), whereas the Monod kinetics parameters q_{max}, and K_s were calculated from Monod equation in the Eq. (3.7).

$$q = \frac{d[TIA]}{dt} \qquad \text{Eq. (3.6)}$$

$$q = \frac{q_{max}[TIA]}{K_s + [TIA]} \qquad \text{Eq. (3.7)}$$

Where: q is TIA consumption rate (mg/L·h)

q_{max} is the maximum TIA consumption rate (mg/L·h)

[TIA] is the TIA concentration (mg/L)

K_s is the half saturation coefficient for TIA (mg/L)

t is time (h).

5) Calculation of degradation rate (% TIA removal)

TIA removal efficiency addressed in percentage was calculated based on the following equation (Eq. 3.8)

$$\text{Degradation rate (\%)} = \frac{(C_o - C)}{C_o} \times 100 \qquad \text{Eq. (3.8)}$$

Where: C_o is the TIA concentration (mg/L)

C is the TIA concentration (mg/L) at time t (in hour for bacteria and in day for fungi).

6) Calculation of Shannon diversity index

Shannon diversity index (H) allows us to know not only the number of species but also how the abundance of the species is distributed among all the species in the community.High values of H would be representative of more diverse communities. A community with only one species would have an H value of 0 because P_i would equal 1 and be multiplied by ln P_i which would equal zero. If the species are evenly distributed then the H value would be high. Typical values are generally between 1.5 and 3.5 in most ecological studies, and the index is raely greater than 4.0. The Shannon index increases as both the richness and the evenness of the community increase.

The following formula of Eq. (3.9) was used to calculate the Shannon diversity index of community structures in bacterial enriched cultures and their corresponding treatments with tiamulin at 2.5 and 200 mg/L.

$$H = -\sum_{i=1}^{s}(P_i * \ln P_i) \qquad \text{Eq. (3.9)}$$

Where: H is the Shannon diversity index.

P_i is fraction of the entire population made up of species i

S is numbers of species encountered

$\sum$ is sum from species 1 to species s.

Chapter 4 BIODEGRADATION OF ANTIBIOTIC TIAMULIN BY WOOD ROT FUNGI ISOLATED FROM SWINE FARMS

It has found that tiamulin (TIA) is widely used in Thai swine farms and was persistent in swine manure storage, so enhancing TIA degradation in swine waste treatment process before releasing to the environment is really essential, contributing to prevent the spreading of this antibiotic into surroundings. We hypothesize that wood rot fungi with non-specific enzymes could degrade various recalcitrant compounds will be also capable to remove tiamulin. Fungal isolation was conducted with the decay wood materials and swine manure collected from two swine farms that have long period of utilizing TIA and then identification was performed by sequencing the ITS region of rRNA gene. Ability to synthesize ligninolytic enzymes that might be capable on degrading the antibiotic tiamulin has been preliminary accessed with the decolorization test on solid agar medium containing the brilliant green and crystal violet dyes. Positive strains were further examined the tiamulin removal capability in batch cultivation at 10 mg/L for 12 days under aerobic and dark conditions. Ligninolytic enzymatic activities were simultaneously identified in the test to elucidate the key enzyme involving in tiamulin elimination.

4.1 Fungal isolation

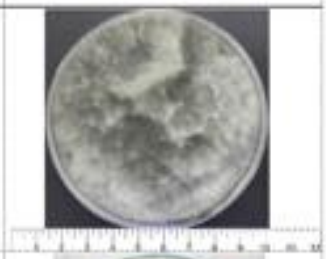
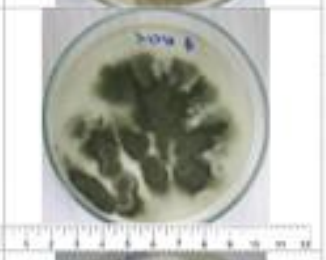
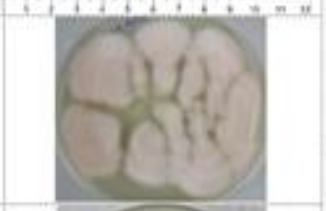
Wood rot fungi were isolated from many rotten wood samples and swine waste materials such as manure, decayed wood, tree trunks, and leaves collected from two swine farms with the covered anaerobic lagoon and stabilizing ponds treatment systems onto PDA plates under aseptic conditions. Based on observing the mycelium characteristics, twelve fungal isolates (strain F1 to F12) with different morphological characteristics were obtained (Table 4.1).

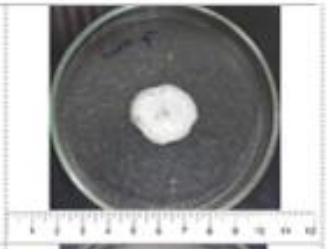
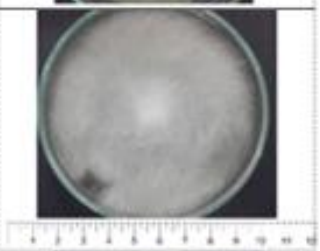
Besides, three reported natural and synthetic dyes degrading white rot fungi including *Trametes hirsuta* AK4 [103], *Trametes versicolor* (TISTR 3224) , *Verticillium* sp. (BCC 27855) were obtained from the Environmental Biotechnology Research Unit of

Prince of Songkla University, Thailand; the Institute of Scientific and Technological Research; and the Thailand Bioresource Research Center, respectively. These strains were selected because of the ability to decolorize some natural and synthetic dyes as well as other recalcitrant pollutants. *Trametes hirsuta* was reported to dephenolize and decolorize treated palm oil mill effluent [103] and endosulfan insecticide [104]; *Trametes versicolor* degraded mixture of metals containing Grey Lanaset G dye, pharmaceuticals, steroid hormones, phytoestrogens, UV-filters, industrial chemicals, and pesticides [102, 156, 157], and *Verticillium* sp. was capable to eliminate pesticide chlorpyrifos [99].

Morphology characteristics and isolation sources of the twelve isolated fungi and three white rot fungi are presented in Table 4.1. Strain F1, F2, and F4 had black and rough mycelia. On the contrary, strain F7 - F12 displayed white color mycelia in which strain F7, F10, and F12 were rough, F9 and F11 were slimy, and F8 was smooth and soft. Strain F3, F5, and F6 had different mycelial color compared with above-mentioned fungi. Indeed, F3 was brownish; F5 was lightly pink, rough, and hard mycelium; and F6 showed yellow, dark blue, and rough. The three obtained white rot fungi also exhibited different appearance characteristics. For example, *Trametes hirsuta* AK4 and *Trametes versicolor* white rot fungi have white rough mycelia, while *Verticillium* sp. has brownish rough and hard mycelia.

Table 4.1 Morphological characteristics and isolation sources of fungi used in this study

Fungal isolate	Source	Mycelial characteristic	Image
F1	Dried grass	Black, rough and thin	
F2	Swine manure	Black and rough	
F3	Green leaf	Brownish mycelium	
F4	Swine manure	Black, rough mycelium	
F5	Swine manure Dried coconut leaf	Lightly pink, rough and hard mycelium	
F6	Decayed wood	Yellow, dark blue, and rough mycelium	

F7	Decayed wood	White, rough mycelium	
F8	Swine manure	White, smooth, and soft mycelium	
F9	Dried banana leaf	White, slimy mycelium	
F10	Dried coconut fruit	White, rough, and aerial mycelium	
F11	Decayed wood	White, slimy mycelium	
F12	Swine manure	White, rough and hard mycelium	

Scale bar on image indicates 1 cm.

4.2 Molecular identification of isolated fungi

Twelve fungal strains with different appearance characteristics were obtained and cultured on PDA plates for further experiment. The quality of genomic DNA extracts was confirmed on 2% agarose gel electrophoresis (Figure 4.1). All fungi displayed their genomic DNA on the gel with the similar bands size larger than 1500 bp.

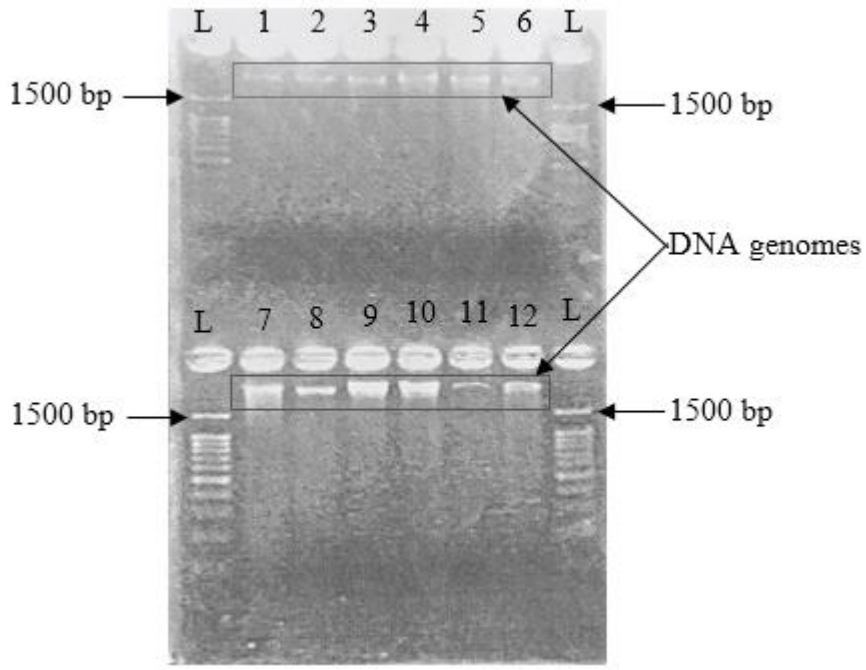


Figure 4.1 Gel documentation of fungal genomic DNA

(L: 100 bp ladder, 1 - 12: Isolated strains F1 to F12)

The internal transcribed spacer in the ribosomal rRNA genes (ITS-rRNA gene) of all twelve isolated fungal genomes were amplified, purified, and sequenced using the universal primers ITS1 and ITS4. The fragments of PCR products obtained were of approximately 400 - 650 bp (Figure 4.2). Strain F8 and F9 showed band length about 400 bp; F1, F5, and F11 had similar bands with approximately 550 bp in length; F2 - F4, F7 and F12 were ~ 600 bp; F6 and F10 showed 650 bp bands. That was in the same range with 400 - 475 bp of fungal DNA amplification using the same pair of primers in previous study [158]. No band appeared in negative control well.

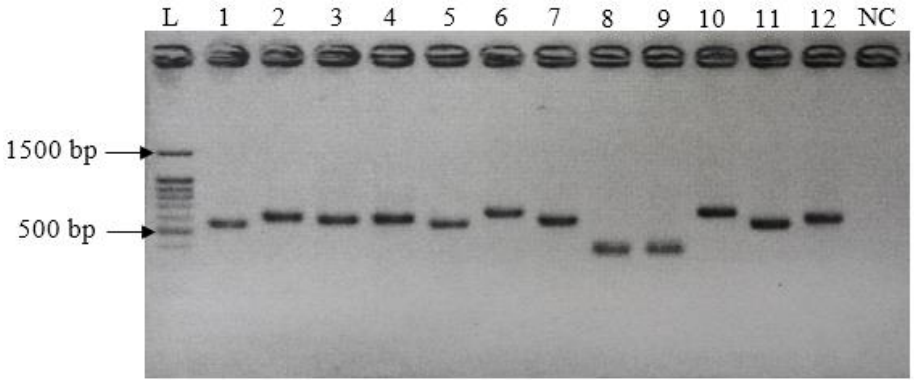


Figure 4.2 PCR products of fungal genomic DNA with ITS1 and ITS4 primers

(L: 100 bp ladder, 1 - 12: Twelve isolated fungi from F1 to F12, NC: Negative control)

The sequencing products of ITS of rRNA gene of all twelve isolated fungal DNA after assembling into one completed sequence were 756, 928, 1029, 661, 721, 1710, 1112, 655, 687, 1089, 1359, and 1144 bp, respectively for F1 - F12 in order (Table 4.2). The DNA sequence information was subsequently compared with that of the database of the National Center for Biotechnology Information (NCBI, USA) using the basic local alignment search tool (BLAST). The closest matched reference sequences of strains F1 to F12 with their corresponding accession numbers are demonstrated in Table 4.4. The nucleotide base sequence similarities were among 91% - 100%.

Based on the BLAST search of the complete sequence of ITS region, the twelve fungal isolates (F1 - F12) were found to be in closest homology of 100% with *Lasiodiplodia theobromae*, 100% with *Curvularia lunata*, 99% with *Aspergillus* sp., 100% with *Penicillium oxalicum*, 100% with *Fusarium* sp., 99% with *Trichoderma* sp., 98% with *Hypocreales* sp., 98% with *Galactomyces geotrichum*, 91% with *Geotrichum* sp., 100% with *Mucor irregularis*, 100% with *Trichosporon asahii*, and 99% with *Penicillium* sp., respectively (Table 4.4). The relationship between the twelve isolated fungi and their reference taxa in phylogenetic tree also revealed the

same information (Figure 4.5). Therefore, the cultures from F1 to F12 were identified as *Lasiodiplodia* sp. F1, *Curvularia* sp. F2, *Aspergillus* sp. F3, *Penicillium* sp. F4, *Fusarium* sp. F5, *Trichoderma* sp. F6, *Hypocreales* sp. F7, *Galactomyces* sp. F8, *Geotrichum* sp. F9, *Mucor* sp. F10, *Trichosporon* sp. F11, and *Penicillium* sp. F12, respectively, with accession numbers from KX224478 to KX224489 in NCBI database. The percent similarities of strain F9, however, were relatively poor only 91%. Since the sequence similarity greater than 97% suggested the possibility of the same genus level [159], this strain might be other genus.

Table 4.2 Identification of isolated fungi

Fungal strain	**Closest matched strains**	**Accession number**	**Identity (%)**
F1 (756 bp)	*Lasiodiplodia theobromae* R1736	KT968475.1	100 (520/520)
	Lasiodiplodia hormozganensis CMM3983	JX464069.1	100 (536/536)
	Lasiodiplodia theobromae Mht-5	JQ658976.1	100 (520/520)
	Lasiodiplodia theobromae CMW28626	GQ469934.1	100 (566/566)
F2 (928 bp)	*Curvularia lunata*	KC462186.1	100 (578/578)
	Curvularia lunata A991	KU232946.1	100

Fungal strain	Closest matched strains	Accession number	Identity (%)
			(573/573)
	Curvularia lunata SQU-MA35	KU945935.1	100 (561/561)
	Curvularia lunata L2858	KC288113.1	99 (588/591)
F3 (1,029 bp)	*Aspergillus aculeatus* NZD-mf39	KM278100.1	99 (521/526)
	Aspergillus japonicus A5	KR708634.1	99 (523/529)
	Aspergillus fijiensis ATCC 20611	KU729079.1	99 (522/528)
	Aspergillus aculeatus AM3	KP418576.1	99 (522/528)
F4 (661 bp)	*Penicillium oxalicum* HP7-1	KJ619601.1	100 (564/564)
	Penicillium oxalicum BM16-3	KJ619590.1	100 (563/563)
	Penicillium oxalicum DB8-2	KJ619596.1	100 (567/567)
	Penicillium oxalicum ME28-1	HM130515.1	100 (552/552)

Fungal strain	Closest matched strains	Accession number	Identity (%)
F5 (721 bp)	*Fusarium* sp. BAB-4835	KU571544.1	100 (526/526)
	Fusarium equiseti	KP026922.1	99 (534/537)
	Fusarium sp. 56GP/F	GQ352485.1	99 (612/618)
	Fusarium chlamydosporum VKM	KM076600.1	99 (542/547)
F6 (1,710 bp)	*Trichoderma* sp. BAB-4696	KR349646.1	99 (602/609)
	Trichoderma longibrachiatum T161	HQ596974.1	99 (599/608)
	Trichoderma longibrachiatum CEN501	KC576707.1	99 (599/607)
	Trichoderma longibrachiatum H09-095	KC009811.1	99 (759/767)
F7 (1,112 bp)	*Hypocreales* sp. TA26-23	JF819145.1	98 (501/513)
	Hypocreales sp. LM141	EF060505.1	89 (440/494)
	Hypocreales sp. BAB-3884	KM066546.1	89 (473/532)
	Hypocreales sp. TA26-55	JF819170.1	89 (457/513)
F8	*Galactomyces geotrichum* UBOCC-A-	KF225047.1	98 (357/366)

Fungal strain	Closest matched strains	Accession number	Identity (%)
(655 bp)	108081		
	Galactomyces geotrichum LMA-38	JF262185.1	98 (375/384)
	Galactomyces geotrichum LMA-20	JF262181.1	98 (375/384)
	Galactomyces sp. SDCF17	HQ657282.1	99 (331/336)
F9 (687 bp)	*Geotrichum candidum* AUMC 10284	KX233856.1	90 (330/366)
	Geotrichum citri-aurantii	JQ083433.1	91 (331/364)
	Geotrichum candidum 2.8	KM265458.1	90 (328/366)
	Geotrichum candidum WLL1	KJ817904.1	90 (331/368)
F10 (1,089 bp)	*Mucor irregularis* CMFCCC B 50m	JX976252.1	100 (591/591)
	Mucor irregularis CBS 654.78	JN206151.1	100 (580/580)
	Mucor irregularis HZ640	KC461926.1	99 (618/620)
	Mucor irregularis AZ1	KM871188.1	99 (614/616)

Fungal strain	Closest matched strains	Accession number	Identity (%)
F11 (1,359 bp)	*Trichosporon asahii* V22	KT900131.1	100 (512/512)
	Trichosporon asahii	AM900369.1	99 (531/533)
	Trichosporon asahii AP.MSU6	KT282395.1	99 (529/532)
	Trichosporon asahii XJU-1	DQ871599.1	99 (522/523)
F12 (1,144 bp)	*Penicillium simplicissimum* PUXX-FS21	KR296890.1	99 (575/579)
	Penicillium simplicissimum MFPX-FS03	KR296877.1	99 (562/565)
	Penicillium simplicissimum DFNP-FS03	KR296860.1	99 (556/560)
	Penicillium sp. 19DL/L	GU066613.1	99 (578/583)

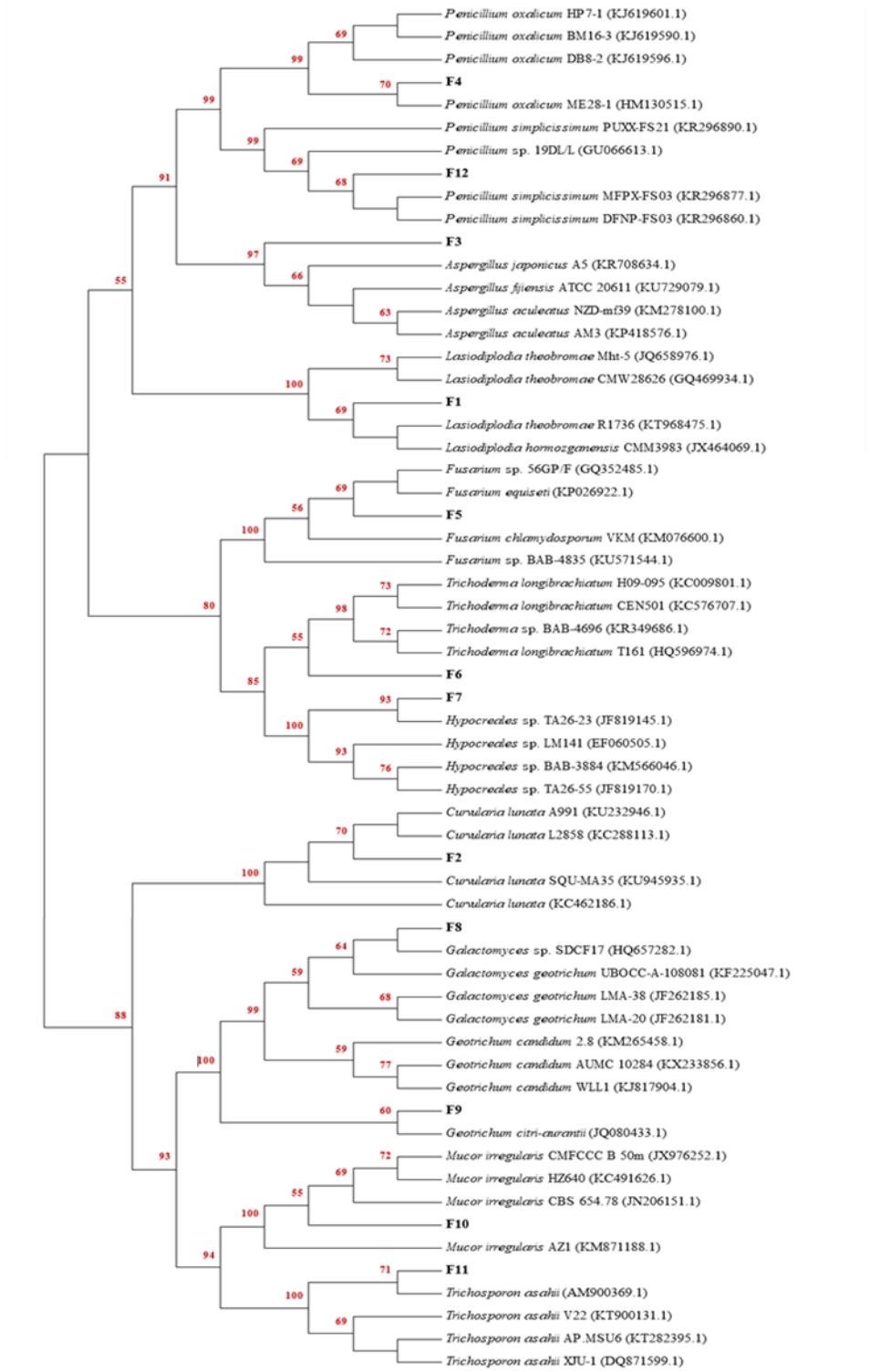


Figure 4.3 Phylogenetic tree of ITS of rDNA sequencing of the twelve swine farm fungal isolates obtained by neighbor-joining analysis with 500 bootstrap numbers

4.3 Dye decolorization capability

Fifteen fungal strains including twelve swine farm isolated fungi (F1 - F12) and three reported dye-degrading white rot fungi (*Trametes hirsuta* AK4, *Trametes versicolor*, and *Verticillium* sp.) were examined the decolorization capabilities on PDA plates containing either brilliant green or crystal violet dyes. Since the diameter of the clear zone suggested the ability of the fungi to produce ligninolytic enzymes, we applied this experiment to prescreen fungal ability on degrading tiamulin. Diameters of fungal mycelia and decolorization zone were measured in mm unit every two days of the 12 days incubation period. The results of 15 fungal developments on PDA and PDA containing brilliant green or crystal violet dyes are presented in Figure 4.4.

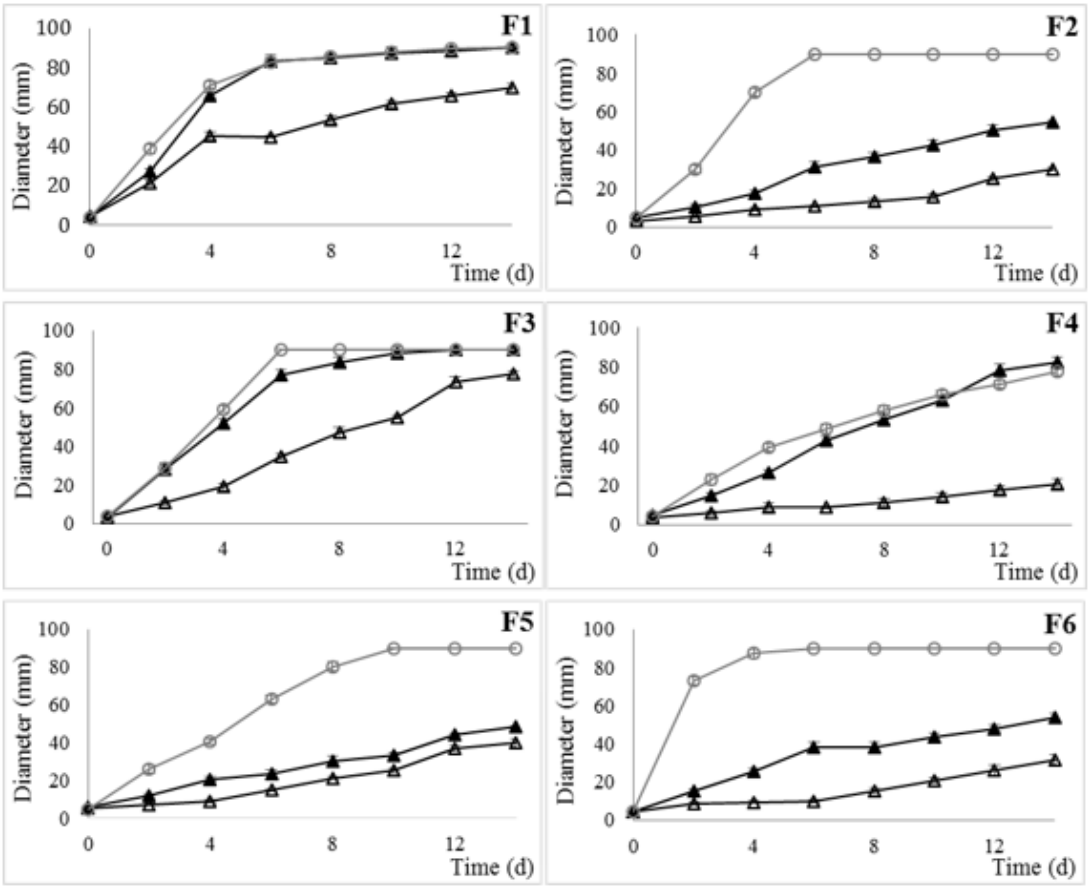


Figure 4.4 Fungal mycelia developed on PDA containing synthetic dyes

(F1 - F12: Twelve swine farm isolated fungi. Error bar indicates the standard error for the triplicate testing).

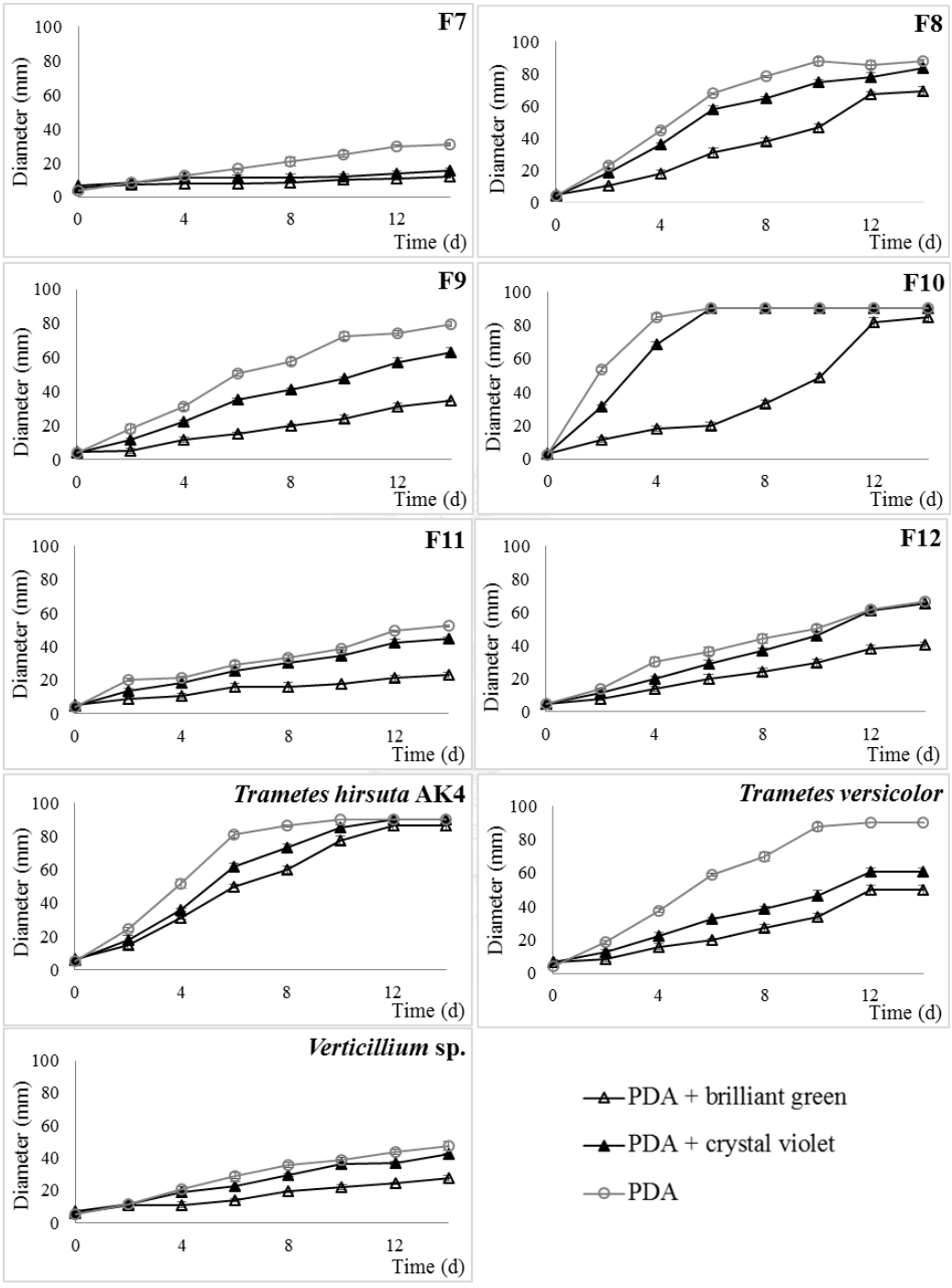


Figure 4-4 (cont.) Fungal mycelia developed on PDA containing synthetic dyes (F1 - F12: Twelve swine farm isolated fungi. Error bar indicates the standard error for the triplicate testing).

All tested strains produced mycelia with diameters ranging from 14 - 90 mm on PDA with crystal violet, and 11 - 87 mm on PDA with brilliant green, and 30 mm to 90 mm on PDA after 12 days of incubation. The growth of all fungal mycelia on PDA plates without dyes was significantly larger than the plates containing either brilliant green or crystal violet dyes (*t*-test *p* value = 0.01). PDA plates supplementing crystal violet had larger fungal mycelia than PDA plates containing brilliant green with statistically meaningful ($p < 0.01$). These results demonstrate the extent of the dyes' inhibitory effects on the growth performance of the fungi, and it was found that brilliant green had a greater inhibitory effect than crystal violet (Figure 4.4 and Figure 4.5).

Ten of fifteen tested strains developed a clear zone with diameter around 28 - 90 mm (Table 4.3 and Figure 4.5). Three pure white rot fungi (*Trametes hirsuta* AK4, *Trametes versicolor*, and *Verticillium* sp.) and three isolated fungi (F1, F5, and F8) highly decolorized both dyes ranging in diameter from 70 mm to 90 mm. Three strains including F3, F6, and F10 only produced clear zones on brilliant green (69, 26, and 28 mm, respectively), while F2 only developed a clear zone of 52 mm on crystal violet. In contrast, F4, F7, F9, F11, and F12 did not produce any clear zones on PDA plates with both dyes.

Size of fungal developments on agar with brilliant green or crystal violet dyes had rather weak correlation to the size of clear zone with the Pearson coefficient of $r = 0.599$ and 0.173, and the significance values of $p = 0.009$ and 0.268, respectively. Brilliant green ($p < 0.01$) showed more significant correlation than crystal violet ($p > 0.01$) in this genuine relationship. This suggested that fungi grew with large mycelial diameters tentatively produced the larger clear zone diameters.

Table 4.3 Decolorization capabilities of wood rot fungi

Fungal strain	Clear zone size			
	Brilliant green		Crystal violet	
	Diameter (mm)	Level	Diameter (mm)	Level
F1	89.6	+++	88.8	+++
F2	0.0	-	50.4	+
F3	69.4	++	0.0	-
F4	0.0	-	0.0	-
F5	90.0	+++	78.6	++
F6	26.4	+	0.0	-
F7	0.0	-	0.0	-
F8	70.2	++	78.5	++
F9	0.0	-	0.0	-
F10	28.1	+	0.0	-

Fungal strain	Clear zone size			
	Brilliant green		Crystal violet	
	Diameter (mm)	Level	Diameter (mm)	Level
F11	0.0	-	0.0	-
F12	0.0	-	0.0	-
Trametes hirsuta AK4	86.9	+++	90.0	+++
Trametes versicolor	82.5	+++	70.5	++
Verticillium sp.	80.0	+++	71.8	++

Decolorization level: +++: 8-9 cm, ++: 7-8 cm, +: < 7 cm, -: No decolorization zone

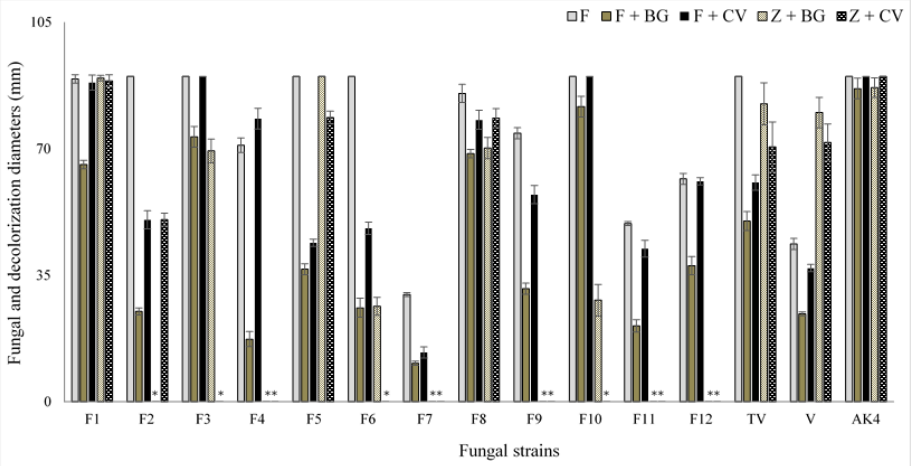


Figure 4.5 Size of fungal mycelia and decolorization zones

F: Fungal mycelial diameters, Z: Clear zone diameter, BG: Brilliant green, CV: Crystal violet,*No clear zone formation, F1 - F12: Isolated fungi, AK4: *Trametes hirsuta* AK4, TV: *Trametes versicolor*, V: *Verticillium* sp. Error bar indicates standard error for the triplicate analyses.

Table 4.4 Growth rates of fungal strains on PDA and PDA containing dyes

Fungal strain	Growth rate (mm/day)		
	PDA	**PDA + BG**	**PDA + CV**
F1	18	10	15
F2	16	2	4
F3	14	6	13
F4	9	1	6
F5	11	3	4
F6	21	2	6
F7	2	1	1
F8	11	5	9
F9	8	2	5
F10	21	7	16
F11	8	2	5

Fungal strain	Growth rate (mm/day)		
	PDA	PDA + BG	PDA + CV
F12	6	3	5
Trametes hirsuta AK4	13	7	9
Trametes versicolor	9	4	5
Verticillium	4	2	3

PDA: Potato dextrose agar, BG: Brilliant green, CV: Crystal violet, F1 - F12: Twelve swine farm isolated fungi.

The growth rates of all wood rot fungi on PDA and the plates containing either brilliant green or crystal violet dye were determined at room temperature (Table 4.4). Strain F6 and F10 fungal remarkably developed their mycelia on PDA plates with the same growth rates of 21 mm/day, but they showed lower growth rates on PDA with dyes (2 and 7 mm/day, 6 and 16 mm/day on PDA with brilliant green or crystal violet). In contrast, strain F7 was the slowest development strain as it only accounted for 2, 1 and 1 mm/day of its growth rate upon the three media. Following F6 and F10, nine remained fungi exhibited their mycelial development in descending order, in which the mean of growth rates of F1, F2, F3, F5, F8, F4, F9, F11, and F12 fungi are 18, 16, 14, 11, 11, 9, 8, 8, and 6 mm/day, respectively on PDA. The growth rates of these strains also showed lower on PDA with crystal violet and lowest on brilliant green PDA.

Three obtained white rot fungi displayed the similar trend of their growth rates with twelve isolated fungi. *Trametes hirsuta* AK4 had the highest growth rate (13, 7, and 9

mm/day on PDA, PDA with brilliant green and crystal violet, respectively), followed by *Trametes versicolor* (9, 4, and 5 mm/day), and *Verticillium* sp. was the lowest growth rate of 4, 2, and 3 mm/day.

All fifteen fungal strains exhibited the same growth pattern, in which fungi growing on PDA without dyes had significantly greater growth rates than fungi living on PDA supplementing dyes (p *t*-test < 0.01). Particularly the growth rates of all wood rot fungi on PDA containing crystal violet were significantly higher than strains on brilliant green-containing PDA plates ($p < 0.01$). These are consistent with the development of fungal diameters on such media, illustrating that dyes, especially brilliant green, were remarkable inhibitory factors on the growth of wood rot fungi.

Finally, fungal strains developing a clear zone larger than 70 mm on the PDA plate with both brilliant green and crystal violet dyes were selected for further experiment to examine their abilities in degrading tiamulin. For this study, the strains of F1, F5, F8, *Trametes hirsuta* AK4, *Trametes versicolor*, and *Verticillium* sp. were obtained for the next tiamulin biodegradation test.

4.4 Tiamulin degradation

The tiamulin degradation efficiency was determined by aqueous cultivation of 10 mg/L tiamulin during 12 days with fungal strains that were capable of degrading dyes, including *Lasiodiplodia* sp. F1, *Fusarium* sp. F5, and *Galactomyces* sp. F8, *Trametes versicolor*, *Verticillium* sp., and *Trametes hirsuta* AK4. Tiamulin concentration was measured along the incubation period as presented in Figure 4.6 - 4.7. The initial concentrations were approximately 10.5 ± 0.1 mg/L. The TIA concentrations of the sorption control and medium control remained stable at around 9.8 and 10.9 mg/L, respectively. The t-test p value of approximately 0.92 ($p > 0.05$) illustrates that the differences of the tiamulin concentrations from the beginning to the end of experiment in the sorption and medium controls were not statistically meaningful. Tiamulin in the tests were not considerably absorbed in the mycelia or degraded abiotically.

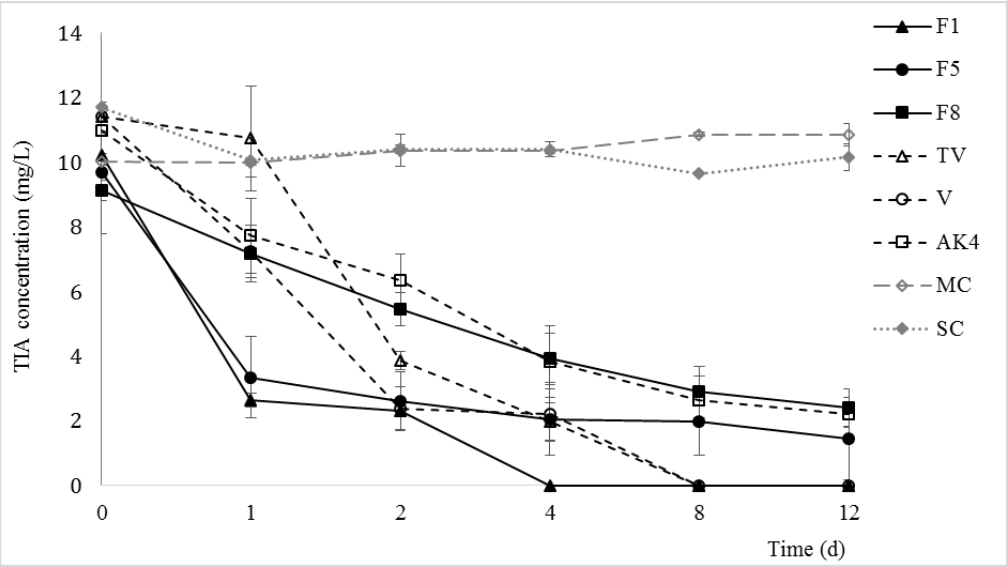


Figure 4.6 Tiamulin degradation during 12-day incubation

Error bar indicates the standard error for the triplicate analyses. F1: *Lasiodiplodia* sp. F1, F5: *Fusarium* sp. F5, F8: *Galactomyces* sp. F8. TV: *Trametes versicolor*, V: *Verticillium* sp., AK4: *Trametes hirsuta* AK4, MC: Medium control, SC: Sorption control, TIA: Tiamulin, d: day.

On another hands, *Lasiodiplodia* sp. F1, *Trametes versicolor*, and *Verticillium* sp. completely transformed the TIA amount to below the detection limit (0.09 mg/L), whereas *Trametes hirsuta* AK4, *Galactomyces* sp. F8, and *Fusarium* sp. F5 removed TIA only 66.8%, 73.8%, and 82.4%, respectively (Table 4.5). The tiamulin concentrations in the fungal test were significantly reduced ($p < 0.05$), indicating the role of fungi in the TIA degradation. The two known white rot fungi (*Trametes versicolor* and *Verticillium* sp.) and one isolated fungus *Lasiodiplodia* sp. F1 were the most effective in TIA removal.

The three fungal isolates with high performance dye decolorization and tiamulin degradation in the current work have been also reported to degrade a variety of pollutants. *Lasiodiplodia* sp. was highly effective in the degradation of endosulfan [105], phenolic compounds [106], plastics [107], and PAHs [108]. *Fusarium* sp. has been able to dissipate PAH [108-110]. *Galactomyces* sp. was shown capable of decolorizing wastewater effluent from the dye industry and removing lincomycin antibiotic residuals [50, 51, 111]. These previous results support our findings on the abilities of the six wood rot fungi to remove tiamulin. It is possible that these fungi might be able to remove other antibiotics and pollutants.

The results of correlation analysis between the formation of decolorization zones and the efficiency of tiamulin degradation revealed the slight negative relationship of r = -0.063 and p = 0.452 for both dyes. *Trametes hirsuta* AK4 exhibited the second largest clear zone development of 88.5 mm average on PDA plate in both dyes but it showed the lowest TIA removal percentage (66.8%), while *Verticillium* sp., *Trametes versicolor*, *Fusarium* sp. F5, and *Galactomyces* sp. F8 with lower clear zone formation (75.9, 76.5, 84.3, and 74.4 mm) displayed higher TIA elimination efficiency (89.3%, 86.1%, 82.4%, and 73.8%). However, this relation could not be applied to *Lasiodiplodia* sp. F1 as it simultaneously exhibited the largest clear zone diameter on the PDA with dyes (89.6 and 88.8 mm for brilliant green and crystal violet, respectively) and the highest effectiveness (93.2%) in getting rid of tiamulin antibiotic. This study suggests that the ability of fungi to degrade dyes does not necessarily correlate with their TIA degradation efficiency.

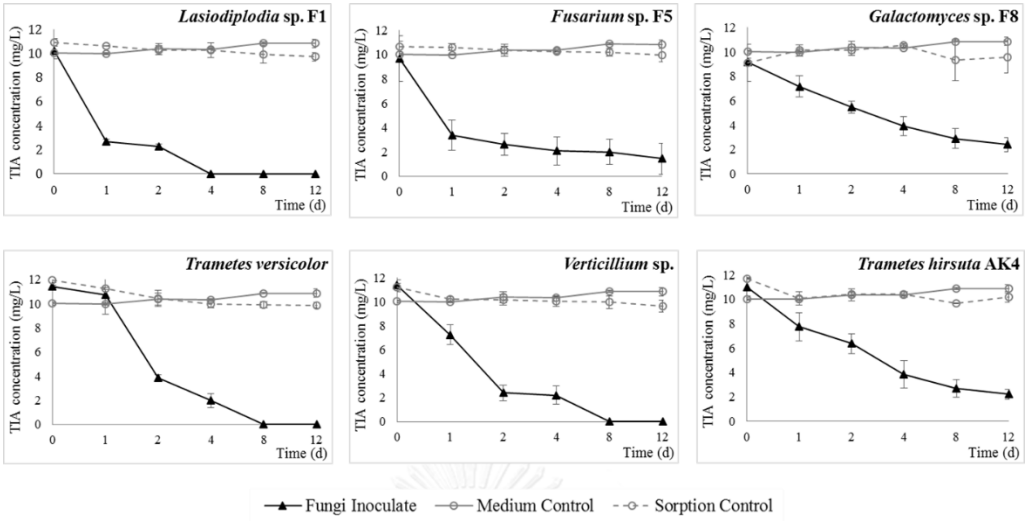


Figure 4.7 Tiamulin degradation using six wood-rot fungi

Error bar indicates the standard error for the triplicate analyses.

The pseudo first-order specific TIA degradation rate was analyzed to compare the tiamulin degradation abilities, as shown in Table 4.5. Different strains of fungi removed tiamulin at different rates. Among all the fungi, the removal rate was the highest with *Lasiodiplodia* sp. F1 with the K_{biol} value of 56.7 mL/g·d. It could remove TIA to below the detection limit (0.09 mg/L) within 4 days with the highest tiamulin removal efficiency of 93.2%. Followed by *Verticillium* sp., *Trametes versicolor* exhibited relatively high effectiveness in tiamulin degradation (89.3 and 86.1%, respectively) that were consistent with tiamulin removal rate values (58.6 and 56.0 mL/g·d, respectively). *Fusarium* sp. F5, *Galactomyces* sp. F8 and *Trametes hirsuta* AK4 displayed low efficiency of tiamulin removal, in which the K_{biol} value was 6.5, 5.6 and 6.85 mL/g·d, correlating with percentage of tiamulin elimination of 82.4, 73.8 and 66.8%.

Table 4.5 Pseudo first-order specific TIA degradation rate and removal efficiency

Fungal strain	**K_{biol} (mL/g fungi·d)**	**TIA removal efficiency (%)**
Lasiodiplodia sp. F1	56.7	93.2 ± 0.0
Fusarium sp. F5	6.5	82.4 ± 10.2
Galactomyces sp. F8	5.6	73.8 ± 5.6
Verticillium sp.	58.6	89.3 ± 0.0
Trametes versicolor	56.0	86.1 ± 0.0
Trametes hirsuta AK4	6.8	66.8 ± 4.8

The average value of the tiamulin removal rates of three wood-rot fungal isolates (22.9 mL/g·d) was lower than that of three pure white-rot fungi (40.5 mL/g·d) The known white-rot fungi removed tiamulin at rates ranging from 6.8 to 58.6 mL/g·d, while the isolates removed tiamulin at rates ranging from 5.5 to 56.7 mL/g·d. Our results demonstrate the fungi found in farms can degrade tiamulin slightly less effectively than pure white-rot fungi.

4.5 Enzymatic activity of fungi

The activity of laccase (Lac), manganese peroxidase (MnP) and lignin peroxidase (LiP) in *Lasiodiplodia* sp. F1, *Fusarium* sp. F5, *Galactomyces* sp. F8, *Trametes versicolor*, *Verticillium* sp., and *Trametes hirsuta* AK4 on degrading of 10 mg/L of

tiamulin are presented in Figure 4.8. MnP activity was found in all tested strains with the maximum MnP activity of 55.8, 51.3, 59.3, 47.9, 43.9, and 33.1 U/L of crude extract, respectively, during the incubation period of 12 days. The MnP activity tended to increase during the first four days, but decrease later on. Only the *Trametes hirsuta* AK4 strain showed an increase of MnP activity on day 12.

The maximum Lac activities were low in all above strains (i.e. 4.9, 2.6, 0.9, 0.9, 0.7, and 0.4 U/L for *Trametes hirsuta* AK4, *Fusarium* sp. F5, *Lasiodiplodia* sp. F1, *Galactomyces* sp. F8, *Verticillium* sp., and *Trametes versicolor*). LiP was also found at low activity in strains *Trametes versicolor*, *Verticillium* sp., *Fusarium* sp. F5, and *Galactomyces* sp. F8, in which the maximum LiP activity was 3.3, 0.5, 0.3 and 0.1 U/L, respectively. LiP activity was not found in *Lasiodiplodia* sp. F1 and *Trametes hirsuta* AK4. It is obviously that MnP activity was more dominant than Lac and LiP activities

This circumstance was also observed in previous experiments in which MnP from white rot fungus *Phanerochaete chrysosporium* was the main enzyme that could degrade 72.5% and 84.3% of tetracycline and oxytetracycline antibiotics [49]. Furthermore, other authors have revealed *Phanerochaete chrysosporium* and *Trametes versicolor*, with low MnP levels (5 - 48 U/L), to be effective in the elimination of anti-inflammatory drugs [94, 116]. In a recent study, the *Trametes hirsuta* strain AK4 illustrated the greatest MnP activity at 3,046 U/L when eliminating phenolic compounds and color [103]. These results demonstrate that MnP likely plays a crucial role in TIA degradation.

However, many previous studies have indicated that Lac and other enzymes are related to recalcitrant compounds degradation. One study, for instance, reports that tetracycline antibiotics could be mainly degraded by the Lac enzyme of *Trametes hirsuta* [44, 118]. *Lasiodiplodia* sp. also exhibited a major contribution of the Lac enzymes on plastic and phenolic-compound degradations [106, 107, 119]. *Lasiodiplodia* sp. and *Fusarium* sp. can utilize PAHs owing to their lipase productions [108]. The production of ligninolytic enzymes can be considerably affected by

different fungal inducers, cultivation conditions, and target compounds. Therefore, the dominant role of MnP on degrading tiamulin in this study might have been related to the culturing characteristic of this study.

All of six fungi that could manufacture ligninolytic enzymes (MnP dominantly) to eliminate tiamulin could also exhibit such enzymatic activities in condition of tiamulin absence (Figure 4.9). The maximum MnP activities of strains *Lasiodiplodia* sp. F1, *Fusarium* sp. F5, *Galactomyces* sp. F8, *Trametes versicolor*, *Verticillium* sp., and *Trametes hirsuta* AK4 in TIA absent test were 34.2, 25.1, 33.1, 39.3, 33.6, and 42.7 U/L, respectively, while they were 55.8, 51.3, 59.3, 47.9, 43.9, and 33.1 U/L in the TIA present condition. The MnP activity reduced in cultivation without the tiamulin in all tested strains, except only *Trametes hirsuta* AK4 that had an increase. In general, the amounts of MnP produced in the presence of tiamulin were significantly greater than those in without TIA conditions ($p < 0.05$, Two-factors ANOVA analysis). This suggested that MnP production was induced by tiamulin. While the Lac and LiP activities in the degradation tests under presence and absence of tiamulin were not statistically significant ($p = 0.2$). Though MnP might be constitutive enzyme, it was likely to play a major role in TIA degradation. Further study is needed to clarify.

MnP activity was found significant increase in degradation of recalcitrant pollutants with supplementation of glucose as cosubstrate. Color removal rate of high strength landfill leachate *Trametes versicolor* fungus was proportional to the ligninolytic enzymatic activity in treatment with glucose [160]. Another study indicated that *Trametes trogii* extracellular fluids obtained in the medium containing glucose which rendered the highest ligninolytic production of MnP, also showed the greatest ability to degrade dyes [161]. In our case, glucose also present in the basal medium suggesting that relationship between tiamulin and glucose might be as a co-substrate using ligninolytic enzymes.

Catalysis of organic or xenobiotic compounds by ligninolytic enzymes was descrised by many previous authors. The ligninolytic enzymes performed a one-electron

oxidation, thereby generating cation radials of the contaminants. The cation radicals might undergo spontaneous chemical reactions such as C–C cleavage or hydroxylation resulting in more hydrophilic products. These products were taken up by the fungal cells and cometabolized in the presence of a proper carbon source to carbon dioxide [162]. The most common ligninolytic peroxidases produced by almost all white-rot basidiomycetes and by various litter-decomposing fungi are manganese peroxidase. MnP preferentially oxidizes Mn^{2+} into Mn^{3+}, which is stabilized by chelators, itself also excreted by the fungi. Mn^{3+} acts as a highly reactive low molecular weight, diffusible redox-mediator. Thus MnP were able to oxidize and depolymerize their natural substrate such as lignin and recalcitrant xenobiotics [115]. MnP was found to simultaneously decompose organic acids, excreted by fungal organisms, oxidatively and oxidized Mn^{2+} to Mn^{3+} even in the absence of H_2O_2. Thus, organic acids are postulated to be the origin of carbon-centered radicals (acetic acid radicals, $COOH–C{\cdot}H_2$), peroxyl radicals ($COOH–CH_2OO{\cdot}$), superoxide ($O_2{\cdot}^-$), formate radicals ($CO_2{\cdot}^-$). Such radicals could be a source of peroxides, which can be used by MnP as substrates instead of H_2O_2. Consequently, even fungi obviously lacking H_2O_2-generating oxidases could be efficient lignin-degraders and, by extension, useful in the degradation of xenobiotics suchas dyes [115, 163].

The fungal isolates and tiamulin degradation characteristics obtained in this work are useful for developing practicable applications for eliminating tiamulin or other antibiotics in contaminated sites. Fungal mycelia or crude enzyme solutions might be directly inoculated onto swine manure or supplemented into the storing or composting process. This will potentially create viable high efficiency, low-cost solutions for the bioremediation of antibiotic tiamulin and other recalcitrant pollutants.

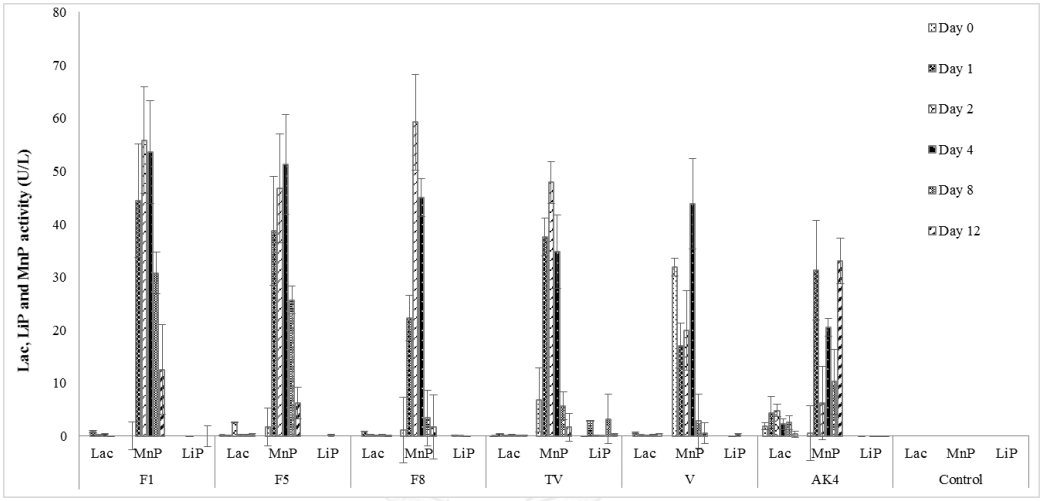


Figure 4.8 Enzymatic activities of six fungi during TIA degradation experiment

Lac: laccase, MnP: manganese peroxidase, LiP: lignin peroxidase, F1: *Lasiodiplodia* sp. F1, F5: *Fusarium* sp. F5, F8: *Galactomyces* sp. F8, TV: *Trametes versicolor*, V: *Verticillium* sp., AK4: *Trametes hirsuta* AK4. Error bar indicates the standard error for the triplicate analyses.

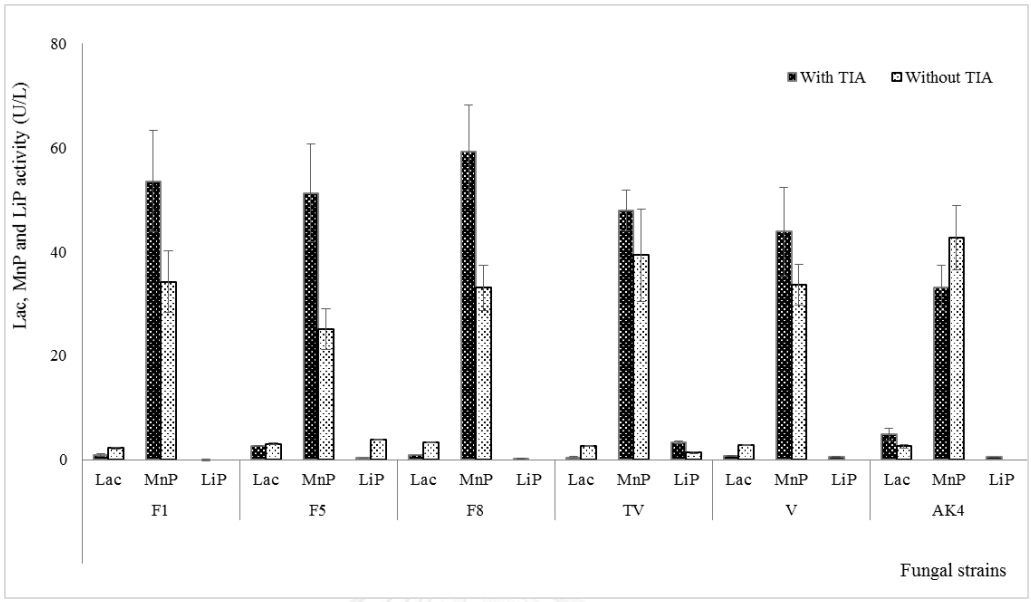


Figure 4.9 Enzymatic activities of six fungi in basal medium with and without tiamulin substrate

Lac: laccase, MnP: manganese peroxidase, LiP: lignin peroxidase, TIA: Tiamulin, F1: *Lasiodiplodia* sp. F1, F5: *Fusarium* sp. F5, F8: *Galactomyces* sp. F8, TV: *Trametes versicolor*, V: *Verticillium* sp., AK4: *Trametes hirsuta* AK4. The error bar indicates the standard error for the triplicate analyses.

4.6 Chapter conclusions

This chapter attempted to obtain wood rot fungi that are capable of degrading tiamulin as they generally produce nonspecific lignolytic enzymes capable of degrading various recalcitrant compounds. Decolorization of brilliant green and crystal violet dyes has been applied for prescreening process of enzyme production. Among twelve fungal isolates from swine farms, *Lasiodiplodia* sp. strain F1, *Fusarium* sp. strain F5 and *Galactomyces* sp. strain F8 produced a clear zone to both dyes and degraded 10 mg/L of tiamulin at 93.2%, 82.4%, and 73.8% with rate 56.7, 6.5, and 5.6 mL/g·d over 12-day period. The tiamulin degradation by other reported dye degrading cultures including *Verticillium* sp., *Trametes versicolor*, and *Trametes hirsuta* AK4

were also observed with efficiency of 89.3%, 86.1%, and 66.8% and rate of 56.0, 58.6, and 6.8 mL/g·d. The higher clear zone diameters exhibited the higher TIA removal rates. The enzymatic activity test illustrated that MnP was mainly involved in degradation of tiamulin. Three fungal strains *Lasiodiplodia* sp. F1, *Verticillium* sp., and *Trametes versicolor* with more than 85% of tiamulin removal efficiency are potentially be used for enhancing the removal of tiamulin in contaminated sites. Manganese peroxidase was largely produced suggesting it main role in tiamulin degradation.

Chapter 5 BIODEGRADATION OF ANTIBIOTIC TIAMULIN BY BACTERIAL ENRICHMENT CULTURES FROM SWINE WASTEWATER

Tiamulin could be degraded by wood rot fungi isolated from swine farms, but it required up to 12 days to be eliminated completely. Subsequently, Tiamulin biodegradation was examined with bacterial cultures enriched from swine wastes as they may accelerate the degradation process. After seven months of enrichments at two cultivations (without and with other carbon source nutrient broth) under aerobic and dark conditions, the enriched cultures were examined for their tiamulin biodegradation efficiencies in mineral salt medium supplementing tiamulin at 2.5 - 200 mg/L for 16h period. Moreover, the kinetics of tiamulin degradation and the cell growth rates were estimated as well. Tiamulin removal efficiencies between different bacterial enriched cultures and different tiamulin concentrations were compared in this chapter.

5.1 Enrichment cultures of tiamulin-degrading bacteria

Mixture of swine waste samples collected from various locations of the two swine farms (i.e. stabilization ponds and the covered anaerobic lagoon processes) was used as sources of TIA-degrading bacteria. The enrichment of TIA-degrading bacteria was performed at tiamulin concentration increasing from 4 to 20 mg/L in two cultivated conditions (with and without nutrient broth). Enriched cultures were transferred to new medium containing with increasing TIA concentration and/or reducing nutrient broth concentration when cell numbers were approximately 10^5 CFU/mL. After seven months with fourteen times subcultures, four enrichment cultures including of A, AN, S, and SN were obtained. Cultures A and AN originated from a covered anaerobic lagoon system supplied with tiamulin only or with nutrient broth, respectively. S and SN cultures had brought from a stabilization ponds system which further cultivate in

tiamulin or with nutrient broth addition. Such four bacterial enriched cultures were used for experiments of tiamulin biodegradation.

5.2 Biodegradation of tiamulin by bacterial enrichment cultures

The abilities of four bacterial enriched cultures A, AN, S, and SN on degrading tiamulin at various initial concentrations of 2.5, 5, 10, 20, 30, 50, 70, 100, 150, and 200 mg/L were examined and demonstrated in Figure 5.1. It was obviously presented that the tiamulin concentrations in all treatments were significantly reduced over the 16-hour experimental period. Particularly the culture S eliminated tiamulin only a half of day in all concentrations. Average tiamulin concentrations of day 0 were 2.7, 5.1, 10, 20, 30.1, 50.3, 70, 100.8, 150.3, 210.6 mg/L, respectively; however, these concentrations greatly reduced to 0.3, 0.3, 0.8, 1.1, 0.3, 4.3, 5.8, 15.3, 34.8, and 42.3 mg/L, respectively on day 16. Student's *t*-test showed *p* value of 0.005 indicating the differences of tiamulin concentrations between the beginning and the end of tiamulin degradation test were statistically meaningful. In contrast, tiamulin at all initial concentrations were constant in all of the abiotic controls ($p = 0.5$, *t*-test) after the 16 hours testing. This indicated that the biosorption, hydrolysis, and other abiotic mechanisms did not obviously contribute to remove of tiamulin.

Growth of bacteria were observed at initial, middle and at the end of the experimental periods along with tiamulin degradation experiments (Figure 5.1). The bacterial enumeration results showed that the numbers of microbes at the beginning of approximately 1.1×10^8 CFU/mL slightly increased after 8 hours (~ 5.6×10^8 CFU/mL) and significantly increased (approximate to 3 orders of magnitude, $p < 0.05$, *t*-test) after 16 hours (3.2×10^{10} CFU/mL, 1.8×10^{11} CFU/mL, 2.7×10^{11} CFU/mL, and 1.6×10^{10} CFU/mL for cultures A, AN, S, and SN, respectively). Analysis of Student's *t*-test indicated that the numbers of colonies significantly increased after 16h of the experimental period with $p < 0.05$.

In combination between the measurement of tiamulin concentrations and the enumeration of bacterial numbers throughout the tiamulin degradation test, it was found that the tiamulin concentrations decreased, while the microbial density

increased. The inverse relationship between tiamulin concentrations and density of bacteria in the experiments implied that bacteria performed growth during the experimental periods. Therefore, it can be concluded that the degradation of tiamulin by the four bacterial enriched cultures from swine wastes was performed by microbial catabolism.

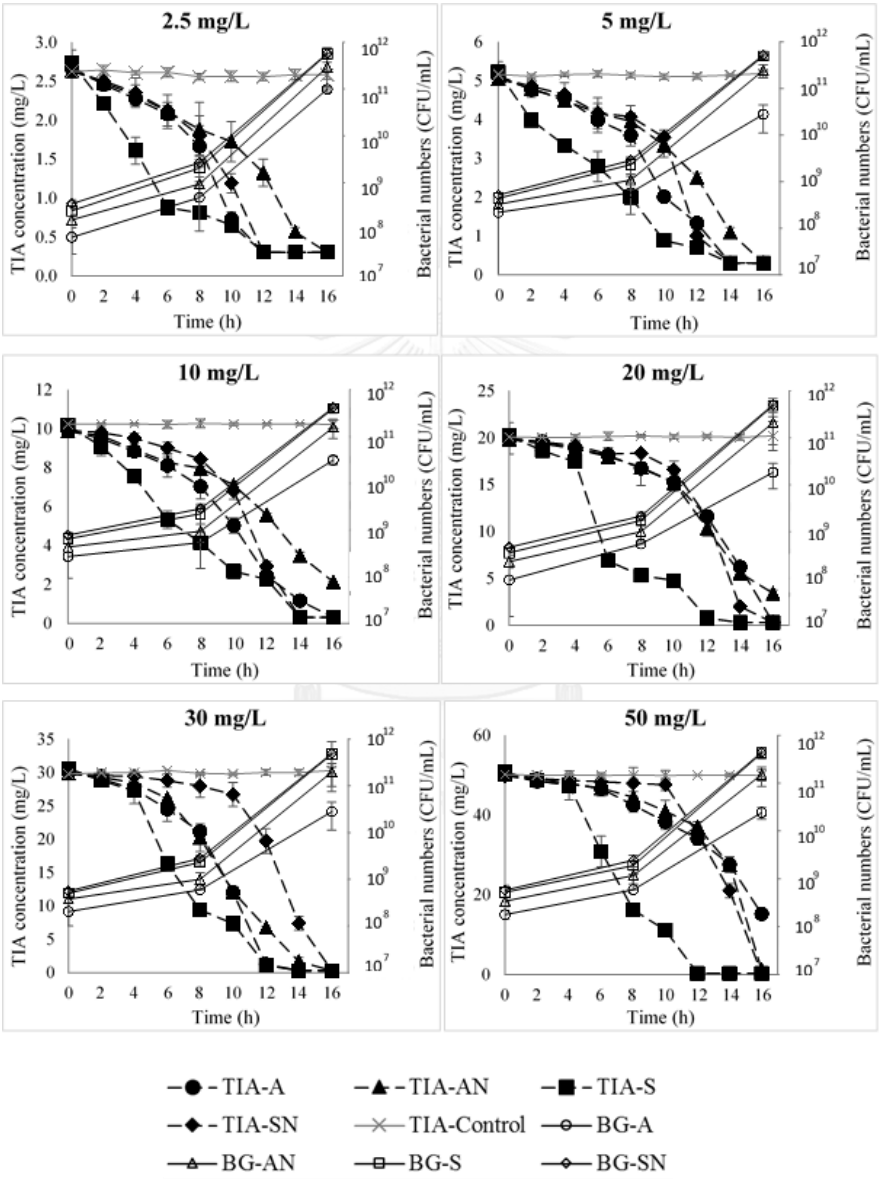


Figure 5.1 Tiamulin degradation by bacterial enriched cultures during 16 h period

The error bar indicates the standard error for the triplicate testing.

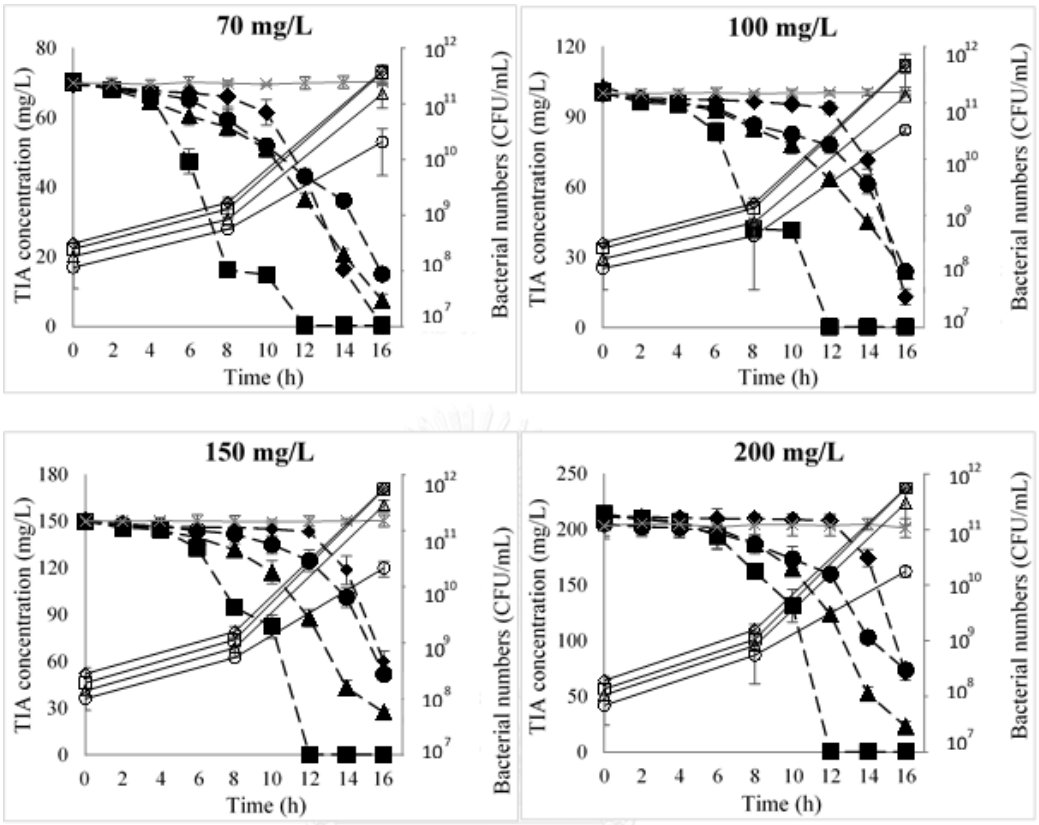


Figure 5-1 (Cont.) Tiamulin degradation by bacterial enriched cultures during 16 h period

Current results were in agreement with one study that revealed 100 mg/kg tiamulin could be efficiently degraded to 8.75 mg/kg (91%) in aerobic composting at high temperature (60^{o}C) [164]. However, this finding was inconsistent with other studies that found the tiamulin was persistent in liquid manure with half-life more than 200 days [27] and it was degraded very slowly in anaerobic conditions [42].

It can be seen in Figure 5.1 that the tiamulin removal rates were slow at the beginning stage (0 - 8 h) and was faster at later stage (8 - 16 h). The slow biodegradation rates at the beginning of the experiment showed that lag-phase was possibly needed for the microbes to pre-adapt to different tiamulin concentrations. The pre-adaptation period of bacteria in experiment performing antibiotic degradation was previously recorded;

for instance, the degradation of salinomycin, tylosin, and tetracycline needs a certain period of time (from 12 days to 6 months) to adapt to presence of these antibiotic [52, 165, 166].

The microbial cultures in this study were enriched by gradually increased tiamulin concentration and were conducted under aerobic condition. Tiamulin could definitely degraded more rapidly in the current work than in the previous research at room temperature. This difference may result from deviation in experimental conditions such as the microbial community structure, chemical structure, and experimental conditions.

Experimental conditions, particularly oxygen availability, considerably promoted biodegradation of recalcitrant pollutants in previous studies. Bacteria isolated from tylosin-containing solid waste could completely degrade 50 mg/L of tylosin within 72 hours under aerobic condition at pH 6.0 and temperature of 30^{o}C [166]. Tylosin is an antibiotic belonging to macrolide which is in the same group with tiamulin [21]. This study showed that tiamulin at initial concentration of 50 mg/L was removed at the rates ranging from 97.4% to 99.4% within 16 hours under pH 7.5 and temperature of 28 - 30^{o}C. This rate was more effective than the above rate of tylosin removal.

Effectiveness of antibiotic removal has also influenced by bacterial community structure. For instance, tylosin was removed by 99% in 40 days composting [167], but it was not removed at all by activated sludge process [168]. Additionally, previous study found that tetracycline was partially degraded (46%) under methanogenic condition, but it was not removed under nitrate- and sulfate- reducing conditions due to different susceptibilities of microbes to tetracycline [127].

Structures of antibiotic compounds have definitely effect on degradation efficiencies. Tetracycline, oxytetracycline, and chlortetracycline were in the same antibiotic groups, but they have different degradable degrees. Oxytetracycline and chlortetracycline were totally removed, whereas tetracycline was remained at 7.1% after 20 days incubation with bacterial enriched cultures [52]. This point explains why

tylosin and tiamulin were degraded differently although they are in the same group of macrolides.

Thus, the dissimilarities of experimental conditions, bacterial community structure, and chemical structure are main drivers for differences in biodegradation rates of antibiotic in among the previous studies and the current study.

5.3 Comparison of tiamulin removal efficiencies by different enriched cultures

Figure 5.2 shows tiamulin degradation at ten different tiamulin concentrations of four bacterial enriched cultures of A, AN, S and SN. Culture S that enriched without nutrient broth effectively degraded tiamulin from all of the ten initial tiamulin concentrations to lower than the limit of detection (0.3 mg/L) within 12 hours. For culture A and SN, tiamulin was reduced completely from 2.5 mg/L within 12-hour, while culture AN needed 16 hours. In treatment of 50 mg/L, tiamulin concentration decreased to 0.3, 1.2, and 15.2 mg/L after 16 hours in culture SN, AN, and A, respectively. For the treatment of 100 mg/L, culture SN, AN and A removed tiamulin down to 13.1, 23.9, and 24.1 mg/L at the end of experiment, respectively. At 200 mg/L, culture AN displayed higher efficiency in tiamulin degradation as compared to culture SN and A since the residual concentrations at 16-hour were 23.2, 71.7, and 73.9 mg/L for the three cultures, respectively. In case of stabilization pond samples, the enriched culture not containing nutrient broth (S) always eliminated TIA at faster rate than the one containing nutrient broth (SN). In contrast, for the cases of anaerobic lagoon samples, the one without nutrient broth (A) could degrade faster than the one with nutrient broth (AN) only in 2.5 mg/L concentration. AN was more efficient in other higher concentrations but was not obviously different. Therefore, it could not conclude that the supplementing broth in enrich process could enhance tiamulin removal. This was depended on sample source.

Tiamulin at all concentrations was largely removed after 16 h of the experimental period. Tiamulin was removed at the rates of 65.5 - 99.7%, 76.8 - 99.0%, 88.9 - 99.9% and 60.1 - 99.6% in the kinetics experiments using consortium of microbes from culture A, AN, S, and SN, respectively. At low tiamulin concentrations of 2.5

and 5 mg/L, it was removed with the similarly high efficiencies (~ 89% and ~ 94%) for all of the four cultures; however, tiamulin removal efficiencies were different at higher concentrations, particularly at 150 mg/L and 200 mg/L of tiamulin. For example, cultures AN and S were still able to degrade tiamulin with high removal efficiencies of 81.8% and 99.8% at 150 mg/L, and of 89.1% and 99.8% at 200 mg/L tiamulin, while tiamulin removal effectiveness of cultures A and SN was much lower at the two highest tiamulin concentrations (approximately 60 - 66%) in compared to those at lower tiamulin concentrations (~ 86.9% to 99.6%). This is in accordance with the growth of bacteria of all of the four cultures in kinetic experiment at these different tiamulin concentrations. Bacterial numbers only increased approximate to 2 orders of magnitude for cultures A and SN, while cultures AN and S significantly developed their bacterial number almost 3 orders of magnitude after 16 hours of tiamulin degradation tests at the two highest tiamulin concentrations. Those make indicate that tiamulin at high concentrations (150 mg/L and 200 mg/L) might inhibit microbial growth of cultures A and SN leading to restrict tiamulin degradation. Similar result was also reported for the inhibitory effect in the previous study. For example, tylosin and sulfamethoxazole antibiotics resulted in inhibitory effect on biodegradation ability of microbes at higher concentration [125, 166]. In order to overcome inhibitory impact, the enriched cultures should be gradually acclimated to target tiamulin concentration. The acclimation could improve removal efficiency of tiamulin.

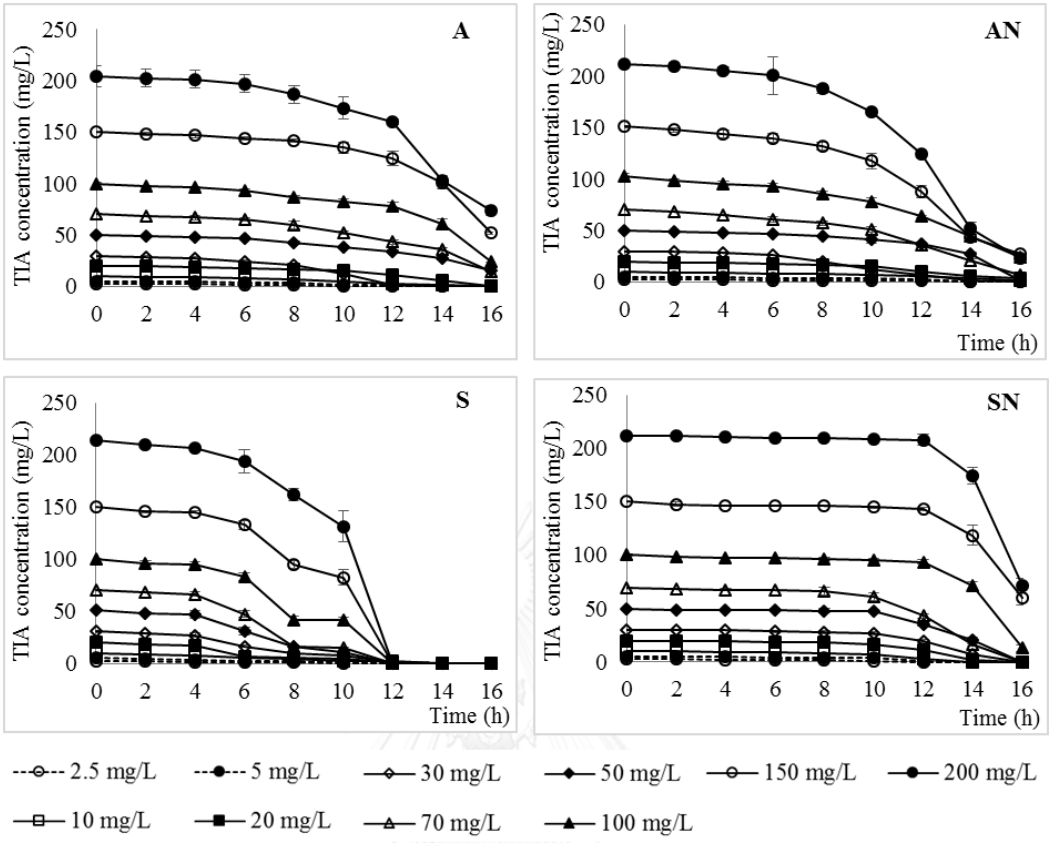


Figure 5.2 Tiamulin degradation by four bacterial enriched cultures

The error bar indicates the standard error for the triplicate testing.

5.4 Tiamulin degradation kinetics

In order to describe relationship between growth of microbes in the enriched cultures who use tiamulin as substrate, Monod model should be used. However, the kinetics experiments were only conducted within 16h. This was a short-term experiment, monitoring of growth was difficult. Therefore, concentrations of tiamulin versus tiamulin degradation rates were used to fit into Monod model to describe the ability of microbes in the enriched cultures in using tiamulin.

The kinetics of tiamulin biodegradation using the four bacterial enriched cultures was performed by varying initial tiamulin concentrations ranging from 2.5 to 200 mg/L. Tiamulin is the main carbon source for microbes in the enriched cultures, when

tiamulin concentration increased, rate of tiamulin consumption also increased from 2.5 - 200 mg/L. The plot of initial tiamulin concentration versus tiamulin consumption rate was addressed by Monod kinetics.

Two important kinetic parameters are the half-saturation constant (K_s) and the maximum tiamulin consumption rate (q_{max}). Half-saturation constant is the Monod kinetic parameter used to indicate the affinity between microbes and substrate utilization. It is also used to predict environmental conditions especially substrate concentration where microorganisms are occupying and functioning.

In this study, half-saturation constant of K_s values were 190.9, 469.6, 2001.0, and 206.7 mg/L for cultures A, AN, S, and SN, respectively (Figure 5.4 and Table 5.1). These K_s values were very high which means that testing microbes in all of four mixed cultures exhibited a low affinity to tiamulin, indicating that the microbes in the cultures could degrade tiamulin at high concentration. Moreover, values of maximum tiamulin consumption rate (q_{max}) of such four above-mentioned cultures were 30.7, 63.5, 269.2, and 48.5 mg/L·h, respectively, making more evidently to conclude that tiamulin could be eliminated by all four bacterial enrichment cultures at high concentration.

When comparing the half-saturation constant (K_s) between two cultures from swine waste samples of the covered anaerobic lagoon system, culture AN (enriched with adding nutrient broth as other carbon source) had higher K_s than culture A (enriched with only tiamulin as sole carbon source) indicating that the culture A can used tiamulin at lower concentration than culture AN. The maximum tiamulin consumption rates (q_{max}) in these two cultures showed such consistent tendency as q_{max} of culture AN (63.5 mg/L·h) was higher than q_{max} of culture A (30.7 mg/L·h). This revealed that tiamulin could be removed at higher concentration by bacteria in culture AN than those in culture A.

Two bacterial cultures (S and SN) enriched from swine stabilization ponds source under two different conditions (without and with nutrient broth supplementation) showed a contrary trend with cultures from covered anaerobic lagoon process. Culture

of no addition of nutrient broth (S) had higher half-saturation constant (K_s: 2001.0 mg/L) and maximum tiamulin consumption rate (q_{max}: 269.2 mg/L·h) than the culture without nutrient broth supplementation in which both values were 206.7 mg/L and 48.5 mg/L·h, respectively. This illustrated that culture S was more effective than culture SN in biodegradation of tiamulin at higher concentration.

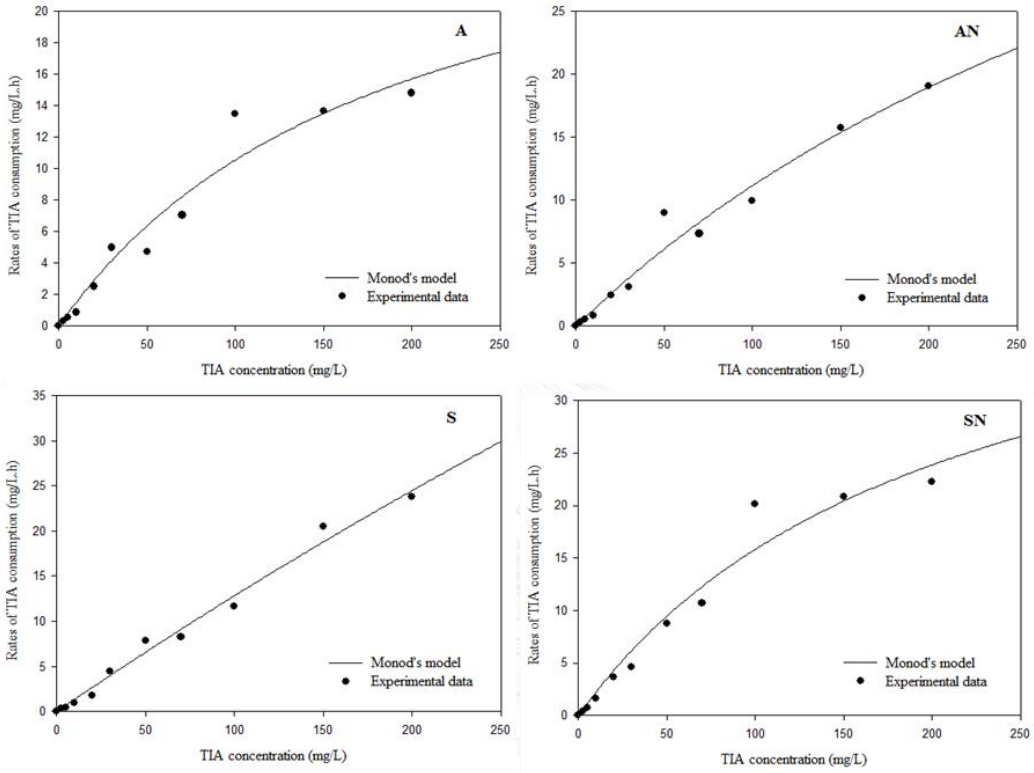


Figure 5.3 Monod curves for enriched cultures in degradation of tiamulin

Table 5.1 Kinetics values of four enriched cultures

Culture	K_s (mg/L)	q_{max} (mg/L·h)	R^2
A	190.9	30.7	0.96
AN	469.6	63.5	0.97
S	2001.0	269.2	0.99
SN	206.7	48.5	0.96

(K_s: Half-saturation constant, q_{max}: Maximum tiamulin consumption rate)

Bacteria were capable to degrade various complex pollutants such as hydrocarbon, aromatic compounds [169], and pesticides [170] in aerobic condition. Under oxic conditions, in most degradation pathways, oxygen was used as a co-substrate in mono- or dioxygenase reactions. Tiamulin with cyclic pleuromutilin structure and hydrocarbon chain [58] may be oxidized by monooxygenases to form alcohols. The alcohol is then converted to the ketone by a dehydrogenase. The ketone is oxidized by a monooxygenase like in subterminal oxidation of alkanes and a cyclic ester (lactone) is formed. Finally, the lactone can be hydrolysed to form a hydroxyl acid [171].

5.5 Comparison of tiamulin degradation by wood-rot fungi and bacterial enriched cultures

Efficiency of tiamulin degradation by using wood-rot fungi was compared with that by using bacterial enriched cultures at the initial concentration of 10 mg/L tiamulin. It can be seen from the Table 5.2 that six wood-rot fungi, including three swine farm fungal isolates (*Lasiodiplodia* sp. F1, *Fusarium* sp. F5 and *Galactomyces* sp. F8) and

three white-rot fungi (*Trametes hirsuta* AK4, *Trametes versicolor* and *Verticillium* sp.) could degrade 66.8 - 93.2% of tiamulin at rates of 5.6 - 58.6 mL/g fungi·d, while four bacterial enrichment cultures (A, AN, S, and SN) were able to remove tiamulin at initial concentrations of 10 mg/L with the rates of 78.4 - 97.0% and 103.4 - 288.4 mL/g bacteria·d. In combination, it is obviously concluded that bacterial mixed cultures are capable to eliminate tiamulin with higher removal efficiencies and at higher tiamulin concentration than wood-rot fungi.

Table 5.2 Comparison of TIA degradation by wood-rot fungi and bacterial enriched cultures

Parameter	**Wood-rot fungi**	**Bacterial enriched cultures**
TIA removal efficiency	66.8 - 93.2%	78.4 - 97.0%
TIA consumption rate	5.6 - 58.6 mL/g fungi·d	103.4 - 288.4 mL/g bacteria·d

Previous finding demonstrated that bacteria make excellent players in biodegradation of any given substrate provided with right conditions because bacteria are widely diverse organisms and usually show superior results in their numerous advantages ranging from their highly specific biochemical reactions to their capabilities of breaking down pollutants efficiently. So bacteria are most commonly used in current applications of biodegradation [172].

Fungi, in contrast, are primarily underutilized for biotechnological purposes because of their lower efficiencies in term of pollutant elimination in comparing with bacteria. However, in some cases, fungi have an advantage over bacteria not just in metabolic versatility but also their environmental resilience. Actually, fungi are able to oxidize a diverse amount of chemicals and tolerate extreme environmental conditions such as low moisture and high pollutant concentration. While many microbial organisms that are used for bioremediation require pre-conditioning of the environment for them to

survive in, fungi can be directly applied into most systems because they degrade based upon nutrient deprivation [173, 174].

5.6 Chapter conclusions

Four microbial enrichment cultures included A (covered anaerobic lagoon source), AN (covered anaerobic lagoon source, Nutrient broth), S (stabilization pond source), and SN (stabilization pond source, Nutrient broth) were obtained under aerobic and dark conditions after seven months of acclimation with 14 subculturing times. These four cultures were able to remove tiamulin at concentrations of 2.5 mg/L - 200 mg/L with the rates of 65.5 - 99.7%, 76.8 - 99.0%, 88.9 - 99.9% and 60.1 - 99.6%, respectively within 16 h. Kinetics values of K_s (190.9, 469.6, 2001.0, and 206.7 mg/L, respectively) and q_{max} (30.7, 63.5, 269.2, and 48.5 mg/L·h) suggested that these microbial cultures could degrade wide range of tiamulin concentration. The removal process was accomplished by microbial activity since the degradation was not observed in abiotic controls. Numbers of cell growth followed the pattern of tiamulin reduction as cell numbers significantly climbed from 1.1×10^{8} CFU/mL to approximately 2.7×10^{11} CFU/mL after 16-hour period. Culture S and AN were effective in degradation of tiamulin than culture A and SN that was consistent with the higher bacterial numbers in culture S and AN than culture A and SN. The findings in this study are crucial for potential bioremediation of tiamulin-contaminated sites which are more effective than fungi in term of high tiamulin degradation rate and efficiency.

Chapter 6 STRUCTURAL COMMUNITIES OF BACTERIAL ENRICHMENT CULTURES FROM SWINE WASTEWATER

In chapter 5, all of the four bacterial enriched cultures exhibited their capabilities of degrading tiamulin from the lowest concentration of 2.5 mg/L to 200 mg/L of tiamulin during 16-hour period. In order to clarify bacterial populations contributing tiamulin degradation, 16S rRNA amplicon sequencing approach has been applied to indicate the bacterial community structures of stable enrichment cultures originated from different farms and culturing conditions. The corresponding treatments of the four enrichment cultures at the lowest and highest tiamulin concentrations (2.5 and 200 mg/L, respectively) were also selected to analyze metagenomes to understand the structures of bacterial communities in different concentrations. DNA of all samples were pre-selected with polymerase chain reaction (PCR) amplifying 16S rRNA and denaturing gradient gel electrophoresis (DGGE), and then analyzed all bacteria in the cultures with the next-generation sequencing.

6.1 Selection of tiamulin-degrading bacterial cultures

Previous findings indicated that bacterial community structures were affected by antibiotic stress, nutrient, and geography. In fact, tetracycline treatments at different concentrations were found to pose great effects on bacterial community composition of sludge in sewage treatment plants [141]. Other author demonstrated that the microbial communities were different in several beach zones with different nutrient levels and various geographic locations [140]. Mixed cultures in current study originated from two different swine waste treatment systems and had been cultivated under two nutrition conditions were subjected to tiamulin degradation tests at several concentrations. Bacterial communities in enriched cultures and their corresponding treatments were analyzed for diversity and abundance.

There are four enriched cultures, consisting of cultures A (covered anaerobic lagoon source), AN (covered anaerobic lagoon source, Nutrient broth), S (stabilization ponds

source), and SN (stabilization ponds source, Nutrient broth). Tiamulin concentrations in the range of 2.5 to 200 mg/L were tested for degradation efficiency in this study. Among all treatments, four original mixed cultures and treatments at three different tiamulin concentrations, including the lowest, medium, and the highest concentrations of 2.5 mg/L, 50 mg/L, and 200 mg/L tiamulin, respectively were obtained for further molecular analysis of PCR to amplify 16S rRNA gene and DGGE in order to select appropriate and meaningful samples for study of bacterial community structures.

6.1.1 16S rRNA gene amplification

Genomic DNA of all sixteen treatments were extracted, measured DNA concentration, and visualized 2% agarose gel (Figure 6.1). Concentration of bacterial DNA extracts fluctuated from 6.2 ± 0.4 ng/µL to 349 ± 5.3 ng/µL which are sufficient for further analysis. All sixteen genomic DNA showed one band at more than 1.5 kb in electrophoresis analysis, confirming they were available for further molecular analysis.

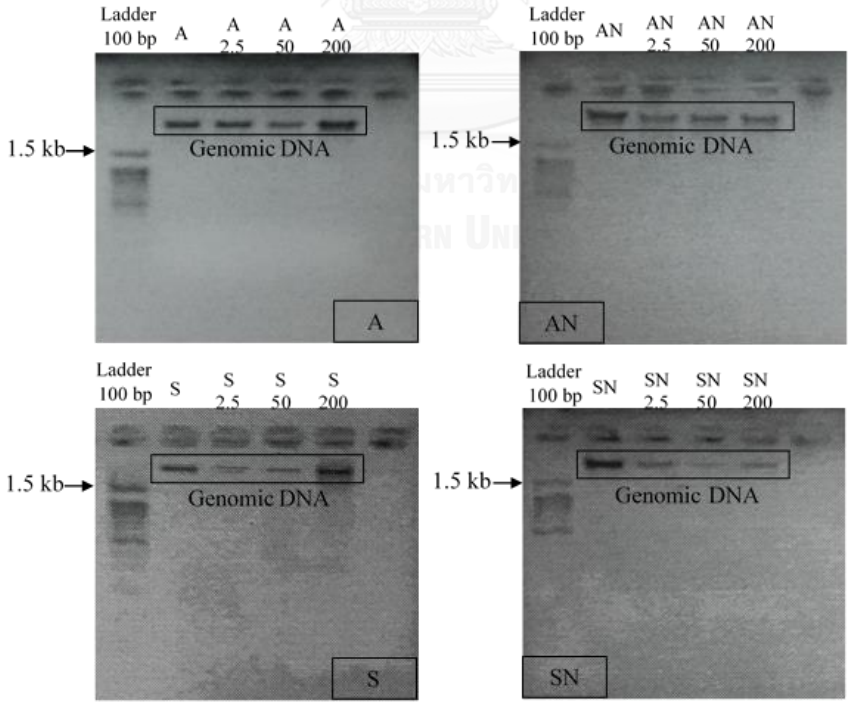


Figure 6.1 Gel documentation of all 16 bacterial genomic DNA

Genomic DNA samples of all of 16 treatments were amplified with universal bacterial primer pairs (27-F/1492-R). Electrophoresis analysis indicates only one band, in which the lengths of amplified 16S rRNA gene fragments were approximately 1.5 kb (Figure 6.2). These bands are in the same position with a positive control of *Mycobacterium* sp. PO1 strain. In contrast, negative control had no any band. This indicates that all 16 cultures contain DNA genomes of bacterial kingdom. Previous authors also used such pairs of 27-F and 1492-R as 16S rRNA technology to identify bacteria [175, 176].

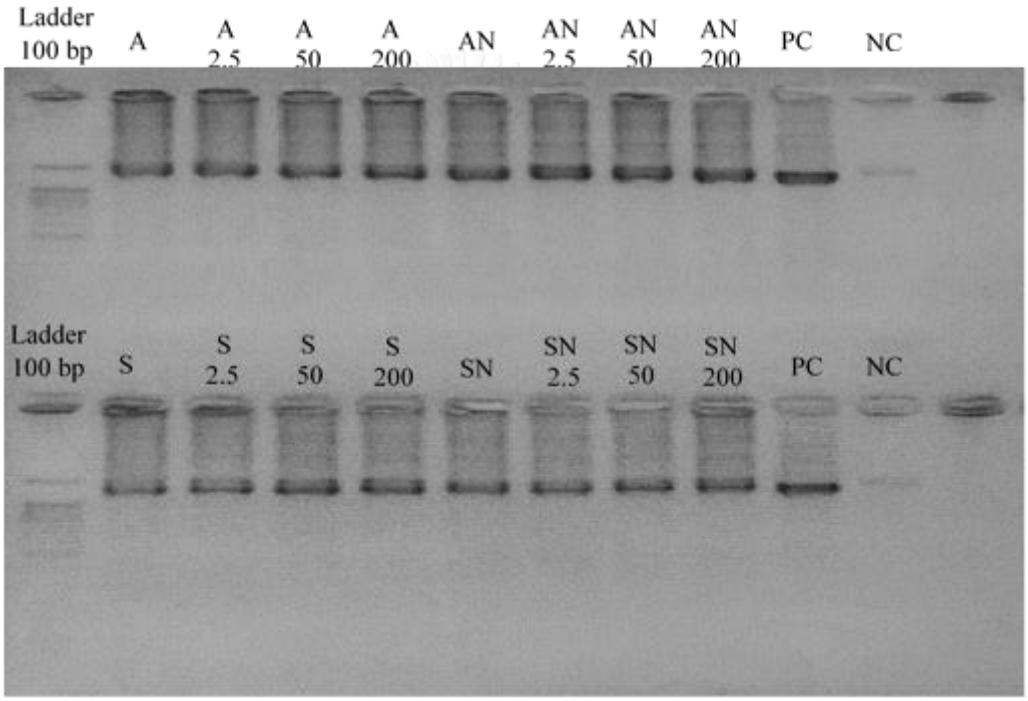


Figure 6.2 PCR products of bacterial DNA with 16S rRNA primer

6.1.2 Denaturing Gradient Gel Electrophoresis (DGGE)

PCR amplification products for denaturing gradient gel electrophoresis (DGGE) primers, 341-F-GC and 520-F, have been identified prior with agarose gel in Figure 6.3. Many bands appeared in all treatments and positive control (strain *Mycobacterium* sp. PO1), while the negative control had no any band. The previous use of such universal primers was well documented in the literature for amplification of a broad range of bacteria, in which the expected product size for the PCR

amplification with these primers was a single band of approximately 200 bp in length; however, there was some nonspecific DNA fragments occurred in agarose gel of PCR products for DGGE [177].

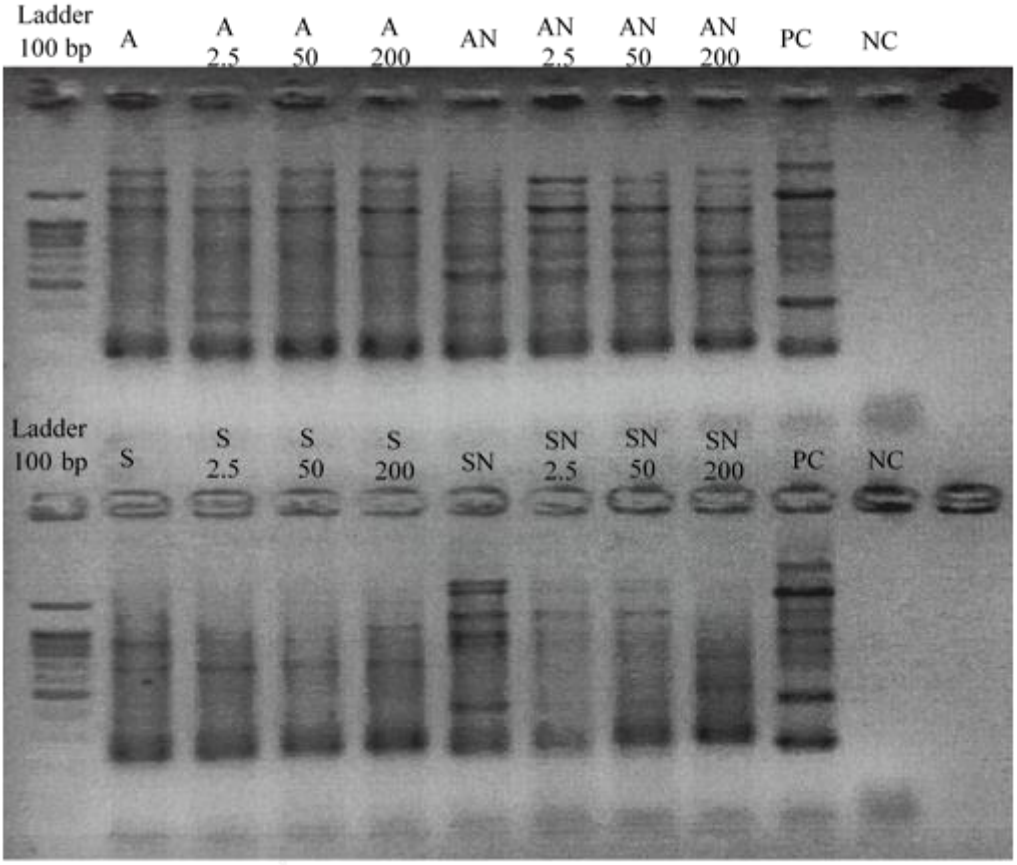


Figure 6.3 PCR products of bacterial DNA for DGGE

The individual PCR products were continuously separated by DGGE. The result was a pattern of bands, for which the number of bands corresponded to the number of predominant members in the microbial communities [178]. The DGGE pattern obtained in current work gave an overall bacterial diversity within the sixteen mixed cultures at different tiamulin concentration treatments (Figure 6.4). All of 16 patterns of DGGE resulted in many bands, including a lot of weak bands and some strong and clear bands. This revealed that bacterial enriched cultures are very diverse and some strong bands may be representing the dominant bacterial group in the enrichments.

The strong bands were labelled with numbers from 1 to 15, indicating these bacteria were dominant in the four bacterial enriched cultures and their corresponding treatments at different tiamulin concentrations. It can be seen that bands 9, 14 and 15 occurred in cultures originated from both swine waste treatment systems, demonstrating that these microbes presented even in various wastewater sources. Bands 1, 5 and 10 only appeared in cultures A and AN, bands 8 and 11, in contrast, occurred only in cultures S and SN. This indicated that the presence of these microbes depended on the sources of swine wastes collected for enrichments.

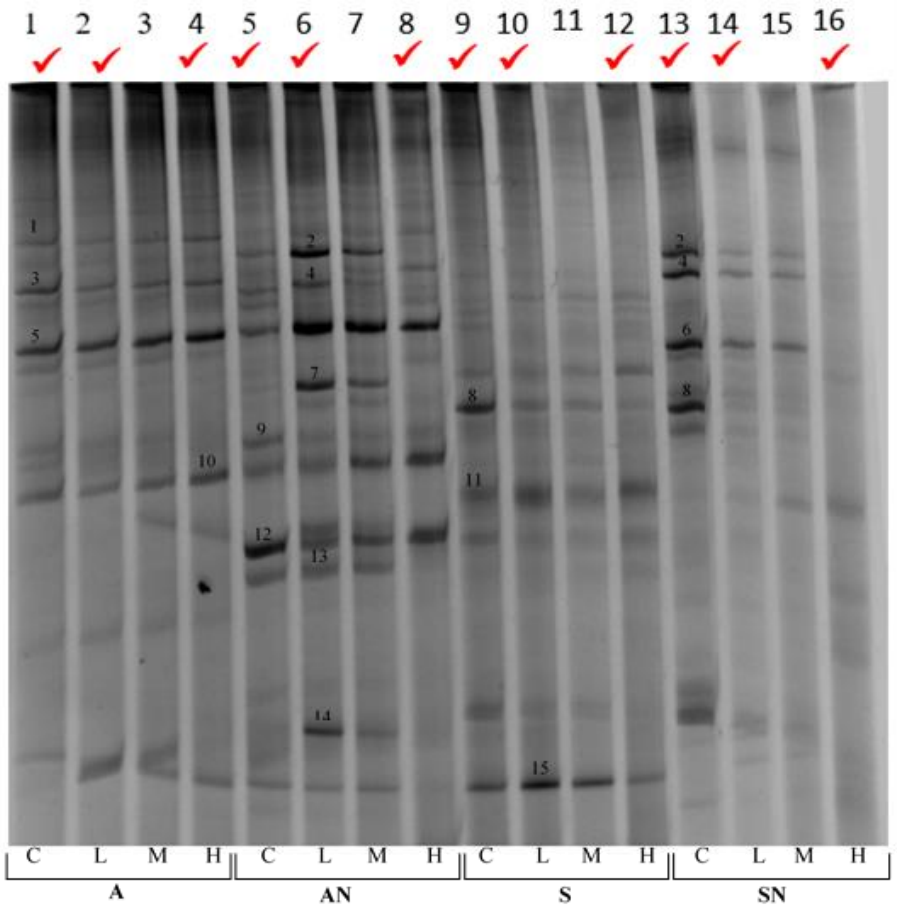


Figure 6.4 DGGE result of bacterial enriched cultures with three different tiamulin concentrations

A, AN, S, and SN: 4 enriched cultures; C: Enriched culture; L, M, and H: Low, medium, and high tiamulin concentrations.

In general, four original enriched cultures (A, AN, S, and SN) at wells 1, 5, 9, and 13 have different DGGE patterns. Since comparison of the DGGE patterns between each original mixed cultures and its corresponding treatments at three different tiamulin concentrations, it is obvious that the pattern of each culture at 2.5 mg/L of tiamulin (well 2, 6, 10, and 14) is almost similar with one at 50 mg/L of tiamulin (well 3, 7, 11, and 15), but it is differed from the one at 200 mg/L of tiamulin (well 4, 8, 12, and 16). High concentration (200 mg/L) rather has strong impact on microbial community. In conclusion, total of twelve samples, including the four mixed cultures (A, AN, S, and SN) and two corresponding treatments of each, at the lowest and highest tiamulin concentrations (A 2.5, A 200, AN 2.5, AN 200, S 2.5, S 200, SN 2.5, and SN 200) were chosen for further next-generation sequencing to compare variation of bacterial community between four mixed cultures as well as the high and low tiamulin concentrations of each culture.

6.2 Bacterial community structures of enrichment cultures

6.2.1 Taxon composition profile of bacterial community

Next-generation sequencing allows to assess the relative diversity of microbial populations that are the most active during pollutant removal [179]. The next-generation sequencing of 16S rRNA gene with the pair of 515F-806R bacterial/archaeal primer in this study showed that almost microbes in twelve enriched cultures were in the bacterial kingdom (99.9% - 100%). This indicated that bacteria were dominant in all of four enriched cultures and their corresponding treatments at low and high tiamulin concentrations and primarily involved in degradation of tiamulin. Only 0.1% of microbes in cultures AN, S 2.5, SN, and SN 2.5 were classified as virus, archaea, and unclassified microorganisms, demonstrating that archaea insignificantly presented in all treatments. Virus, even though very few numbers, was identified in some treatments by the next-generation sequencing of 16S rRNA gene, suggesting that virus might uptake 16S rDNA of bacteria or archaea in the cultures.

As shown in Figure 6.5 and Table 6.1, phylum Proteobacteria predominated in all of the four mixed cultures (99.3% in A, 95.2% in AN, 92.8% in S, and 91.5% in SN). This phylum was also the most abundant in the corresponding treatments of such the four cultures at the low and high tiamulin concentrations (i.e. 88% - 99.2% and 94.6% - 98.9% for treatments of 2.5 mg/L and 200 mg/L of tiamulin, respectively). Previous studies also indicated that Proteobacteria was dominant in activated sludge and increased the abundance under other antibiotic (tetracycline) treatment [141, 180].

Followed by Proteobacteria, Bacteroidetes phylum occurred with higher abundant in cultures from swine waste source of stabilization ponds and their corresponding treatments at 2.5 and 200 mg/L of tiamulin (2.9 - 11.0%) than cultures from the covered anaerobic lagoon source (0.1 - 3.8%). Firmicutes, Actinobacteria, and others phyla were found in all treatments at lower percentage for the total effective reads (0.1% - 0.5%). Bacteroidetes, Firmicutes, and Actinobacteria phyla were also found in activated sludge with low abundance of 1.8, 0.5, and 11.2%, respectively [141].

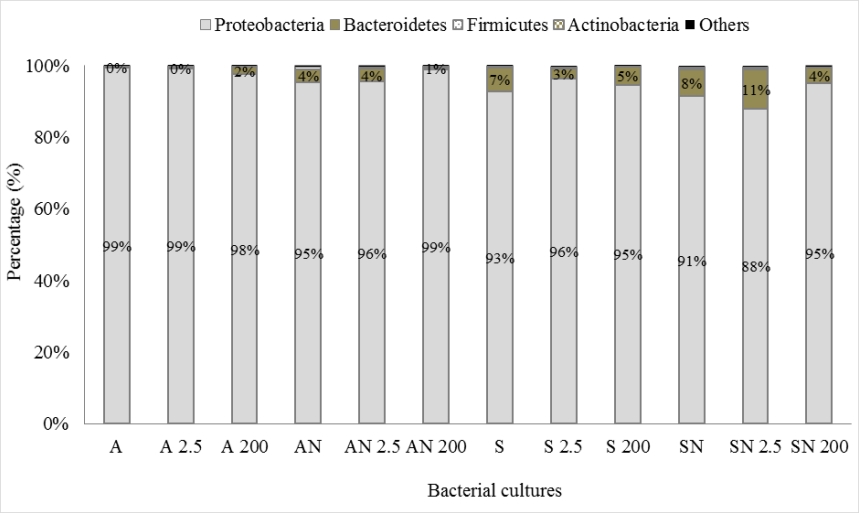


Figure 6.5 Classification at phylum level of four enriched cultures and their corresponding treatments at two different tiamulin concentrations

The relative abundance is presented as the percentage of each phylum in total effective reads of the corresponding sample.

Table 6.1 Classification at phylum level of four enriched cultures and their corresponding treatments at two different tiamulin concentrations

Phylum	A			AN			S			SN		
	0	2.5	200	0	2.5	200	0	2.5	200	0	2.5	200
Proteobacteria	99.3%	99.2%	97.6%	95.2%	95.5%	98.9%	92.8%	96.3%	94.6%	91.5%	88.0%	95.1%
Bacteroidetes	0.1%	0.2%	2.0%	3.8%	3.6%	0.6%	6.7%	2.9%	5.1%	7.5%	11.0%	4.3%
Firmicutes	0.3%	0.3%	0.2%	0.5%	0.5%	0.3%	0.2%	0.3%	0.1%	0.4%	0.3%	0.2%
Actinobacteria	0.1%	0.1%	0.1%	0.1%	0.2%	0.1%	0.1%	0.3%	0.0%	0.2%	0.3%	0.1%
Others	0.2%	0.2%	0.2%	0.3%	0.3%	0.2%	0.2%	0.3%	0.2%	0.4%	0.4%	0.2%

The relative abundance is presented as the percentage of each phylum in total effective reads of the corresponding sample.

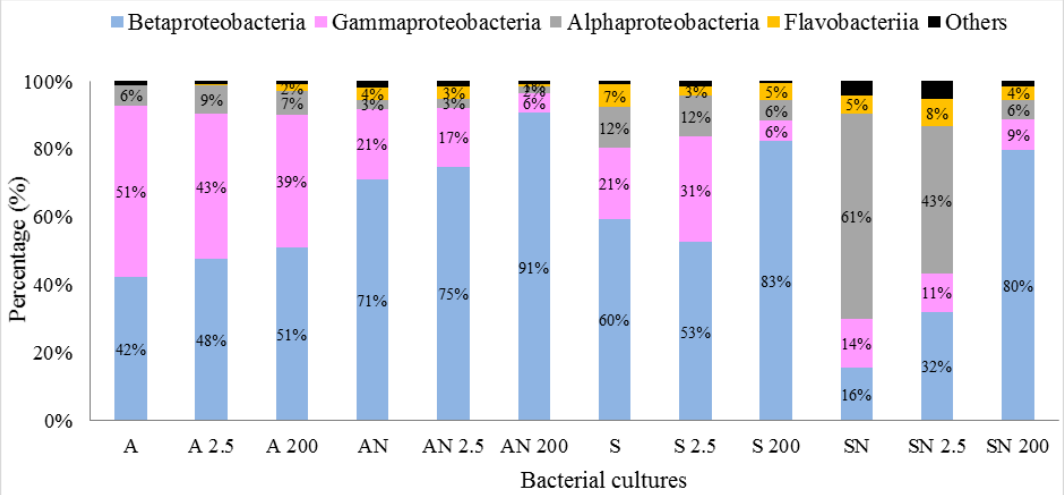


Figure 6.6 Classification at class level of four enriched cultures and their corresponding treatments at two different tiamulin concentrations

The relative abundance is presented as the percentage of each class in total effective reads of the corresponding sample.

At the level of class, enriched bacteria consisted of Betaproteobacteria, Gammaproteobacteria, Alphaproteobacteria, and Flavobacteria, accounting for 58.4%, 22.4%, 14.1%, and 3.5% of the total bacterial community in all of twelve cultures, respectively, while other classes of mixed bacteria were only 1.7% (Figure 6.6). This demonstrated that Betaproteobacteria was the main class of all cultures under tiamulin antibiotic treatment. This was supported by a previous study indicating that Betaproteobacteria dominated in an oxytetracycline production wastewater treatment plant [181] and an aerobic reactor treating high-concentration antibiotic wastewater [182].

A top seven genera in Betaproteobacteria class included *Achromobacter* (25.1%) that had the highest abundance, followed by *Delftia* (11.6%), *Pandoraea* (9.2%), *Burkholderia* (2.8%), *Methylobacillus* (1.8%), *Lautropia* (0.6%), and *Curvibacter* (0.3%). *Ochrobactrum* was the main genus of Alphaproteobacteria class, accounting for the highest percentage of 25.1% of the total bacterial community. *Pedomicrobium*, *Shinella*, and *Pseudaminobacter* genera of the class of Alphaproteobacteria also occurred in the cultures with low abundance (1.1%, 0.9%, and 0.5%, respectively). Two genera belong to Gammaproteobacteria class, comprising of *Pseudomonas* and *Stenotrophomonas*, were identified with same abundant percentages (10.6% and 10.0%, respectively). Only 0.6% of *Chryseobacterium* genus was found in the class of Flavobacteria (Table 6.2).

At species level, three species of *Achromobacter insolitus* (11.7%), *Achromobacter arsenitoxydans* (3.2%), and *Achromobacter xylosoxidans* (0.5%) were dominant in the highest abundance of *Achromobacter* genus. *Stenotrophomonas* and *Delftia* genera also had three representing species that consisted of *Stenotrophomonas maltophilia* (4.0%), *Stenotrophomonas pavanii* (1.7%), *Stenotrophomonas acidaminiphila* (1.3%), *Delftia lacustris* (5.7%), *Delftia tsuruhatensis* (3.0%), and *Delftia acidovorans* (0.2%), respectively. Three genera of *Burkholderia*, *Methylobacillus*, and *Pedomicrobium* had two dominant species of each, including *Burkholderia ubonensis* (0.8%), *Burkholderia phenoliruptrix* (0.4%), *Methylobacillus flagellatus* (1.4%), *Methylobacillus glycogenes* (0.2%), *Pedomicrobium manganicum* (0.3%), and

Pedomicrobium ferrugineum (0.4%), respectively. There is only one species represented for each of all eight remaining genera. These species were *Pseudomonas panipatensis* (7.2%), *Pandoraea apista* (2.0%), *Lautropia mirabilis* (0.6%), *Curvibacter gracilis* (0.3%), *Ochrobactrum thiophenivorans* (0.4%), *Pseudaminobacter salicylatoxidans* (0.2%), *Shinella granuli* (0.9%), and *Chryseobacterium joostei* (0.4%).

However, these bacterial species differently presented in the enriched cultures and their tiamulin degradation treatments (Figure 6.7). For examples, eight strains, including *Achromobacter insolitus*, *Achromobacter arsenitoxydans*, *Achromobacter xylosoxidans*, *Stenotrophomonas maltophilia*, *Stenotrophomonas pavanii*, *Stenotrophomonas acidaminiphila*, *Pseudomonas panipatensis*, and *Ochrobactrum thiophenivorans* were found in all twelve samples. *Pandoraea apista*, *Lautropia mirabilis*, *Burkholderia ubonensis*, *Burkholderia phenoliruptrix*, *Methylobacillus flagellatus*, *Methylobacillus flagellatus*, *Methylobacillus glycogenes*, and *Chryseobacterium joostei* only occurred in cultures A, AN, and their treatments at 2.5 and 200 mg/L of tiamulin. On the contrary, *Delftia lacustris*, *Delftia tsuruhatensis*, *Delftia acidovorans*, *Curvibacter gracilis*, *Pseudaminobacter salicylatoxidans*, *Shinella granuli*, *Pedomicrobium manganicum*, and *Pedomicrobium ferrugineum* presented only in cultures S, SN, and their corresponding treatments with two different tiamulin concentrations.

Table 6.2 Taxon composition profile of bacterial community at genus level in the four enriched cultures and their corresponding treatments

Class	Genus	Percentage of each genus in total effective reads (%)											
		A	A 2.5	A 200	AN	AN 2.5	AN 200	S	S 2.5	S 200	SN	SN 2.5	SN 200
Betaproteobacteria	*Achromobacter*	34.1	39.9	42.6	9.3	11.4	48.1	24.3	21.1	32.1	9.3	14.5	14.9
	Delftia	0.0	0.0	0.0	0.0	0.0	0.0	24.3	20.5	37.1	1.8	9.2	45.9
	Pandoraea	3.9	3.5	3.9	42.6	28.2	28.3	0.1	0.1	0.1	0.3	0.1	0.1
	Lautropia	0.7	0.8	1.0	0.2	0.5	0.9	0.5	0.4	0.7	0.3	0.3	0.6
	Burkholderia	1.2	1.1	1.2	10.8	11.7	7.8	0.1	0.1	0.0	0.1	0.0	0.1
	Curvibacter	0.0	0.0	0.0	0.0	0.0	0.0	0.7	0.8	0.6	0.1	0.2	0.9
	Methylobacillus	0.0	0.0	0.0	2.2	18.8	0.4	0.0	0.0	0.0	0.0	0.0	0.0
Gammaproteobacteria	*Stenotrophomonas*	30.2	33.3	25.3	2.5	3.0	1.2	1.7	1.2	0.4	11.4	4.7	5.5
	Pseudomonas	17.8	7.5	12.2	16.1	12.6	3.4	17.7	27.8	4.7	0.7	5.1	1.7
Alphaproteobacteria	*Ochrobactrum*	2.1	1.8	2.7	0.8	0.5	0.5	0.7	0.4	0.5	4.1	1.1	0.5
	Pseudaminobacter	0.0	0.0	0.0	0.0	0.0	0.0	1.8	1.1	0.7	1.3	0.3	0.1
	Shinella	0.1	0.0	0.1	0.0	0.0	0.0	0.1	0.1	0.1	6.6	2.9	1.0
	Pedomicrobium	0.1	0.9	0.1	0.0	0.0	0.0	1.3	1.2	0.5	6.1	2.3	0.3
Flavobacteria	*Chryseobacterium*	0.0	0.0	0.0	3.7	3.1	0.5	0.0	0.0	0.0	0.0	0.0	0.0

The relative abundance is presented as the percentage of each genus in total effective reads of the corresponding sample. Color scales from red to white indicate the percentage of taxon from high to low.

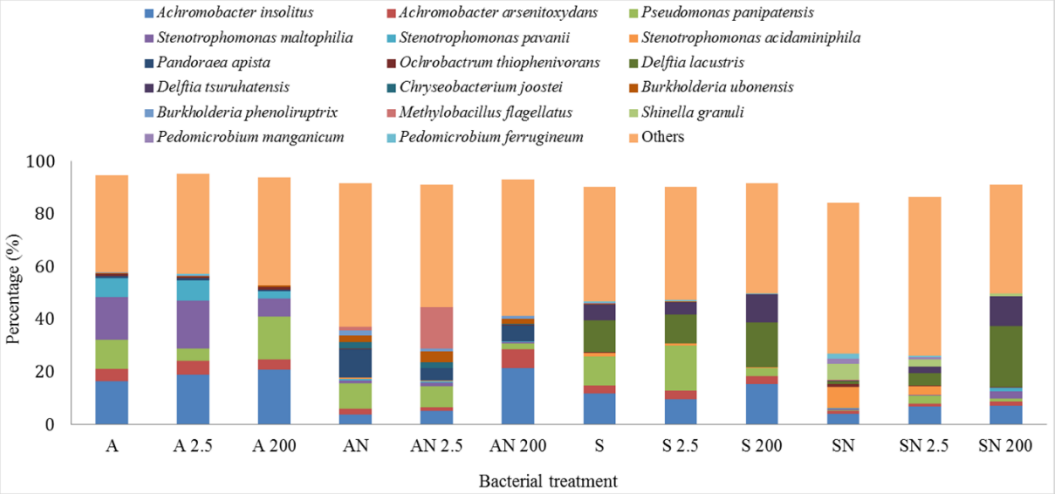


Figure 6.7 Classification at species level of four enriched cultures and their corresponding treatment at two different tiamulin concentrations

The relative abundance is presented as the percentage of each species in total effective reads of the corresponding sample.

6.2.2 Bacterial community from different sources

In Figure 6.8, *Achromobacter*, *Stenotrophomonas*, *Pseudomonas*, *Ochrobactrum*, and *Defluvibacter* genera were found at large numbers in all four enriched cultures from both two farms (21.7%, 16.3%, 16.9%, 1.5%, and 1.0% in covered anaerobic lagoon system; 16.8%, 6.5%, 9.2%, 2.4%, and 4.4% in stabilization pond system). This indicated that five bacterial genera simultaneously occurred in enriched cultures to carry out tiamulin degradation, even though they resulted from various swine waste sources of two different wastewater treatment processes.

At species level, eight bacterial strains were found dominantly in all twelve samples of both different farms that include *Achromobacter insolitus* (11.7%), *Achromobacter arsenitoxydans* (3.2%), *Achromobacter xylosoxidans* (0.5%), *Stenotrophomonas maltophilia* (4.0%), *Stenotrophomonas pavanii* (1.7%), *Stenotrophomonas acidaminiphila* (1.3%), *Pseudomonas panipatensis* (7.2%), and *Ochrobactrum*

thiophenivorans (0.4%) (Figure 6.7). This illustrated that the eight mentioned microbes might contributed as main bioremediators on tiamulin degradation process.

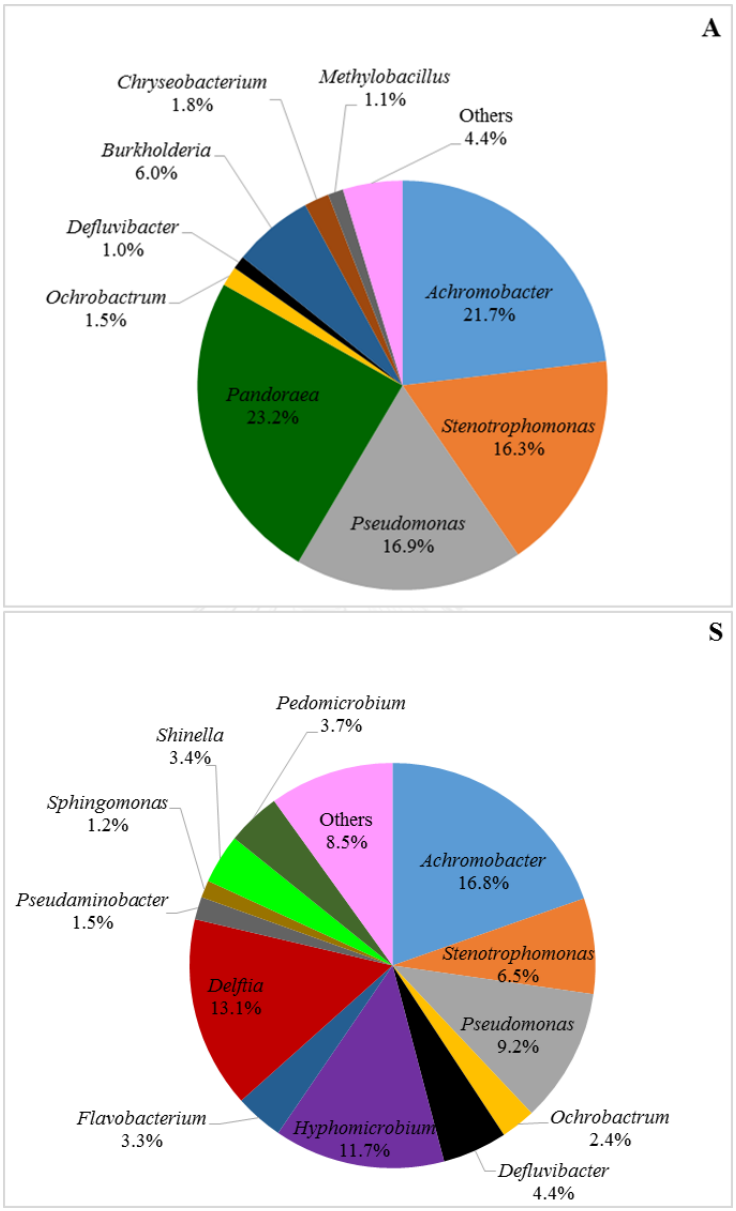


Figure 6.8 Abundance of bacterial genera in the enriched cultures at two different swine wastewater sources

However, genus *Pandoraea* (23.2%), *Burkholderia* (6.0%), *Chryseobacterium* (1.8%), and *Methylobacillus* (1.1%) significantly presented in cultures from the covered anaerobic digester, while *Delftia* (13.1%), *Hyphomicrobium* (11.7%), *Pedomicrobium* (3.7%), *Shinella* (3.4%), *Flavobacterium* (3.3%), *Pseudaminobacter* (1.5%), and *Sphingomonas* (1.2%) were largely observed in cultures from swine stabilization ponds (Figure 6.8). The microbial consortiums in these enriched cultures were rather different under similar enrichment conditions demonstrating the different swine waste treatments result in various diversity of microbes in enrichment of cultures.

Seven species, comprising *Pandoraea apista* (3.9%), *Lautropia mirabilis* (0.7%), *Burkholderia ubonensis* (1.6%), *Burkholderia phenoliruptrix* (0.8%), *Methylobacillus flagellatus* (2.9%), *Methylobacillus glycogenes* (0.4%), and *Chryseobacterium joostei* (0.9%) presented only in swine farm with covered anaerobic lagoon treatment systems. On the contrary, *Delftia lacustris* (11.5%), *Delftia tsuruhatensis* (6.0%), *Delftia acidovorans* (0.4%), *Curvibacter gracilis* (0.5%), *Pseudaminobacter salicylatoxidans* (0.5%), *Shinella granuli* (1.7%), *Pedomicrobium manganicum* (0.6%), and *Pedomicrobium ferrugineum* (0.6%) bacterial consortiums only occurred in the swine stabilization pond farm (Figure 6.7). This suggested their roles in eliminating tiamulin antibiotic in different farms.

6.2.3 Bacterial community at different cultivating substrates

Achromobacter, *Stenotrophomonas*, *Pseudomonas* and *Delftia* were more numerous in enrichment without nutrient broth (29.2%, 15.9%, 17.7%, and 12.2%, respectively) than those in the culture supplementing nutrient broth (9.3%, 7.0%, 8.4%, and 0.0%, respectively), revealing that these three genera could degrade tiamulin as major carbon source without major adaptation. In contrast, *Pandoraea*, *Hyphomicrobium*, *Burkholderia*, *Defluvibacter*, *Shinella*, *Pedomicrobium*, *Ochrobactrum*, *Chryseobacterium*, *Methylobacillus*, and *Sphingomonas* genera were dominant in the mixed cultures with nutrient broth (Figure 6.9), indicating that they were enriched by

nutrient broth and later on they continuously adapted to use tiamulin as a major carbon source during the enrichment period.

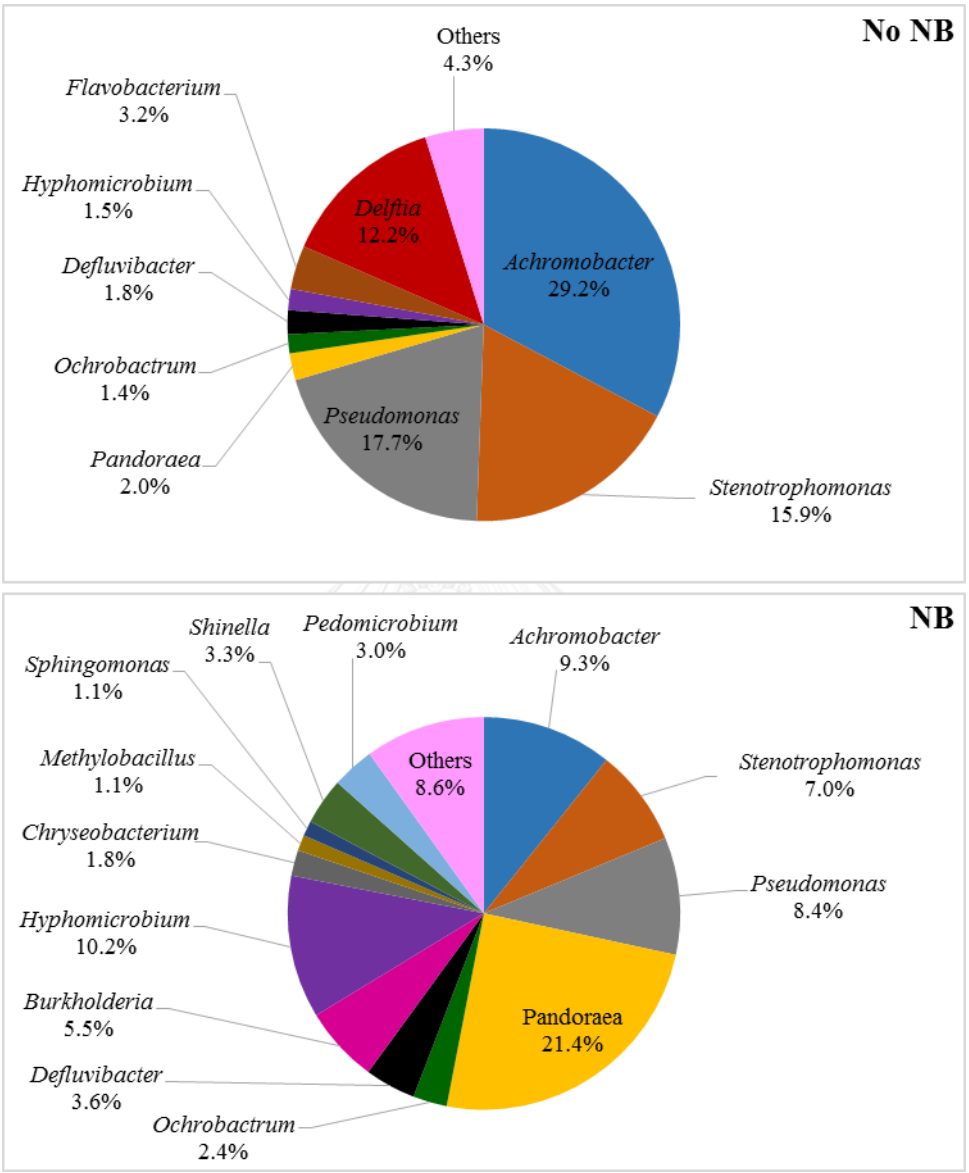


Figure 6.9 Abundance of various bacterial genera in the enriched cultures at two different cultivation conditions

Table 6.3 Abundance of various bacterial species in the enriched cultures at two different cultivation conditions

Genus	**Species**	**No NB**	**NB**
Stenotrophomonas	*Stenotrophomonas maltophilia*	6.9%	1.0%
	Stenotrophomonas pavanii	2.9%	0.6%
	Stenotrophomonas acidaminiphila	0.4%	2.1%
Pseudomonas	*Pseudomonas panipatensis*	10.5%	4.0%
Achromobacter	*Achromobacter insolitus*	15.4%	8.0%
	Achromobacter arsenitoxydans	3.8%	2.5%
	Achromobacter xylosoxidans	0.7%	0.4%
Delftia	*Delftia lacustris*	6.7%	4.8%
	Delftia tsuruhatensis	3.6%	2.4%
	Delftia acidovorans	0.3%	0.1%
Burkholderia	*Burkholderia ubonensis*	0.2%	1.4%
	Burkholderia phenoliruptrix	0.1%	0.7%

Genus	Species	No NB	NB
Pandoraea	*Pandoraea apista*	0.4%	3.6%
Shinella	*Shinella granuli*	0.1%	1.6%
Methylobacillus	*Methylobacillus flagellatus*	0.0%	2.9%
	Methylobacillus glycogenes	0.0%	0.4%
Pedomicrobium	*Pedomicrobium manganicum*	0.1%	0.5%
	Pedomicrobium ferrugineum	0.3%	0.4%
Chryseobacterium	*Chryseobacterium joostei*	0.0%	0.9%

NB: Nutrient broth

All species of *Achromobacter*, *Stenotrophomonas*, *Pseudomonas* and *Delftia* genera displayed more dominant in all treatments of enrichments without nutrient broth supplementation than those of cultures with the presence of other carbon source except *Stenotrophomonas acidaminiphila* of *Stenotrophomonas* genus. It accounted for only 0.4% in no nutrient broth cultivation, while the abundance of this species was approximately 2.1% in cultures adding nutrient broth (Table 6.3). On the contrary, the numbers of *Pandoraea*, *Burkholderia*, *Methylobacillus*, *Shinella*, *Pedomicrobium,* and *Chryseobacterium* genera were greater numbers in all tiamulin degradation treatments of cultures supplementing nutrient broth as other carbon source.

6.2.4 Bacterial community under different tiamulin concentrations

It can be seen from Table 6.4 that Proteobacteria was the most abundant phylum in both the enriched culture and corresponding treatments with tiamulin at low and high concentrations, accounting for 94.7%, 94.7%, and 96.5%, respectively. This predominant phylum tended to increase its abundance from 94.7% in mixed culture and under the low tiamulin stress (2.5 mg/L) to 96.5% with 200 mg/L of tiamulin. On the contrary, Bacteroidetes, Firmicutes, Actinobacteria, and other phyla seemed susceptible to tiamulin since their abundance evidently decreased with the increase of tiamulin concentration. These demonstrated that tiamulin treatment dramatically increased the abundance of Proteobacteria, making this phylum was dominant in all of the enrichment cultures.

Table 6.4 Abundance of bacterial phyla in the enriched cultures under the low and high tiamulin concentrations

Phylum	**Enriched culture**	**TIA 2.5**	**TIA 200**
Proteobacteria	94.7%	94.7%	96.5%
Firmicutes	0.4%	0.3%	0.2%
Bacteroidetes	4.5%	4.4%	3.0%
Actinobacteria	0.1%	0.2%	0.1%
Others	0.3%	0.3%	0.2%

The relative abundance is presented as the percentage of each phylum in total effective reads of the corresponding sample; TIA: Tiamulin; 2.5 and 200: Two different tiamulin concentrations

Figure 6.10 shows that at the level of class, Betaproteobacteria dominated in both enrichment culture and treatments of low and high tiamulin concentrations. Betaproteobacteria exceptionally exhibited capabilities of growth under stress of tiamulin concentration when its abundance increased from 47.2% in enrichment culture to 51.8% and 76.0% at 2.5 mg/L and 200 mg/L of tiamulin, respectively. Gammaproteobacteria, Alphaproteobacteria, Flavobacteriia, and other classes seemed to have higher abundance in enriched structures, but had slightly and significantly lower abundance at 2.5 mg/L and 200 mg/L of tiamulin. 16S rRNA coding sequences analysis previously showed that the majority of bacteria (75%) in degrading PAHs were affiliated to the Betaproteobacteria and Gammaproteobacteria classes such as *Achromobacter* sp., *Pseudomonas* sp., and *Stenotrophomonas* sp. [183].

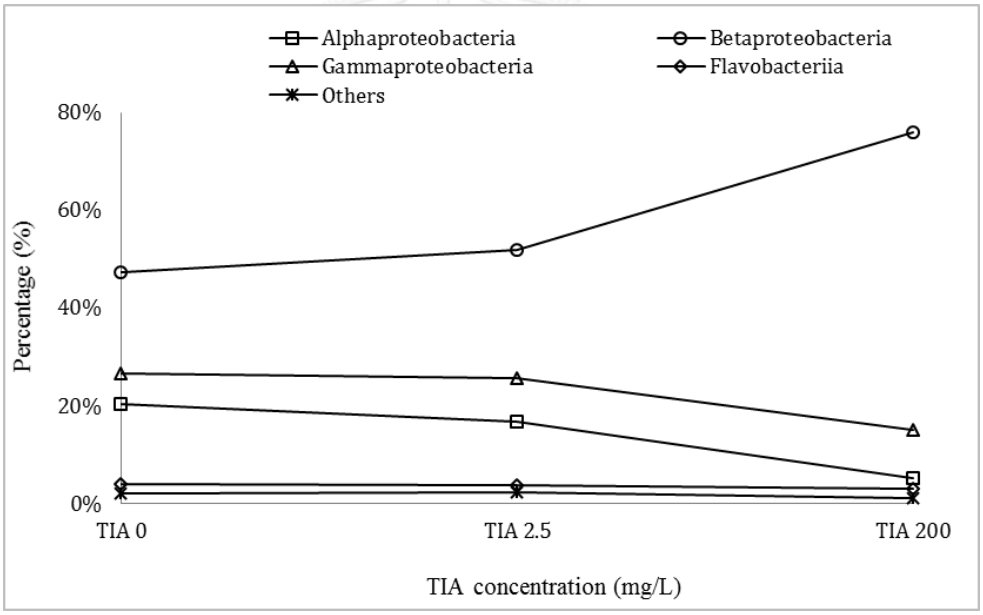


Figure 6.10 Abundance of various bacterial classes in the enriched cultures at two different tiamulin concentrations

TIA: Tiamulin; 0: Enriched culture; 2.5 and 200: Tiamulin concentrations.

Moving to the genus level of bacterial community, *Achromobacter*, *Stenotrophomonas*, and *Pseudomonas* genera were found at large numbers in all four enriched cultures and their corresponding treatments with tiamulin at 2.5 mg/L and

200 mg/L (Figure 6.11 and Figure 6.12); however, three genera had two different tendencies of bacterial growth. Genus *Achromobacter* dominated in the enrichments (34.1%, 9.3%, 21.1%, and 9.3% for cultures A, AN, S, and SN, respectively) and tended to increase their abundance slightly at 2.5 mg/L tiamulin treatment (39.9%, 11.4%, 24.3%, and 14.5%) and continuously to dramatically increase the abundance at 200 mg/L of tiamulin concentrations (42.6%, 48.1%, 32.1%, and 14.9%). *Delftia* genus only occurred in cultures of stabilizing pond process, in which culture S, taking up 20.5% of total abundance that was more than 20 times of those in culture SN (only 1.8%). However, this genus had the similar tendency in the growth of genus *Achromobacter* in both treatments with the low and high tiamulin concentrations. For instance, *Delftia* had a slight growth with abundance of 24.3% and 9.2% in cultures S and SN, respectively, in 2.5 mg/L treatment, and dramatically grew with huge numbers (37.1% and 45.9%) in 200 mg/L culture. It could be implied that *Achromobacter* and *Delftia* genera of Betaproteobacteria class were not effect by increasing of tiamulin concentration, indicating that two genera considerably contributed to process of tiamulin degradation, particularly at high concentration.

Previous studies have found that *Achromobacter* and *Delftia* were identified with high abundance or used in degradation of various recalcitrant pollutants, comprising antibiotics. For example, *Achromobacter* has been reported as sulfamethoxazole scavenger at high concentrations of 200 ppm in the anodic chamber of microbial fuel cell reactors [184]. *Achromobacter* was also reported that it could degrade sulfamethoxazole, sulfamethazine and other sulfonamide antibiotics [126, 185, 186], PAHs [183], triclosan [187], and bromophenol (including 2,4,6-tribromophenol and chlorophenols) [188]. *Delftia* genus has been mentioned with crucial role in removal of xenobiotic compounds; for instance, toxic triphenylmethane crystal violet dyes [189]. *Delftia acidovorans* could completely degrade the products of partial photocatalytic mineralization of antimicrobial solutions containing ampicillin, doxycycline, tylosin or sulfathiazole [190] as well as became important in biodegradation of photolysis products of sulfamethoxazole and dissolved organic carbon [191].

In contrast with *Achromobacter* and *Delftia*, abundance of *Stenotrophomonas* and *Pseudomonas*, two genera of Grammaproteobacteria class, was increased at low tiamulin treatment, after that remarkably reduced at high tiamulin concentration. This figure also occurred with *Burkholderia* and *Methylobacillus* genera of Betaproteobacteria, and *Sphingomonas* genus of Alphaproteobacteria classes. These obviously demonstrated that *Stenotrophomonas*, *Pseudomonas*, *Burkholderia*, *Methylobacillus*, and *Sphingomonas* were susceptible to tiamulin. The sub-inhibitory dose treatment of tiamulin tended to increase the abundance of four genera, but high tiamulin (200 mg/L) might inhibit these genera growth. Similarly, genus *Pandoraea* of Betaproteobacteria class that significantly presented in cultures from anaerobic digester farm enriched with nutrient broth (culture AN) had a remarkable drop of bacterial abundance from 42.6% in enriched culture to approximately 28% of the total effective reads at both 2.5 mg/L and 200 mg/L of tiamulin. The pattern of this trend was found with six other genera of Alphaproteobacteria class, including *Shinella*, *Defluvibacter*, *Pedomicrobium*, *Ochrobactrum*, *Hyphomicrobium*, and *Pseudaminobacter* (occupying approximately 1.8% - 7.0% in mixed culture, falling to less than 1.0% in the total of effective reads) and genus *Chryseobacterium* of Flavobacteria class (dropping from 3.7% to 3.1% and < 1.0% in treatments of low and high tiamulin, respectively). This evidently illustrated that tiamulin could inhibit the growth of *Pandoraea* and other above mentioned genera of bacterial communities (Figure 6.11).

Comparatively, antibiotic treatment, particularly at sub-inhibitory concentrations can pose effects on the bacterial community structures. This study showed tiamulin at high concentration of 200 mg/L marked declining of four cultures. This is consistent with previous findings; for example, tetracycline treatment greatly affected bacterial community structure in sewage treatment plant sludge as showing low abundance at high tetracycline concentration [141, 181].

Some previous findings indicated that besides *Achromobacter* and *Delftia*, other above bacterial genera were also important degraders in getting rids of various recalcitrant pollutants. Actually, *Pseudomonas* and *Stenotrophomonas* could degrade

sulfamethoxazole and other antibiotics such as ampicillin, doxycycline, and tylosin [184, 190], and PAHs [183, 192-194]. *Hyphomicrobium* sp. can grow on media with chloromethane, methanol, methylamine and ethanol as sole carbon and energy sources, and the microorganism has been used for bioremediation of gasoline-contaminated site [195].

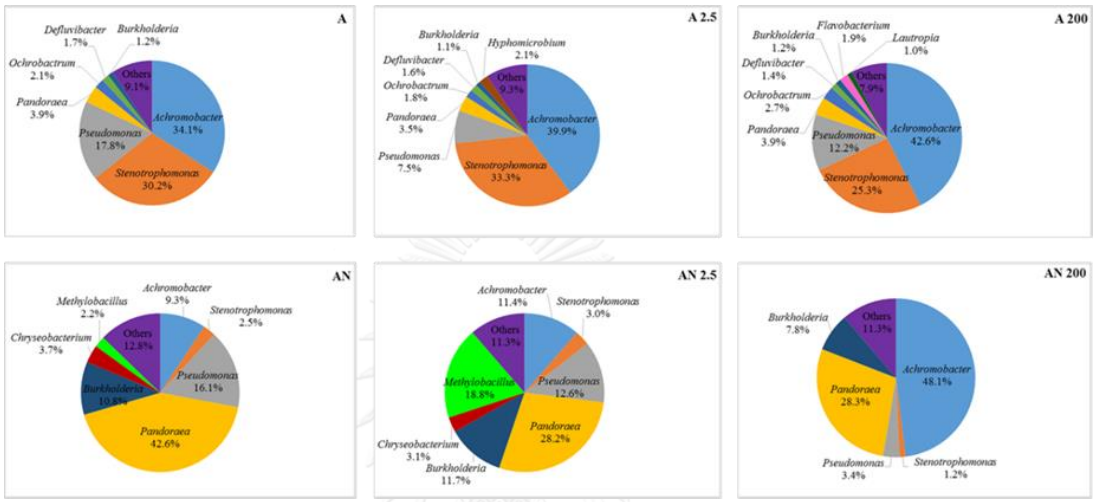


Figure 6.11 Classification at genus level of two enriched cultures from covered anaerobic lagoon system and their corresponding treatment at two different tiamulin concentrations

The relative abundance is presented as the percentage of each genus in total effective reads of the corresponding sample; A: Anaerobic digester, N: Nutrient broth; 2.5 and 200: Two different tiamulin concentrations.

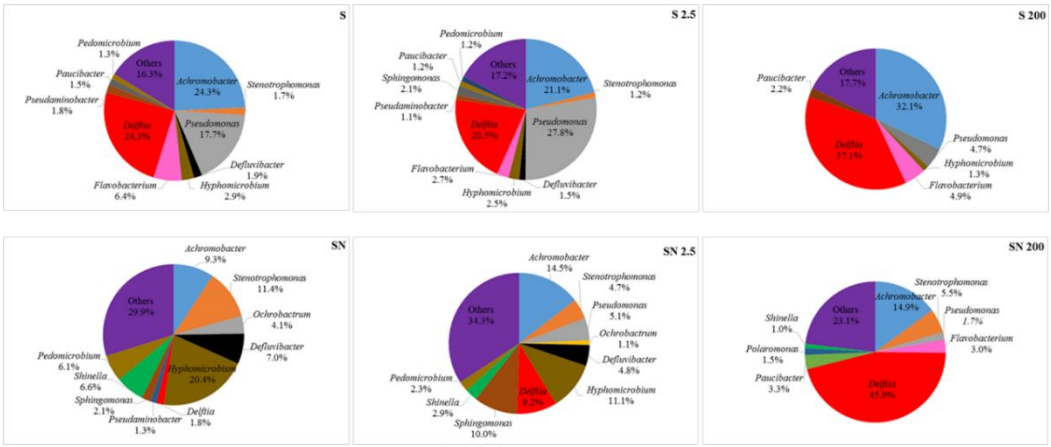


Figure 6.12 Classification at genus level of two enriched cultures from stabilizing pond system and their corresponding treatment at two different tiamulin concentrations

The relative abundance is presented as the percentage of each genus in total effective reads of the corresponding sample; S: Stabilization pond, N: Nutrient broth; 2.5 and 200: Two different tiamulin concentrations.

Table 6.5 Number of 16S rRNA gene sequences analyzed, identified species numbers, and Shannon species diversity for each sample of the four enriched cultures

Cultures	**Tiamulin concentrations**	**Numbers of analyzed sequences**	**Number of species identified**	**Shannon species diversity**
A	Enriched culture	157512	508	2.1
	2.5 mg/L	138390	469	2.0
	200 mg/L	118383	444	1.9
AN	Enriched culture	120153	479	2.0
	2.5 mg/L	225606	619	2.2
	200 mg/L	93454	353	1.8
S	Enriched culture	166060	547	2.1
	2.5 mg/L	136844	580	2.2
	200 mg/L	286095	519	2.0
SN	Enriched	130299	605	2.2

Cultures	Tiamulin concentrations	Numbers of analyzed sequences	Number of species identified	Shannon species diversity
	culture			
	2.5 mg/L	192989	773	2.1
	200 mg/L	145647	525	2.1

Next-generation sequencing of 16S rRNA gene showed that numbers of identified species of the three enriched cultures, including culture AN, S, and SN, tended to increase in treatment with 2.5 mg/L of tiamulin (from 479, 587, and 605 to 619, 580, and 773, respectively), but they had a tendency of decrease in tiamulin treatment 200 mg/L (down to 353, 519, and 525, respectively) after 16-hour period. This is consistent with the patterns of Shannon species diversity in Table 6.5. The cultures treated with 2.5 mg/L of tiamulin had high numbers of identified species, revealing that sub-inhibitory concentrations of tiamulin stress may favor enhancement of bacterial species richness [196]. However, bacterial species numbers were low in cultures fed with 200 mg/L of tiamulin, indicating that the growth of microbes was inhibited under the high tiamulin stress. Previous studies also found that the antibiotic stresses seemed not effective in reduction of the bacterial diversities of river water [197]. Culture A displayed dissimilar pattern with such three above cultures. Its identified species numbers tended to reduce in both treatments with low and high tiamulin concentrations. The reason might be that tiamulin, even though high concentration, has not an inhibition of enriched bacterial growth.

Combination of bacterial community structures at species level and tiamulin degradation efficiencies (Figure 6.13), over 16-hour period tiamulin was effectively degraded in all treatments with 2.5 mg/L (~ 89%). At treatments with 200 mg/L of tiamulin, two cultures of AN and S almost completely removed tiamulin with efficient

percentage of approximately 89% and 100%, respectively, while cultures of A and SN only eliminated tiamulin around 66%. *Achromobacter insolitus*, *Achromobacter arsenitoxydans*, *Pseudomonas panipatensis*, and *Stenotrophomonas pavanii* were dominant in all of eight treatments. *Delftia lacustris* and *Delftia tsuruhatensis* had enormous numbers in cultures from stabilizing pond systems at both low and high tiamulin. *Stenotrophomonas maltophilia* was large numbers in culture A and SN at 200 mg/L of tiamulin treatment that had low effectiveness in eliminating tiamulin, demonstrating that this species insignificantly helped to reduce tiamulin. On the contrary, *Pandoraea apista* and *Burkholderia ubonensis* which only occurred with high abundance within culture AN at both 2.5 mg/L and 200 mg/L of tiamulin might contribute to improve efficiency of tiamulin elimination. However, next-generation sequencing results have not showed any dominantly bacterial species definitely involved in creation of great tiamulin removal efficiency (almost 100%) of culture S at 200 mg/L, hypothetically illustrating important species related to tiamulin degradation at very high concentration in culture from stabilizing pond sources might include in ~ 47% of other and unclassified species.

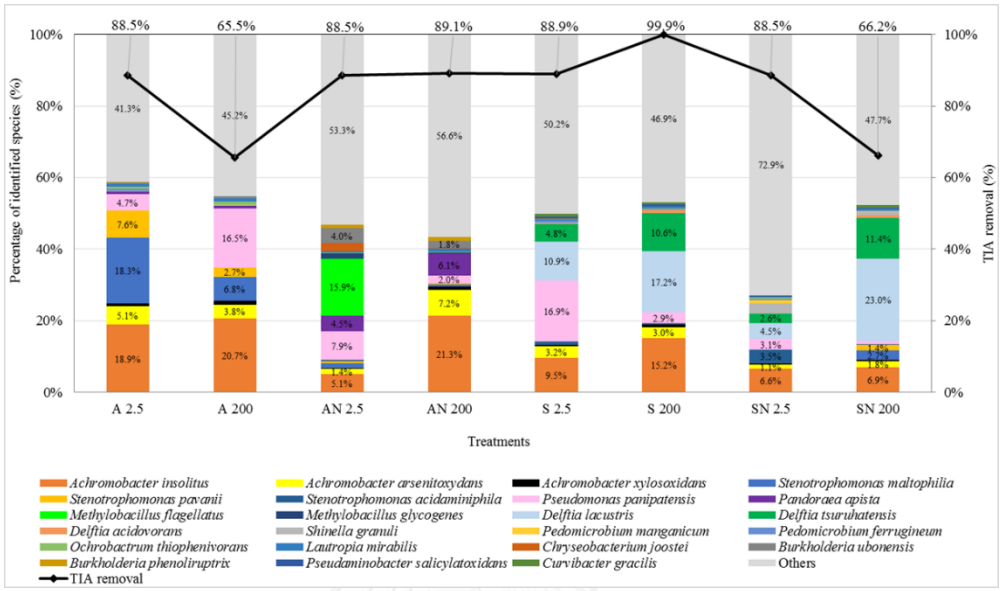


Figure 6.13 Tiamulin removal efficiency and bacterial community structures at species level of four enriched cultures with two different tiamulin concentrations

The relative abundance is presented as the percentage of each species in total effective reads of the corresponding sample; A: Covered anaerobic lagoon, S: Stabilization pond, N: Nutrient broth; 2.5 and 200: Two different tiamulin concentrations.

6.3 Chapter conclusions

Four enrichment cultures, including A (covered anaerobic lagoon source), AN (covered anaerobic lagoon source, Nutrient broth), S (stabilization pond source), and SN (stabilization pond source, Nutrient broth), obtained after long time acclimation to aerobically degrading tiamulin were able to remove tiamulin at concentrations of 2.5 mg/L - 200 mg/L with the rates of 60.1 - 99.9% within 16 h. Bacterial community structures of all treatments were discovered by using the next-generation sequencing technique. The metagenomics analysis illustrated that bacterial community structures had various abundance and diversity as different swine waste sources, cultivation conditions, and tiamulin concentrations. Combination of bacterial community structures at genus level and tiamulin degradation efficiencies revealed that

Achromobacter, and *Delftia*, *Pandoraea* (Betaproteobacteria class) as well as *Pseudomonas* and *Stenotrophomonas* (Gammaproteobacteria class) genera dominated and considerably contributed to improve tiamulin elimination process. The findings in this study are crucial for potential bioremediation of tiamulin-contaminated sites. Future studies on tiamulin degradation by each isolate and combinations of isolates at a field site should be proposed.

Chapter 7 CONCLUSIONS AND RECOMMENDATIONS

7.1 General discussion and conclusions

The antibiotic tiamulin (TIA) is widely used in swine industries not only in Thai but also all around the world. It is persistent during manure storage causing concern on spreading of the compound and the evolution of resistant bacteria and genes into environment through the fertilizer application and wastewater discharge. Therefore, TIA antibiotic removal in swine farm wastes before releasing should be enhanced which importantly contributes to minimize TIA and TIA resistant gene pollution. This study successfully isolated wood-rot fungi and enriched TIA-degrading mixed bacterial cultures from Thai swine farms that are capable on removing TIA antibiotic efficiently. The major findings in the preceding chapters are summarized as follows.

7.1.1 Fungal isolation and bacterial enrichment cultures

- Twelve morphological different wood-rot fungi were isolated from several rotten wood and manure samples collected in swine farms using Potato dextrose agar (PDA). The sequencing of the internal transcribed spacer region in rRNA genes revealed their taxonomic affiliation as *Lasiodiplodia* sp. F1, *Curvularia* sp. F2, *Aspergillus* sp. F3, *Penicillium* sp. F4, *Fusarium* sp. F5, *Trichoderma* sp. F6, *Hypocreales* sp. F7, *Galactomyces* sp. F8, *Geotrichum* sp. F9, *Mucor* sp. F10, *Trichosporon* sp. F11, and *Penicillium* sp. F12, respectively.

- Four bacterial mixed cultures were enriched from two swine waste treatment systems namely covered anaerobic lagoon (A) and stabilization pond (S). Two tiamulin media which were tiamulin fumarate in mineral solution and tiamulin fumarate mixed with nutrient broth in mineral solution (N) were introduced. When the cell number reached designated level, a portion of enriched culture was transferred to new medium. The acclimatization period of seven months covering fourteen subcultures was needed to obtain stable growth rate cultures. The stable enriched cultures consisted of A (covered anaerobic lagoon source), AN (covered anaerobic

lagoon source, nutrient broth), S (stabilization pond source), and SN (stabilization pond source, nutrient broth).

7.1.2 Biodegradation of tiamulin by wood-rot fungi

- Three of the twelve swine farm wood-rot fungal isolates (*Lasiodiplodia* sp. F1, *Fusarium* sp. F5 and *Galactomyces* sp. F8) and three white-rot fungi (*Trametes hirsuta* AK4, *Trametes versicolor* and *Verticillium* sp.) that decolorized brilliant green and crystal violet dyes (0.05 w/v) were tested for tiamulin degradation at 10 mg/L over 12-day period. Six wood-rot fungi degraded 66.8 - 93.2% of tiamulin, at rates of 5.6 - 58.6 mL/g fungi·d. The fungi showed larger dye decolorization diameters exhibited the higher tiamulin removal rates. Three fungal strains *Lasiodiplodia* sp. F1, *Verticillium* sp., and *Trametes versicolor* were the most efficient with more than 85% of tiamulin removal efficiency, making them potential candidates for eliminating tiamulin in contaminated sites.

- Six such wood-rot fungi also exhibited capability to decolorized variety of dyes and several other recalcitrant pollutants. *Lasiodiplodia* sp., *Fusarium* sp., and *Galactomyces* sp. could decolorize and remove pesticide, phenolic compounds, plastics, PAHs, wastewater effluent, and lincomycin antibiotic residuals as well based on literature review.

7.1.3 Role of ligninolytic enzymes in tiamulin degradation

Fungi possess ligninolytic enzymes, including laccase (Lac), lignin peroxidase (LiP), and manganese peroxidase (MnP), involved in the elimination of diverse recalcitrant pollutants and antibiotics according to previous reports. These lignin-degrading enzymes were monitored throughout the tiamulin degradation in all six wood-rot fungi. Manganese peroxidase was detected in all the fungi at the highest level with maximum activity ranging from 33.1 U/L to 59.3 U/L, in comparison to laccase and lignin peroxidase that detected only in maximum activities at 0.4 - 4.9 U/L and 0.0 - 3.3 U/L. MnP was the main enzyme involved in tiamulin removal. As glucose contained in basal medium, it might be a cosubstrate in tiamulin degradation.

7.1.4 Biodegradation of tiamulin by bacterial enriched cultures

- Four microbial enrichment cultures were able to remove tiamulin at concentrations of 2.5 - 200 mg/L with the rates of 65.5 - 99.7%, 76.8 - 99.0%, 88.9 - 99.9% and 60.1 - 99.6%, respectively for culture A, AN, S, and SN within 16 h. Monod kinetic parameters of K_s (190.9, 469.6, 2001.0, and 206.7 mg/L, respectively) and q_{max} (30.7, 63.5, 269.2, and 48.5 mg/L· h) suggested that these microbial cultures could degrade tiamulin at high concentration. Culture S and AN were effective in degradation of tiamulin.

- Cell numbers in the kinetics experiments significantly climbed from 1.1×10^{8} CFU/mL to approximately 2.7×10^{11} CFU/mL after 16-hour period and TIA concentrations were stable in all abiotic controls. These were evidently supported and confirmed that TIA removal process was performed by microbial activity or biodegradation.

7.1.5 Community structures of bacterial enriched cultures

- Bacterial community structures of such the four enriched cultures (A, AN, S, and SN) and their corresponding treatments treated with the lowest (2.5 mg/L) and highest tiamulin concentrations (200 mg/L) were discovered by using the next-generation sequencing technique. The amplicon sequencing illustrated that bacterial community structures had various abundance and diversity as different swine waste sources, cultivation conditions, and tiamulin concentrations.

- Microbes in all four enriched cultures and their corresponding treatments were in bacterial kingdom (99.9 - 100%). Phylum Proteobacteria predominated in all treatments, accounted for 99.3% in A, 95.2% in AN, 92.8% in S, 91.5% in SN, 88% - 99.2% in 2.5 mg/L tiamulin treatments and 94.6% - 98.9% in 200 mg/L tiamulin treatment.

- Combination of bacterial community structures at genus level and tiamulin degradation efficiencies revealed that *Achromobacter*, and *Delftia*, *Pandoraea* (Betaproteobacteria class) as well as *Pseudomonas* and *Stenotrophomonas*

(Gammaproteobacteria class) genera dominated and considerably contributed to improve tiamulin elimination process.

7.1.6 Comparison of tiamulin degradation by wood-rot fungi and bacterial mixed cultures

Enriched bacterial cultures were able to remove tiamulin at initial concentrations of 10 mg/L with the rates of 78.4 - 97.0% and 103.4 - 288.4 mL/g bacteria·d. These which were higher than TIA removal efficiency of wood-rot fungi ranging 66.8 - 93.2% with the rates of 5.6 - 58.6 mL/g fungi·d). Therefore, tiamulin-degrading bacterial enriched cultures had an advantage over tested fungi in term of the degradation speed.

7.2 Recommendations for future research

7.2.1 Application of wood-rot fungi and bacterial enriched cultures in tiamulin degradation

- This study clearly revealed the excellent capacity of enriched bacterial cultures over isolated fungi on eliminating tiamulin. However, conventionally using bacterial mixed cultures to directly treat swine wastewater in pig farms might spread antibiotic resistant bacteria to surrounding environments. Bioencapsulation technique may help to enhance field application efficacy of bacterial enriched cultures as immobilization of microorganisms can control microbial release from formulations [130]. Though there may be some concern over the release of the antibiotic resistance gene with the cell lysis, this might not be a critical issue as generally DNA is degraded easily in environment. Bioencapsulation might retard DNA into some extent. Therefore, the bioencapsulation of tiamulin-dedrading enriching cultures treated tiamulin in swine wastewater at lab-scale and pig farms should be study in future.

- Fungi is more appropriate for cases of dry material like fertilizer. Degradation of tetracyclines was accelerated during pig manure composting with rice straw in greenhouses [198]. Wood-rot fungal isolates in this study can be further used to add into composting process of TIA-contaminated swine manure to decrease the potential

environmental risk of tiamulin-contaminated swine manure and make composted residues safer for field application. Pure manganese peroxidase extract is also promising technology that should be applied for improving rates of tiamulin degradation as it can eliminate tiamulin at high speed and can be used in both solid and liquid conditions, without concerning growth factor. Further study should evaluate the efficiency of enzyme treatment compared to bacterial enrichment culture.

- Antibiotics including tetracycline, chlortetracycline, oxytetracycline, sulfathiazole, and ampicillin were effectively biodegraded and biotransformed through immobilized bacterial enrichments supplementing microencapsules containing ligninolytic (laccase) enzyme-extract from spent mushroom compost of *Pleurotus eryngii* in bioreactors [52]. TIA-degrading bacterial enriched cultures and wood-rot fungi in this study may be practicably applied following this application to treat tiamulin-contaminated swine waste sites. Mixed microbes will be encapsulated or immobilized in microencapsulation and supplemented in the sites. In order to enhance effectiveness and speed of tiamulin degradation as well as restrict tiamulin release, immobilized bacterial cells and crude enzymes of wood-rot fungi shall be combined.

7.2.2 Determination of tiamulin residues and setup of tiamulin remediation criteria

- Tiamulin was detected from swine manure and wastewater with concentrations of 43 µg/kg and 5 µg/L, respectively [24], and it was detected in environments such as soil at 0.7 µg/kg, surface water at 0.77 - 18.77 ng/L, and groundwater at 29 ng/L [25]. There is no any research about tiamulin residues discovered in swine farms and environments in Thailand. In addition, there is no environmental quality standard or regulation for tiamulin. Therefore, it is difficult to determine whether such above-mention residual tiamulin is harmful or acceptable. Risks assessment of tiamulin should be investigated to determinate and evaluate which levels of this compound to be toxic or safe on microrganisms, animal, human health, and ecological balance, thus to conduct criteria of tiamulin remediation goal. Furthermore, residues of tiamulin in swine farms and environments in Thailand should be also studied to know data of tiamulin concentrations in swine waste so as to appropriately applied wood-rot fungi and bacterial enriched cultures in this study to treat tiamulin-contaminated sites.

- Biochemical pathway though degradation of refractory organic compounds is an important consideration in evaluating feasibility and benefits of any treatment system. Eliminating xenobiotic contaminants is desirable as ensuring that the end products or intermediates are non-hazardous [199]. Several TIA metabolites were produced during its degradation (2β-hydroxy-N-deethyl-tiamulin and 8α-hydroxy-N-deethyl-tiamulin) and its metabolism processes (2β-hydroxy-tiamulin, 8α-hydroxytiamulin and N-deethyl-tiamulin) [129]. These metabolites may be continuously introduced, spread and posed risks into the environments. Hence, biodegradation of tiamulin should be studied together with production of its metabolites. These tiamulin metabolites should be determinated during degradation tests by LC-MS/MS to evidently confirm the biodegradation process and definitely elucidate the mechanisms or pathway of tiamulin degradation.

- In combination between metabolites and remediating criteria of tiamulin, the standard of tiamulin antibiotic should be created with specific criteria of acceptable tiamulin and its intermediates concentration for the environments. This will contribute to effectively manage environment.

7.2.3 Optimization of tiamulin degradation

- Microbial growth and activity are affected by several factors including organic matters, oxygen concentration, nitrogen, pH, and temperature. In order to improve effectiveness of tiamulin degradation, the favoring condition for tiamulin degrading cultures should be studied thoroughly. The modification of environmental conditions at the contaminated sites specific to tiamulin degraders might enhance the tiamulin degradation.

- In swine production, enormous amount and sorts of antibiotics have simultaneously used for growth promotion, disease prevention and treatment, so swine waste contains various residual antimicrobial substances [200]. For instance, several types of antibiotics have been introduced in swine production such as amoxicillin, chlortetracycline, colistin, doxycycline, enrofloxacin, erythromycin, oxytetracycline, sulfamethazine, tetracycline, tiamulin, and tylosin [4-6]. The TIA-degrading microbes

could tolerate to tiamulin, but they may be sensitive to other antibiotics. Further research on this topic, thus, should pay attention to co-effect of other antibiotics on TIA-degrading bacterial growth and tiamulin biodegradation effectiveness as well. Moreover, examination of other antibiotics degradation efficiency in TIA-degrading bacterial cultures & wood-rot fungi in order to apply for sample containing multiclass antibiotics.

7.2.4 Isolation and identification of specific tiamulin-degrading bacteria

One limitation of current work is that it is unable to prove which bacterial strains can uptake tiamulin. These suggest that tiamulin-degrading bacterial communities in four enriched cultures should be isolated and identified to species level to exactly determine which bacterial strains mainly involve in tiamulin degradation process. Some in situ microbial functional analysis such as DNA stable-isotope probing (DNA-SIP) that can identify active microorganisms assimilating particular carbon substrates or nutrients into cellular biomass [201], might be useful to solve this problem.

7.2.5 Genetic characterization of tiamulin biodegradation

Genetic analysis provides new information to undertake the evolution of degradation pathways of recalcitrant compounds and help to apply molecular adaptation strategies to changing environmental conditions. The metabolism of some refractory pollutants degradation, such as ibuprofen and nicotine by *Sphingomonas* and *Pseudomonas putida* S16, respectively, were molecularly characterized to gain insight into the mechanism responsible for degradation process [202, 203] that would be useful for further improving the biological degradation efficiency.

7.2.6 Identification of antibiotic resistance genes

Antibiotic resistance is the acquired ability of a microorganism to grow in the presence of an antibiotic to which the microorganism is typically sensitive. Antibiotic resistance is becoming a greater concern on a global scale, even leading some members of the European Union to ban the use of agricultural antibiotics [204].

Agricultural use of antibiotics is potentially contributing to a population of antibiotic resistant organisms that might spread resistance to pathogenic bacteria. Identifying antibiotic resistance genes and DNA mobile elements in the bacteria should be focused to monitor the spread of antibiotic resistant bacteria, contributing to manage the environment.

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APPENDIX

APPENDIX A RAW DATA

1 Tiamulin degradation results

1.1 Tiamulin concentrations in biodegradation test using wood-rot fungi

The raw data results of tiamulin degradation by using six wood-rot fungi in aqueous basal medium at 10 mg/L of tiamulin over 12-day experimental period is presented in Table A1.

Table A1 Raw data of tiamulin concentrations in biodegradation test using wood-rot fungi

Treatment	Time (d)	TIA concentration (mg/L)					
		F1	**F5**	**F8**	**TV**	**V**	**AK4**
Fungal inoculation	0	10.2	9.7	9.1	11.4	11.4	11.0
	1	2.7	3.4	7.2	10.7	7.2	7.7
	2	2.3	2.6	5.5	3.9	2.4	6.4
	4	0.0	2.1	3.9	2.0	2.2	3.8
	8	0.0	2.0	2.9	0.0	0.0	2.7
	12	0.0	1.4	2.4	0.0	0.0	2.2
Sorption control	0	10.9	10.7	9.1	11.9	11.2	11.7
	1	10.6	10.6	10.1	11.2	10.2	10.1
	2	10.3	10.4	10.2	10.5	10.1	10.4
	4	10.3	10.2	10.5	9.9	10.0	10.4
	8	9.9	10.2	9.3	9.9	10.0	9.7
	12	9.7	10.0	9.6	9.9	9.6	10.2

Treatment	Time (d)	TIA concentration (mg/L)					
		F1	F5	F8	TV	V	AK4
Medium control	0	10.0	10.0	10.0	10.0	10.0	10.0
	1	10.0	10.0	10.0	10.0	10.0	10.0
	2	10.4	10.4	10.4	10.4	10.4	10.4
	4	10.3	10.3	10.3	10.3	10.3	10.3
	8	10.9	10.9	10.9	10.9	10.9	10.9
	12	10.9	10.9	10.9	10.9	10.9	10.9

1.2 Tiamulin concentrations in biodegradation test using bacterial enriched cultures

The changes of tiamulin concentrations in biodegradation experiment using four bacterial mixed cultures (A, AN, S, and SN) at various initial tiamulin concentrations ranging from 2.5 to 200 mg/L are showed in Table A2.

Table A2 Raw data of tiamulin concentrations in biodegradation test using bacterial mixed cultures

Treatment	Time (h)	TIA concentration (mg/L)				
		A	AN	S	SN	Control
2.5 mg/L	0	2.7	2.6	2.7	2.6	2.6
	2	2.5	2.5	2.2	2.5	2.6
	4	2.3	2.3	1.6	2.4	2.6
	6	2.1	2.1	0.9	2.1	2.6
	8	1.7	1.9	0.8	1.8	2.6
	10	0.7	1.7	0.7	1.2	2.6
	12	0.3	1.3	0.3	0.3	2.6

Treatment	Time (h)	TIA concentration (mg/L)				
		A	AN	S	SN	Control
	14	0.3	0.6	0.3	0.3	2.6
	16	0.3	0.3	0.3	0.3	2.6
5 mg/L	0	5.1	5.1	5.2	5.1	5.2
	2	4.8	4.8	4.0	4.9	5.1
	4	4.6	4.5	3.3	4.6	5.2
	6	4.0	4.2	2.8	4.2	5.2
	8	3.6	4.0	2.0	4.1	5.1
	10	2.0	3.3	0.9	3.5	5.1
	12	1.3	2.5	0.7	1.0	5.1
	14	0.3	1.1	0.3	0.3	5.1
	16	0.3	0.3	0.3	0.3	5.2
10 mg/L	0	9.9	9.9	10.2	9.9	10.2
	2	9.5	9.6	9.1	9.8	10.2
	4	8.8	8.9	7.5	9.5	10.2
	6	8.1	8.3	5.3	9.0	10.2
	8	7.0	8.0	4.1	8.4	10.3
	10	5.0	7.1	2.7	6.8	10.2
	12	2.4	5.6	2.3	2.9	10.2
	14	1.2	3.5	0.3	0.3	10.2
	16	0.3	2.1	0.3	0.3	10.2
20 mg/L	0	19.9	19.8	20.2	19.9	20.1
	2	19.6	19.4	18.6	19.6	20.1

Treatment	Time (h)	TIA concentration (mg/L)				
		A	AN	S	SN	Control
	4	18.9	18.8	17.4	19.2	20.1
	6	18.1	18.0	6.9	18.3	20.1
	8	16.8	16.9	5.3	18.4	20.2
	10	15.3	15.2	4.8	16.6	20.1
	12	11.6	10.3	0.8	11.5	20.2
	14	6.2	5.6	0.3	2.0	20.1
	16	0.3	3.4	0.3	0.3	20.2
30 mg/L	0	30.0	29.9	30.6	30.1	29.8
	2	29.0	29.3	28.8	29.5	30.0
	4	27.5	28.5	27.2	29.4	30.0
	6	24.5	26.1	16.3	28.8	30.3
	8	21.1	20.2	9.4	28.0	29.9
	10	12.0	12.1	7.4	26.7	29.8
	12	1.2	6.7	1.1	19.7	30.0
	14	0.3	1.7	0.3	7.4	30.0
	16	0.3	0.3	0.3	0.3	30.3
50 mg/L	0	50.0	50.3	50.8	49.9	50.1
	2	48.5	49.2	48.6	49.1	50.0
	4	47.8	47.9	47.4	48.7	50.1
	6	46.6	46.8	30.9	48.4	50.0
	8	42.5	44.4	16.2	48.0	50.0
	10	38.3	41.0	11.1	47.6	50.0

Treatment	Time (h)	TIA concentration (mg/L)				
		A	AN	S	SN	Control
	12	34.1	37.1	0.3	35.3	50.0
	14	27.6	27.6	0.3	21.0	50.1
	16	15.2	1.2	0.3	0.3	50.0
	0	70.1	70.1	70.4	69.5	69.9
	2	68.6	68.2	68.2	68.6	69.9
	4	67.2	64.7	66.5	67.7	69.5
	6	65.3	60.5	47.4	67.1	70.2
70 mg/L	8	59.3	57.3	16.2	66.1	69.9
	10	51.9	51.0	14.9	61.5	69.5
	12	43.2	36.4	0.3	43.1	70.0
	14	36.1	20.7	0.3	16.4	70.2
	16	15.0	7.4	0.3	0.3	70.2
	0	99.8	102.8	100.5	100.1	100.0
	2	97.2	98.1	96.2	98.1	100.0
	4	96.1	95.6	95.4	97.6	100.2
	6	93.7	92.8	83.6	97.3	100.3
100 mg/L	8	86.6	85.2	42.0	96.6	100.0
	10	82.6	78.2	41.6	95.4	100.2
	12	78.0	63.6	0.3	93.8	100.3
	14	61.3	45.0	0.3	71.5	100.5
	16	24.1	23.9	0.3	13.1	100.6
150 mg/L	0	150.0	151.0	150.0	150.2	150.2

Treatment	Time (h)	TIA concentration (mg/L)				
		A	AN	S	SN	Control
	2	148.3	147.7	145.7	147.7	150.0
	4	146.7	143.9	145.4	146.3	150.3
	6	144.0	139.8	132.8	146.3	150.0
	8	141.8	131.7	94.8	146.0	149.9
	10	135.0	117.4	82.4	145.1	149.8
	12	124.7	87.8	0.3	143.3	150.0
	14	101.0	43.2	0.3	118.6	150.1
	16	51.8	27.3	0.3	60.0	150.5
200 mg/L	0	204.7	211.7	213.8	212.1	203.0
	2	202.3	209.7	209.4	211.3	205.5
	4	201.5	205.4	207.2	210.7	204.7
	6	197.3	200.5	193.9	209.8	202.0
	8	186.8	187.4	161.9	209.7	204.2
	10	173.5	164.8	131.5	208.9	204.2
	12	160.0	124.1	0.3	208.0	203.6
	14	103.1	52.5	0.3	174.4	204.8
	16	73.9	23.2	0.3	71.7	201.3

2 Fungal diameters on synthetic dye-containing PDA plates

Table A3 showed the growth of fungi on PDA plates supplementing with brilliant green and crystal violet dyes, compared to fungal diameters on control of PDA plates.

Table A3 Fungal diameters growing on PDA containing synthetic dyes

Fungal strain	Time (d)	Fungal diameter (mm)		
		PDA + brilliant green	**PDA + crystal violet**	**PDA**
F1	0	4.7	4.3	3.7
	2	21.3	27.0	38.7
	4	45.0	65.7	71.0
	6	44.7	83.3	82.7
	8	53.3	84.7	85.3
	10	61.3	87.3	87.7
	12	65.7	88.3	89.3
F2	0	3.3	4.7	4.7
	2	5.3	10.3	30.0
	4	9.0	17.7	70.0
	6	10.7	31.3	90.0
	8	13.0	36.7	90.0
	10	15.7	42.7	90.0
	12	25.0	50.3	90.0
F3	0	3.7	3.3	3.7
	2	10.7	28.3	28.7
	4	19.0	52.0	59.3
	6	34.7	76.7	90.0
	8	47.0	83.7	90.0
	10	55.0	88.3	90.0

Fungal strain	Time (d)	Fungal diameter (mm)		
		PDA + brilliant green	PDA + crystal violet	PDA
	12	73.3	90.0	90.0
F4	0	3.3	5.0	4.3
	2	5.7	14.7	23.0
	4	8.7	26.0	39.0
	6	8.7	42.3	48.7
	8	11.0	53.3	57.7
	10	14.0	63.0	66.0
	12	17.3	78.3	71.0
F5	0	5.0	5.7	4.7
	2	7.3	12.0	25.7
	4	9.0	20.7	40.7
	6	14.7	23.3	63.0
	8	21.3	30.3	80.3
	10	25.0	33.3	90.0
	12	36.7	44.0	90.0
F6	0	4.3	4.3	4.3
	2	8.7	15.0	73.3
	4	9.3	25.7	87.3
	6	10.0	38.3	90.0
	8	15.3	38.3	90.0
	10	20.7	43.3	90.0

Fungal strain	Time (d)	Fungal diameter (mm)		
		PDA + brilliant green	PDA + crystal violet	PDA
	12	26.0	48.0	90.0
F7	0	5.3	6.7	3.7
	2	7.5	8.5	8.3
	4	8.0	11.3	12.3
	6	8.0	11.3	16.7
	8	8.7	11.7	21.0
	10	10.0	12.0	25.0
	12	10.7	13.7	29.7
F8	0	5.0	4.3	4.3
	2	10.3	18.7	23.0
	4	17.7	36.0	44.7
	6	31.0	58.0	67.7
	8	38.0	64.7	78.3
	10	46.3	75.0	87.7
	12	67.3	78.0	85.3
F9	0	4.7	3.7	4.0
	2	5.3	11.3	18.0
	4	11.7	22.3	31.0
	6	15.0	35.3	50.3
	8	20.0	41.0	57.7
	10	24.0	47.3	72.7
	12	31.3	57.3	74.3

Fungal strain	Time (d)	Fungal diameter (mm)		
		PDA + brilliant green	PDA + crystal violet	PDA
F10	0	3.0	3.0	2.7
	2	11.7	31.0	53.3
	4	18.3	68.3	85.0
	6	20.0	90.0	90.0
	8	33.3	90.0	90.0
	10	48.7	90.0	90.0
	12	81.7	90.0	90.0
F11	0	5.3	4.3	4.0
	2	8.7	13.7	20.0
	4	10.3	18.3	21.3
	6	15.7	25.3	29.3
	8	16.0	30.3	33.3
	10	17.7	34.3	38.7
	12	21.0	42.3	49.3
F12	0	4.7	4.7	4.3
	2	7.7	11.0	13.7
	4	13.7	20.0	30.0
	6	19.7	29.0	36.3
	8	24.0	36.7	44.0
	10	29.3	45.7	50.3
	12	37.7	61.0	61.7
Trametes versicolor	0	6.7	6.7	4.7

Fungal strain	Time (d)	Fungal diameter (mm)		
		PDA + brilliant green	PDA + crystal violet	PDA
	2	8.7	13.0	18.7
	4	16.0	22.7	37.7
	6	20.0	32.3	59.0
	8	27.0	38.7	70.0
	10	34.0	46.7	87.7
	12	50.0	60.7	90.0
Verticillium sp.	0	6.0	7.3	5.3
	2	10.7	11.7	11.7
	4	11.0	19.0	20.7
	6	14.0	22.7	29.0
	8	19.3	29.3	35.7
	10	22.0	36.3	38.7
	12	24.3	37.0	43.7
Trametes hirsuta AK4	0	6.3	5.7	5.0
	2	15.0	18.0	24.3
	4	31.0	36.0	51.7
	6	50.0	61.7	81.0
	8	60.0	73.0	86.3
	10	77.7	85.0	90.0
	12	86.7	90.0	90.0

3 Fungal enzymatic activities

3.1 Ligninolytic enzyme activity of wood-rot fungi in tiamulin degradation

Activities of manganese peroxidase (MnP), lignin peroxidase (LiP), and laccase (Lac) in three isolated fungi (F1, F5 and F8) and three white-rot fungi (*Trametes versicolor*, *Verticillium* sp., and *Trametes hirsuta* AK4) used in degradation of 10 mg/L tiamulin are showed with raw data in Table A4.

Table A4 Raw data of fungal enzymatic activities in tiamulin degradation test

Fungal strain	Enzyme	Time (d)					
		0	**1**	**2**	**4**	**8**	**12**
F1	Lac	0.0	0.9	0.2	0.4	0.0	0.0
	MnP	0.0	44.4	55.8	53.6	30.8	12.5
	LiP	0.0	0.0	0.0	0.0	0.0	0.0
F5	Lac	0.2	0.2	2.6	0.2	0.3	0.3
	MnP	1.7	38.7	46.7	51.3	25.6	6.3
	LiP	0.0	0.0	0.0	0.3	0.0	0.0
F8	Lac	0.0	0.9	0.3	0.1	0.2	0.1
	MnP	1.1	22.2	59.3	45.0	3.4	1.7
	LiP	0.0	0.1	0.1	0.0	0.0	0.0
TV	Lac	0.0	0.4	0.1	0.3	0.1	0.2
	MnP	6.8	37.6	47.9	34.8	5.7	1.7
	LiP	0.0	2.9	0.1	0.1	3.3	0.4
V	Lac	0.0	0.7	0.2	0.2	0.3	0.4
	MnP	31.9	17.1	19.9	43.9	2.8	0.6
	LiP	0.0	0.0	0.0	0.5	0.0	0.0
AK4	Lac	1.8	4.4	4.9	2.2	2.5	0.4
	MnP	0.6	31.3	6.3	20.5	10.3	33.0
	LiP	0.0	0.0	0.0	0.0	0.0	0.0
Control	Lac	0.0	0.0	0.0	0.0	0.0	0.0
	MnP	0.0	0.0	0.0	0.0	0.0	0.0
	LiP	0.0	0.0	0.0	0.0	0.0	0.0

3.2 Ligninolytic enzyme production in basal medium without tiamulin substrate

Three ligninolytic enzymes are also produced in basal medium with no supplementation of tiamulin substrate (Figure A5).

Table A5 Raw data of fungal enzymatic activities in tiamulin degradation test

Fungal strain	**Enzyme**	**Time (d)**					
		0	**1**	**2**	**4**	**8**	**12**
F1	Lac	0.0	0.4	0.1	0.3	0.9	0.2
	MnP	0.0	34.2	10.8	13.1	17.7	10.8
	LiP	0.0	0.0	0.0	0.0	0.0	0.0
F5	Lac	0.1	0.3	1.3	1.9	1.5	0.3
	MnP	0.0	2.8	5.1	8.0	22.2	25.1
	LiP	0.0	0.0	0.3	0.2	0.9	0.0
F8	Lac	0.0	0.9	0.6	0.3	0.6	0.2
	MnP	0.0	5.7	6.3	20.5	10.3	33.0
	LiP	0.0	0.0	0.0	0.0	0.0	0.0
TV	Lac	0.0	0.9	0.8	0.9	0.6	0.2
	MnP	0.0	17.7	39.3	7.4	2.3	2.3
	LiP	0.0	0.0	0.9	1.3	0.7	0.4
V	Lac	0.0	0.5	0.9	0.8	0.7	0.1
	MnP	0.0	2.8	0.6	33.6	32.5	10.8
	LiP	0.0	0.0	0.0	0.0	0.0	0.0
AK4	Lac	0.0	0.4	0.3	0.4	1.0	2.6
	MnP	0.0	8.5	20.5	10.3	42.7	29.6
	LiP	0.0	0.0	0.0	0.0	0.0	0.0

Fungal strain	Enzyme	Time (d)					
		0	1	2	4	8	12
Control	Lac	0.0	0.0	0.0	0.0	0.0	0.0
	MnP	0.0	0.0	0.0	0.0	0.0	0.0
	LiP	0.0	0.0	0.0	0.0	0.0	0.0

4. Bacterial numbers in tiamulin degradation test

Cell numbers of bacteria at the beginning, the middle, and the end of tiamulin degradation test by using in four enriched cultures (A, AN, S, and SN) at different tiamulin concentrations (2.5 - 200 mg/L) are showed in Table A6.

Table A6 Raw data of bacterial numbers in tiamulin biodegradation test at different tiamulin concentrations

Treatment	Time (h)	TIA concentration (mg/L)			
		A	AN	S	SN
2.5 mg/L	0	6.8×10^{7}	9.2×10^{7}	8.9×10^{7}	1.0×10^{8}
	8	4.8×10^{8}	4.5×10^{8}	1.1×10^{9}	6.1×10^{8}
	16	9.9×10^{10}	1.9×10^{11}	2.7×10^{11}	1.0×10^{10}
5 mg/L	0	2.2×10^{8}	1.1×10^{8}	1.1×10^{8}	7.8×10^{7}
	8	5.8×10^{8}	4.9×10^{8}	1.3×10^{9}	5.3×10^{8}
	16	2.8×10^{10}	2.2×10^{11}	2.5×10^{11}	2.2×10^{10}
10 mg/L	0	2.7×10^{8}	1.6×10^{8}	2.1×10^{8}	1.3×10^{8}
	8	5.3×10^{8}	4.0×10^{8}	1.3×10^{9}	6.5×10^{8}
	16	3.1×10^{10}	1.3×10^{11}	2.4×10^{11}	2.1×10^{10}
20 mg/L	0	9.2×10^{7}	1.4×10^{8}	1.2×10^{8}	1.1×10^{8}

Treatment	Time (h)	TIA concentration (mg/L)			
		A	AN	S	SN
	8	5.5×10^{8}	4.5×10^{8}	7.2×10^{8}	4.3×10^{8}
	16	1.8×10^{10}	1.9×10^{11}	2.8×10^{11}	2.3×10^{10}
30 mg/L	0	2.0×10^{8}	1.7×10^{8}	1.3×10^{8}	5.3×10^{7}
	8	6.0×10^{8}	4.1×10^{8}	1.3×10^{9}	5.0×10^{8}
	16	2.8×10^{10}	1.7×10^{11}	2.8×10^{11}	1.7×10^{10}
50 mg/L	0	1.8×10^{8}	1.6×10^{8}	1.8×10^{8}	5.1×10^{7}
	8	5.9×10^{8}	5.8×10^{8}	7.3×10^{8}	5.6×10^{8}
	16	2.4×10^{10}	1.3×10^{11}	2.8×10^{11}	1.3×10^{10}
70 mg/L	0	1.1×10^{8}	6.7×10^{7}	6.6×10^{7}	6.2×10^{7}
	8	5.6×10^{8}	2.8×10^{8}	4.5×10^{8}	3.8×10^{8}
	16	2.1×10^{10}	1.3×10^{11}	2.1×10^{11}	1.9×10^{10}
100 mg/L	0	1.1×10^{8}	5.3×10^{7}	9.0×10^{7}	6.9×10^{7}
	8	4.3×10^{8}	3.1×10^{8}	6.0×10^{8}	2.9×10^{8}
	16	3.3×10^{10}	9.9×10^{10}	3.3×10^{11}	1.8×10^{10}
150 mg/L	0	1.0×10^{8}	3.9×10^{7}	5.2×10^{7}	8.8×10^{7}
	8	5.4×10^{8}	2.7×10^{8}	3.2×10^{8}	4.3×10^{8}
	16	2.2×10^{10}	2.6×10^{11}	2.7×10^{11}	1.0×10^{10}
200 mg/L	0	6.9×10^{7}	3.8×10^{7}	3.0×10^{7}	5.7×10^{7}
	8	5.5×10^{8}	2.7×10^{8}	2.5×10^{8}	5.2×10^{8}
	16	1.8×10^{10}	2.8×10^{11}	2.5×10^{11}	6.7×10^{9}

5. Bacterial diversity in enriched cultures

5.1 Sample information

All four bacterial enriched culture and their corresponding treatments at 2.5 and 200 mg/L of tiamulin samples were analyzed with the next-generation sequencing technique to determine bacterial community structures. All information of these samples from the next-generation sequencing is indicated in Table A7.

Table A7 Sample information in metagenomics analysis

Sample	Total reads	Reads Passing Quality Filtering	% Reads Passing Quality Filtering
A	202,033	157,512	77.96%
A 2.5	172,436	138,390	80.26%
A 200	150,509	118,383	78.66%
AN	150,575	120,153	79.80%
AN 2.5	287,985	225,606	78.34%
AN 200	116,455	93,454	80.25%
S	201,692	166,060	82.33%
S 2.5	166,979	136,844	81.95%
S 200	344,749	286,095	82.99%
SN	166,317	130,299	78.34%
SN 2.5	241,746	192,989	79.83%
SN 200	183,193	145,647	79.50%

5.2 Classification statistics

5.2.1 Read numbers classified to taxonomic level

Bacterial communities in all samples were classified to taxonomic level of kingdom, phylum, class, order, family, genus, and species with corresponding read numbers (Table A8).

Table A8 Reads classified to taxonomic level

Sample	Taxonomic level						
	Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**
A	157,475	157,288	156,748	155,664	154,365	152,754	99,503
A 2.5	138,347	138,182	137,826	137,106	136,133	134,800	85,941
A 200	118,344	118,224	117,917	117,321	116,436	115,556	69,957
AN	120,088	119,838	119,265	117,330	116,029	113,164	54,651
AN 2.5	225,497	225,118	224,197	221,026	219,326	215,472	120,741
AN 200	93,432	93,307	93,042	91,975	90,893	89,377	45,155
S	166,012	165,848	165,299	163,705	162,783	156,800	93,453
S 2.5	136,745	136,558	136,054	134,414	133,599	128,762	78,338
S 200	286,011	285,822	285,256	282,963	281,611	269,186	166,182
SN	130,183	129,877	128,817	127,731	126,062	115,457	55,752
SN 2.5	192,750	192,373	190,606	189,318	187,491	167,217	76,700
SN 200	145,583	145,389	144,893	143,188	142,460	131,664	85,674

5.2.2 Percentage of total reads classified to taxonomic level

Total reads classified to taxonomic level was also showed in percentage in Table A9.

Table A9 Percentage of reads classified to taxonomic level

Sample	Taxonomic level						
	Kingdom	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**
A	100.0%	99.9%	99.5%	98.8%	98.0%	97.0%	63.2%
A 2.5	100.0%	99.9%	99.6%	99.1%	98.4%	97.4%	62.1%
A 200	100.0%	99.9%	99.6%	99.1%	98.4%	97.6%	59.1%
AN	100.0%	99.7%	99.3%	97.7%	96.6%	94.2%	45.5%
AN 2.5	100.0%	99.8%	99.4%	98.0%	97.2%	95.5%	53.5%
AN 200	100.0%	99.8%	99.6%	98.4%	97.3%	95.6%	48.3%
S	100.0%	99.9%	99.5%	98.6%	98.0%	94.4%	56.3%
S 2.5	99.9%	99.8%	99.4%	98.2%	97.6%	94.1%	57.3%
S 200	100.0%	99.9%	99.7%	98.9%	98.4%	94.1%	58.1%
SN	99.9%	99.7%	98.9%	98.0%	96.8%	88.6%	42.8%
SN 2.5	99.9%	99.7%	98.8%	98.1%	97.2%	86.7%	39.7%
SN 200	100.0%	99.8%	99.5%	98.3%	97.8%	90.4%	58.8%

5.3 Classification results by taxonomic level

The results of metagenomics analysis showed bacterial communities in all samples were classified to taxonomic level form kingdom to species (Table A10 - Table A14).

5.3.1 Top kingdom classification results

Table A10 Number of reads in top kingdom classification results

Sample	Classification			
	Bacteria	**Viruses**	**Archaea**	**Unclassified at kingdom level**
A	157,457	17	1	37
A 2.5	138,334	13		43
A 200	118,333	11		39
AN	120,073	15		65
AN 2.5	225,476	21		109
AN 200	93,429	3		22
S	166,000	12		48
S 2.5	136,739	6		99
S 200	286,002	8	1	84
SN	130,175	8		116
SN 2.5	192,731	17	2	239
SN 200	145,566	16	1	64

5.3.2 Top phylum classification results

Table A11 Number of reads in top phylum classification results

Classification	Sample											
	A	A 2.5	A 200	AN	AN 2.5	AN 200	S	S 2.5	S 200	SN	SN 2.5	SN 200
Proteobacteria	156,488	137,264	115,498	114,402	215,510	92,390	154,147	131,766	270,748	119,160	169,804	138,522
Bacteroidetes	179	289	2,412	4,544	8,016	516	11,072	3,944	14,453	9,797	21,262	6,320
Firmicutes	412	484	188	652	1,064	254	358	371	328	575	586	290
Actinobacteria	128	79	65	151	355	103	121	349	133	223	504	152
Tenericutes	36	25	28	59	109	29	76	63	81	68	83	51
Unclassified	224	208	159	315	488	147	212	286	273	422	616	258

5.3.3 Top class classification results

Table A12 Number of reads in top class classification results

Classification	Sample											
	A	A 2.5	A 200	AN	AN 2.5	AN 200	S	S 2.5	S 200	SN	SN 2.5	SN 200
Betaproteobacteria	79,610	58,930	46,402	24,919	39,194	5,478	34,536	42,524	16,906	18,683	21,743	12,850
Gammaproteobacteria	66,825	66,053	60,413	85,568	168,892	84,737	98,863	71,966	236,098	20,293	61,997	116,405
Alphaproteobacteria	9,301	11,786	8,247	3,151	6,150	1,811	19,999	16,557	16,966	79,085	83,717	8,589
Bacilli	321	408	151	550	930	203	280	278	241	374	367	223
Deltaproteobacteria	171	109	117	146	310	53	191	184	212	314	523	204
Flavobacteriia	128	256	2,373	4,498	7,682	501	10,990	3,874	14,379	6,853	15,411	5,767
Actinobacteria	126	78	65	148	349	102	121	347	131	219	492	151
Epsilonproteobacteria	65	46	29	76	130	60	66	79	62	264	1,178	93
Sphingobacteriia	45	30	33	41	275	15	48	42	40	2,415	4,722	447
Clostridia	76	64	30	77	107	39	61	81	69	189	207	60
Mollicutes	36	25	28	59	109	29	76	63	81	68	83	51
Unclassified	764	564	466	888	1,409	412	761	790	839	1,482	2,383	754

5.3.4 Top genus classification results

Table A13 Number of reads in top genus classification results

Classification	Sample											
	A	A 2.5	A 200	AN	AN 2.5	AN 200	S	S 2.5	S 200	SN	SN 2.5	SN 200
Achromobacter	53,715	55,176	50,487	11,233	25,698	44,935	40,326	28,823	91,732	12,128	28,001	21,712
Stenotrophomonas	47,497	46,126	29,950	3,043	6,767	1,119	2,827	1,664	1,084	14,816	8,978	8,052
Pseudomonas	28,025	10,338	14,404	19,304	28,381	3,134	29,310	38,048	13,471	916	9,924	2,483
Pandoraea	6,083	4,840	4,583	51,166	63,597	26,412	159	144	152	332	100	73
Ochrobactrum	3,356	2,427	3,188	943	1,085	443	1,095	614	1,373	5,309	2,049	667
Defluvibacter	2,602	2,237	1,608	377	839	269	3,082	1,994	2,152	9,081	9,249	1,248
Burkholderia	1,864	1,480	1,425	12,948	26,348	7,286	134	146	119	172	92	112
Hyphomicrobium	344	2,890	271	27	35	13	4,781	3,354	3,769	26,629	21,465	1,383
Flavobacterium	15	191	2,271	9	19	5	10,681	3,715	14,142	224	753	4,385
Chryseobacterium	10	1	2	4,410	6,950	470	6	4	5	11	33	3
Methylobacillus	73	48	27	2,685	42,372	401	30	24	8	29	14	8
Lautropia	1,155	1,055	1,160	267	1,033	839	848	482	1,961	347	626	802
Delftia	1	5	4	5	24	12	40,422	28,094	106,104	2,388	17,822	66,887
Pseudaminobacter	26	35	11	8	15	3	2,990	1,504	2,127	1,691	656	180
Sphingomonas	77	46	41	51	55	31	376	2,849	275	2,772	19,306	372
Paucibacter	98	64	38	15	158	19	2,561	1,628	6,171	210	1,023	4,786
Polaromonas	7	0	19	2	10	6	1,070	922	2,629	105	668	2,178
Shinella	87	68	81	46	107	17	185	110	313	8,652	5,565	1,492
Pedomicrobium	189	1,299	129	4	14	5	2,085	1,647	1,440	7,925	4,495	499
Unclassified	4,758	3,590	2,827	6,989	10,134	4,077	9,260	8,082	16,909	14,842	25,772	13,983

5.3.5 Top species classification results

Table A14 Number of reads in top species classification results

Classification	Sample											
	A	A 2.5	A 200	AN	AN 2.5	AN 200	S	S 2.5	S 200	SN	SN 2.5	SN 200
Stenotrophomonas maltophilia	25,753	25,373	8,032	1,082	2,886	504	6	2	1	648	355	3,953
Achromobacter insolitus	25,675	26,135	24,482	4,528	11,501	19,948	19,293	13,068	43,492	5,205	12,733	10,054
Pseudomonas panipatensis	17,350	6,464	19,476	11,645	17,759	1,895	18,137	23,182	8,343	358	5,933	1,335
Stenotrophomonas pavanii	11,220	10,473	3,145	812	1,495	303	32	38	21	415	127	2,059
Achromobacter arsenitoxydans	7,259	7,081	4,507	2,605	3,196	6,719	5,286	4,423	8,706	1,431	2,165	2,644
Pandoraea apista	1,346	1,047	888	12,849	10,087	5,657	25	33	24	73	22	22
Ochrobactrum thiophenivorans	1,254	942	1,235	383	423	161	135	59	112	1,474	486	164
Achromobacter xylosoxidans	1,057	1,124	1,156	211	542	953	794	623	2,156	250	713	411
Lautropia mirabilis	1,155	1,055	1,160	267	1,033	839	848	482	1,961	347	626	802
Chryseobacterium joostei	0	0	0	3,009	5,035	346	0	0	0	0	0	0
Burkholderia ubonensis	577	457	483	2,899	9,110	1,674	44	34	33	49	11	26
Burkholderia phenoliruptrix	254	180	133	2,554	2,152	1,125	11	24	14	35	11	24
Methylobacillus flagellatus	10	3	1	1,530	35,932	99	10	9	1	8	0	2
Methylobacillus glycogenes	60	43	26	906	3,328	281	20	14	7	21	14	6
Delftia lacustris	1	4	4	5	20	8	20,165	14,927	49,103	1,359	8,623	33,544
Delftia tsuruhatensis	0	1	0	0	2	1	10,402	6,579	30,411	503	4,988	16,661
Stenotrophomonas acidaminiphila	0	2	0	519	892	77	2,345	1,296	921	10,274	6,713	322
Pseudaminobacter salicylatoxidans	5	6	1	2	0	0	1,652	788	1,331	860	389	100
Curvibacter gracilis	1	3	8	4	13	7	1,210	1,122	1,767	78	352	1,245
Delftia acidovorans	0	0	0	0	2	1	960	462	2,404	47	78	1,111
Shinella granuli	77	57	71	5	1	2	167	97	286	8,041	5,218	1,381
Pedomicrobium manganicum	22	127	27	2	4	4	285	141	163	2,595	1,921	214
Pedomicrobium ferrugineum	83	639	46	2	9	0	923	782	549	2,396	882	119
Unclassified	58,009	52,449	48,426	65,502	104,865	48,299	72,607	58,506	119,913	74,547	116,289	59,973

APPENDIX B SUPPLEMENTARY PROTOCOL AND METHODS

1 DNA extraction protocol

1.1 Genomic DNA of swine farm isolated fungi

Genomic DNA of the fungal isolated strains was extracted using Fast DNA Spin extraction kit for soil (QBiogene, Solon, Ohio, USA) according to the manufacturer's instructions. The protocol of fungal DNA extraction was following steps:

1) Add 100 mg of fungal mycelia to a Lysing Matrix E tube;

2) Add 978 µL sodium phosphate buffer and 122 µL MT buffer to sample in Lysing Matrix E tube;

3) Homogenize in the FastPrep® instrument at a speed of 6.0 for 40 minutes;

4) Centrifuge at 14,000 x g for 10 minutes to pellet debris;

5) Transfer supernatant to a clean 2.0 mL microcentrifuge tube, adding 250 µL PPS (protein precipitation solution) and mixing 10 times by hand shaking;

6) Centrifuge at 14,000 x g for 5 minutes to pellet precipitate, transferring supernatant to a clean 2.0 mL tube;

7) Resuspend and add 1.0 mL of Binding Matrix suspension to supernatant in 2.0 mL tube;

8) Invert by hand or place on rotator for minutes to allow binding of DNA and place tube in a rack for 3 minutes to allow setting of silica matrix;

9) Remove and discard 500 µL of supernatant being careful to avoid settled Binding Matrix, resuspend Binding Matrix in the remaining amount of supernatant, transfer

approximately 500 µL of the mixture to a SPIN^{TM} filter and centrifuge at 14,000 x g for 1 minute, empty the catch tube and add the remaining mixture to the SPIN^{TM} filter and centrifuge as before, empty the catch tube again;

10) Add 500 µL prepared SEWS-M (already added ethanol) and gently resuspend the pellet using the force of the liquid from the pipet tip;

11) Centrifuge at 14,000 x g for 1 minute, empty the catch tube and replace;

12) Without any addition of liquid, centrifuge a second time a 14,000 x g for 2 minutes to dry the matrix of residual wash solution, discard the catch tube and replace with a new, clean catch tube;

13) Air dry the SPIN^{TM} filter for 5 minutes at room temperature;

14) Gently resuspend Binding Matrix (above the SPIN^{TM} filter) in 50 µL of DES (DNase/Pyrogen-Free Water); and

15) Centrifuge at 14,000 x g for 1 minute to bring eluted DNA into the clean catch tube, discard the SPIN^{TM} filter. The DNA products were stored at - 20^{o}C for extended periods or until further molecular analysis.

1.2 Genomic DNA of bacterial mixed enriched cultures

Genomic DNA of the bacterial mixed enriched cultures was also extracted using Fast DNA Spin extraction kit for soil (QBiogene, USA) with the same protocol of fungal DNA extraction of 15 steps according to the manufacturer's instructions. However, 10 mL of enriched samples were centrifuged in succession in 2 mL tubes at 14,000 x g for 20 minutes to get bacterial cells at the bottom of the tubes before adding to a Lysing Matrix E tube in the first step.

2 Determination of DNA concentration

The nucleic acid concentrations of all bacterial DNA samples of enriched cultures were measured by using NanoDrop Spectrophotometer (Thermo scientific, USA) with five steps of the protocol.

1) Cleaning the upper and lower optical surfaces of the microspectrophotometer sample retention system as follows. Pipet 1 to 2 µL of clean DI water onto the lower optical surface. Close the lever arm and tap it a few times to bathe the upper optical surface. Lift the lever arm and wipe off both optical surfaces with a Kimwipe;

2) Opening the NanoDrop software and select the nucleic acids module;

3) Initializing the spectrophotometer by placing 1 µL clean water onto the lower optical surface, lowering the lever arm, and selecting the "initialize" in the NanoDrop software. Once initialization is complete (approximately 10 s), clean both optical surfaces with a Kimwipe;

4) Performing the blank measurement by loading 1 µL DI water and selecting "blank". Once the blank is complete, clean both optical surfaces with a Kimwipe;

5) Measuring the nucleic acid sample by loading 1 µL and selecting "measure". Once the measurement is complete, clean both optical surfaces with a Kimwipe.

3 Denaturing gradient gel electrophoresis (DGGE) protocol

3.1 Chemicals preparation

All-important chemicals used in DGGE were prepared following Table B1.

Table B1 Preparation of necessary chemicals used in DGGE

Chemicals	Preparation	Note

Chemicals	Preparation	Note
Acry/Bis 37.5:1, 40% (w/v) solution	187.5 g Acrylamide + 5 g Bis-Acrylamide + 500 mL distilled H_2O	From Amresco
Deionized formamide		From Amresco
50 X TAE buffer	484.4 g Trizma-Base + 272.0 g Sodium-Acetate + 37.2 g Trisodium EDTA + 2 L H_2O Adjust pH to 7.4 with about 230 mL of glacial acetic acid	Check pH carefully the second day after preparation
7 M urea	42.042 g Urea + 100 ml H_2O	
Glycerol		From Pacific Sci.
10% Ammonium persulfate (APS)	0.1 g APS + 1 mL H_2O	Make fresh when used
TEMED		From Pacific Sci.

3.2 DGGE protocol

3.2.1 Building the gel assembly

1) Using lint-free tissues, wash glass plates, spacers and combs thoroughly with isopropanol.

2) Assemble the gel sandwich by placing the small glass plate on top of the large plate, being sure to correctly place a 1 mm spacer along each edge of the plate assembly. Attach the plate clamps (tight enough to hold everything together) and place the entire assembly into the rear slot of the pouring stand. Loosen the clamps slightly and use the spacing card to assure the proper spacer alignment. Tighten the plate clamps (snug, as if you were trying to prevent "leakage") and remove the plate assembly from the pouring stand. Inspect the plate assembly to ensure that the two glass plates and the spacers form a flush surface across bottom of the assembly. If the surface is not flush, re-set the plate assembly, as breaches in the seal of the plates assembly with the bottom of the pouring stand will result in leaking gel solutions.

3) Place a foam gasket into one of the two front slots of pouring stand, insert the plate assembly, and clamp into place. Place the well comb firmly in between the plates and draw a line on the glass plate where the bottom of the wells is located. This will be a future reference point when pouring the gel.

4) Set up the gradient former by inserting 30 mL syringes into a "closed" gradient wheel. Secure the syringes and 'back-out" the syringe plungers by reversing the gradient wheel. If the syringes do not move smoothly, replace them with new syringes. Note the resulting volume measured on each syringe (between 12 - 13 mL), which will be the amount of solution that the system will deliver to create the new gel.

5) Attach the small (10 cm) delivery tubing to the syringes.

3.2.2 Pouring the gel

Using the Table B2 to determine the appropriate composition of the denaturing gradient gel. The DGGE gel was poured following steps as follows.

1) Make two solutions of total 15.8 mL volume each that are a "low" denaturant concentration solution of 30% and a "high" denaturant concentration solution of 70%

following the order in Table 3.11 and swirl gently to mix both solutions. It is noted that two solutions were put in the ice box during making time. The gradient wheel was combined these during the pouring of the gel to create the gradient within the gel matrix.

2) Add 10 µL loading dye to the high denaturant solution making easily and clearly to see the gradient when pouring the gel.

3) Draw each solution into the syringes. The low denaturant syringe was on the front side of the gradient maker and the high denaturant was in on the backside. Make sure air was removed from syringes (by tapping on the side) before placing them into the gradient maker. Attach the "Y" connector to each tube and attach the final long (15 cm) tube to the "Y" connector.

4) Place the delivery tube in between the two plates near the center of the top edge of the plate assembly. Slowly and consistently turn the wheel until the gel is poured to the level of the line drawn. It should take between two to three minutes to pour the gel.

5) Carefully, without disturbing the gel solution, add approximately 2 mL of 1 X TAE buffer to the gel solution to form a layer on top of the gel solutions approximately 5 mm thick. This layer will help the top boundary of the gel to be smooth.

6) Let the gel polymerize for about 30 minutes. Then, remove the 1 X TEA buffer with a syringe and with the same syringe add approximately 3 mL of "cap" solution to the top of the polymerized gel. Carefully place the comb at top at a slight angle between the plates. Be sure to avoid trapping any air bubbles as the comb was lowered into the cap solution. Let polymerize at least two hours to overnight before running the gel.

3.2.3 Running the gel

1) Prepare approximately 7 liters of 1 X TAE buffer and fill the buffer chamber, put about 0.5 L aside for later use. Preheat the buffer in the DCODE apparatus to 65°C; this will take about 2 hours.

2) When the temperature is about 50°C interrupt heating and attach the gel plates to the core assembly. Two sets of plates must be attached. Therefore, if only one gel is to be run, the other set of plates will be an assembly of two plates with no spacers or gel. Place the core assembly in the buffer chamber and the fill top reservoir with remaining buffer.

3) Flush each well with buffer to remove any unpolymerized acrylamide. Failure to do this might result in uneven well floors and unresolved bands. Continue heating until 65°C is reached. Do not add plate assembly to buffer that is too hot (> 55^{o}C). This will cause the plates to crack.

4) Flush each well with buffer again and load approximately 30 µL of PCR product containing loading dye to each well. The volume loaded depends on the success of the PCR and the number of expected products. Soil samples produce many products; therefore, the maximum volume should be loaded. Conversely, when running single isolate PCR products, a few microliters will suffice. Then run at 130 V for 4.5 hours (270 min).

3.2.4 Staining the gel

1) When the electrophoresis is complete, take apart the apparatus and remove the glass plates from the gel clamps. Carefully separate the plates, leaving the gel exposed on the large plate. Use the edge of the small plate to trim the well walls, but be sure to leave the left-most wall slightly higher than the others for use as a reference. Also, trim off any portions of the gel that do not contain used lanes. For easy manipulation, the gel should be transferred to, stained on, and transported on plastic wrap.

2) Stain the gel for 15 minutes in 200 mL of 10% Ethidium bromide diluted 20 µL: 200 mL in DI water. Remember wear gloves (powder-free) in this step. Use a

container that is slightly larger than the gel. The container should be plastic and not glass.

3) Slide the gel off of the plastic wrap onto a UV transilluminator and view the gel.

Table B2 DGGE gel compositions

Reagents	Final Conc.	Cap	20%	25%	30%	35%	40%	45%	50%	55%	60%	65%	70%
H_2O	N/A	3.6 mL	9.5 mL	9.0 mL	8.5 mL	8.0 mL	7.5 mL	7.0 mL	6.5 mL	6.0 mL	5.5 mL	5.0 mL	4.5 mL
40% acrylamide:bis (37:1)	8%	1.3 mL	3 mL	3 mL	3 mL	3 mL	3 mL	3 mL	3 mL	3 mL	3 mL	3 mL	3 mL
50 X TAE buffer	1X	0.1 mL	0.3 mL	0.3 mL	0.3 mL	0.3 mL	0.3 mL	0.3 mL	0.3 mL	0.3 mL	0.3 mL	0.3 mL	0.3 mL
Urea	Variable	0	1.3 mL	1.6 mL	1.9 mL	2.2 mL	2.5 mL	2.8 mL	3.1 mL	3.4 mL	3.7 mL	4.0 mL	4.3 mL
Formamide	Variable	0	1.2 mL	1.5 mL	1.8 mL	2.1 mL	2.4 mL	2.7 mL	3.0 mL	3.3 mL	3.6 mL	3.9 mL	4.2 mL
Glycerol	2%	0	0.3 mL	0.3 mL	0.3 mL	0.3 mL	0.3 mL	0.3 mL	0.3 mL	0.3 mL	0.3 mL	0.3 mL	0.3 mL
10% APS		40 µL	81 µL	81 µL	81 µL	81 µL	81 µL	81 µL	81 µL	81 µL	81 µL	81 µL	81 µL
TEMED		2.5 µL	4.5 µL	4.5 µL	4.5 µL	4.5 µL	4.5 µL	4.5 µL	4.5 µL	4.5 µL	4.5 µL	4.5 µL	4.5 µL

4 Measurement of fungal enzymatic activity by UV spectrophotometer

4.1 Preparation of chemicals

All chemicals used in measuring enzymatic activities of fungi including 40 mmol/L Veratrylalcohol (VA), 10 mmol/L Hydrogen peroxide (H_2O_2), 10 mmol/L Manganese (II) sulfate ($MnSO_4$), 10 mmol/L Guaiacol, and 250 mmol/L Sodium succinate buffer (pH 4.5 and pH 5.0) were prepared following Table B3.

Table B3 Preparation of chemicals used in measurement of fungal enzymatic activity

Chemicals	**Concentration**	**Preparation**
Guaiacol	10 mmol/L	0.11 mL Guaiacol + 99.89 mL DI water
Hydrogen peroxide	10 mmol/L	78.2 µL H_2O_2 30% + 100 mL DI water
Manganese (II) sulfate	10 mmol/L	33.8 mg $MnSO_4 \cdot H_2O$ + 100 mL DI water
Veratrylalcohol	40 mmol/L	0.581 mL VA + 99.419 mL DI water
Sodium succinate buffer	pH 4.5 and 5.0	25 mL 0.2 M solution of succinic acid (2.36 g succinic acid + 100 mL DI water) + 17 mL or 26.7 mL 0.2 M NaOH
Crude enzyme	Samples collected from tiamulin degradation experiment by fungi	

4.2 Procedure of absorbent measurement

The protocol of measurement of absorbance of six fungal crude enzymes using UV spectrophotometer (Thermo Electron Corporation, USA) consists of some following steps. The volume of all components of reaction mixture as well as wavelength and time of measurement of each enzyme are presented in Table B4.

Table B4 Ingredients of reaction mixture, measured wavelength and time in absorbance measurement of three enzymes in tiamulin degradation test with fungi

Enzyme	**Reaction mixture**	**Wavelength**	**Time**
Lac	200 µL 250 mmol/L Sodium succinate buffer (pH 4.5) + 40 µL 10 mmol/L Guaiacol + 760 µL crude enzyme	465 nm	1 min (30 min reaction)
MnP	400 µL 250 mmol/L Sodium succinate buffer (pH 5.0) + 50 µL 2 mmol/L $MnSO_4$ + 10 µL 10 mmol/L H_2O_2 + 540 µL crude enzyme	238 nm	10 s
LiP	250 µL 250 mmol/L Sodium succinate buffer (pH 4.5) + 50 µL 40 mmol/L Veratrylalcohol + 40 µL 10 mmol/L H_2O_2 + 660 µL crude enzyme	310 nm	2 min

1) Operating and optimizing the UV spectrophotometer at 465 nm, 238 nm, and 310 nm for Lac, MnP, and LiP activity, respectively. Use DI water to zero instrument;

2) Performing the blank measurement by adding 1 mL DI water into cuvette and selecting “blank”, then measuring DI water is sample and recording zero value. Once the blank is complete, clean cuvette with a Kimwipe;

3) Measuring absorbance of mixture containing all reagents of reaction mixture of each sort of enzyme except the crude enzyme with or without DI water as two

controls. Recording the change in the absorbance from the beginning to the end of the measurement period. Then cleaning cuvette with DI water and a Kimwipe;

4) Measuring absorbance of three kinds of enzymes by adding all necessary substrates of reaction mixture and crude enzyme in total 1 mL into the cuvette and selecting "measure". Recording the absorbance changes: starting the stopwatch as the initial absorbance, ending the measurement period as the final ones. Cleaning the cuvette by using DI water and a Kimwipe after each measurement.

5 Tiamulin standard curves and tiamulin residues in biodegradation test

5.1 Standard curve and residues of tiamulin in biodegradation test with wood rot fungi

5.1.1 Tiamulin standard curve

A stock standard tiamulin solution was prepared by dissolving 100 mg of the compound in 100 mL ultrapure water 18 Ω and stored at 4^{o}C refrigerator. Working standard solutions, including 0.1, 0.5, 1, 5, and 10 mg/L of tiamulin concentrations were created by diluting stock with ultrapure water for making tiamulin standard curve in tiamulin degradation experiment with wood rot fungi.

After analyzing all working standard samples with HPLC-DAD Agilent 1100 series diode array and multiple wavelength detectors system [205] with autosampler, the chromatogram of all working tiamulin standards is showed in figure B1. The Hypersil ODS C18 column (250 × 4.0 mm, particle size 5 µm) with column guard was used to separate chromatograms. Mobile phases of HPLC analysis were 10% grade methanol (A) and 90% HPLC water acidified to pH 2.2 with sulphuric acid (B). Injection volume was 20 µL standard solutions. Mobile phases were run at the flow rate of 0.25 and 0.5 mL/min. Tiamulin was detected at 208 nm with approximately 20 minutes' retention time with controlling temperature at 25^{o}C. TIA standards were always run at the same time with samples.

Standard curve was built based on HPLC-DAD results with specific concentrations of tiamulin. Standard curves used to calculate the tiamulin concentrations in liquid media during the biodegradation test (Figure B2).

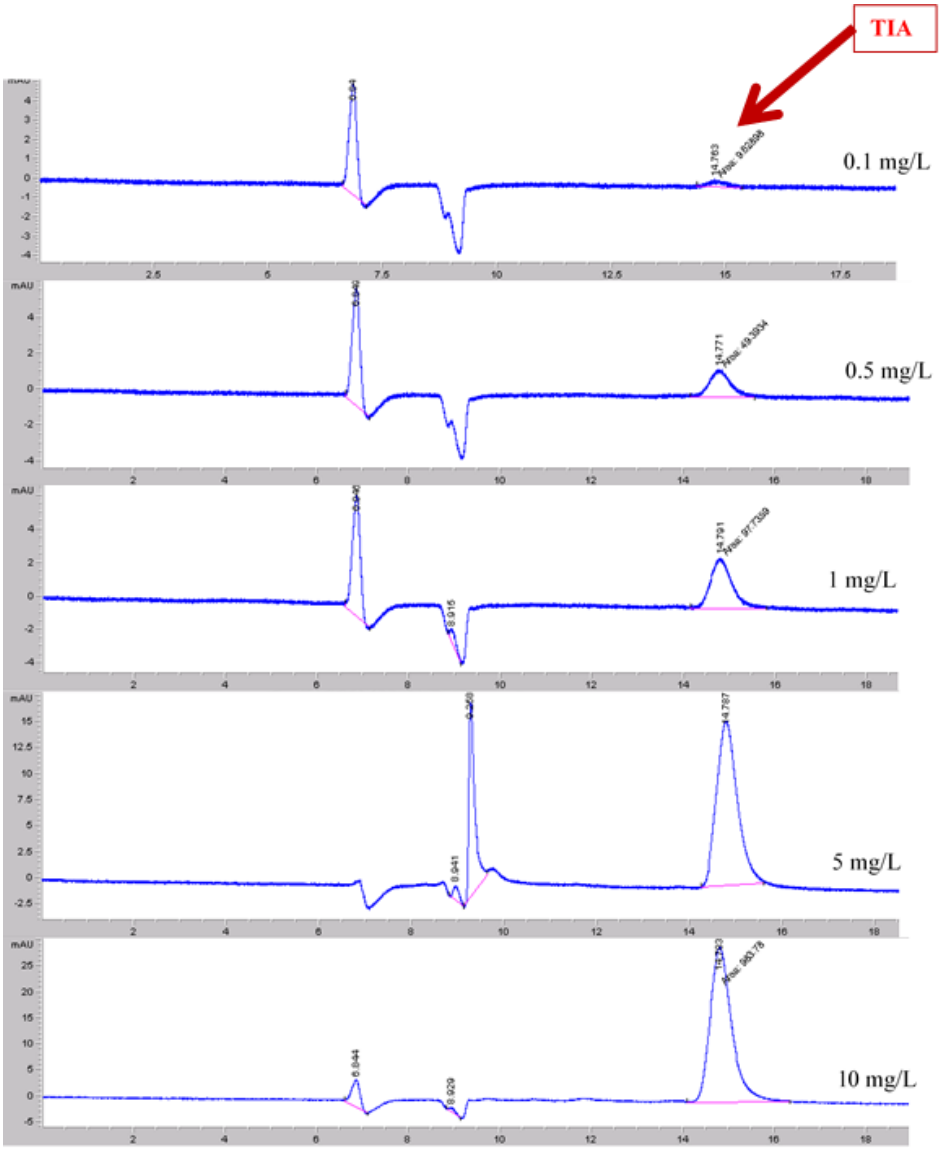

Figure B1 Chromatogram of tiamulin standards

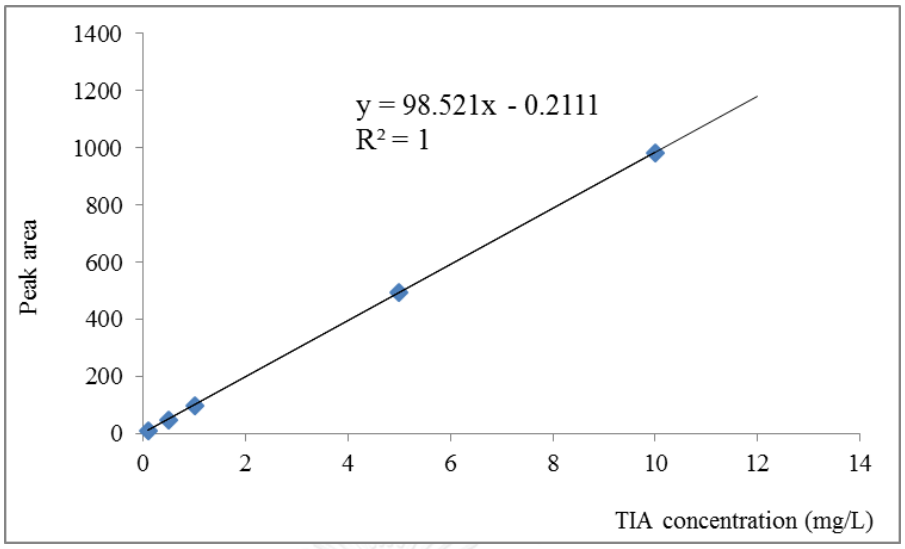

Figure B2 Tiamulin standard curve

5.1.2 Detection of tiamulin in liquid medium of biodegradation test

Based on the retention time of tiamulin detection in tiamulin standards (around 15 minute), residual tiamulin in all liquid samples at different time periods of biodegradation test was detected in the chromatograms (Figure B3).

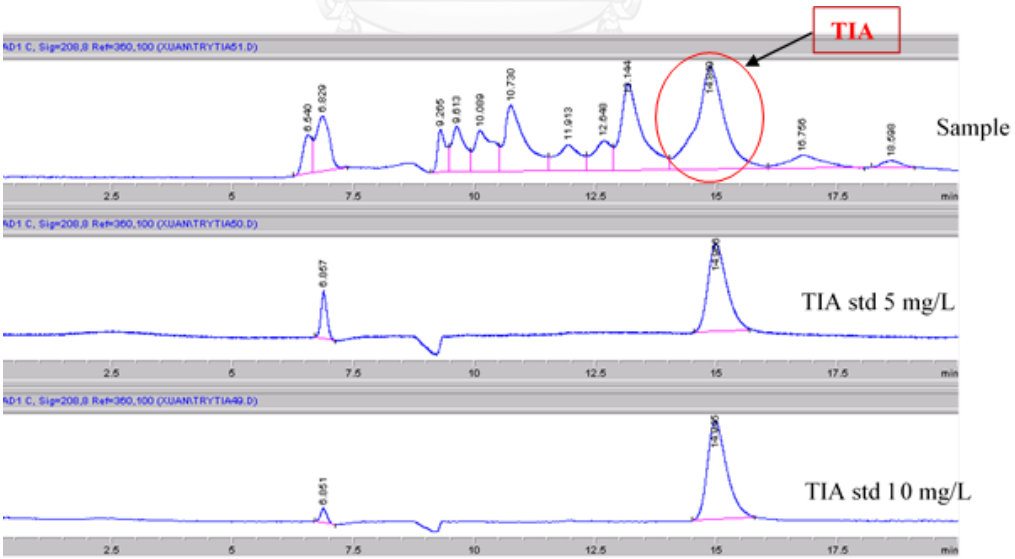

Figure B3 Tiamulin standards and tiamulin residues detected in liquid sample

5.2 Standard curve and residues of tiamulin in biodegradation test with bacterial enriched cultures

5.2.1 Tiamulin standard curve

Standard curve of tiamulin in biodegradation test with bacterial enriched cultures was similarly built with that using wood rot fungi (Figure B4). Besides five above-mentioned concentrations, tiamulin standard curve, however, was established with more five working standard solutions at higher concentrations (i.e. 20, 30, 50, 70, and 100 mg/L)

HPLC-DAD conditions to detect tiamulin standard residues in liquid medium of biodegradation test with bacterial cultures were 0.5 mL/min of mobile phase's flow rate and 10 minutes of retention time.

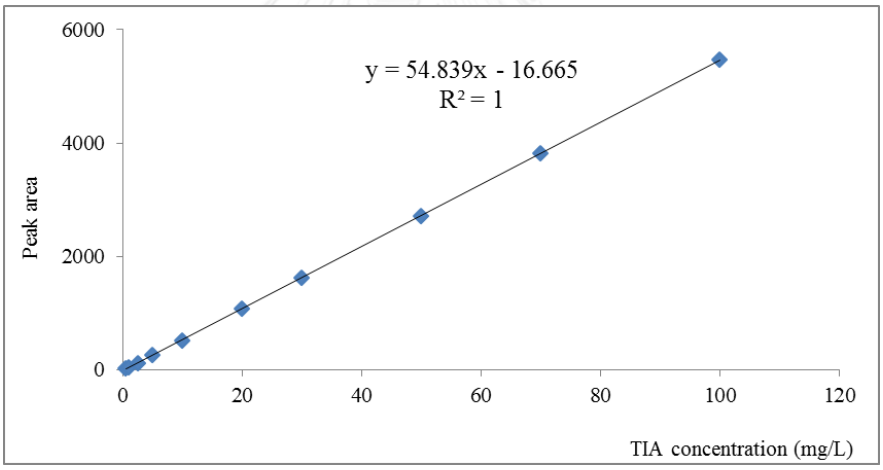

Figure B4 Tiamulin standard curve

5.2.2 Detection of tiamulin in liquid medium of biodegradation test

TIA residues in biodegradation test with bacterial enriched cultures is detected based on the peak area in chromatogram (Figure B5).

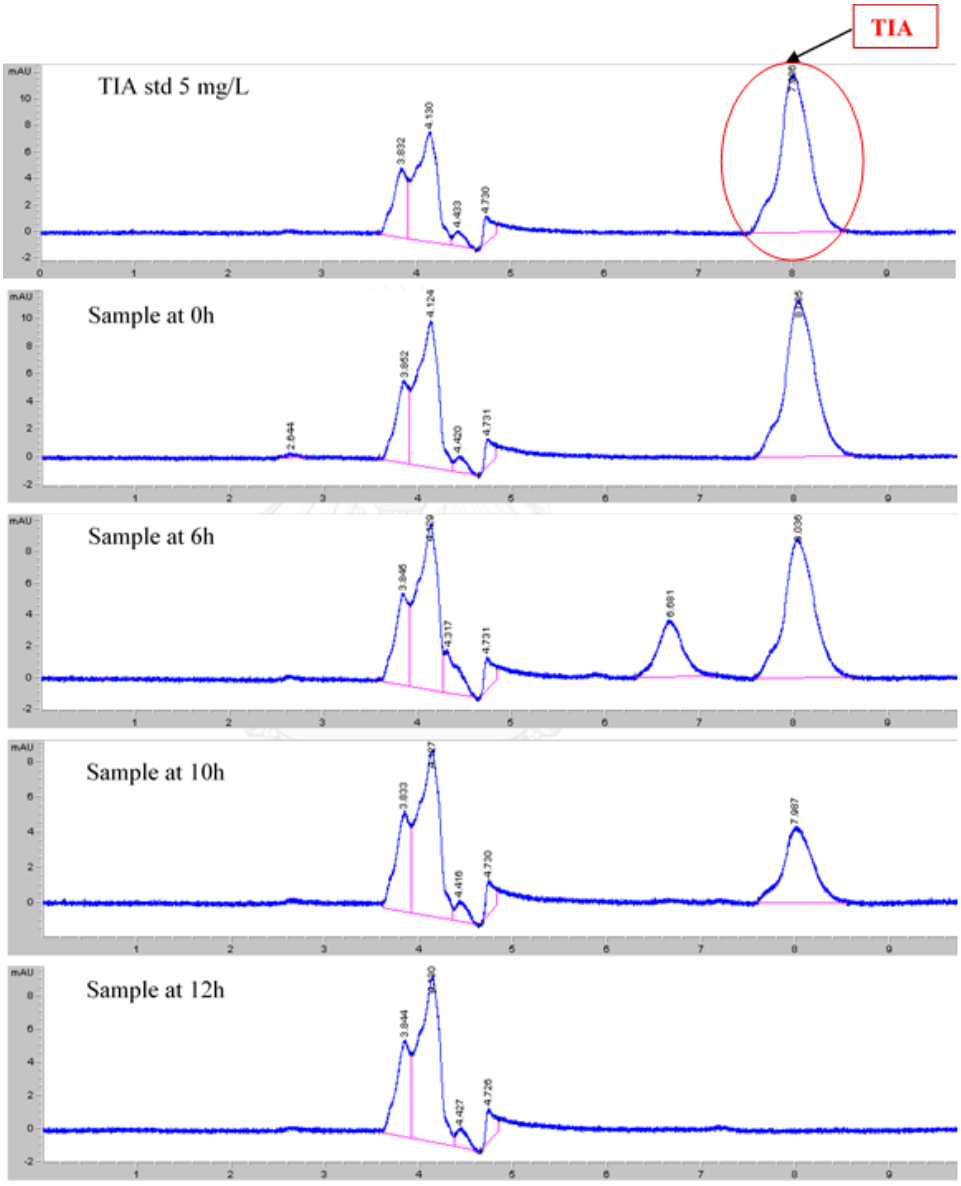

Figure B5 Tiamulin standards and tiamulin residues detected in liquid sample

VITA

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