PHENOLS AND COLOR REMOVAL FROM PALM OIL MILL WASTEWATER BY IMMOBILIZED BACTERIA AND WHITE ROT FUNGI

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Environmental Management
(Interdisciplinary Program)
Graduate School
Chulalongkorn University
Academic Year 2013

การกำจัดสารประกอบฟีนอลิคและสีในน้ำเสียของโรงงานสกัดน้ำมันปาล์มโดยแบคทีเรียตรึงและราไวท์รอท

นางสาววิภาพร เอกอมรธนกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาการจัดการสิ่งแวดล้อม (สหสาขาวิชา)
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2556

Thesis Title	PHENOLS AND COLOR REMOVAL FROM PALM OIL MILL WASTEWATER BY IMMOBILIZED BACTERIA AND WHITE ROT FUNGI
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Field of Study	Environmental Management
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วิภาพร เอกอมรธนกุล : การกำจัดสารประกอบฟีนอลิคและสีในน้ำเสียของโรงงานสกัดน้ำมันปาล์มโดยแบคทีเรียตรึงและราไวท์รอท (PHENOLS AND COLOR REMOVAL FROM PALM OIL MILL WASTEWATER BY IMMOBILIZED BACTERIA AND WHITE ROT FUNGI) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.ดร.เอกวัล ลือพร้อมชัย, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม :ดร.อรมาศสุทธินุ่น, 117หน้า.

งานวิจัยนี้ใช้แบคทีเรียผสม *Methylobacterium* sp. NP3 และ *Acinetobacter* sp. PK1และเชื้อราไวท์รอทหรือเชื้อราผุขาว *Trametes hirsuta* AK4 เพื่อลดความเข้มข้นของสารประกอบฟีนอลิคและสีของน้ำทิ้งจากโรงงานสกัดน้ำมันปาล์ม โดยเริ่มจากนำน้ำทิ้งจากบ่อปรับเสถียรบ่อสุดท้ายของโรงงานสกัดน้ำมันปาล์มแห่งหนึ่งในจังหวัดสุราษฎร์ธานีไปวิเคราะห์ พบว่า pHสารประกอบฟีนอลิคและสีมีค่าเท่ากับ8 - 9, 259 - 338 มิลลิกรัมต่อลิตรและ 95 -117 หน่วยสีตามลำดับ หลังจากนั้นได้เปรียบเทียบประสิทธิภาพของแบคทีเรียตรึง 2 แบบ คือ แบคทีเรียผสมตรึงในซิลิกาและแบคทีเรียผสมตรึงบนเม็ดพลาสติกใช้ซ้ำ และเปรียบเทียบเชื้อราผุขาว 2 ลักษณะ คือ แบบก้อนกลมและเชื้อราผุขาวตรึงบนเส้นใยปาล์ม เมื่อใช้จุลินทรีย์ชนิดใดชนิดหนึ่ง พบว่าแบคทีเรียผสมตรึงในซิลิกามีประสิทธิภาพในการลดสารประกอบฟีนอลิคและสีได้มากกว่าแบคทีเรียผสมตรึงบนเม็ดพลาสติกใช้ซ้ำ โดยแบคทีเรียผสมตรึงในซิลิกาสามารถลดสารประกอบฟีนอลิคและสีได้ 38% และ 40% ตามลำดับ ในน้ำทิ้งโรงงานน้ำมันปาล์มที่ไม่ได้เจือจาง ในขณะที่แบคทีเรียผสมตรึงบนเม็ดพลาสติกใช้ซ้ำสามารถลดสารประกอบฟีนอลิคและสีได้ 33% และ 1% ตามลำดับ สำหรับการบำบัดด้วยเชื้อราผุขาวก้อนกลม พบว่าปริมาณรา 250 กรัมต่อลิตร ให้ประสิทธิภาพสูงที่สุดในการบำบัดสี การศึกษาประสิทธิภาพในการบำบัดสีของน้ำที่ผ่านการเจือจางพบว่าเชื้อราผุขาวก้อนกลมในปริมาณ 250 กรัมต่อลิตร สามารถลดสีของน้ำที่ผ่านการเจือจาง (25%)และน้ำที่ไม่ผ่านการเจือจาง(100%) ได้ถึง82% และ38% ตามลำดับ อย่างไรก็ตามเชื้อราผุขาวก้อนกลมจะแตกเมื่อใช้บำบัดไปแล้ว 7 วัน ดังนั้นจึงตรึงเชื้อราผุขาวตรึงบนเส้นใยปาล์ม พบว่าราตรึงสามารถลดสีได้ถึง 55% ภายใน 4 วัน เนื่องจากมีรายงานถึงพิษของสารประกอบฟีนอลิคต่อรา ดังนั้นจึงทดสอบการบำบัดแบบสองขั้นตอน ซึ่งได้เปรียบเทียบกระบวนการ 3 แบบ คือ การบำบัดด้วย(1) แบคทีเรียตรึงในซิลิกาและบำบัดต่อด้วยเชื้อราก้อนกลม (2) แบคทีเรียตรึงในซิลิกาและบำบัดต่อด้วยเชื้อราตรึงบนเส้นใยปาล์ม และ (3) แบคทีเรียตรึงบนเม็ดพลาสติกใช้ซ้ำและบำบัดต่อด้วยเชื้อราตรึงบนเส้นใยปาล์ม พบว่าการบำบัดแบบสองขั้นตอนที่ประกอบด้วยแบคทีเรียตรึงในซิลิกาและบำบัดต่อด้วยเชื้อราก้อนกลมสามารถลดสารประกอบฟีนอลิคและสีได้มากที่สุด นอกจากนี้เชื้อราตรึงบนเส้นใยปาล์มสามารถบำบัดสีจากน้ำเสียที่ไม่ผ่านการบำบัดด้วยแบคทีเรียมาก่อน ซึ่งคาดว่าเพราะเส้นใยปาล์มช่วยปกป้องเซลล์ราจากสารพิษ

สาขาวิชา การจัดการสิ่งแวดล้อม ลายมือชื่อนิสิต .....

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5487586620 : MAJOR ENVIRONMENTAL MANAGEMENT

KEYWORDS : PHENOLS / COLOR / TREATED PALM OIL EFFLUENT / IMMOBILIZED BACTERIA / WHITE ROT FUNGI

WIPAPORNEKAMORNTHANAKUL:PHENOLSANDCOLOR REMOVAL FROM PALM OIL MILL WASTEWATER BY IMMOBILIZED BACTERIA AND WHITE ROT FUNGI. ADVISOR : ASST. PROF. EKAWANLUEPROMCHAI, Ph.D., CO-ADVISOR : ORAMAS SUTTINUN, Ph.D., 117pp.

This study usedbacterial co-culture of *Methylobactrium* sp.NP3 and *Acinetobacter* sp. PK1 and white rot fungus, *Trametes hirsuta* AK4 to remove phenols and color from treated palm oil mill effluent (POME). Initially, the quality of effluent from the last stabilization pond of a palm oil mill in Surat Thani province was investigated. The values of pH, phenols and color of these treated POME samples were ranged 8 - 9, 264 - 338 mg/L and 95 -117 color units, respectively. After that, the efficiencies of two immobilized bacteria, i.e. silica- and recycled plastic immobilized co-culture bacteria and two kinds of *Trametes hirsuta* AK4, i.e.pellets and immobilized on palm pericarp fiber (PF) were compared. The result showed that silica immobilized co-culture bacteria had higher phenols and color removal efficiency than recycled plastic immobilized co-culture bacteria. The immobilized silica removed 38% and 40% of phenols and color from 100% treated POME, respectively while immobilized recycled plastics removed 33% and 1% of phenols and color removal, respectively. For white rot fungal treatment, 250 g/L of *Trametes hirsuta* AK4 pellets had the highest color removal efficiency. The study with diluted treated POME found that 250 g/L of white rot fungal pellets could remove color up to 82% and 38% in 25% (diluted) and 100% (undiluted) treated POME, respectively. However, white rot fungal pellets werebroken after 7 days. Therefore, *Trametes hirsuta* AK4 was immobilized on palm pericarp fiber (PF) to solve the problem. The immobilized fungus removed color up to 55% within 4 days. Since many researchers reported the toxicity of phenolic compounds to fungi, two-stage treatment was conducted. There were three types of two-stage treatment i.e.the systems with(1) silica immobilized bacteria and fungal pellets, (2) silica immobilized bacteria and PF immobilized fungus and (3) recycled plastic immobilized bacteria and PF immobilized fungi. The result showed that two-stage treatment of silica immobilized bacteria and white rot fungal pellets had the highest phenols and color removal efficiency. In addition, PF immobilized white rot fungus could remove color from wastewater that had not been treated by the bacteria. This is probably due to the protection of fungal cells by palm pericarp fiber.

Field of Study: Environmental Management Student's Signature .....
Advisor's Signature .....
Academic Year: 2013 Co-advisor's Signature .....

ACKNOWLEDGMENTS

I would like to express my honestthankfulness to Assistant Professor Dr. Ekawan Luepromchai; my thesis advisor for her valuable advice, useful comments, great encouragement, big thoughtfulness and helpfulness throughout this research work and also big thanks for Dr. Oramas Suttinun; my thesis co-advisor for her helpful suggestions and supervision.

I would like to extend my sincere gratitude to the thesis committee chairman, Assistant Professor Dr. Tawan Limpiyakorn, and thesis committee members, Assistant Professor Dr. Onruthai Pinyakong and Dr. Chalermchai Ruangchainikom for their reviews and constructive criticism. Moreover, I wish to manifest my big thanks to the National Center of Excellence for Environmental and Hazardous Waste Management (NCE-EHWM) for providing me the full scholarship, research funding and supporting facilities to complete this work.

I am also thankful to palm oil mill industry in Surat Thani province for providing the funding, facilitiesabout this study and collecting palm oil mill effluent that used in this study as well.

I would also trustworthy thank EHWM program members and laboratory staffs at Department of Microbiology, Faculty of Science, Chulalongkorn University for assistance, useful advice and providing equipment.

I would like to truthful appreciation to my all of laboratory members for teaching, helpful advice, superintendence, consideration, support, and encouragement throughout in this study.

I would like to faithful thankfulness my family and my friends for their love, cheerful and supporting always.

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LIST OF ABBREVIATIONS

POME	Palm oil mill effluent
COD	Chemical oxygen demand
BOD	Biochemical oxygen demand
DO	Dissolved oxygen
TP	Total phosphorus
TKN	Total Kjeldahl nitrogen
TSS	Total suspended solid
PF	Palm pericarp fiber
CFMM	Carbon free mineral medium
PDA	Potato dextrose agar
GYEB	Glucose yeast extract broth
SEM	Scanning Electron Microscopy

CHAPTER I

INTRODUCTION

1.1 Theoretical background

The palm oil mill industry is a major industry in the Southern of Thailand. Although, this industry produces a lot of money to Thailand, but it also causes an environmental problem such as dirty water, solid waste and air pollution (Chavalparit *et al*., 2006). The oil palm industry discharges large amount of wastewater that relevant to palm oil mill effluent (POME) (Rupani *et al*., 2010). The palm oil mill effluent is the liquid waste from sterilization and clarification processes (Zinatizadeh et al., 2006). Normally, 5-7.7 tons of water is used for 1 ton of produced gross palm oil and about 50% of the water remains in thePOME (Ma, 1999). POME comes from three main sources of dirty water including clarification (60%), sterilization (36%) and hydrocyclone (4%) units.

POME is generally treated by using ponding system. The ponding system is the conventional method extensively used for the palm oil mill effluent treatment because of low operating cost, for example, anaerobic stabilization pond and nowadays the treated POME is usually used for watering the palm plantation or allowing it to evaporate from ponds naturally. But this solution is not permanent and incomplete. In rainy season, it is difficult to control the wastewater quantity and the wastewater can overflow to water resource. However, the disgusting smell from anaerobic stabilization pond can

disturbnearby community and the treated POME still has COD, BOD, phenols and color (dark brownish) (Wu *et al*., 2010; Zahrim *et al*., 2009). The water quality standards specify phenols concentration at less than 1 mg/L and color to be not disgusting, so they will be concerned before releases (Notification the Ministry of Science, Technology and Environment, No. 3, B.E.2539 (1996) issued under the Enhancement and Conservation of the National Environmental Quality Act B.E.2535 (1992), published in the Royal Government Gazette, Vol. 113 Part 13 D, dated February 13, B.E.2539 (1996)). Phenols will be discharged during palm oil extraction process because phenols are plant composition for example tannic and humic acids and they can be injurious to living organisms and ecosystem (Barron *et al.* 2002; Wu *et al.,* 2010). Besides phenols will be react with oxygen to become melanin (complex brown polymers) in enzymatic browning reaction, so this is a factor that causes treated POME to have dark-brownish color.

Phenols and color can be removed by both chemical and physical processes including adsorption, coagulation-flocculation oxidation and electrochemical method (Kapdan and Kargi, 2002). However, these processes are very expensive, require high energy and potentially harmful to the environment (Couto, 2009). Therefore, biological processes are selected because they are considered as cheap, easy operation and environmental friendly (Zahrim *et al*., 2009). From the previous study by Khongkhaem *et al.* (2011), free cells of *Methylobacterium* sp. NP3 and *Acinetobacter* sp. PK1 degraded phenol up to 2,500 mg/L. When the co-culture bacteria were immobilized in silica, they could degrade phenol concentration at 5,000 mg/L. The immobilization can protect cells

from severe environmental conditions or high concentration of toxic substances (Soltmann and Bottcher, 2008).

The application of *Methylobacterium* sp. NP3 and *Acinetobacter* sp. PK1 for removal of phenols in the treated palm oil mill effluent were investigated here.This study compared the efficiency of silica- and recycled plastic-immobilized bacterial cells. Recycled plastic was selected as another immobilizing material because it has lower cost than silica. Since bacteria usually have low color removal efficiency, this study further investigated the efficiency of fungi in the treated POME. *Trametes hirsuta* AK4, a white rot fungus screened from the bark of the timber, was used (Kietkwanboot *et al.,* 2013). White rot fungi usually produce ligninolytic enzymes that can degrade xenobiotic compounds and dyes (Hofrichter, 2002; Songulashvili *et al.*, 2007). Nonetheless, phenols have been reported to inhibit white rot fungi (Abd El-Zaher *et al.,* 2011). This study therefore conducted a sequential treatment system that started with phenol removal of by bacteria and then color removal by fungi.It is expected that the two-stage treatment by silica-/recycled plastic- immobilized cells and white rot fungus would remove phenols and color from the treated POME at higher efficiency than either microorganism alone.

1.2 Hypotheses

The removal of phenols by immobilized bacteria can enhance the efficiency of *Trametes hirsuta* AK4on removing color from treated palm oil mill effluent. The bacteria and fungi can be applied together in a two-stage treatment.

1.3 Objectives

The main objective is to remove both phenols and color from treatedpalm oil mill effluent by bacteria (co-culture of *Methylobacterium* sp. NP3and *Acinetobacter* sp. PK1) and/or white rot fungus(*Trametes hirsuta* AK4), respectively. Three sub-objectives are:

1.3.1 To determine the efficiency of immobilized *Methylobacterium* sp. NP3and *Acinetobacter* sp. PK1 on removing phenols and colors from treated palm oil mill effluent.

1.3.2 To compare the efficiency of *Trametes hirsuta* AK4 pellets and *Trametes hirsuta* AK4 immobilized on palm pericarp fiber on removing phenols and colors from treated palm oil mill effluent.

1.3.3 To develop a two-stage treatment consisted of immobilized bacteria and *Trametes hirsuta* AK4for removing phenols and colors from treated palm oil mill effluent.

1.4 Scopes of the study

1.4.1 Samples collection and characterization

Wastewaters from the last stabilization pond of a large-scale palm oil mill in Surat Thani province were collected. Properties of samples, pH, COD, BOD, TSS, nitrogen, phosphorus, oil and grease, phenols and color, were analyzed.

1.4.2 Activitiesof the immobilized bacteria and white rot fungi

1) Phenols removal by using immobilized bacteria

This study uses the co-culture bacteria (*Methylobacterium* sp. NP3 and *Acinetobacter* sp. PK1) to immobilize with the different materials including silica and recycled plasticsfor phenols removal from treated palm oil mill effluent in batch experiment.

2) Color removal by using white rot fungi

White rot fungi were selected to use in this study because they have the ability for color removal. Kietkwanboot *et al.* (2013) showed that *Trametes hirsuta* AK4 has the best ability for removing color. Moreover, in this study interests in comparing between*Trametes hirsuta* AK4 pellets and immobilized on palm pericarp fiber in batch experiment.

1.4.3 Two-stage treatment

Bacterial and white rot fungal treatments were combined as a two-stage treatment for sequential phenols and color removal.In this study, immobilized bacterial treatment was first used for phenols removal and follows by white rot fungal treatment for color removal. There were 3 experiments of two-stage treatment consisted of silica immobilized co-culture bacteria and *Trametes hirsuta* AK4 pellets, silica immobilized the co-culture bacteria and *Trametes hirsuta* AK4 immobilized on palm pericarp fiber and recycled plastic immobilized co-culture bacteria and *Trametes hirsuta* AK4 immobilized

on palm pericarp fiber. All of two-stage treatments were compared between silica and recycled immobilized bacterial cells and between white rot fungal pellets and immobilized white rot fungus.

All experiments were carried out in laboratory scale. The analyses of phenols and color were conducted by spectrophotometry method.

1.5 The benefits of the study

1.5.1 Different immobilization matrices were studied for enhancing the activities of bacteria and fungi.

1.5.2 Immobilized cells were effective for phenols and color removal from palm oil mill wastewater.

1.5.3 Two-stage treatment consisted of immobilized bacteria and fungi could be used for phenols and color removal from palm oil mill industry.

1.5.4 These treatment could be applied for phenols and color removal in the effluent from other industries.

CHAPTER II

LITERATURE REVIEW

2.1 Palm oil mill effluent (POME) process and oil palm residues

The oil palm fruit produces two types of oil including freshly mesocarp and kernel. Palm oil is extracted from freshly mesocarp that is edible oil. The palm kernel oil is obtained from kernel which is widely used in oleochemical manufacture (Sundram *et al.,* 2003). The crude palm oil extraction process can be divided into two types: (1) standard method or wet production and (2) dry production. These processes produce many oil palm residues including empty fruit bunches, palm pericarp fiber, palm kernel cake, palm shell, sludge and POME (Prasertsan *et al*., 1990). Moreover, in wet production, 1 ton of gross palm oil uses 5-7.7 tons of water and about 50% of the water remains in the POME (Ma, 1999). POME is generated at 0.87 cubic meters per ton of fresh fruit bunches (Hanpongkittitkul *et al.,* 1994). The POME contains three main sources of dirty water including clarification (60%), sterilization (36%) and hydrocyclone (4%) units as shown in Figure2.1 (Wu *et al*., 2010). Large volumes of wastewater are generated from palm oil mills, of which there are higher organic compounds, pH, and COD than the level of the water quality standards (Notification the Ministry of Science, Technology and Environment, No. 3, B.E.2539 (1996) issued under the Enhancement and Conservation of the National Environmental Quality Act B.E.2535 (1992), published in

the Royal Government Gazette, Vol. 113 Part 13 D, dated February 13, B.E.2539 (1996)).

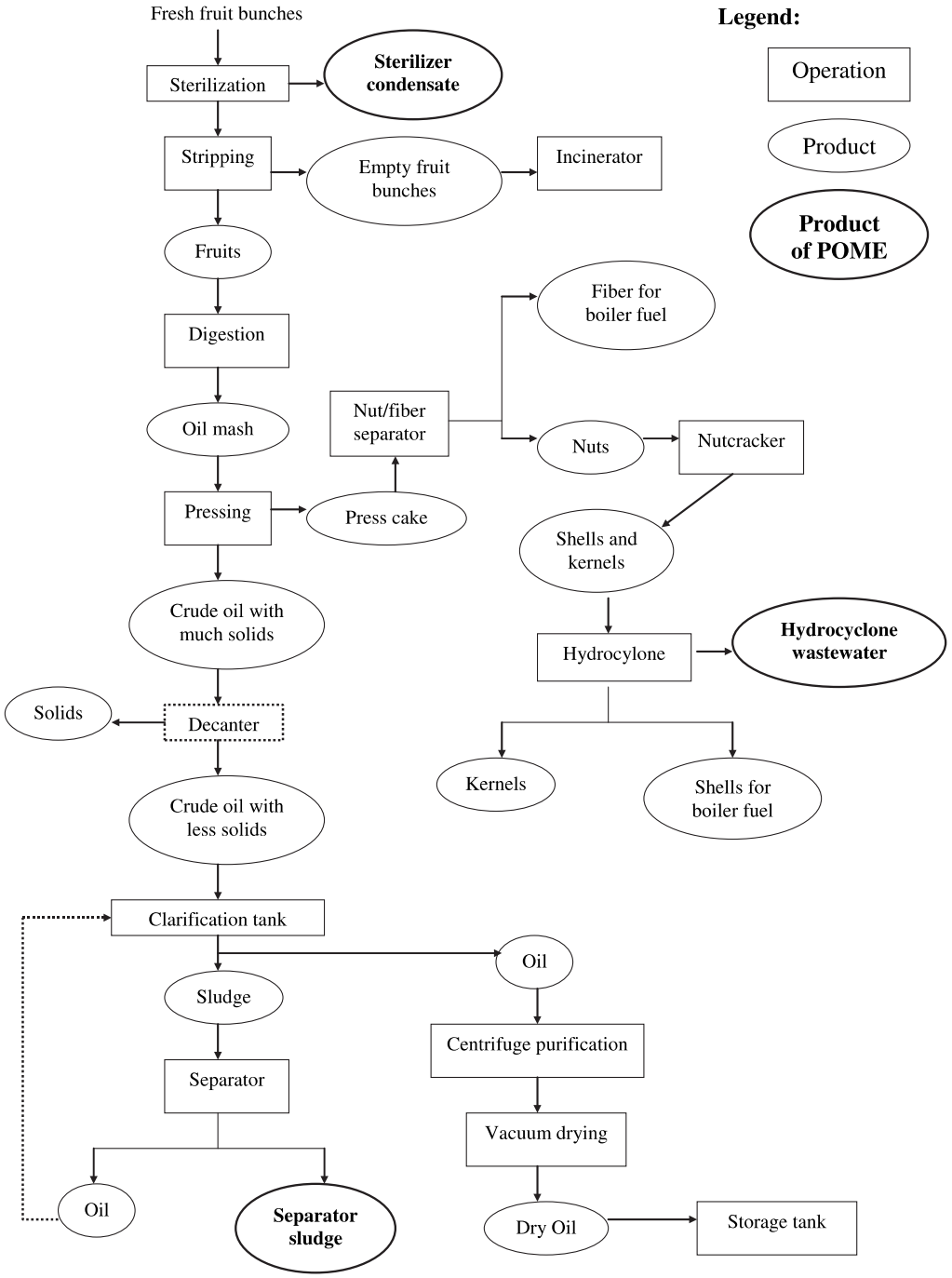


Figure 2. 1 Palm oil mill process (Wu *et al.,* 2010)

At the present,POME was treated by using ponding system. It is also used as animal feed and biogas because palm oil is high in nutrients (Prasertsan, 1999). However, the treated POME still has high COD, phenols and dark brown color (Poh and Chong, 2009; Zahrim *et al*., 2009).

Table 2. 1 The properties ofthe final anaerobic pond in palm oil mill effluent (Rakamthong and prasertsan, 2011)

Parameters	Effluent
Appear color	Dark-brown
Color (OD_{475})	2.417
pH	9.5
COD (mg/L)	1.586
Total solids (TS) (mg/L)	3.840
Suspended solids (SS) (mg/L)	2.170
Total phenol (mg/L)	43.01

2.2 Phenols and biodegradation of phenols

Phenols are pollutants in wastewaters and effluents that can be harmful to living organisms and ecosystem. Phenols affect to the liver, kidneys, lungs and blood circulatory system (Barron *et al.* 2002). Phenols have been found from many industrial factories, for example, ceramic plants, steel plants, coal conversion processes, phenolic resin industries, pesticide, paint, pharmaceutics, paper and pulp industries and petroleum plants (Aksu, 2005; Yan *et al*., 2006). The derivatives of phenol such as cinnamic acid,

benzoic acid, and flavonoids, are mostly found inPOME while sterilization. Sterilization process inactivates polyphenol oxidases enzyme that cause phenolic acids and flavonoids are maintain in treated POME because they are water-soluble phenolic compounds (Halimoon, 2006; Sundram *et al.*, 2003). So we need to remove phenols to a satisfactory standard before discharge.

Phenols consist of aromatic ring and at least 1 hydroxyl group (-OH). Phenolic compounds are divided into three types: (i) Monocyclic phenols are commonly found in plants, for example, phenol (see Figure 2.2), catechol, tyrosol and gallic acid, (ii) dicyclic phenols have two phenol rings, for example, flavonoids and lignans and (iii) polycyclic phenols have more than two phenol rings, for example, lignins, catechol melanins, flavolans and condensed tannins.

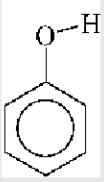
Formula	C_6H_5OH 
Molecular weight (g/mol)	94.11
T_{melt} (°C)	40.9
T_{eb} (°C)	181.75
Water solubility (r.t.)	9.3 g_{phenol} / 100 ml_{H_2O}
pK_a	9.89
Flammability limits in air (vol%)	1.7 (lower) 8.6 (higher)
Flash point (°C)	79 (closed cup)
Autoignition temperature (°C)	715

Figure 2. 2 Example of chemical and physical properties of phenol

(Busca *et al.*, 2008)

Some microorganisms are able to grow on phenols under both aerobic and anaerobic conditions but it is generally found that the degradation of phenols occur under aerobic condition (Melo *et al*., 2005). Two aerobic pathways for phenol biodegradation are: (1) ortho-pathway and (2) meta-pathway. In the first step, phenol hydroxylase transforms phenol to catechol by oxygenation reaction. Catechol can be degraded using meta-pathway or ortho-pathway depending on microorganisms. For ortho-pathway (1), catechol ring is cleaved to cis,cis-muconic acid by 1,2-dioxygenase enzyme. For meta-pathway (2), catechol ring is cleaved to 2-hydroxymuconic semialdehyde by 2,3-dioxygenase enzyme. These compounds are metabolites through Krebs cycle as shown in Figure 2.3(Van Schie and Young, 2000).

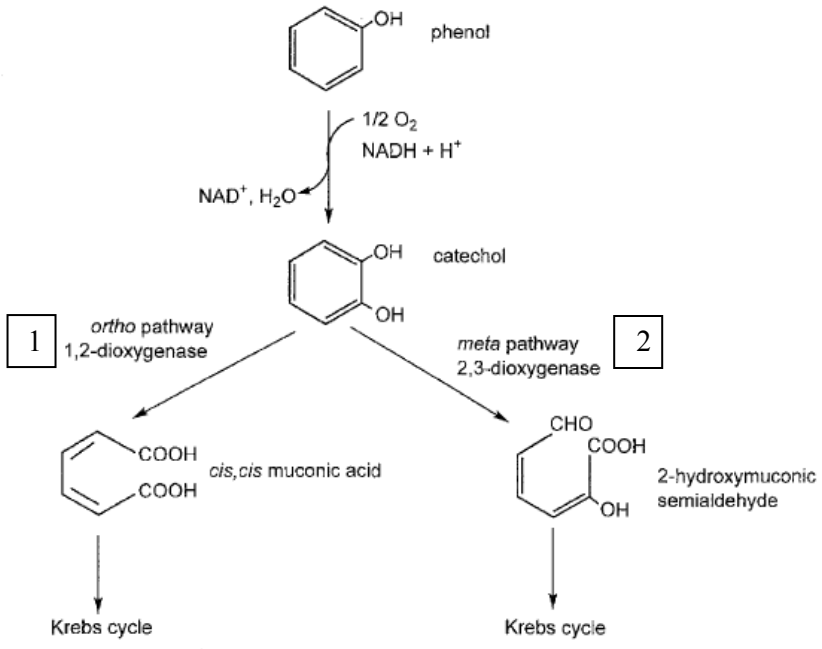


Figure 2. 3 Pathway of phenol degradation by aerobic microorganism (Van Schie and Young, 2000)

2.3 Phenols degrading bacteria

Phenols are toxic to several organisms, but some bacteria are resistant to phenols and can use these compounds as a carbon and energy source. Example of phenols-degrading bacteria is below (Table 2.2).

Table 2. 2 Example of phenols-degrading bacteria

Phenols-degrading bacteria	Phenols	References
Pseudomonas putida	Phenol	Zilli, 1993
Pseudomonas sp. CF600	Phenol	Powlowski and Shingler, 1994
Pseudomonas pickettii	2-chlorophenol 3-chlorophenol 4-chlorophenol	Fava *et al.,* 1995
Bacillus subtilis	2,4,6-trichlorophenol	Daughney and Fein, 1998
Acinetobacter sp.	Phenol and chlorophenol	Hao *et al.,* 2002
Nocardia hydrocarbonoxydans	Phenol	Shetty *et al.,* 2007
Bacillus amyloliquefaciens	Phenol	Lu *et al.,* 2012

From Khongkhaem *et al.* (2011) study, they used the co-culture of *Methylobacterium* sp. NP3 and *Acinetobacter* sp. PK1 for phenol degradation. The co-culture bacteria degraded phenol more quickly than the individual strains. Therefore, this study will use the mixed culture of *Methylobacterium* sp. NP3 and *Acinetobacter* sp. PK1 for phenols degradation.

2.3.1 *Methylobacterium*

Methylobacterium is dark-pink colony, strictly aerobic, facultative methylotrophic, Gram-negative, and rod-shaped bacterium and can be found in environment. They can use many of organic compounds as carbon source, for example, methanol and methylamine as well as a variety of C_2, C_3 and C_4 (Green, 1992). Genus *Methylobacterium* can be found in a many kinds of natural and man-made environments (Hiraishi *et al*., 1995; Trotsenko *et al*., 2001).

2.3.2 *Acinetobacter*

Acinetobacter is white colony, aerobic and gram-negative bacilli bacterium. They can be found in water, soil, living organisms and on human skin. They can use many compounds as sources of carbon and energy; for example, phenol and benzoate and they can grow easily in simple media (Abd El-Haleem 2003; Caposio *et al*., 2002).

2.4Source of colors in treated POME

In fact, there is no chemical addition in the palm oil procedure so dark-brown color comes from splitting of organic compounds and destruct of pigments (anthocyanins and carotene) in palm fruit because heating from sterilization and pressing (Hartley, 1977). Moreover, color comes from phenols. Phenols are plant constituents for example tannic and humic acids from the materials. Phenols are contaminated in treated POME during sterilization process (Sundram *et al*., 2003).

Figure 2. 4 Example of stabilization pond in palm oil mill

When the palm fruits are destroyed by heat of the sterilization process, phenols in palm fruit are extracted with oil and steam and phenols are dissolved in water than oil. Phenols are extracted into contact with oxygen in the air causes oxidation reaction. Polyphenoloxidase (PPO) is oxidized to quinone and polymerized to melanin (brown pigment) as shown in Figure2.5 (Hudson, 1998; Wu *et al*., 2010) and the respond of melanoidins from Maillard reaction of sugars (carbohydrates) with proteins (amino groups) might provide the color of wastewater (Ho et al., 1984; Kort, 1979). The color appearance obstructed the water clearness of sunlight, blocked photosynthesis and affected to aquatic animals growth (Venkata-Mohan *et al*., 1997).

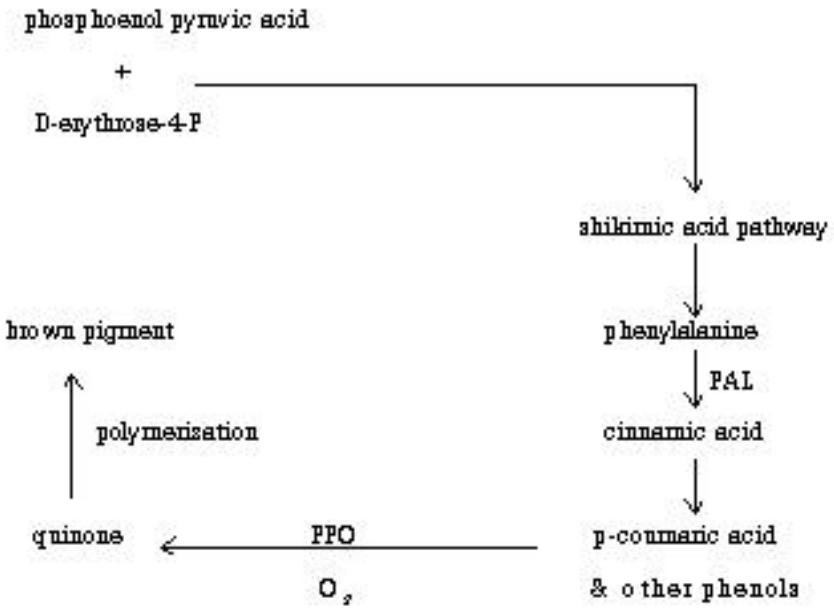


Figure 2. 5 Enzymatic browning reaction (Hudson, 1998)

2.5 Color removal by white rot fungi

The colors come from phenols. Lignin, cellulose and hemicellulose are the components of cell wall of wood. Fungi have well-known of wood decay that can be divided into three types: (1) soft-rot fungi, (2) brown-rot fungi and (3) white-rot fungi by they can insert themselves into the secondary walls of wood cells and decay. (1) soft-rot fungi can well degrade polysaccharides but slowly lignin degradation (Kirk, 1984). (2) brown-rot fungi can degrade polysaccharides and change the structure of lignin (Buswell and Odeis, 1987). However, both of soft-rot and brown-rot can degrade lignin slower than white rot fungi. White rot fungi are classified in class Basidiomycetes. The name of white rot came from the appearance of wood that changed from brown to white color.

White rot fungi emits the extracellular ligninolytic enzyme and has the degradation efficiency both of polysaccharides and lignin (Hatakka, 1986). Moreover, it has ability to degrade recalcitrant organic compounds, for example, dichloroaniline, DDT, hexachlorocyclohexane, pentachlorophenol, polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (Pointing, 2001; Kapdan and Kargi, 2002).

Table 2. 3 Example of white rot fungi for pollutants degradation

White Rot Fungi	Pollutants	References
Coriolopsis polyzona	Polychlorinated biphenyls (PCBs)	Novotny *et al.,* 1997
Coriolus versicolor	Textile dye stuff	Kapdan and Kargi, 2002
Phanerochaete chrysosporium	Olive mill wastewater (OMW)	Kissi *et al.,* 2001
	Synthetic dyes	Cripps *et al.* 1990
	2,4,6-trinitrotoluene (TNT)	Fernando, 1990
	Polychlorinated biphenyls (PCBs)	Yadav *et al.,* 1995
	Polycyclic aromatic hydrocarbons (PAH)	Bumpus, 1989
	Pentachlorophenol (PCP)	Mileski *et al.,* 1988
Pleurotus sp.	PAH (Polycyclic aromatic hydrocarbons)	Morgan *et al.,* 1991
Pleurotus ostreatus	Polychlorinated biphenyls (PCBs)	Zeddel *et al.,* 1993
Pycnoporus sanguineus	Azo and triphenylmethane dyes	Pointing *et al.,* 2000
Trametes hirsuta	Textile dye	Abadulla *et al.,* 2000
Trametes versicolor	Polychlorinated biphenyls (PCBs)	Zeddel *et al.,* 1993
	Pentachlorophenol (PCP)	Ricotta *et al.,* 1998

Ligninolytic enzyme is enzyme that catalyzes the degradation of lignin including generally three oxidative enzymes (1) Lignin peroxidase, (2) Manganese peroxidase and (3) Laccase (Niladevi, 2009).

(1) Lignin peroxidase was found that the first enzyme can degrade lignin by using hydrogen peroxide (H_2O_2) by cleaving C_α-C_β of lignin and veratryl alcohol is a mediator (Renganathan and Gold, 1986).

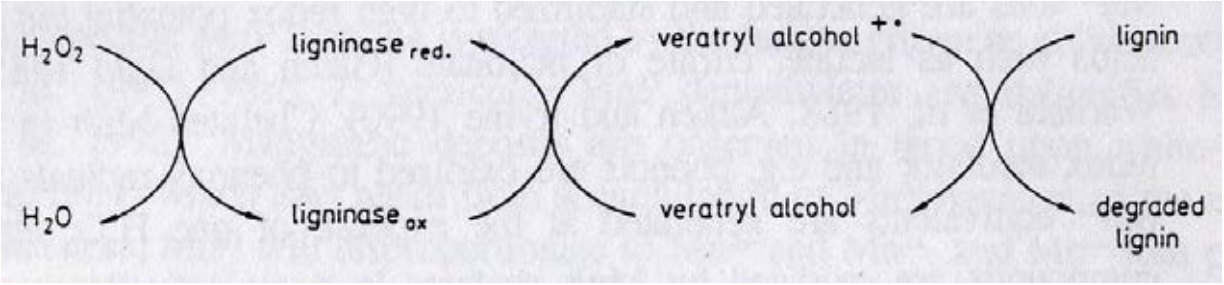


Figure 2. 6 The role of veratryl alcohol as mediator (Harvey *et al.,* 1986)

(2) Manganese peroxidase is a peroxidase enzyme by using hydrogen peroxide and has manganese(II) as the reducing substrate (Wariishi *et al.,* 1992,1988).

$$MnP + H_2O_2 \longrightarrow MnPI + H_2O \quad (1)$$

$$MnPI + Mn^{II} \longrightarrow MnPII + Mn^{III} \quad (2)$$

$$MnPII + Mn^{II} \longrightarrow MnP + Mn^{III} \quad (3)$$

$$Mn^{III} + RH \longrightarrow Mn^{II} + R^{\cdot} + H^{+} \quad (4)$$

(3) Laccase is an enzyme that uses oxygen (O_2) different from lignin peroxidase and manganese peroxidase. Laccase can degrade lignin model compounds and phenolic hydroxyl to phenoxy redicals by using O_2 as electron acceptor (Hatakka, 2001).

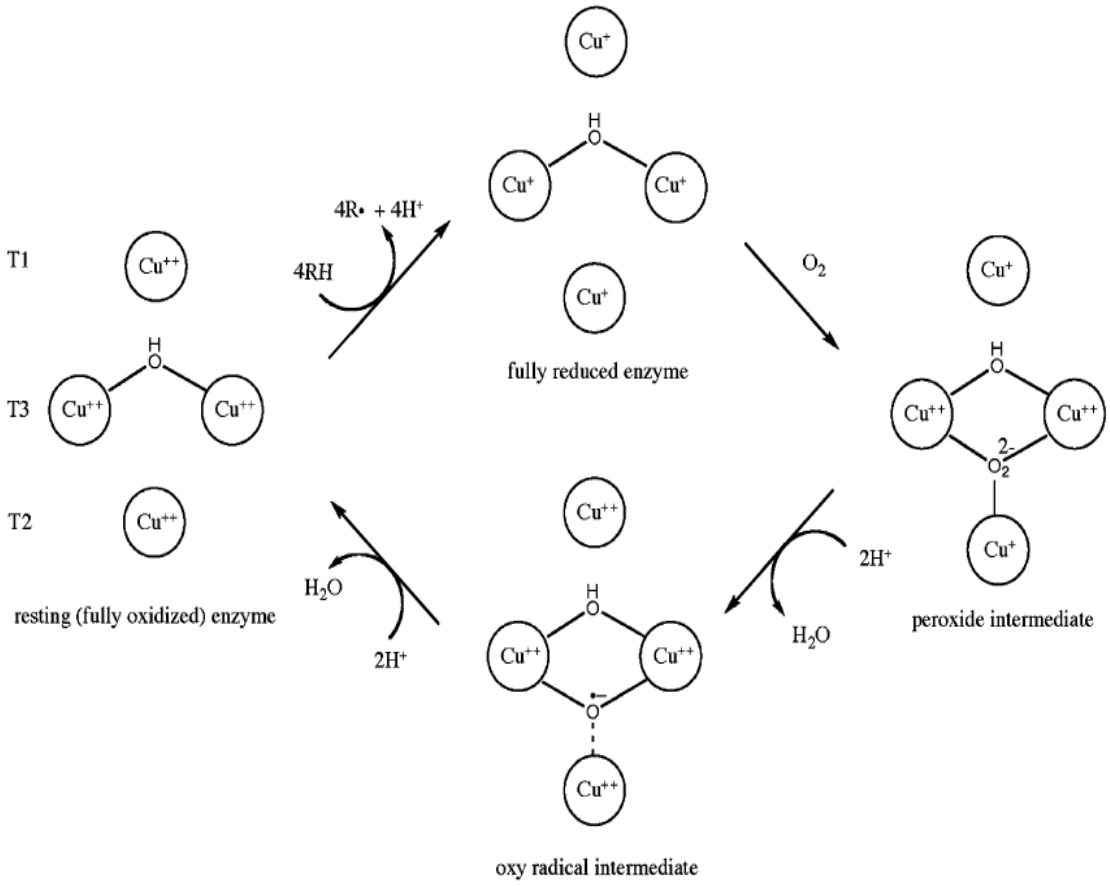


Figure 2. 7 Catalytic mechanism of laccase (Huang *et al.,* 1999)

Table 2. 4 Main reaction of ligninolytic enzymes (Hatakka, 2001)

Enzyme and abbreviation	Cofactor	Substrate, mediator	Reaction
Lignin peroxidase, LiP	H_2O_2	Veratryl alcohol	Aromatic ring oxidized to cation radical
Manganese peroxidase, MnP	H_2O_2	Mn, organic acids as chelators, thiols, unsaturated fatty acids	Mn(II) oxidised to Mn(III); chelated Mn(III) oxidises phenolic compounds to phenoxyl redicals; other reactions in the presence of additional compounds
Versatile peroxidase, VP	H_2O_2	Mn, veratryl alcohol, compounds similar to LiP and MnP	Mn(II) oxidised to Mn(III), oxidation of phenolic and non phenolic compounds, and dyes
Laccase	O_2	Phenols, mediators, e.g.,hydroxybenzotriazole or ABTS	Phenols are oxidized to phenoxyl radicals; other reactions in the presence of mediators
Glyoxal oxidase, GLOX	-	Glyoxal, methyl glyoxal	Glyoxal oxidised to glyoxal acid; H_2O_2 production
Aryl alcohol oxidase, AAO	-	Aromatic alcohols (anisyl, veratryl alcohol)	Aromatic alcohols oxidised to aldehydes; H_2O_2 production
Other H_2O_2-producing enzymes	-	Many organic compounds	O_2 reduced to H_2O_2

In this study, *Trametes hirsuta* AK4 from Kietkwanboot *et al.* (2013) was isolated from the bark of timber from Songkhla province that was screened from 10 strains.*Trametes hirsuta* AK4 showed the highest capability of 66.5% and 64.7% of phenolic compounds removal and decolorization of diluted (50%) treated POME, respectively.

2.6 Immobilization

Immobilization is a technique that used to fix the cells, which may be physical or chemical immobilization on a solid supporting material or substance to increase the cell stability and reusable. Immobilization has many types (see Figure2.8) but they can be divided into two types: (1) self-attachment and (2) artificial immobilization for example encapsulation in polymer gel (Cohen *et al.,* 2001). Self-attachment is the immobilization by simple adsorption of the cells on the conductive material with electrostatic interactions, covalent bond formation or hydrophobic interactions. It is an easy technique that had has limitation of diffusion of a substance less than artificial immobilization. However, the attached cells may be loss easily because of a relatively weak adsorption (Cheetham *et al.*, 1979; Lee and Palsson, 1994). Artificial immobilization or encapsulation has high stability, withstands the physical and chemical force and utilizes in a wide pH range. Moreover, organic solvents are not influence with this technique and low cost of synthesis (Alvarez *et al.*, 2007). However, the technique of attachment is considered as an effective immobilization because it can increase cell numbers easier than in artificial immobilization (Lee and Palsson, 1994).

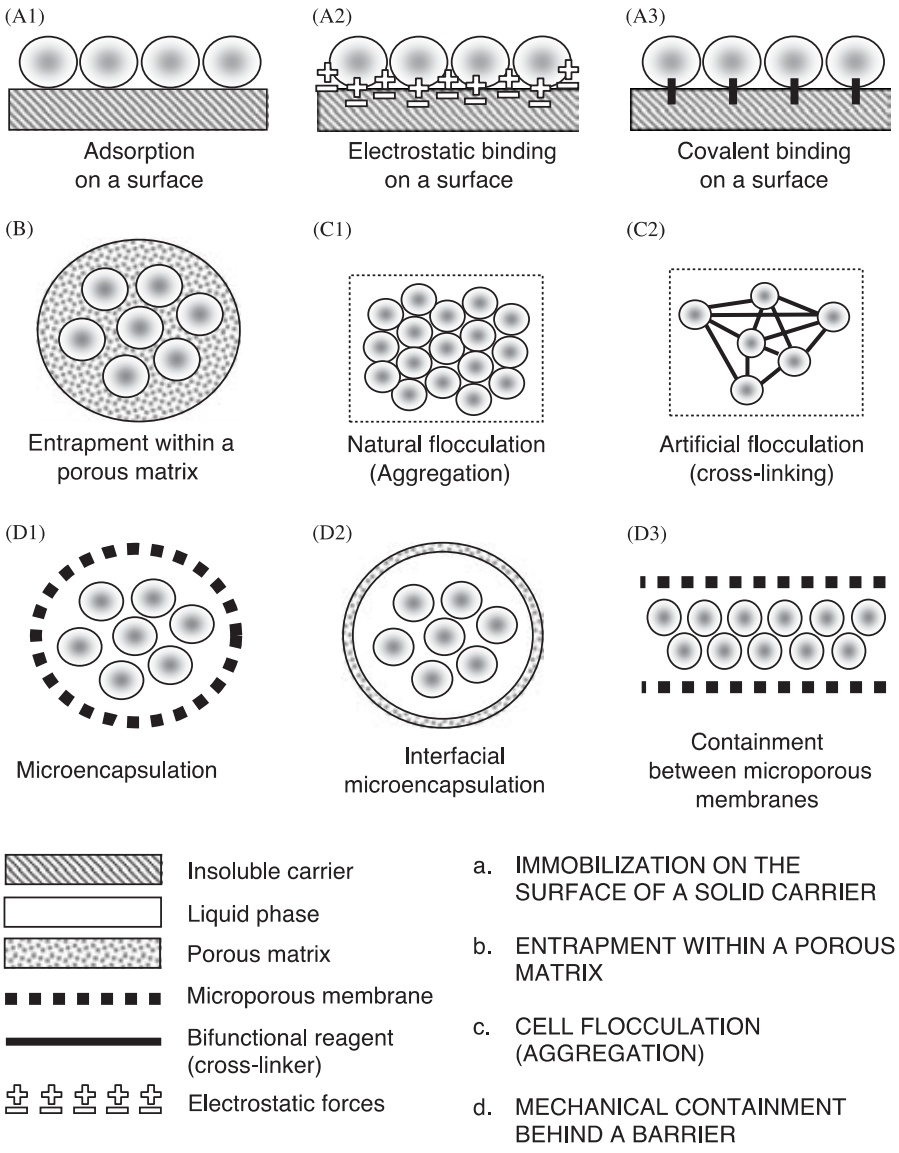


Figure 2. 8 Types of immobilization (Kourkoutas *et al.,* 2004)

In this study, immobilization is used for both of bacteria and white rot fungus. There are two immobilized materials for bacteria in this study including silica and recycled plastic. Silica is synthetic material by using sol-gel process (encapsulation immobilization) (Uo *et al.,* 1992). From previous study, Khongkhaem *et al.* (2011) used

silica-immobilized the co-culture of *Methylobacterium* sp. NP3 and *Acinetobacter* sp. PK1 for phenol degradation.Silica-immobilized the co-culture bacteria degraded phenol up to 2,500 mg/L in batch scale. For recycled-plastic, it comes from waste of plastic and is attachment immobilization.

White rot fungi immobilization has many advantages; easy to separate between solid (immobilized cells) and liquid (wastewater), reusable, reducing clogging in the continuous-flow systems, durable for unsuitable condition and increasing ligninolytic enzyme activity (Tieng and Sun, 2000, Shin *et al.,* 2002). In this study interests in palm oil residues because they can be carbon source to fungi when immobilization and make zero waste in palm oil mill process. Previous study, Kietkwanboot (2013) investigatedthe highest degradability of 80.6% phenolic compounds removal and 94% of color removal diluted (50%) treated POME by *Trametes hirsuta* AK4 immobilized on palm pericarp fiber.

2.7 Related Articles

2.7.1 Phenols removal by immobilized bacteria

Biological process is an effective method to treat phenol completely, cost effectively and environmental-friendly (Banerjee *et al*., 2001). However, from the previous study free cells can tolerate only low concentration of phenols (Ruiz-Ordaz *et al*., 2001), so the efficiency of biodegradation is also low. This study interests in immobilization of bacteria to treat phenols in wastewater because immobilized cells will

be resistant to high concentration of phenols and have high survival rates even in unsuitable condition (Obuekwe *et al.,* 2001).

Ehrhardt and Rehm (1985) found that the free cells of *Candida* sp. and *Pseudomonas* sp. did not degrade phenol when phenol concentration more than 1.5 g/L, but both of them can degrade phenol when they are immobilized on activated carbon.

Pazarlioglu *et al.* (2005) demonstrated that activated pumice immobilized *Pseudomonas* sp. could degrade phenol at 1 g/L within 22 hours and could be reused.

Lee et al. (2009) found that immobilizedthe consortium of phenol-decomposing cells in resin could degrade phenol up to 95% and tolerates wide ranges of pH than free cells. Free cells are inhibited when phenol concentration at 3,000 mg/L. On the other hand, immobilized the consortium of phenol-decomposing cells can degrade phenol with up to 95% removal efficiency.

Lu *et al.* (2011) showed that alginate-chitosan-alginate (ACA) microcapsules immobilized *Bacillus amyloliquefaciens* strain WJDB-1 could degrade phenol at 200 mg/L within 36 hours.

Khongkhaem *et al.* (2011) showed that silica-immobilized *Methylobacterium* sp. NP3 and *Acinetobacter* sp. PK1 could degrade phenol more than 2,500 mg/L in batch scale and silica immobilized cells could be reused for more than 2 months.

From the previous researches, immobilized bacteria can be used to treat phenols effectively. Therefore, this study immobilized the co-culture bacteria in silica and recycled-plastic to treat phenols in the treated POME.

2.7.2 Phenols removal by white rot fungi

Fountoulakis *et al.* (2002) found that *Pleurotus ostreatus* could remove phenols in different conditions from the culture medium, The initial phenol concentration was reduced from 4.05 to 0.88 g/L or 78.3% phenols removal efficiency of sterilized 50% diluted olive mill wastewaters (OMW) after 21 days and for thermally processed (100 °C) OMW with 50% dilution the initial phenol concentration was reduced from 5.25 to 1.75 g/L or 66.7% phenols removal efficiency after 19 days and 64.7% (10.35 to 3.65 g/L of initial phenol concentration) phenols removal efficiency of OMW without dilution after 21 days.

Gusse *et al.* (2006) showed that the white-rot fungi, *Phanerochaete chrysosporium* could degrade phenolic resins (phenol and formaldehyde polymers) by producing chromatic transformation in culture medium (yellow to pink).

Udayasoorian *et al.* (2007) demonstrated that *Phanerochaete chrysosporium*, isolated from pulp and paper mill effluent degraded 85% of pentachlorophenol after 8 days when grown in media containing ammonium lignosulphonate.

Justino *et al.* (2010) proved that *Trametes versicolor* and *Pleurotus sajor caju* could treat phenols 74% and 76% (18 mg/L of the initial phenol concentration), respectively.

From the previous study fungi could degrade phenols but if high concentration of phenols, they could be toxic to fungi. Annibale *et al.* (2004) studied about organic load, phenols and color removal efficiency of *Panus tigrinus* CBS 577.79 on high strength (5.5 g/L of the initial phenol concentration) and low strength (2.9 g/L of the initial phenol

concentration) olive-mill wastewater that presence and absence of supplement and total suspended solids removal. The results showed that high phenols affected on *Panus tigrinus* CBS 577.79 growth. The phenols removal efficiency was 88% in high strength wastewater that were removed total suspended solids and added supplement with 9 days. On the other hand, *Panus tigrinus* CBS 577.79 could remove 89% phenols efficiency removal in low strength wastewater within 3 days. So, in this study focuses on decreasing phenol concentrations in wastewater before applying to fungal treatment.

2.7.3 Color removal by white rot fungi

Color is another problem in palm oil mill effluent that must be solved. There are many researches about color removal by white rot fungi.

Annibale *et al.,* 2004 investigated that *Panus tigrinus* CBS 577.79 could not remove color in high strength olive mill wastewater containing high concentration of phenols. Meanwhile 72.4% color removal efficiency in low strength olive mill wastewater with added supplement by *Panus tigrinus* CBS 577.79.

Bibi *et al.* (2009) found that *Ganoderma lucidum* IBL-05 could degrade Solor golden yellow R that used in the textile industry at 83.78±5% at acidic pH and 30 °C and it removed color better when adding 1% of starch as carbon source.

Rakamthong and Prasertsan (2011) showed that *Phanerochaete chrysosporium* ATCC 24725 could degrade 83.4% of phenol (43.01 mg/L of the initial phenol concentration) and 61.22% of color from the mixture of the final effluent and the decanter effluent at ratio 1:1 from the palm oil mill effluent industry.

Zapata-Castillo *et al.* (2012) showed that *Trametes hirsuta* Bm-2 produces the major laccase (Lacl) from wheat bran for synthetic dye and textile effluent decolorization.

From the above researches, various white rot fungi could remove color effectively. In this study, *Trametes hirsuta* AK4, a local white rot funguswas used for color and phenols removal.

CHAPTER III

METHODOLOGY

The overall experiments are below (Figure 3.1).

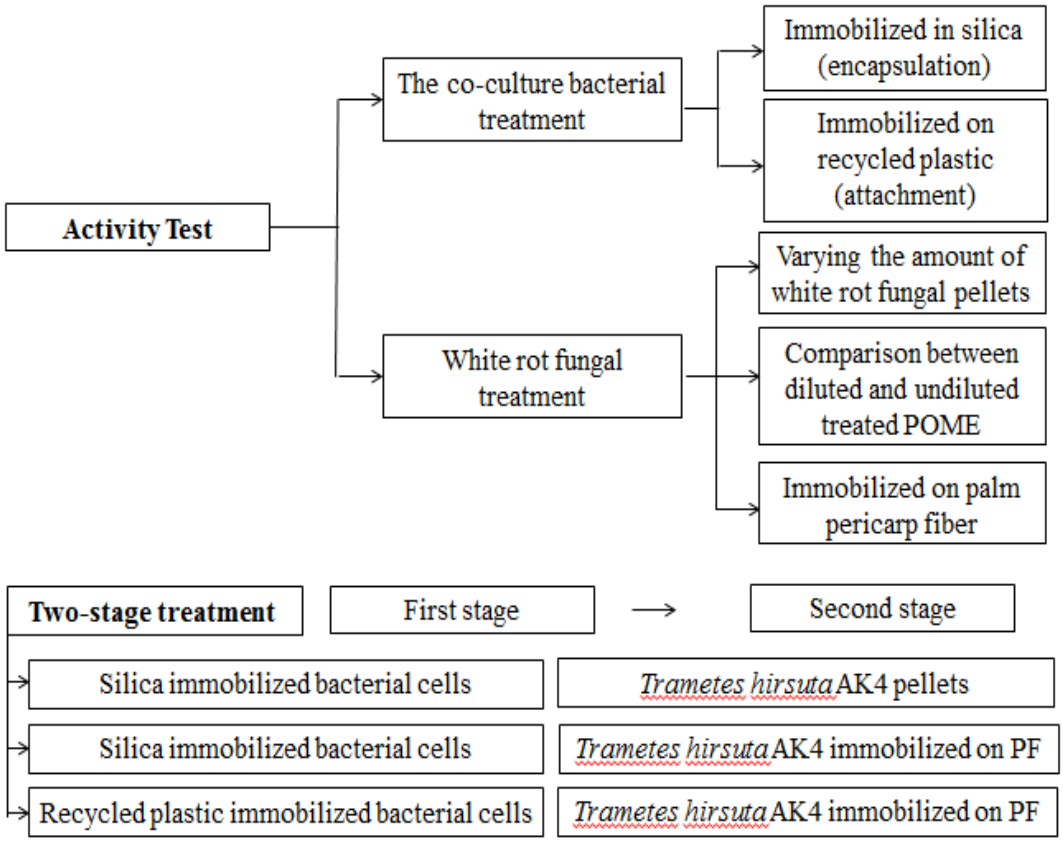


Figure 3. 1 Overview of this research

3.1 Treated palm oil mill effluent (POME) samples

Treated POME samples used in this study were collected from a palm oil mill in Surat Thani province. Itis a large-scale palm oil mill which has production capacity of 45 tons palm oil/hour. Samples were the effluent from the last stabilization pond as shown inFigure 3.2. They were analyzed according to the quality of water in analysis of treated POME (3.6.1).

Figure 3. 2 Stabilization pond of the palm oil industry

3.2 Bacterial preparation and immobilization

3.2.1 Cultivation of bacteria

Methylobacterium sp. NP3 and *Acinetobacter* sp. PK1 were isolated locally by Khongkhaem*et al.* (2011). Each strain was separately cultivated in 100 ml of carbon free mineral medium (CFMM) (see in appendix A) containing 4% glucose and was incubated in room temperature with shaking at 200 rpm for 3-5 days. Before immobilization, the

culture was harvested and washed with normal saline solution and resuspended in fresh CFMM containing 10 mg/L of phenol to induce phenol-degrading enzymes.

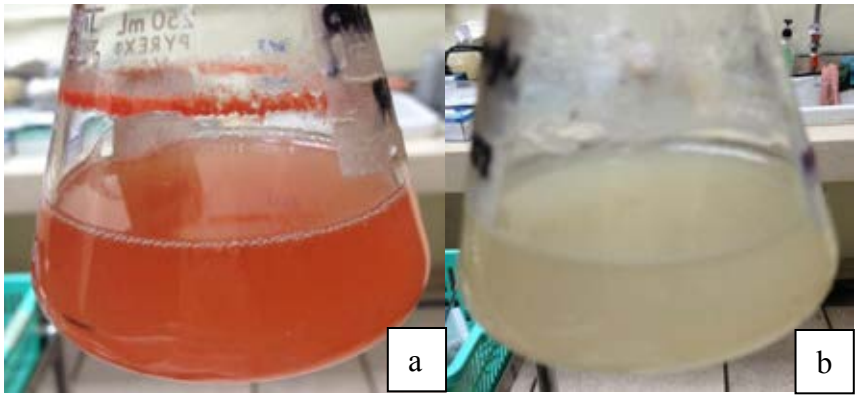


Figure 3. 3*Methylobacterium* sp. NP3 (a) and *Acinetobacter* sp. PK1 (b) in CFMM containing 4% glucose broth

3.2.2 Encapsulation of bacteria on silica

Encapsulated silica was synthesized by sol-gel process. Briefly, 7 mL tetraethoxysilane (TEOS,Merck)was vigorously mixed with 5.6 mL of HCl until the turbid mixture is clear. The solution was kept at 4 °C for 72 hours. The silica solution (12.6 mL) was added in plate for 15 minutes to allow ethanol in TEOS evaporation. Then, the solution was quickly mixed with 1.64 mL KOH (pH 8) to adjust its pHto neutral beforeadding 12 mL of each bacterial inoculum. The encapsulation silica was cut into 1.0×1.0×0.5 cm pieces as shown in Figure 3.4 and dried at room temperature for 1 day (Khongkhaem *et al.,* 2011). The amount of cells in silica (CFU/g of silica) was analyzed as in 3.6.4.

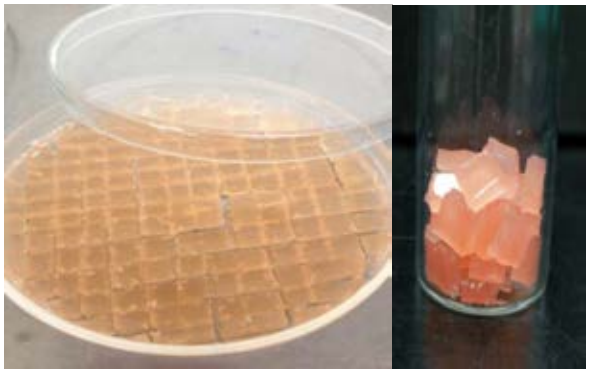


Figure 3. 4 Example of silica immobilized bacteria in this study

3.2.3 Preparation of recycled plastics for attachment

To prepare attachment culture medium, 6.7 g recycled plastic (Figure 3.5)was added to 100 mL carbon free mineral medium (CFMM) containing 0.1% glucose and 4% phenol. Then, 10% inoculum of *Methylobacterium* sp. NP3 and *Acinetobacter* sp. PK1 was added and incubated at room temperature with shaking for 200 rpm, 4 days. The amount of cells was be analyzed by plate count technique before and after immobilization (3.6.4). The surface of recycled plastic granules was investigated by using zoom stereomicroscopic SMZ1000. These granules had rough surface, which were suitable for bacterial attachment (Figure 3.6).

Figure 3. 5 Recycled plastic granules

Table 3. 1 Properties of recycled plastic

Properties	**Recycled plastic**
Size	0.02 cm^3
Type of plastic	Mixing of many types of plastic
Location when put in water	Submerge

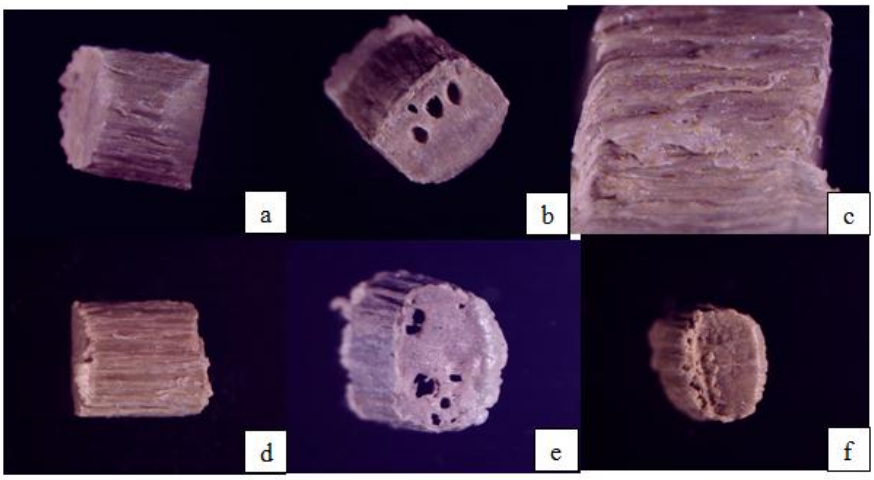


Figure 3. 6 Pictures of recycled plastic granules surface (a-f); magnification (150x) of a, b, d, e, f and magnification (300x) of c by using zoom stereomicroscope SMZ1000

3.2.4Attachmentof bacteria on recycled plastic

After the co-culture bacteria cultivation was well grown, they were inoculated in CFMM containing 6.7 g recycled plastic, 0.1% glucose and 4% phenol for immobilization. Then, a piece of recycled plastic was collected every day for 5 days for estimating the amount of attached co-culture bacteria by plate count technique. The result showed that day 4 had the highest the amount of the co-culture bacteria of 9 Log CFU/g of recycled plastic (Figure 3.7). Thus, the attachment process was carried out by incubating bacterial cells with recycled plastic granules for 4 days.

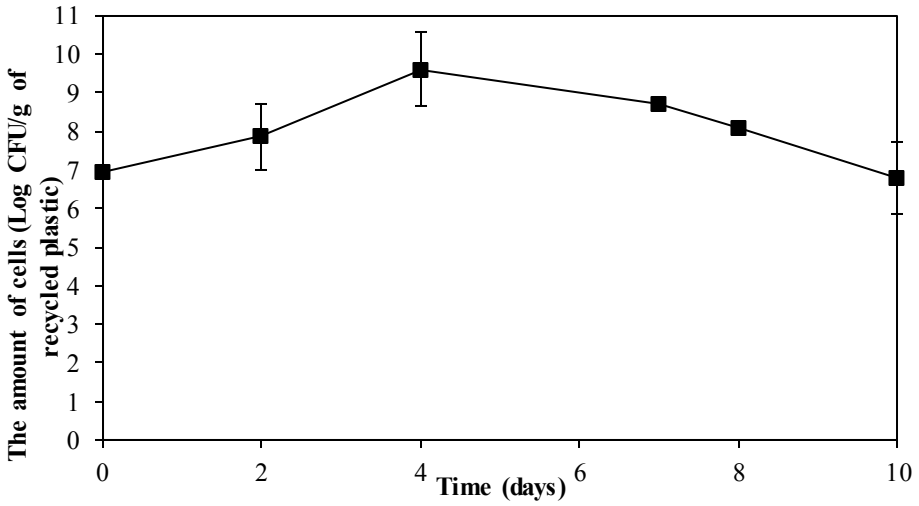


Figure 3. 7 Changes in number of attached bacteria on recycled plastic after incubation

3.3 *Trametes hirsuta*AK4 preparation

Trametes hirsuta AK4 (Figure 3.8) was isolated from the bark of timber from Songkhla province(Kietkwanboot *et al.,* 2013). It wascultivated in potato dextrose agar (PDA) (see in appendix A) and incubated at room temperature for 5-7 days. The culture was maintained on PDA plates at 4 °C and sub-cultured every three months.

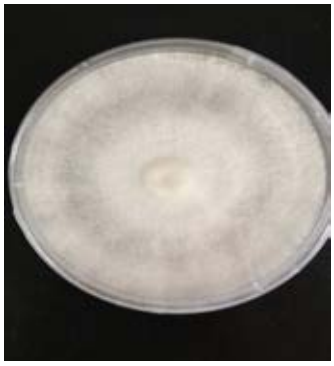


Figure 3. 8*Trametes hirsuta* AK4 on PDA plate

3.3.1 Free cells (White rot fungal pellets)

Trametes hirsuta AK4was cultivated in potato dextrose agar (PDA) and incubated at room temperature for 5-7 days. The mycelia of *Trametes hirsuta* AK4 was cut by a cork borer number 1 (diameter 0.5 cm) before added to glucose yeast extract broth (GYEB) pH 4.5 (see in appendix A) and incubated at room temperature with shaking at 120 rpm for 5-7 days to obtain white rot fungal pellets (Figure 3.9).

Figure 3. 9*Trametes hirsuta* AK4 pellets in GYEB broth

3.3.2Immobilization of fungi on palm pericarp fiber (PF)

Palm pericarp fiber (PF) was chosen to use as fungal immobilizing matrix in this study. It came from palm oil residues in palm oil industry, Surat Thani province.

Table 3. 2 The compositions of palm pericarp fiber (Tosu, 2012)

Compositions	**Palm pericarp fiber (PF)**
Nitrogen (%)	2.16
Phosphorus (%)	0.21
TOC (%)	35.93
Total pore volume (cc/g)	0.0068
Specific surface area (m^2/g)	5.45
Average pore diameter (nm)	5.01

Before immobilization, PF was washed by clean water and sterilized following Kietkwanboot *et al.* (2013). For sterilization, 10 g of PF was put in a plastic bag and autoclaved twice at 121 °C for 20 minutes. The sterilized PF was later added to 100 mL glucose yeast extract broth (GYEB) and autoclaved again at 121 °C for 20 minutes. To immobilize the fungi, 5 pieces of *Trametes hirsuta* AK4 mycelia after cut with cork borer number 1 (diameter 0.5 cm) were added to GYEB containing PF and incubated at room temperature without shaking for 6 days.

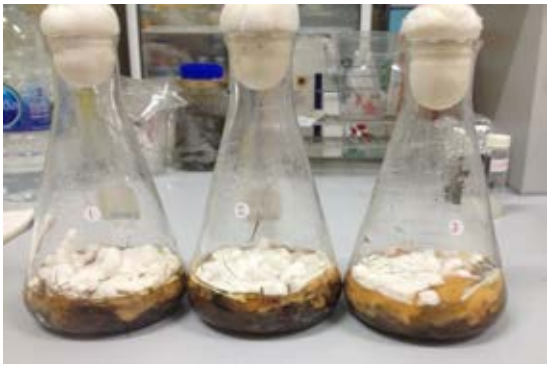


Figure 3. 10*Trametes hirsuta* AK4 immobilized on palm pericarp fiber in GYEB broth

3.4 Activity test

3.4.1 Silica-immobilized co-culture bacteria

Silica-immobilized co-culture bacteria at 25 g were added to 100 mL 100% treated POME samples and incubated at room temperature with shaking at 200 rpm for 7 days. To study abiotic phenols and color removal process, the control set contained POME only and cell-free silica with POME. All samples were triplicated and collected to analyze for the remaining of phenols concentration and color units (3.6.2, 3.6.3 and 3.6.4).

3.4.2 Recycled plastic-immobilized co-culture bacteria

Before starting the experiment, the CFMM medium from the attachment process was poured out of the flask. Then, 100 mL of 100% treated POME samples was added and incubated at room temperature with shaking at 200 rpm for 7 days. To study abiotic phenols and color removal process, the control set contained POME only and cell-free

recycled plastic with POME. All samples were triplicated and collected to analyze for the remaining phenols concentration and color (3.6.2, 3.6.3 and 3.6.4).

3.4.3 *Trametes hirsuta* AK4 pellets

The amounts of pellets for POME treatment was varied at 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 g for the suitable amount of pellets studying.To study the effect of POME concentration, the white rot fungal pellets were added to 10 mL 25% treated POME or 100% treated POME and were incubated in room temperature with shaking at 120 rpm for 3 days.

After chosen a suitable amount of white rot fungal pellets, the efficiency of fungal pellets were investigated in series of diluted treated POME samples starting from 25%, 50%, 75% and 100% treated POME for acclimatization and collected at 0 to 3 days. To study abiotic phenols and color removal process, the control set contained killed cells of *Trametes hirsuta* AK4 pellets and treated POME only. All samples were triplicated and collected to analyze for the remaining phenols concentration and color units (3.6.2, and 3.6.3).

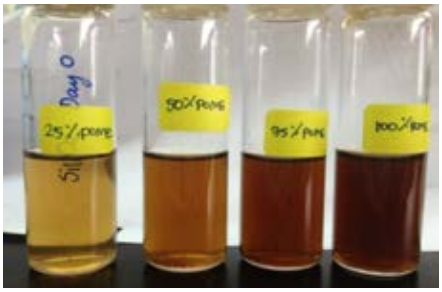


Figure 3. 11 Color of diluted treated POME from 25%, 50% 75% and 100% treated POME

3.4.4 *Trametes hirsuta* AK4 immobilized on palm pericarp fiber (PF)

After 6 days of immobilization, GYEB medium was removed and 100% POME was added to the flask. The immobilized *Trametes hirsuta* AK4 was reversed from the top to the bottom of the flask and incubated at room temperature with shaking at 200 rpm for 8 days. For control, PF with treated POME and treated POME only were used. All samples were triplicated and collected to analyze for the remaining phenols concentration and color units (3.6.2 and 3.6.3).

3.5 Two-stage treatment

Bacterial and white rot fungal treatments were combined as a two-stage treatment for sequential phenols and color removal.Basically, the bacterial treated wastewater was applied to the white rot fungal treatment. There were 3 experiments of two-stage treatment consisted of silica immobilized co-culture bacteria and *Trametes hirsuta* AK4 pellets, silica immobilized the co-culture bacteria and *Trametes hirsuta* AK4 immobilized on palm pericarp fiber and recycled plastic immobilized co-culture bacteria and *Trametes hirsuta* AK4 immobilized on palm pericarp fiber. All of the two-stage treatments used 100% POME to start the experiment.All samples were triplicated and collected to analyze for the remaining phenols concentration and color units (3.6.2 and 3.6.3). This experiment was plotted by mixing data from each stage over time.

3.6 Analytical methods

3.6.1 Analysis of treated POME

Treated POME samples were analyzed following the Standard Methods for Examination of Water and Wastewater (APHA, AWWA and WEF, 2005).

Table 3. 3 Analytical methodology

Parameters	Method
1. pH	pH meter
2. Temperature	Thermometer
3. BOD	Azide modification method
4. COD	Potassium Dichromate
5. DO	Azide modification method
6. TP	Persulfate Dihestion/ Ascobic acid Method
7. TKN	Kjeldahl Method
8. TSS	Dried at 103-105 °C at least 1 hour
9. Oil and Grease	Soxhlet Extraction

3.6.2 Analysis of total phenols

Analysis of total phenolic compounds used Folin-Ciocalteau method modified from Barlocher and Graca (2005). Briefly, 100 µL of sampleswere centrifuged at 10,000 rpm for 7 minutes to remove sediment. The sample was diluted 1 time before adding 1 mL 2%

Na_2CO_3. After waiting for 5 minutes, 100 µL of Folin-Ciocalteau reagent was added and incubation for 30 minutes to get blue products. The absorbance at 760 nm was measured by spectrophotometer. The remaining phenolic compound was calculated by comparing to the standard curve.

3.6.3 Analysis of color (APHA, AWWA and WEF, 2005)

The analysis of color in wastewater samples was done by centrifuging 100 µL samples at 10,000 rpm for 7 minutes to remove sediment. Then, the sample wasdiluted before measuring the absorbance at 475 nm using distilled water as a blank. The absorbance was compared with Platinum-Cobalt standard of 0-500 color units. Calculate color units by the following equation:

$$\text{Color units} = \frac{A \times 50}{B}$$

where: A = estimated color of a diluted sample and

B = mL sample taken for dilution

3.6.4 Measurement of bacteria

Immobilized cells at 0.1 g were added to 0.9 mL normal saline solution and sonicated with an ultrasonic bath for 2 minutes, 3 times to separate cells. The sonicated samples were used to make ten-fold dilution. Then, 10 µL of the cell suspensions was dropped into CFMM containing 4% glucose agar plate to estimate total bacteria concentration (CFU/g of silica and Log CFU/g of recycled plastic).

3.6.5 Scanning Electron Microscopy (SEM) morphology

Immobilized cells and silica morphology were investigated by scanning electron microscopy. The samples were prepared by fixation of 2.5% (v/v) glutaraldehyde in phosphate buffer pH 7.2 for 1-2 hours and washed by phosphate buffer 2 times for 10 minutes and washed by distilled water 1 time and dehydrate by ethanol concentration at 30%, 50%, 70% and 95% and 100%, respectively. Dehydration at critical point by critical point dryer and fix samples on stub and coated with gold by Ion sputter.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Characteristics of treated POME

The treated POME was collected by grab sampling from the last stabilization pond in a palm oil mill, Surat Thani province. The palm oil mill generated large volumes of dark brown wastewater with high phenols and COD. In Table 4.1 showed that phenols and color concentrations were in the range of264-338 mg/L and 95-117 color units, respectively.

Table 4. 1 Characteristics of the treated POME from the last stabilization pond of a palm oil mill in Surat Thani province

Parameters	Standard*	Treated POME
pH***	5.5-9.0	8-9
COD (mg/L)***	120	19,800
BOD (mg/L) ***	20	224
TSS (mg/L) ***	50	623
Oil & Grease (mg/L) ***	5	19.3
Total Phosphorous (mg/L) ***	-	1.30
Total Nitrogen (mg/L) ***	100	25.7
Phenols (mg/L) **	1	338
Phenols (mg/L) ***	1	264
Color (color units) **	Do not be disgusting	117
Color (color units) ***	Do not be disgusting	95

* Water quality standards (Notification the Ministry of Science, Technology and Environment, No. 3, B.E.2539 (1996) issued under the Enhancement and Conservation of the National Environmental Quality Act B.E.2535 (1992), published in the Royal Government Gazette, Vol. 113 Part 13 D, dated February 13, B.E.2539 (1996))

** Sample was collected in June 2012., ***Sample was collected in January 2013.

4.2 Activity Test

4.2.1 Co-culture bacterial treatment

This study interested to immobilize bacterial cells in two materials i.e. silica and recycled plastic. Both of them had different immobilizing approaches. For silica, bacterial cells were encapsulated in immobilized material. Silica has a porous structure, which protects bacterial cells but allows oxygen and nutrients to pass through the structure (Branyik and Kuncova, 1998; Meunier *et al.,* 2010). Recycled plastic was used for attachment immobilization, of which bacterial cells adhered on the surface of immobilized material**.** The attachment technique can increase the amount of bacterial cells (Lee and Palsson, 1994).There were two phenol-degrading bacteria in this study; *Methylobacterium* sp. NP3 and *Acinetobacter* sp. PK1. When these bacterial cells were mixed together, they supported each other for phenol removal (Khongkhaem *et al.,* 2011). In this experiment, 100% (undiluted)treated palm oil mill effluent (POME) was used. However, the initial phenols concentrations in treated POME from different sampling times were varied. There was 264 mg/L phenols in silica-immobilized the co-culture bacteria treatmentwhen using 100% treated POME collected in June 2012, while there was 129 mg/L phenols in recycled plastic immobilized the co-culture bacteria treatment when using 100% treated POME collected in January 2013.

The co-culture bacteria immobilized in silica decreased phenols from 264 mg/L to 164 mg/L or approximately 38% phenols removal within 7 days in batch experiment when the co-culture starters were 3.41×10^{8} CFU/g silica (Figure 4.1a). The recycled plastic immobilized co-culture bacteria decreased phenolsfrom 129 mg/L to 87 mg/L or

33% phenols removal within 7 days when the co-culture culturestarter were 3.10×10^{8} CFU/g recycled plastic **(**Figure 4.1a**).** The initial phenols concentrations in 100% treated POME treatment for cell free silica and cell free recycled plastic treatments were 248 mg/L and 159 mg/L, respectively. Phenols concentrations in cell free silica in treatment decreased from 248 mg/L to 186 mg/L or 25% of phenols removal and phenols concentration of cell free recycled plastic treatment decreased from 159 mg/L to 109 mg/L or 31% of phenols removal. These results showed that small amount of phenols decreased due to absorption and abiotic factors, for example, photo-oxidation. Nonetheless, the extent of phenol removal was higher in the presence of added co-culture.

For color removal(Figure 4.1b**.)**, the initial color units in 100% treated POME that collected in June 2012and January 2013were 95 and 96 color units, respectively. The silica immobilized co-culture bacteria removed color from 95 to 57 units or 40% of color removal, whilethe plastic immobilized co-culture bacteria removed color from 96 to 95 units or 1% of color removal. The results showed that recycled plastic did not absorb color and the immobilized cells could not degrade color. Nonetheless, color reduction in treatment with silica immobilized cells might be due to both absorption and degradation because color in the treated POME containing cell free silica (control) decreased from 54 to 51 colorunits or 6% of color removal.

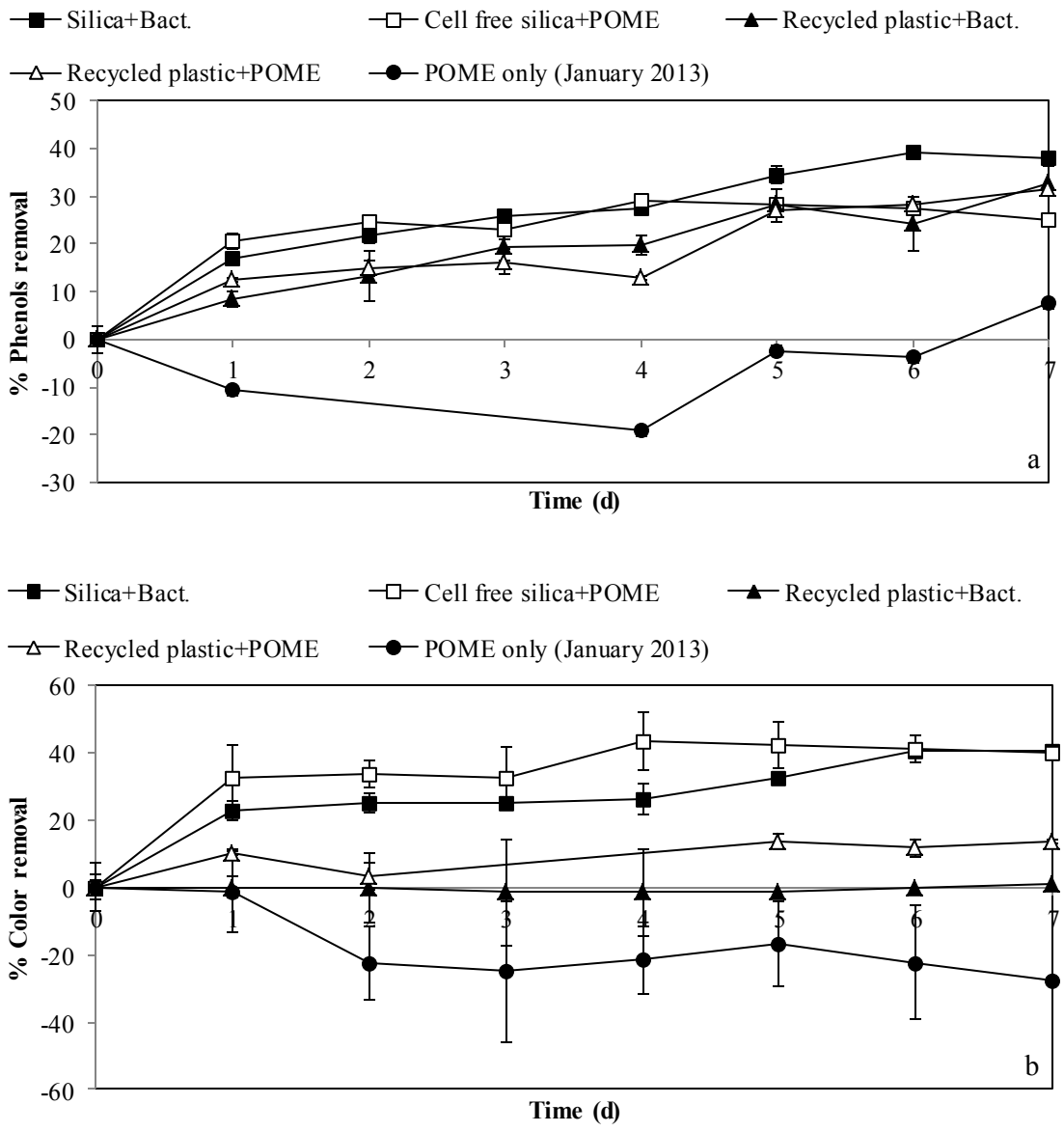


Figure 4. 1 Phenols removal (a) and colorremoval (b) from 100% treated POME after treatment by silica- and recycled plastic-immobilized *Methylobacterium* sp. NP3 and *Acinetobacter* sp. PK1 in batch experiment

Scanning electron microscope (SEM) showed that the amounts of cells in cross-sectional silica before used (Figure 4.2a) were more than after used (Figure 4.2b). The results confirmed the data from plate count technique that the cells in silica before used were 3.41×10^8 CFU/g silica and after used were 6.33×10^7 CFU/g of silica. Similarly, the bacterial cells in recycled plastic before used were 3.10×10^8 CFU/g recycled plastic and after used were 7×10^7 CFU/g recycled plastic. The reduction of bacteria cells suggested that other components in 100% treated POME might be toxic to the cells in silica and recycled plastic and caused cell death.

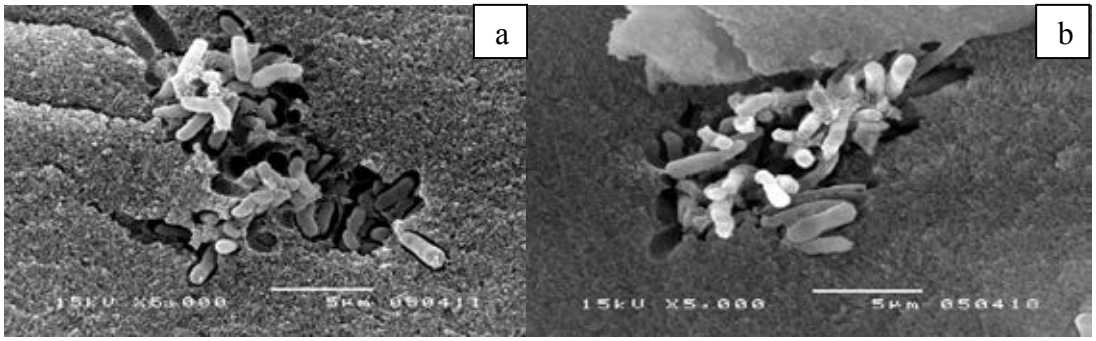


Figure 4. 2SEM pictures of an encapsulation silica cross-section before used (a) and after used (b) by using scanning electron microscope technique; magnification (5000x) ofa and b

From the results, silica immobilized bacterial cells had higher efficiency for phenols removal than recycled plastic immobilized bacterial cells. This was corresponded toKhongkhaem *et al*. (2011), which reported that the co-culture of*Methylobacterium* sp. NP3 and *Acinetobacter* sp. PK1 encapsulated in silica had higher phenol removal efficiency than the attachment of silica.

4.2.2 White rot fungal treatment

1) Phenols and color removal efficiency ofTrametes hirsuta AK4 pellets

To find the optimum amount of fungal pellets, this experiment first varied the amount of *Trametes hirsuta* AK4 pellets at 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 g in 10 mL of 25% treated POME. The initial phenols concentration and color in 25% treated POME of *Trametes hirsuta* AK4 pellets were 95 mg/L and 56 color units, respectively. The result showed that the percent of color removal was higher when more *Trametes hirsuta* AK4 pellets were used. The highest color removal was 82% or decreased from 56 color units to 10 color units when used 2.5 g *Trametes hirsuta* AK4 pellets within 2 days (Figure 4.3 (b)). For control (killed cells), the color wasdecreased from 39 to 32 color units or approximately 18% of color removal. Thismight cause froma little absorption or abiotic factors. Consequently, the next experiment used 2.5 g of white rot fungal pellets per 10 mL or 250 g/L. For phenol removal, only 100 g/L and 150 g/L of *Trametes hirsuta* AK4 pellets removed phenols in day 1 to day 3 as shown in Figure 4.3 (a). Killed white rot fungal pellets in 25% treated POME (Control) decreased phenol concentration from 122 mg/L to 102 mg/L or 16% of phenols removal. It was possible that white rot fungal pellets might absorb small amount of phenols into themselves. The ability of *Trametes hirsuta* AK4 pellets to remove color was corresponded to Yesilada *et al.* (2010), which reported that white rot fungal pellets had the ability for color removal in dye and textile industry wastewaters. In their study, *Trametes versicolor*, the fungus within the same genus of *Trametes hirsuta* that used in this study, was used as fungus pellets.

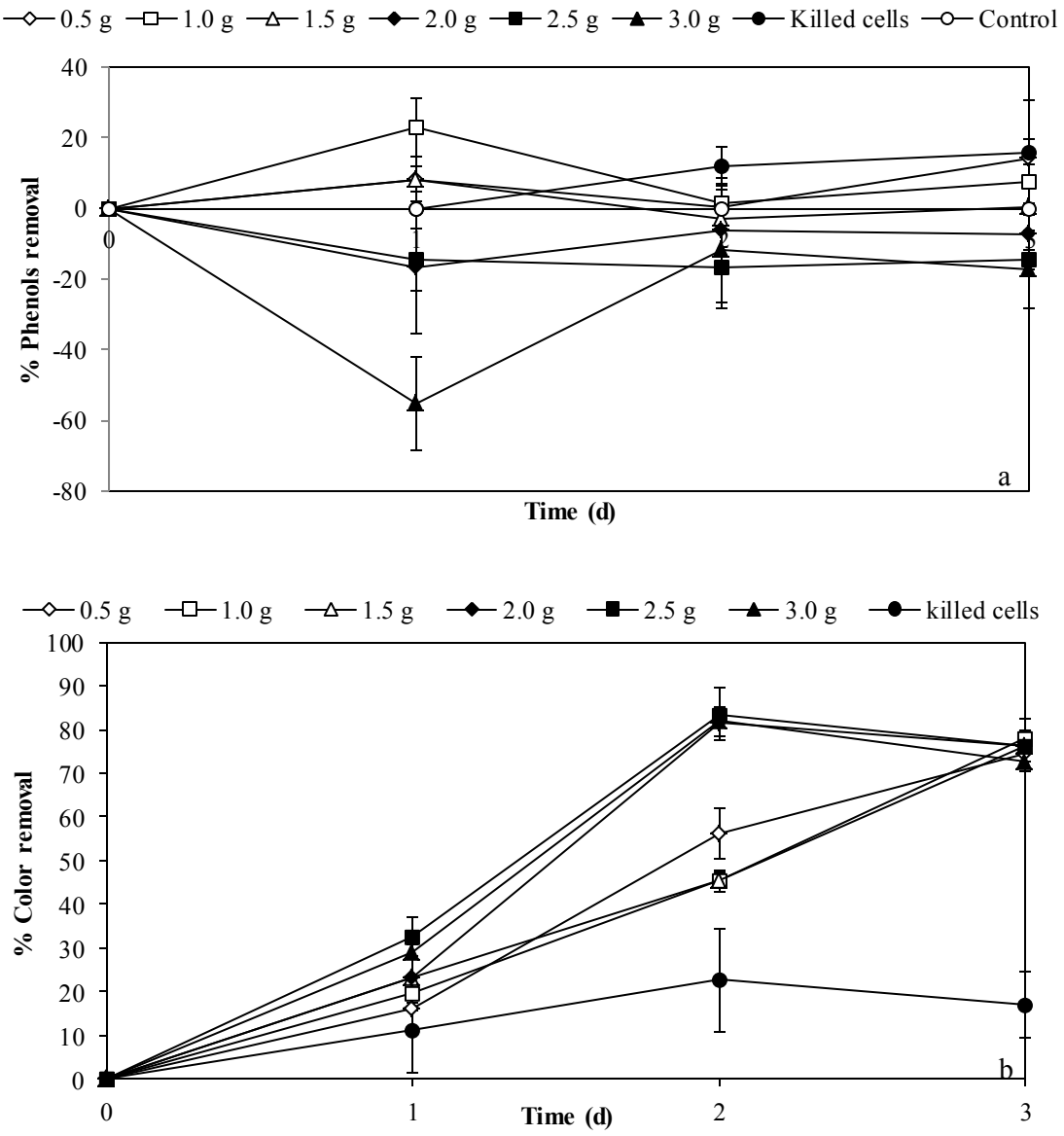


Figure 4. 3 Phenols removal (a) and color removal (b) of *Trametes hirsuta* AK4 pellets at various concentrations when using 25% treated POMEin batch experiment. The experiments compared between sterilized fungal pellets (Killed cells) and various concentrations of*Trametes hirsuta* AK4 pellets (0.5-3.0 g).

2) Efficiency of Trametes hirsuta AK4 pellets for color removal in diluted and undiluted POME samples

This experiment compared *Trametes hirsuta* AK4 pelletsbetween diluted (25% treated POME) and undiluted treated POME (100% treated POME). The 25% (Figure 4.4a) and 100% (Figure 4.4b) treated palm oil mill effluent (POME) samples had the initial color of56 and 115 color units, respectively. The highest color removal efficiencies were 82% from 25% treated POME within 2 days. For 100% treated POME, the colorwas decreased from 115 to 71 units or 38% color removal.Control consisted of 25% treated POME only and killed cells or killed white rot fungal pellets in 25% treated POME as shown in Figure 4.4a and Figure 4.4b was 100% treated POME only. For killed fungal pellets (control), the color in treatment with killed *Trametes hirsuta* AK4 pellets decreased from 39 to 32 color units or approximately 18% of color removal, so the fungus absorbed a little color. When the concentration of treated POME increased, the efficiency of white rot fungal pellets was low. Similar to Hadibarata and Kristanti (2011), high concentration of phenolic compounds was affected the decolorization of dyes. When the concentrations of phenolic compounds increased, decolorization was inhibited.

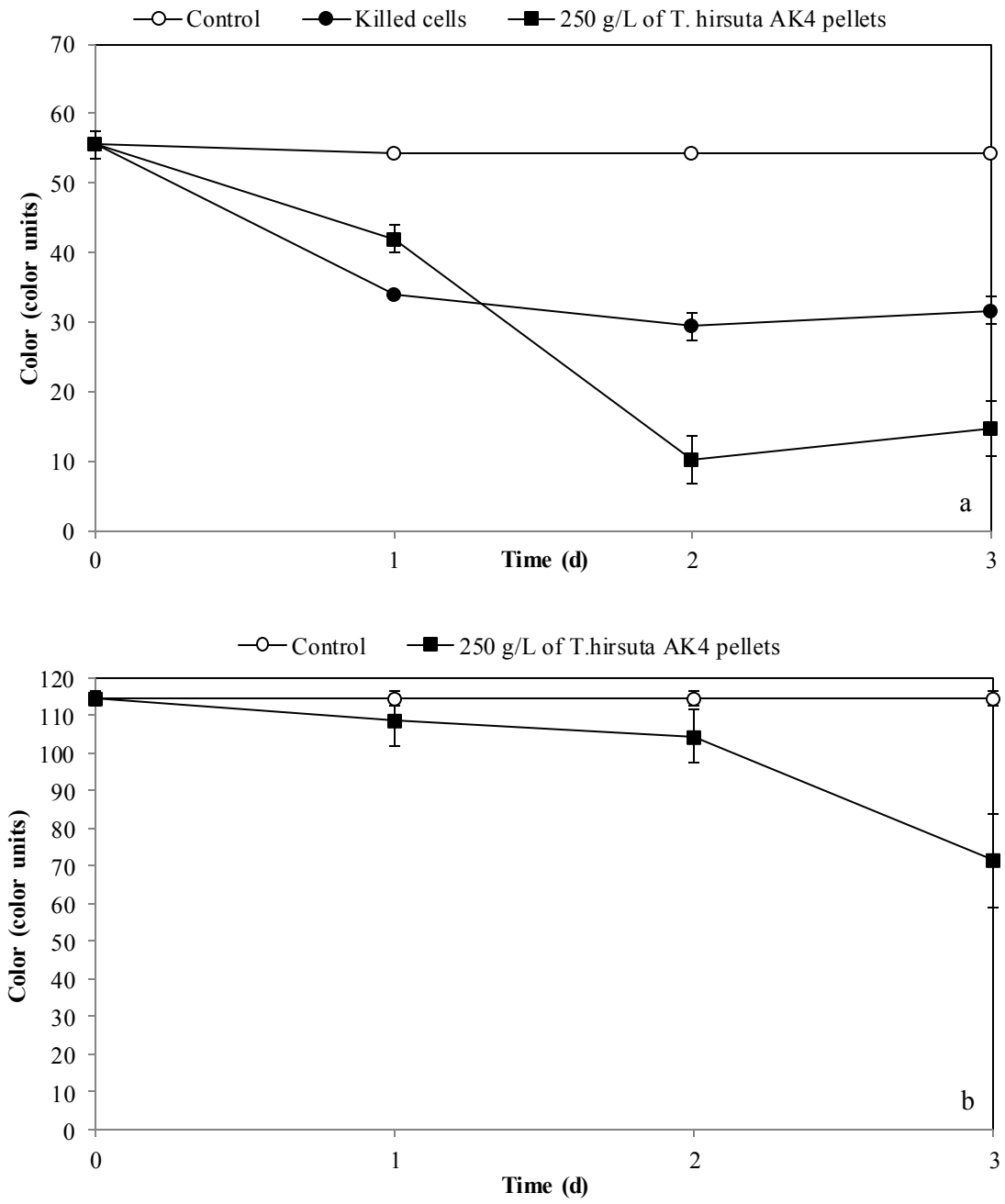


Figure 4. 4 Changes in color units with 25% treated POME (a) and 100% treated POME (b) by *Trametes hirsuta* AK4 pellets. The experiments compared between treated POME only (Control), sterilized fungal pellets (Killed cells) and *Trametes hirsuta* AK4 pellets (250 g/l).

3) Acclimatization of Trametes hirsuta AK4 pellets in diluted POME for color removal

Free cells of white rot fungi might be sensitive to phenols and other components in POME, therefore this study acclimatized the fungi in series of diluted POME. Guo *et al.* (2005) investigated that the acclimatization of salt-tolerant bacteria enhanced their decolorization activity in high salinity colored wastewater. The initial phenols concentration of 25%, 50%, 75% and 100% were 95, 161, 213 and 293 mg/L, respectively. The initial color units of 25%, 50%, 75% and 100% were 56, 62, 86 and 115 color units, respectively. The acclimatization experiment was conducted by incubating 250 g/L of *Trametes hirsuta* AK4 pellets in a series of diluted treated POME samples starting from 25%, 50%, 75% and 100% treated POME. The fungi had the highest color removal in 25% treated POME and its activity was not increased after acclimatization (Figure 4.5b). For phenols, the acclimatizedfungi slightly reducethephenols concentration (Figure 4.5a). So, it indicatedthat acclimatization did not work. Phenols were suspected to be the color agents and could exert toxic effects to the white rot fungi. Gray (1946) reported that alcohol tolerance of yeast could not be acclimatized because of low tolerance when increasing alcohol concentrations. It is similar to this study, when acclimatization did not help the white rot fungus to adapt with high concentrations of treated POME.

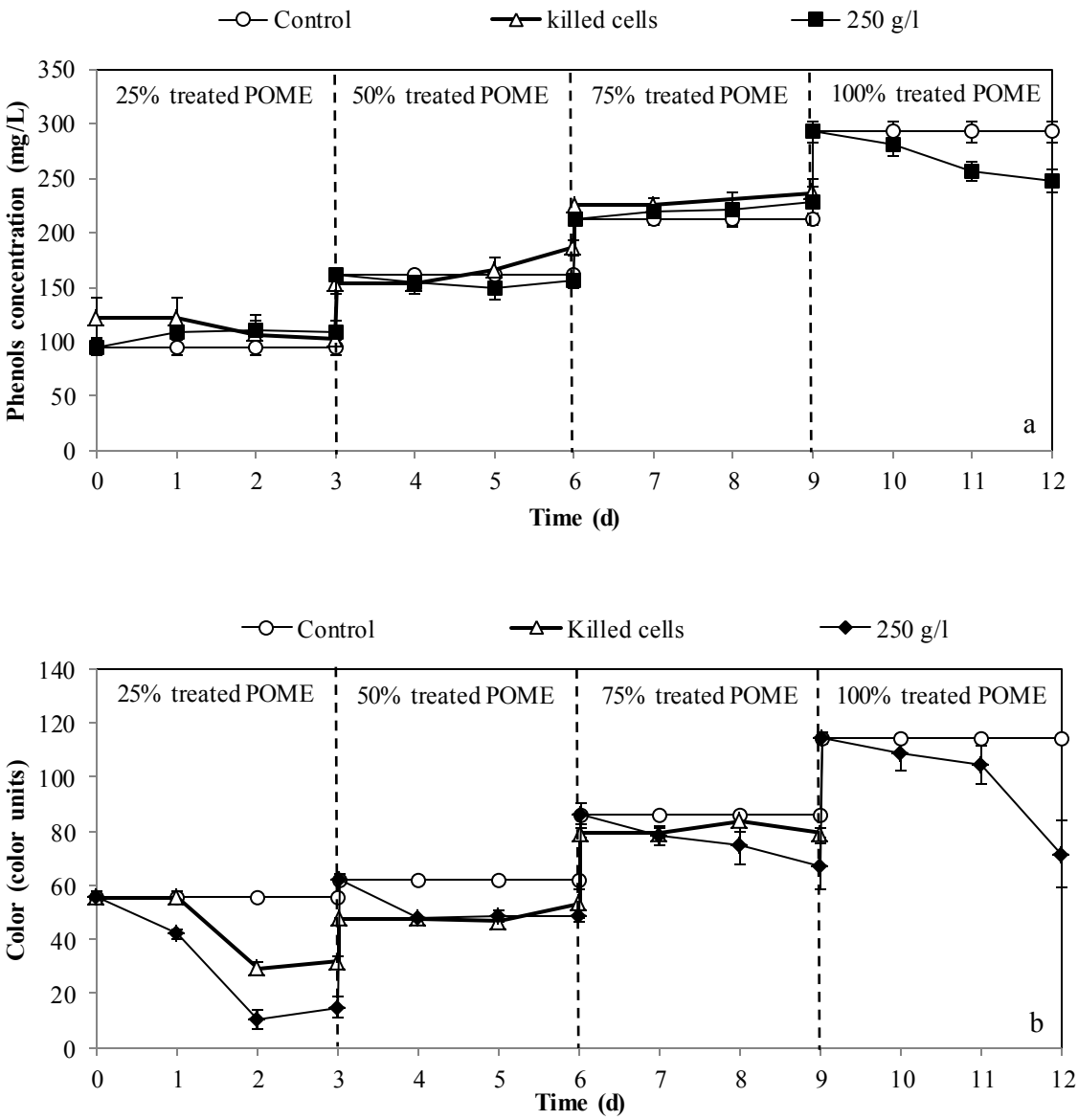


Figure 4. 5 Changes in phenols concentration (a) and color units (b) during incubation of *Trametes hirsuta* AK4 pellets in a series of diluted treated POME (acclimatization). The experiments compared between treated POME only (Control), sterilized fungal pellets(Killed cells) and *Trametes hirsuta* AK4 pellets (250 g/l).

Although*Trametes hirsuta* AK4 pellets removed color up to 82% in 25% treated POME however they had low efficiency in 100% treated POME. It is impossible to use undiluted POME in the real site or in continuous system. Besides *Trametes hirsuta* AK4 pellets broke after7 days and their color returned to the treated POME (Figure 4.6b). Thereby,*Trametes hirsuta* AK4 immobilization waschosen to use for the next experiment.

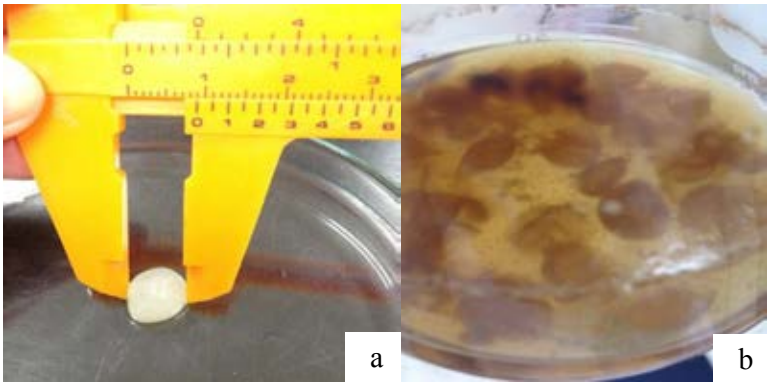


Figure 4. 6*Trametes hirsuta* AK4 pellets before (a) and after (b) used for 7 days

4) Phenols and color removal efficiency by Trametes hirsuta AK4 immobilized on palm pericarp fiber (PF)

Palm pericarp fiber (PF) was chosen as immobilization material in this study. Pant and Adholeya (2007) supported that the agro-residues can be used for fungus immobilization because they are environmentally harmless and do not add carbon and/or nitrogen source in the media. Besides PF is the waste from palm oil industry, an adoption of palm pericarp fiber as immobilized material is the way for zero-waste.

This experiment immobilized *Trametes hirsuta* AK4 on PF and tested with 100% (undiluted) treated POME. The initial phenols concentration and color units were 231 mg/L and 85 color units. Figure 4.7 (a) showed that the treatment with*Trametes hirsuta* AK4 immobilized on PF had higher phenol concentrations than both of controls because phenols were probably left over from palm pericarp fiber although it was washed before used. Moreover, phenolic compounds could be produced during transformation of lignin in the fiber. When white rot fungus immobilized on palm pericarp fiber, it probably emitted ligninolytic enzyme to degrade lignin more than in the form of fungal pellets. The enzyme induction was also reported by Wong (2009).On the other hand, color was declined from 85 to 38 color units or approximately 55% color removal within 4 days as shown in Figure 4.7 (b). This study corresponded to Ghasemzadeh *et al.* (2011) that *Phanerochate chrysosporium*, white rot fungus, could degrade 45 mg/L of divers the structure dyes within 7-15 days.

It could be concluded that immobilized white rot fungi had higher color removal efficiency than white rot fungal pellets. It might be because palm pericarp fiber has nutrients especially lignin that the immobilized white rot fungi produced high amounts of ligninolytic enzymesto degrade lignin and color. In addition, the immobilized fungus worked well in 100% POME. This was probably because the palm pericarp fiber protected fungal cells from toxic phenols.

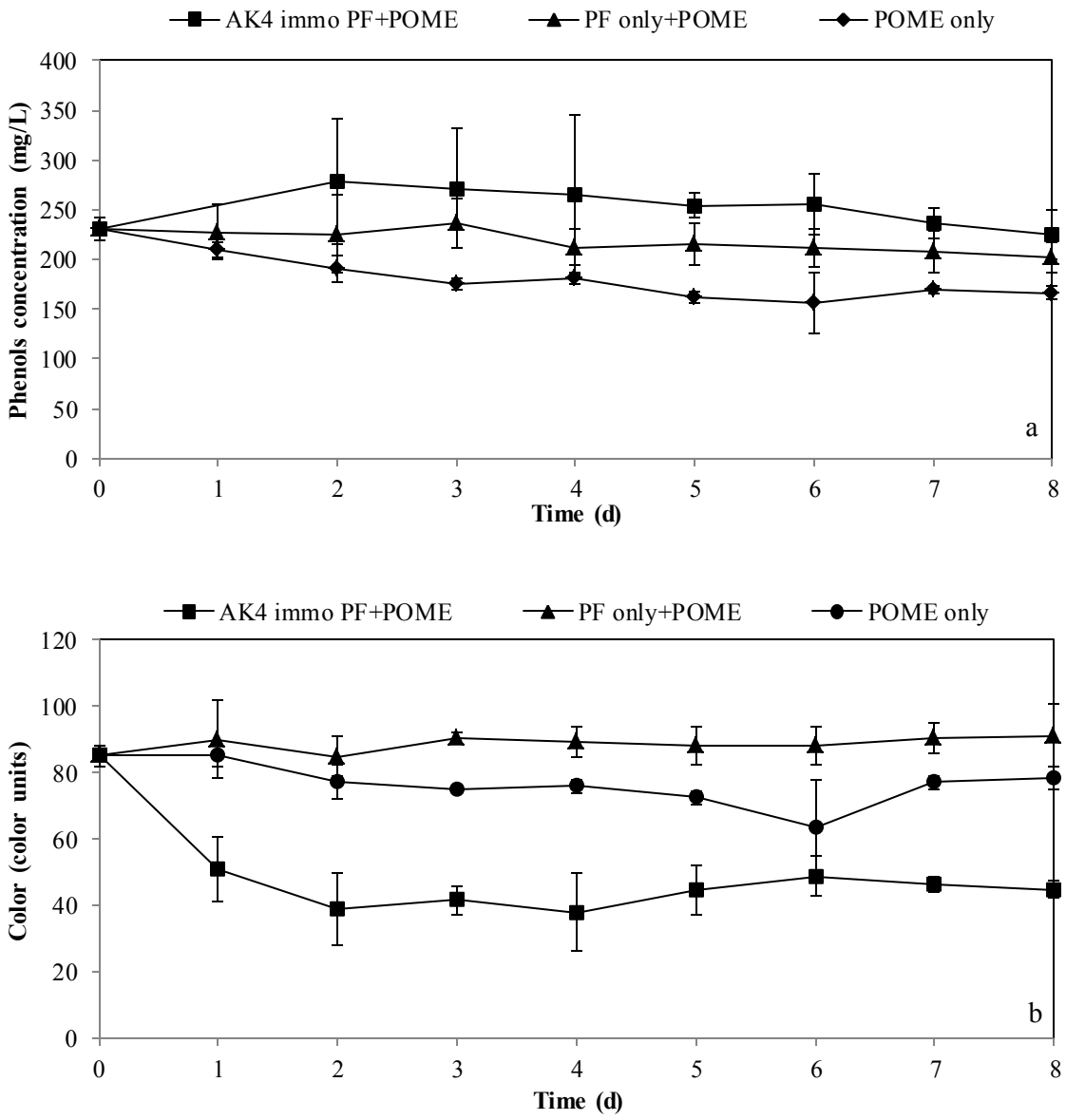


Figure 4. 7 Phenols concentration (a) and color units (b) removal efficiency of *Trametes hirsuta* AK4 immobilized on PFin 100%treated POME. The experiments compared between POME only (100% treated POME), PF only+POME (palm pericarp fiber only) and AK4 immo PF+POME (*Trametes hirsuta* AK4 immobilized on PF)

4.3 Two-stage treatment

Due to the high concentration of phenols in treated palm oil mill effluent (POME) and phenols are parts of color as seen in section4.1. Therefore, this study used bacterial treatment to remove phenols before using white rot fungi for color removal. Besides, the previous study showed that the presence of phenolic compounds could inhibit color removal (Hadibarata and Kristanti, 2011). All of the experiments used 100% treated POME and treated by immobilized co-culture bacteria from day 0 to day 7. Then, *Trametes hirsuta* AK4 was applied to the bacterial treated wastewater from day 8 to day 14

4.3.1Two-stage treatment by silica-immobilized bacteria and fungal pellets

The phenols and color removal of two-stage treatment by pre-treatment with silica immobilized co-culture bacteria in 100% treated POME and followed by *Trametes hirsuta* AK4 pellets in bacterial treated wastewater as shown in Figure 4.8a and 4.8b. The initial phenols concentration and color units in wastewater before bacterial treatment were 264 mg/L and 95 color units. The initial phenols concentration and color units in wastewater before fungal treatment were 164 mg/L and 57 color units. The silica immobilized co-culture bacteria could remove phenols from 264 mg/L to 164 mg/L or 38% of phenols removal. When the *Trametes hirsuta* AK4 pellets were applied to the bacterial treated wastewater, it removed phenols from 164 mg/L to 119 mg/L or 27% of phenols removal. For color removal, silica immobilized co-culture bacteria in 100% treated POME could remove color from 95 to 57 color units or 40% of color removal.

When the *Trametes hirsuta* AK4 pellets were applied to the bacterial treated wastewater, it removed color from 57 to 43 color units or 25% of color removal.

In conclusion, total phenols removal from 100% treated POME were 55% from the beginning to the end of two-stage treatment. For color removal, there were 55% of total color removal from the beginning to the end of two-stage treatment. When compared between activity test and two-stage treatment, two-stage treatment had higher color removal efficiency than activity test. White rot fungal pellets could not remove color in 100% treated POME because they had lower activity when phenols were still in system. In the two-stage treatment, phenols were removed before fungal treatement, so the toxicity of phenols were low and not damage the fungi.

Two-stage systems similar to this study have been conducted. For example, Martinez-Garcia *et al.* (2007) developed a two-stage system that consisted of aerobic pre-treatment by using *Candida tropicalis* for phenolic degradation prior to anaerobic digestion of methane production. The removal of phenol is necessary because it inhibit methanogenesis. By removing the inhibitory factors in the first treatment, the researchers can increase the efficiency of second treatment. Similarly, this study aimed to remove phenols that are toxic to fungi and also cause the color. The bacterial treatment with silica immobilized bacteria and followed by white rot fungal pellets could decrease partially phenols concentration and phenols did not toxic to white rot fungal pellets. Thereby, white rot fungal pellets had more color efficiency.

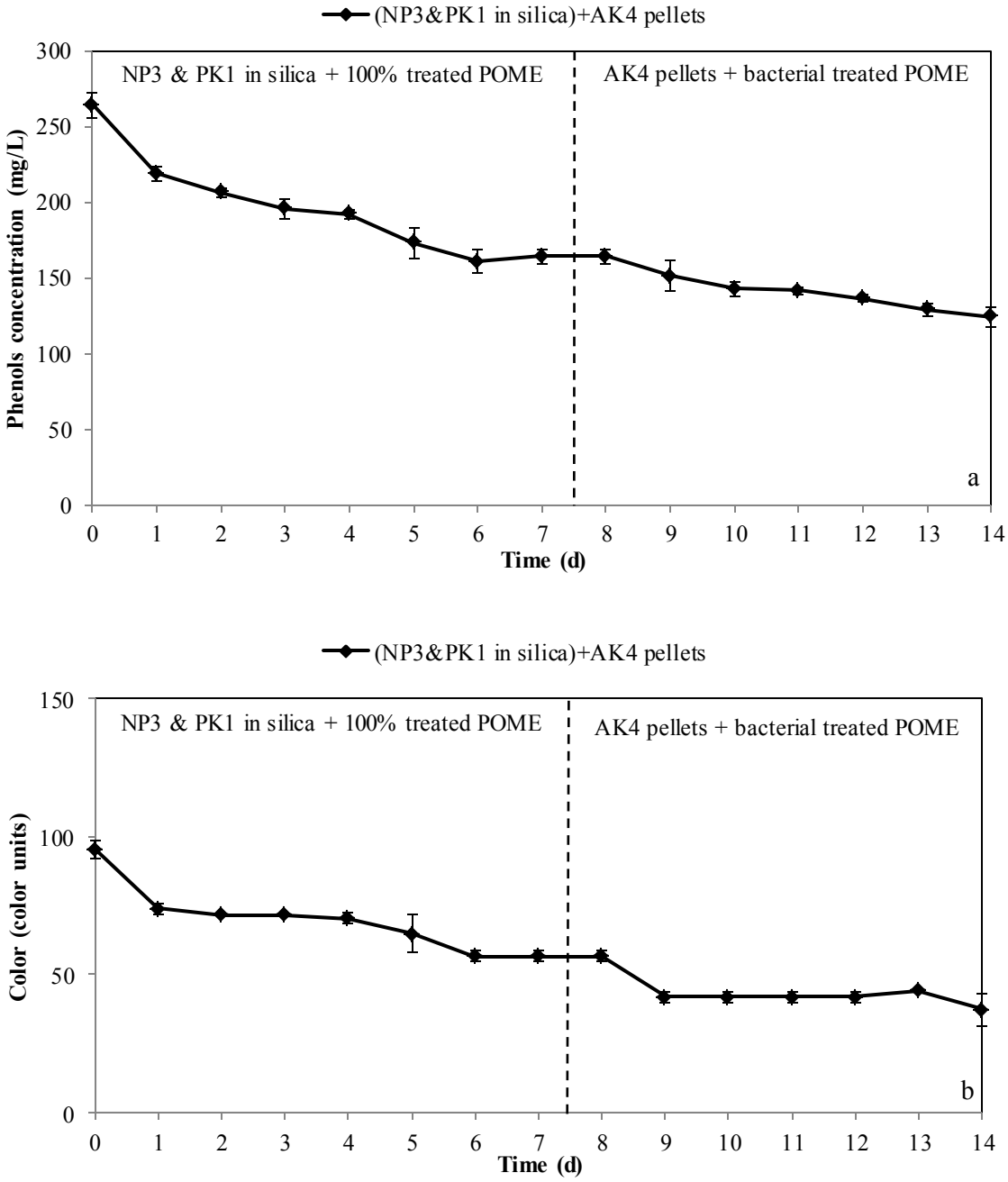


Figure 4. 8 Changes intotal phenols concentration (a) and color units (b) of two-stage treatment by silica immobilized co-culture bacteria and *Trametes hirsuta* AK4 pelletswithin 14 days.

4.3.2Two-stage treatment by silica-immobilized bacteria and palm pericarp fiber-immobilized fungi

Figure 4.9 showed the phenols (a) and color removal (b) of two-stage treatment consisting of silica immobilized co-culture bacteria and palm pericarp fiber immobilized *Trametes hirsuta* AK4. The initial phenols concentration and color units were 121 mg/L and 85 color units in wastewater before bacterial treatment. The initial phenols concentration and color units were 58 mg/L and 73 color units in wastewater before fungal treatment. For phenols removal, silica immobilized co-culture bacteria could remove phenols from 121 mg/L to 58 mg/L or 52% of phenols removal. *Trametes hirsuta* AK4 immobilized on palm pericarp fiber were applied to the bacterial treated wastewater and found that it slightly increased phenols concentration in the wastewater.

For color removal, silica immobilized co-culture bacteria in 100% treated POME could remove color from 85 color units to 73 color units or 14% of color removal. Bacterial treated wastewater was applied by *Trametes hirsuta* AK4 immobilzied on palm pericarp fiber and found that it hardly increased color in the wastewater.

The color removal efficiency of palm pericarp fiber immobilized white rot fungus in the treated POME without bacterial treatment (Figure 4.7) was higher than in two-stage treatment. This was probably because there was lower nutrients in the bacterial treated POME. Moreover, the mass transfer of nutrient of immobilized cells was difficult than free cells. It concluded that low nutrient and difficult mass transfer of immobilized fungus were led to low color removal efficiency.So, the addition of nutrients in bacterial treated POME might be used for incresing the efficiency of fungal treatment.

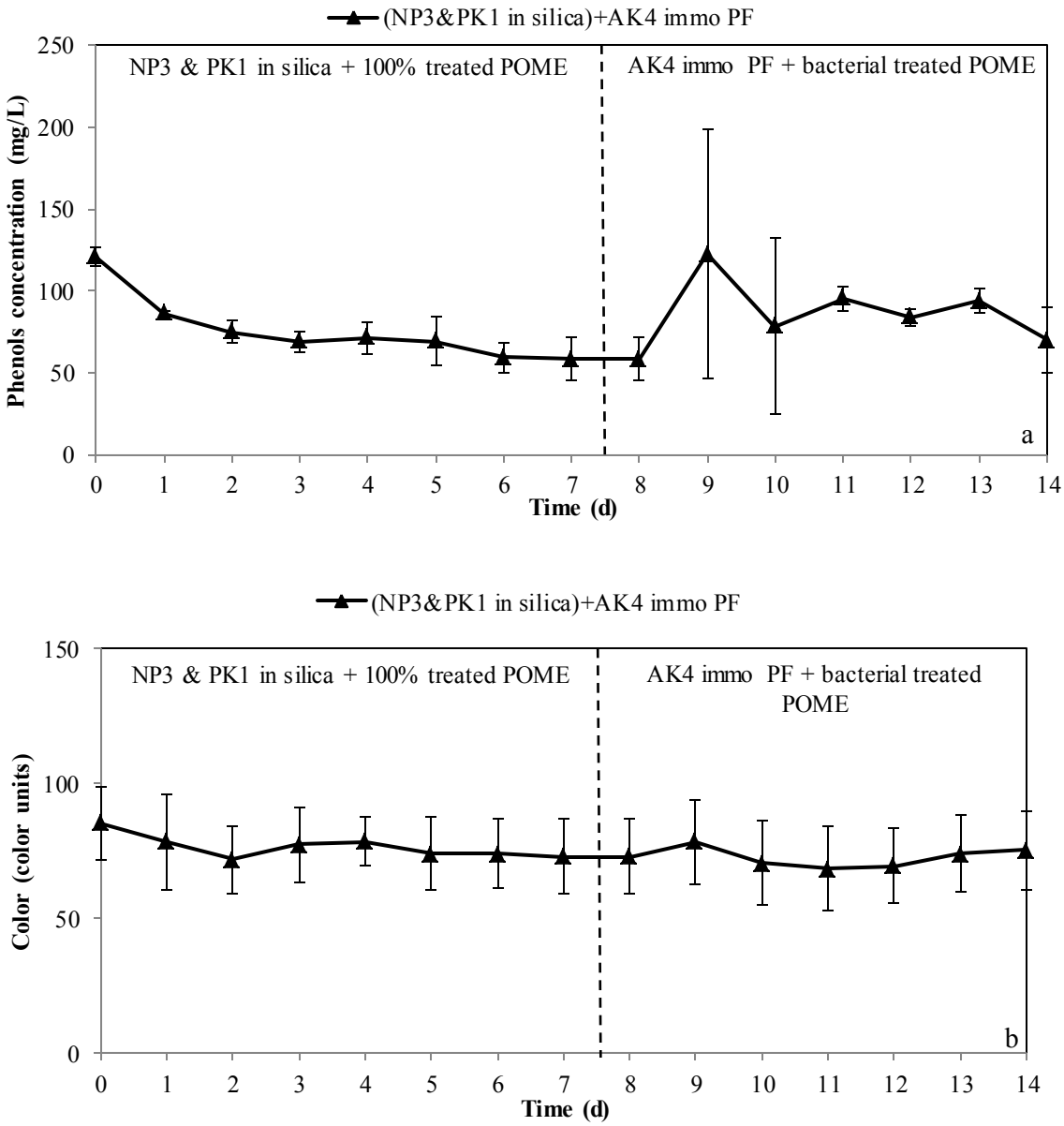


Figure 4. 9 Changes intotal phenols concentration (a) and color units (b) of two-stage treatment by silica immobilized co-culture bacteria and palm pericarp fiber immobilized *Trametes hirsuta* AK4in within 14 days.

4.3.3 Two-stage treatment by recycled plastic-immobilized bacteria and palm pericarp fiber-immobilized fungi

Figure 4.10 showed the phenols (a) and color removal (b) of two-stage treatment consisting of recycled plastic immobilized co-culture bacteria and palm pericarp fiber immobilized *Trametes hirsuta* AK4. The initial phenols concentration and color units were 129 mg/L and 96 color units in wastewater before bacterial treatment. The initial phenols concentration and color units were 87 mg/L and 95 color units in wastewater before fungal treatment. For phenols removal, recycled plaatic immobilized co-culture bacteria could remove phenols from 129 mg/L to 87 mg/L or 33% of phenols removal. *Trametes hirsuta* AK4 immobilized on palm pericarp fiber were applied to the bacterial treated wastewater and found that it increased phenols concentration in the wastewater.

For color removal, recycled plastic immobilized co-culture bacteria in 100% treated POME could remove color from 96 color units to 95 color units or 1% of color removal. Bacterial treated wastewater was applied by *Trametes hirsuta* AK4 immobilzied on palm pericarp fiber could remove color from 95 color units to 77 color units or 19% color removal .

When compared to 4.3.2, this experiment had higher color removal efficiency. In addition, the efficiency of palm pericarp fiber immobilized white rot fungus in this two-stage treatment was similar to the system without bacterial treatment (Figure 4.7). This was probably because the recycled plastic immobilized bacteria had lower efficiency than silica immobilized bacteria. Consequently, the treated POME from recycled plastic

immobilized bacteria had more nutrients, which could support the activities of immobilized fungus.

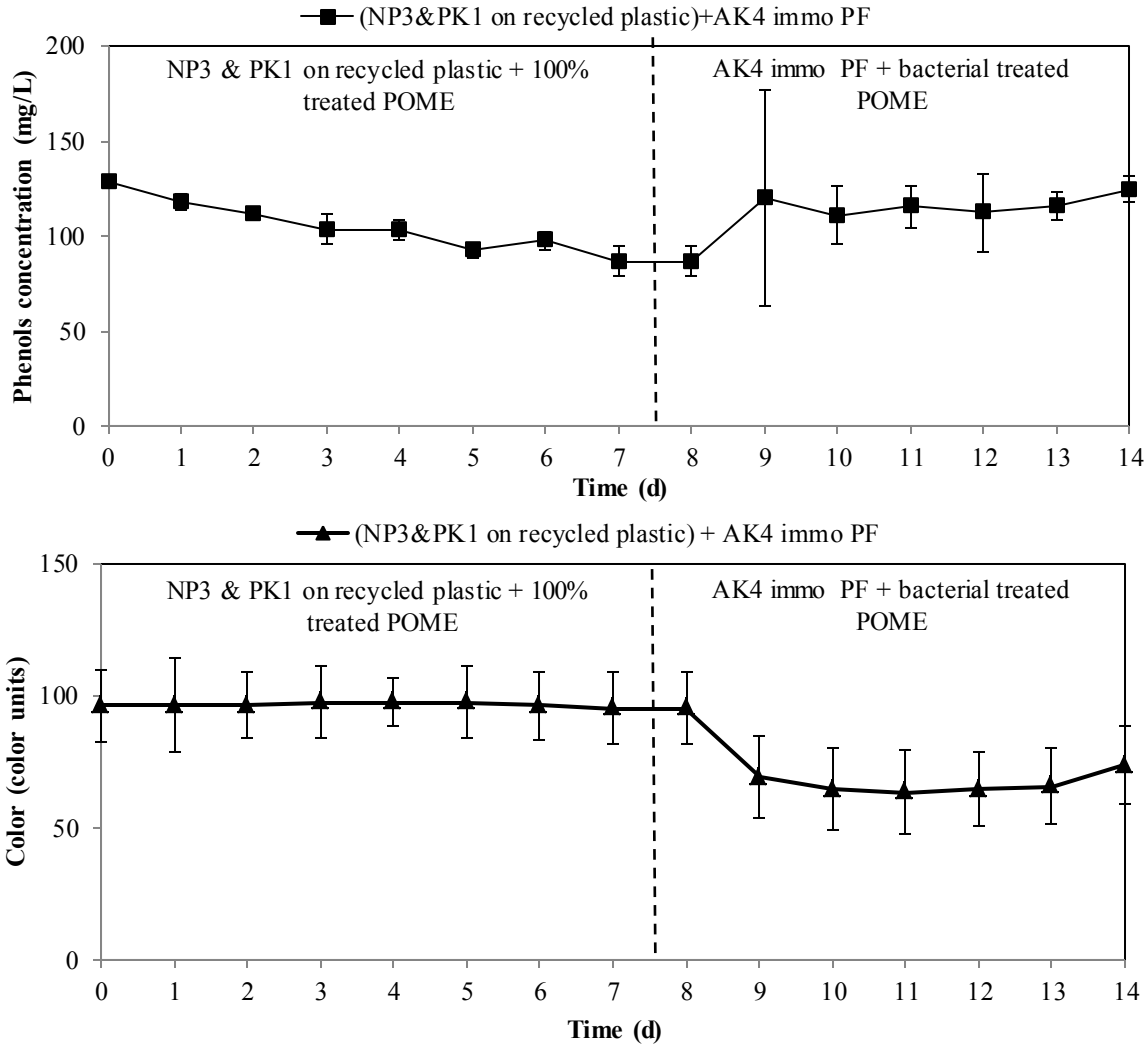


Figure 4. 10 Changes in total phenols concentration (a) and color units (b) of two-stage treatment of recycled plastic immobilized bacteria and *Trametes hirsuta* AK4 immobilized on palm pericarp fiber within 14 days

Three experiments of two-stage treatment were investigated; it concluded that silica-immobilized the co-culture bacteria and followed by *Trametes hirsuta* AK4 pellets were the suitable for using two-stage treatment. Moreover, *Trametes hirsuta* AK4 immobilized on palm pericarp fiber showed high color removal efficiency even though phenols still in the treated POME as shown in Figure 4.11. The color appearance was changed from dark-brown color to light-yellow color within 14 days.

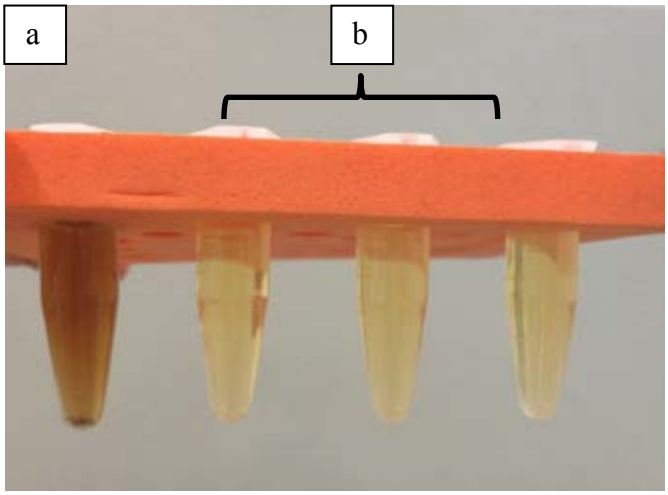


Figure 4. 11 Color removal of before treatment (a) and after treatment for 14 days (b) by recycled plastic immobilized co-culture bacteria and palm pericarp fiber immobilized *Trametes hirsuta* AK4 in the two-stage treatment

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Wastewater samplein this study wascollectedfrom the last stabilization pond of a palm oil mill in Surat Thani province. Phenols and color are concerned because phenols are toxic to living organisms and color is disgusting. There are physical and chemical treatment for removing phenols and color, for example, adsorption, coagulation-flocculation oxidation and electrochemical method. However, these treatments are high cost and injurious harmful to the environment. Therefore, biological treatment was used because it is cheap and safe to living organisms.

Consequently, the aims of this study were to remove phenols and color by using immobilized bacteria and white rot fungus. The study compared the activity of bacterial or fungal treatment and then two-stage of sequential bacterial and fungal treatment was conducted. Initially, two materials including silica- and recycled plastic granules were used for immobilizing co-culture of *Methylobacterium* sp. NP3 and *Acinetobacter* sp. PK1 bacteria. These bacteria had high ability to remove phenols from previous research. The resulted showed that silica-immobilized co-culture bacteria had higher phenol removal efficiency than recycledplastic-immobilized co-culture bacteria within 7 days. However, silica-immobilized co-culture bacteria removed color gradually as same as recycled plastic-immobilized co-culture bacteria.

For fungal treatment, *Trametes hirsuta* AK4 was used in this study because it had high ability for color removal. Firstly, *Trametes hirsuta* AK4 was prepared as pellets and varied the suitable amount of pellets from 50 mg/L to 300 mg/L. The result showed that 250 mg/L of*Trametes hirsuta* AK4 pellets could remove color up to 82% within 2 days in 25% treated POME. After that, 250 mg/L of pellets were applied to determine their efficiency in undiluted and diluted treated POME, the result showed that they could remove color in 25% treated POME better than 100% treated POME. So, *Trametes hirsuta* AK4 pellets were acclimatized in a series of 25%, 50%, 75% and 100% treated POME, the result showed the acclimatization did not work because*Trametes hirsuta* AK4 pellets could remove color well only in 25% treated POME. Moreover, when using white rot fungal pellets more than 7 days, the pellets broken and color was released back to the wastewater. Thereby, immobilization was chosen to improve the efficiency of *Trametes hirsuta* AK4. Palm pericarp fiber is the one of palm oil residues that contains some nutrients. So, it can be used as immobilized material. *Trametes hirsuta* AK4 immobilized on palm pericarp fiber could remove color up to 55% within 4 days but it did not remove phenols. On the contrary, they slightly increased the phenols concentration in treated POME.

Since phenols were toxic to white rot fungi and also caused the color. Two-stage treatment was chosen to improve the process efficiency. Two-stage treatment in this study was using bacterial treatment for phenols removal and followed by white rot fungal treatment for color removal. There were 3 experiments consisted of silica immobilized the co-culture bacteria and *Trametes hirsuta* AK4 pellets, silica immobilized the co-

culture bacteria and *Trametes hirsuta* AK4 immobilized on palm pericarp fiber and recycled plastic immobilized the co-culture bacteria and *Trametes hirsuta* AK4 immobilized on palm pericarp fiber. The results showed that a two-stage treatment of silica-immobilized the co-culture bacteria and followed by *Trametes hirsuta* AK4 pellets gave the highest phenol and color removal efficiency. The color appearance was changed from dark-brown color to light-yellow color within 14 days. Although*Trametes hirsuta* AK4 immobilized on palm pericarp fiber had high color removal efficiency, it should not apply after bacterial treatment. This was due to the low nutrient concentrations in bacterial treated wastewater and the limitation of nutrient transfer in immobilized fiber.

In conclusion, the highest phenols and color removal efficiencies of bacteria and fungus were found with silica-immobilized the co-culture bacteria and *Trametes hirsuta* AK4 immobilized on palm pericarp fiber, respectively. For two-stage treatment, the highest phenols and color removal were silica-immobilized bacterial cells and white rot fungal pellets, respectively. Although, phenols concentration in this study did not passed the standard but this study showed the possibility of phenols removal by bacteria. In addition, the dark-brown color in palm oil mill effluent was changed to light-yellow color in the end of treatment using white rot fungus.

Moreover, this study proved that *Methylobacterium* sp. NP3 and *Acinetobacter* sp. PK1 immobilized cells had high ability for phenols removal and *Trametes hirsuta* AK4 had high ability for color removal from treated POME.

5.2 Recommendations

5.2.1 The amounts of bacteria and fungal enzymes in wastewater during treatment should be studied so the treatment could be optimized accordingly.

5.2.2 For further application, a sequential batch bioreactor (SBR) containing silica-immobilized bacteria and fungal pellets might be used for wastewater treatment. A previous study reported that this bioreactor could remove COD, BOD and total suspended solids (TSS) in palm oil mill effluent by using sequencing batch reactor (Chan *et al.,* 2010). SBR is a bioreactor that consists of 5 steps including FILL, REACT, SETTLE, DRAW and IDLE. SBR is easy to control and suitable for environmental treatment. The advantages of this bioreactor are (1) SBR combines idle tank, aeration tank and sedimentation tank in the same tank to reduce the space, (2) the system is able to control the organic loading by the dilution of treated wastewater and (3) system can be changed to suit the operating cycle, (6) the growth of fungi can be controlled when FILL step (Subramaniam *et al.,* 1994; Yu *et al.,* 1996; Sombatsompob, 2008).

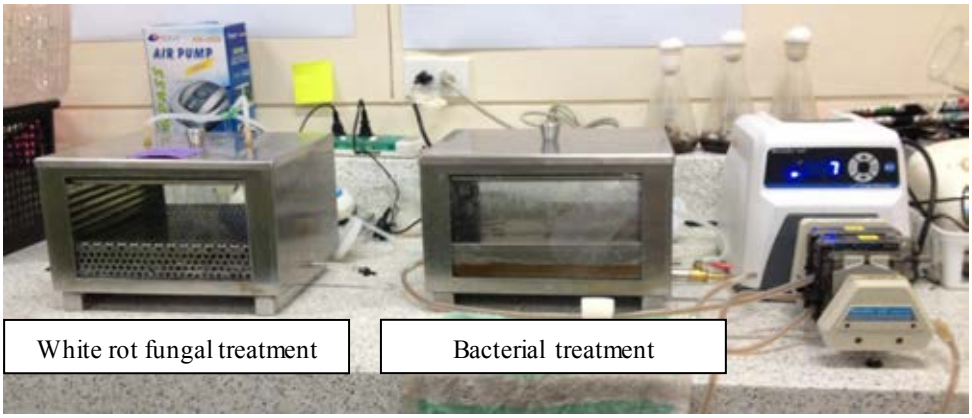


Figure 5. 1 Example oftwo-stage treatment that consisted of batch bioreactors of immobilized bacteriaand white rot fungi

5.2.3 Alcian Blue adsorption assay should be studied to measure the amount of exopolysaccharide during recycled plastic immobilization. The knowledge could be used to find the optimum bacterial attachment period.

5.2.4 Ligninolytic enzymes assay should be analyzed to measure the ligninolytic enzymes that released from white rot fungi for assuring the color removal.

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APPENDICES

APPENDIX A

Media preparation

1. Carbon Free Mineral Medium (CFMM)

Solution A.(1 L.)

-	NH_4NO_3	3.0	g
-	Na_2HPO_4	2.2	g
-	KH_2PO_4	0.8	g

Solution B. (1 mL)

-	$MgSO_4 \cdot 7H_2O$	1.0	g
-	$FeCl_3 \cdot 6H_2O$	0.5	g
-	$CaCl_2 \cdot 2H_2O$	0.5	g

Solution A was sterilized by autoclavingwith pressure 15 lb/inch^2 at 121 °C for 15 minutes and added solution B that was filter through cellulose acetate filter paper pore size 0.45 μm.

2. Potato Dextrose Agar (PDA) 1 L.

-	Potato Starch (from infusion)	4.0	g
-	Dextrose	20.0	g
-	Agar	15.0	g

Suspended 39 g of PDA to distilled water 1,000 mL and sterilized by autoclaving with pressure 15 lb/inch^2 at 121 °C for 15 minutes.

3. Glucose Yeast Extract broth (GYEB) 1 L.

-	Glucose	100	g
-	Yeast Extract	10	g

Suspended glucose and yeast extract in 1,000 mL of distilled water and sterilized by autoclaving with pressure 15 lb/inch^2 at 121 °C for 15 minutes.

APPENDIX B

The standard curved was plotted between absorbance and phenols concentration which is gallic acid.

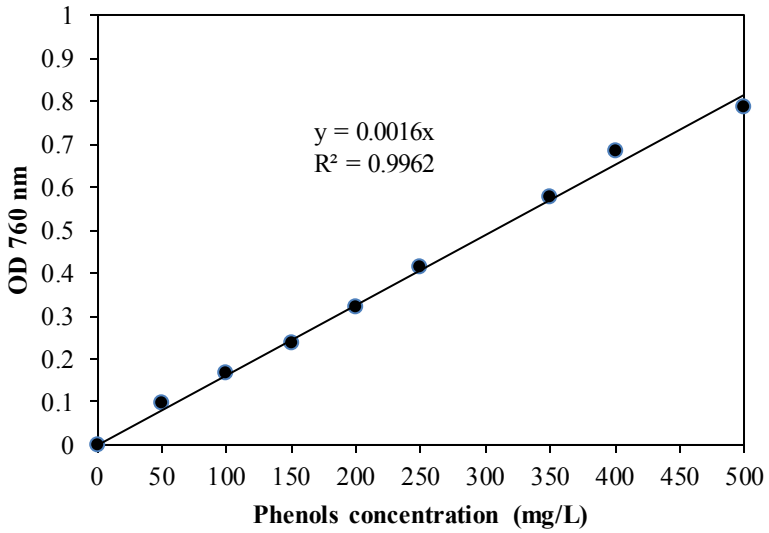

Figure B- 1 Standard curve of absorbance (λ_{760})of varied gallic acid concentration that was diluted 2 times.

2. The standard curved of color units was plotted between absorbance and color units

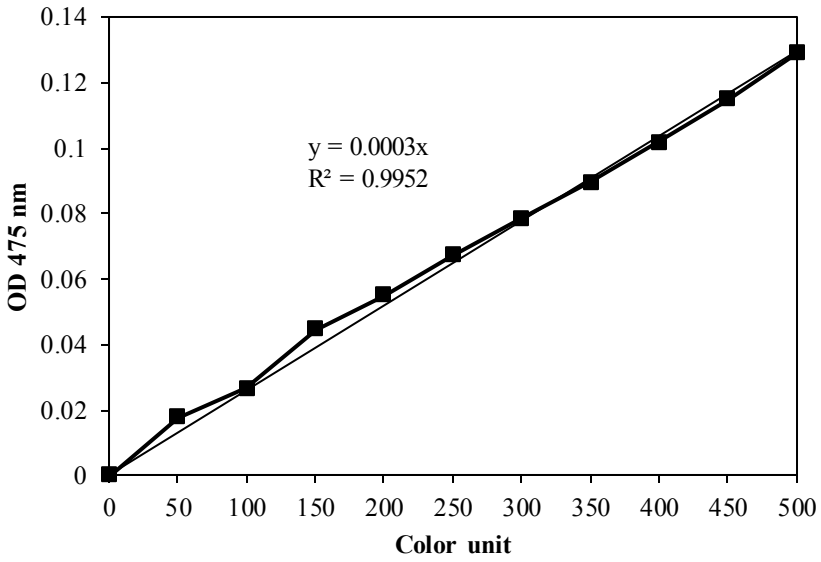

Figure B- 2 Standard curve of absorbance (λ_{475})of varied color concentration 0-500 color units.

APPENDIX C

Table C- 2 The study of the suitable day for recycled plastic immobilization (Log CFU/g of recycled plastic)

Day		1	2	3	Average	SD	Average	SD
0	1	7.039	7.059	7.112	7.070	0.038		
	2	6.697	6.697	7.174	6.856	0.275	6.963	0.151
1	1	6.980	6.980	7.059	7.007	0.046		
	2	7.105	7.105	7.503	7.238	0.230		
	3	8.406	8.503	8.503	8.471	0.056		
	4	8.785	8.672	8.672	8.709	0.066	7.856	0.858
4	1	8.814	8.981	9.046	8.947	0.120		
	2	9.060	9.185	9.185	9.144	0.072		
	3	10.627	10.706	10.773	10.702	0.073	9.598	0.962
7	1	8.535	8.593	8.690	8.606	0.078		
	2	8.690	8.690	8.991	8.791	0.174	8.699	0.130
8	1	7.775	8.252	8.252	8.093	0.275	8.090	0.000
10	1	5.618	5.810	5.840	5.756	0.120		
	2	6.563	6.513	6.747	6.608	0.123		
	3	6.668	6.785	6.664	6.705	0.069		
	4	7.664	8.141	8.266	8.023	0.318	6.773	0.936

1. Activity Test

1.1 The co-culture bacteria immobilized in silicaand on recycled plastic in batch experiment

Table C- 3 Phenols concentration of 100% POME only in January 2013 (control)

Time (days)	λ_{760}			Phenols concentration (mg/L)			Average	SD
	1	2	3	1	2	3		
0	0.145	0.154	0.128	59.184	62.857	52.245	58.095	5.389
1	0.202	0.146	0.128	82.449	59.592	52.245	64.762	15.752
2	0.226	0.189	0.187	92.245	77.143	76.327	81.905	8.964
3	0.233	0.144	0.175	95.102	58.776	71.429	75.102	18.440
4	0.183	0.164	0.161	74.694	66.939	65.714	69.116	4.870
5	0.142	0.144	0.149	57.959	58.776	60.816	59.184	1.472
6	0.156	0.140	0.146	63.673	57.143	59.592	60.136	3.299
7	0.143	0.100	0.155	58.367	40.816	63.265	54.150	11.804

Table C- 4 Phenols concentrationof cell-free silica in 100% POME (control)

Time (days)	λ_{760}			Phenols concentration (mg/L)			Average	SD
	1	2	3	1	2	3		
0	0.593	0.614	0.616	242.041	250.612	251.429	248.027	5.200
1	0.480	0.477	0.488	195.918	194.694	199.184	196.599	2.321
2	0.437	0.467	0.473	178.367	190.612	193.061	187.347	7.872
3	0.479	0.463	0.462	195.510	188.980	188.571	191.020	3.894
4	0.413	0.428	0.450	168.571	174.694	183.673	175.646	7.596
5	0.434	0.434	0.442	177.143	177.143	180.408	178.231	1.885
6	0.448	0.435	0.439	182.857	177.551	179.184	179.864	2.718
7	0.452	0.459	0.457	184.490	187.347	186.531	186.122	1.472

Table C- 5 Phenols concentrationof cell-free recycled plastic in 100% POME (control)

Time (days)	λ_{760}			Phenols concentration (mg/L)			Average	SD
	1	2	3	1	2	3		
0	0.387	0.389	0.393	157.959	158.776	160.408	159.048	1.247
1	0.346	0.341	0.336	141.224	139.184	137.143	139.184	2.041
2	0.330	0.333	0.331	134.694	135.918	135.102	135.238	0.623
3	0.320	0.336	0.322	130.612	137.143	131.429	133.061	3.558
4	0.326	0.351	0.340	133.061	143.265	138.776	138.367	5.114
5	0.285	0.285	0.284	116.327	116.327	115.918	116.190	0.236
6	0.273	0.283	0.281	111.429	115.510	114.694	113.878	2.160
7	0.269	0.265	0.266	109.796	108.163	108.571	108.844	0.850

Table C- 6 Phenols concentration of silica-immobilized cells in 100% POME

Time (days)	λ_{760}			Phenols concentration (mg/L)			Average	SD
	1	2	3	1	2	3		
0	0.624	0.653	0.665	254.694	266.531	271.429	264.218	8.604
1	0.525	0.537	0.548	214.286	219.184	223.673	219.048	4.695
2	0.504	0.499	0.514	205.714	203.673	209.796	206.395	3.117
3	0.463	0.479	0.496	188.980	195.510	202.449	195.646	6.736
4	0.462	0.470	0.479	188.571	191.837	195.510	191.973	3.471
5	0.447	0.429	0.397	182.449	175.102	162.041	173.197	10.337
6	0.372	0.405	0.405	151.837	165.306	165.306	160.816	7.777
7	0.397	0.395	0.415	162.041	161.224	169.388	164.218	4.496

Table C- 7 Phenols concentration of recycled plastic-immobilized cells in 100% POME

Time (days)	λ_{760}			Phenols concentration (mg/L)			Average	SD
	1	2	3	1	2	3		
0	0.312	0.317	0.318	127.347	129.388	129.796	128.844	1.312
1	0.297	0.294	0.276	121.224	120.000	112.653	117.959	4.636
2	0.270	0.282	0.269	110.204	115.102	109.796	111.701	2.953
3	0.258	0.271	0.233	105.306	110.612	95.102	103.673	7.883
4	0.239	0.258	0.262	97.551	105.306	106.939	103.265	5.016
5	0.230	0.234	0.217	93.878	95.510	88.571	92.653	3.628
6	0.230	0.253	0.235	93.878	103.265	95.918	97.687	4.937
7	0.192	0.216	0.231	78.367	88.163	94.286	86.939	8.030

Table C- 8 Color units of 100% POME only in January 2013 (control)

Time (days)	λ_{475}			Color units			Average	SD
	1	2	3	1	2	3		
0	0.027	0.025	0.011	91.837	85.034	37.415	71.429	29.652
1	0.027	0.026	0.011	91.837	88.435	37.415	72.562	30.486
2	0.031	0.029	0.015	105.442	98.639	51.020	85.034	29.652
3	0.031	0.031	0.015	105.442	105.442	51.020	87.302	31.420
4	0.030	0.027	0.016	102.041	91.837	54.422	82.766	25.072
5	0.029	0.029	0.014	98.639	98.639	47.619	81.633	29.457
6	0.032	0.028	0.015	108.844	95.238	51.020	85.034	30.232
7	0.034	0.028	0.016	115.646	95.238	54.422	88.435	31.174

Table C- 9Color units of cell-free silica in 100% POME (control)

Time (days)	λ_{475}			Color units			Average	SD
	1	2	3	1	2	3		
0	0.029	0.027	0.027	98.639	91.837	91.837	94.104	3.928
1	0.020	0.017	0.019	68.027	57.823	64.626	63.492	5.196
2	0.019	0.018	0.018	64.626	61.224	61.224	62.358	1.964
3	0.019	0.020	0.017	64.626	68.027	57.823	63.492	5.196
4	0.016	0.016	0.015	54.422	54.422	51.020	53.288	1.964
5	0.016	0.016	0.016	54.422	54.422	54.422	54.422	0.000
6	0.016	0.016	0.017	54.422	54.422	57.823	55.556	1.964
7	0.017	0.017	0.016	57.823	57.823	54.422	56.689	1.964

Table C- 10 Color unitsof cell-free recycled plastic in 100% POME (control)

Time (days)	λ_{475}			Color units			Average	SD
	1	2	3	1	2	3		
0	0.034	0.032	0.032	115.646	108.844	108.844	111.111	3.928
1	0.032	0.030	0.026	108.844	102.041	88.435	99.773	10.391
2	0.034	0.030	0.031	115.646	102.041	105.442	107.710	7.080
3	0.042	0.033	0.030	142.857	112.245	102.041	119.048	21.241
4	0.043	0.038	0.036	146.259	129.252	122.449	132.653	12.264
5	0.031	0.030	0.024	105.442	102.041	81.633	96.372	12.877
6	0.035	0.029	0.023	119.048	98.639	78.231	98.639	20.408
7	0.033	0.029	0.023	112.245	98.639	78.231	96.372	17.120

Table C- 11 Color unitsof silica-immobilized cells in 100% POME

Time (days)	λ_{475}			Color units			Average	SD
	1	2	3	1	2	3		
0	0.029	0.028	0.027	98.639	95.238	91.837	95.238	3.401
1	0.021	0.022	0.022	71.429	74.830	74.830	73.696	1.964
2	0.021	0.021	0.021	71.429	71.429	71.429	71.429	0.000
3	0.021	0.021	0.021	71.429	71.429	71.429	71.429	0.000
4	0.020	0.021	0.021	68.027	71.429	71.429	70.295	1.964
5	0.021	0.019	0.017	71.429	64.626	57.823	64.626	6.803
6	0.016	0.017	0.017	54.422	57.823	57.823	56.689	1.964
7	0.017	0.017	0.016	57.823	57.823	54.422	56.689	1.964

Table C- 12 Color unitsof recycled plastic-immobilized cells in 100% POME

Time (days)	λ_{475}			Color units			Average	SD
	1	2	3	1	2	3		
0	0.033	0.027	0.025	112.245	91.837	85.034	96.372	14.161
1	0.033	0.027	0.025	112.245	91.837	85.034	96.372	14.161
2	0.031	0.030	0.024	105.442	102.041	81.633	96.372	12.877
3	0.032	0.028	0.026	108.844	95.238	88.435	97.506	10.391
4	0.031	0.030	0.025	105.442	102.041	85.034	97.506	10.934
5	0.031	0.030	0.025	105.442	102.041	85.034	97.506	10.934
6	0.031	0.029	0.025	105.442	98.639	85.034	96.372	10.391
7	0.032	0.028	0.024	108.844	95.238	81.633	95.238	13.605

1.2*Trametes hirsuta* AK4 pellets treatment in varying the amount of white rot fungal pellets in batch experiment

Table C- 13 Phenols concentration of 25% POME (control)

Time (days)	λ_{760}			Phenols concentration (mg/L)			Average	SD
	1	2	3	1	2	3		
0	0.213	0.237	0.246	86.939	96.735	100.408	94.694	6.963
1	0.213	0.237	0.246	86.939	96.735	100.408	94.694	6.963
2	0.213	0.237	0.246	86.939	96.735	100.408	94.694	6.963
3	0.213	0.237	0.246	86.939	96.735	100.408	94.694	6.963

Table C- 14 Phenols concentration of killed pellets in 25% POME (control)

Time (days)	λ_{760}			Phenols concentration (mg/L)			Average	SD
	1	2	3	1	2	3		
0	0.258	0.290	0.347	105.306	118.367	141.633	121.769	18.401
1	0.258	0.290	0.347	105.306	118.367	141.633	121.769	18.401
2	0.227	0.271	0.286	92.653	110.612	116.735	106.667	12.516
3	0.222	0.250	0.277	90.612	102.041	113.061	101.905	11.225

Table C- 15 Phenols concentration of *Trametes hirsuta* AK4 pellets 25% POME (treatment)

Time (days)	g.	λ_{760}			Phenols concentration (mg/L)			Average	SD
		1	2	3	1	2	3		
0		0.213	0.237	0.246	86.939	96.735	100.408	94.694	6.963
1	0.5	0.212	0.203	0.224	86.531	82.857	91.429	86.939	4.300
	1.0	0.184	0.171	0.179	75.102	69.796	73.061	72.653	2.677
	1.5	0.200	0.207	0.231	81.633	84.490	94.286	86.803	6.636
	2.0	0.293	0.265	0.249	119.592	108.163	101.633	109.796	9.090
	2.5	0.263	0.271	0.260	107.347	110.612	106.122	108.027	2.321
	3.0	0.328	0.401	0.351	133.878	163.673	143.265	146.939	15.234
2	0.5	0.222	0.224	0.246	90.612	91.429	100.408	94.150	5.435
	1.0	0.228	0.228	0.229	93.061	93.061	93.469	93.197	0.236
	1.5	0.212	0.272	0.234	86.531	111.020	95.510	97.687	12.389
	2.0	0.241	0.266	0.228	98.367	108.571	93.061	100.000	7.883
	2.5	0.258	0.247	0.309	105.306	100.816	126.122	110.748	13.502
	3.0	0.268	0.229	0.279	109.388	93.469	113.878	105.578	10.724
3	0.5	0.217	0.204	0.170	88.571	83.265	69.388	80.408	9.906
	1.0	0.202	0.238	0.202	82.449	97.143	82.449	87.347	8.484
	1.5	0.216	0.236	0.243	88.163	96.327	99.184	94.558	5.719
	2.0	0.235	0.269	0.243	95.918	109.796	99.184	101.633	7.256
	2.5	0.237	0.276	0.284	96.735	112.653	115.918	108.435	10.264
	3.0	0.276	0.258	0.279	112.653	105.306	113.878	110.612	4.636

Table C- 16 Color units of 25% POME (control)

Time (days)	λ_{475}			Color units			Average	SD
	1	2	3	1	2	3		
0	0.017	0.016	0.016	57.823	54.422	54.422	55.556	1.964
1	0.017	0.016	0.016	57.823	54.422	54.422	55.556	1.964
2	0.016	0.017	0.016	54.422	57.823	54.422	55.556	1.964
3	0.016	0.016	0.017	54.422	54.422	57.823	55.556	1.964

Table C- 17 Color units of killed *Trametes hirsuta* AK4 pellets in 25% POME (control)

Time (days)	λ_{475}			Color units			Average	SD
	1	2	3	1	2	3		
0	0.012	0.012	0.010	57.823	54.422	54.422	55.556	1.964
1	0.010	0.010	0.010	57.823	54.422	54.422	55.556	1.964
2	0.008	0.009	0.009	27.211	30.612	30.612	29.478	1.964
3	0.010	0.009	0.009	34.014	30.612	30.612	31.746	1.964

Table C- 18 Color units of *Trametes hirsuta* AK4 pellets in 25% POME (Treatment)

Time (days)	g.	λ_{475}			Color units			Average	SD
		1	2	3	1	2	3		
0		0.017	0.016	0.016	57.823	54.422	54.422	55.556	1.964
1	0.5	0.016	0.015	0.015	54.422	51.020	51.020	52.154	1.964
	1.0	0.016	0.014	0.014	54.422	47.619	47.619	49.887	3.928
	1.5	0.014	0.015	0.013	47.619	51.020	44.218	47.619	3.401
	2.0	0.015	0.014	0.013	51.020	47.619	44.218	47.619	3.401
	2.5	0.013	0.012	0.012	44.218	40.816	40.816	41.950	1.964
	3.0	0.013	0.013	0.013	44.218	44.218	44.218	44.218	0.000
2	0.5	0.007	0.009	0.008	23.810	30.612	27.211	27.211	3.401
	1.0	0.011	0.01	0.009	37.415	34.014	30.612	34.014	3.401
	1.5	0.01	0.01	0.01	34.014	34.014	34.014	34.014	0.000
	2.0	0.004	0.003	0.003	13.605	10.204	10.204	11.338	1.964
	2.5	0.004	0.003	0.002	13.605	10.204	6.803	10.204	3.401
	3.0	0.003	0.003	0.004	10.204	10.204	13.605	11.338	1.964
3	0.5	0.005	0.005	0.004	17.007	17.007	13.605	15.873	1.964
	1.0	0.004	0.004	0.004	13.605	13.605	13.605	13.605	0.000
	1.5	0.003	0.005	0.005	10.204	17.007	17.007	14.739	3.928
	2.0	0.005	0.004	0.004	17.007	13.605	13.605	14.739	1.964
	2.5	0.005	0.003	0.005	17.007	10.204	17.007	14.739	3.928
	3.0	0.005	0.005	0.005	17.007	17.007	17.007	17.007	0.000

1.3*Trametes hirsuta* AK4 pellets treatment in 250g/L of *Trametes hirsuta* AK4 pellets in diluted and undiluted treated POME in batch experiment

Table C- 19 Color units of 25% POME (control)

Time (days)	λ_{475}			Color units			Average	SD
	1	2	3	1	2	3		
0	0.017	0.016	0.016	57.823	54.422	54.422	55.556	1.964
1	0.017	0.016	0.016	57.823	54.422	54.422	55.556	1.964
2	0.016	0.017	0.016	57.823	54.422	54.422	55.556	1.964
3	0.016	0.016	0.017	57.823	54.422	54.422	55.556	1.964

Table C- 20 Color units of killed *Trametes hirsuta* AK4 pellets in 25% POME (control)

Time (days)	λ_{475}			Color units			Average	SD
	1	2	3	1	2	3		
0	0.012	0.012	0.010	40.816	40.816	34.014	38.549	3.928
1	0.010	0.010	0.010	34.014	34.014	34.014	34.014	0.000
2	0.008	0.009	0.009	27.211	30.612	30.612	29.478	1.964
3	0.010	0.009	0.009	34.014	30.612	30.612	31.746	1.964

Table C- 21 Color units of 250g/L *Trametes hirsuta* AK4 pellets in 25% POME (Treatment)

Time (days)	λ_{475}			Color units			Average	SD
	1	2	3	1	2	3		
0	0.017	0.016	0.016	57.823	54.422	54.422	55.556	1.964
1	0.013	0.012	0.012	44.218	40.816	40.816	41.950	1.964
2	0.004	0.003	0.002	13.605	10.204	6.803	10.204	3.401
3	0.005	0.003	0.005	17.007	10.204	17.007	14.739	3.928

Table C- 22 Color units of 100% POME (control)

Time (days)	λ_{475}			Color units			Average	SD
	1	2	3	1	2	3		
0	0.034	0.033	0.034	115.646	112.245	115.646	114.512	1.964
1	0.034	0.033	0.034	115.646	112.245	115.646	114.512	1.964
2	0.034	0.034	0.033	115.646	115.646	112.245	114.512	1.964
3	0.033	0.034	0.034	112.245	115.646	115.646	114.512	1.964

Table C- 23 Color units of 250g/L *Trametes hirsuta* AK4 pellets in 100% POME (Treatment)

Time (days)	λ_{475}			Color units			Average	SD
	1	2	3	1	2	3		
0	0.034	0.034	0.033	115.646	115.646	112.245	114.512	1.964
1	0.030	0.034	0.032	102.041	115.646	108.844	108.844	6.803
2	0.030	0.033	0.029	102.041	112.245	98.639	104.308	7.080
3	0.025	0.020	0.018	85.034	68.027	61.224	71.429	12.264

1.4Acclimatization of 250g/L *Trametes hirsuta* AK4 pellets treatment in batch experiment

Table C- 24 Phenols concentration of series of treated POME (Control)

POME conc.	Time (days)	λ_{760}			Phenols concentration (mg/L)			Average	SD
		1	2	3	1	2	3		
25%	0	0.213	0.237	0.246	86.939	96.735	100.408	94.694	6.963
	1	0.213	0.237	0.246	86.939	96.735	100.408	94.694	6.963
	2	0.213	0.237	0.246	86.939	96.735	100.408	94.694	6.963
	3	0.213	0.237	0.246	86.939	96.735	100.408	94.694	6.963
50%	0	0.391	0.398	0.394	159.592	162.449	160.816	160.952	1.433
	1	0.391	0.398	0.394	159.592	162.449	160.816	160.952	1.433
	2	0.391	0.398	0.394	159.592	162.449	160.816	160.952	1.433
	3	0.391	0.398	0.394	159.592	162.449	160.816	160.952	1.433
75%	0	0.535	0.521	0.508	218.367	212.653	207.347	212.789	5.511
	1	0.535	0.521	0.508	218.367	212.653	207.347	212.789	5.511
	2	0.535	0.521	0.508	218.367	212.653	207.347	212.789	5.511
	3	0.535	0.521	0.508	218.367	212.653	207.347	212.789	5.511
100%	0	0.706	0.703	0.746	288.163	286.939	304.490	293.197	9.799
	1	0.706	0.703	0.746	288.163	286.939	304.490	293.197	9.799
	2	0.706	0.703	0.746	288.163	286.939	304.490	293.197	9.799
	3	0.706	0.703	0.746	288.163	286.939	304.490	293.197	9.799

Table C- 25 Phenols concentration of killed *Trametes hirsuta* AK4 pellets (Control)

POME conc.	Time (days)	λ_{760}			Phenols concentration (mg/L)			Average	SD
		1	2	3	1	2	3		
25%	0	0.258	0.290	0.347	105.306	118.367	141.633	121.769	18.401
	1	0.258	0.290	0.347	105.306	118.367	141.633	121.769	18.401
	2	0.227	0.271	0.286	92.653	110.612	116.735	106.667	12.516
	3	0.222	0.250	0.277	90.612	102.041	113.061	101.905	11.225
50%	0	0.350	0.396	0.377	142.857	161.633	153.878	152.789	9.435
	1	0.350	0.396	0.377	142.857	161.633	153.878	152.789	9.435
	2	0.438	0.388	0.393	178.776	158.367	160.408	165.850	11.240
	3	0.472	0.439	0.460	192.653	179.184	187.755	186.531	6.818
75%	0	0.549	0.550	0.553	224.082	224.490	225.714	224.762	0.850
	1	0.549	0.550	0.553	224.082	224.490	225.714	224.762	0.850
	2	0.466	0.423	0.381	190.204	172.653	155.510	172.789	17.347
	3	0.539	0.600	0.597	220.000	244.898	243.673	236.190	14.035

Table C- 26 Phenols concentration of *Trametes hirsuta* AK4 pellets (Treatment)

POME conc.	Time (days)	λ_{760}			Phenols concentration (mg/L)			Average	SD
		1	2	3	1	2	3		
25%	0	0.213	0.237	0.246	86.939	96.735	100.408	94.694	6.963
	1	0.263	0.271	0.260	107.347	110.612	106.122	108.027	2.321
	2	0.258	0.247	0.309	105.306	100.816	126.122	110.748	13.502
	3	0.237	0.276	0.284	96.735	112.653	115.918	108.435	10.264
50%	0	0.391	0.398	0.394	159.592	162.449	160.816	160.952	1.433
	1	0.362	0.391	0.381	147.755	159.592	155.510	154.286	6.013
	2	0.336	0.389	0.373	137.143	158.776	152.245	149.388	11.096
	3	0.369	0.407	0.377	150.612	166.122	153.878	156.871	8.177
75%	0	0.535	0.521	0.508	218.367	212.653	207.347	212.789	5.511
	1	0.502	0.560	0.551	204.898	228.571	224.898	219.456	12.741
	2	0.530	0.587	0.511	216.327	239.592	208.571	221.497	16.144
	3	0.536	-	0.584	218.776	-	238.367	228.571	13.854
100%	0	0.706	0.703	0.746	288.163	286.939	304.490	293.197	9.799
	1	0.665	0.715	0.684	271.429	291.837	279.184	280.816	10.302
	2	0.622	0.609	0.650	253.878	248.571	265.306	255.918	8.552
	3	0.580	0.612	0.632	236.735	249.796	257.959	248.163	10.706

Table C- 27 Color units of series of treated POME (Control)

POME conc.	Time (days)	λ_{475}			Color units			Average	SD
		1	2	3	1	2	3		
25%	0	0.017	0.016	0.016	57.823	54.422	54.422	55.556	1.964
	1	0.017	0.016	0.016	57.823	54.422	54.422	55.556	1.964
	2	0.017	0.016	0.016	57.823	54.422	54.422	55.556	1.964
	3	0.017	0.016	0.016	57.823	54.422	54.422	55.556	1.964
50%	0	0.018	0.018	0.019	61.224	61.224	64.626	62.358	1.964
	1	0.018	0.018	0.019	61.224	61.224	64.626	62.358	1.964
	2	0.018	0.018	0.019	61.224	61.224	64.626	62.358	1.964
	3	0.018	0.018	0.019	61.224	61.224	64.626	62.358	1.964
75%	0	0.024	0.026	0.026	81.633	88.435	88.435	86.168	3.928
	1	0.024	0.026	0.026	81.633	88.435	88.435	86.168	3.928
	2	0.024	0.026	0.026	81.633	88.435	88.435	86.168	3.928
	3	0.024	0.026	0.026	81.633	88.435	88.435	86.168	3.928
100%	0	0.034	0.034	0.033	115.646	115.646	112.245	114.512	1.964
	1	0.034	0.034	0.033	115.646	115.646	112.245	114.512	1.964
	2	0.034	0.034	0.033	115.646	115.646	112.245	114.512	1.964
	3	0.034	0.034	0.033	115.646	115.646	112.245	114.512	1.964

Table C- 28 Color units of killed *Trametes hirsuta* AK4 pellets (Control)

POME conc.	Time (days)	λ_{475}			Color units			Average	SD
		1	2	3	1	2	3		
25%	0	0.017	0.016	0.016	57.823	54.422	54.422	55.556	1.964
	1	0.017	0.016	0.016	57.823	54.422	54.422	55.556	1.964
	2	0.008	0.009	0.009	27.211	30.612	30.612	29.478	1.964
	3	0.010	0.009	0.009	34.014	30.612	30.612	31.746	1.964
50%	0	0.014	0.014	0.014	47.619	47.619	47.619	47.619	0.000
	1	0.014	0.014	0.014	47.619	47.619	47.619	47.619	0.000
	2	0.013	0.014	0.014	44.218	47.619	47.619	46.485	1.964
	3	0.014	0.016	0.017	47.619	54.422	57.823	53.288	5.196
75%	0	0.024	0.023	0.023	81.633	78.231	78.231	79.365	1.964
	1	0.024	0.023	0.023	81.633	78.231	78.231	79.365	1.964
	2	0.026	0.024	0.024	88.435	81.633	81.633	83.900	3.928
	3	0.024	0.023	0.023	81.633	78.231	78.231	79.365	1.964

Table C- 29 Color units of *Trametes hirsuta* AK4 pellets (Treatment)

POME conc.	Time (days)	λ_{475}			Color units			Average	SD
		1	2	3	1	2	3		
25%	0	0.017	0.016	0.016	57.823	54.422	54.422	55.556	1.964
	1	0.013	0.012	0.012	44.218	40.816	40.816	41.950	1.964
	2	0.004	0.003	0.002	13.605	10.204	6.803	10.204	3.401
	3	0.005	0.003	0.005	17.007	10.204	17.007	14.739	3.928
50%	0	0.018	0.018	0.019	61.224	61.224	64.626	62.358	1.964
	1	0.014	0.014	0.014	47.619	47.619	47.619	47.619	0.000
	2	0.014	0.015	0.014	47.619	51.020	47.619	48.753	1.964
	3	0.015	0.014	0.014	51.020	47.619	47.619	48.753	1.964
75%	0	0.024	0.026	0.026	81.633	88.435	88.435	86.168	3.928
	1	0.022	0.024	0.023	74.830	81.633	78.231	78.231	3.401
	2	0.020	0.024	0.022	68.027	81.633	74.830	74.830	6.803
	3	0.020	0.017	0.022	68.027	57.823	74.830	66.893	8.560
100%	0	0.034	0.034	0.033	115.646	115.646	112.245	114.512	1.964
	1	0.030	0.034	0.032	102.041	115.646	108.844	108.844	6.803
	2	0.030	0.033	0.029	102.041	112.245	98.639	104.304	7.080
	3	0.025	0.020	0.018	85.034	68.027	61.224	71.429	12.264

1.5 *Trametes hirsuta* AK4 immobilized on palm pericarp fiber in batch experiment

Table C- 30 Phenols concentration of 100% treated POME only (Control)

Time (days)	λ_{760}			Phenols concentration (mg/L)			Average	SD
	1	2	3	1	2	3		
0	0.583	0.583	0.531	237.959	237.959	216.735	230.884	12.254
1	0.537	0.502	0.507	219.184	204.898	206.939	210.340	7.726
2	0.485	0.429	0.484	197.959	175.102	197.551	190.204	13.080
3	0.433	0.445	0.416	176.735	181.633	169.796	176.054	5.948
4	0.452	0.430	0.446	184.490	175.510	182.041	180.680	4.642
5	0.388	0.387	0.415	158.367	157.959	169.388	161.905	6.484
6	0.411	0.438	0.296	167.755	178.776	120.816	155.782	30.779
7	0.426	0.408	0.413	173.878	166.531	168.571	169.660	3.792
8	0.399	0.397	0.430	162.857	162.041	175.510	166.803	7.552

Table C- 31 Phenols concentration of palm pericarp fiber only in 100% treated POME (Control)

Time (days)	λ_{760}			Phenols concentration (mg/L)			Average	SD
	1	2	3	1	2	3		
0	0.316	0.334	0.330	128.980	136.327	134.694	133.333	3.858
1	0.339	0.319	0.304	138.367	130.204	124.082	130.884	7.167
2	0.336	0.341	0.277	137.143	139.184	113.061	129.796	14.529
3	0.342	0.339	0.318	139.592	138.367	129.796	135.918	5.337
4	0.308	0.293	0.298	125.714	119.592	121.633	122.313	3.117
5	0.295	0.325	0.293	120.408	132.653	119.592	124.218	7.317
6	0.310	0.274	0.314	126.531	111.837	128.163	122.177	8.992
7	0.307	0.277	0.294	125.306	113.061	120.000	119.456	6.141
8	0.281	0.295	0.281	114.694	120.408	114.694	116.599	3.299

Table C- 32 Phenols concentration of *Trametes hirsuta* AK4 immobilized on palm pericarp fiber in 100% treated POME (Treatment)

Time (days)	λ_{760}			Phenols concentration (mg/L)			Average	SD
	1	2	3	1	2	3		
0	0.814	0.996	0.900	332.245	406.531	367.347	368.707	37.162
1	0.545	0.448	0.622	556.122	457.143	634.694	549.320	88.971
2	0.457	0.292	0.394	466.327	297.959	402.041	388.776	84.964
3	0.621	0.382	0.392	506.939	311.837	320.000	379.592	110.361
4	0.582	0.601	0.636	356.327	367.959	389.388	371.224	16.771
5	0.517	0.655	0.567	316.531	401.020	347.143	354.898	42.775
6	0.552	0.620	0.581	337.959	379.592	355.714	357.755	20.891
7	0.479	0.543	0.594	293.265	332.449	363.673	329.796	35.279
8	0.451	0.516	0.570	276.122	315.918	348.980	313.673	36.480

Table C- 33 Color units of 100% treated POME only (Control)

Time (days)	λ_{475}			Color units			Average	SD
	1	2	3	1	2	3		
0	0.026	0.025	0.024	88.435	85.034	81.633	85.034	3.401
1	0.026	0.025	0.024	88.435	85.034	81.633	85.034	3.401
2	0.023	0.021	0.024	78.231	71.429	81.633	77.098	5.196
3	0.022	0.022	0.022	74.830	74.830	74.830	74.830	0.000
4	0.022	0.022	0.023	74.830	74.830	78.231	75.964	1.964
5	0.021	0.022	0.021	71.429	74.830	71.429	72.562	1.964
6	0.020	0.022	0.014	68.027	74.830	47.619	63.492	14.161
7	0.023	0.022	0.023	78.231	74.830	78.231	77.098	1.964
8	0.024	0.022	0.023	81.633	74.830	78.231	78.231	3.401

Table C- 34 Color units of palm pericarp fiber only in 100% treated POME (Control)

Time (days)	λ_{475}			Color units			Average	SD
	1	2	3	1	2	3		
0	0.029	0.026	0.023	98.639	88.435	78.231	88.435	10.204
1	0.034	0.026	0.023	115.646	88.435	78.231	94.104	19.341
2	0.029	0.027	0.022	98.639	91.837	74.830	88.435	12.264
3	0.030	0.028	0.025	102.041	95.238	85.034	94.104	8.560
4	0.031	0.027	0.024	105.442	91.837	81.633	92.971	11.945
5	0.030	0.028	0.023	102.041	95.238	78.231	91.837	12.264
6	0.030	0.028	0.023	102.041	95.238	78.231	91.837	12.264
7	0.031	0.028	0.024	105.442	95.238	81.633	94.104	11.945
8	0.033	0.028	0.023	112.245	95.238	78.231	95.238	17.007

Table C- 35 Color units of *Trametes hirsuta* AK4 immobilized on palm pericarp fiber in 100% treated POME (Treatment)

Time (days)	λ_{475}			Color units			Average	SD
	1	2	3	1	2	3		
0	0.029	0.025	0.028	98.639	85.034	95.238	92.971	7.080
1	0.013	0.016	0.020	44.218	54.422	68.027	55.556	11.945
2	0.010	0.015	0.012	34.014	51.020	40.816	41.950	8.560
3	0.012	0.013	0.015	40.816	44.218	51.020	45.351	5.196
4	0.011	0.015	0.010	37.415	51.020	34.014	40.816	8.999
5	0.012	0.015	0.016	40.816	51.020	54.422	48.753	7.080
6	0.014	0.016	0.017	47.619	54.422	57.823	53.288	5.196
7	0.016	0.013	0.016	54.422	44.218	54.422	51.020	5.891
8	0.014	0.014	0.015	47.619	47.619	51.020	48.753	1.964

2. Two-stage treatment

2.1 Two-stage treatment by silica-immobilized bacteria and white rot fungal pellets

Table C- 36 Phenols concentration of silica-immobilized bacteriaand white rot fungal pellets (Treatment)

Times (Days)	λ_{760}			Phenols concentration (mg/L)			Average	SD
	1	2	3	1	2	3		
0	0.624	0.653	0.665	254.694	266.531	271.429	264.218	8.604
1	0.525	0.537	0.548	214.286	219.184	223.673	219.048	4.695
2	0.504	0.499	0.514	205.714	203.673	209.796	206.395	3.117
3	0.463	0.479	0.496	188.980	195.510	202.449	195.646	6.736
4	0.462	0.47	0.479	188.571	191.837	195.510	191.973	3.471
5	0.447	0.429	0.397	182.449	175.102	162.041	173.197	10.337
6	0.372	0.405	0.405	151.837	165.306	165.306	160.816	7.777
7	0.397	0.395	0.415	162.041	161.224	169.388	164.218	4.496
8	0.347	0.369	0.396	141.633	150.612	161.633	151.293	10.017
9	0.337	0.35	0.362	137.551	142.857	147.755	142.721	5.103
10	0.341	0.353	0.348	139.184	144.082	142.041	141.769	2.460
11	0.33	0.332	0.341	134.694	135.510	139.184	136.463	2.392
12	0.318	0.324	0.305	129.796	132.245	124.490	128.844	3.964
13	0.289	0.302	0.322	117.959	123.265	131.429	124.218	6.785
14	0.287	0.292	0.296	117.143	119.184	120.816	119.048	1.841

Table C- 37 Color units of silica-immobilized bacteriaand white rot fungal pellets (Treatment)

Times (Days)	λ_{475}			Color units			Average	SD
	1	2	3	1	2	3		
0	0.029	0.028	0.027	98.639	95.238	91.837	95.238	3.401
1	0.021	0.022	0.022	71.429	74.830	74.830	73.696	1.964
2	0.021	0.021	0.021	71.429	71.429	71.429	71.429	0.000
3	0.021	0.021	0.021	71.429	71.429	71.429	71.429	0.000
4	0.020	0.021	0.021	68.027	71.429	71.429	70.295	1.964
5	0.021	0.019	0.017	71.429	64.626	57.823	64.626	6.803
6	0.016	0.017	0.017	54.422	57.823	57.823	56.689	1.964
7	0.017	0.017	0.016	57.823	57.823	54.422	56.689	1.964
8	0.012	0.012	0.013	40.816	40.816	44.218	41.950	1.964
9	0.013	0.012	0.012	44.218	40.816	40.816	41.950	1.964
10	0.012	0.013	0.012	40.816	44.218	40.816	41.950	1.964
11	0.012	0.013	0.012	40.816	44.218	40.816	41.950	1.964
12	0.013	0.013	0.013	44.218	44.218	44.218	44.218	0.000
13	0.01	0.01	0.013	34.014	34.014	44.218	37.415	5.891
14	0.014	0.012	0.012	47.619	40.816	40.816	43.084	3.928

2.2 Two-stage treatment by silica-immobilized bacteria and PF-immobilized whiterot fungus

Table C- 38 Phenols concentration of silica-immobilized bacteria and PF-immobilized white rot fungus (Treatment)

Time (Days)	λ_{760}			Phenols concentration (mg/L)			Average	SD
	1	2	3	1	2	3		
0	0.310	0.295	0.283	126.531	120.408	115.510	120.816	5.522
1	0.214	0.213	0.206	87.347	86.939	84.082	86.122	1.779
2	0.179	0.201	0.168	73.061	82.041	68.571	74.558	6.858
3	0.153	0.172	0.181	62.449	70.204	73.878	68.844	5.834
4	0.197	0.150	0.176	80.408	61.224	71.837	71.156	9.610
5	0.210	0.155	0.143	85.714	63.265	58.367	69.116	14.582
6	0.122	0.145	0.168	49.796	59.184	68.571	59.184	9.388
7	0.120	0.179	0.129	48.980	73.061	52.653	58.231	12.974
8	0.892	0.513	0.999	364.082	-	407.755	385.918	30.882
9	0.640	0.833	0.559	261.224	340.000	228.163	276.463	57.454
10	0.706	0.672	0.647	288.163	274.286	264.082	275.510	12.087
11	0.725	0.651	0.624	295.918	265.714	254.694	272.109	21.343
12	0.710	0.602	0.585	289.796	245.714	238.776	258.095	27.672
13	0.617	0.542	0.613	251.837	221.224	250.204	241.088	17.222
14	0.580	0.695	0.456	236.735	283.673	186.122	235.510	48.787

Table C- 39 Color units of silica-immobilized bacteriaand PF-immobilized white rot fungus (Treatment)

Time (Days)	λ_{475}			Color units			Average	SD
	1	2	3	1	2	3		
0	0.029	0.025	0.021	98.639	85.034	71.429	85.034	13.605
1	0.029	0.020	0.020	98.639	68.027	68.027	78.231	17.674
2	0.025	0.020	0.018	85.034	68.027	61.224	71.429	12.264
3	0.027	0.022	0.019	91.837	74.830	64.626	77.098	13.746
4	0.026	0.022	0.021	88.435	74.830	71.429	78.231	8.999
5	0.026	0.021	0.018	88.435	71.429	61.224	73.696	13.746
6	0.026	0.020	0.019	88.435	68.027	64.626	73.696	12.877
7	0.025	0.022	0.017	85.034	74.830	57.823	72.562	13.746
8	0.024	0.018	0.027	81.633	61.224	91.837	78.231	15.587
9	0.021	0.016	0.025	71.429	54.422	85.034	70.295	15.338
10	0.021	0.015	0.024	71.429	51.020	81.633	68.027	15.587
11	0.021	0.016	0.024	71.429	54.422	81.633	69.161	13.746
12	0.023	0.017	0.025	78.231	57.823	85.034	73.696	14.161
13	0.025	0.017	0.024	85.034	57.823	81.633	74.830	14.826
14	0.023	0.020	0.026	78.231	68.027	88.435	78.231	10.204

2.3 Sequential treatment by recycled plastic-immobilized bacteria and PF-immobilized white rot fungus

Table C- 40 Phenols concentration of recycled plastic-immobilized bacteriaand PF-immobilized white rot fungus (Treatment)

Time (Days)	λ_{760}			Phenols concentration (mg/L)			Average	SD
	1	2	3	1	2	3		
0	0.312	0.317	0.318	127.347	129.388	129.796	128.844	1.312
1	0.297	0.294	0.276	121.224	120.000	112.653	117.959	4.636
2	0.270	0.282	0.269	110.204	115.102	109.796	111.701	2.953
3	0.258	0.271	0.233	105.306	110.612	95.102	103.673	7.883
4	0.239	0.258	0.262	97.551	105.306	106.939	103.265	5.016
5	0.230	0.234	0.217	93.878	95.510	88.571	92.653	3.628
6	0.230	0.253	0.235	93.878	103.265	95.918	97.687	4.937
7	0.192	0.216	0.231	78.367	88.163	94.286	86.939	8.030
8	0.188	0.241	0.453	76.735	98.367	184.898	120.000	57.235
9	0.297	0.291	0.228	121.224	118.776	93.061	111.020	15.601
10	0.308	0.254	0.289	125.714	103.673	117.959	115.782	11.180
11	0.304	0.305	0.216	124.082	124.490	88.163	112.245	20.856
12	0.293	0.264	0.297	119.592	107.755	121.224	116.190	7.351
13	0.290	0.325	0.302	118.367	132.653	123.265	124.762	7.259
14	0.29	0.304	0.298	118.367	124.082	121.633	121.361	2.867

Table C- 41 Color units of recycled plastic-immobilized bacteriaand PF-immobilized white rot fungus (Treatment)

Time (Days)	λ_{475}			Color units			Average	SD
	1	2	3	1	2	3		
0	0.033	0.027	0.025	112.245	91.837	85.034	96.372	14.161
1	0.033	0.027	0.025	112.245	91.837	85.034	96.372	14.161
2	0.031	0.030	0.024	105.442	102.041	81.633	96.372	12.877
3	0.032	0.028	0.026	108.844	95.238	88.435	97.506	10.391
4	0.031	0.030	0.025	105.442	102.041	85.034	97.506	10.934
5	0.031	0.030	0.025	105.442	102.041	85.034	97.506	10.934
6	0.031	0.029	0.025	105.442	98.639	85.034	96.372	10.391
7	0.032	0.028	0.024	108.844	95.238	81.633	95.238	13.605
8	0.025	0.022	0.014	85.034	74.830	47.619	69.161	19.341
9	0.023	0.020	0.014	78.231	68.027	47.619	64.626	15.587
10	0.023	0.020	0.013	78.231	68.027	44.218	63.492	17.454
11	0.024	0.020	0.013	81.633	68.027	44.218	64.626	18.938
12	0.024	0.021	0.013	81.633	71.429	44.218	65.760	19.341
13	0.027	0.024	0.014	91.837	81.633	47.619	73.696	23.153
14	0.028	0.028	0.012	95.238	95.238	40.816	77.098	31.420

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