การขจัดสารประกอบฟีนอลิกและสีจากน้ำเสียของโรงงานสกัดน้ำมันปาล์มโดยใช้หญ้าและแบคทีเรียบริเวณราก

นายพงไพบูน พอนปะเสิด

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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REMOVAL OF PHENOLIC COMPOUNDS AND COLOR FROM PALM OIL MILL EFFLUENT BY USING GRASSES AND THEIR RHIZOSPHERE BACTERIA

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A Thesis Submitted in Partial Fulfillment of the Requirements
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พงไพบูน พอนปะเสิด : การขจัดสารประกอบฟีนอลิกและสีจากน้ำเสียของโรงงานสกัดน้ำมันปาล์มโดยใช้หญ้าและแบคทีเรียบริเวณราก (REMOVAL OF PHENOLIC COMPOUNDS AND COLOR FROM PALM OIL MILL EFFLUENT BY USING GRASSES AND THEIR RHIZOSPHERE BACTERIA) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. เอกวัล ลือพร้อมชัย, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อ. ดร. บุญลือ คะเชนทร์ชาติ, 84 หน้า.

สารประกอบฟีนอลิกและสีในน้ำทิ้งจากบ่อปรับเสถียรโรงงานสกัดน้ำมันปาล์มมีค่าสูงกว่ามาตรฐาน การนำน้ำทิ้งไปใช้รดสวนปาล์มสามารถลดปริมาณน้ำได้ แต่ส่งผลเสียต่อพืช เนื่องจากเกิดการสะสมของสารประกอบฟีนอลิกในดิน ดังนั้นการศึกษานี้มีวัตถุประสงค์เพื่อลดความเข้มข้นของสารประกอบฟีนอลิคและสีในดินที่รดด้วยน้ำทิ้งจากโรงงานสกัดน้ำมันปาล์ม โดยใช้แบคทีเรียบริเวณรากของหญ้าอาหารสัตว์จำนวนสามสายพันธุ์ คือ หญ้ามูลาโต้ II (หญ้าพันธุ์ลูกผสมระหว่าง *Brachiaria ruziziensis* x *Brachiaria decumbens* x *Brachiaria brizantha*) หญ้าซิกแนลเลื้อย (*Brachiaria humidicola*) และหญ้ากินี *(Panicum maximum* Jacq) ทำการทดลองภายในกระถางภายใต้สภาวะของเรือนเพาะชำ ผลการทดลองพบว่าสารประกอบฟีนอลิคและสีลดลงอย่างมากในดินที่ปลูกหญ้าทั้งสามสายพันธุ์และมีประสิทธิภาพสูงในรอบที่ 1-4 ของการรดด้วยน้ำทิ้งจากบ่อปรับเสถียรบ่อสุดท้ายของโรงงานสกัดน้ำมันปาล์ม จังหวัดสุราษฎร์ธานี โดยหญ้ามูลาโต้ หญ้าซิกแนลเลื้อย และหญ้าหญ้ากินี สามารถกำจัดสารประกอบฟีนอลิกได้ 87-93%, 90-95% และ 65-83% และสีได้ 84-92%, 90-96% และ 82-96% ตามลำดับ ในขณะที่ชุดดินควบคุมสามารถกำจัดสารประกอบฟีนอลิกและสีได้เพียง 39-72% และ 59-71% ตามลำดับ สารประกอบฟีนอลิกที่สะสมในส่วนต่างๆ ของหญ้ามีปริมาณน้อยมาก โดยเมื่อเปรียบเทียบระหว่างชุดทดลองที่รดด้วยน้ำทิ้งจากบ่อปรับเสถียรบ่อสุดท้ายและชุดทดลองที่รดน้ำด้วยประปา พบว่าไม่มีความแตกต่างกันอย่างมีนัยสำคัญ จำนวนของแบคทีเรียย่อยสลายฟีนอลในดินมีจำนวนเพิ่มขึ้นอย่างต่อเนื่องตลอดระยะเวลาที่ปลูกหญ้า โดยมีจำนวนเพิ่มขึ้นจาก 5.89 log CFU ต่อกรัมดิน เป็น 8.16, 7.52 และ 7.27 log CFU ต่อกรัมดิน ภายหลังจากปลูกหญ้ามูลาโต้ หญ้าซิกแนลเลื้อย และหญ้าหญ้ากินี ตามลำดับ ในทางกลับกันจำนวนของแบคทีเรียในชุดดินควบคุมเพิ่มขึ้นเพียงเล็กน้อยเป็น 6.14 log CFU ต่อกรัมดิน ดังนั้นการย่อยสลายของสารประกอบฟีนอลิกจึงสัมพันธ์กับการเจริญของแบคทีเรียบริเวณรากพืช น้ำชะจากชุดทดลองที่ปลูกหญ้ามูลาโต้มีความเป็นพิษต่ำสุดเมื่อเทียบกับชุดทดลองอื่นๆ จึงได้เลือกหญ้าดังกล่าวเพื่อใช้ในการทดลองต่อไป การเติมแบคทีเรียย่อยสลายฟีนอล *Acinetobacter* sp. OPB เพิ่มในดินที่ปลูกหญ้ามูลาโต้ พบว่าสามารถเพิ่มประสิทธิภาพในการกำจัดสารประกอบฟีนอลิกเพียงเล็กน้อย เมื่อขยายขนาดของระบบที่ปลูกหญ้ามูลาโต้เป็นถังปฏิกรณ์ พบว่าสามารถลดสารประกอบฟีนอลิกได้ 77% หลังจากรดด้วยน้ำทิ้งจากบ่อปรับเสถียรบ่อสุดท้ายสองรอบ ซึ่งค่าการขจัดสูงกว่าดินประมาณ 30% ดังนั้นสามารถนำหญ้ามูลาโต้ไปปลูกเพื่อลดความเป็นพิษของสารประกอบฟีนอลิกในดินได้

ภาควิชา จุลชีววิทยา	ลายมือชื่อนิสิต .....
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PHONGPHAYBOUN PHONEPASEUTH: REMOVAL OF PHENOLIC COMPOUNDS AND COLOR FROM PALM OIL MILL EFFLUENT BY USING GRASSES AND THEIR RHIZOSPHERE BACTERIA. ADVISOR: ASST. PROF. EKAWAN LUEPROMCHAI, Ph.D., CO-ADVISOR: BOONLUE KACHENCHART, Ph.D., 84 pp.

Palm oil mill effluent (POME) in stabilization ponds contains phenolic compounds and color higher than standard. Soil irrigation is usually used to reduce the wastewater volume; however the accumulation of phenolic compounds can be toxic to plants. This study aimed to reduce the concentration of phenolic compounds in soil by using rhizosphere bacteria of pasture grasses. Three grass cultivars namely Mulato or Brachiaria hybrid (*B. ruziziensis x B. Decumbens x B. brizantha*), Creeping signal (*Brachiaria humidicola*) and Guinea grasses (*Panicum maximum* Jacq) were compared in pot experiments under greenhouse conditions. Phenolic compounds and color significantly reduced in planted soil and highly effective in the 1 - 4 cycles of irrigation using POME from the last stabilization pond of a palm oil mill in Surat Thani province. Mulato, Creeping signal and guinea grasses removed 87-93%, 90-95% and 65-83% of phenolic compounds and 84-92%, 90-96% and 82-96% of color, respectively. At the same time, the control soil removed only 39-72% and 59-71% of phenolic compounds and color, respectively. Phenolic compound accumulation was minimal since their concentrations in grasses watered with POME and tap water were not significantly different. The number of phenol degrading bacteria in soil increased from 5.89 log CFU g^{-1} soil to 8.16, 7.52 and 7.27 log CFU g^{-1} soil after planted with Mulato, Creeping signal and Guinea grasses, respectively. On the other hand, the bacterial number in control soil was 6.14 log CFU g^{-1} soil. Thus, the degradation of phenolic compounds was corresponded to the growth of rhizosphere bacteria. The soil leachate from Mulato grass had the lowest toxicity to seed germination, thus Mulato grass was selected for further experiment. The addition of phenol-degrading bacteria, *Acinetobacter* sp. strain OPB to soil with Mulato grass slightly increased the efficiency of phenolic compounds removal. In the scale-up reactor, phenolic compounds in Mulato reactor were decreased around 77% after two irrigation cycles of POME from the last stabilization pond. The reduction was around 30% higher than the unplanted soil reactor. Thus, Mulato grass could be cultivated for reducing toxicity of phenolic compounds in soil.

Department: Microbiology Student's Signature .....
Field of Study: Industrial Microbiology Advisor's Signature .....
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LIST OF ABBREVIATIONS

POME	Palm oil mill effluent
CFMM	Carbon free mineral medium
CFU	Colony forming unit
H	Hour
μ	Micro
mm	Millimeter
mL	Milliliter
mg	Milligram
%	Percent

CHAPTER 1

INTRODUCTION

1.1 State of problem

Oil palm is one of the world's most rapidly expanding tropical crops. Indonesia, Malaysia and Thailand are the three largest palm oil producing countries in the world (Rupani et al., 2010). Palm oil processing releases large quantities of wastewater. They are concerned as environmental problems when discharged to natural water because they are toxic to aquatic plants and animals. Several chemical and physical technologies have been utilized for the treatment of this palm oil mill effluent (POME) such as adsorption (Shavandi et al., 2012), electrocoagulation (Nasution et al., 2011), membrane technology (Ahmad et al., 2003), electrochemical method and ultrafiltration (Said et al., 2013). However, some of them have limitations related to its low efficiency, high construction and operation costs, and possible more toxic by-products. In palm oil mill wastewater treatment process, stabilization ponds are usually used as the final treatment for POME after using for biogas production. The biogas effluent contains bioflocs, anaerobic microorganisms, macrofibrils, while the soluble fraction consist of carbohydrate, pectin, lignin, tannin, humic and fulvic acid like substance, melanoidin and phenolic compounds (Zahrim, 2014).

The phenolic compounds and color of wastewater in stabilization ponds are usually higher than the standard of industrial wastewater. In addition, the volume of this wastewater can exceed the pond capacity in rainy season. Thus, soil irrigation is commonly used to reduce the wastewater volume. The POME amended soil can enhance soil microbiological activities which ultimately increase soil fertility (Nwoko and Ogunyemi, 2010a) and plant biomass (Akinyele et al., 2013). In contrast, the accumulation of toxic compounds such as phenolic compounds and other organic

acids in olive oil mill effluent (OMEs) and palm oil mill effluent (POME) amended soil is reported (Barbera et al., 2013; Umeugochukwu and Ezeaku, 2012).

Recently, grasses have been used in technology such as phytoremediation (Ibanez et al., 2012), rhizoremediation and constructed wetlands for the treatment of phenols polluted environments (Kurzbaum et al., 2010). They are considered as low cost, easily to operate, environmental friendly and strong potential for application (Kivaisi, 2001). Rhizoremediation is a new biological treatment technique for treatment of difference pollutants (Gonzalez et al., 2013) and also known as an environmental friendly technology. Rhizoremediation uses microbial activity in the plant root zone to breakdown contaminants (Gaskin and Bentham, 2010). Specific plants may promote degradation of specific type of pollution; however they have limitation when there are high concentrations of pollutants (Phillips et al., 2012). Thus, bioaugmentation with efficient bacteria could overcome the limitation of plants used (Glick, 2010). Chavan and Dhulap (2012) reported that Guinea grass (*Panicum maximum* Jacq) and their root associated bacteria could reduce sewage wastewater parameters up to 50% after 100 days. However, research on the decolorization and phenols removal from palm oil mill effluent by rhizoremediation especially animal feed grasses is limited.

Pasture grass, *Brachiaria* spp. especially, *Brachiaria humidicola* (Rendle) Schweick releases substantial amounts of Biological Nitrification Inhibitor compound (BNIs) from its roots, ranging from 17 to 50 ATU per gram of root dry weight per day (Subbarao et al., 2006). The BNIs have structures similarly with phenolic compounds. Thus, it might promote growth of phenol degrading bacteria in rhizosphere and enhance phenolic compound removal from wastewater. According to Viroj Rakkiatsakul (2013), three *Brachiaria* spp. grasses were cultivated in experiment pots for POME treatment. Among them, *Brachiaria humidicola* was the most effective cultivar to enrich for phenol-degrading bacteria and enhance phenolic compound removal. Although, this grass specie is effective in rhizoremediation, it has limitations. Currently, *Brachiaria humidicola* is not popular to use as animal feed because the percent of grass seed germination is low and the grass biomass is also lowest when

compares to other *Brachiaria* cultivars. This could be a drawback to apply this grass in field. Thus, this study selected two more popular animal feed grass species i.e. Mulato grass and Guinea grass to compare with Creeping signal grass (*Brachiaria humidicola*). The efficiency of three animal feed grasses on rhizoremediation of POME irrigated soil was compared. In addition, a phenol-degrading bacterium was isolated to examine the ability to enhance rhizoremediation. Finally, the selected grass was tested for its potential for field application by upscaling to a rhizoreactor. The application of these grasses in rhizoreactor would be beneficial for rhizoremediation as well as for animal feeds after harvested.

1.2 Objectives

(1) To isolation of phenol-degrading bacteria from plant roots and examine their efficiency;

(2) To compare the efficiency of three animal feed grasses on removal of phenolic compounds and color from palm oil mill effluent (POME);

(3) To examine ability of phenol-degrading bacteria bioaugmentation to enhance rhizoremediation;

(4) To develop a rhizoreactor for removal of phenolic compounds and color from POME.

1.3 The benefits of the study

The selected grass and their rhizosphere bacteria could be applied as a rhizoremediation approach for treatment of phenol, phenolic compounds and dark browned color containing in POME.

Grass cultivation could prevent soil erosion in rainy season. In addition, the harvested grasses could be used for animal feed or bioethanol production as cellulosic biomass.

CHAPTER 2

LITERATURE REVIEW

2.1 Phenol and phenolic compounds

Phenol is an aromatic hydrocarbon containing a hydroxyl group (OH) attached to the benzene ring; it is a basic structural unit for a variety chemical synthetic organic compounds structure of common phenolic compounds as shown in Figure 2.1. Phenols are regarded as pollutants in wastewaters and can be harmful to living organisms and ecosystem even at low concentrations. Phenols affect to the liver, kidneys, lungs and blood circulatory system (Ibáñez et al., 2013). They 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 (Coniglio et al., 2008).

2.2 Biodegradation of phenols

Phenols are highly toxic and harmful to several organisms, but some microorganisms are able to use phenol as carbon and energy source for grow under both aerobic and anaerobic conditions such as *Acinetobacter calcoaceticus*, *Pseudomonas aeruginosa* T1, *Streptomyces setonii*, and *Trichosporon cutaneum* (van Schie and Young., 2000), but it is generally found that the degradation of phenols occur under aerobic condition (Melo et al., 2005).

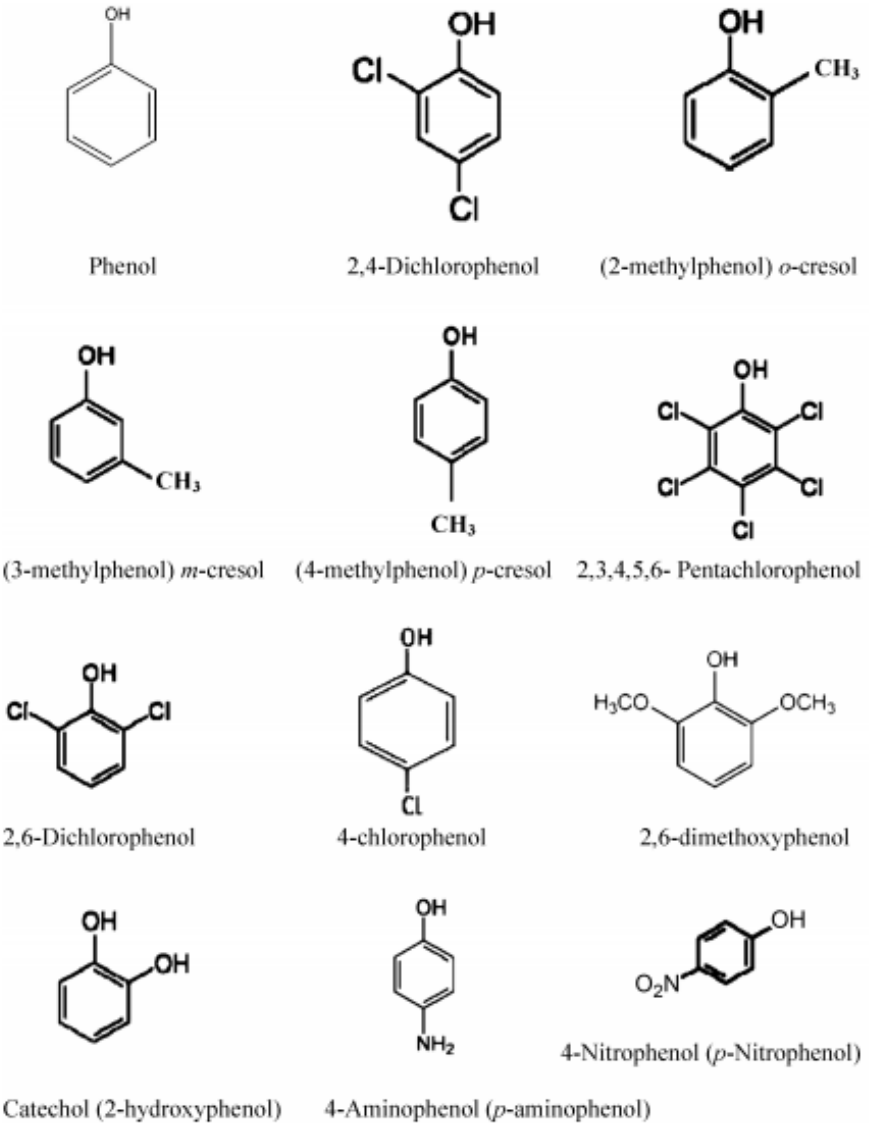


Figure 2.1 Chemical structure of common phenolic compound (Al-Khalid and El-Naas, 2012)

2.3 Aerobic biodegradation of phenol

In the first step, enzyme phenol hydroxylase transforms phenol to catechol by oxygenation reaction. Catechol can be degraded using meta-pathway or ortho-pathway depending on microorganisms (van Schie and Young, 2000). In the meta-pathway, ring fission occurs adjacent to the two hydroxyl groups of catechol

(extradiol fission). The enzyme catechol 2, 3-dioxygenase transforms catechol to 2-hydroxymuconic semialdehyde. This compound is metabolized further to intermediates of the Krebs cycle. For the ortho- or β-ketoadipate pathway, the aromatic ring is cleaved between the catechol hydroxyls by a catechol 1, 2-dioxygenase (intradiol fission). The resulting cis,cis muconate is further metabolized via β-ketoadipate to Krebs cycle. Some example of meta and ortho pathway was show in table 2.1.

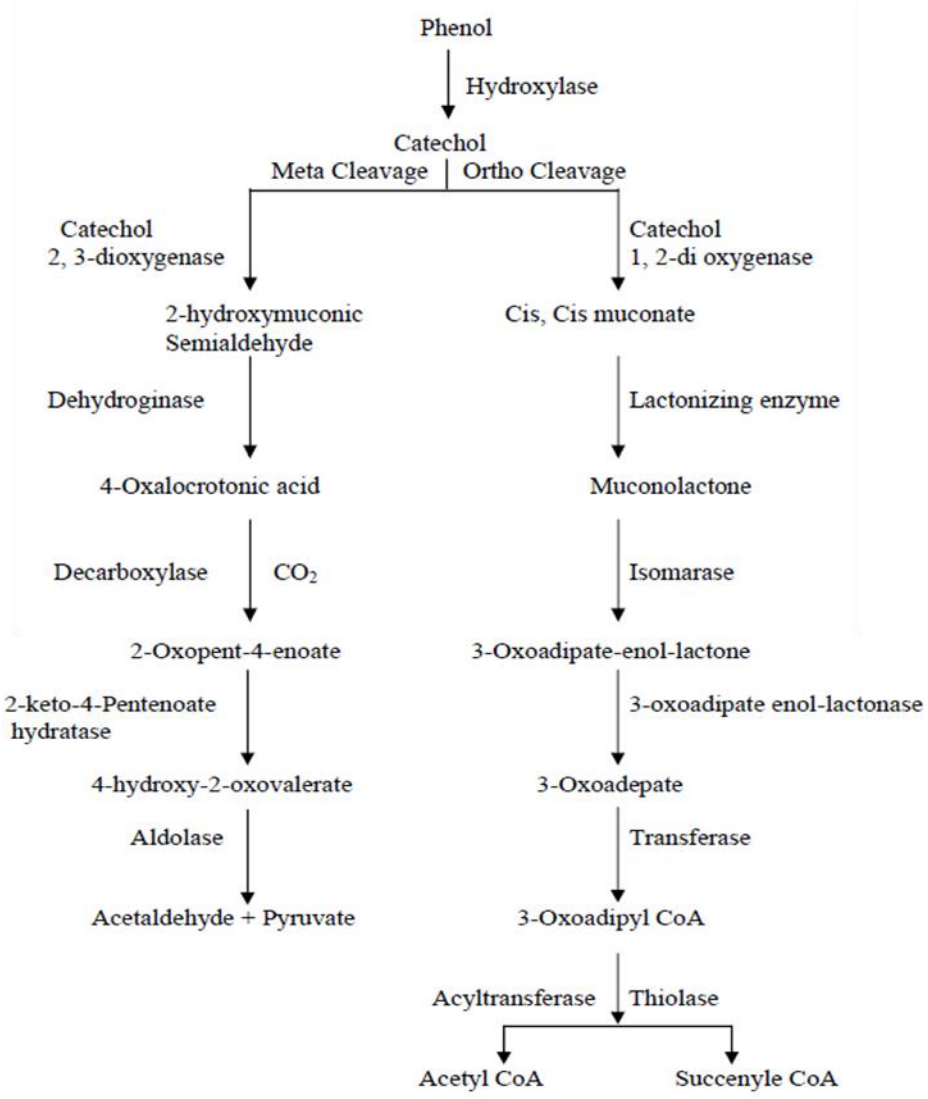


Figure 2.2 Flow chart of aerobic degradation pathway for phenol by aerobic microorganisms (Basha et al., 2010)

Table 2.1 Example of phenols-degrading bacteria

Phenols-degrading bacteria	Pathway	Phenol Conc.	Time require (h)	References
Acinetobacter sp. PK1	Ortho-cleavage	500	>48	Khongkhaem et al. (2011)
Bacillus amyloliquefaciens WJDB-1	-	200	36	Lu et al. (2012)
Bacillus cereus WJ1	-	500	>60	Yan et al. (2013)
Stenotrophomonas maltophilia (GU358076)	Meta-cleavage	500	48	Basak et al. (2013)
Acinetobacter sp. RTE1.4	Ortho-cleavage	200	>72	(Paisio et al., 2014)

2.4 Phenol degradation by Genus Acinetobacter

Acinetobacter are white colony, aerobic and Gram-negative bacilli bacterium, ubiquitously distributed free-living saprophytic bacteria and as a consequence are often isolated from soils, seawater, freshwater, estuaries, sewage, contaminated foods, the mucosa and skin of animals and humans. They can use many compounds as carbon and energy sources such as phenol and benzoate and they can grow easily in simple media (Abdel-El-Haleem, 2003). Example of *Acinetobacter* strains for phenol degradation are below.

Acinetobacter sp. PD12 was isolated from the activated sludge. This strain was capable of removing 500 mg L^{-1} phenol in liquid minimal medium by 99.6% within 9 h and metabolizing phenol at concentrations up to 1100 mg L^{-1}. They found that immobilized this bacteria in PVA could maintain their phenol degrading activity during 50 days storage while free cell activity remain 10% (Wang et al., 2007).

Acinetobacter calcoaceticus. P23 is the first growth-promoting bacterium identified from Lemna aoukikusa rapidly colonized on the surface of sterilized

duckweed roots and formed biofilms. This bacterial stain capable to degrade phenol 20 mg L^{-1} in BM medium at around 5 h (Yamaga et al., 2010).

Acinetobacter sp. strain AQ5NOL encapsulated in gellan gum completely degrade phenol at 1,100, 1,500 and 1,900 mg L^{-1} within 108, 216 and 240 h, respectively. The immobilized cells showed no loss in phenol degrading activity after being used repeatedly for 45 cycles of 18 h cycle (Ahmad et al., 2012).

2.5 Palm oil mill effluent

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 and Prasertsan, 1996). The POME contains three main sources of dirty water including clarification (60%), sterilization (36%) and hydrocyclone (4%) units as shown in Figure 2.3 (Wu et al., 2010). Large volumes of wastewater are generated from palm oil mills, of which there are higher organic compounds, pH, COD, phenols and color consider as toxic if this wastewater was release directly to environment. In the process of palm oil production, they are no chemical addition. These colors are natural organic matter, tannins, phenolic compounds, and melanoidin (generated from heating of organic in oil extraction process). When the palm fruits are destroyed by heat of the steam process, phenols in palm fruit are extracted with oil and steam and phenols are dissolved in water than oil. They contact with oxygen in the air and causes oxidation reaction. Polyphenoloxidase (PPO) is oxidized to quinone and polymerized to melanin (brown pigment) (Wu et al., 2010). The color effluent blocked sunlight and aquatic plants to photosynthesis and also affected to aquatic animals growth. The removal of colored compounds from industrial wastewater has become an important problem. Several chemical and physical technologies have been utilized for the treatment of this colored effluent such as adsorption (Shavandi et al., 2012), electrocoagulation (Nasution et al., 2011), membrane technology (Ahmad et al., 2003) electrochemical method and ultrafiltration (Said et al., 2013). However, some of them have limitations related to its low efficiency, high construction and operation

costs, and lead to more toxic by-products. At the present, final treated POME is treated by using ponding system. It is also used as animal feed (Famurewa and Olarewaju, 2013) and biogas production because palm oil is high in nutrients. However, the treated POME still has high COD, phenols and dark brown color (Zahrim, 2014).

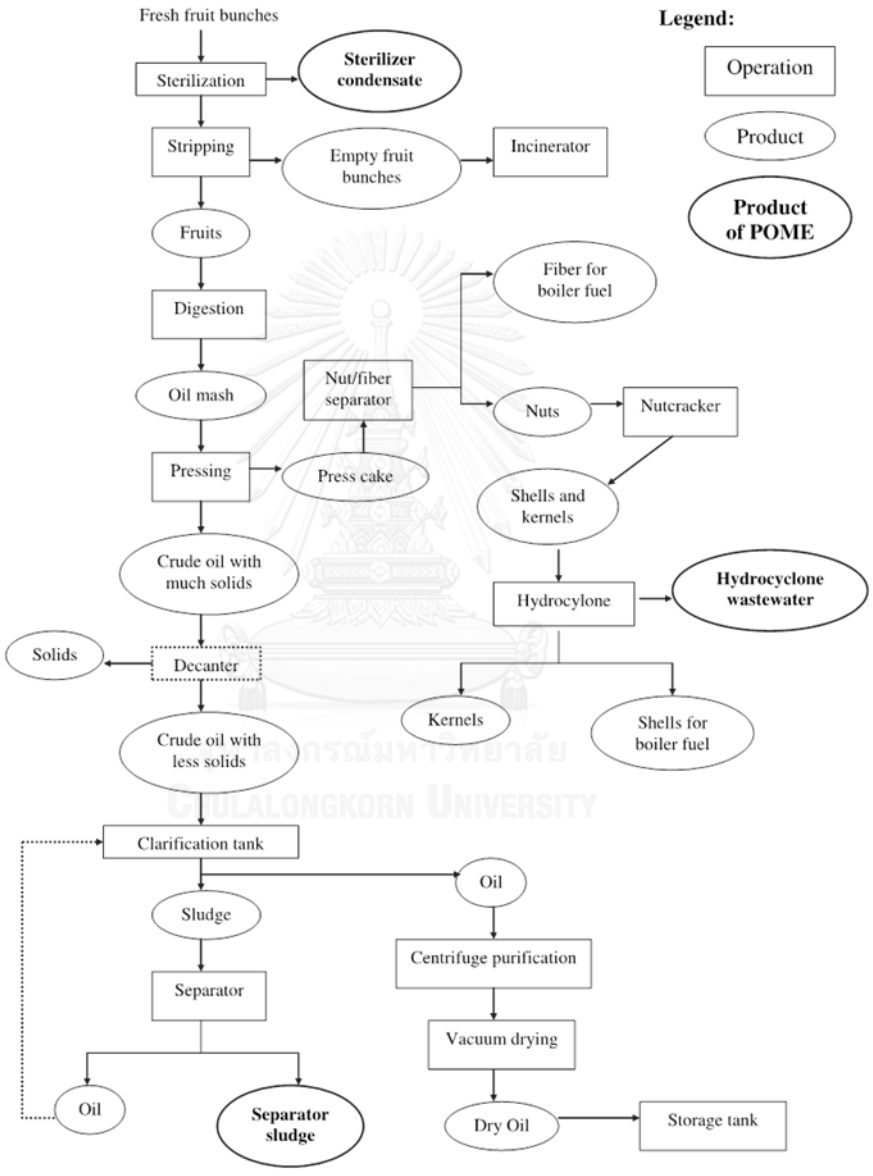


Figure 2.3 Palm oil mill process operations and products (Wu et al., 2010)

2.6 Rhizoremediation

Rhizoremediation is the use of microorganisms to degrade or remove pollutants in plant rhizosphere (Gaskin and Bentham, 2010). In the rhizoremediation, plant roots exudate sugars, organic acids, and amino acids as main components, consequently create a nutrient-rich zone that stimulate the microbial activity (Phillips et al., 2012). In the same time, microbes enhance plant growth by decreasing pollutants concentration and secreting some phytohormones for plants (Zelicourt et al., 2013; Hrynkiewicz and Baum, 2012). These rhizosphere bacteria are known as plant-growth promoting rhizobacteria (PGPR). The example of PGPR is Bacillus subtilis strain SJ-101, which is capable of producing the phytohormone indole acetic acid (IAA) and solubilising inorganic phosphates for stimulated Brassica juncea growth on nickel polluted soil (Zaidi et al., 2006). Huo et al. (2012) found that inoculated *Pantoea* sp. Jp3-3 significantly reduced Cu uptake and enhance guinea grass growth. Plant also derived enzymes with the potential to contribute to the degradation of organic pollutants such as peroxidases, laccase that degrading aromatic pollutants in the rhizosphere (Rodríguez et al., 2004). Other examples of plant-bacterial interactions for pollutant removal are shown in Table 2.2.

Table 2.2 Example of plant-bacterial interactions for pollutant removals

Plants	Bacteria	Type of pollutants	References
Italian ryegrass	*Pantoea* sp. ITSI10	Diesel oil	(Afzal et al., 2012)
Lollium multiflorum	*Bacillus* pumilus C2A1	Chlorpyrifos	(Ahmad, Iqbal, et al., 2012)
Cytisus striatus	*Sphingomonas* sp. D4	Hexachlorocyclohexane (HCH)	(Becerra-Castro et al., 2013)
Brassica napus	*Burkholderia kururiensis* KP 23 and *Agrobacterium rhizogenes* LBA	Phenolic compounds	Gonzalez et al. (2013)
Chrysopogon zizanioides	*Achromobacter xylosoxidans* F3B	Toluene	(Ho et al., 2013)

Vicia sativa	*Bacillus* sp	Phenol	(Ibáñez et al., 2013)
Glandularia pulchella (Sweet) Tronc	*Pseudomonas monteilii* ANK	Textile effluent	(Kabra et al., 2013)
Testuca arundinacea L.	*Pseudomonas* sp. SB	Oily-sludge	(Liu et al., 2013)

2.7 Grass species used in this research

2.7.1 Guinea grass (Panicum maximum Jacq)

Guinea grass (Panicum maximum Jacq) is an important multicut forage grass with ease of propagation, fast growth, available at local level and high quality forage for livestock. P. maximum grows in most soil types providing they are well-drained, moist and fertile, although some varieties are tolerant of lower fertility and poorer drainage. Tolerance of low soil pH and high Aluminum saturation is also variable.

(Source: http://www.tropicalforages.info/key/Forages/Media/Html/Panicum_maximum.htm Access date 22/10/2014)

Figure 2.4 Guinea grass *(Panicum maximum* Jacq)

(Source: http://info.agri.ubu.ac.th/~ubuforage/photoactivity.html Access date 22/10/2014)

It has been used for wastewater treatment by rhizoremediation technology through constructed wetland (Chavan and Dhulap, 2012). The strengths of this grass are more leaves making it a high quality feed for cattle. This grass is highly tolerant to

droughts. It is distributed worldwide, mostly grows or adapted in the tropics. This grass grows naturally in open grasslands, usually under or near trees, around shrubs, along riverbanks and in sugarcane fields, due to its ability to grow under shaded conditions. Subbarao et al. (2006) reported that this grass could release Biological Nitrification Inhibitor (BNI) 2–5 ATU/g root dry wt/d.

2.7.2 Creeping signal grass (Brachiaria humidicola (Rendle) Schweick)

This grass grows on a very wide range of soil types from very acid-infertile (pH 3.5), high Al soils, to heavy cracking clays, to high pH coralline sands. It is grows well in infertile soils with low Phosphate levels, but will respond to Nitrogen and Phosphate. It has a low Calcium requirement. Tolerant of poor drainage and often found on seasonally wet clays in valley bottoms.

(Source: http://www.tropicalforages.info/key/Forages/Media/Html/Brachiaria_humidicola.htm Access date 22/10/2014)

Figure 2.5 Creeping signal grass

(Source: http://nutrition.dld.go.th/Nutrition_Knowlage/ARTICLE/Pro6.htm Access date 22/10/2014)

Subbarao et al. (2006) reported that the amount of BNIs from Brachiaria humidicola roots were ranged from 17 to 50 ATU/g of root dry weight/d, which were higher than other Brachiaria spp. The grass played important role in nitrogen conservation in soil.

Gopalakrishnan et al. (2007) reported that *Brachiaria* species, produced two methylated phenolic acids, i.e. methyl-*p*-coumarate and methyl ferulate; which are Biological Nitrification Inhibitor compounds (BNIs).

Fernandes et al. (2011) studied three forage species (*Brachiaria brizantha*, *B. ruziziensis* and *B. decumbens*), and found that *Brachiaria* species reduced the $N\text{-}NO_3^-$ level in the soil, independently of the N rate, whereas the $N\text{-}NH_4^+$ level was increased, principally at lower N rates.

2.7.3 Mulato II or Brachiaria hybrid (*B. ruziziensis* x *B. decumbens* x *B. brizantha*)

Mulato II is a high yielding and vigorous. Produces 10–25% more dry mass than *B. brizantha* or *B. decumbens*. In Tabasco, Mexico, yields of up to 25 t/ha dry mass have been reported.

(Source: http://www.tropicalforages.info/key/Forages/Media/Html/Brachiaria_spp._hybrids.htm Access date 22/10/2014)

Figure 2.6 Brachiaria hybrid (Mulato II)

(Source: http://info.agri.ubu.ac.th/~ubuforage/photoMulatoII.html Access date 22/10/2014)

These grass species have not been report in phytoremediation of phenolic compounds contaminated site.

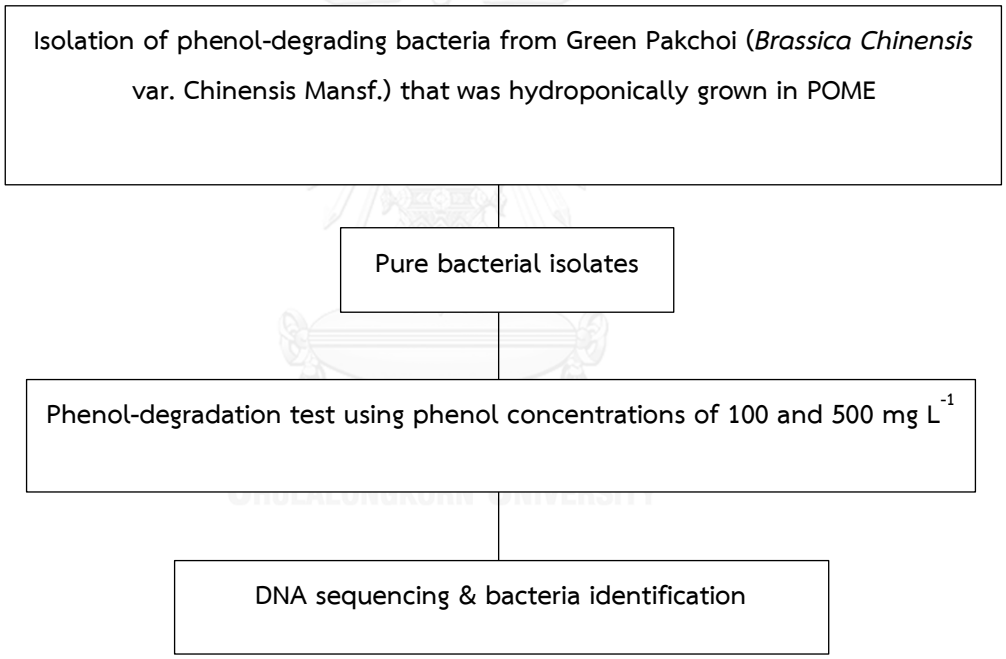
CHAPTER 3

MATERIALS AND METHODOLOGY

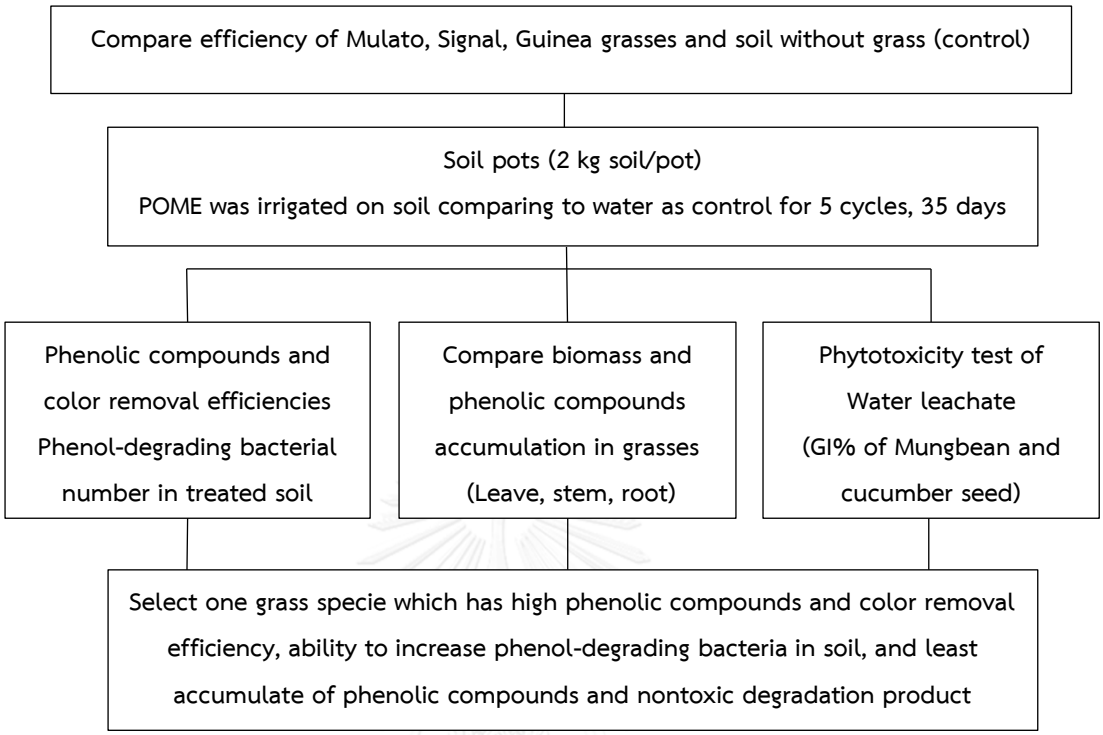
3.1 Flow chart of experimental procedure

The experiment was conducted in 4 phases to investigate the potential of bacteria and grass on removal of phenol, phenolic compounds and color from palm oil mill effluent

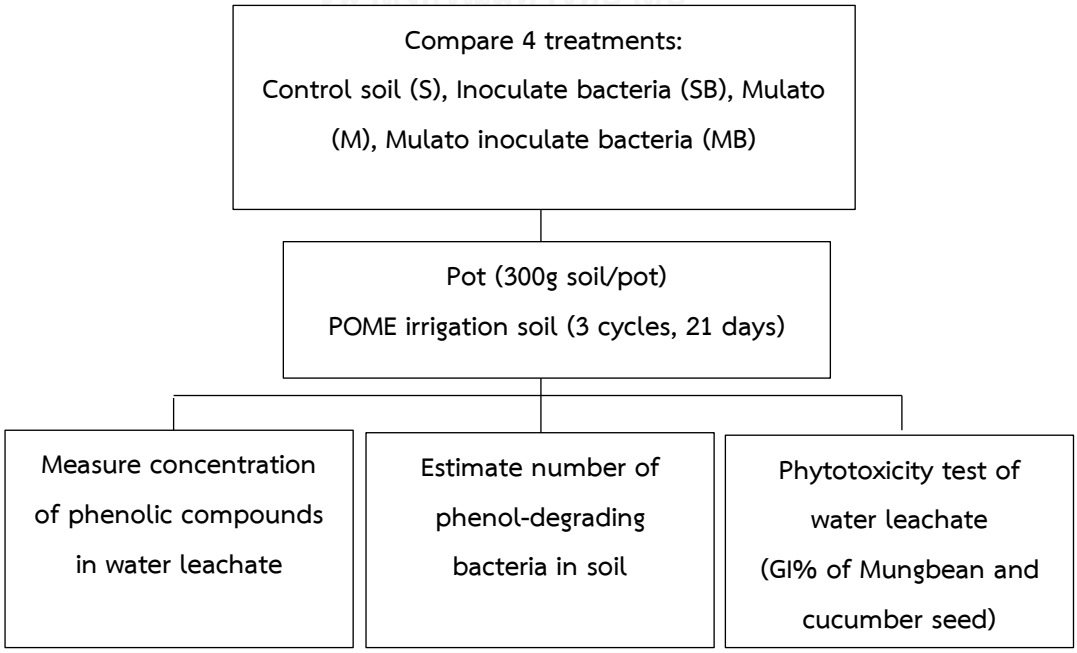
Phase 1 Isolation of phenol-degrading bacteria



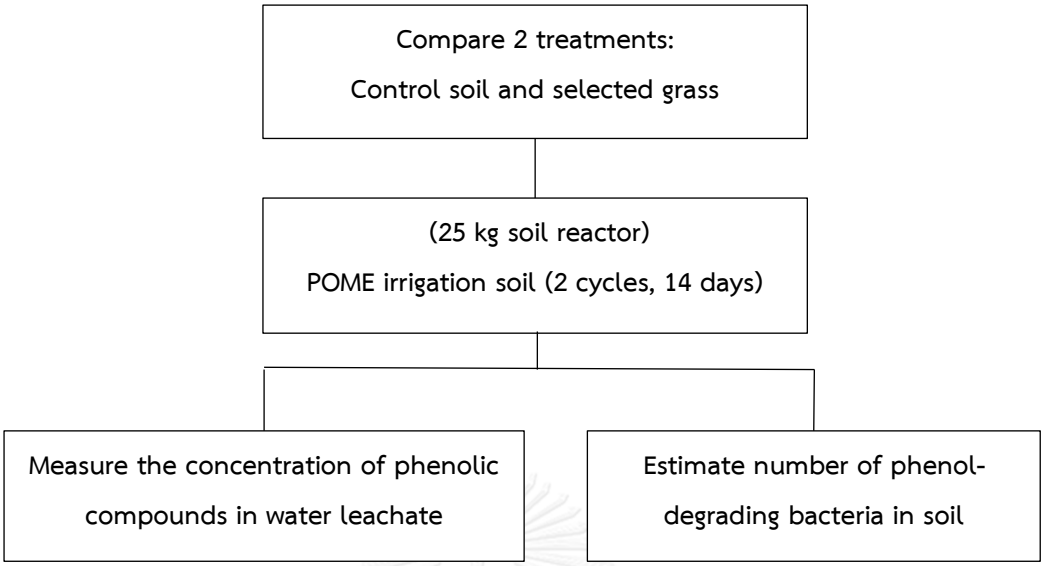
Phase 2 Selection of grass species



Phase 3 Examination of augmented phenol-degrading bacteria (phase 1) and selected grass (phase 2) to remove phenolic compounds from POME irrigated soil



Phase 4 Examination of rhizoreactor to treated POME irrigated soil



3.1.1 Palm oil mill effluent (POME)

Palm oil mill effluent (POME) samples were collected from the last stabilization pond of a palm oil mill in Surat Thani province, Thailand, as shown in Figure 3.1. It is a large-scale palm oil mill, which has production capacity of 45 tons palm oil/hour. Although this effluent is previously treated by anaerobic process, it still contains phenolic compounds and color higher than standard of industrial wastewater. The samples were collected and stored in 20 L bottles and kept at 4°C prior to use to avoid changes of wastewater properties.

Figure 3.1 The last stabilization pond of the palm oil industry

3.1.2 Soil material

Soil sample for planting experiment was excavated from planting area of the palm oil mill in Surat Thani province. Soil was screened through a 2 mm screen to separate slivers and grains, and then homogenized. Samples of the homogenized soil will be analyzed for water filled pore space before adding in pots. The soil was loamy sand, which contained sand (83.8%), silt (12.3%) and clay (3.9%) and has initial pH 7.8. The soil moisture was adjusted at 60% of water holding capacity before put in the pots.

Figure 3.2 Soil screening and (B) Pot filling

3.1.3 Grasses cultivation

Mulato II or Brachiaria hybrid (*B. ruziziensis* x *B. Decumbens* x *B. brizantha*) seeds and Guinea seeds were provided by Prof. Dr. Michael Hare, Ubon Ratchathani University, Creping Creeping signal grass (*Brachiaria humidicola*) stolon were provided from the Suratthani Animal Nutrition Research and Development Center. In this experiment, grass seeds and stolon were grown under greenhouse condition which temperature control around 30°C and soil humidity around 60% of water holding capacity. When the grasses were 2 week old, they were transplant to pots for later one month aging. The 45 days which healthier plant was select to further experiment.

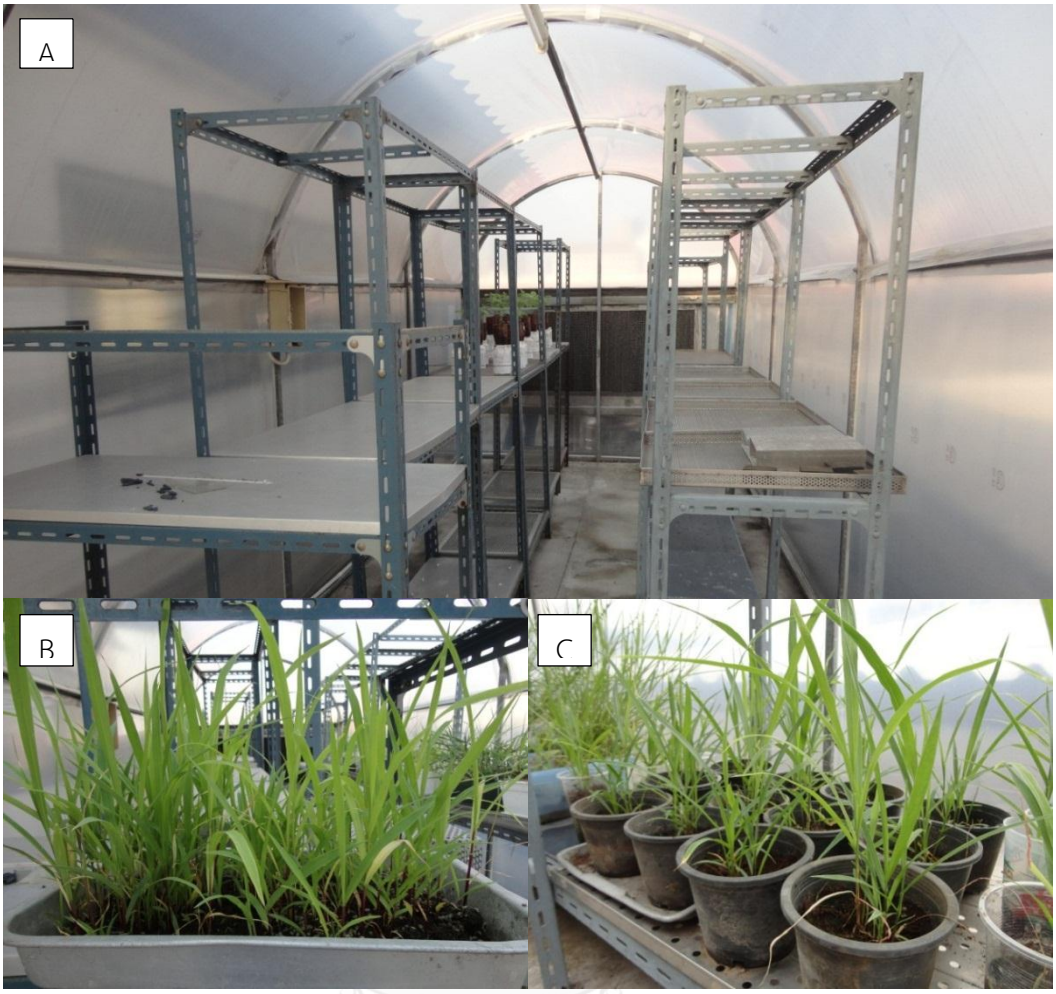


Figure 3.3 Grasses cultivation in greenhouse. (A) Greenhouse, (B) grasses 2 weeks old, and (C) transplant grasses to pots

3.2 Isolation of phenol-degrading bacteria and phenol degradation test

3.2.1 Isolation of phenol-degrading bacteria

Phenol-degrading bacteria were isolated from Green Pakchoi (*Brassica Chinensis* var. Chinensis Mansf.), which was previously grown hydroponically in palm oil mill effluent (POME). Plant seeds were sterilized with ethanol for 2 minute, and then seeds were washed by deionized water for twice. After that, each seed was put in sterilized 2 cm x 2 cm x 2 cm Polyurethane foam, which placed in a container and watered every day for a week. Then, POME was use instead of water.

After one week, only one plant was survived, which led to a hypothesis that "the survived plant will have some bacteria from POME associated with its root to reduce toxicity of POME". Thus, the plant was used to isolate phenol-degrading bacteria.

The survived plant was sterilized through deionized water twice then was put into 100 mL flask containing 50 mL of Carbon Free Mineral Medium (CFMM) supplemented with 100 mg L^{-1} phenol as the sole carbon and energy source, the flask was shaken at 200 rpm at room temperature for 7 days. Five mL of culture broth was transferred to fresh CFMM containing and 200 mg L^{-1} phenol and incubated under the same condition. The culture was spreaded on CFMM agar plate supplemented with 200 mg L^{-1} phenol for single colony isolation. The plates were incubated at room temperature for 7 days. The purity of isolate was streak on 25% Luria Bertani (LB) agar plate. The pure bacterium colony plate was sent to DNA sequence at Macrogen Korea for identify the organism.

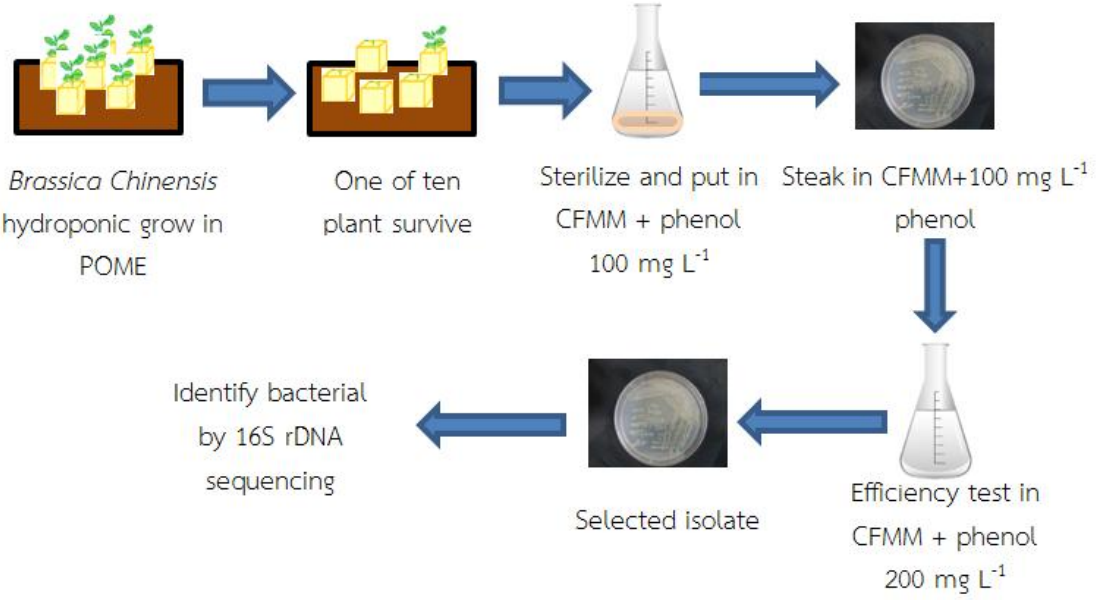


Figure 3.4 Process of phenol-degrading bacteria isolation

3.2.2 Efficiency of the isolated bacteria on degrading phenol

Phenol-degrading efficiency of the isolated bacterium was tested in 250 mL flask containing 100 mL carbon free mineral medium (CFMM); content NH_4NO_3 3 g L^{-1}, Na_2HPO_4 2.2 g L^{-1}, KH_2PO_4 0.8 g L^{-1} with add 1 mL L^{-1} trace metal solution. The stock phenol solution (10,000 mg L^{-1}) was filter-sterilized and individually added to different flasks containing sterilized CFMM medium to provide final concentrations of 100 and 500 mg L^{-1}. Then, 10% of inoculum (10^7 CFU mL^{-1}) was added to the medium and placed on shaker at 200 rpm for 24 h at room temperature. CFMM with phenol only at each concentration were used as control. Each treatment was set for triplicates. Every 3 h, samples were collected to measure bacterial growth by spectrophotometer at absorbance 600 nm and analyze for phenol remaining using Folin-Ciocalteau method. Phenol used in all experiments was purchased from Merck. Solutions were prepared with deionized water and all substances used were of analytical reagent grade.

Figure 3.5 Phenol degradation test in CFMM containing phenol 100 and 500 mg L^{-1} phenol and control.

3.3 Efficiency of grass on phenolic compounds and color removal

The abilities of three animals feed grasses on the removal of both phenolic compounds and color in soil were examined after irrigating with palm oil mill effluent (POME). Pot experiment containing 2 kg soil/pot and three plantlets were conducted for 5 weeks under greenhouse conditions. Three grass cultivars namely Mulato (M), Creeping signal (S) and Guinea (G) grasses were compared. Twenty one pots were separated in to 3 condition viz. bare soil watered by POME as control soil (C) representing natural attenuation, planting grasses pots with watered by tap water (MW, SW and GW) represent grasses grow under normal condition, planting grass pots with watered by POME (MP, SP and GP) represent grass rhizoremediation. Three replicates for each treatment. Every week, the soil was irrigated with POME for 3 times with soil moisture content at approximately 60% (w/w) of water holding capacity, which was counted as one cycle. All experiment plots represent arranged in a randomized complete block design for statistical comparisons of treatments. At the end of each cycle, soil was collected to measure phenol-degrading bacterial number. Then each pot was flushed with about 600 ml of tap water to collect approximately 200 ml of leachate for estimate residual phenolic compounds, color and phytotoxicity.

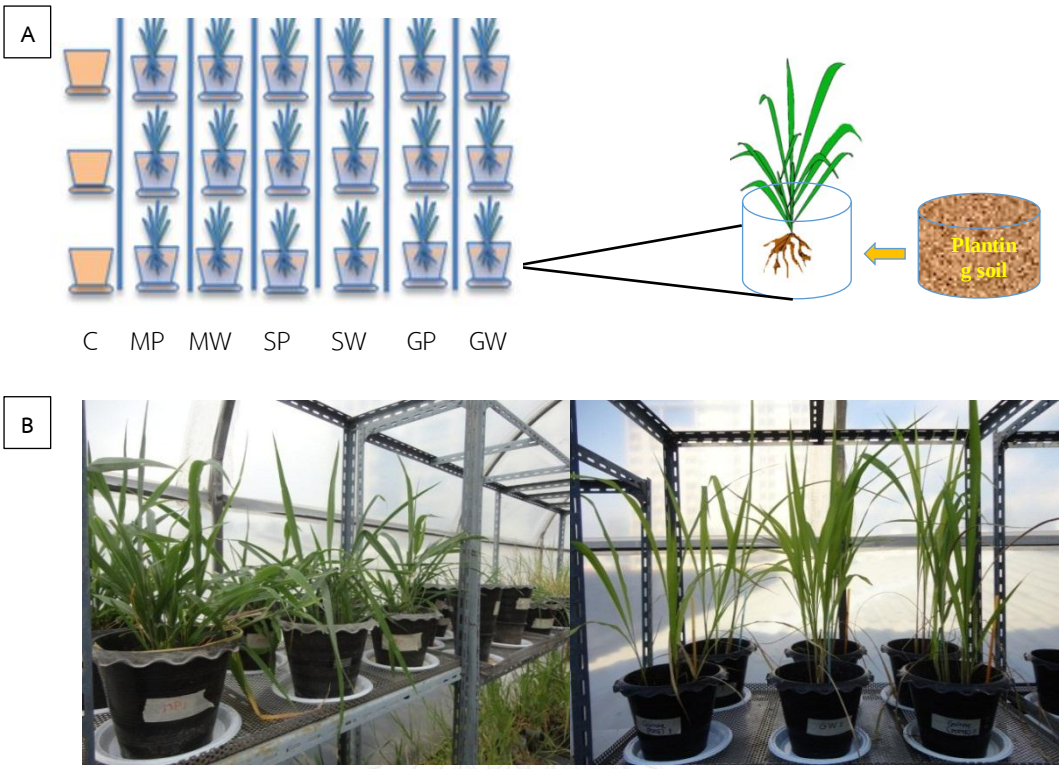


Figure 3.6 (A) Rhizoremediation treatments, (B) Experiment pots inside greenhouse

3.4 Efficiency of selected grass and added bacteria on removal of phenolic compounds from POME irrigated soil

There were four treatments viz. soil without plant and bacteria (C), soil with inoculated bacteria but without plant (SB), soil with plant but without inoculated bacteria (M) and soil with both plant and inoculated bacteria (MB). Triplicates were set for each treatment and pots were randomized in greenhouse.

For soil bioaugmentation, bacteria were cultured in 250 mL flasks containing 100 mL of 25% LB medium on rotary shaker at 200 rpm at room temperature (30°C) overnight to reach the mid-log growth phase. Then, cultures were centrifuged (8000 rpm), and pellets were washed twice with 0.85% NaCl. After this, the pellets were re-suspended in sterile DI. Next, 50 mL of this suspension was poured into pots (SB and MB) resulting in the initial bacterial number of 10^8 CFU g soil^{-1} and allowed the soil to settling for one day before start the experiment. POME was irrigated at 50 mL per pot

every 3 day interval as one cycle. At the end of each irrigation cycle, soil was collect to measure bacterial number and each pot were flushed with about 100 mL of tap water to collect approximately 20 mL of leachate. The leachate was examined for residual phenolic compounds and phytotoxicity.

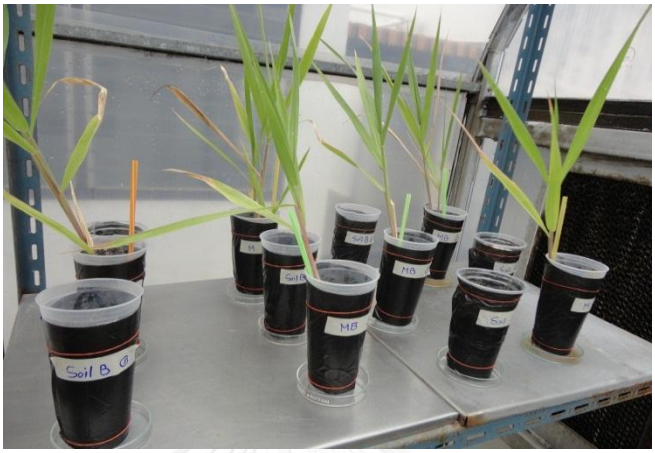


Figure 3.7 Experiment pots of four treatments were random placed inside greenhouse

3.5 Rhizoreactor test

To examine the potential of upscale rhizoremediation, 50x37x28 cm buckets were used to construct a rhizoreactor for 2-week operation under greenhouse conditions. Mulato grass as selected grass from previous experiment was used in these rhizoractor. Three treatments viz. soil reactor, Mulato reactor irrigate with POME, and control grass reactor irrigate with water. In each reactor, gravels (5 cm in height) were placed in the bottom of reactor to prevent fouling in outlet vault, then 25 kg of planting soil were added to form upper layer of 18-20 cm height. Two-week old of 25 healthy grasses were transplanted into each reactor. This grasses number was calculated according to plant/area that give best phenol removal efficiency in pot experiment (5 grasses per pot) from Viroj Rakkiatsakul (2013). Two sampling tubes (PVC) were added for collect samplings POME by syringe. One month after transplant, 6 L of POME was irrigate to provide water exceed the saturated soil, for control plant reactor 6L of tap water was irrigate instead of POME. POME was sampling at day 0, 3

and 7 to analyze phenolic compounds residual and soil was collect at day 7 for estimate phenol-degrading bacteria.

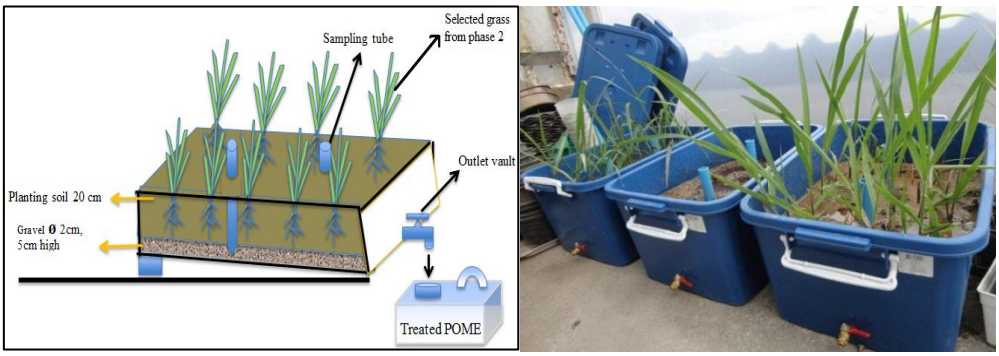


Figure 3.8 Transverse section of basic infrastructure of rhizoreactors and soil reactors

3.6 Analytical methods

3.6.1 Analysis of phenol and total phenolic compounds

Analysis of phenol and total phenolic compounds was modified from Bärlocher and Graça (2005) using Folin-Ciocalteau method. Briefly, 1 mL of liquid solution or water leachate sample was centrifuged at 10,000 rpm for 10 min to separate supernatant and sediment. For analysis, 100 μL of supernatant was adding to 150 μL DI water followed by 1 mL of 2% Na_2CO_3. After 5 min of incubation, 50 μL of Folin-Ciocalteau reagent was added and mixed. The sample was incubated for 1 h at room temperature (30°C), measure absorbance 760 nm. The remaining phenolic compounds is calculated by comparing to the standard curve and reported as gallic acid equivalents.

The percent removal of phenolic compounds can be calculated from the equation below:

$$\text{Percent removal (\%)} = \frac{\text{Initial phenolic compounds (mg)} - \text{Final phenolic compounds (mg)}}{\text{Initial phenolic compounds (mg)}} \times 100$$

Where the initial phenolic compounds were the total mass of phenol in irrigated POME and the final phenolic compounds were the total mass of phenol in water leachate from soil. Mass of phenolic compounds (mg) was calculated from the concentration in the sample (mg L^{-1}) time the total volume of the sample (mL).

Figure 3.9 Water leachate collection from grass pot

3.6.2 Total number of phenol-degrading bacteria in soil

Five gram of soil (from the top 5-10 cm of each pot) was collected using a 6 mm sterilized cork borer. The soil was sampled three times for each pot. The sampling protocol is adapted from (Lamichhane et al., 2012). The samples from all replicates of each treatment were combined and mixed. One gram of the mixed soil from each treatment was used to estimate the phenol degrading bacteria by drop plate technique. Briefly, one gram mixture of soil sample from each treatment added to 9 ml of normal 0.85% saline solution and mixed by vortex mixer then do ten-fold dilution. Ten μL of the cell suspensions was dropped into CFMM supplement with 100 mg L^{-1} phenol as carbon source, the agar plates were incubated at 30°C, after 5 days phenol-degrading bacteria was counted and reported as log CFU/g soil.

3.6.3 Phytotoxicity test

Ecotoxicity of treated pollutant was recommended to assess due to some degradation product are more toxic than untreated pollutant (Anastasi et al., 2011). Mungbean (*Vigna radiata* (L) Wilczek) and cucumber *(Cucumis sativus* L.var) were

used to determine whether the leachate from soil was toxic or not compare to control (untreated POME) and DI (positive control). All of these plant seeds represent common vegetable seeds, which are sensitive to organic and inorganic pollutants (Lin and Xing, 2007; Nisha and Sreedevi, 2008). The experiments were conducted in triplicates by placing ten seeds in separate Petri dishes and adding 5 mL sample daily. Percent of germination index (GI %) and the length of radicle (root) were recorded after 4 days.

Percent of germination index (GI %) was calculated according to the following formula:

$$GI\% = (Gt \times Lt)/(Gc \times Lc) \times 100$$

Where Gt is the mean number of germinated seeds in the treatment sample, Lt is the mean root length of the treatment sample, Gc is the mean number of germinated seeds in the control (DI water), and Lc is the mean root length of the control.

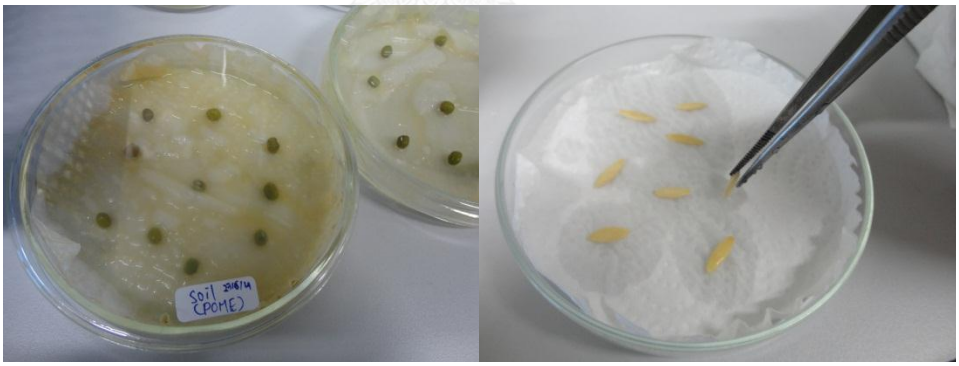


Figure 3.10 Seed germination phytotoxicity test using Mungbean seeds (left) and cucumber seeds (right).

3.6.4 Grass biomass and phenolic compounds accumulation

Criteria to select grasses among three species were their biomass and the phenolic compounds accumulation. In the end of experiment, all grasses were harvested. Each specie was cut and separate into three parts (leave, stem and root) then clean dirt particle by washing with clean water, after that air dry in greenhouse for 7 days. After 7 days, their dry mass was measured and compared.

Figure 3.11 Grass root after POME irrigation for 5 cycles, from left to right Mulato, Creeping signal and Guinea grasses

Phenolic compound extraction from each part of grasses was modified from Hancock and R. Dean (1997). Briefly, 10 mL of methanol/water (60/40 v/v) was added to one gram of sample. The homogenate was mixed and sonicated for 1 h. The mixed was centrifuged at 10,000 rpm for 10 min. The supernatant was analyzed for a total phenolic by Folin-Ciocalteau method.

Figure 3.12 Process of phenolic compounds extraction; (A) measurement of grass dry weight, (B) grinding by mortar and pestle (C) grinded biomass, (D) phenolic extraction by methanol/water (60/40v/v), (E) sonication for 1 h, and (F) Folin-Ciocalteau reagent was added for phenolic compounds measurement.

3.7 Statistical analysis

Statistical test were performed using IBM SPSS software (Statistic Version 20 for Window 7). All dependent variables were analysis of variance (ANOVA) and multiple comparison analysis using a Turkey test with $P<0.05$.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Isolation of phenol-degrading bacteria from plant roots

Three bacterial strains were initially isolated from plant roots on CFMM agar plate supplemented with phenol. Each isolate was tested for its ability to utilize phenol at concentrations 100 mg L^{-1} and 200 mg L^{-1}. Among these isolates, the strain OPB was selected for further studies because this strain had the highest degradation rate which was capable of removing 200 mg L^{-1} phenol in CFMM after 10 h (Appendix C).

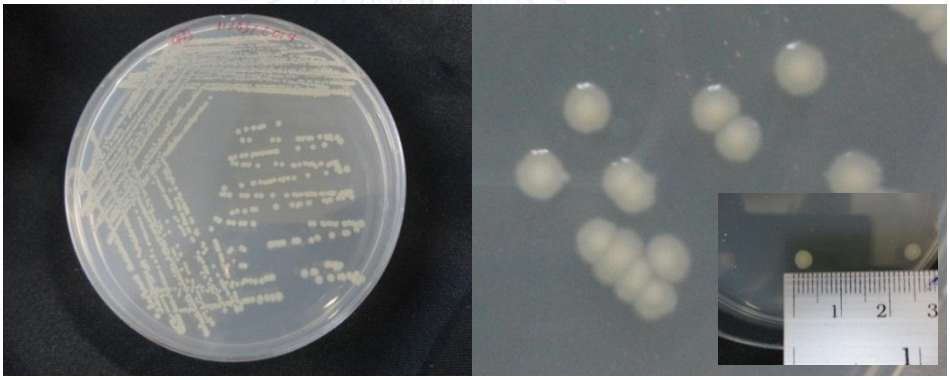


Figure 4.1 (A) The colony of *Acinetobacter* sp. OPB on 0.25% LB agar and (B) colony size

The selected bacterial isolate was identified using 16S ribosomal DNA (16S rDNA) sequence analysis. The result showed that the 16S rDNA had 99% sequence similarity to those of the genus *Acinetobacter* sp. Thus, OPB was identified as *Acinetobacter* sp. strain OPB.

4.1.1 Phenol degradation and growth of *Acinetobacter* sp. strain OPB

The results of batch studies for phenol degradation in CFMM by Acinetobacter sp. strain OPB are shown in Figure 4.2 (A). The bacterium could remove phenol completely after 6 and 15 h incubation for 100 and 500 mg L^{-1} phenol,

respectively. The complete degradation of phenol was showed as clear reaction of liquid medium with Folin-Ciocalteau reagent as in Figure 4.3. The increasing of bacterial growth (OD 600 nm) was correlated with the decreasing of phenol as in Figure 4.2 (B). This result indicated that the bacterium could grow with phenol as sole carbon and energy source. Similarly, Ahmad et al., (2012) and Wang et al., (2007) reported that Acinetobacter sp. strains could rapidly degrade phenol at concentrations between 100-500 mg L^{-1} within 3-16 h. Thus, Acinetobacter sp. strain OPB could be applied to enhance phenol removal in contaminated site.

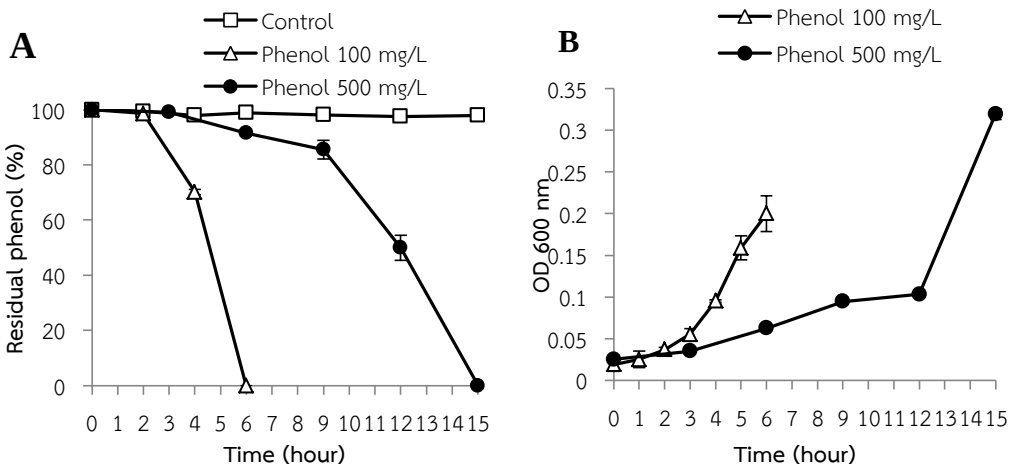


Figure 4.2 (A) Degradation profile of phenol and (B) growth curves of *Acinetobacter* sp. OPB in CFMM with phenol concentrations (100 and 500 mg L^{-1}). Control was the treatment without added bacteria.

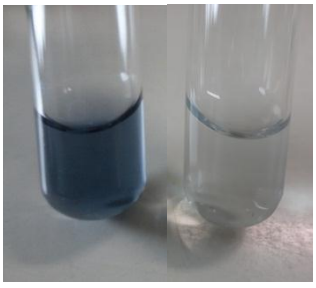


Figure 4.3 Reaction of phenol with Folin-Ciocalteau reagent in control (left) and *Acinetobacter* sp. OPB treatments (right)

4.1.2 Comparison of phenol degrading efficiency between *Acinetobacter sp.* strains OPB and *Acinetobacter sp.* strains PK1

Acinetobacter sp. PK1 was previously isolated as a contaminant during cultivation of *Methylobacterium* sp. NP3 in the presence of high phenol concentrations (Khongkhaem et al. 2011). They also reported that the co-culture of *Acinetobacter* sp. PK1 and *Methylobacterium* sp. NP3 had higher phenol-degrading activity than either strain alone. This study used *Acinetobacter* sp. PK1 as a reference strain for comparison with *Acinetobacter* sp. strain OPB.

According to the results, phenol degradation of both *Acinetobacter* sp. strains (OPB and PK1) was associated with growth, which indicated that phenol was used as carbon and energy sources for the bacteria (Figure 4.4). The degradation of 100 mg L^{-1} phenol showed a similar trend in both strains with only 6 hours for a complete degradation. At 500 mg L^{-1} phenol, there was a significant different in phenol degradation between OPB and PK1 which was shown in Figure 4.4 (B). Bacterial strain OPB completely degraded phenol after 18 hrs, while PK1 required 48 hrs.

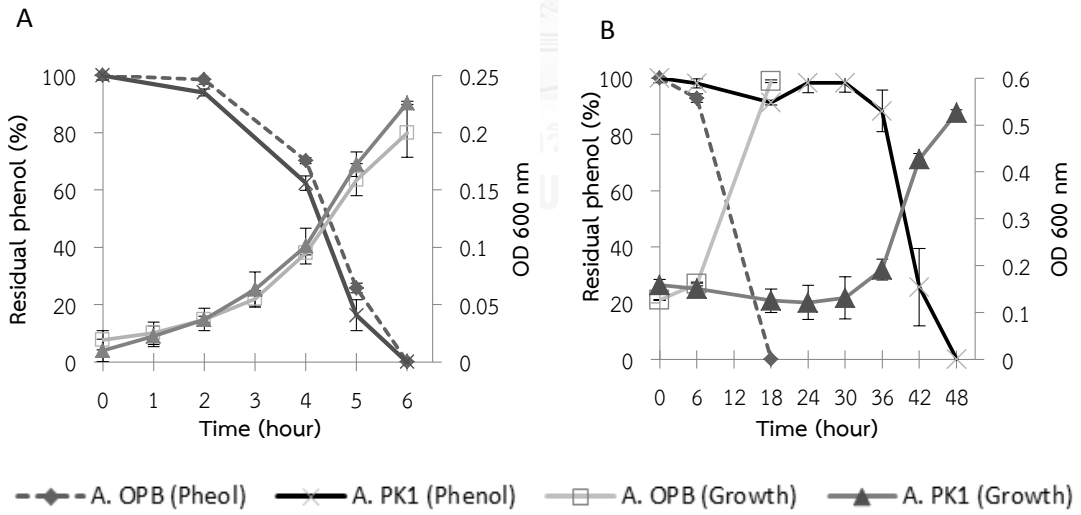


Figure 4.4 Comparison of Acinetobacter sp. strains OPB and PK1 on phenol degradation at (A), 100 mg L^{-1} and (B) 500 mg L^{-1}.

This result confirmed that bacterium strain OPB had high phenol degradation ability and required shorter time than PK1 for phenol adaptation and degradation.

4.2 Selection of grass for phenolic compounds and color removal

This study examined the abilities of three animal feed grasses to remove both phenolic compounds and color in soil after irrigating with palm oil mill effluent (POME). Phenolic compounds concentrations and color unit in this experiment were in the range of 360-420 mg L^{-1} and 4,000-5,000 Pt/Co units, respectively. The criteria to select grass were 1) to provide the highest removal of phenolic compounds and color from POME, 2) to have the lowest accumulation of phenolic compounds in biomass, 3) to increase the number of phenol-degrading bacteria in soil, and 4) to produce nontoxic products in soil leachate.

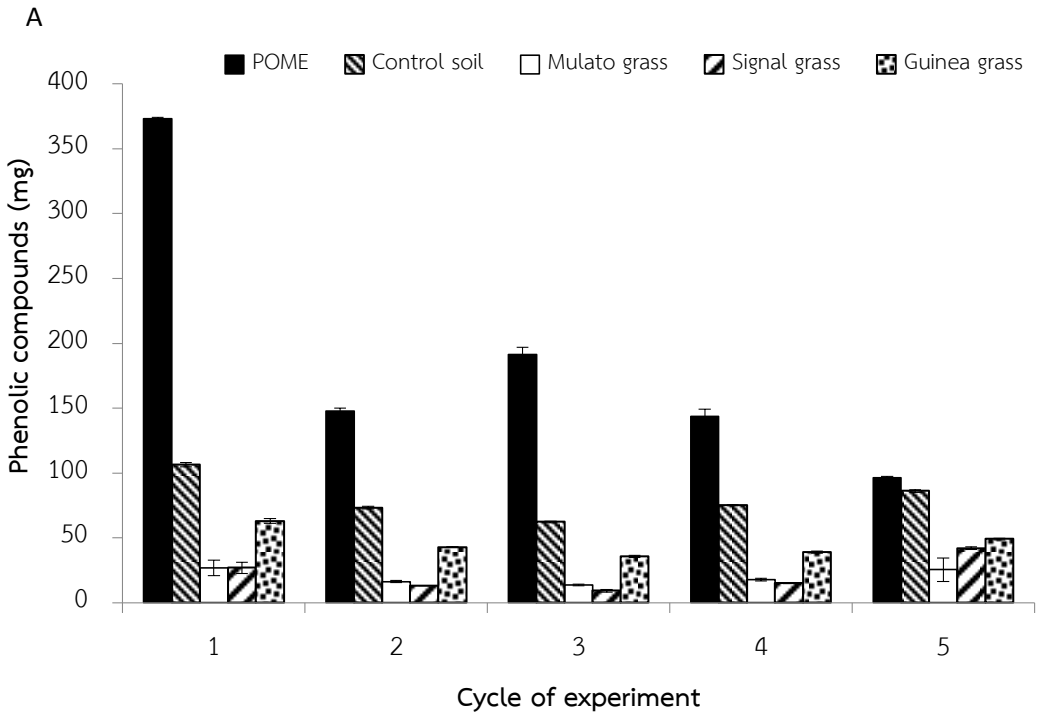
4.2.1 Phenolic compounds and color removal by three grass cultivars

Pot experiment was conducted for 5 weeks under greenhouse conditions. Three grass cultivars namely Mulato, Creeping signal and Guinea grasses were compared. Every week, the soil was irrigated with POME for 3 times, which was counted as one cycle. The different phenolic compounds represent in each irrigation cycle was due to the different volume of POME irrigation, which was added to maintain 60% of water holding capacity to each pot. To determine the effect of POME irrigation, the mass of phenolic compounds in Figure 4.5 were normalized by comparing between grass cultivar which irrigated with POME and their control that irrigate with water. The values in graph were equal to values from treatment (POME) subtracted with values from grass control treatment (root exudate phenolic) as in Appendix D.

From the results in Figure 4.5 (A) phenolic compounds was reduced in Mulato and Creeping signal pots more than Guinea pot between 1^{st} to 4^{th} irrigation cycles. When compare between Mulato and Creeping signal grasses pots, the phenolic compounds removal was not significant different. In this experiment we found that phenolic compounds and color removal were in same trend which significantly reduced in planted soil more than unplanted soil. These were mostly effective in the 1^{st} - 4^{th} cycles with 87-93%, 90-95% and 65-83% for phenolic compounds and 84-92%, 90-96% and 82-96% for color removal by Mulato, Creeping signal and guinea grasses, respectively (Table 4.1). At the same time, the control soil removed 39-72%

and 59-71% of phenolic compounds and color, respectively (Table 4.1). The color reduction also clearly observes in Figure 4.6.

However, phenolic compounds were clearly increased in the 5th irrigation cycle. These might be due to the accumulation of more complex structures of phenolic compounds, which were more difficult to degrade. Other possible reason was the increasing in soil pH from the initial pH of 7.9 to 8.9 in the end of experiment (Appendix G). Zieslin and Abolitz (1994) explained that more phenolic compounds could leak from plant roots due to the increasing of soil pH, which was similar to our result on soil pH. The increasing of soil pH was corresponded with pH of POME from the last stabilization pond at 8.9-9.3.



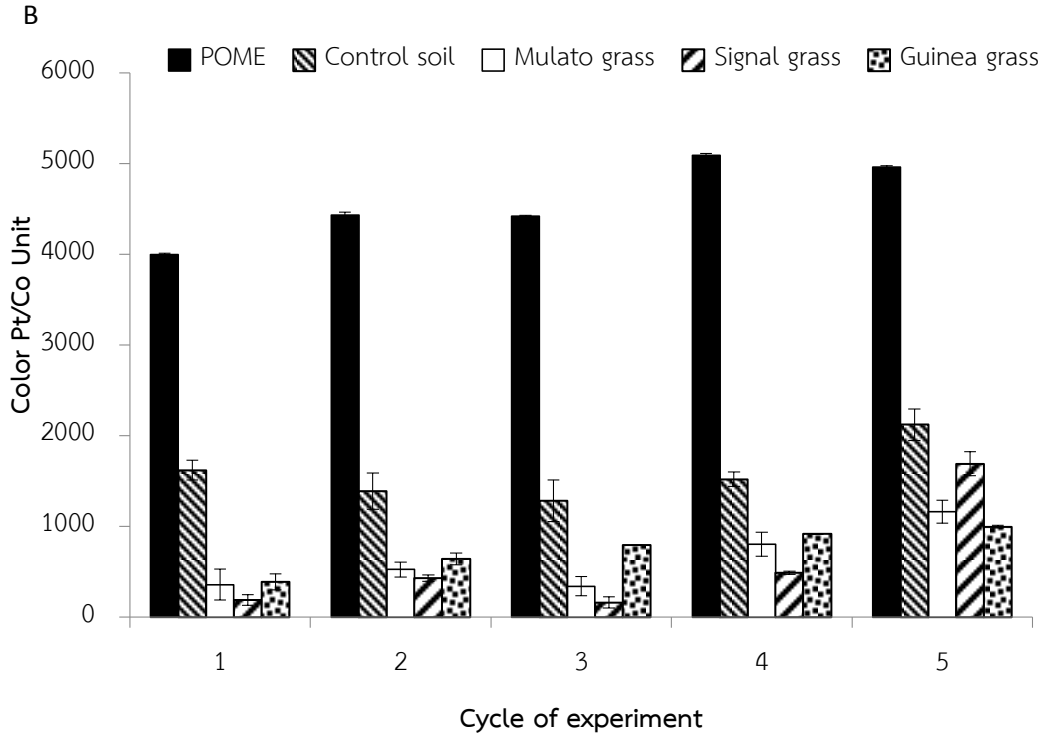


Figure 4.5 (A) Residual mass of phenolic compounds (mg) and (B) color (Pt/Co unit) in POME and soil leachates after five cycles of POME irrigation. Error bar represent the standard deviation of three sample pots.

Table 4.1 Comparison of phenolic compounds and color removal (%) of each treatment

Phenolic compounds removal (%)					
Treatments	First cycle	Second cycle	Third cycle	Forth cycle	Fifth cycle
Control soil	71.5±0.4	39.4±1.3	67.3±1.2	57.9±1.9	31.0±0.7
Mulato grass	92.8±0.3	86.5±0.6	92.8±0.2	90.1±0.2	79.6±0.4
Creeping signal grass	92.8±1.9	89.2±0.3	95.2±0.3	91.6±0.8	66.3±1.0
Guinea grass	83.1±0.4	64.7±0.1	81.2±0.2	78.2±0.4	60.7±1.1

Color removal (%)					
Treatments	First cycle	Second cycle	Third cycle	Forth cycle	Fifth cycle
Control soil	59.4±2.7	68.7±2.5	71.0±3.7	70.1±2.0	57.2±3.9
Mulato grass	89.3±0.2	88.1±2.0	92.2±2.6	84.2±3.1	76.6±2.5
Creeping signal grass	95.3±1.4	90.1±3.6	96.3±1.6	90.3±0.4	65.9±2.6
Guinea grass	90.3±2.2	85.5±1.6	81.9±0.1	82.0±0.1	79.4±1.1

The percent removal of phenolic compounds or color in Table 4.1 could be calculated from the equation below:

$$\text{Percent removal (\%)} = \frac{\text{Initial concentration (POME)} - \text{Final concentration(treatment)}}{\text{Initial concentration (POME)}} \times 100$$

Where the initial concentration and final concentration of phenolic compounds of each cycle were the total mass of phenolic in irrigated POME (mg) and the total mass of phenolic in water leachate from treatment (mg), respectively. While color concentration was show as Pt/Co unit. The percent removal represent the efficiency of treatment.

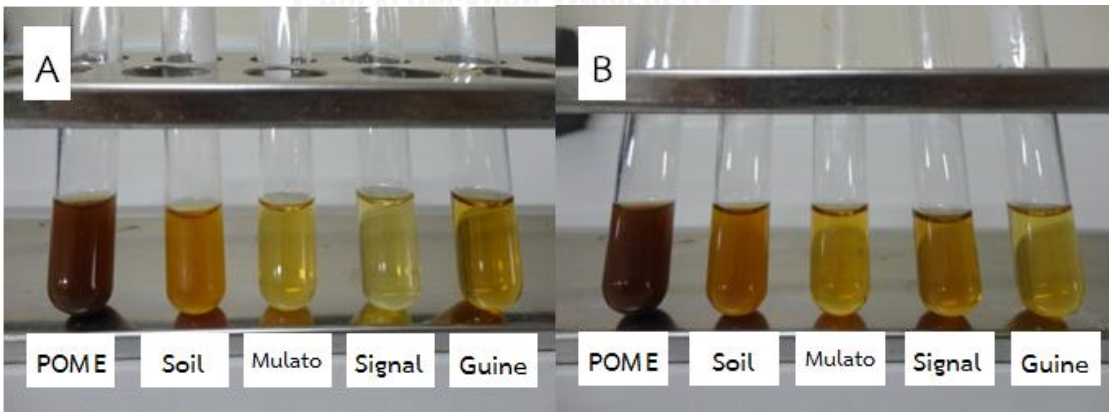


Figure 4.6 Color of POME and soil leachates after treatment for the third irrigation (A) and the fifth irrigation (B)

4.2.2 Phenol-degrading bacteria in rhizosphere of three grass cultivars and control soil

Phenol degrading bacteria were counted every cycle of experiment and numbers of bacteria are show in Figure 4.7. After 5 cycles of experiment, the results show that phenol-degrading bacteria of the soil planted with Mulato, Creeping signal and Guinea were increased from 5.89 log CFU g^{-1} soil to 8.16 log CFU g^{-1} soil, 7.52 log CFU g^{-1} soil and 7.27 log CFU g^{-1} soil, respectively. The numbers of rhizosphere bacteria were significantly higher than bacterial number in control soil (6.14 log CFU g^{-1} soil) (Figure 4.7). These results were in agreement with the findings by Baneshi et al, (2014) the bacterial population in the rhizosphere was 9 log CFU g^{-1} soil, while in the non-rhizosphere soils was 7 log CFU g^{-1} soil. When compared between grass cultivars, phenol degrading bacteria could be more stimulated by plant roots of Mulato, which had the number of phenol-degrading bacteria higher than that soil with Creeping signal and Guinea grasses (Figure 4.7). These result may due to the more root was presented in Mulato plot than other two cultivars as shown in Figure 4.9.

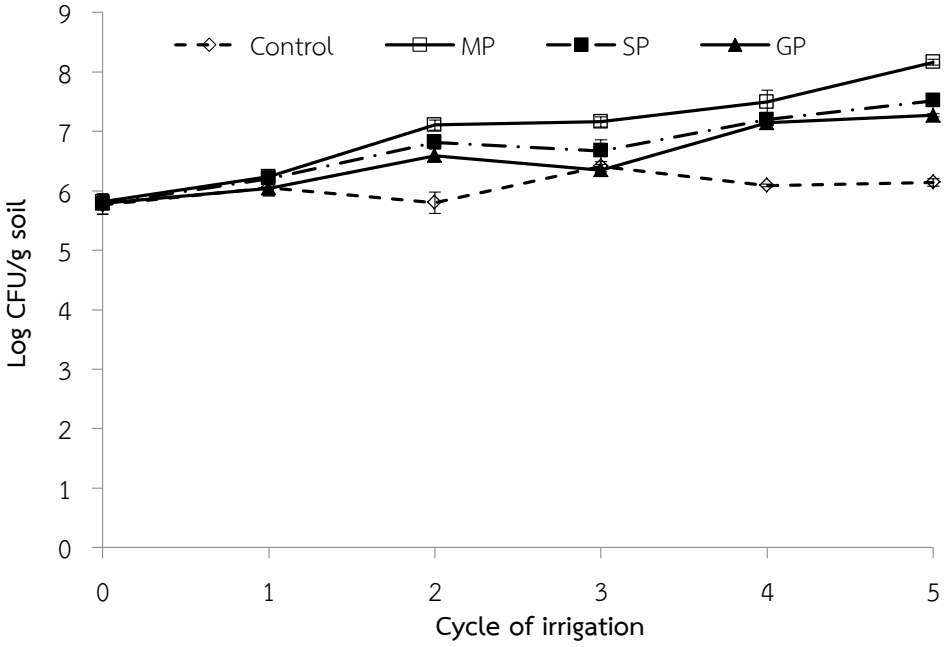


Figure 4.7 Phenol degrading-bacteria in soil from control, Mulato (MP), Creeping signal (SP) and Guinea (GP) pots.

To investigate the correlation between phenolic compounds and color removal with phenol-degrading bacteria, the average of % phenolic compound and color removal from each treatment of the fifth irrigation cycle were plotted against the number of phenol-degrading bacteria in Figure 4.8. The graph shows a positive significant correlations with R^2 = 0.936 and 0.809 for phenolic compounds and color respectively. These results confirmed that phenol-degrading bacteria were responsible for both phenolic compounds and color removal from POME irrigated soil.

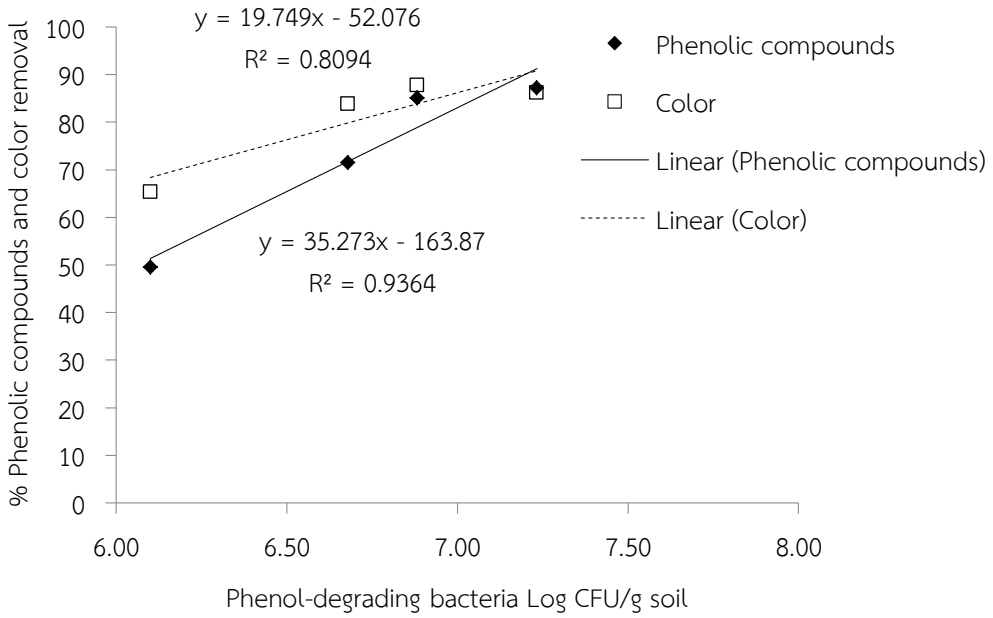


Figure 4.8 Correlations between percentage of phenolic compounds and color removal and phenol-degrading bacteria populations in soil.

4.2.3 Plant biomass and phenolic compounds accumulation in each part of 3 grass cultivars

Plant biomass and phenolic compounds accumulation in plant leaves, stems and roots of each grass cultivar were compared between the pots irrigated with POME and with tap water at the end of experiment. From the results, the changes in biomass of leaves, stems and roots from each grass cultivar were in the same trend (Figure 4.9 A, B, C). Mulato and Creeping signal grasses from pots that irrigated with

POME had decreased biomass when compare to the pots irrigated with water (Figure 4.9 A, B, C). This could be explained that both *Brachiaria* grasses might be more sensitive to toxic compounds in POME than Guinea grass. The Guinea grass biomass seem to be increased but was not significantly different when compared to its control. Their physiology could be observed in Figure 4.10. In the end of experiment, some of Mulato and Guinea grasses leaves were clearly changed to yellow color in treatment with irrigated POME. However, the control grasses that irrigated with tap water were healthy. The toxicity was not clearly observe in Creeping signal grass. This is probably because Mulato and Guinea grasses had leaves more than stems, while the Creeping signal had more stem than leave. This phenomenon shows the toxic of POME to grass physiology.

To reduce the toxicity of POME, Nwoko and Ogunyemi (2010b) suggested that POME should be aerobically-fermented before apply as plant fertilizer. The procedure includes mixing fresh POME with 0.8 g/L of urea to facilitate microbial activity and N mineralization, stirring at least once a day to provide aeration, and controlling temperature at 30°C for 20 days. The fermented POME has reduce toxicity and give positive effect of plant growth. In the application, we have to mixed urea as fertilizer with treated POME from stabilized ponds in separate containers, this will help well mixing before use as irrigate.

Phenolic compounds accumulation of all three grass species in pots with POME were equal or less than control pots with tap water for leaves part (Figure 4.9 D). While in stem part (Figure 4.9 E), phenolic compounds in Creeping signal grasses seem to be higher than the control grasses. In root part (Figure 4.9 F), phenolic compounds accumulation in all three grass cultivars were not significant different when compare to the control. These results suggest that the aerial parts of Mulato and Guinea grasses could accumulate phenolic compounds at lesser extent than Creeping signal grass. Thus, it might cause public concerns when apply Creeping signal grass as animal feed, but not other grasses.

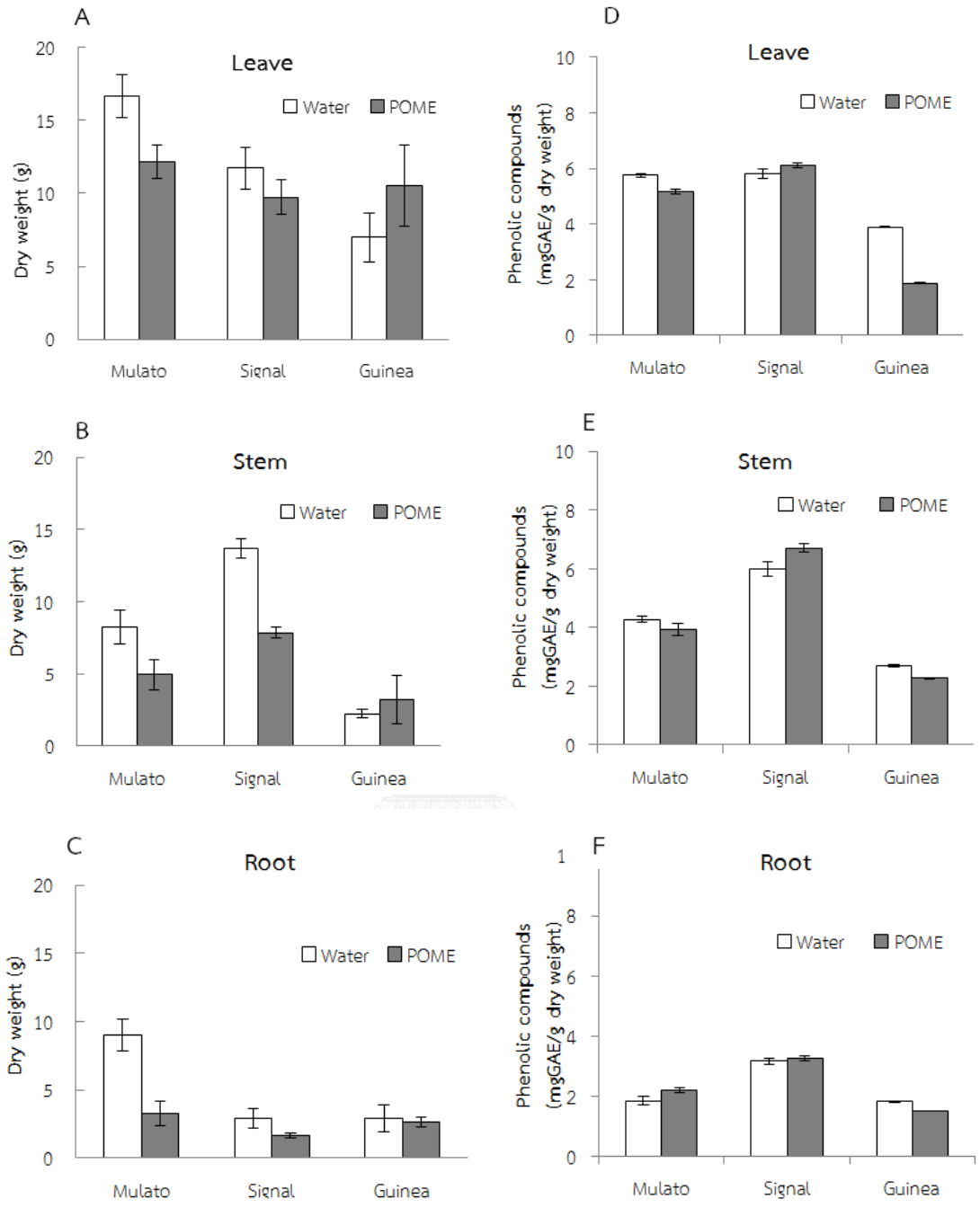


Figure 4.9 Biomass (A-C) and (D-F) phenolic compounds concentration in Mulato, Creeping signal and Guinea grasses leaves, stems, and root, respectively.

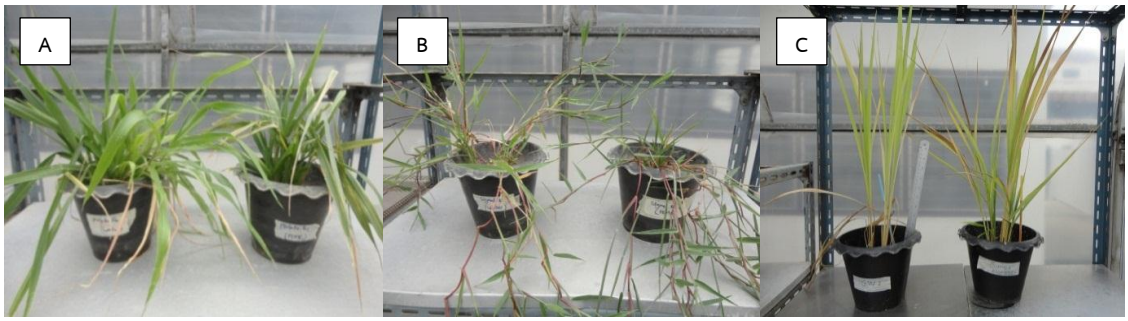


Figure 4.10 Grasses after treatment (A) Mulato, (B) Creeping signal, and (C) Guinea. The left pot was irrigated by tap water, while the right pot was irrigated by POME.

4.2.4 Phytotoxicity test of leachates from grass rhizoremediation

Toxicity of leachates containing degradation products from each treatment was examined. Sample with GI % value more than 50 % were consider as non-toxic Anastasi et al. (2011). The water leachate from the third and fifth cycles were tested for their toxicity to mungbean and cucumber seeds germination index percent (GI %). The results were compared to untreated POME (negative control) and deionized water (positive control). Results from Table 4.2 showed GI% of water leachate from Guinea and Mulato pots were equal or more than 50%, which indicate that they were nontoxic to the seed germination. On the other hand, GI% of water leachate from the final cycle of Creeping signal treatments were only 9.2% and 0% for mungbean and cucumber seeds, respectively. This percent of germination index were reduced when compare to the third cycle. This result indicated that more toxic by-products were accumulated in soil when more treated cycle were conducted. This was similar with result found by Viroj Rakkiatsakul (2013).

Among three grass species, the leachates from Creeping signal grasses pots were the most toxic to mungbean and cucumber seeds than that from Guinea and Mulato pots. The result was corresponded with the higher phenolic and color compounds remaining in soil after POME irrigation for 5 cycles (Figure 4.5). Nonetheless, the water leachate from control soil (unplanted) as natural attenuation was the most toxic as none of mungbean and cucumber seeds could be germinated.

Table 4.2 Phytotoxicity test of water leachate from three grass cultivars and their control based on mungbean and cucumber seed germination index (GI%)

Treatments	Germination index (%)			
	Third irrigation		Fifth irrigation	
	Mungbean	Cucumber	Mungbean	Cucumber
Deionize water	100	100	100	100
POME	25.0±3.4	21.6±1.3	26.3±2.5	14.1±2.9
Control soil	31.0±4.3	24.7±2.8	0.0±0.0	0.0±0.0
Mulato (POME)	59.5±2.9	51.5±2.5	64.5±3.5	60.0±3.9
Mulato (water)	79.8±0.8	83.5±2.6	92.1±4.3	87.5±4.2
Creeping signal (POME)	44.0±4.3	40.2±2.3	9.2±2.2	0.0±0.0
Creeping signal (water)	69.0±1.3	60.8±3.7	75.0±4.8	71.3±3.7
Guinea (POME)	51.2±2.9	44.3±1.9	52.6±2.0	50.0±2.5
Guinea (water)	79.3±1.1	83.5±0.9	85.3±2.9	75.0±3.7

Values represent mean ± standard deviation

In Viroj Rakkiatsakul (2013), creeping signal (*Brachiaria humidicola*) has the highest phenolic compounds removal efficiency. However, when compare to Mulato in this experiment, the phenolic compounds removal efficiency was not significant difference between both grass cultivars. On the other hand, in phytotoxicity test, water leachate from Creeping signal rhizoremediation pots were more toxic after the fifth irrigation cycle, while the leachate from Mulato pots were consider as non-toxic. This result may correlate with phenol-degrading bacteria that found in rhizosphere of Mulato in fifth irrigation cycle has higher than that found in Guinea and Creeping signal pot (Figure 4.7). From these results, Mulato grass was selected for further study.

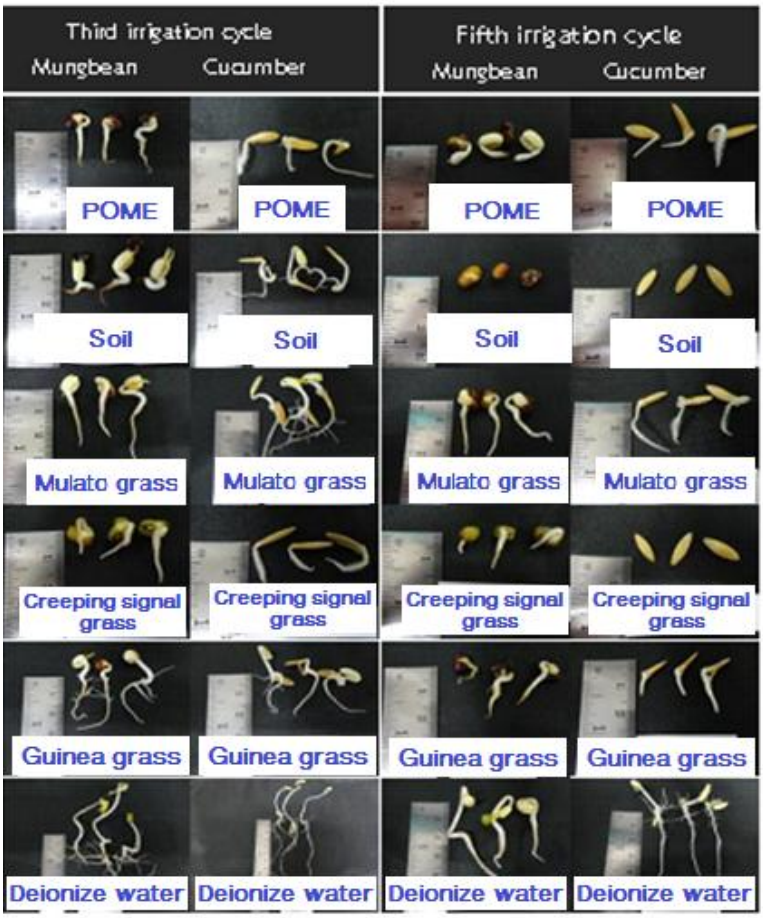


Figure 4.11 Effect of water leachate from the third and fifth irrigation cycles to mungbean and cucumber seeds germination. The tested samples were POME as negative control, control soil, Mulato grass, Creeping signal grass, Guinea grass and Deionize water as positive control.

From our results, after 1 month of POME irrigation, the phenolic compounds remaining in soil with Mulato grass cultivation were increased but at lesser extent than soil alone. To extend the ability of Mulato grass and its rhizosphere bacteria, we suggest that several cultivate areas should be constructed and alternatively irrigated them with wastewater and natural water. This will allow the grass and its rhizosphere bacteria to recover from toxic compounds in the wastewater. Another way to improve the rhizoremediation could be to inoculate the soil with plant-growth promoting rhizobacteria (PGPR) to enhance the plant growth and thereby restore the activity of rhizosphere bacteria (Glick, 2010).

4.3 Efficiency of selected grass and added bacteria on phenolic compounds from POME irrigated soil

4.3.1 Phenolic compounds removal by Mulato grass with bacterial inoculation and their phenol-degrading bacterium number

Since, Mulato grass could not completely remove phenolic compounds and color from soil irrigated with POME. This experiment examined the enhancing ability of *Acinetobacter* sp. OPB for rhizoremediation of phenolic compounds in soil. *Acinetobacter* sp. OPB was inoculated in both planted and unplanted soil. Phenolic compounds removal (%) from water leachate at the end of each irrigation cycle is shown in Figure. 4.12 (A). The initial phenolic compounds in POME were 25 mg, 26 mg and 24 mg in the first, second and third irrigation, respectively. Phenolic compounds removal (%) was significantly increased in both planted and unplanted soil with inoculated bacteria, but more noted in soil that plant roots were presented. Phytoremediation alone (M) and bioaugmentation alone (SB) had the removal efficiency about 72-75% and 71-76%, respectively. The most effective of phenolic compounds removal were the treatment with both Mulato grass and inoculated bacteria (MB) with 78-82% of phenolic compounds removal, which was significantly higher than that of control soil (S) (55-65%). From this result, the bioaugmentation of *Acinetobacter* strain OPB could enhance the phytoremediation of soils contaminated with phenolic compounds by 7%.

Similar results were also observed by Baneshi et al. (2014). They found that phytoremediation alone had the removal efficiency of pyrene and phenanthrene from the contaminated soil samples about 63% and 74.5%, respectively. In the combined mode, bioaugmentation can significantly enhance the phytoremediation of soils contaminated with 22% of pyrene and 16% of phenanthrene. In other study, Cordova-Rosa et al (2009) reported that textile sludge-contaminated soil contained 19.48 mg kg^{-1} phenol inoculated with *Acinetobacter calcoaceticus* var. *anitratus*, and bacterial consortium of the activated sludge could remove phenol up to 81% when compare to control soil. From these results, bioaugmentation of mixed bacterial strains during rhizoremediation could be a more effective tool. Glick (2010) also

suggested that bioaugmentation with biodegradative bacteria, plant growth-promoting bacteria and bacteria that facilitated phytoremediation could overcome some limitation such as changing pollutant form to increase bioavailability for plant and increasing plant tolerate to various environmental stresses.

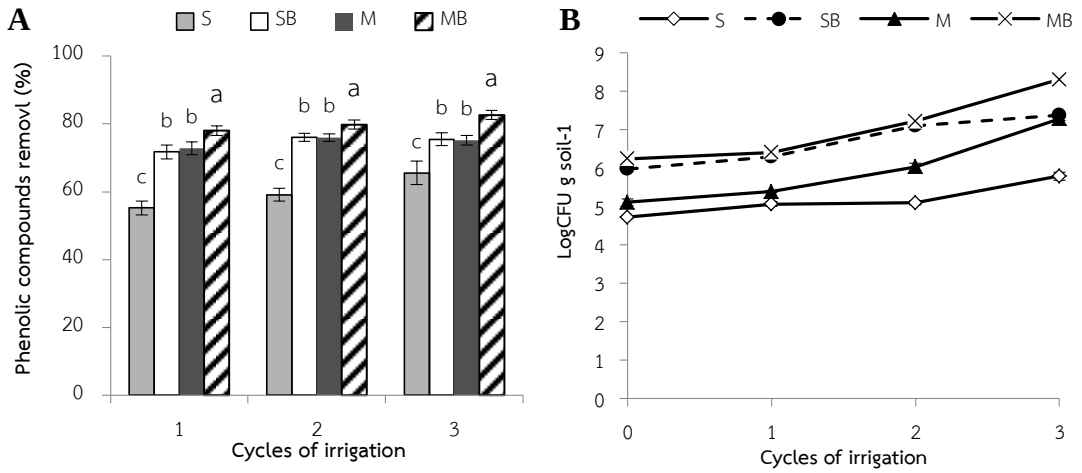


Figure 4.12 (A) Phenolic compounds removal (%) and (B) number of phenol degrading bacteria in Soil (S), Soil with bacteria inoculation (SB), Mulato grass (M) and Mulato inoculated with bacteria (MB). The error bar with alphabets a, b and c indicate the significant difference between treatments in the same irrigation cycle at $P<0.05$.

The phenol-degrading bacteria count in different treatments was show in figure 4.12 (B). The bacterial number was increased from 4.7 to 5.8 Log CFU g^{-1} soil in control at the end of experiment, while 7.3, 7.2, and 8.3 Log CFU g^{-1} soil were observed in the soil inoculated with *Acinetobacter* sp. OPB (SB), soil planted Mulato (M) and soil with both planted and inoculated bacterium (MB), respectively. From this result we can see that only planted Mulato grass alone also increase phenol-degrading bacteria similar as inoculated bacterium and their phenolic compounds removal efficiency was not significant different as in Figure 4.12 (A).

4.3.2 Phytotoxicity of the leachate from bioaugmentation of *Acinetobacter* sp. OPB and Mulato grass rhizoremediation

Toxicity of the leachate from each treatment was examined. The water leachate from three cycles of irrigation were tested with mungbean and cucumber seeds germination and compared to untreated POME (negative control) and deionize water (positive control). Results from Table 4.3 showed the GI % was increased from 15-18% (untreated POME) to 41-76%, 65-85%, 66-89% after treat with inoculated bacteria pot (SB), planted Mulato pot (M), and bacterium inoculation to Mulato grass pot (MB), respectively. Whereas, water leachate from uninoculated soil (S) were only 33-42 % and 16-21% for mungbean and cucumber seeds, respectively. From this result, bioaugmentation of this bacterium in soil irrigated with POME in both planted and unplanted soil not only increased the efficiency of phenolic compounds removal but also decreased toxicity of degradation product when compared to control soil (Figure 4.13). However, when compare the bacterium inoculation to Mulato grass pot (MB) and pot with only Mulato (M), the inoculated bacteria could improve phenolic compounds removal only around 7%. Thus, inoculation of this bacterium with Mulato grass might not necessary for rhizoremediation.

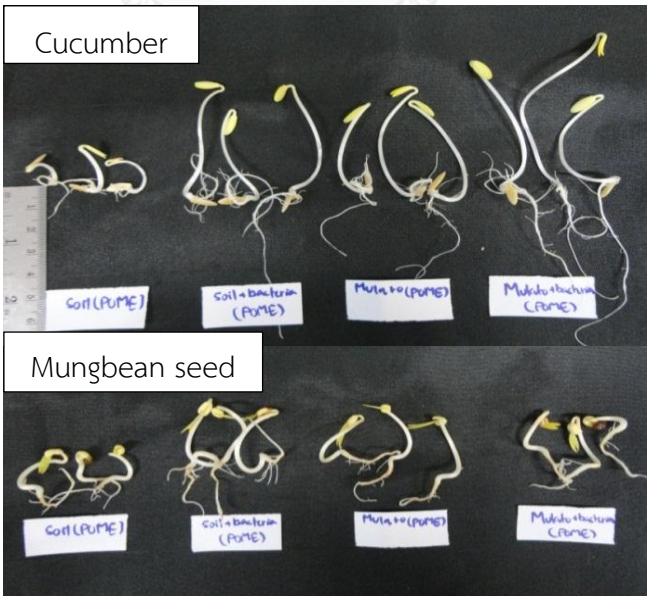


Figure 4.13 Phytotoxicity test of water leachate from treatments

Table 4.3 Phytotoxicity test of water leachate from inoculated and uninoclate bacteria treatment based on mungbean and cucumber seed germination index (GI%)

Treatments	Germination index (%)					
	First irrigation		Second irrigation		Third irrigation	
	Mungbean	Cucumber	Mungbean	Cucumber	Mungbean	Cucumber
DI	100	100	100	100	100	100
POME	15.9±0.6	11.4±0.3	18.3±0.3	11.9±0.3	17.6±0.3	12.5±0.2
S	39.5±0.7	16.9±1.3	42.9±0.6	21.4±0.2	33.6±0.6	16.7±1.3
SB	75.0±0.8	41.1±0.7	76.5±0.4	41.5±0.6	66.4±0.9	50.2±0.5
M	85.2±0.8	65.0±0.6	82.6±0.5	65.2±0.4	83.2±1.0	65.6±0.4
MB	86.1±0.9	67.3±0.4	87.6±0.5	69.4±0.5	89.2±0.4	68.8±0.5

POME = palm oil mill effluent before treat, DI = deionized water, S = Control soil uninoculated, SB = soil inoculated with bacteria, M = Mulato, and MB = Mulato inoculated with bacteria. Values represent mean ± standard deviation

4.4 Efficiency of rhizoreactor on removal of phenolic compounds and color from POME irrigated soil

To examine the potential of upscale rhizoremediation. Three treatment viz. soil reactor, Mulato reactor irrigate with POME and control grass reactor irrigate with water was constructed. In this experiment, the bacterium was not added according to our finding from the previous experiment (section 4.3) that the adding bacteria could enhance only 7% of phenolic compounds removal. Six liter of POME (1.95±0.04 g of phenolic compounds) was added to the soil and Mulato grass reactor. The volume of POME led to soil saturation (water holding capacity >100%) at day 0 and 9, which used to represent large irrigation volume in field. The initial concentration of phenolic compounds in irrigated POME was 325 mg L^{-1}. After irrigation, phenolic compounds in leachates from both control soil and grass reactor

were 250±10 mg L^{-1}, which was corresponded to around 20% reduction of phenolic compounds (Figure 4.14). The fast removal of phenolic compounds might cause by soil absorption. Previously, we examined the effect of abiotic process on phenolic compounds removal by comparing sterilized and non-sterilize samples of both POME and soil. We found that when both POME and soil were sterilized, only 20-25% of phenolic compounds were removed by sorption process (Appendix H1 B). Consequently, soil sorption was a minor process for phenolic compounds removal.

After six days of the first irrigation and 15 days of the second irrigation, phenolic compounds (Figure 4.14) and color (Figure 4.15) in leachates were clearly reduced in rhizoreactor more than in soil reactor. The similar result of phenolic compounds and color removal was also observed in the previous pot experiment (Figure 4.5).

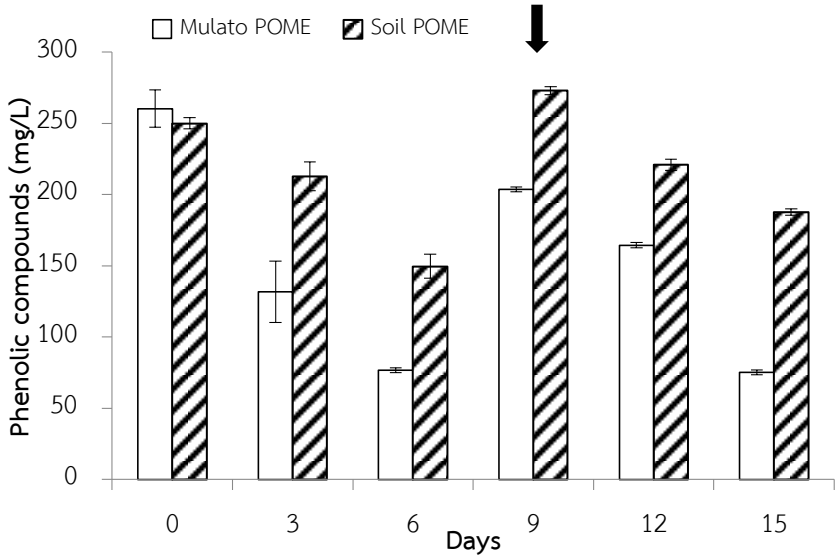


Figure 4.14 Concentrations of phenolic compounds in leachates from rhizoreactor and soil reactor after irrigated with POME. An arrow indicate the second irrigation of POME.

Percent phenolic compounds removal for Mulato reactor were 76.4±0.5 and 77.4±0.6 and for soil reactor were 53.9±1.1 and 43.9±0.5 at the first and second irrigation cycles, respectively (Table 4.4). The efficiency of phenolic compound

removal in rhizoreactors was lower than that of the previous experiment in pots (Table 4.1). These might be because of a large amount of POME was irrigated and this amount was exceed water holding capacity that suitable for plant growth and microbial activities. Thus, it can be concluded that the optimal amount of wastewater for grass irrigation should be around 60% of the soil water holding capacity. The practice would lead to the best result of phenolic compounds removal.

Table 4.4 Comparison of Mulato rhizoreactor and soil reactor on phenolic compounds removal

Treatments	First irrigation	Second irrigation
Soil reactor	53.9±1.1	43.7±0.5
Mulato reactor	76.4±0.5	77.4±0.6

Values represent mean ± standard deviation

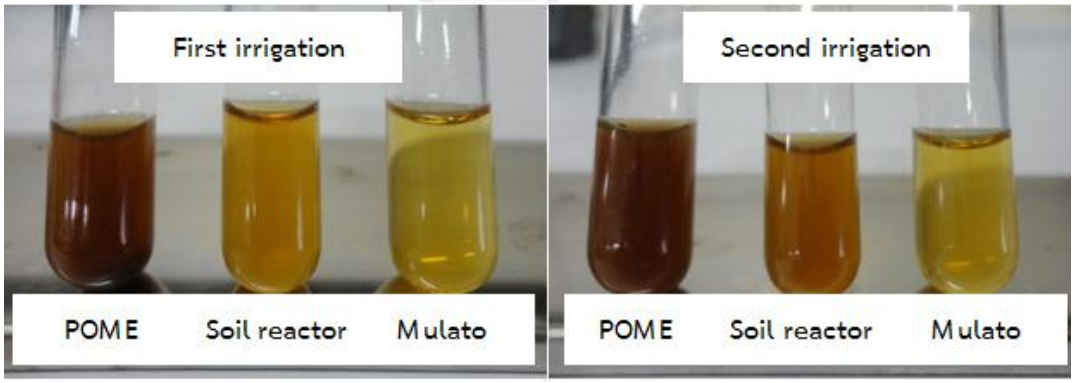


Figure 4.15 Comparison of color of the leachates from soil reactor and Mulato rhizoreactor in the first and second irrigation cycles.

Phenol degrading bacteria were counted at day 0, 7 and 14 of experiment. Phenol-degrading bacteria in rhizoreactor (Mulato POME) and control rhizoreactor (Mulato Water) were slightly increased from 6.4 log CFU g^{-1} soil to 9.2 log CFU g^{-1} soil and 6.8 log CFU g^{-1} soil to 7.3 log CFU g^{-1} soil, respectively (Figure 4.16). The numbers were significantly higher than that of control soil that increase from 5.2 to 6.3 log

CFU g^{-1} soil (Figure 4.16). These results were similar to previous finding in pots experiment (Figure 4.7). However, it was interesting to note that the bacterial population in the rhizosphere of rhizoreactor was greater than that found in pots experiment. The possible reason might be the increasing number of grass plantlets, which led to more root exudates to stimulate the growth of rhizosphere bacteria.

Although the rhizoreactor had high number of phenol-degrading bacteria, its efficiency of phenolic compound removal was lower than that of the previous experiment in pots. This might be due to the different in water holding capacity. The high amounts of irrigated POME in rhizoreactor might cause anaerobic condition in soil. Normally, the phenol-degradation occurs under aerobic condition faster than anaerobic condition (Melo et al., 2005). Thus, phenol-degrading bacteria in rhizoreactor condition might utilize other compounds instead of phenolic compounds. Consequently, the number of phenol-degrading bacteria was still increased as in Figure 4.16. The results confirmed that Mulato could enhance the growth of phenol-degrading bacteria in rhizosphere soil.

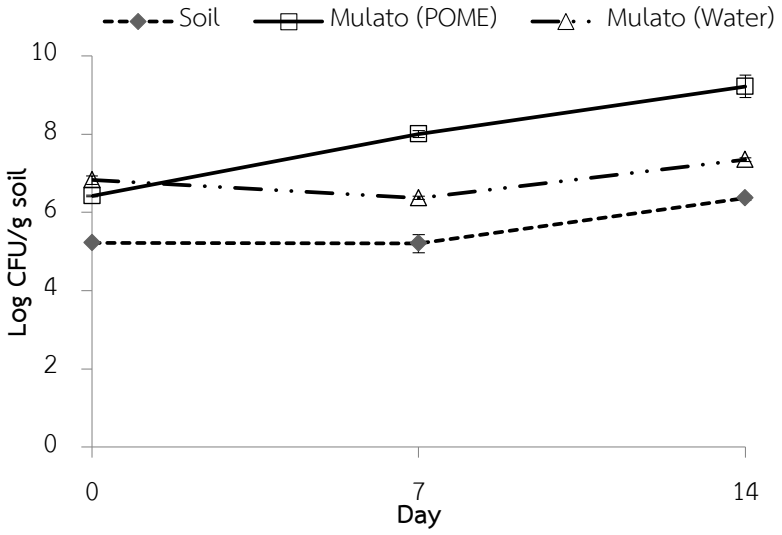


Figure 4.16 Phenol degrading-bacteria in control soil reactor, control Mulato rhizoreactor (Water) and Mulato rhizoreactor (POME).

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

A newly isolated *Acinetobacter* sp. strain OPB completely degraded 100 and 500 mg L^{-1} phenol in synthetic medium after 6 and 15 h incubation, respectively. The degradation efficiency of this bacterium was higher than *Acinetobacter* sp. strains PK1 from Khongkhaem et al. (2011) in our laboratory. Bioaugmentation of this bacterium in soil irrigated with POME in both planted and unplanted soil not only increased the efficiency of phenolic compounds removal but also decreased toxicity of degradation products when compared to control soil as show in Figure 4.12. The combination of bacterial inoculation and grass rhizoremediation could improve nearly 10% of phenolic compounds removal. *Acinetobacter* sp. strain OPB could be considered as a potential bioaugmentation strain to enhance phenol removal in contaminated site.

In rhizoremediation experiment, phenolic compounds and color significantly reduced in planted soil when compare to unplanted soil. The most effective phenolic compounds and color removal was found in the 1^{st} - 4^{th} irrigation cycles. After the forth irrigation cycles, both phenolic compounds and color were increased. This result might be due to the accumulation of phenolic compounds with complex structures as well as the leakage of rhizosphere phenolic compounds due to an increasing of soil pH. The number of phenol-degrading bacteria in planted soil was significantly higher than that in unplanted soil. This result suggested that plant roots supported conditions that stimulated the growth of rhizosphere microorganisms. The reduction of both phenolic compounds and color was positively correlated with the increasing of phenol-degrading bacteria in rhizosphere with R^2 = 0.936 and 0.809 for phenolic compounds and color, respectively. These results confirmed that phenol-degrading bacteria were involved in both phenolic compounds and color removal from POME irrigated soil. Among 3 species of animal feed grasses namely Mulato,

Creeping signal and Guinea, Mulato grass had the highest efficiency and meet our criteria on grass selection for rhizoremediation of POME irrigated soil. This grass was selected as for further examination in an upscale rhizoreactor.

In the upscale rhizoremediation, the percent removal of phenolic compounds by Mulato reactor was 25-30% significantly higher than that of soil reactor. Although, the efficiency of rhizoreactor was 10-15% less than rhizoremediation in the pot experiment, this Mulato grass still had the potential to apply as rhizoremediation in field. The rate of phenolic compounds removal calculated from the size of rhizoreactor size showed that this approach could treat POME in the final stabilize pond at 4-6 L per 0.25 m^2 of grass planting reactor per week or 16-24 L/m^2/week. The rate was equal to 160-240 m^3/ha/week (1ha=10,000m^2). Planting grasses not only aim to reduce the concentration of phenolic compounds in soil irrigation with POME but also reduce the POME toxicity and can prevent soil erosion in rainy season. In addition, the harvested grasses after treatment could be used for animal feed or bioethanol production as cellulosic biomass.

5.2 Recommendations

1. *Acinetobacter* sp. strains OPB could degrade 1-500 mg L^{-1} phenol but this strain had decreased degradation activity at the phenol concentrations above 500 mg L^{-1}. Thus, when apply this stain in higher phenol concentration environment, the bacterium should be immobilized to improve their survival and degradation rate as in Khongkhaem et al. (2011), which showed that encapsulation of a mixed culture of *Methylobacterium* sp. NP3 and *Acinetobacter* sp. PK1 could degrade high concentration of phenol (up to 5,000 mg L^{-1}). In addition, (Ahmad, Shamaan, et al., 2012) encapsulated *Acinetobacter* sp. Strain AQ5NOL in gellan gum and found that it completely degraded phenol within 108, 216 and 240 h at 1,100, 1,500 and 1,900 mg l^{-1} phenol, respectively. The immobilized cells showed no loss in phenol degrading activity after being used repeatedly for 45 cycles.

2. For rhizoremediation using grasses, the indigenous phenol-degrading rhizosphere bacteria had the potential to degrade phenolic compounds in POME.

They only demanded some stimulations from grasses and their root exudates. Thus, selecting grasses with complex root system such as Vetiver grass (Ho et al., 2013) or grasses with the ability to release phenolic compounds would be a strategy for remediation of phenolic compounds contaminated in soil

3. From the overall results, the researchers should grow grasses in several cultivate areas and alternatively irrigated them with the wastewater. This will allow the grass and its rhizosphere bacteria to recover from toxic compounds in the wastewater. Another point to concern is cultivation season, grass would have slow growth after the cultivation season and it will lead to lower root mass and root exudates and consequently lower number of rhizosphere bacteria in soil. In addition, (Ibe et al. (2014)) found that when soil irrigated with POME, the total heterotrophic bacterial population decreased in the dry season more than in the rainy season. This was due to the increasing POME viscosity in dry season, which could reduce oxygen from soil surface and inhibit aerobic microbial activity at higher extant than in rainy season.

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APPENDIX

APPENDIX A

Media preparation

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 autoclaving with 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.

Appendix B

Standard curve of phenolic and color

Table B.1 standard phenolic compounds (Gallic acid) concentration 0-1000 mg/L

Conc. (mg/L)	OD 760 nm					Average	SD
	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5		
0	0	0	0	0	0	0.000	0.000
100	0.087	0.086	0.087	0.086	0.089	0.087	0.001
200	0.176	0.179	0.173	0.174	0.176	0.176	0.002
300	0.276	0.275	0.271	0.27	0.269	0.272	0.003
400	0.415	0.408	0.413	0.423	0.405	0.413	0.007
500	0.531	0.539	0.54	0.54	0.539	0.538	0.004
600	0.649	0.647	0.65	0.652	0.647	0.649	0.002
800	0.841	0.847	0.846	0.85	0.845	0.846	0.003
1000	0.985	0.988	0.989	0.986	0.987	0.987	0.002

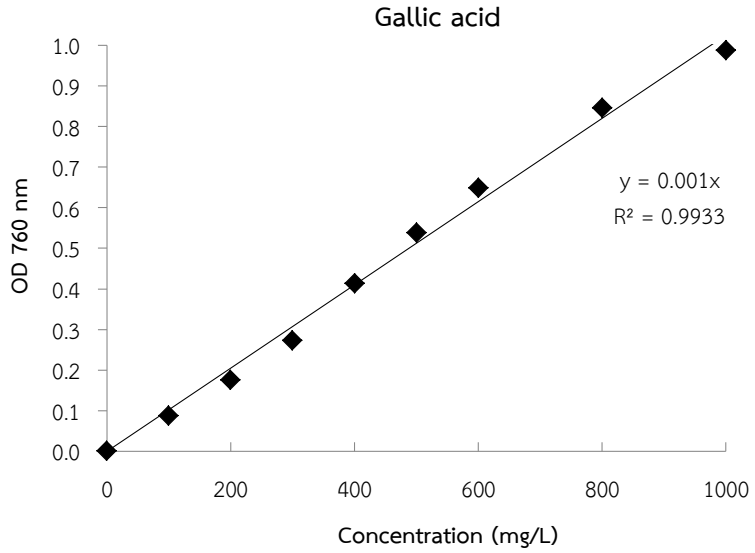

Figure B.1 Standard curve of phenolic compounds (Gallic acid) concentration 0-1000 mg/L

Standard color 0-500 Pt/Co unit

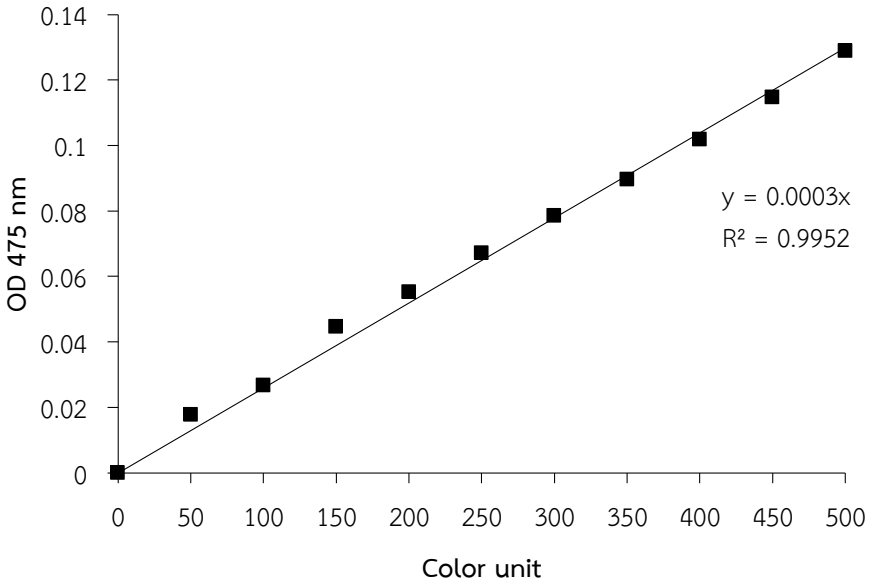

Figure B.2 Standard curve of absorbance (OD_{475})of varied color concentration 0-500 color units.

Appendix C

Comparison of three phenol degrading isolates

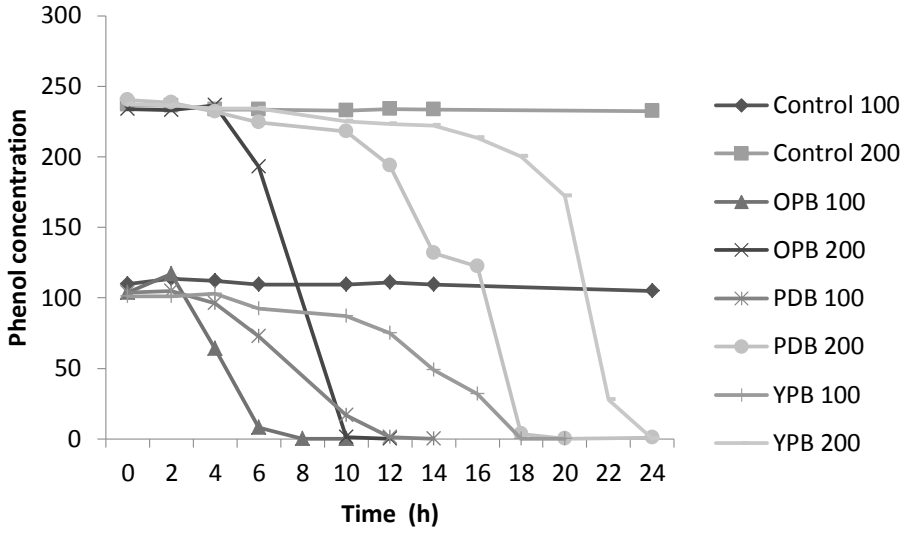

Figure C.1 Phenol degradation by isolate OPB, PDB and YPB at concentration 100 and 200 mg L^{-1}

Appendix D

Phenolic compounds remaining in each treatment

Table D.1 Phenolic compounds from water leachate (mg) in treatments with and without planting in pot experiment

Phenolic compounds remaining in treatments (mg) (1st cycle of experiment)					
Treatments	Replicate 1	Replicate 2	Replicate 3	Average	SD
POME	373.12	374.44	371.80	373.12	1.32
Soil (POME)	107.07	105.40	106.53	106.33	1.74
Mulato (POME)	99.47	96.27	97.60	97.78	2.15
Mulato (Water)	79.87	66.40	66.80	71.02	7.97
Creeping signal (POME)	88.80	87.33	89.47	88.53	5.46
Creeping signal (Water)	61.20	62.00	61.73	61.64	1.22
Guinea (POME)	67.33	68.00	69.60	68.31	2.92
Guinea (Water)	5.47	4.67	5.87	5.33	1.02
Phenolic compounds remaining in treatments (mg) (2nd cycle of experiment)					
Treatments	Replicate 1	Replicate 2	Replicate 3	Average	SD
POME	147.90	150.10	145.40	147.80	2.35
Soil (POME)	73.59	72.89	73.36	73.28	0.98
Mulato (POME)	59.92	62.53	60.39	60.95	1.66
Mulato (Water)	44.80	44.89	44.33	44.68	0.73
Creeping signal (POME)	55.53	56.56	55.35	55.81	0.75
Creeping signal (Water)	42.28	42.93	43.03	42.75	0.68
Guinea (POME)	48.09	48.37	47.32	47.93	0.56

Guinea (Water)	5.88	4.95	4.76	5.20	0.72
Phenolic compounds remaining in treatments (mg) (Pt/Co unit) (3rd cycle of experiment)					
Treatments	Replicate 1	Replicate 2	Replicate 3	Average	SD
POME	185.13	196.35	192.28	191.25	5.68
Soil (POME)	62.26	62.70	61.75	62.24	0.56
Mulato (POME)	54.19	55.81	54.19	54.73	1.33
Mulato (Water)	40.63	41.29	41.14	41.02	0.64
Creeping signal (POME)	52.51	52.29	51.77	52.19	1.60
Creeping signal (Water)	42.83	43.34	42.75	42.97	0.48
Guinea (POME)	35.26	36.25	36.03	35.84	0.77
Guinea (Water)	0.15	0.07	0.07	0.10	0.04
Phenolic compounds remaining in treatments (mg) (4th cycle of experiment)					
Treatments	Replicate 1	Replicate 2	Replicate 3	Average	SD
POME	137.60	146.80	147.12	143.84	5.41
Soil (POME)	75.17	74.87	75.17	75.07	0.49
Mulato (POME)	51.77	55.73	53.68	53.73	2.13
Mulato (Water)	35.05	35.86	37.25	36.06	1.12
Creeping signal (POME)	43.85	45.54	43.56	44.32	1.10
Creeping signal (Water)	29.19	29.77	29.11	29.36	0.79
Guinea (POME)	38.45	39.60	38.89	38.98	0.75
Guinea (Water)	0.07	0.00	0.07	0.05	0.04

Phenolic compounds remaining in treatments (mg) (5th cycle of experiment)					
Treatments	Replicate 1	Replicate 2	Replicate 3	Average	SD
POME	96.84	94.80	97.02	96.22	1.23
Soil (POME)	85.76	85.52	86.92	86.07	0.95
Mulato (POME)	51.52	52.24	34.29	46.02	10.64
Mulato (Water)	19.28	21.60	20.80	20.56	1.64
Creeping signal (POME)	69.76	67.36	69.76	68.96	1.92
Creeping signal (Water)	27.44	27.28	26.16	26.96	0.95
Guinea (POME)	48.48	48.96	49.68	49.04	0.75
Guinea (Water)	0.00	0.00	0.00	0.00	0.00

Table D.2 Phenolic compounds from water leachate (mg) in bioaugmentation pot experiment, treatments with and without inoculate *Acinetobacter* sp. OPB.

Phenolic compounds remaining (mg) (1st cycle of experiment)					
Treatments	Replicate 1	Replicate 2	Replicate 3	Average	SD
Soil	4.65	4.97	4.99	4.87	0.23
Soil+bacteria	2.93	3.32	2.97	3.08	0.22
Mulato	3.17	2.79	2.91	2.96	0.21
Mulato+bacteria	2.28	2.51	2.39	2.39	0.15
POME added	10.68	11.09	10.87	10.88	0.20
Phenolic compounds remaining (mg) (2nd cycle of experiment)					
Treatments	Replicate 1	Replicate 2	Replicate 3	Average	SD
Soil	2.86	2.93	3.11	2.97	0.14
Soil+bacteria	1.69	1.76	1.75	1.73	0.09
Mulato	1.71	1.79	1.72	1.74	0.08
Mulato+bacteria	1.39	1.51	1.49	1.46	0.10
POME added	7.12	7.39	7.25	7.25	0.14

Phenolic compounds remaining (mg) (3rd cycle of experiment)					
Treatments	Replicate 1	Replicate 2	Replicate 3	Average	SD
Soil	2.61	2.33	2.55	2.50	0.25
Soil+bacteria	1.72	1.75	1.87	1.78	0.14
Mulato	1.84	1.71	1.87	1.80	0.10
Mulato+bacteria	1.31	1.17	1.29	1.26	0.10
POME added	7.12	7.39	7.25	7.25	0.14

Appendix E

Color remaining in pot rhizoremediation experiment

Table E.1 Color from water leachate (Pt/Co unit) in treatments with and without planting in pot experiment

Color remaining in treatments (Pt/Co unit) (1st cycle of experiment)					
Treatments	Replicate 1	Replicate 2	Replicate 3	Average	SD
POME	4000.00	3980.00	4010.00	3996.67	15.28
Soil (POME)	1724.80	1628.80	1510.40	1621.33	107.39
Mulato (POME)	1903.33	2083.33	1876.67	1954.44	112.41
Mulato (Water)	1646.67	1503.33	1366.67	1505.56	140.01
Creeping signal (POME)	1766.67	1993.33	1910.00	1890.00	114.65
Creeping signal (Water)	1746.67	1720.00	1636.67	1701.11	57.38
Guinea (POME)	806.67	906.67	1023.33	912.22	108.44
Guinea (Water)	546.67	520.00	503.33	523.33	21.86
Color remaining in treatments (Pt/Co unit) (2nd cycle of experiment)					
Treatments	Replicate 1	Replicate 2	Replicate 3	Average	SD
POME	4420.00	4473.33	4403.33	4432.22	36.57
Soil (POME)	1826.67	1746.67	1706.67	1760.00	61.10
Mulato (POME)	2016.67	1670.00	1686.67	1791.11	195.51
Mulato (Water)	1583.33	1066.67	1143.33	1264.44	278.81
Creeping signal (POME)	1443.33	2033.33	2036.67	1837.78	341.60
Creeping signal (Water)	1573.33	1420.00	1256.67	1416.67	158.36
Guinea (POME)	1123.33	1126.67	1140.00	1130.00	8.82
Guinea (Water)	416.67	480.00	563.33	486.67	73.56

Color remaining in treatments (Pt/Co unit) (3rd cycle of experiment)					
Treatments	Replicate 1	Replicate 2	Replicate 3	Average	SD
POME	4420.00	4433.33	4413.33	4422.22	10.18
Soil (POME)	1453.33	1540.00	1570.00	1521.11	60.58
Mulato (POME)	1616.67	1753.33	1920.00	1763.33	151.91
Mulato (Water)	1666.67	1443.33	1153.33	1421.11	257.39
Creeping signal (POME)	1436.67	1760.00	2083.33	1760.00	323.33
Creeping signal (Water)	2223.33	1760.00	1786.67	1923.33	260.15
Guinea (POME)	1396.67	1426.67	1453.33	1425.56	28.35
Guinea (Water)	606.67	620.00	660.00	628.89	27.76
Color remaining in treatments (Pt/Co unit) (4th cycle of experiment)					
Treatments	Replicate 1	Replicate 2	Replicate 3	Average	SD
POME	5100.00	5070.00	5106.67	5092.22	19.53
Soil (POME)	1570.00	1580.00	1613.33	1587.78	22.69
Mulato (POME)	2006.67	1833.33	2386.67	2075.56	283.03
Mulato (Water)	1440.00	1150.00	1220.00	1270.00	151.33
Creeping signal (POME)	1380.00	1720.00	1993.33	1697.78	307.27
Creeping signal (Water)	1223.33	1486.67	906.67	1205.56	290.41
Guinea (POME)	1506.67	1520.00	1493.33	1506.67	13.33
Guinea (Water)	606.67	593.33	570.00	590.00	18.56

Color remaining in treatments (Pt/Co unit) (5th cycle of experiment)					
Treatments	Replicate 1	Replicate 2	Replicate 3	Average	SD
POME	4976.67	4953.33	4966.67	4965.56	11.71
Soil (POME)	1984.00	2320.00	2068.00	2124.00	174.86
Mulato (POME)	1880.00	1706.67	1850.00	1812.22	92.64
Mulato (Water)	900.00	500.00	546.67	648.89	218.72
Creeping signal (POME)	2543.33	2543.33	2456.67	2514.44	50.04
Creeping signal (Water)	1026.67	673.33	766.67	822.22	183.10
Guinea (POME)	1573.33	1630.00	1643.33	1615.56	37.17
Guinea (Water)	613.33	576.67	593.33	594.44	18.36

Appendix F

Phenol-degrading bacteria

Table F.1 Phenol-degrading bacteria in rhizosphere of three grasses species and control soil after irrigation with POME in five irrigation cycle.

Phenol-degrading bacteria Log CFU/ g soil (day 0)					
Treatments	Replicate 1	Replicate 2	Replicate 3	Average	SD
Soil (POME)	5.60	5.78	5.90	5.76	0.15
Mulato (POME)	5.90	5.85	5.70	5.82	0.11
Mulato (Water)	5.95	5.78	5.70	5.81	0.13
Creeping signal (POME)	5.60	5.78	5.95	5.78	0.18
Creeping signal (Water)	5.78	5.78	5.85	5.80	0.04
Guinea (POME)	5.70	5.90	5.78	5.79	0.10
Guinea (Water)	5.78	5.60	5.70	5.69	0.09
Phenol-degrading bacteria Log CFU/ g soil (First irrigation, day 7)					
Treatments	Replicate 1	Replicate 2	Replicate 3	Average	SD
Soil (POME)	5.90	6.08	6.18	6.05	0.14
Mulato (POME)	6.23	6.34	6.15	6.24	0.10
Mulato (Water)	6.18	6.20	6.11	6.16	0.05
Creeping signal (POME)	6.30	6.15	6.18	6.21	0.08
Creeping signal (Water)	6.08	6.00	6.11	6.06	0.06
Guinea (POME)	6.15	6.08	5.90	6.04	0.13
Guinea (Water)	6.00	6.11	5.95	6.02	0.08

Phenol-degrading bacteria Log CFU/ g soil (Second irrigation, day 14)					
Treatments	Replicate 1	Replicate 2	Replicate 3	Average	SD
Soil (POME)	5.60	5.85	5.95	5.80	0.18
Mulato (POME)	7.08	7.20	7.04	7.11	0.09
Mulato (Water)	5.95	6.00	6.15	6.03	0.10
Creeping signal (POME)	6.70	6.85	6.90	6.82	0.11
Creeping signal (Water)	6.60	6.70	6.60	6.63	0.06
Guinea (POME)	6.60	6.70	6.48	6.59	0.11
Guinea (Water)	6.30	6.48	6.60	6.46	0.15
Phenol-degrading bacteria Log CFU/ g soil (Third irrigation, day 21)					
Treatments	Replicate 1	Replicate 2	Replicate 3	Average	SD
Soil (POME)	6.45	6.41	6.38	6.41	0.03
Mulato (POME)	7.26	7.15	7.08	7.16	0.09
Mulato (Water)	7.04	7.18	7.11	7.11	0.07
Creeping signal (POME)	6.48	6.70	6.85	6.67	0.19
Creeping signal (Water)	6.40	6.46	6.43	6.43	0.03
Guinea (POME)	6.32	6.34	6.38	6.35	0.03
Guinea (Water)	6.26	6.15	6.30	6.23	0.08

Phenol-degrading bacteria Log CFU/ g soil (Forth irrigation, day 28)					
Treatments	Replicate 1	Replicate 2	Replicate 3	Average	SD
Soil (POME)	6.08	6.11	6.08	6.09	0.02
Mulato (POME)	7.48	7.48	7.70	7.55	0.13
Mulato (Water)	7.15	7.23	7.32	7.23	0.09
Creeping signal (POME)	7.28	7.20	7.11	7.20	0.08
Creeping signal (Water)	6.95	6.70	6.90	6.85	0.14
Guinea (POME)	7.15	7.11	7.18	7.15	0.03
Guinea (Water)	6.90	7.04	7.08	7.01	0.09
Phenol-degrading bacteria Log CFU/ g soil (Fifth irrigation, day 35)					
Treatments	Replicate 1	Replicate 2	Replicate 3	Average	SD
Soil (POME)	6.08	6.15	6.20	6.14	0.06
Mulato (POME)	8.18	8.20	8.11	8.16	0.05
Mulato (Water)	7.32	7.38	7.41	7.37	0.05
Creeping signal (POME)	7.60	7.48	7.48	7.52	0.07
Creeping signal (Water)	7.18	7.26	7.15	7.19	0.06
Guinea (POME)	7.26	7.26	7.30	7.27	0.03
Guinea (Water)	6.95	7.08	7.11	7.05	0.08

Table F.2 Phenol-degrading bacteria in rhizosphere of inoculate and uninoculate *Acinetobacter* sp. OPB and control soil after irrigation with POME in bioaugmentation pot experiment.

Phenol-degrading bacteria Log CFU/ g soil (day 0)					
Treatments	Replicate 1	Replicate 2	Replicate 3	Average	SD
Soil (POME)	4.70	4.70	4.78	4.73	0.05
Mulato (POME)	5.90	6.04	6.00	5.98	0.07
Mulato (Water)	5.08	5.20	5.04	5.11	0.09
Creeping signal (POME)	6.26	6.20	6.28	6.25	0.04
Phenol-degrading bacteria Log CFU/ g soil (day 7)					
Treatments	Replicate 1	Replicate 2	Replicate 3	Average	SD
Soil (POME)	5.08	5.00	5.11	5.06	0.06
Mulato (POME)	6.30	6.32	6.28	6.30	0.02
Mulato (Water)	5.41	5.36	5.40	5.39	0.03
Creeping signal (POME)	6.43	6.41	6.38	6.41	0.03
Phenol-degrading bacteria Log CFU/ g soil (day 14)					
Treatments	Replicate 1	Replicate 2	Replicate 3	Average	SD
Soil (POME)	5.15	5.04	5.11	5.10	0.05
Mulato (POME)	7.08	7.20	7.04	7.11	0.09
Mulato (Water)	5.95	6.00	6.15	6.03	0.10
Creeping signal (POME)	7.18	7.23	7.26	7.22	0.04
Phenol-degrading bacteria Log CFU/ g soil (day 21)					
Treatments	Replicate 1	Replicate 2	Replicate 3	Average	SD
Soil (POME)	5.90	5.78	5.70	5.79	0.10
Mulato (POME)	7.36	7.40	7.36	7.37	0.02
Mulato (Water)	7.23	7.30	7.32	7.28	0.05
Creeping signal (POME)	8.34	8.28	8.30	8.31	0.03

Appendix G

Grasses biomass and phenolic compounds in three grasses species

Table G.1 Grasses biomass in each part (leave, stem and root)

Leave mass (g)					
Treatments	Replicate 1	Replicate 2	Replicate 3	Average	SD
Mulato (POME)	12.18	12.15	12.16	12.16	0.03
Mulato (Water)	16.64	16.67	16.64	16.65	0.02
Creeping signal (POME)	9.73	9.72	9.73	9.73	0.02
Creeping signal (Water)	11.72	11.73	11.77	11.74	0.04
Guinea (POME)	10.30	10.30	10.32	10.30	0.02
Guinea (Water)	6.96	6.99	7.00	6.98	0.03
Stem mass (g)					
Treatments	Replicate 1	Replicate 2	Replicate 3	Average	SD
Mulato (POME)	4.91	4.94	4.96	4.94	0.03
Mulato (Water)	8.27	8.25	8.26	8.26	0.03
Creeping signal (POME)	7.82	7.85	7.84	7.84	0.02
Creeping signal (Water)	13.57	13.89	13.56	13.68	0.19
Guinea (POME)	3.52	3.52	3.54	3.53	0.02
Guinea (Water)	2.23	2.24	2.23	2.23	0.01

Root mass (g)					
Treatments	Replicate 1	Replicate 2	Replicate 3	Average	SD
Mulato (POME)	12.18	12.15	12.16	12.16	0.03
Mulato (Water)	16.64	16.67	16.64	16.65	0.02
Creeping signal (POME)	9.73	9.72	9.73	9.73	0.02
Creeping signal (Water)	11.72	11.73	11.77	11.74	0.04
Guinea (POME)	10.30	10.30	10.32	10.30	0.02
Guinea (Water)	6.96	6.99	7.00	6.98	0.03

Table G.2 Phenolic compounds each grasses part (leave, stem and root)

Phenolic compounds in leave (mg/g dry weight)					
Treatments	Replicate 1	Replicate 2	Replicate 3	Average	SD
Mulato (POME)	5.15	5.21	5.17	5.17	0.09
Mulato (Water)	5.75	5.78	5.74	5.76	0.06
Creeping signal (POME)	6.10	6.18	6.10	6.13	0.09
Creeping signal (Water)	5.69	5.95	5.76	5.80	0.16
Guinea (POME)	2.54	2.54	2.56	2.55	0.03
Guinea (Water)	3.89	3.91	3.87	3.89	0.02
Phenolic compounds in stem (mg/g dry weight)					
Treatments	Replicate 1	Replicate 2	Replicate 3	Average	SD
Mulato (POME)	3.71	4.07	4.01	3.93	0.20
Mulato (Water)	4.19	4.38	4.26	4.28	0.10
Creeping signal (POME)	6.60	6.86	6.67	6.71	0.16
Creeping signal (Water)	5.93	6.08	5.98	6.00	0.26
Guinea (POME)	2.40	2.43	2.46	2.43	0.03
Guinea (Water)	2.69	2.70	2.72	2.70	0.03
Phenolic compounds in root (mg/g dry weight)					
Treatments	Replicate 1	Replicate 2	Replicate 3	Average	SD
Mulato (POME)	2.13	2.23	2.25	2.20	0.08
Mulato (Water)	1.69	1.93	1.93	1.85	0.14
Creeping signal (POME)	3.24	3.30	3.30	3.28	0.09
Creeping signal (Water)	3.12	3.16	3.26	3.18	0.11
Guinea (POME)	1.62	1.61	1.61	1.61	0.02
Guinea (Water)	1.84	1.84	1.83	1.84	0.02

Appendix H

Soil pH changed during pot rhizoremediation experiment

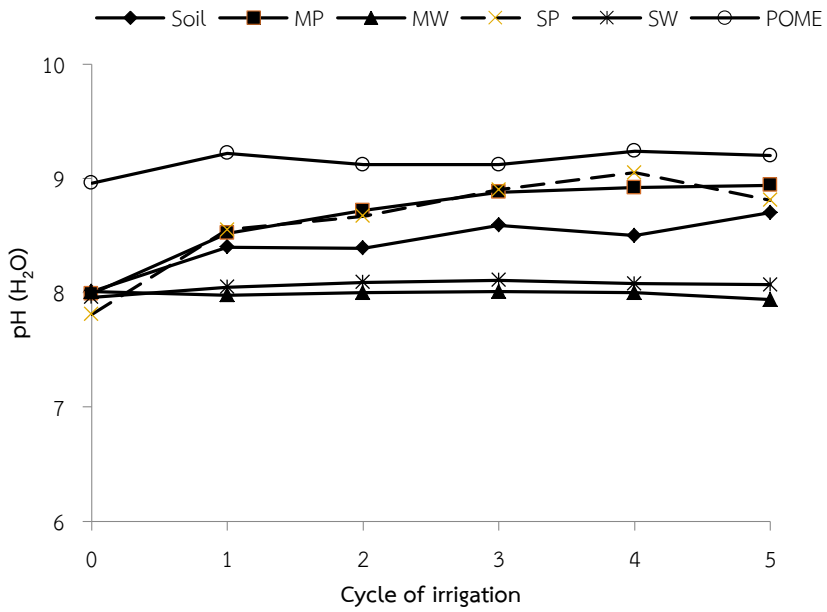

Figure H.1 Changes of soil pH (H_2O) of each treatment during five irrigation cycles

Appendix I

Phenolic compounds removal by sterilized and non-sterilized soil

Table I.1 Percent phenolic compounds remaining in satirized and nonsterilized soil and POME

	First irrigation		Second irrigation	
Treatments	Average	SD	Average	SD
POME	100	0.00	100	0.00
SNPN	22.73	1.53	28.64	1.10
SSPN	61.95	1.04	66.96	1.55
Claved POME	100	0.00	100	0
SNPS	22.21	0.60	39.82	0.81
SSPS	72.95	1.39	83.15	2.89

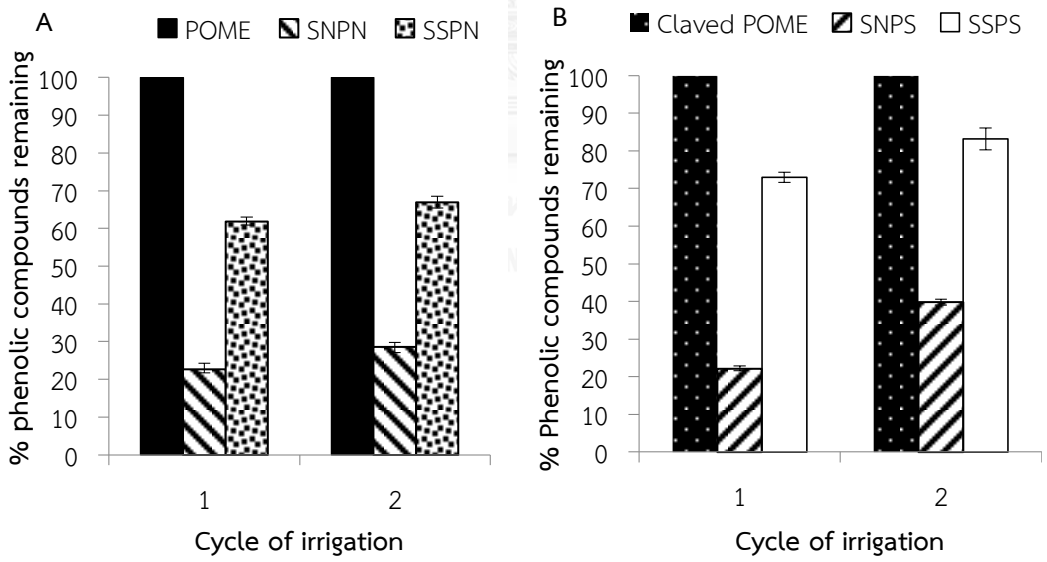

Figure I.1 Phenolic compounds remaining (%). (A) nonsterilized POME, (B) sterilized POME. Nonsterilized soil and POME (SNPN), sterilized soil but nonsterilized POME (SSPN), Nonsterilized soil but sterilized POME (SNPS), sterilized soil and POME (SSPS)

Appendix J

BlastN and DNA sequencing report of Acinetobacter sp. OPB

>140320-36_E14_OPB_phong_518F.ab1 991

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CTAACACTGACGCTGAGGTGCGAAAGCATGGGGAGCAAACAGGATTAGAT
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>140320-36_G14_OPB_phong_800R.ab1 794

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CTGCTGGCACAGAGTTAGCCGGTGCTTATTCTGCGAGTAACGTCCACTAT
CTCTAGGTATTAACTAAAGTAGCCTCCTCCTCGCTTAAAGTGCTTTACAA
CCATAAGGCCTTCTTCACACACGCGGCATGGCTGGATCAGGCTTGCGCCC
ATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTC
TCAGTCCCAGTGTGGCGGATCATCCTCTCAGACCCGCTACAGATCGTCGC
CTTGGTAGGCCTTTACCCCACCAACTAGCTAATCCGACTTAGGCTCATCT
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AAGCATTACTCACCCGTCCGCCGCTAAGATCAGTAGCAAGCTACCTCTCT
CCGCTCGACTTGCATGTGTTAAGCCTGCCGCCAGCGTTCAATCTGAGCAA
GGGAAAAAAACCCTAAAAAACCGGAAAACGGGAAAAAAGTGGGA

- Query name : OPB_phong_contig_1
- Query length : 1492

Query		Subject					Score			Identities			
Start	End	Description	AC	Length	Start	End	Bit	Raw	EV	Match	Total	Pct.(%)	Strand
1	1463	Acinetobacter sp. VKPM 3780 16S ribosomal RNA gene, partial sequence	JF891388.1	1480	7	1468	2695	1459	0.0	1462	1463	99	Plus/Plus
1	1463	Acinetobacter sp. 532 16S ribosomal RNA gene, partial sequence	HQ200407.1	1530	20	1481	2695	1459	0.0	1462	1463	99	Plus/Plus
1	1463	Acinetobacter sp. KL5(2010) 16S ribosomal RNA gene, partial sequence	GU566321.1	1530	21	1482	2695	1459	0.0	1462	1463	99	Plus/Plus
1	1463	Acinetobacter sp. JD2(2010) 16S ribosomal RNA gene, partial sequence	GU566314.1	1533	22	1483	2695	1459	0.0	1462	1463	99	Plus/Plus
1	1463	Acinetobacter sp. MK4(2010) 16S ribosomal RNA gene, partial sequence	GU566310.1	1531	20	1481	2695	1459	0.0	1462	1463	99	Plus/Plus
1	1463	Uncultured bacterium clone G10-41 16S ribosomal RNA gene, partial sequence	GQ487956.1	1500	20	1481	2695	1459	0.0	1462	1463	99	Plus/Plus
1	1463	Acinetobacter [calcoaceticus] NCCB 22016 strain : NCCB 22016 16S ribosomal RNA, partial sequence	NR_042387.1	1516	11	1472	2695	1459	0.0	1462	1463	99	Plus/Plus
1	1462	Acinetobacter genomosp. 3 strain LUH 8131 16S ribosomal RNA gene, partial sequence	FJ860873.1	1480	20	1480	2693	1458	0.0	1461	1462	99	Plus/Plus
1	1462	Acinetobacter genomosp. 3 strain LUH 5779 16S ribosomal RNA gene, partial sequence	FJ860872.1	1480	20	1480	2693	1458	0.0	1461	1462	99	Plus/Plus
2	1462	Acinetobacter genomosp. 3 strain LUH 6127 16S ribosomal RNA gene, partial sequence	FJ867362.1	1460	1	1460	2691	1457	0.0	1460	1461	99	Plus/Plus

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Conferences:

Phonepaseuth, P., Kachenchart, B. and Luepromchai, E. 2014. Removal of phenolic compounds from soil irrigated with palm oil mill effluent using Acinetobacter sp. OPB and grass rhizosphere bacteria. Poster Presentation. The 26th Annual Meeting of the Thai Society for Biotechnology and International Conference. Mae Fah Lunag University, Chiang Rai, Thailand. 26th-29th November, 2014. (Best Poster award)

Phonepaseuth, P., Kachenchart, B. and Luepromchai, E. 2014. Rhizoremediation of phenolic compounds and color in soil after palm oil mill wastewater irrigation. The 19th Biological Sciences Graduate Congress. Department of Biological Science, National University of Singapore, Singapore, 12-14 December 2014.