USING VETIVER GRASS TO REMEDIATE WATER AND GROUNDWATER CONTAMINATED WITH PHENOL FROM ILLEGAL DUMPING AT NONG NAE SUB-DISTRICT, PANOMSARAKARM DISTRICT, CHACHOENGSAO PROVINCE

PIMPAWAT TEERATITAYANGKUL

A Thesis Submitted to the Graduate School of Naresuan University
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Thesis entitled "Using Vetiver Grass to Remediate Water and Groundwater Contaminated with Phenol from Illegal Dumping at the Nong-Nea Sub district, Panomsarakarm District, Chachoengsao Province"

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ABSTRACT

The illegal dumping of phenol containing wastewater in February 2012 at Nong Nae village, Chachoengsao Province, Thailand caused chemical contamination of wells, agricultural ponds and water supplies .Once phenol entered the ground water, it spread and caused further contamination as it was carried by the local ground water and other water sources. This problem poses a danger to the local ecology and public health due to its biocidal property. Here we employed phytoremediation using vetiver grass (*Vetiveria zizanioides* (L.) Nash) to degrade phenol with the help of vetiver's oxidative stress and rhizomicrobial activity. The experiments divided to 2 parts: phenol containing wastewater and groundwater treatments. Two-phase mechanism of phenol degradation using vetiver root system has been proposed in phenol containing wastewater. The phase I (PI) was driven by the oxidative stress, POD and H_2O_2 in phyto-oxidation and phyto-polymerization followed by the phase II (PII), a combination of phase I with rhizo-microbial activities. Using the vetiver plantlet, the maximum rate of phenol degradation, in aeration system, was $13x10^{-3}$ hr^{-1} from the 100 vetiver plantlets in 35 liters of 500 mg/L, phenol concentration. While, the mature

vetiver root system achieved the rate of phenol degradation of $60x10^{-3}$ hr^{-1} which was about 4.6 times greater within 12 days comparing to vetiver plantlets in the same condition which achieved the MCL within 2 months. To compare with natural attenuation, it was required about a year.

The phytoremediation of phenol by vetiver grass is also an effective approach of remedial action for shallow groundwater. The study of phenol containing groundwater treatment demonstrated in the laboratory-scaled experiments. Permeable reactive barrier made by vetiver root system (PRVRB) were applied at various conditions i.e .the hydraulic retention time (HRT) and the bioaugmentation as microbial enhancement to evaluate the most effective system to degrade phenol (500 mg/L. The PRVRB with bioaugmentation could degrade 91.08% of phenol in 14 days (at the flow rate of 7 cm/d). The contributions of abiotic and biotic were assessed. 61.31% of phenol was supposed to be gradated by phytochemicals while 29.77% was degraded by biotic transformation of phenol: about 91.08% with the degradation rate via the advection-dispersion-reaction model and the first order rate law $5.04x10^{-3}$ hr^{-1}.

These results revealed that the presence of vetiver root is effective in phenol degradation as providing the phytochemicals for phyto-oxidation and phyto-polymerization as well as the phenol degradation by rhizomicrobiological processes.

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CHAPTER I

INTRODUCTION

Research Rationale and Background

The industrialization of the Thai economy includes development of special economic zones, the establishment of industrial parks and estates, and the granting of permission for factories to be established in many parts of the country (Wei, 2015). Effective environmental management is therefore, imperative and laws, policies, and practices need to be established to control industrial pollution of the environment.

In the past industrial practices and factory locations have not been properly managed, resulting in heavy pollution of large areas of the country. This has caused significant health problems in neighboring populations. Remediation has become a matter of great urgency.

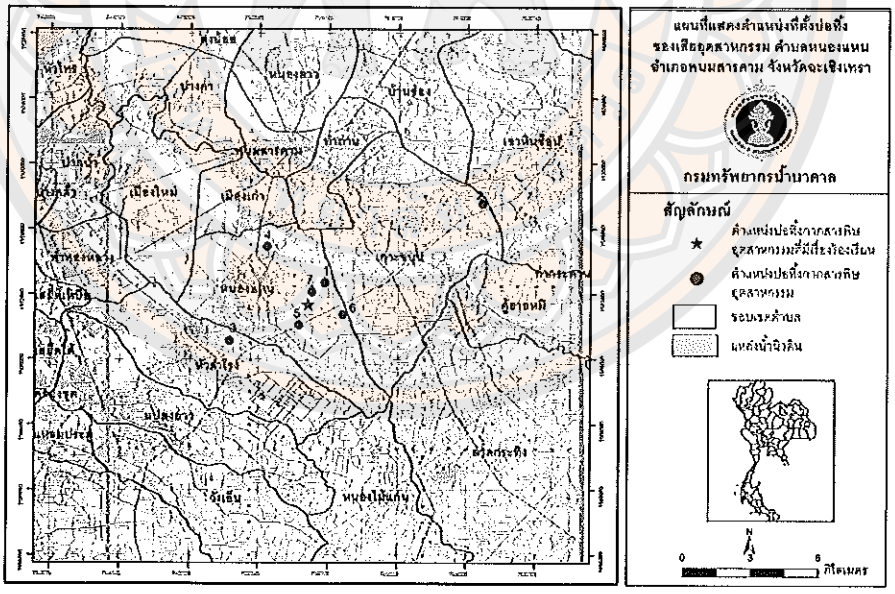

Figure 1 The location of Nong Nae sub-district

Source: http://www.gwcr.org/wp-content/uploads/2014/06/slide4-12.jpg

Nong Nae sub-district of Chachoengsao province is an area that has been heavily polluted with phenol. The area consists of a number of villages where the farming community lives. They grow rice, produce rubber from plantations, vegetables, mangoes and papaya, and raise pigs, chickens and other livestock (Green World Foundation, 2013). They sell their products to markets in Bangkok and other neighboring countries. Over the last few decades, large tracts of land have been purchased by outside businesses that have quarried the area for laterite for use in construction. This has resulted in a number of open pits. The land was, subsequently, sold to other business interests that have used it as a dumping ground. Much of the dumping has been the illegal disposal of chemical and other toxic waste. Local authorities failed to deal with the illegal dumping. Recycling plants for industrial waste and the recycling of waste such as batteries, electronic equipment, motor oil and other toxic substances have been established in the area. The dumping grounds and recycling plants have been established close to inhabited areas. The surrounding area has become severely polluted with chemical contaminants. Water sources used for household and agricultural purposes, have become polluted, affecting human and animal health. Nong Nae sub-district is close to the industrial estates of Rayong and Chonburi. This means that the area is convenient as a waste disposal site. The illegal dumping of toxic waste means resulted in the spread of these harmful substances throughout the area (Green World Foundation, 2013). Over the last five years, the spread of toxins has led to water resources being effected (Thaipublica, 2015b). Land that is watered, using the contaminated water has also been affected. The qualities of crops produced as well as yields have declined. Decreased agricultural output, reproduction problems in sows and weak and sick piglets are now common. The business of small farmers is threatened, and in many cases, destroyed (Thairathonline, 2015; The National Health Commission Office, 2013a, 2013c). The health of many villagers has been badly affected by using polluted water. Skin infections and irritation with red eruptions and itching is a clear sign of the effect of chemical pollution, with children being most affected. Evidence of these problems has been collected by local and central government officials, with soil and water samples being collected and tested. The main hazardous substance identified, was phenol, which damages the liver, kidneys and reproductive system in both humans and other creatures (Ministry of

Public Health, 2012; Thairathonline, 2012). The contaminated situation is spread from upstream to downstream.

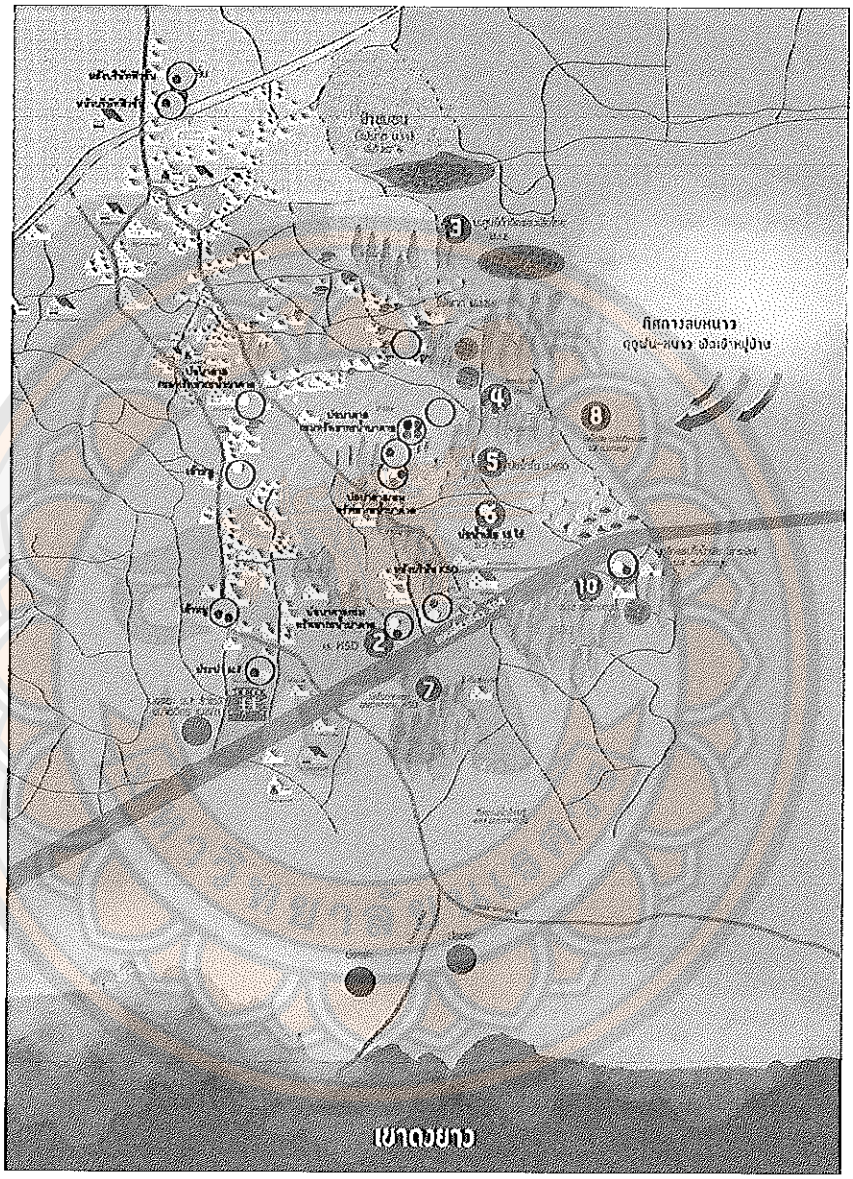

Figure 2 The map shows contaminated area around Nong Nae sub-district (Red points show phenol contaminated are)

Source: http://www.thia.in.th/uploads/image/July%202013/photo_hilight/NongNae/NongNae-Community-Map-for-5-Senimar-13-at-CU.jpg

Phenol and other hazardous waste products such as Phthalate group, Bis phenol A and some heavy metals were found in and around Nong Nae village (Thaipublica, 2015b). As shown in figure 1.2, the red dots are the represent of phenol contaminated area. Phenol concentrations were higher than the maximum level concentration (MLC).

Due to the seriousness of the problem, urgent action to degrade the phenol has been taken. In the planned first phase, the contaminated water needs to be treated with ozone gas to render it safe for human consumption. The next phase is the development of a multidimensional approach to remove all hazardous substances from the local area. At this time local villagers approached us at the Faculty of Engineering at Naresuan University for advice in dealing with this problem (The National Health Commission Office, 2013b). After examining the area, we suggested Phytoremediation using vetiver grass as a solution and a research project was established. The project involves academic staffs and me, as a graduate student in the faculty. The success of the remediation project is being monitored, and steps are being taken against the further dumping of hazardous waste in the area.

Research Aims

1. To evaluates the use of vetiver grass in degrading phenol in contaminated wastewater and groundwater.

2. To compare the efficiency of vetiver plantlets and mature vetiver on degrading phenol in wastewater using floating platforms.

3. To degrade phenol in groundwater by permeable reactive vetiver root barrier (PRVRB) and also use PRVRB as a buffer zone to reduce the phenol concentration from source zone to consumption water resources.

4. To identify and characterize microorganism strains including indigenous and selective microorganisms, from contaminated wastewater and vetiver root, that can tolerate to toxicity of phenol.

5. To study the effect of the selected microorganisms as rhizo-microbes on improving the efficiency of phenol removal on groundwater treatment.

6. To investigate and gain a further understanding of the mechanisms by which vetiver grass degrades and mineralizes phenol in the environment.

Research Scope

1. This study utilized experimental work under laboratory conditions to evaluate the effectiveness of vetiver grass to decontaminate wastewater and groundwater contaminated with phenol.

2. Phenol contaminated wastewater was obtained from contaminated sites. Additional phenol might be added to the wastewater for experimental purposes.

3. All experiments were carried out at room temperature, rather than controlled environments.

4. All experiments had been continued until the phenol concentration is lower than 1 mg/L, the legal standard as an acceptable concentration of phenol in the environment ("ราชกิจจานุเบกษา," ๖ มิถุนายน ๒๕๕๙; "ราชกิจจานุเบกษา," ๗ มิถุนายน ๒๕๖๐)

Research Hypothesis

Vetiver grass occurs naturally in the environment which makes it an ideal choice if it is able to neutralize environmental pollutants. It is also possible, by monitoring the growth and fertility of the grass, to use it as a meter of environmental indicator.

In this study, the ability of vetiver grass as a contaminant removal tool is under investigation. The hypothesis is that, vetiver grass is a viable means of phytoremediation and can be used to degrade phenol in the environment.

Moreover, vetiver root may be the suitable habitation of several microorganisms which promote and support the remediation mechanism by degrading phenol from various media including wastewater and soil. This association may enhance the potential of the phytoremediation using vetiver grass. Finally, the achievement of this study will be useful and applicable at Nong Nae sub-district therefore, this is the model of site remediation forward.

Keywords

Vetiver grass, Phenol degradation, Phytochemicals, Rhizo-microorganisms, Groundwater treatment, Permeable reactive root barrier.

CHAPTER II

LITERATURE REVIEW

This chapter aims to describe the fundamental and related knowledge about what were we focus on for example, phenol and its properties, phytoremediation technics and their application and our selected plant of phytoremediation which was vetiver grass.

Phenol

Phenol, also called phenic acid or carbolic acid, is aromatic compound discovered in 1834 by Friedlieb Ferdinand Runge, German chemist. The first discovery of phenol was appeared by extracting some constitutes from coal tar in refinery process (Franck, & Stadelhofer, 2012). In 1841, phenol was purified as white crystals with disinfectant smell as one of its properties. Since early of 1900's phenol began to be produced from many type of benzene derivation for example, the sulfonation of benzene, cumene hydropeoxidation, toluene oxidation, cyclohexane oxidation. By the way, Isopropylbenzene or cumene by hydroperoxidation reaction is the most important process in the production of phenol because it is the most economical substance among other benzene derivations. The demand of phenol has been increasing widespread used as an important raw material of phenolic resins, caprolactam and bisphenol A production. Bisphenol A and phenolic resins and are mainly used in the early state of plastic manufacturing while, caprolactam is used in synthetic polymer and nylon production. In addition, phenol can be transformed to other secondary intermediates in the industrial processes of surfactants, explosive, paints, fertilizers, textiles, rubber and so forth (Busca, Berardinelli, Resini, & Arrighi, 2008; Schmidt, 2005). However, phenol is also found naturally in natural decomposition of organic material likes animal waste (Bruce, R. M., Santodonato, & Neal, 1987).

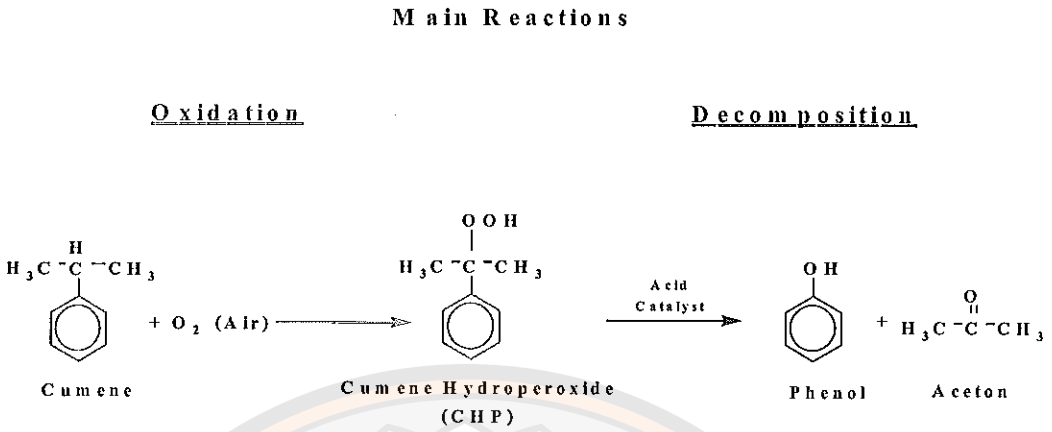

Figure 3 Main reactions for phenol and acetone production via cumene

Source: Schmidt, 2005

1. Production and Consumption of Phenol.

Phenol consumption has increased in every year since, 1985 the production of phenol was about 3.5 billion tons (Franck, & Stadelhofer, 2012) and surpass to 8.3 billion tons in 2012 (Merchance Research and Consulting ltd., 2014). There is no surprising that phenol ranks in the top 50 in production volumes for chemicals produced in the United States (ATSDR, 2008).

Possible stricter environmental regulations concerning formaldehyde emissions from phenolic resins used in wood adhesives and controversy over the health effects of trace amounts of bis-phenol A in polycarbonate resins for food-contact applications may limit phenol consumption growth. Overall consumption of phenol is expected to grow at just around 3% per year over the next five years (Peacock, 2016).

2. Chemical and Physical Properties of Phenol.

To considering the chemical and physical properties of phenol, it consists of a benzene ring with single hydrogen replaced by a hydroxyl group. It is found both in liquid and solid forms. Most of derivative phenols are color compounds but pure phenol has colorless. However, phenol is oxidized and can be changed to red color. In solution phase, phenol solution may be colorless to slightly pink with a distinctive phenol odor which is sharp burning taste. Aqueous solution will be acidic and act as

such (The National Institute for Occupational Safety and Health). Some chemical and physical properties of phenol are presented in table 1 (Busca et al., 2008).

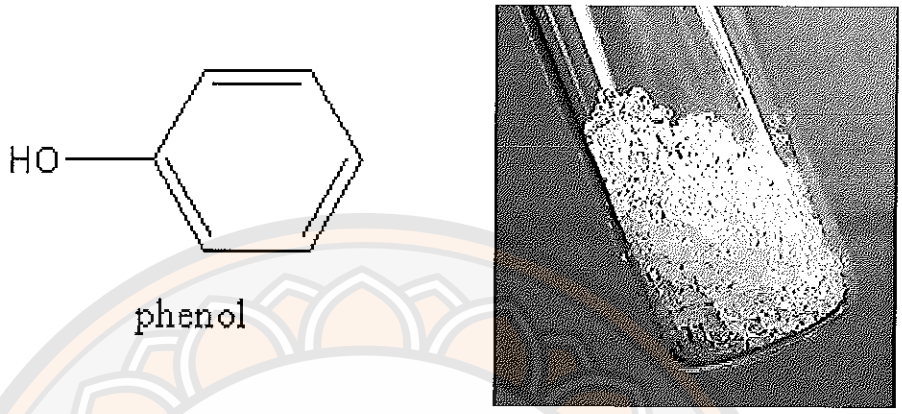

Figure 4 Phenol

Source: https://en.wikipedia.org/wiki/Phenol

Table 1 Phenol characteristic

	Chemical Properties
Formula	C_6H_5OH
Molecular weight (g/mol)	94.11
Melting point (°C)	40.9
Boiling point (°C)	181.75
Vapor pressure (mmHg)	0.4
Water solubility (r.t.)	9.3 g phenol/100 ml H_2O
pK_a	9.89
Flammability limits in air (vol%)	1.7 (lower)
	8.6 (higher)
Flash point (°C)	79 (closed container)
Auto ignition temperature (°C)	715
Specific gravity	1.06

3. Sources, Fates and Contamination of Phenol in Environments.

Phenols are synthetic organic substances, obtained on large, industrial and commercial scales by coal tar extraction in petroleum refinery. Refinery wastewater is identified by high chemical oxygen demand (COD) and high concentrations of phenol and phenolic derivatives by the refinery processes i.e. cooling, cleaning, steam generation, distillation and hydro-treating (El-Naas, Surkatti, & Al-Zuhair, 2016). The reduction of these contaminants is needed before releasing to water resources. For phenol, the maximum concentration level of industrial effluent is limited at 1 mg/L ("ราชกิจจานุเบกษา," ๖ มิถุนายน ๒๕๕๙; "ราชกิจจานุเบกษา," ๗ มิถุนายน ๒๕๖๐). Attempt to deal with reducing phenol concentration is inevitable. Accidentally or intentionally, phenol contaminated in wastewater can occurs by the industrial wastewater release to water resources before the qualities reach the wastewater discharge standard. Sometimes, the characteristic of phenol that can damage cells as disinfectants leads to the illegal wastewater discharge because the high concentration of phenol may fail the biological treatment processes (Villegas et al., 2016). The environmental threats caused by phenols are from processes in which anthropogenic inputs of their application for instance, pesticides, disinfectants, antiseptics or even waste incineration. At normal environmental concentrations, phenols are unlikely to harm wildlife or plants. Leakage of phenols by human usually occur to water or soil, where they can persist (particularly if there is limited oxygen, such as in landfills). Phenols also accumulate in aquatic organisms. Due to the fact that they do not easily evaporate, phenols are not usually found at high concentrations in the atmosphere (SEPA, 2016). Low-level naturally occurring phenols are unlikely to harm human health. But long-term exposure to higher levels of some types (such as chlorophenols) released due to human activities may cause cancer and have other adverse effects on health. These are the incidents of phenol leakage to the environments.

In January 1984, the River Dee in north Wales was contaminated with phenol, with subsequent contamination of the tap water received by about two million consumers. The principal pollutants in the tap water during this incident were phenol and chlorophenols which were produced during the treatment of water supply (about 80% were 2,4,6-trichlorophenol). Poisoning at high concentrations produces corrosive local damage and subsequent systemic shock, with a fatal dose being 80-1,300 mg/kg.

The most concern was 2,4,6-trichlorophenol which was the most poisonous isomeric form and appreciable carcinogenic properties in rats and mice. Due to the production of chlorophenols, the principal criterion is on which international and local standards for the permitted concentrations of phenol in treated drinking water are set as the baseline of 1 ppb (Jarvis, Straube, Williams, & Bartlett, 1985).

The Nakdong river, the source of water supply at Taeku city in South Korea suffered from the contamination of phenol about 30 tons in 1991. The source zone was from a manufacturing industry located 50 kilometers away at upstream of the river. Phenol leakage posed the worse when it met the chlorination process of the central water supply. Phenols transformed to chlorophenol that is more dangerous. The concentration of chlorophenols in tap water was 0.0084 ppm which exceeded 0.005 ppm, the maximum contaminant level (MCL) limited by the Korean Environmental Protection Agency for phenols in drinking water. The 6,931 individuals were undertaken to determine associated with illness such as nausea (4 times greater than that unexposed to phenol), vomiting, diarrhea (4.5 times greater than that unexposed to phenol) or abdominal pain (5.3 times greater than that unexposed to phenol) (Sanger, 1991).

Therefore, phenol and chlorophenols have been identified as "priority substances" for action to prevent harm to the environment and human health as listed in table 2. Internationally, phenols are controlled through the OSPAR and Helsinki conventions which protect the marine environments of the north-east Atlantic and Baltic sea respectively; the Basel convention on transboundary movement and disposal of hazardous wastes; and the Rotterdam convention on the trade of certain hazardous chemicals and pesticides.

4. Toxicity of Phenol.

Toxicity of phenol is concerned for a long time due to health effect to human Phenol poisoning can occur by skin absorption, vapor inhalation or ingestion that can result in detrimental health effects (Parikh, 2015).

4.1 Skin absorption

Phenol is a powerful corrosive poison to human skin. It is rapidly absorbed through the skin causing both of local and systemic toxicity (Bentur et al., 1998; Conning, & Hayes, 1970), but because of its anesthetic qualities, it will usually

numb rather than cause burning pain on contact. However, dermal contact with phenolic compounds may result in irritation at low concentration (greater than 1.5% solution), dermatitis and abnormal pigmentation in chronic exposure to skin, and burns immediately after directly contact. If skin contact with concentrated phenol, second degree burns appeared on patient who contact with spilled phenol 15% dilution and third-degree burns will follow because the reaction between phenol and amino acids in keratin and collagen of epidermis and inner skin (hydrophobic effect of phenol) (Michałowicz, & Duda, 2007). However, systemic toxicity and poisoning usually occur by skin absorption, the main route of entry for solid, liquid or vapor phenol; the result can be caused death (Lin, Lee, Lai, & Lin, 2006). Moreover, phenol performs upon the central nervous system causing excitation and convulsions followed by sudden collapse and unconsciousness. Death can be very rapid and is due to toxic effects on the central nervous system, the heart, lungs, kidneys, and blood vessels. Also reported that people who had skin exposure to high amounts of phenol had skin burns, liver damage, dark urine, irregular heartbeat, and some died (ATSDR, 2008). The level of phenol that resulted in laboratory animals were shown,

Rat LD_{50}	669	mg/kg
Rabbit LD_{50}	850	mg/kg

LD_{50} = Dose that is lethal to 50% of a given species by a given route of exposure.

4.2 Inhalation

The melting temperature of phenol is about 41 °C so that, bring phenol evaporate easily in normal temperature. A phenol vapor is easily absorbed through both the lungs and the skin by resulting in lung irritation and pulmonary edema (ATSDR, 2008; US.EPA, 2002). Phenol absorbed through the lungs is excreted rapidly in urine in its free and conjugated forms. Within 24 hours after human subjects inhaled phenol at concentrations of 6–20 mg/m^3 (1.5–5.1 ppm), 99±8% of the phenol retained in the lungs was excreted (Piotrowski, 1971). The evaporation of phenol can cause many results of headache, loss of body weight, weakness, muscle pain and anorexia. Urinary excretion of total phenol (free and conjugates) is considered a biomarker of exposure for phenol. The biological exposure index (BEI) for phenol, for exposure to 5 ppm in air (for phenol: 1 ppm = 3.85 mg/m^3 at 25 °C), is 250 mg/g

creatinine when measured at the end of the exposure time (Acgih, 2001). The dark urine may be a result of an oxidation product of phenol or hemoglobin or hemoglobin breakdown products. Acute-and intermediate-duration inhalation studies in animals are available (US.EPA, 2002). The effect of phenol exposure was observed in rats but rats did not show the significant of toxicity due to phenol was oxidized efficiently and readily exposed to a 25 ppm phenol concentration (Hoffman et al., 2001). Although, the death of rats and rabbits were not observed but not for death in guinea pigs which were died by exposure inhalation of phenol vapor concentration as low as 26 ppm or as high as 52 ppm for 41 days (Deichmann, 1944) thus, the lower limit of the exposure range, 26 ppm, is recorded as a serious LOAEL.

LOAEL = the lowest-observed-adverse-effect level

4.3 Ingestion

Ingestion directly leads to nausea, vomiting, burning of the mouth, mouth sores, diarrhea, and marked abdominal pain as well as respiratory and cardiovascular effects. Some symptom in showed in painless on white/brown skin and mucosal burns because of the destruction of nerve ending. Internally, it damages to the kidney, livers, pancreas, and spleen when death is delayed (*Summary review of the health effects associated with phenol [microform] : health issue assessment*, 1986).

Accidental and intentional ingestions of phenol have been reported. As small as 50 to 500 mg has been fatal in infants. Deaths in adults have resulted after ingestions of 1 to 32 g (ATSDR, 2008).

Rat LD_{50}	530	mg/kg
Cat LD_{50}	100	mg/kg
Dog LD_{50}	500	mg/kg
Human LD_{LO}	140	mg/kg
Infant LD_{LO}	10	mg/kg

LD_{LO} = Lowest lethal dose in a given species by a given route of exposure.

LD_{50} = Dose that is lethal to 50% of a given species by a given route of exposure.

4.4 Eye contact

Phenol and its vapor can absorb not only on skin but also on eye, it causes severe corrosive and burn. The irritation takes place with tearing and followed by corneal injury then numbness, severe burns, eschar formation and permanent blindness (Lin et al., 2006).

5. Chlorophenols and Their Toxicity: Pentachlorophenol (PCP).

Chlorophenols are synthetic organic compounds by chlorinating phenol or hydrolysing chlorobenzenes. Chlorophenols found to be ubiquitous in the environment from a variety of sources for example, industrial waste, pesticides, and insecticides, or by degradation of complex chlorinated hydrocarbons by biological process. Chlorophenols are able to pose adversely affect to biological systems. These compounds are recalcitrant and persistent in the environments. In aquatic ecosystems, their potential to contaminate aquatic ecosystems (ground and surface waters) and therefore, resulting in great risk to humans and other organisms associated with the food chain of the aquatic eco-biota by accumulation. Bioaccumulation coefficients (BCF) for 2,4-dichlorophenol are: 1.00 for trout, 1.53 for gold fish and 2.41 for algae. Higher BCF are discovered for PCP, and they are: 1000 for gold fish, 324 for mussels and 78 for oysters (Czaplicka, 2004; Hooived et al., 1998). The compounds are toxic in humans and animals by being the causes of histopathological changes, mutagenic, and carcinogenic effects. Moreover, it could be worse by the fact that chlorophenols are recalcitrant that they may maintain high toxicity levels within the environments for a very long time due to they favour with the accumulation and sorption especially, in bottom sediments and on suspended matter (Czaplicka, 2004; Igbinosa et al., 2013).

Based on their toxicity, stability, and bioaccumulation, the European Union (EU) and US.EPA classify the priority contaminants of some chlorophenols in table 2.

Table 2 Priority contaminants established by EU and U.S. EPA

EU	U.S. EPA
2-Amino-4-chlorophenol	Phenol
2-Chlorophenol	2-Chlorophenol
3-Chlorophenol	2,4-Dichlorophenol
4-Chlorophenol	4-Chloro-3-methylphenol
4-Chlorophenol-3-methylphenol	2,4,6-Trichlorophenol
2,3,4-Trichlorophenol	Pentachlorophenol
2,4,5-Trichlorophenol	
2,4,6-Trichlorophenol	
3,4,5-Trichlorophenol	
3,5,6-Trichlorophenol	
Pentachlorophenol	

Source: Igbinosa et al., 2013

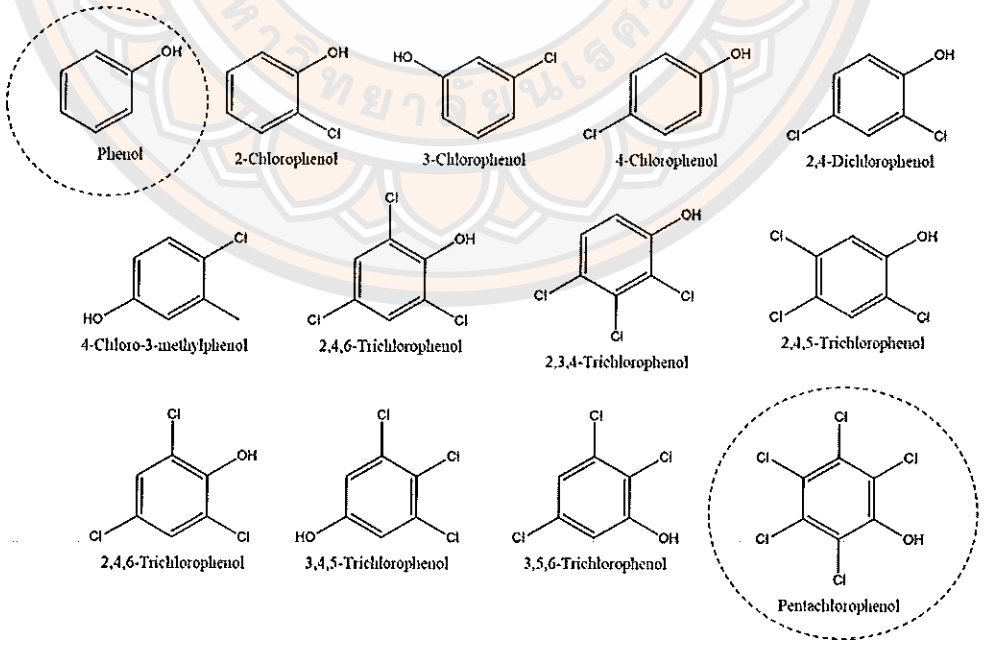

Figure 5 illustrated the scheme of some chlorohenols which are on the List Priority Contaminants of EU and U.S. EPA.

By the degree of chlorination and the position of chlorine atoms, toxicity of chlorophenols decreases with the number of chlorine substituents. These suggest that pentachlorophenol is more toxic than other chlorophenols. Hence, we emphasize on PCP.

PCP was used in the preservation and treatment of wood. In certain applications (fence poles, housing substructures), pentachlorophenol color is relatively unimportant. In many other uses in the woodworking industry, particularly where painting of the treated product is necessary, a light or water-white colored pentachlorophenol was highly desirable (Christena, 1975). Although pentachlorophenol was widely used as a pesticide, its use has been restricted so that only people with special certification or a license can purchase and use it.

The production of PCP is based on the chlorination of phenol at a temperature between 65 and 200 °C in the presence of properly prepared tellurium and its compounds as chlorination catalysts. The process consists in a successive replacement of five hydrogen atoms combined with the five carbon atoms of the benzene ring by five chlorine atoms. PCP is extremely toxic to humans from acute (short-term) ingestion and inhalation exposure. Acute inhalation exposures in humans have resulted in neurological, blood, and liver effects, and eye irritation. Chronic (long-term) exposure to pentachlorophenol by inhalation in humans has resulted in effects on the respiratory tract, blood, kidney, liver, immune system, eyes, nose, and skin. Human studies are inconclusive regarding pentachlorophenol exposure and reproductive effects. Human studies suggest an association between exposure to pentachlorophenol and cancer. Oral animal studies have reported increases in liver tumors and two uncommon tumor types. US.EPA has classified PCP as a Group B2, probable human carcinogen (US.EPA, 2010b).

Due to the extremely toxicity of pentachlorophenol, the maximum contaminant level goal (MCLG) for pentachlorophenol is zero. US.EPA has set this level of protection based on the best available science to prevent potential health problems. U.S.EPA has set an enforceable regulation for pentachlorophenol, called a maximum contaminant level (MCL), at 0.001 mg/L or 1 ppb. MCLs are set as close to the health goals as possible, considering cost, benefits and the ability of public water

systems to detect and remove contaminants using suitable treatment technologies (US.EPA, 2009).

The formation of chlorophenols in Nong Nae sub-district may occur if the phenol contaminated water is purified and disinfected by chlorine.

6. Phenol Removal.

Through the hazard of phenol and its derivations, the typical potential treatments are required since its occurrences in various industries in many mediators like wastewater, air vapors and soil. Before discharge of these mediators which are contaminated with phenol to environment. The principal methods and technologies are promoted to the effective phenol removal.

6.1 Abatement of phenol from wastewater

Phenol and its derivatives are mainly determined as the hazardous organic pollutants in the serious discharge of wastewater because of the highly toxic even at low concentrations. Besides, phenol in natural water leads to the formation of other toxic compounds during disinfection and oxidation processes. The demands of phenol as substrate in various industries are brought into the contamination in water effluent (Busca et al., 2008). To illustrate, petroleum refinery (13-500 mg/L) (Abdelwahab, Amin, & El-Ashtoukhy, 2009; El-Naas, Al-Zuhair, & Alhaija, 2010), pesticides (100–42,000 mg/L)(Y. Zhang, & Pagilla, 2010) , pulp-paper (0.5-1,600 mg/L) (Uğurlu, Gürses, Doğar, & Yalçın, 2008), coke industrial (333-1,200mg/L) (Vázquez, Rodríguez-Iglesias, Marañón, Castrillón, & Álvarez, 2007), foundries (330 mg/L) (Cooper, & Nicell, 1996), resin plants (12-300 mg/L) (Mukherjee, Kumar, Misra, & Fan, 2007).

Phenol has been set as priority pollutants by the U.S. Environmental Protection Agency with a permissible level less than 0.1 mg/L in natural water (Brandes, Newton, Owens, & Southerland, 1985). By the way, the world health organization (WHO) is stricter on phenol regulation. It limits a 0.001 mg/L as the limit of phenol concentration in potable water (World Health Organization, 1958). In Thailand, PCD agrees to set the water purification standard of discharged water from industries to natural water resources is 1 mg/L. Several treatments have been used to eliminate phenol from wastewater, including phenol separation and destruction.

6.2 Phenol separation from water solutions

6.2.1 Extraction

Extraction of phenol from wastewater is a quick, reagent-saving treatment technique for phenolic wastewater. This method is suitable for phenol-containing wastewater which has concentration over than 3,000 mg/L.

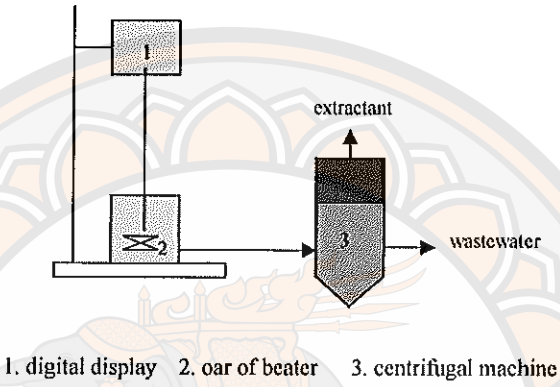

Figure 6 Schematic of liquid-liquid extraction

Source: Jiang, Fang, Fu, & Guo, 2003

Some chemical plant in southern of China discharged the wastewater was containing 6,000 mg/L phenol used alcohols, amines and acids as extractants. The result shows $C_8H_{17}OH$ is the best extractant with phenol removal efficiency 99.6% in pilot-scale due to its shorter hydrocarbon chains but alcohols with lower than 8 carbon atoms have greater solubility in water, so the extraction efficiencies are lower than alcohols with upper than 8 carbon atoms (octanol). Extraction is a method available to reuse the phenol and water by separating phenol and salt from wastewater (Jiang et al., 2003).

6.2.2 Adsorption

Adsorption is a well-known and effective method of phenol removal in wastewater from various industries. This method is included in advance wastewater treatment. It is the process of collecting adsorbate which is the substances that are in solution from liquid phase on the appropriate interface. Adsorbate will be

removed and attached on adsorbent that can be in solid, liquid, gas phase. The solid adsorbents are widely used because of two main properties include its greatly large surface area to volume ratio and its special affinity for certain components in the liquid phase.

Most of the solid adsorbents of the industrial applications create a large complex porous structure that consists of pores of different sizes and shapes. In term of adsorption science, the total surface properties are classified into three groups. These three group are identified below

Macropores are identified as pores of a size greater than 25 nm

Mesopores are identified as pores of a size between 1 to 25 nm

Micropores are identified as pores of a size not exceeding 1 nm

The fundamental concepts in adsorption science are described in four steps:

1. Bulk solution transport is the first step that the organic substances move and adsorb through the bulk liquid to the boundary layer of fixed film around the adsorbent by advection and dispersion.

2. Film diffusion transport implies the transport by diffusion of the adsorbate to the entrance of the pores of adsorbent through the stagnant liquid film.

3. Pore transport involved the conjunction of molecular diffusions through the pore liquid and/or diffusion along the surface of adsorbent adsorbs the organic substances.

4. Adsorption involves the connection of the adsorbate to be adsorbed at an accessible area. It can take place on outer space include macropores, mesopores, micropores and sub-micropores of the adsorbent but the amount of adsorbates which are adsorbed on the surface area of the macro- and mesopores are usually considered negligible because its small area compares with the surface area of micropores and sub-micropores.

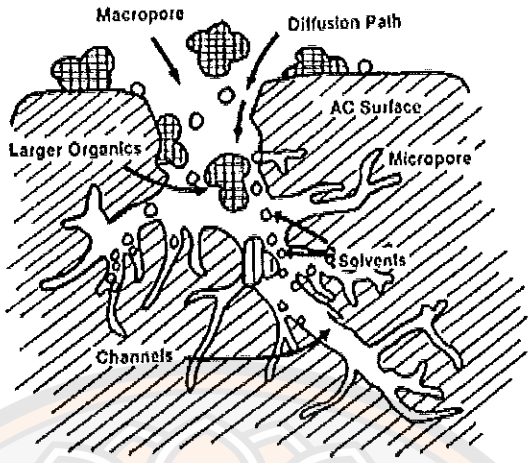

Figure 7 Pore structure of selected adsorbent (activated carbon)

Source: http://207.56.82.96/ENGLISH/WT/filtration/pu.htm

The function of the characteristics and concentration of adsorbate relate to the quantity of adsorbate which is attached on adsorbent. The characteristics of the adsorbate that are considering are its molecular structure, molecular weight, polarity, solubility and hydrocarbon saturation.

From various studies, it can be shown the potential of adsorption process as the secondary process of phenol-containing wastewater treatment.

6.2.3 Membrane

Membrane separation, which is the physical-chemical process, is a capable method with low-energy consumption for example, membrane pervaporation (Hoshi, Kogure, Saitoh, & Nakagawa, 1997) and membrane-based solvent separation (Lazarova, & Boyadzhieva, 2004).

6.3 Phenol destruction in water solution

6.3.1 Oxidation of phenol by air or oxygen in aqueous solution

This process is well established process for wastewater treatment, especially when these are too dilute to incinerate and too toxic to use biological treatment. The standard circumstances of this process are 180°C and 2 MPa to 315°C and 15 MPa. Retention time is about 15-120 min. The advantage is Insoluble organic substance will be converted to simple organic substances without emission of

SO_2, HCl, dioxins, furans, fly ash, NO_x, etc. (Luck, 1999). Wet air oxidation is grounded on the concept of air's oxygen properties oxidation.

$$O_2 + 4H^+ + 4e^- \quad 2H_2O \quad E^o = +1.23\ V \quad \text{(Eq 2.1)}$$

$$O_2 + 2H_2O + 4e^- \quad 4OH^- \quad E^o = +0.40\ V \quad \text{(Eq 2.2)}$$

For eliminated phenol from wastewater, it is significantly controlled by reaction temperature and oxygen partial pressure and pH as well. At 220 °C and P_{O2} = 3MPa while, 88% TOC conversion occurs compared to the conditions of 130 °C and P_{O2} = 0.5 MPa, about 5% TOC conversion occurs after 2 hr. In addition, pH 2-7 and over than 10, phenol destruction is significantly presented. The intermediates and by-products observed by different authors were formic acid, acetic acid, maleic acid, oxalic acid, succinic acid, acetone, and acetaldehyde. The *ortho* and *para* dihydroxy-benzenes (catechol and hydroquinone) and *ortho* and *para* benzoquinones are presumed to act as prior substances for aromatic ring opening.

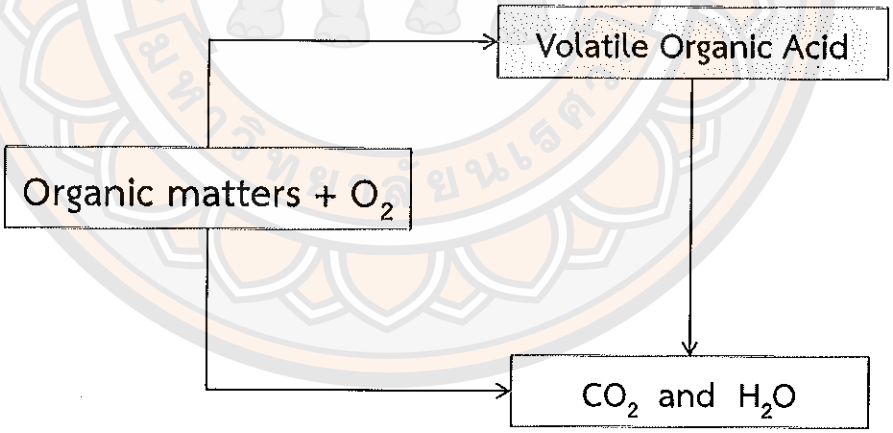

Figure 8 Model for wet air oxidation

Source: Luck, 1999

Organic feed destruction rates are usually higher than 99.99% producing CO_2 and water. Nevertheless, the real behavior of phenol oxidation under hydrothermal conditions is hard to explain since many factors include oxygen/phenol ratio, different geometry or/and materials of reactor, operating order and etc.

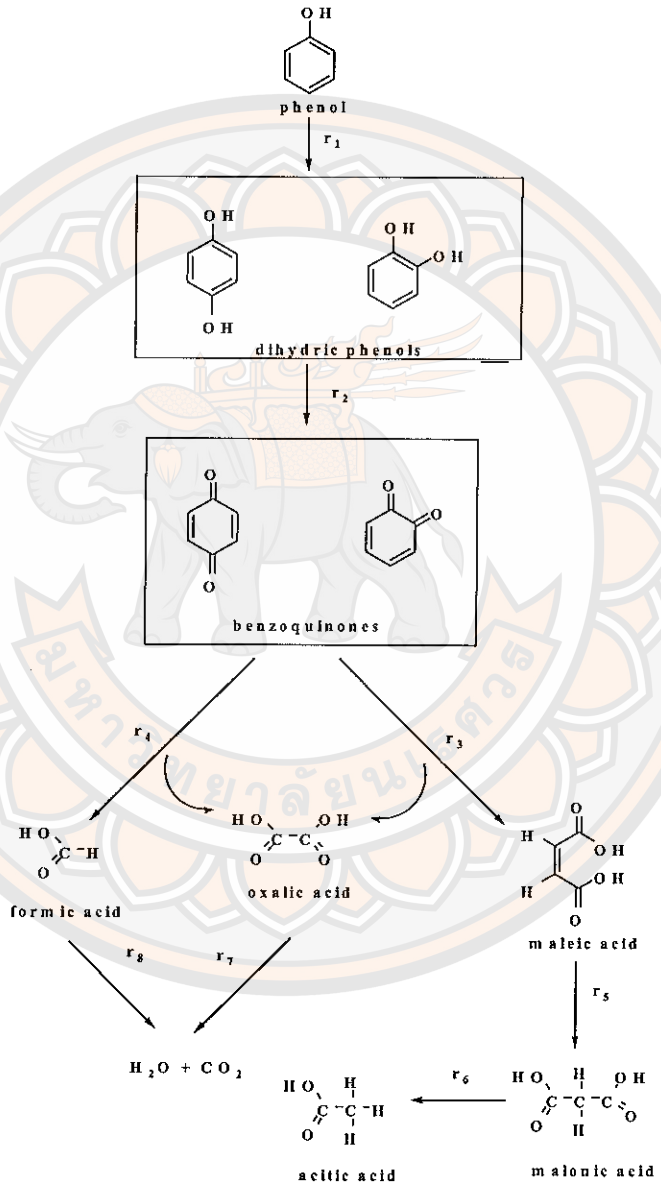

Figure 9 Simplified reaction pathways for catalytic wet air oxidation of phenol

Source: Eftaxias et al., 2001

Figure 9 shows the oxidation of phenol conducts considerably via two routes. First, directly to carbon dioxide and water, and second, through intermediates which are difficult to oxidize over the catalysts studied.

6.3.2 Oxidation with chemical oxidants

Chemical oxidants have been used to be active in the oxidation of phenol in aqueous phase. The most commonly oxidants used in the process are ozone and hydrogen peroxide.

6.3.3 Ozone oxidation process

Ozone is the one of the dominate oxidants technically applied according to some reasons as describes below (Sano et al., 2007).

1. Reactivity of ozone is quite strong so that organic compounds can be degraded effectively.

2. Ozone can be generated easily by use of ultraviolet light or electric discharge.

3. Ozone itself can be converted to O_2 easily.

4. Ozone reaction can be combined with other method to achieve enhanced water purification.

Ozone reacts directly through the nucleopillic sites and unsaturated bonds of the organic pollutants consistent with the high reduction potentials both at acid and basic pHs.

$$O_3 + 2H^+ + 2e^- \rightarrow O_2 + H_2O \qquad E^o = 2.07\ V \qquad \text{(Eq 2.3)}$$

$$O_3 + H_2O + 2e^- \rightarrow O_2 + 2OH^- \qquad E^o = 1.24\ V \qquad \text{(Eq 2.4)}$$

Ozone reacts with aqueous solutions in two ways include direct reactions of the molecular ozone with the compounds; and indirect reactions of the radicals resulting from the decomposition of ozone with the compounds. The direct reactions are more important in acidic solutions, while the indirect free radical reactions establish a significant portion of the ozonation at basic pH (Rice, 1982). It can be seen that OH radical is one of the most reactive species in ozonation of water and the amount of OH radical determines the efficiency of ozonation (Hoigné, 1988).

6.3.4 Oxidation with hydrogen peroxide

Hydrogen peroxide is a clear, colorless liquid which is completely miscible with water. Concentrated aqueous solutions are considerably more soluble in organic solvents than water (Strukul, 2013). Hydrogen Peroxide (H_2O_2) is one of the most powerful oxidizers due to a number of advantages compared to other common low cost oxidants, safe storage, operation method, effective oxygen content and an environmentally friendly nature. H_2O_2 can be converted into hydroxyl radicals with reactivity second only to fluorine and can be decomposed into water and oxygen by enzymatic and non-enzymatic routes. Standard potential is 1.80 V and 0.87 V at pH 0 and 14, respectively.

$$H_2O_2 + 2H^+ + 2e^- \rightarrow 2H_2O \qquad \underline{E^o = 1.77V} \qquad \text{(Eq 2.5)}$$

$$HO^{2-} + H_2O + 2e^- \rightarrow 3OH^- \qquad E^o = 0.87V \qquad \text{(Eq 2.6)}$$

Depending on the purposes, hydrogen peroxide is used either alone or in combined applications with other processes to improve the performance. Improvements can be achieved by using transition metal salts (e.g. iron salts) or ozone and UV-light can activate H_2O_2 to form hydroxyl radicals, which are strong oxidants. Oxidation processes utilizing activation of H_2O_2 by iron salts, conventionally referred to as Fenton's reagent is known to be very effective in the destruction of many hazardous organic pollutants in water (Neyens & Baeyens, 2003). Transition of iron salts ($FeSO_4$) and hydrogen peroxide are presented below as Fenton reaction;

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^- \qquad \text{(Eq 2.7)}$$

$$H_2O_2 + Fe^{3+} \rightarrow Fe^{2+} + OOH^{\bullet} + H^+ \qquad \text{(Eq 2.8)}$$

The ferrous ion (Fe^{2+}) initiates and catalyzes the decomposition of H_2O_2, resulting in the generation of hydroxyl radicals (Eq 2.7). Hydroxyl radicals (oxidation potential: 2.8 V) are stronger oxidants than ozone and H_2O_2. In addition, the lately formed ferric ion (Fe^{3+}) may catalyze hydrogen peroxide, causing it to be decomposed into water and oxygen. Ferrous ions and radicals are also formed in the

reactions (Eq 2.8). Hydroxyl radicals non-specifically oxidize target compounds at high reaction rates with the optimal pH range are between 2.7 and 3.5.

This is a very simple way of producing OH radicals neither special reactants nor special apparatus being required due to the fact that iron is very abundant and non-toxic element and hydrogen peroxide is easy to handle and environmentally safe (Andreozzi, Caprio, Insola, & Marotta, 1999).

The phenol degradation has been confirmed by hydroxyl radical reaction which is given as:

$$\text{Phenol} + \bullet OH^{-} \rightarrow \text{oxidized products} \qquad \text{(Eq 2.9)}$$

Zazo, et al. described the chemical pathway of phenol degradation by oxidation of Fenton's reagent (Zazo, Casas, Mohedano, Gilarranz, & Rodriguez, 2005). The conditions of this study were conducted by phenol-containing water 100 mg/L, H_2O_2 as oxidants 500 mg/L and Fe^{2+} as catalysts 1 mg/L at temperature 25 °C, pH 3. The results showed the performance of this process under these experimental conditions that close to 90% of phenol is converted to dihydroxybenzenes, catechol, hydroquinone (para-hydroxylation) and resorcinol (meta-hydroxylation) in about 30 min. Ring-opening of the aromatic intermediates leads to the formation of organic acids. As a result, a pH decrease takes place, although it remained always within the optimum range, above 2. These compounds are intermediates and/or final oxidation products. Maleic, acetic, and formic acids were the earlier and more abundant products of the acid formation stage.

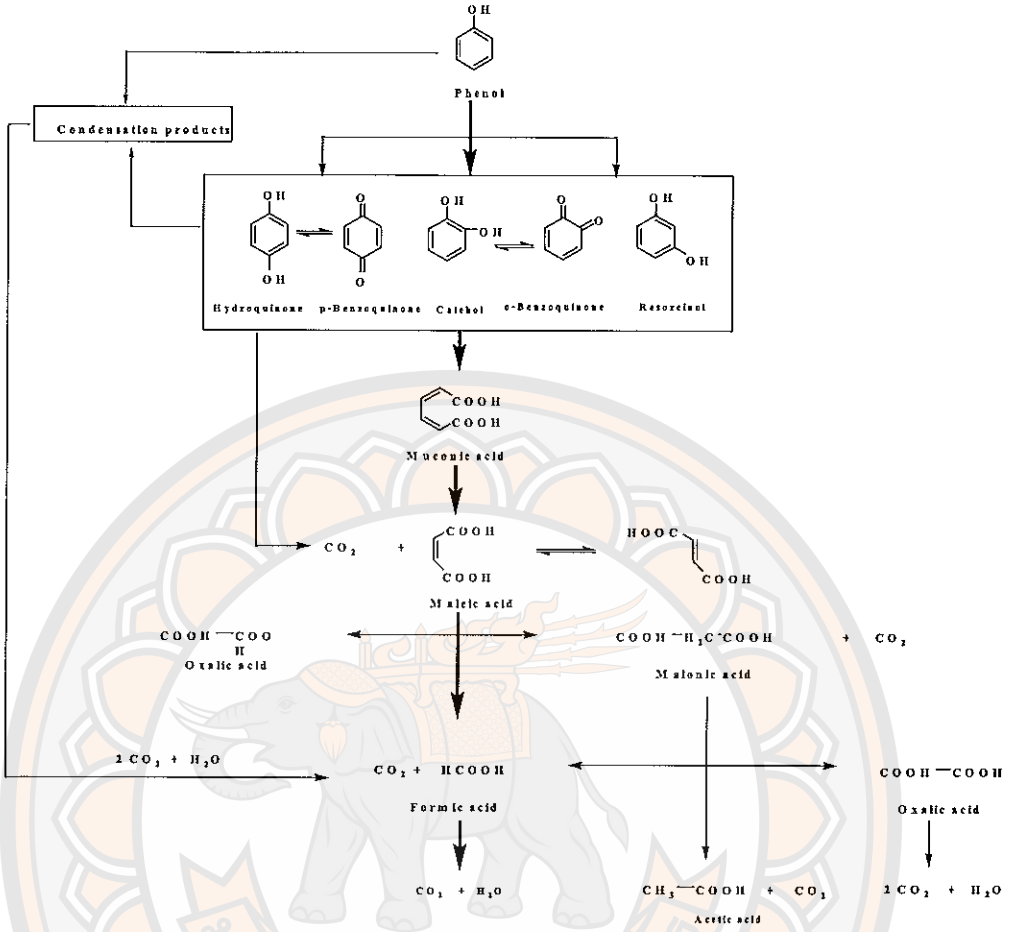

Figure 10 Proposed routes for phenol oxidation by Fenton's reagent

Source: Zazo et al., 2005

6.3.5 Biochemical abatement

Many microbial strains capable of degrading phenol have been cited such as *Pseudomonas putida*, *Pseudomonas fluoroescens*, *Acinetobacter*, *Trichosporon cutaneum* and *Candida tropicalis* (Ren, Wang, Zhao, & Cai, 2008). Most of the cultures tested are capable of degrading phenol at low concentrations. However, phenol is toxic to most types of microorganisms at sufficiently high concentration and can be a growth rate inhibitory to even those species, which have the metabolic capability of using it as a substrate for growth.

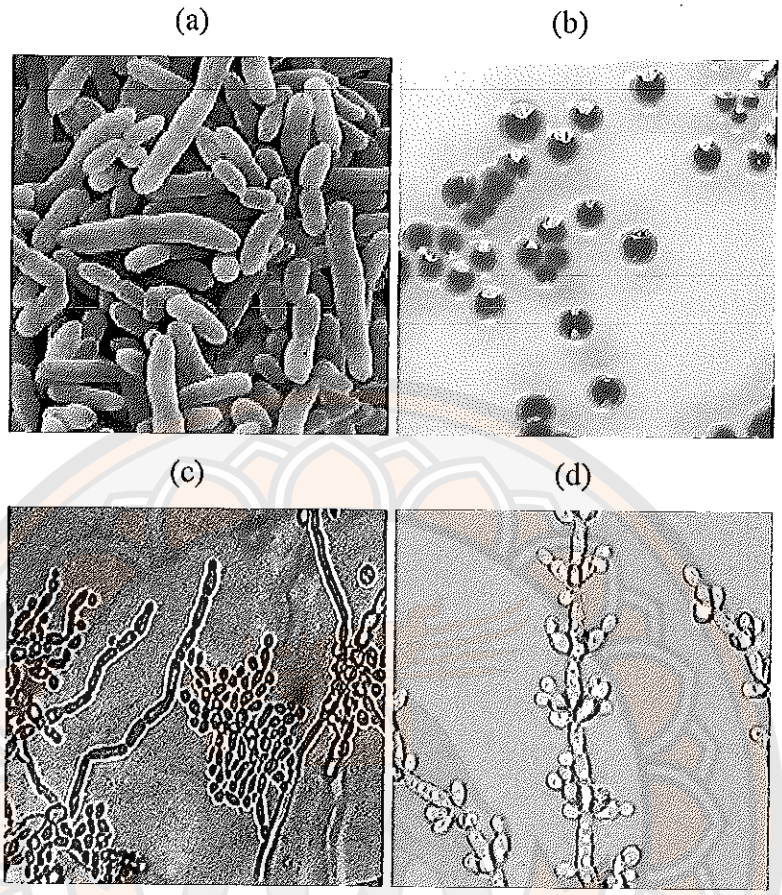

Figure 11 some species of microorganism in phenol degradation by bio-treatment

Note: (a) *Pseudomonas putida, n.d.* is gram negative aerobic bacteria known as super bug (http://bioremediationmadesimple.weebly.com/)

(b) *Acinetobacter* is a gram negative aerobic coccobacillary rod bacterium (http://www.chromagar.com)

(c) *Trichosporon cutaneum* is anamorphic fungi and typically isolated from soil (http://www.tk.de/rochelexikon/pics/p39215.000-1.html)

(d) *Candida tropicalis* is a species of yeast in the genus Candida (http://www.life-worldwide.org/)

Phenol is a recalcitrant compound, which exerts toxicity to microorganisms during biological treatment, leading to failure of the whole wastewater treatment system, if the microbial flora is not adapted to phenol. Phenol is growth inhibitory to bacteria at concentrations above 0.05 g/L and is bactericidal at concentrations of about 2 g/L, if not adapted to phenol (Heipieper, Keweloh, & Rehm, 1991).

The potential of microorganism strains in phenol degradation have been reported in several studies for example; Phenol biodegradation using a pure culture of *Candida tropicalis* was studied through activated sludge. The initial phenol concentration varied from 0 to 2,000 mg/L, the temperature of 30 °C, and the initial pH of 6.0. *Candida tropicalis* shows the high phenol degradation potential to eliminate phenol concentration about 2,000 mg/L within 66 hr. in the mineral salt medium. However, for a certain starting inoculum, with the step increase of phenol concentration, substrate inhibition was obviously enhanced, and more phenol consumption was not assimilated by cell for growth, but was used to detoxify strong substrate inhibition (Yan, Jianping, Hongmei, Suliang, & Zongding, 2005).

Annadurai, et al. conducted the application of bio-degradation and adsorption used in degradation of phenol by chitosan-immobilized *Pseudomonas putida* (NICM 2174) in batch reactor (Annadurai, Babu, Mahesh, & Murugesan, 2000). Cell growth contributed significantly to phenol removal rates especially when the degradation medium was supplemented with a utilizable carbon source. The optimization of initial phenol concentration, temperature and pH were found to be 200 mg/L, 7.0, and 30 °C respectively. The results show the process followed first order rate kinetics and achieve the phenol degradation rate 0.1021/min.

Bio-filtration is the common process performs in removing phenol from waste gases. For the preliminary batch tests, carried out in order to test the effective feasibility of the process. The continuous experiments have been conducted during a period of about 1 year in a laboratory-scale column, packed with a mixture of peat and glass beads, and utilizing the pure culture of *Pseudomonas putida* as microflora and varying the inlet phenol concentration from 50 up to 2000 mg/m^3. The results obtained show that high degrees of conversion can be obtained (0.93/0.996) operating at a residence time of 54 s (Zilli, Converti, Lodi, Borghi, &

Ferraiolo, 1993). This technique is becoming widely applied due to its efficiency (in particular for Sulphur compounds if the feed is not excessively concentrated), to the moderate capital costs, and to the very low maintenance costs. In particular, biofiltration seems to be the choice technique for the treatment of the odorous emissions arising from wastewater treatments (Busca et al., 2008).

Phytoremediation

The definition of phytoremediation is defined by the US.EPA which is "the direct use of green plants and their associated microorganisms to stabilize or reduce contamination in soils, sludge, sediments, surface water, or ground water" (US.EPA, 2000).

The basic idea that plants can be used for environmental remediation is very old and cannot be traced to any particular source. According to Cunningham et al., the goal of phytoremediation is to completely mineralize organic contaminants into relatively nontoxic constituents, such as carbon dioxide, nitrate, chlorine, and ammonia (Cunningham, & Ow, 1996). However, a series of fascinating scientific discoveries combined with an interdisciplinary research approach have allowed the development of this idea into a promising, cost-effective, and environmentally friendly technology. It is more cost-effective than alternative mechanical or chemical methods of removing hazardous waste from the soil (Bollag, Mertz, & Otjen, 1994). It is socially accepted by surrounding communities and regulatory agencies as a potentially elegant and beautiful technology (Ensley, 1997).

Phyto-technology is an excellent cleanup technology with various advantages:

1. Low-maintenance, passive, in situ, self-regulating, solar-driven system
2. Potentially applicable in remote locations without utility access
3. Decreased air and water emissions as well as secondary waste
4. Control of soil erosion, surface water runoff, infiltration, and fugitive dust emissions
5. Applicable to simultaneously remediate sites with multiple or mixed contaminants
6. It can be used in conjunction with other remediation methods and may be more beneficial than a stand-alone technology

7. Habitat creation or restoration provides land reclamation upon completion
8. Favorable public perception, increased aesthetics, and reduced noise
9. Increasing regulatory approval and standardization
10. Carbon dioxide and greenhouse gas sequestration
11. Cost-effective and environmentally friendly technology

Also, there are several disadvantages of this technology, which include the following (Prasad, 2007; Schnoor, Light, McCutcheon, Wolfe, & Carreia, 1995):

1. Growth habit of the planted system
2. Slow/shallow root penetration of the selected plant(s)
3. Limited to available land
4. Susceptible to infestation and disease
5. Bioaccumulation of contaminants in vegetation
6. By-products may be more toxic
7. Limited capability for contaminant mass transfer to the treatment zone or root zone
8. Unfamiliarity by public or regulatory communities
9. May be difficult to establish or maintain vegetation
10. Relatively slow in comparison to more active remediation technologies
11. Dependence on local climatic conditions and growth season

Despites, constraints referenced above, plants have many features that result in a high potential for environmental cleanup. Plants are adapted to a wide range of environmental conditions and are capable of modifying conditions of the environment to some extent.

Phytoremediation may be applicable for the remediation of metals, pesticides, solvents, explosives, crude oil, PAHs, and landfill leachates. Some plant species have the ability to store metals in their roots. They can be transplanted to sites to filter metals from wastewater. As the roots become saturated with metal contaminants, they can be harvested. Hyper-accumulator plants may be able to remove and store significant amounts of metallic contaminants. Currently, trees are under investigation to determine their ability to remove organic contaminants from groundwater, for translocation and transpiration, and possibly to metabolize them either to CO_2 or plant tissue. Phytoremediation can be used to clean up organic contaminants from surface

water, groundwater, leachate, and municipal and industrial wastewater. Plants also produce enzymes, such as dehalogenase and oxygenase, which help catalyze degradation. Constructed wetlands have most commonly been used in wastewater treatment for controlling organic matter; nutrients, such as nitrogen and phosphorus; and suspended sediments. The wetlands process is also suitable for controlling trace metals and other toxic materials. Additionally, the treatment has been used to treat acid mine drainage generated by metal or coal mining activities. These wastes typically contain high metal concentrations and are acidic. The process can be adapted to treat neutral and basic tailing solutions (Anjum et al., 2012). Thus, phytoremediation can be applied to both organic and inorganic pollutants, present in solid substrates (e.g. soil), liquid substrates (e.g. water), and the air (Salt, Smith, & Raskin, 1998). Phytoremediation is currently divided into the following mechanisms in Figure 12.

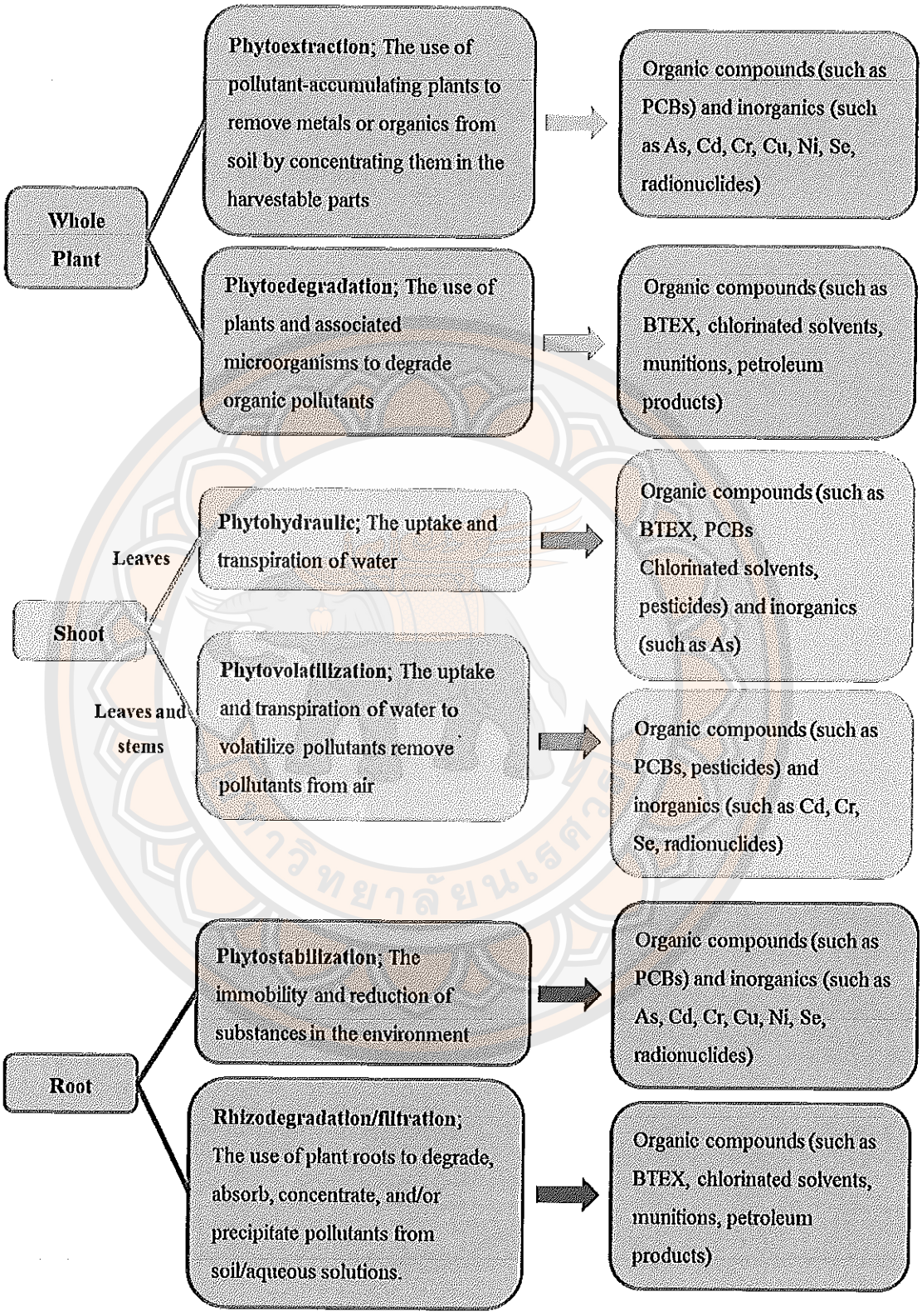

Figure 12 Schematic representations of major phyto-technology mechanisms

Source: Anjum et al., 2012

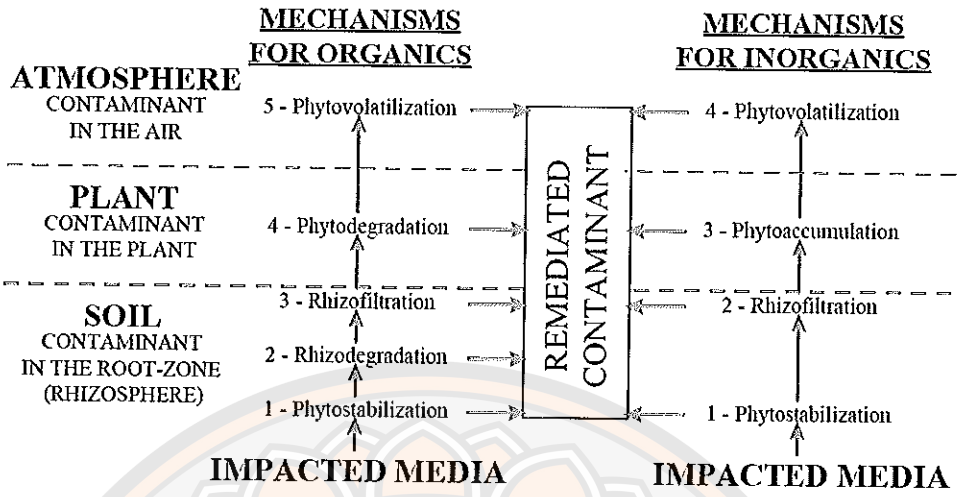

Figure 13 Contaminant fates in the Soil-Plant-Atmosphere continuum

Source: Team, 1999

1. Phytoextraction

The use of plants to remove contaminants from the environment and concentrate them in above ground plant tissue is known as phytoextraction. Phytoextraction is also called phytoaccumulation. Phytoextraction is the ability of plants to remove pollutants particularly metals and other compounds in the range of 100- to 1000-fold the levels normally found in most plant species and translocate them into the above-ground harvestable biomass (leaves, stems and other plant tissues) most especially through a process termed hyper-accumulation because they accumulate appreciable quantities of metal in their tissues regardless of the concentration of metal in the soil (or other contaminated media), as long as the metal in question is present (Erakhrumen, 2012; Prasad, 2007).

Hyper-accumulation of noxious substances such as heavy metals is a complex phenomenon which involves several steps such as

1. Transport of metals across plasma membrane of root cells,
2. Xylem loading and translocation, and
3. Detoxification and sequestration of metals at the whole plants and cellular levels (Lombi, Zhao, Dunham, & McGrath, 2001).

Different types of phytoextraction methods have been investigated but two basic strategies are mostly reported. These are 1) continuous phytoextraction or natural hyperaccumulation in which the removal of contaminants depends on the genetic and physiological capacity of specialized plants to accumulate, translocate, and resist high amounts of metals. Major disadvantages of using naturally occurring metal hyperaccumulators for continuous phytoextraction are their relatively low biomass, slow growth rates, and the lack of any hyperaccumulators for the most environmentally important metallic pollutants (e.g. lead, cadmium, arsenic, and uranium).

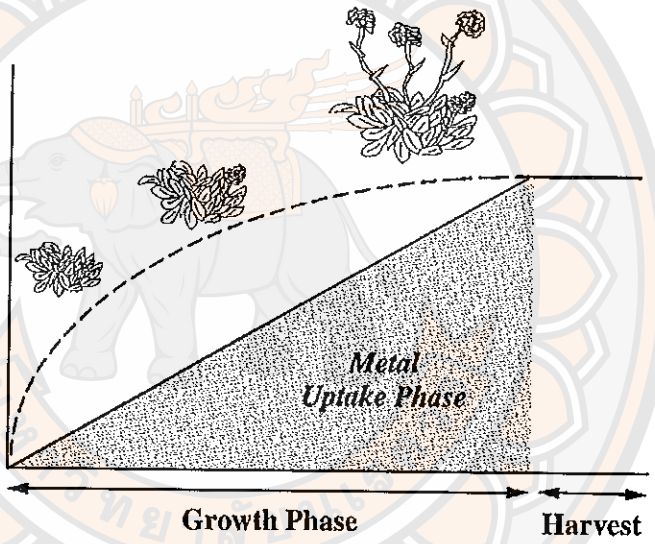

Figure 14 Schematic representation of continuous phytoextraction

Solid line represents metal concentration in shoot biomass; dashed line represents shoot biomass. adapted from (Salt et al., 1998). 2) induced or chelate assisted phytoextraction or induced or assisted hyper-accumulation in which a conditioning fluid with artificial chelates or other acidifying agents are added to increase the mobility and easy uptake of metal contaminants by the plant to be used for phytoremediation. Chelate-assisted phytoextraction consists of two basic processes—release of bound metals into soil solution combined with transport of metals to the harvestable shoot. The role of chelates in increasing the soluble metal

concentration in the soil solution can be explained using well-established equilibrium principles.

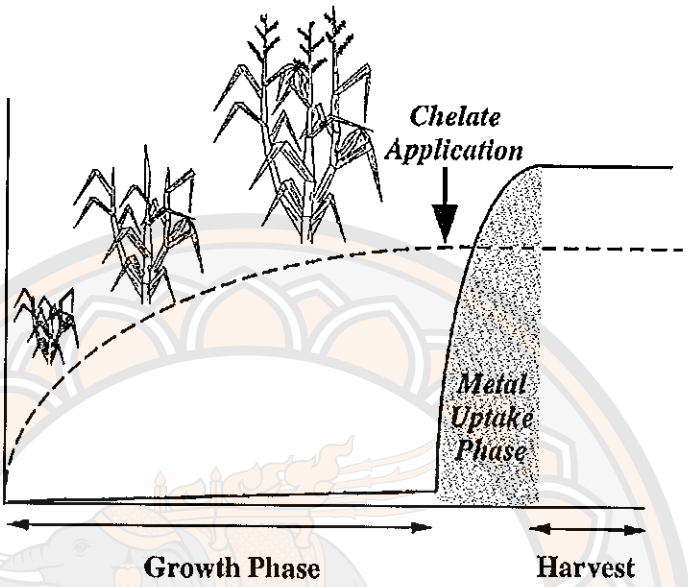

Figure 15 Schematic representation of chelate-assisted phytoremediation. Solid line represents metal concentration in shoot biomass; dashed line represents shoot biomass

Source: Salt et al., 1998.

It can be summarized the main characteristics of these two phytoextraction systems in table 3.

Table 3 Main characteristics of the two strategies of phytoextraction of metals from soils

Natural Phytoextraction	Chemically Assisted Phytoextraction
Plant naturally hyperaccumulate metals.	Plants are normally metal excluders.
Slow growing, low biomass production.	Fast growing, high biomass plants.
Natural ability to extract high amount of metals from soils.	Synthetic chelators and organic acids are used to enhance metal uptake.
Efficient translocation of metals from roots to shoots.	Chemical amendments increase the metal transfer from roots to shoots.
High tolerance; survival with high concentrations of metals in tissues.	Low tolerance to metals; the increase in absorption leads to plant death.
No environmental drawback regarding leaching of metals.	Risk of leaching of metal chelates to groundwater.

2. Phytodegredation.

The phytodegradation, also called phytotransformation, concept is based on the well-known ability of plants and their associated rhizospheres to degrade highly dilute contaminants and uptake organic contaminants from soil, sediments, and water with a subsequent transformation by the plant. Some of the non-volatile compounds can be degraded or rendered non-toxic via enzymatic modification and sequestration *in planta.* Microbe-assisted phytoremediation, including rhizodegradation, appears to be particularly effective for removal and/or degradation of organic contaminants from impacted soils, particularly when used in conjunction with appropriate agronomic techniques. The synergism of phytoremediation and microbial remediation strategies influenced a more successful approach to remediation of toxic contaminants, especially organic compound.

Rhizoremediation, defined as degradation of contaminants in the rhizosphere degrades particularly, large recalcitrant organic compounds. In this case, complex interactions involving roots, root exudates, rhizosphere soil and microbes result in degradation of organics to non-toxic, or less-toxic, compounds. In soil

containing large volumes of roots, microbial populations can reach titers of about 10^{12} cells/g of soil.

These microbial consortia can provide various benefits to plants, including the synthesis of compounds that protect the plants by decreasing plant stress hormone levels; chelators for delivering key plant nutrients; protection against plant pathogens; and degradation of contaminants before they can negatively impact the plants (Gerhardt, Huang, Glick, & Greenberg, 2009). The rhizo-microbes may also accelerate remediation processes by volatizing organics such as PAHs or by increasing the production of humic substances from organic matters (Cunningham, & Ow, 1996). Phytodegradation works well for organics that are mobile in plants such as herbicides, TNT, and trichloroethylene (TCE).

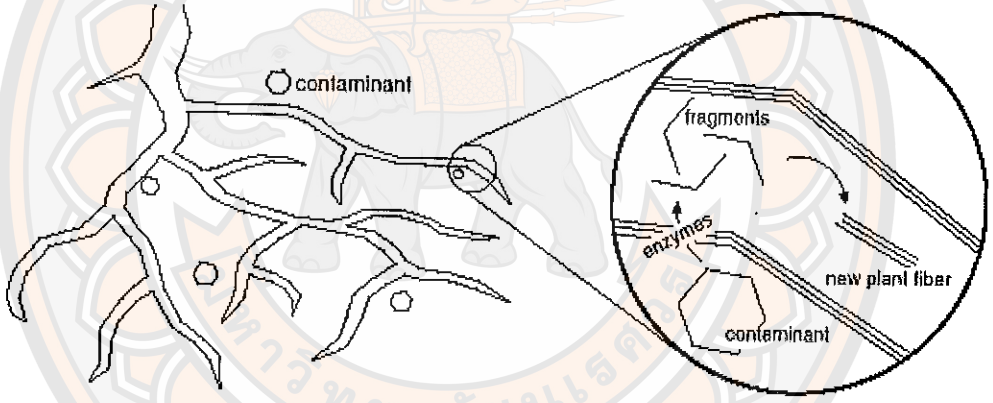

Figure 16 Destruction of Organic Contaminants by Phytodegradation: Enzymes in plant roots break down (degrade) organic contaminants. The fragments are incorporated into new plant material

Source: http://www.oocities.org/razanoor/biophyto.html

3. Phytooxidation

Phytooxidation is the oxidation reaction observed in hairy root cultures. Hairy root cultures of several other plant systems demonstrated potential to perform the oxidation reaction. Due to, hairy root cultures are preferred over plant cell/callus and suspension cultures as biocatalyst due to their genetic/biochemical stability,

hormone autotrophy, multi-enzyme biosynthetic potential mimicking that of the parent plants and relatively low-cost cultural requirements (Banerjee, Singh, & Rahman, 2012).

The plant enzymatic systems in hairy root cultures involved in phytoremediation include nitroreductases, glycosyl and glutathione transferases, oxidases, phosphatases, etc. These enzymes are implicated in the transformation of toxic xenobiotic compounds such as explosives, pesticides and halogenated organic compounds. In addition, plants and many microorganisms contain several oxidases, such as laccases and peroxidases, which are involved in the removal of different pollutants (González, Capozucca, Tigier, Milrad, & Agostini, 2006). In particular, peroxidases are constitutive and inducible enzymes that are expressed as soluble, extracellular and membrane bound proteins from some plant tissues, where oxidation of aromatic substrates occur using H_2O_2 as a co-substrate. Moreover, oxidation by other enzymes like laccases can oxidize the organic substances such as phenols by using O_2 instead H_2O_2 as electron acceptor. Sometimes, the enzymes mediated oxidative coupling of the pollutants, followed by precipitation of the formed polymers from the aqueous phase (Dec, & Bollag, 1994).

The removal of phenol from water using tomato hairy root cultures as source of enzymes, particularly peroxidases, which were capable to oxidize these compounds was reported (González et al., 2006). Phenol and H_2O_2 were fixed at 100 mg/l and 5 mM, respectively. A significant elimination of phenol within the first 5–15 min of incubation was observed and high removal was achieved during the first hour reaching 95% of efficiency after 5 h of treatment. This fast reaction rate during the first hour was advantageous with respect to other methods, such as biological oxidation performed by microorganisms. The reaction was catalyzed mainly by peroxidase iso-enzymes present in the root tissues as shown in Eq 2.10.

$$C_6H_6O + H_2O_2 \xrightarrow{\text{Peroxidase}} H_2O + \text{Carboxylic acid} \qquad \text{(Eq 2.10)}$$

The removal of phenol from water using *Brassica napus* hairy roots as a source of enzymes, such as peroxidases, which were able to oxidize phenol was studies. Roots grew normally in medium containing phenol in concentrations not exceeding 100 mg/L, without the addition of H_2O_2. However, roots were able to remove phenol concentrations up to 500 mg/L, in the presence of H_2O_2, reaching high removal efficiency, within 1 h of treatment and over a wide range of pH (4–9). Phenol oxidation caused a change in the color of the roots; they turned from white to brown and the reduction in growth (Coniglio et al., 2008).

Removal efficiency increased with the increment in H_2O_2 concentration, until an optimal value. This behavior demonstrates that the hydrogen peroxide concentration is a limiting factor. Therefore, it is important to avoid, control and/or prevent the suicide inactivation of peroxidases during the phenol removal process and one way could be the use of H_2O_2 concentrations below the inhibitory range, which must be determined for each system.

4. Phytostabilization

Phytostabilization, also known as in-place inactivation, involves the use of certain plant species to immobilize contaminants in the soil and groundwater through absorption onto the surface of roots or by the formation of insoluble compounds and accumulation by roots, or precipitation within the root zone (Sarma, 2011). It is applicable to the cleanup of both organic and inorganic contaminants. It uses certain plant species to immobilize contaminants in the soil, sediments, and groundwater. This mechanism cannot use plants to remove contaminants out of the site area but it effectively makes them unavailable for entry into the food chain. In addition, it decreases the potential migration of contamination through wind erosion, transport of exposed surface soils and leaching of soil contamination to ground water (Vara Prasad, & de Oliveira Freitas, 2003).

Phytostabilization has proved useful for the treatment of Pb, As, Cd, Cr, Cu, and Zn (Etim, 2012) and has been successful in addressing metals and other inorganic contaminants in soil and sediments (US.EPA, 2015).

Phytostabilization is most effective for fine- textured soils with high organic-matter content, but it is suitable for treating a wide range of sites where large areas are subject to surface contamination (Cunningham, & Ow, 1996). Thus,

phytostabilization ability can be enhanced by the addition of manure in the contaminated soil (Pérez-Esteban, Escolástico, Ruiz-Fernández, Masaguer, & Moliner, 2013). However, some highly contaminated sites are not suitable for phytostabilization because plant growth and survival are impossible. The root zone is the primary area affecting chemically moderated immobilization or root precipitation. Plants can be selected for their root depth; for example, poplars can be used for remediation of soil to a depth of 5 to 10 feet.

5. Phytovolatilization

Phytovolatilization is the absorption of a contaminant by a plant from the soil through its roots followed by the release of the contaminant or a modified form of the organic and inorganic contaminants with water and pass on to the leaves and volatilize into the atmosphere (Anjum et al., 2012; Team, 1999). Many organic compounds transpired by a plant are subject to more effective or rapid natural degradation processes such as photodegradation and released to the atmosphere.

Direct volatilization and phytovolatilization are expected to be moderate for hydrophilic compounds such as acetone and phenol (Polprasert, Dan, & Thayalakumaran, 1996). In contrast, volatilization may be an essential removal process for volatile hydrophobic compounds such as lower chlorinated benzenes, chlorinated ethenes and BTEX compounds (McCUTCHEON, & Schnoor, 2004). Uptake by the transpiration stream and subsequent phytovolatilization through the stems and leaves may be a major removal process and significantly contribute to contaminant mass loss; additionally, the vegetation increases the upward movement of water into the unsaturated zone, where enhanced volatilization occurs (Hong et al., 2001).

6. Rhizofiltration

Rhizofiltration is similar in concept to Phytoextraction but is concerned with the remediation of contaminated water and groundwater rather than the remediation of polluted soils. Hydroponically grown plant roots can also directly absorb, precipitate and concentrate toxic metals from polluted effluents in a process termed (Dushenkov, Kumar, Motto, & Raskin, 1995). So, this mechanism has shown promise for dealing with metals contamination in water. In greenhouses, hydroponically cultivated plants have developed a large root system; contaminated

water is collected from a waste site and brought to the plants where it is substituted for their water source and rapidly remove heavy metals from water and concentrate them in their roots and shoots. Rhizofiltration has proved useful for industrial discharge, agricultural runoff, acid mine drainage, and radioactive contamination. To illustrate, the uptake of uranium by sunflower (*Helianthus annuus L.*) and bean (*Phaseolus vulgaris L.* var. vulgaris) grown in hydroponic conditions were observed (Lee, M., & Yang, 2010). An artificially uranium contaminated solution and three genuine groundwater samples were used in the experiments. More than 80% of the initial uranium in solution and genuine groundwater, respectively, was removed within 24 h by using sunflower and the residual uranium concentration of the treated water was lower than 30 g/L (USEPA drinking water limit). For bean, the uranium removal efficiency of the rhizofiltration was roughly 60–80%. The results of SEM and EDS analyses indicated that most uranium accumulated in the roots of plants. The results suggested that the uranium removal capacity of two plants evaluated in the clean-up system was about 25 mg/kg of wet plant mass. Notably, the removal capacity of the root parts only was more than 500 mg/kg.

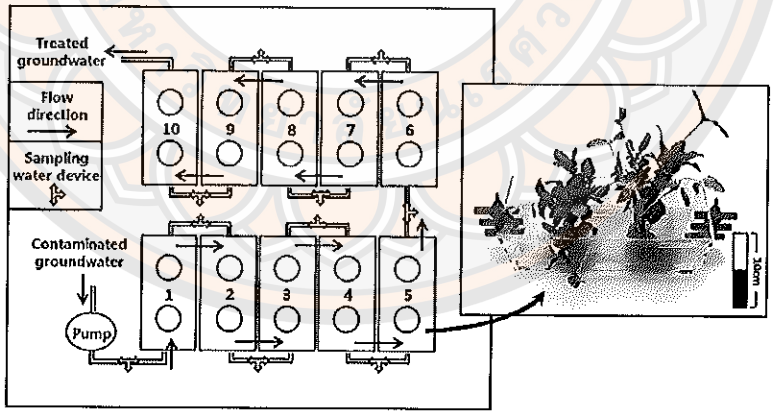

Figure 17 Schematic and photograph of the continuous clean-up system for rhizofiltration

Source: Lee, M., & Yang, 2010

Reactive Oxidative Stress (ROS) and Enzymes Responsible for the Biodegradation

One of the major plant responses to stresses is generation of reactive oxygen species (ROS). ROS perform the role in phytooxidation as the substrate/electron donor in the chemical reactions degrading toxic stresses include, toxic compounds. ROS are both produced by cellular metabolic activities and environmental factors. Endogenous production of ROS are continuously as by-products of various metabolic pathways including respiration and photosynthesis localized in different cellular compartments such as in plastids, peroxisomes, mitochondria, the cytosol and the apoplast (Apel, & Hirt, 2004; Birben, Sahiner, Sackesen, Erzurum, & Kalayci, 2012; R. Singh et al., 2016; Tripathy, & Oelmüller, 2012). Exogenous ROS can also be generated by cell exposure to harsh environmental conditions and become a sources of ROS production (Kim, & Park, 2014; Liu et al., 2011). The major members of the ROS family include free radicals like $O_2^{\bullet-}$, $OH^{\bullet}$ and non-radicals like H_2O_2 and $^1O_2$. A common feature among the different ROS types is their capacity to cause oxidative damage to proteins, DNA, and lipids.

Superoxide radical ($O_2^{\bullet-}$) is the primary ROS formed in the cell which initiates a cascade of reactions to generate "secondary" ROS, either directly or prevalently through enzyme catalyzed processes. $O_2^{\bullet-}$ is a moderately reactive, short-lived ROS with a half-life. $O_2^{\bullet-}$ can accept one electron and two protons to form to H_2O_2. It is easily transform to H_2O_2 either non-enzymatically or by enzyme superoxide dismutase (SOD) catalyzed reaction to H_2O_2 (Sharma, Jha, Dubey, & Pessarakli, 2012).

Hydrogen peroxide (H_2O_2), a moderately reactive ROS is formed when $O_2^{\bullet-}$ undergoes both univalent reduction as well as protonation. It can occur both non enzymatically by being dismutated to H_2O_2 under low pH conditions, or mostly by a reaction catalyzed by enzyme superoxide dismutase (SOD). Also, H_2O_2 is generated in the cells under normal as well as wide range of stressful conditions. At low intracellular concentrations, it acts as a regulatory signal for essential physiological processes like senescence (Pengetal, 2005), photorespiration and photosynthesis (Noctor et al., 2002), stomatal movement (Brightetal., 2006), cell cycle, growth and development (Tanouetal, 2009a, 2009b). H_2O_2 at high intracellular concentration oxidizes both cysteine (-SH) and methionine ($-SCH_3$) residues and inactivates Calvin

cycle enzymes, Cu/ZnSOD and Fe-SOD by oxidizing their thiol groups (Halliwell,2006). It causes 50% loss in activity of different enzymes like fructose1, 6 bisphosphatase, sedoheptulose 1, 7 bisphosphatase and phosphoribulokinase, at concentrations of 10 μM H_2O_2 and is also responsible for programmed cell death at high cellular concentrations (Datetal, 2000). H_2O_2 is stimulated by enzyme peroxidase (POD) under stressful. H_2O_2, a more stable ROS, is an oxidizing agent interacts with phenol via POD, break down phenol molecules to phenol radical (Das & Roychoudhury, 2014). Among its family members, hydroxyl radical ($OH^{\bullet}$) is the most reactive and the most toxic ROS known. It is generated at neutral pH by the Fenton reaction between H_2O_2 and $O_2^{\bullet-}$ catalyzed by transition metals like Fe ($Fe^{2+,}$ Fe^{3+}) (Das, & Roychoudhury, 2014).

The role of the ROS family is that of a double edged sword; Under physiological steady state conditions these molecules are scavenged by different anti-oxidative defense components that are often confined to particular compartments. Specific ROS function as signaling molecules and activate signal transduction processes in response to various stresses. On the other hand, imbalance between ROS generation and safe detoxification generates oxidative stress and the accumulating ROS are harmful for the plants. Moreover, the equilibrium between production and scavenging of ROS may be agitated by a number of adverse environmental factors (Tripathy, & Oelmüller, 2012). Environmental stresses such as drought, salinity, chilling, metal toxicity, and UV-B radiation as well as pathogens attack lead to enhanced generation of the higher ROS in plants due to disruption of cellular homeostasis. Here, we presented some ROS characteristics in Table 5 below.

ROS are harmful to cells because they cause the oxidation of lipids, proteins and DNA and other components in their environment. Hence, it is important for a living cell to retain the redox balance, e.g. by antioxidants and/or antioxidant enzyme systems (Tripathy, & Oelmüller, 2012). Antioxidants can be classified in multiple ways. Based on their activity, they can be classified as enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants work by breaking down and removing free radicals. The antioxidant enzymes convert dangerous oxidative products to H_2O_2 and then to water, in a multi-step process in presence of co-factors such as copper, zinc, manganese, and iron. Non-enzymatic antioxidants work by interrupting free radical

chain reactions. Few examples of the non-enzymatic antioxidants are vitamin C, vitamin E, plant polyphenol, carotenoids, and glutathione (Nimse, & Pal, 2015).

Table 4 Members of ROS and their attributes

ROS/Oxidants	$t_{1/2}$	Migration distance	Sources	Mode of action	Reaction with DNA	Reaction with protein	Scavenging systems
Superoxide ($O_2^{\bullet-}$)	1 - 4 µs	30 nm	Membranes, Chloroplasts, Mitochondria	Reacts with double bond containing compounds such as (Fe-S) proteins	No	Via the Fe-center	SOD
Hydroxyl radical ($OH^{\bullet}$)	1 µs	1 nm	Membranes, Chloroplasts, Mitochondria	Extremely reactive with all biomolecules	Rapidly reacting	Rapidly reacting	Flavonoids and Proline
Hydrogen peroxide (H_2O_2)	1 ms	1 µs	Membranes, Chloroplasts, Mitochondria, Peroxisomes	Oxidizes proteins and forms $OH^{\bullet}$ via $O_2^{\bullet-}$	No	Attacks the Cys residue	CAT, POXs and Flavonoids
Singlet Oxygen ($^1O_2$)	1 - 4 µs	30 nm	Membranes, Chloroplasts, Mitochondria	Oxidized proteins, PUFAs and DNA	Reacts with G residue	Attacks Trp, His, Tyr, Met and Cys residues	Carotenoids and α-Tocopherol

Souece: Das, & Roychoudhury, 2014

Enzyme Superoxide dismutase (SOD, EC.1.15.1.1) is central role in defense against oxidative stress in all aerobic organisms. Three isozymes of SOD copper/zinc SOD (Cu/Zn-SOD), manganese SOD (Mn-SOD), and iron SOD (Fe-SOD) are presented in plants. SOD has been found to be up regulated by abiotic stress conditions. SOD converts the $O_2^{\bullet-}$ to H_2O_2. The H_2O_2 is then converted to the O_2 and H_2O in the reaction catalyzed by enzyme Catalase (Das & Roychoudhury, 2014; Scandalios, 1993). OD activity has been noted to increase in plants proved to various environmental stresses, including drought and metal toxicity (Sharma & Dubey, 2005). Increased activity of SOD is often correlated with increased tolerance of the plant against environmental stresses. It was suggested that SOD can be used as an indirect selection criterion for screening drought-resistant plant materials. Overproduction of SOD has been reported to result in enhanced oxidative stress tolerance in plants (Gupta, Heinen, Holaday, Burke, & Allen, 1993).

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \xrightarrow{\text{catalyzed by SOD}} 2H_2O_2 + O_2 \quad \text{(Eq 2.11)}$$

Enzyme Guaiacol peroxidase (GPX, POD, EC.1.11.1.7), a heme containing protein, preferably oxidizes aromatic electron donor such as guaiacol and pyragallol at the expense of H_2O_2. It is extensively found in animals, plants, and microbes. POD is associated with many important biosynthetic processes, including lignification of cell wall, biosynthesis of ethylene, wound healing, and defense against abiotic and biotic stresses. POD are extensively accepted as stress "enzyme." that use H_2O_2 as an electron acceptor. POD expression appears to be a response to oxidative stress, i.e. an increase in the concentration of ROS, which can be created by various combinations of oxygen and nutrient availability (Sharma et al., 2012; Sinsabaugh, 2010). In the other word, POD might be responsible for the production of H_2O_2 (Almagro et al., 2008; Mayaba, Minibayeva, & Beckett, 2002). Not only for the oxidation reaction but polymerization can occur by the catalysis of POD via the degradation of phenol (Kawakita, 2012).

$2RH + H_2O_2 \rightarrow 2R^{\bullet -} + H_2O$ (Eq 2.12)
catalyzed by POD

Enzyme Catalase (CAT, EC.1.11.1.6), in plants, H_2O_2 has been affected in many stress conditions. When cells are stressed for energy and are rapidly generating H_2O_2 through catabolic processes, excess H_2O_2 is degraded by CAT in an energy efficient manner (Mallick, & Mohn, 2000). The excess H_2O_2 can be detoxified by CAT which occur mainly in peroxisomes. CAT catalyzed typically reaction by the dismutation of two molecules of H_2O_2 to water and O_2 as shown in Eq 2.13. Environmental stresses cause either enhancement or depletion of CAT activity, depending on the intensity, duration, and type of the stress (Sharma, & Dubey, 2005). Normally, plants contain several types of H_2O_2-degrading enzymes. These include CAT, ascorbate peroxidases (APX), various types of peroxiredoxins (PRX), glutathione/thioredoxin peroxidases (GPX), and glutathione S-transferases (GST). Especially, CATs are notably unique as they do not require cellular reducing equivalent. It has a very high turnover rate (6×10^6 molecules of H_2O_2 to H_2O and O_2 min^{-1}) (Mhamdi et al., 2010; Sharma et al., 2012). Therefore, CAT is crucial for maintaining the redox balance during the oxidative stress.

$2H_2O_2 \rightarrow 2H_2O + O_2$ (Eq 2.13)
catalyzed by CAT

Herein, I proposed the model of ROS formation and their scavenging enzymes in figure 18.

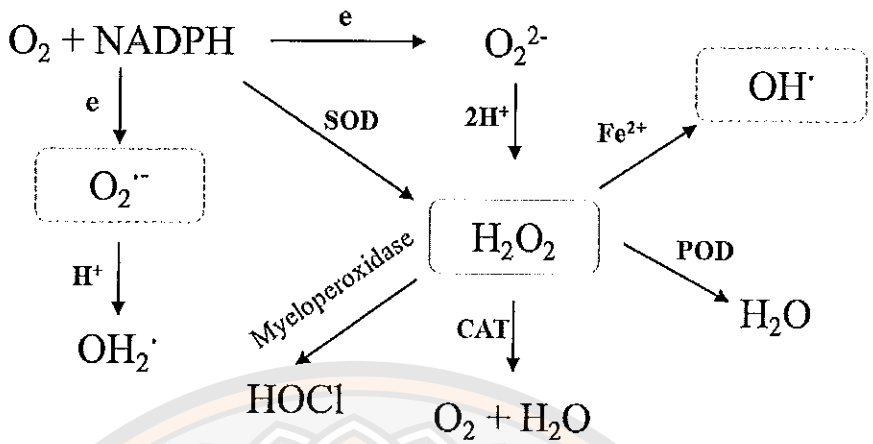

Figure 18 Proposed formation of ROS and scavenging activity of SOD, CAT and POD

Source: Das, & Roychoudhury, 2014; Mhamdi et al., 2010; Nimse, & Pal, 2015; Xu, Duan, Yang, Beeching, & Zhang, 2013

Singh M. et al. conducted the study using in vitro grown vetiver plantlets under aseptic conditions has shown that the vetiver plantlets could remove almost all the phenol from the medium in a period of 4 days when concentration of phenol was less than 200 mg/L. Phenol removal was found to be associated with inherent production of POD and H_2O_2. Coupled with H_2O_2 formation, the levels of antioxidant enzymes like SOD and POD showed an enhancement when plants were exposed to phenol, whereas CAT levels initially showed a decline due to the utilization of H_2O_2 by POD for phenol oxidation. Since the plants were grown under aseptic conditions without the confounding effect of microorganisms, it was demonstrated in the present study that vetiver plants were solely responsible for phenol remediation. This will make vetiver grass as a potential plant for remediation of phenol.

In addition, it was shown that one of the roles that ROS play is the killing of pathogens infected cells, it seems that ROS are required to play two opposing roles: causing and intensifying damage and signaling the activation of defense responses include organic contaminants (Kovalchuk, 2010). In fact, several ROS are continuously produced as byproducts of aerobic metabolism localized in different

cellular compartments (Bhattacharjee, 2010) such as fungi, bacteria, elicitors or even plant root zone. Moreover, a variety of microbial components have been demonstrated to elicit ROS production in plant cells (Baker, & Orlandi, 2002).

In order to enhance the degradation of toxic compounds in the soil by focusing on ROS such as H_2O_2, production in this type of association, plants are known for their contribution in making contaminants of the soil more bioavailable through the release of a series of low molecular weight organic acids, carbon and nitrogen compounds to nourish microbes in the rhizosphere and by releasing exudates that enhance the degradation of soil contaminants through the induction of biochemical pathways within bacteria. Therefore, amendment substances need to be created to assist the growth of the vetiver root system and also increase the microorganism populations, in order to enhance the aerobic metabolism and promote ROS production (Doty, 2008). It was found that the organic amendment substances such as suitable fertilizers often enhance plant growth by improving the structure of the soil and plant nutrition and increasing the population of microorganisms in the root zone. Several types of organic amendments, but for field scale applications, inexpensive and easily available amendments like manure, sewage sludge, compost, bark or other biomass-residues have been most commonly used.

The enhancement of the plant potential in phytoremediation by rhizomicrobes and endophytes

Although, phytoremediation, a strategy that uses plants to degrade, stabilize, and/or remove contaminants in several environments, has been extensively interesting due to their advantages as shown above. But it still has some limitation, one is that high concentrations of contaminants tend to inhibit plant growth, including root growth, in part due to oxidative stress(Chaudhry, Blom-Zandstra, Gupta, & Joner, 2005; X.-D. Huang, El-Alawi, Penrose, Glick, & Greenberg, 2004).The stresses will restrict the rate of phytoremediation in situ(Ferro, Rock, Kennedy, Herrick, & Turner, 1999). Contaminated media such as soil, water also tend to be nutrient poor and/or lack of microbial diversity, which contributes to sub-optimal plant biomass accumulation, as well as impeded rates of remediation (Chaudhry et al., 2005; Ferro et al., 1999; Siciliano, Germida, Banks, & Greer, 2003).

To overcome these conditions which limit the potential of plant in organic contaminant degradation, the microorganisms-assisted phytoremediation are investigated and evaluated through the decreasing of phenol. It is based on the potential of root-associated microorganisms, which are assisted by the organic compounds in root exudates and by a favorable environment in the rhizosphere (Juwarkar, 2012). It can be defined as rhizoremediation, a specific type of phytoremediation that involves both plants and their associated rhizosphere microorganisms, can arise naturally, or can be actuated by purposely introducing specific microorganisms. Microbe-assisted phytoremediation appears to be especially effective for removal and/or degradation of organic contaminants from impacted environments, particularly when used in combination with appropriate agronomic techniques (Kuiper, Lagendijk, Bloemberg, & Lugtenberg, 2004). Other benefits related with the use of microbe-assisted phytoremediation include;

1. quantitative gene expression of bacterial pollutant catabolic genes can be used to assess the efficiency of the remediation process,

2. genetic engineering of the bacterial catabolic pathway is easier to manipulate than a plant catabolic pathway, and

3. hazardous pollutants taken up by plant may be degrade inside by endophytic degraders decreasing the toxic effects of contaminants in environments.

Nonetheless, there are some restriction including;

1. the choice of plant can mean that it is only seasonally effective,

2. it is associated with phytotoxic effects of contaminants, and

3. the by-products from their metabolic pathway enter to the food chain if contaminants are not completely detoxified.

Yet attentive selection of microbe-assisted phytoremediation and culturing them into native plants can sidestep some of the problems related phytotoxicity and the ability of plants to survive at non-native locations (Glass, 1999; Khan, & Doty, 2011).

Selective microorganism's inoculation at such sites increases the better survivability, growth and biomass of the plants in the long-term (Juwarkar, 2012). These microorganisms are also the good candidates of pollutant degraders and/or can support plant growth under stress conditions (Gerhardt et al., 2009). In addition, some

plant species have the qualification to recruit, or selective expand, the necessary microbe-assisted phytoremediation to remove the contaminants or to help deal with the contaminant stress, more than other plants in the same area (Khan, & Doty, 2011). From commonly searching for the diversity of microbes, many researches have turn to adding a specific microbe for a specific contaminant.

In the recent time, several studies demonstrated that bacterial endophytes and microbes aid phytoremediation by direct degradation of contaminants and/or by reducing toxicity to the plants. For example, Germaine *et al.* operated three *Psuedomonas* sp. isolated from the hybrid cotton wood growing on a phytoremediation site in Belgium, were recognized to degrade 2,4-dichlorodiphenoxyacetic acid (2,4 D), toluene, naphthalene (Kieran Germaine et al., 2004). They exhibited successful re-colonization of poplar trees when inoculated with these strains. To developing the research work, the same authors presented that a genetically enhanced endophytic strains of poplar endophyte *Psuedomonas putida* VM1441 could protect inoculated pea plants from the toxic effects of naphthalene and also the inoculation of plants with this strain facilitated higher 40% naphthalene degradation rate compared to uninoculated plants (Kieran Germaine, Keogh, Ryan, & Dowling, 2009). Teng Y., et al. (Teng et al., 2011) presented a pot study which was conducted to examine the effects of inoculation with *Rhizobium meliloti* on phytoremediation by alfalfa grown for 90 days in an agricultural soil contaminated with polycyclic aromatic hydrocarbons (PAHs). Planting with uninoculated alfalfa and alfalfa inoculated with *Rhizobium meliloti* significantly lowered the initial soil PAH concentrations by 37.2% and 51.4% respectively compared with unplanted control soil. By the conclusion, the authors confirm that the symbiotic association between alfalfa and Rhizobium can stimulate the rhizosphere microflora to degrade PAHs (Teng et al., 2011).

Vetiver plant was used in phytoremediation technic for treating oil sludge and total petroleum hydrocarbon (TPHs) from contaminated soil with the stimulation and addition of indigenous and augmented microbial consortium. The results showed that the combination of vetiver for rhizodegradation, bioaugmentation for enhanced microbial degradation, addition of nutrients for stimulation of indigenous and augmented consortium and addition of wheat husks as bulking material to increase porosity and aeration helped to remove maximum oil and TPH from the contaminated

soil very effectively by resulting in 72.8% TPHs removal and a marked decrease of heavy metals such as lead and cadmium (Nanekar, Dhote, Kashyap, Singh, & Juwarkar, 2015).

To consider the microbe-assisted phytoremediation in the aspect of promoting the growth of plant. Barac T., et al. discovered a method of transferring plant endophytes with the performance to degrade toluene by the pTOM plasmic from *Burkholderia cepacia* G4 was naturally enter into the lupine endotypes *Burkholderia cepacia* BU 0072 through bacterial conjugation (Barac et al., 2004). When the lupine plants were inoculated with this endophyte, the plant had higher tolerance of toluene. The lupine plant grew at high concentration of toluene (1,000mg/L) without any toxic effects, but the un-inoculated controls showed growth reduction with as little as 100 mg/L to toluene. Again, the lupine inoculated plant displayed the significantly decrease of 50% - 70% toluene from the aerial portions of the plant through phyto-transpiration. Even though, the heavy metals are resistant compounds. Sheng X, et al. presented the ability of lead resistant endophytes which were isolated from Brassica *napus* that enhanced phytoremediation performance by promoting the plant growth and Pb uptake by rape. Inoculation of plant with the Pb resistant endophytes increased uptake into the shoot from 76% to 131% by *Psuedomonas fluorescens* and from 59% to 80% by *Microbacterium sp.* compared to the uninoculated control plants (Sheng, Xia, Jiang, He, & Qian, 2008).

Utter studies clearly indicate that endophytes and microbe-assisted phytoremediation can be employed to improve phytoremediation of various pollutants. Notwithstanding, there are a lot of challenges to be met both from the fundamental and applied perspectives.

Vetiver Grass: A True Miracle Grass

Vetiver grass, *Chrysopogon zizanioides* (L.) Roberty, syn. *Vetiveria zizanioides* (L.) Nash belongs to the *Poaceae* family, a subfamily of *Panicoideae*, tribe *Andropogonae* and sub-tribe *Sorghina*, and the genus includes ten species. It can be found throughout the tropical and subtropical regions of Africa, Asia, America, Australasia and Mediterranean Europe.

1. Characteristics of Vetiver Grass

The valuable characteristics of vetiver grass which bring the widespread used include;

1.1 Morphological characteristics.

1.1.1 Beautiful fine stem flower heads with light purple flower, turning to mauve color later.

1.1.2 Stiff and erect stems, up to 2 m high, which can be trimmed to form neat hedges.

1.1.3 Vetiver grass has no stolons, very short rhizomes so it does neither invade nor compete with nearby plants.

1.1.4 A massive and penetrating root system that can grow very fast, up to 3-4m in the first year. This massively thick root system can penetrate hardpan, enhances the structural strength and organic content of the soil and at the same time makes it very tolerant to drought.

1.1.5 New shoots develop from the underground crown making vetiver resistant to fire, frosts, traffic and heavy grazing pressure.

1.1.6 New shoots continuously form from the crown so the base of vetiver is always thick, active and resume fast growth.

1.1.7 New roots grow from nodes when buried by trapped sediment. Vetiver will continue to grow up with the deposited silt eventually forming terraces, if trapped sediment is not removed.

1.2 Physiological characteristics.

1.2.1 Tolerance to extreme climatic variation such as prolonged drought, flood, submergence and extreme temperatures (-150 oC to 600 oC). Effective vetiver hedges have been established with annual precipitation levels as low as 300 mm and with up to 6-month drought.

1.2.2 Ability to regrow very quickly after being adversely affected.

1.2.3 Tolerance to a wide range of soil pH (3.0 to 10.5).

1.2.4 Highly tolerant to growing media high in acidity, alkalinity, salinity, sodicity and magnesium.

1.2.5 Highly tolerant to Al, Mn and heavy metals such as As, Cd, Cr, Ni, Pb, Hg, Se and Zn in the soils

1.3 Ecological characteristics

1.3.1 Vetiver is sterile, it flowers but produces no seeds. Therefore, it is not invasive and has no weed potential.

1.3.2 Vetiver planting can also provide wild life sanctuary.

On erodible or unstable ground vetiver first reduces erosion, stabilizing the erodible ground (particularly steep slopes), before improving its micro environment so that other wild or sown plants can establish later. A well-established vetiver grass hedge will slow down rainfall runoff, spreading it out evenly, and will trap runoff sediments to create natural terraces. All this is possible without the use of complex hydrological data and design, and without the aid of high cost consultants and surveyors. Because of these characteristics vetiver can be considered as a pioneer plant on disturbed lands (Grimshaw, 1993; Maffei, 2003).

Figure 19 Young vetiver grass on highly erosive riverbank dense planting Banda Aceh

Source: https://www.flickr.com/photos/normvanthoff/3933833370/in/album-72157622408840550/

2. Phytoremediation Potential of Vetiver Grass

At a time when a great deal of attention is being paid to simple low cost technology for sustainable agriculture, vetiver grass provides one very good, widely and easily applicable technology that is practical, proven, effective, and profitable (Grimshaw, 1993).

Recently, it has received great interest from scientists and the public for its ability to remove a wide range of inorganic and organic contaminants from soil and water. The special characteristics of vetiver grass are evaluated for their phytoremediation potential, and economic incentives to promote application of vetiver grass for phytoremediation (Danh, Truong, Mammucari, Pu, & Foster, 2012; Truong, Hart, Chomchalow, Sombatpanit, & Network, 2001). Plants used for phytoremediation must satisfy the following requirements below. Also, greenhouse and field studies have demonstrated that vetiver grass can fulfill these conditions (Danh et al., 2012):

2.1 Fast growth and high biomass production

Vetiver is a C4 grass that has high growth rates, as indicated by high radiation use efficiency of 18 kg/ha per MJ/m. Its growth rate is more than three times higher than that of C3 grasses such as coastal couch grass (*Cynodon dactylon*). Its biomass is extremely high under tropical hot and wet conditions, producing more than 100 tons of dry matter/ha /year. In sub-tropical weather, vetiver can produce high dry biomass from 10 to 20 tons after 5–6 months of cultivation. This may be due to the fact that vetiver grass retains high activity of the key enzymes involved in photosynthesis (NADP-MDH and NADP-MET) even when cultivated in temperate climates (Bertea, & Camusso, 2002).

2.2 Widely and deeply distributed root system

Vetiver grass possesses a lacework root system that is abundant, complex, and extensive which present in figure 20. This extensive and thick root system binds the soil and at the same time makes it very difficult to dislodge and extremely tolerant to drought.

Figure 20 Vetiver grass and its root

Source: http://online-media.unimarburg.de/biologie/nutzpflanzen/maximilian_sittinger/morphologie.html
https://defeatingpain.wordpress.com/2014/05/16/vetiver-the-root-that-is-dug/

A field trial carried out in Thailand showed that vetiver roots reached the depth of 1.5 and 2.5 m after one and two and a half months of cultivation, respectively; a higher root density was found at the depth 30–100 cm. The roots were found at soil depth of 4 m and 5 m at 10 and 25 months old, respectively. In another field study in Colombia, vetiver grass acquired a total length of 7 meters after 34 months (Tscherning, Leihner, Hilger, Müller-Sämann, & El Sharkawy, 1995). The special characteristics of the vetiver root system allow good contact between vetiver and contaminants, even at deep soil levels.

2.3 Adaptability to a wide range of weather conditions

Field and glasshouse studies have demonstrated that vetiver grass is highly adaptable to extreme environmental and soil conditions. Large numbers of soil microbes have been discovered in the vetiver rhizosphere, ranging from 10^6 to 10^8 cells/g soil (Siripin, Thirathorn, Pintarak, & Aibcharoen, 2000). Vetiver grass not only survives and grows well on infertile soil, but also improves the soil quality, in terms of nutritional, physical and biological properties. Moreover, the cultivation of vetiver grass can increase the microbial populations in such soils. Vetiver grass has been demonstrated to survive and grow on the soils contaminated with high concentrations of a wide range of several conditions showed below in table 5.

Table 5 Adaptability range of vetiver grass in Australia and other countries

Adverse Soil Conditions	Australia	Other Countries
Chemical conditions		
Acidity	pH 3.3	pH 4.2 (with high level soluble aluminium)
Aluminium level (Al sat. %)	Between 68% - 87%	Between 80%-87%
Manganese level	>578 mg/kg	
Alkalinity (highly sodic)	pH 9.5	pH 11.5
Salinity (50% yield reduction)	17.5 mS/cm	
Salinity (survived)	47.5 mS/cm	
Sodicity	33% (exchange Na)	
Magnesicity	2,400 mg/kg (Mg)	
Heavy Metals		
Arsenic	100 - 205 mg/kg	
Cadmium	20 mg/kg	
Copper	35 - 50 mg/kg	
Chromium	200 - 600 mg/kg	
Nickel	50 - 100 mg/kg	
Mercury	>6 mg/kg	

Table 5 (cont.)

Adverse Soil Conditions	Australia	Other Countries
Lead	>1,500 mg/kg	
Selenium	>74 mg/kg	
Zinc	>750 mg/kg	
Location		
	15°S - 37°S	41°N - 38°S
Climate		
Annual Rainfall (mm)	450 - 4,000	
Frost (ground temp.)	-11°C	-22°C
Heat wave	45°C	60°C
Drought (without effective rain)	15 months	
Fertilizer		
Vetiver can be established on very inertial soil due to its strong association with mycorrhiza	N and P (300 kg/ha DAP)	N and P, farm manure
Palatability		
	Dairy cows, cattle, horse, rabbits, sheep, kangaroo	Cows, cattle, goats, sheep, pigs, carp
Nutritional Value		
	N = 1.1% P = 0.17% K = 2.2%	Crude protein 3.3% Crude fat 0.4% Crude fiber 7.1%

2.4 Easy cultivation

Vetiver grass has been cultivated at over 100 countries for different purposes, such as environmental protection and essential oil production. It produces nonviable seeds, so the propagation is only based on vegetative methods. The

establishment of vetiver grass in the field is quite simple. The common way to propagate vetiver is to split mature tillers (shoots) from vetiver clumps or mother plants, which yield bare root slips for immediate planting or propagating in the polybags or plastic containers. When at least three new tillers appear, the plants are ready for transplantation on the field.

2.5 No potential to become a weed

Most hyper-accumulators are native plants, and produce viable seeds that increase the weeding potential in new environments. For vetiver grass, the chance of it becoming a weed is very low, as vetiver grass cultivars from south Indian accessions, deliberately selected for phytoremediation, produce flowers but set no seeds. Vetiver grass can be destroyed easily either by spraying with glyphosate (Roundup) or by cutting off the plant below the crown

Vetiver grass has been shown to enhance the degradation of heavy metals such as aluminum, cadmium, chromium, copper, lead, and nickel and polycyclic aromatic hydrocarbons in the soil. For example, in China, vetiver grass (*Vetiveria zizanioides*) was studied by (Yahua Chen, Shen, & Li, 2004) for its potential use in the phytoremediation of soils contaminated with heavy metals. the uptake and transport of Pb by vetiver from Pb contaminated soils under EDTA application was investigated. The results showed that vetiver had the capacity to tolerate high Pb concentrations in soils. With the application of EDTA, the translocation ratio of Pb from vetiver roots to shoots was significantly increased. The results showed that soil matrix with planted vetiver, could re-adsorb 98%, 54%, 41%, and 88% of the initially applied Pb, Cu, Zn, and Cd, respectively, in 14 days which may reduce the risk of heavy metals flowing downwards and entering the groundwater (Yahua Chen et al., 2004).

In Thailand it was reported that vetiver could decontaminate agrochemicals, especially pesticides and prevented them from accumulating in crops, polluting streams and other ecosystems. Some experiments were also carried out to determine the possibility of using vetiver grass to treat wastewater and it was found that vetiver could uptake significant amounts of N, P, K, Ca, Mg, Pb, Cd and Hg. Laboratory results also showed the ability of vetiver in absorbing heavy metals

(Hengchaovanich, 2000). Not only inorganic compounds but also organic compounds appear significantly as important pollutions which are decreased by vetiver grass.

CHAPTER III

VETIVER PLANTLETS IN AERATED SYSTEM DEGRADE PHENOL IN ILLEGALLY DUMPED INDUSTRIAL WASTEWATER BY PHYTOCHEMICAL AND RHIZOMICROBIAL DEGRADATION CONTAINING PHENOL SYNTHETIC PHENOL-CONTAINING WASTEWATER

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Introduction

Illegal dumping of industrial waste and wastewater is a vexing environmental problem globally affecting environmental and public health (Ichinose & Yamamoto, 2011). Similarly, over the last few years, illegal dumping of industrial waste and wastewater has become a major environmental problem in Thailand. More than 50 cases of illegal industrial waste dumping were found in remote areas close to residential zones throughout the country, but most (40 cases) are in the eastern part of Thailand (Figure 21a).

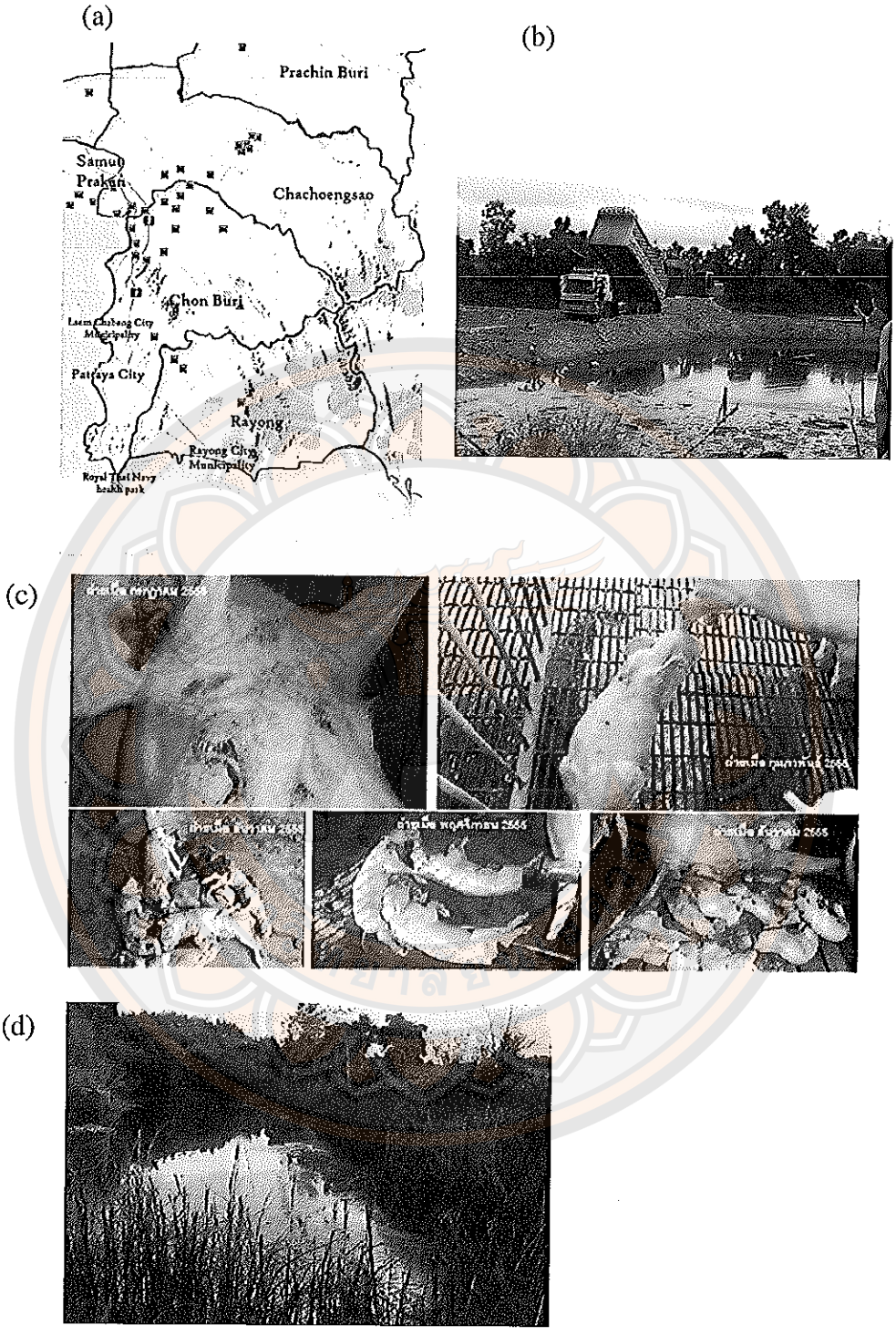


Figure 21 The situations and effects of illegal dumping in Thailand

Note: (a) Forty illegal dumping sites in eastern Thailand,
(b) the "15-rai" site during on-site treatment of illegally dumped wastewater containing high phenol concentrations,
(c) reported strange miscarriages in pigs believed to be caused by phenol contamination of shallow groundwater,
(d) Mr.Manus's pond affected by the illegal dumping of wastewater containing high concentrations of phenol

Nevertheless, the most infamous illegal dumping case in Thailand is probably the dumping of phenol (C_6H_6O)-contaminated wastewater in a 15-rai pond (1 rai = 1600 m^2) in the Nong Nae subdistrict, Phanom Sarakham district, Chachoengsao province. As a result of the illegal dumping, shallow groundwater was contaminated with as much as 225 µg/L of phenol, while the acceptable maximum contamination level (MCL) of pentachlorophenol in drinking water is only 1 µg/L (US.EPA, 2010b). Phenol is a toxic substance causing irritation and kidney inflammation, and disinfection of phenol-contaminated water using chlorine yields carcinogenic pentachlorophenols, which can also affect the human reproductive system (Lee, G.F., & Morris, 1962; US.EPA, 2002; Veeramachaneni, Palmer, & Amann, 2001).

Undoubtedly, a sustainable remediation measure to treat phenol in illegally dumped industrial wastewater (IDIWW) is needed. Phytoremediation is a green technology, gaining more and more popularity these days (Bahraminia, Zarei, Ronaghi, & Ghasemi-Fasaei, 2016; Brandt, Merkl, Schultze-Kraft, Infante, & Broll, 2006; Danh, Truong, Mammucari, Tran, & Foster, 2009; Gopalakrishnan, Burken, & Werth, 2009; Huang, D.Y. et al., 2011; Roongtanakiat & Chairoj, 2001; Singh, Melo, Eapen, & D'Souza, 2008; Singh, Melo, Eapen, & D'Souza, 2006; Truong, Baker, Chonchalow, & Henle, 1998; Truong, Hart, Chomchalow, & Sombatpanit, 2001). In addition to its cost effectiveness and non-environmental disruption, phytoremediation is a great remedial action candidate as its simplicity allows affected villagers to participate in the implementation of the technology. Various kinds of plants and their hairy root cultures (HRC) that are capable of production of peroxidases (PODs) were reported to effectively detoxify phenols via enzyme catalyzed oxidation and polymerization using either external hydrogen peroxide (H_2O_2) or inherent H_2O_2 as the

oxidizing agent (Harvey et al., 2002). In fact, H_2O_2 and horseradish POD or soybean POD were known to treat phenols via polymerization and precipitation years before the phytotreatment of phenols (Klibanov, Tu, & Scott, 1983; Kurnik, Treder, Skorupa-Kłaput, Tretyn, & Tyburski, 2015; Tong et al., 1998; Wright, & Nicell, 1999). Nevertheless, the prohibitive cost of the enzyme makes it economically unfavorable. As a result, relatively low cost of phytoremediation makes this phytodegradation technique promising. For example, the POD extracts from *Daucus carota* and *Solanum aviculare* with the addition of H_2O_2 accelerate the transformation of phenol, 2-chlorophenol, and 2,6-dichlorophenol (De Araujo, Charlwood, & Pletsch, 2002; de Araujo, de Oliveira, Machado, & Pletsch, 2004). Similarly, HRC of *Brassica napus* was able to remove 2,4-dichlorophenol (2,4-DCP) (100 –1000 mg/L) from aqueous solutions in the presence of H_2O_2 (5 to 10 mM) as effectively as 97–98 % within 1 hr. The HRC could be reused to remove 2,4-DCP for six consecutive cycles (Agostini, Coniglio, Milrad, Tigier, & Giulietti, 2003).

Singh et al. (2006) reported that even without the addition of H_2O_2, HRC of Indian mustard (*Brassica juncea* L. Czern and Coss), beet root (*Beta vulgaris* L.), white radish (Raphanus sativus L.), and neem (*Azadirachta indica* L. Juss) successfully degraded phenol via intercellular production of H_2O_2 and peroxidase, presumably due to the self-protection mechanism of the plants against xenobiotic stress. In addition, B. juncea showed the highest phenol removal (97 %) followed by *B. vulgaris* (70 %), *R. sativus* (54 %), and *A. indica* (51 %) (Singh et al., 2006). Some research groups used transgenic hairy root clones of plants, which overexpress the TPX1 gene to increase POD activity. Transgenic tomato (*Lycopersicon esculentum* Mill. cv. Pera) with the addition of 5 mM H_2O_2 removed 85 % of phenol in comparison to the removal of 70 % by wild type (Wevar-Oller et al., 2005) Similarly, transgenic tobacco HRC with basic POD genes from tomato (TPX1 and TPX2) removed as much as 90 % of phenol in 1 hr with the addition of 5 mM H_2O_2. Furthermore, whole plants were also used for phenol treatment. For example, common vetch (*Vicia sativa* L.) removed almost 100%of phenol (100 mg/L) within 6 days by increasing the level of POD and ascorbate POD and maintaining superoxide dismutase activity, malondialdehyde, and H_2O_2 levels (Ibáñez, Alderete, Medina, & Agostini, 2012). Double transgenic tobacco (*Nicotiana tabacum* cv. Wisconsin) with TPX1 and

TPX2 tomato POD genes was reported to degrade as much as 98 % of 2,4-DCP (25 mg/L) in 72 h without the addition of H_2O_2 while the wild type could degrade 84 % of 2,4-DCP (Talano et al., 2012).

For several reasons, vetiver grass (*Vetiveria zizanioides* (L.) Nash), which was introduced to Thailand by his majesty King Bhumibol Adulyadej in 1991, is the plant of choice for phytoremediation of heavy metals and toxic organic contaminants, including phenol (Danh et al., 2009). Vetiver grass can tolerate harsh environments, various toxic metals, and relatively high soil salinity. Vetiver grass is known to yield high root biomass, which is favorable for phytostimulation, phytostabilization, and rhizofiltration. Within a year, the root system can be 3- to 4-m deep and may be as long as 7 m after 3 years, making it an ideal plant for phytoremediation (Troung, Hedrick, & Searle, 2006).

Vetiver was also evaluated for its potential to treat phenol in Murashige and Skoog's liquid medium under aseptic conditions. Within 4 days, vetiver plantlets completely degraded phenol at the concentrations of 50 and 100 mg/L, while they could degrade with 89 to 70 % a high phenol concentration (i.e., 200 to 1000 mg/L) without the addition of H_2O_2. The study pointed out that phenol removal was associated with inherent H_2O_2 and POD produced by the vetiver roots (Singh et al., 2008). Nevertheless, neither a laboratory-scale study nor a field application of vetiver grass with real wastewater has been examined. Real wastewater contains not only phenol but also microbes capable of involving phenol detoxification (González et al., 2006; Kietkwanboot, Tran, & Suttinun, 2015). Thus, currently, no complete mechanistic understanding of phenol degradation by vetiver grass, especially in a complex wastewater system for real application, is available.

This present research aims at evaluating the feasibility of using vetiver plantlets on a floating platform together with aeration to treat phenol in IDIWW. A floating platform as a support for aquatic vegetation to form floating hydroponic root mats is an eco-technological wastewater treatment system used for the treatment of various types of contaminated water, including domestic wastewater, agricultural effluents, and industrial wastewater. Laboratory-scale experiments were performed to evaluate the role of vetiver density on phenol treatment kinetics and to determine the synergetic effect between vetiver and rhizomicrobes in an aerated system on phenol

transformation. A conceptual model of phenol degradation in real wastewater using vetiver plantlets on a floating platform was proposed.

Materials and Methods

1. Vetiver plantlet, Phenol, and Wastewater

Vetiver plantlets (*V. zizanioides* (L.) Nash) used in this study were the bare root Songkhla-3 ecotype obtained from Chachoengsao Land Development Station. In order to develop the root system for the experiment, vetiver plantlets were cultivated in clean soil for 1 month prior to being used in the experiments. The length of the vetiver plantlets' root ready for the experiments was 13.09 ± 5.27 cm. Each vetiver plantlet weighted 0.81 ± 0.15 g (fresh weight). The leaf was cut to be less than 25 cm to stimulate root development. Phenol with 99 % purity was obtained from Panreac (Barcelona, Spain). The IDIWW for the laboratory studies was obtained from Mr.Manus's pond. Table 6 summarizes the basic physical and chemical properties of the wastewater other than phenol, adjusted to be 500 mg/L for this study (i.e., the same concentration as in the beginning of the incident).

Table 6 Chemical characteristics of illegally dumped wastewater at Mr. Manus's pond after the illegal dumping for two years

Depth	pH	COD (mg/L)	TKN (mg/L)	SS (mg/L)	TDS (mg/L)	Sulfide (mg/L)
Middle	8.17	296.30	19.04	28	2188	0.01
Bottom	6.83	3703.70	89.6	9420	15968	0.35
Mixed for the Experiment	7.75	1760	47.05	210	6980	0.15
Effluent Standard	5.5-9	120	100	50	3000	1.00

The IDIWW samples from the middle and the bottom of the pond were mixed together to use in the present study.

2. Phenol Degradation Kinetics by Vetiver Plantlets on a Floating Platform.

In this study, phenol degradation by vetiver plantlets on a floating platform was conducted in glass tanks (40 × 20 × 60 cm) filled with 35 L of real IDIWW from Mr. Manus's pond (Figure 22), in which the initial phenol concentration was adjusted to 500 mg/L, the maximum phenol concentration measured when the incident was discovered.

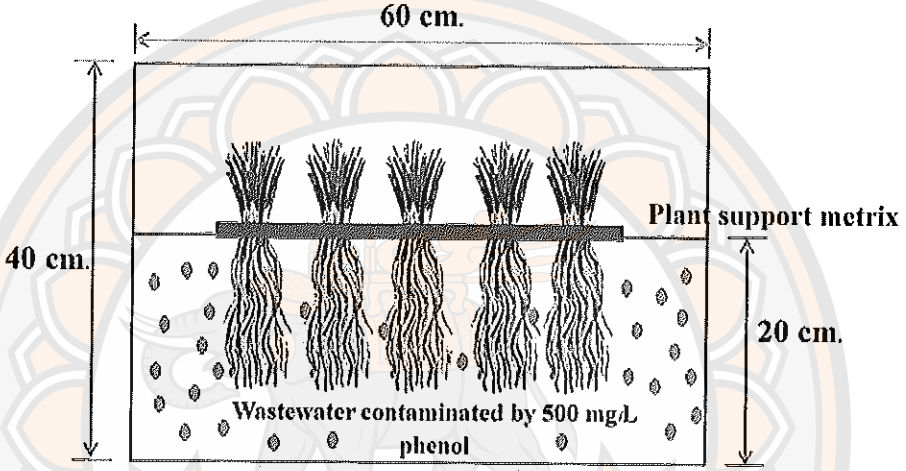


Figure 22 Schematic of vetiver plantlets on a floating platform used in this study

Five different experimental conditions (P1 to P5) using different numbers of vetiver plantlets (P1 = 20, P2 = 40, P3 = 60, P4 = 80, and P5 = 100 plantlets) and different percentages of plant coverage (P1 = 11.38 %, P2 = 22.76 %, P3 = 34.13 %, P4 = 45.51 %, and P5 = 56.98 %) were examined. The experiments were conducted with aeration, where air was pumped through the tank at the rate of 15 L/min using four ceramic diffusers. Two control studies without vetiver grass, but with and without aeration, were also conducted. Phenol degradation kinetics were determined over 1,400 hr. Similarly, the kinetics of chemical oxygen demand (COD) removal, volatile organic acids (VOA) formation, lignin and tannin removal, and H_2O_2 formation were monitored over 1,400 hr to verify the phenol degradation mechanism. The POD formation in the root was examined in a selected experiment and selected time to fulfill the determination of the phenol degradation mechanism. In addition, the vetiver growth kinetics including the weight and root length of vetiver plantlets were

quantified as a function of time. While all the leaves were measured and counted, 10 % of vetiver plantlets in each floating platform were selected to count and measure the root length. The same selected vetiver plantlets were marked to monitor the change of root length over the course of the experiments. To understand the synergistic effects of vetiver grass and microbes, this study isolated and characterized native microorganisms after the phenol treatment. Vetiver roots and treated IDIWW samples from P1 and P5 were collected and examined for microbial content after four different treatment times. The total viable counts of yeasts, molds, and *Pseudomonas* spp. were quantified as described next.

3. Chemical and Physical Analysis

Phenol concentrations in IDIWW samples from the experiments were measured using the 4-amino-antipyrine method 8047 with a Spectroquant® Move 100 mobile colorimeter (Merck KGaA, Darmstadt, Germany). The detection limit in our matrix was 0.5 mg/L. Chemical oxygen demand (COD) was measured by the closed reflux method (Standard Method 5220 C), while tannin and lignin were measured by Standard Method 5550. Volatile organic acids (VOA) were quantified by Standard Method 5560. pH, oxidation-reduction potential (ORP), and conductivity were measured with appropriate probes with proper calibration. Hydrogen peroxide (H_2O_2) was measured by reacting it with dissolved titanium to form yellow pertitanic acid (Lei Wang & Xuanzhen Jiang, 2009). The colored complex was measured by a spectrophotometer at 400 nm POD activity in root was determined at 25°C with a spectrophotometer following the formation of tetraguaiacol as described by Singh et al. (2006). One unit of POD activity (U) represents the amount of enzyme catalyzing the oxidation of 1 μmole of guaiacol in 1 min at 25°C.

4. Microbial and Rhizomicrobial Characterization

To characterize microbes and rhizomicrobes, vetiver roots were collected and surface sterilized by washing with 75% ethanol and 5% NaOCl, followed by rinsing with sterile distilled water. Surface sterile roots were ground and mixed with potassium dihydrogen phosphate buffer pH 6.8. Root and wastewater samples were subjected to ten-fold serial dilutions (Dudeja, Giri, Saini, Suneja-Madan, & Kothe, 2012). The pour plate technique on standard plate count agar was used for total viable counts (TVC), and the spread plate technique was applied to determine yeasts and

molds on Sabouraud dextrose agar and *Pseudomonas* sp. on *Pseudomonas* agar. Standard plate count agar and Sabouraud dextrose agar plates were inverted and incubated for 48 hr at 35°C. Sabouraud dextrose agar plates for yeast and mold counts were upturned and incubated for 5 days at 25°C. Colonies growing on the plates were counted and recorded as colony forming units per g (CFU/g) of each sample. Colonies were purified on standard plate count agar for bacteria and on Sabouraud dextrose agar for yeasts and molds. The pure isolates were stored on the same media at 4°C. Isolated colonies were characterized by Gram staining, cell morphology, and biochemical tests as described in Bergey's Manual of Systematic Bacteriology (Garrity, Boone, & Castenholz, 2001).

Results and Discussions

1. Effect of Phenol on Vetiver Plantlet Growth

The phenol in IDIWW adversely affected the growth of vetiver plantlets, and the degree of severity was a function of remaining phenol concentration in the IDIWW. As shown in Figure 23, over time, the weight of vetiver plantlets decreased exponentially.

(a)

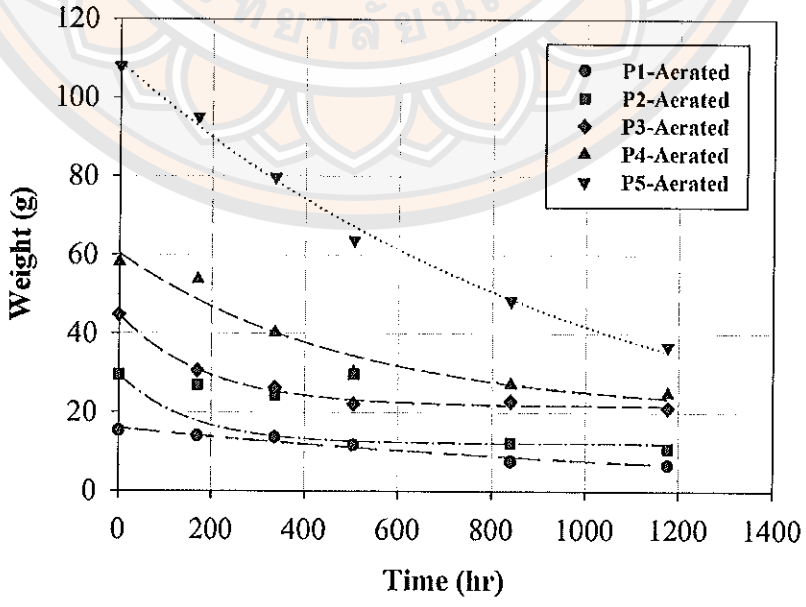
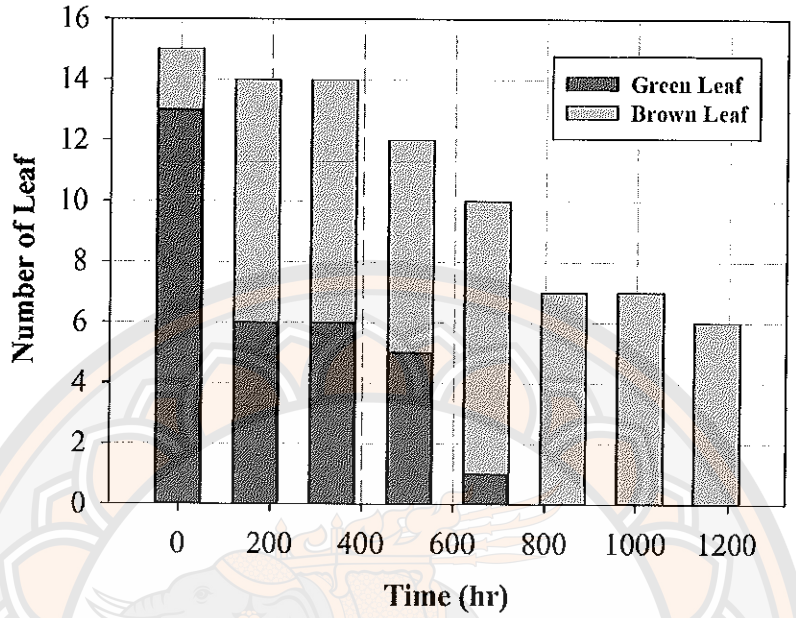


Figure 23 Weigh reduction kinetics of vetiver plantlet in floating platforms

(b)



(c)

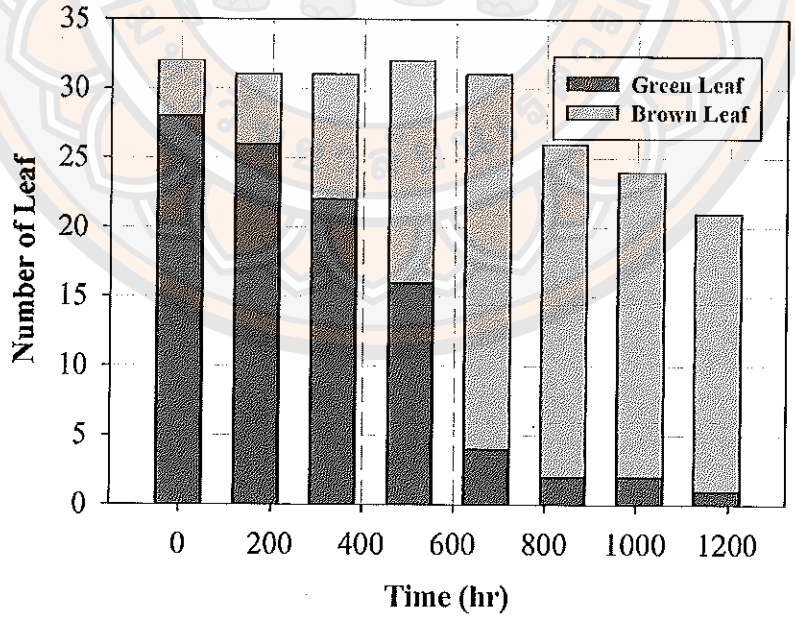
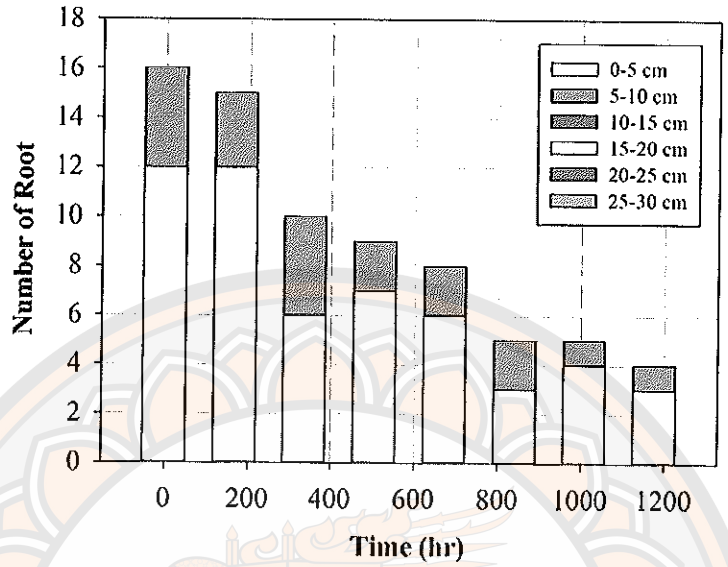


Figure 23 (cont.)

(d)



(e)

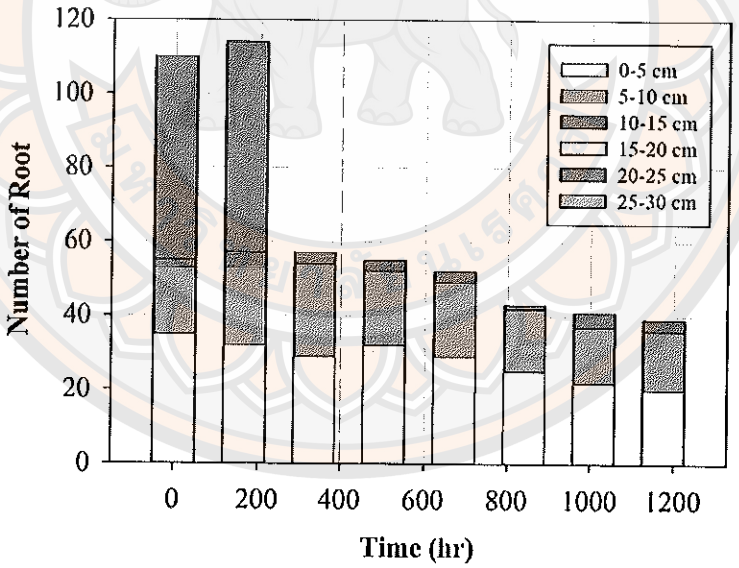


Figure 23 (cont.)

Note: (a) Weight reduction kinetics of vetiver plantlets in floating platforms (P1 to P5), the change of the leaves of vetiver plantlets over time (b) for P1 and (c) for P5, and the change of the roots of vetiver plantlets over time (d) for P1 and (e) for P5.

The rate constants of the weight reduction modelled by pseudo first order were from 0.7 to 5.4 hr^{-1}. Similarly, the leaves of all the vetiver plantlets (P1 to P5) turned more brownish and fell out (see example of P1 and P5 in Figure 23b and 23c, respectively). Furthermore, the same deterioration was also observed for the roots of all the vetiver plantlets (P1 to P5) (see example of P1 and P5 in Figure 23d and 23e, respectively). Noticeably, for all the floating platforms (P1 to P5), the degradation of the root systems was severed from the introduction of high phenol concentration (500 mg/L) up to around 400 to 600 hr (phenol concentration was decreased <303 mg/L) while after 600 hr (phenol concentration was <180 mg/L), the root systems became relatively unaffected. These are clear signs of dose-dependent phenol toxicity on the roots of the vetiver plantlets. They also show phenol detoxification and adaptation process of the vetiver plantlets as discussed in details in the next section. This agrees very well with a recent study reporting that phenol seriously reduced the growth of six economically important agricultural plant species in China including 1) *Amaranthus*, 2) *Cucumis*, 3) *Raphanus*, 4) Cabbage no.19, 5) Yellow leaf cabbage, and 6) Pak Choi (Poon, Hon, & Huang, 2011). They also reported that the effective concentrations of phenol that inhibited 50% of root elongation and seed germination (EC50) ranged from 42.7 mg/L for Cabbage no.19 to 322.7 mg/L for *Cucumis*. As for willow trees (*Salix viminalis*), phenol at the concentration of 250 mg/l was reported to slightly inhibit transpiration while at the concentration greater than 500 mg/L, transpiration dropped to 50% or less, and the trees wilted. If exposed up to 1,000 mg/L, the tree seriously wilted and eventually died (Ucisik & Trapp, 2006). For vetiver, ten percent decrease of growth was reported when exposed to phenol at the concentration of 200 mg/L from 5 to 30 days.

2. Phenol Degradation and COD Removal Kinetics by Vetiver Plantlets with Aeration.

Without vetiver plantlets, phenol degradation in IDIWWwith aeration followed the pseudo first-order rate law (Eq 3.1) (red cycles in Figure 24a), as it has been reported that rates for phenol degradation were first order in substrate concentration and zero order in biomass concentration (Bekins, Warren, & Godsy, 1998; Huang, D.Y. et al., 2011; Hussain, Dubey, & Kumar, 2015; Kietkwanboot et al., 2015; Shibata, Inoue, & Katayama, 2006), as follows:

$$C_t = C_0 e^{-k_p * t} \text{ (Eq 3.1)}$$

where C_t and C_0 are the phenol concentration at time t and 0, respectively, k_p is phenol degradation rate constant (hr^{-1}), and t is the unit in hours.

(a)

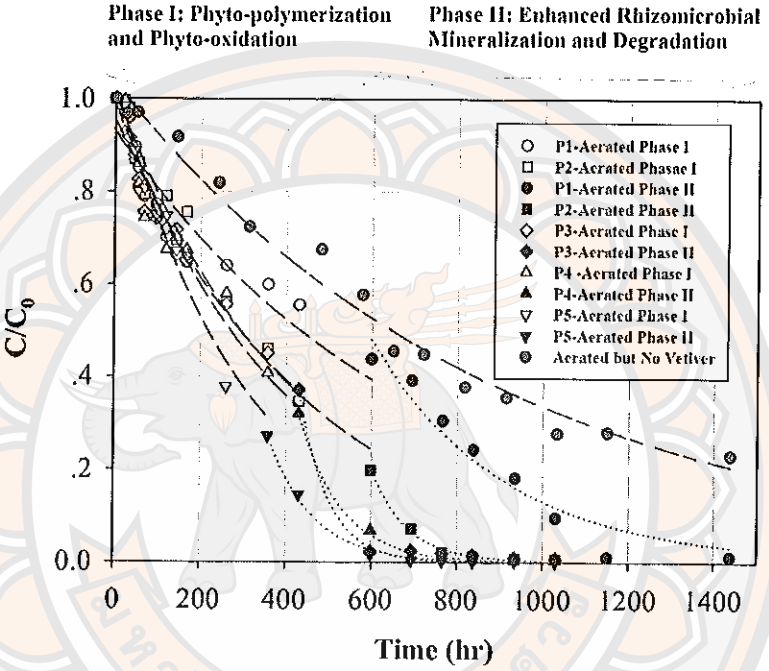


Figure 24 Phenol degradation and COD reduction kinetics

(b)

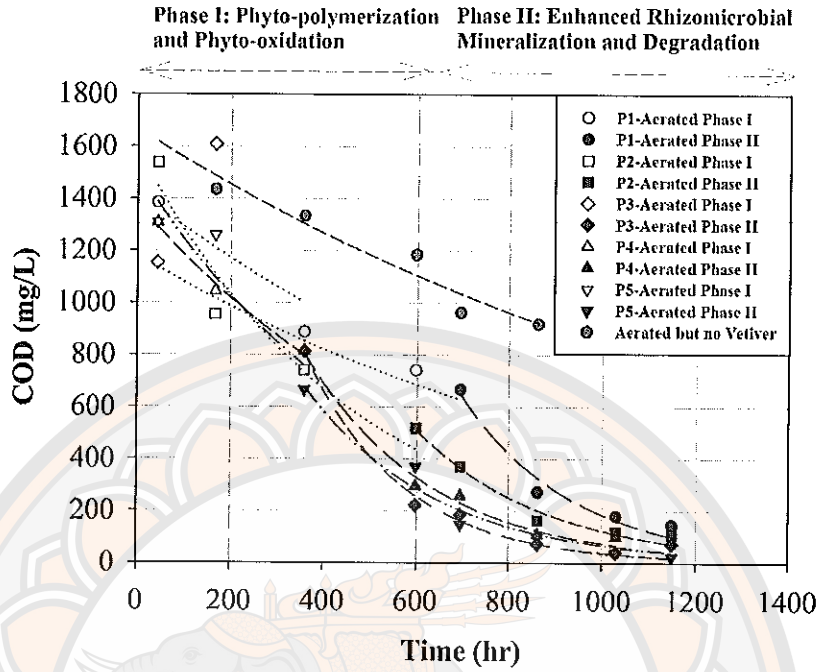


Figure 24 (cont.)

Note: (a) Phenol degradation kinetics and (b) COD removal kinetics modeled by two-phase pseudo first-order reactions (phase I and II)

The rate constant of phenol degradation in IDIWW without vetiver plantlets but with aeration was $1.10 \pm 0.05 \times 10^{-3}$ hr^{-1}, around three times greater than without the aeration in the same IDIWW ($k_p = 0.3 \times 10^{-3}$ hr^{-1}). This also agrees with the literature reporting that aerobic biodegradation of phenol is substantially faster than anaerobic biodegradation (Shibata et al., 2006). The volatilization of phenol was assumed to play a minor role on the removal (Hussain et al., 2015). As discussed in the next section, the IDIWW consisted of various microbes including bacteria, such as *Rhodococcus* sp., *Pseudomonas* sp., *Bacillus* sp., *Enterobacter* sp., and *Alcaligenes* sp. as well as Y&Ms, such as *Trichosporon* sp., *Rhodotorula* sp., and *Fusarium* sp. These microorganisms are capable of phenol transformation via phenol mineralization through meta cleavage and *ortho* cleavage pathways (Al-Khalid, & El-Naas, 2012;

van Schie, & Young, 2000) (Figure 25) and via enzymatic polymerization of phenol (Figure 26) (Pradeep et al., 2015).

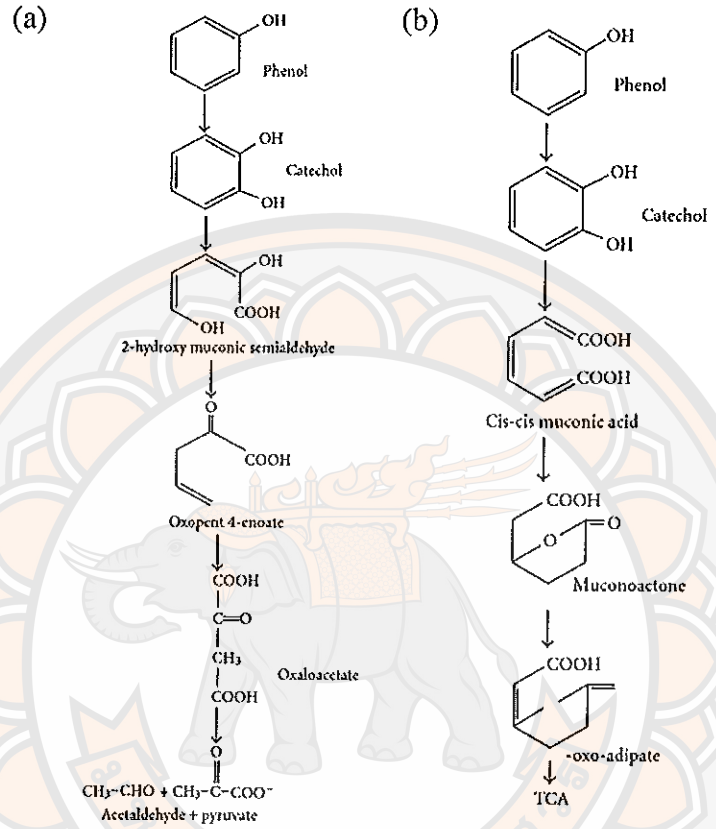


Figure 25 Phenol degradation meta and ortho cleavage pathway

Note: (a) *meta* cleavage pathway and (b) *ortho* cleavage pathway

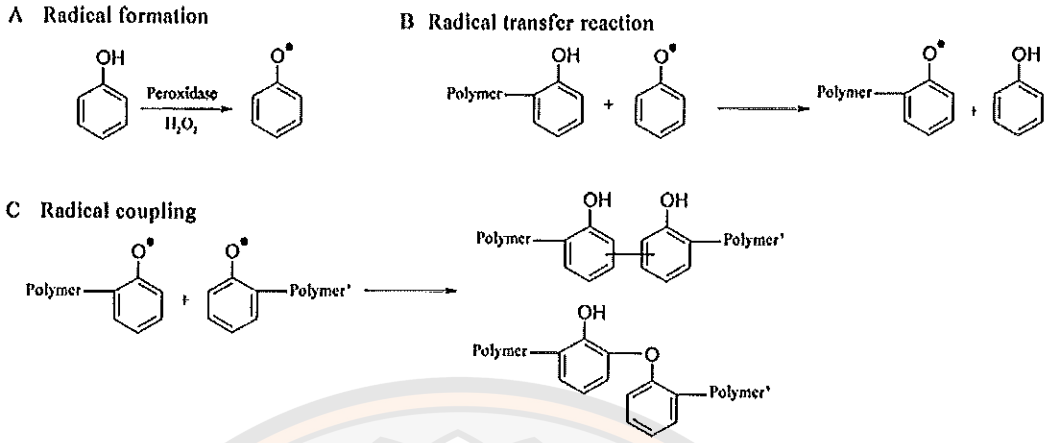


Figure 26 The reactions of phenol with H_2O_2 catalyzed by POD involve radical formation, radical transfer reaction, and radical coupling reaction resulting in the formation of much less toxic polyphenols

No lag phase was observed, as these microorganisms were indigenous in phenol contaminated IDIWW, similar to a recent study reporting that at a phenol concentration of 500 mg/L, acclimatized microbes immediately started phenol degradation (Hussain et al., 2015).

Moreover, COD removal in the IDIWW with aeration but without vetiver plantlets followed the pseudo first-order rate law (red cycles in Figure 24b) with the rate constant (k_{COD}) = 0.70 ± 0.07 × 10^{-3} hr^{-1}. Noticeably, k_{COD} is only around 63.63 % of k_p, suggesting that for every 100 % of phenol removed, only 63.63 % at most was presumably mineralized via *meta*-cleavage and *ortho*-cleavage pathways; the rest was transformed to polyphenols via enzymatic polymerization, which did not substantially decrease COD but decreased phenol concentration.

The presence of vetiver plantlets in an aerated system significantly enhanced phenol degradation. The overall phenol and COD removal rates (Figure 24a and 24b) appear to qualitatively increase with the number of vetiver plantlets used in the treatment (i.e., P1 < P2 < P3~P4 < P5). For example, it took 2,932 hr (122.00 days), 1,597 hr (66.57 days), 1,213 hr (50.55 days), 1,363 hr (56.80 days), and 766 hr (31.92 days) to degrade phenol to be less than 1 mg/L for the case of P1 (20 plantlets), P2 (40 plantlets), P3 (60 plantlets), P4 (80 plantlets), and P5 (100 plantlets), respectively. It will take as long as 5,649 hr (235 days) to degrade phenol to 1 mg/L

without vetiver plantlets but with aeration. Similarly, it took 1796, 1030, 790, 849, and 792 hr to decrease COD to be lower than 120 mg/L for the cases of P1, P2, P3, P4, and P5, respectively.

Additionally, H_2O_2 and POD were detected in IDIWW and vetiver roots, respectively (Figure 27a and 27b). Initially, POD in the root of vetiver plantlets (P5 as an example) was 2.35 U g^{-1} fw.

(a)

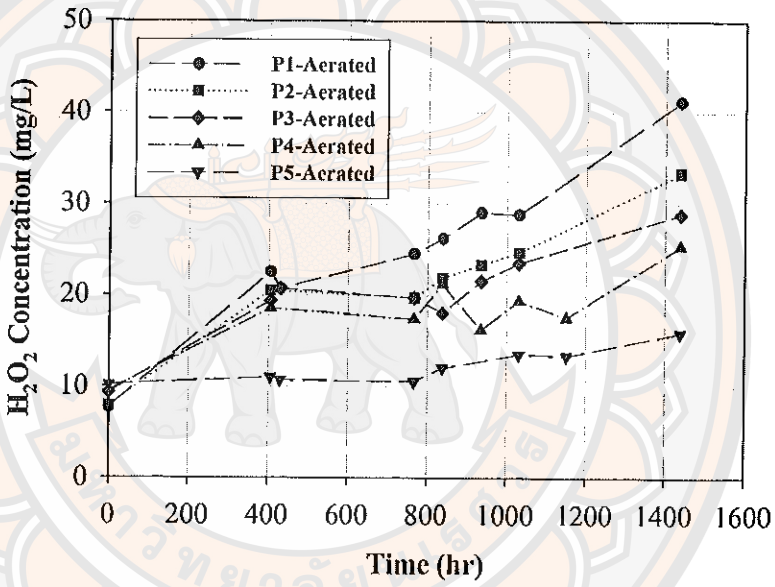


Figure 27 H_2O_2 in IDIWW in P1 to P5, POD in vetiver roots and phenol of P5

(b)

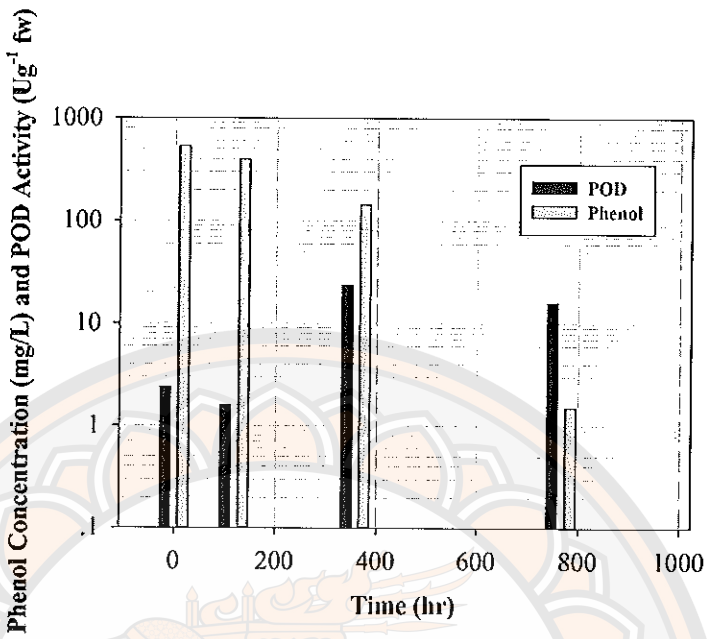


Figure 27 (cont.)

Note: (a) H_2O_2 in IDIWWin P1 to P5 over the course of the experiments,
(b) POD concentration in vetiver roots of P5 and phenol concentration in IDIWW of P5 as a function of selected times

After being exposed to high phenol concentration (500 mg/L), POD in the root increased to 23.55 U g^{-1} fw after 400 hr and decreased to 15.81 U g^{-1} fw at 766 hr when the phenol concentration declined to around 1 mg/L (Figure 27b). This agrees with a recent study reporting the increase of POD up to 20 U g^{-1} fw when vetiver plantlets were exposed to phenol at the concentration of 200 mg/L, but once phenol was degraded to 20 mg/L, the POD decreased to around 15 U g^{-1} fw (Singh et al., 2008). Furthermore, H_2O_2 increased from 10 to 25 or 40 mg/L (i.e., 2.5- to 4-fold enhancement) once exposed to the high phenol concentration in IDIWW. Recent studies reported the same finding in that phenol exposure induced the increase of H_2O_2 excretion by *B. juncea* hairy roots and vetiver plantlets by 2.72 and 5.78 times in comparison to the H_2O_2 concentration without phenol stress (Singh et al., 2008; Singh et al., 2006) It should be noted that the greater the number of vetiver plantlets, the

lower the H_2O_2 concentration in IDIWW (i.e., P5 < P4 < P3 < P2 < P1) (Figure 27a). Presumably, the greater the number of the vetiver plantlets, the great POD released from the roots. The POD consumes H_2O_2 to oxidize phenol to phenol radical (Figure 26), explaining the lower remaining H_2O_2 concentration in IDIWW. This partially contributes to the faster phenol removal for a platform with a greater number of the vetiver plantlets.

The increase of H_2O_2 and POD supports the recent hypothesis that the phytotoxicity and irritation of phenol induces the self-protection mechanism of plants involving the intercellular production of both H_2O_2 and POD, which catalytically transform phenol to non-toxic polyphenol precipitates (PPPs) (Singh et al., 2006). The evidence of phenol phytotoxicity and irritation in the present study is the substantial deterioration of the leaf and root system from the introduction of phenol at t = 0 to t from 400 to 600 hr, as reported in the previous section. The enzyme-assisted oxidation and polymerization mechanism is also supported by the increase of the portion of the particulate COD, presumably caused by PPP, in the total COD in the first 400 hr for P1 to P5 (Figure 28). The greater the number of vetiver plantlets in the floating platform, the more the transformation of phenol to PPP, and the higher the fraction of particulate COD (Figure 28).

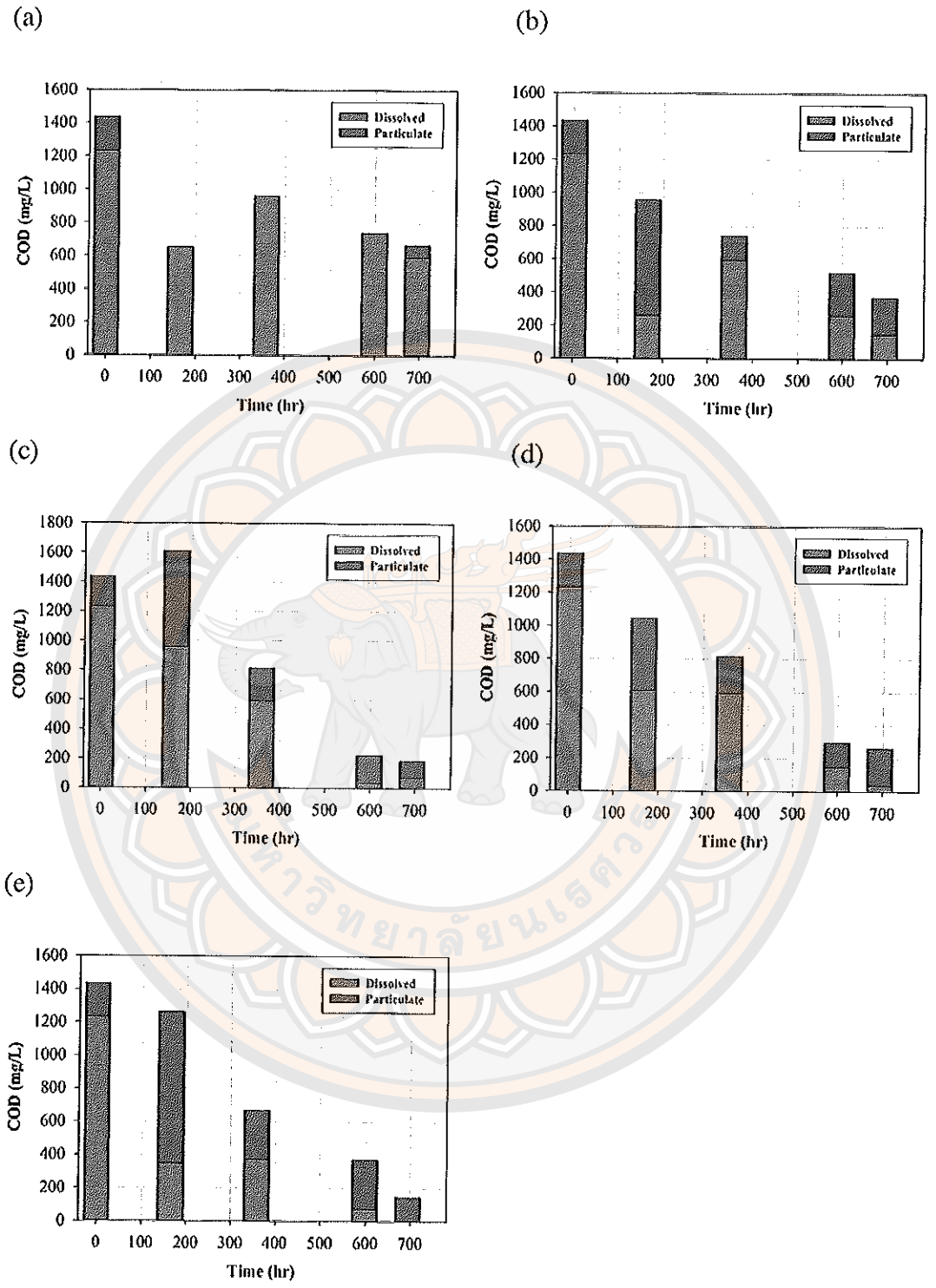


Figure 28 COD fractionation (dissolved and particulate) as a function of treatment time for (a) P1, (a) P2, (b) P3, (c) P4, and (e) P5 with aeration

Nevertheless, unlike previous studies (Esplugas, Giménez, Contreras, Pascual, & Rodríguez, 2002; Hussain et al., 2015; Nielsen, Bjerg, Nielsen, Smith, & Christensen, 1995; Singh et al., 2008; Singh et al., 2006), we were unable to apply the pseudo first-order reaction kinetics to model the entire phenol degradation results with vetiver plantlets in the present study even though the first order kinetics are typically used to model abiotic and biotic phenol degradation reactions, including phytoremediation using vetiver plantlets. Phenol concentrations were exponentially decreased after the interaction with vetiver plantlets in P1 to P5 from the beginning to t between 400 and 600 hr (i.e., the regime of the high phenol concentration treatment). Then, the reaction proceeded even more rapidly in all P1 to P5 from t between 400 and 600 hr to t between 700 and 1400 hr, the regime of the moderate and low phenol concentration treatment (Figure 24a). The same behavior was also observed for COD removal (Figure 24b). Nevertheless, this pattern was not observed in a control study without vetiver plantlets but with aeration (red cycles in Figure 24a and 24b).

Consequently, we needed to divide the kinetic data into two phases to model each phase with the pseudo first-order kinetics (Figure 24a and 24b). With this approach, the first-order kinetics successfully modeled the phenol degradation and COD removal behaviors as shown in Tables 7 and 8, respectively. The phenol concentration ranges for the two phases of P1 to P5 are summarized in Table 7.

Table 7 Phenol degradation kinetics fitting for P1 to P5 with aeration based on the two-phase hypothesis

Experiment	Phase 1			Phase 2			Enhance degradation rate (Time)
	R^2	Concentration Range (mg/L)	k ($x10^{-3}$/hr)	R^2	Concentration Range (mg/L)	k ($x10^{-3}$/hr)	
P1	0.8685	550-120	1.4±0.2	0.9711	120-0.1	3.2±0.5	2.29
P2	0.9855	530-90	2.4±0.2	0.9976	90-0.1	11.4±0.7	4.75
P3	0.9745	533-117	2.2±0.1	0.9912	117-0.1	14.6±2.4	6.64
P4	0.9462	537-104	2.5±0.2	0.995	104-0.1	10.1±0.8	4.04
P5	0.9656	553-83	3.3±0.2	0.997	83-0.1	9.97±0.5	3.02

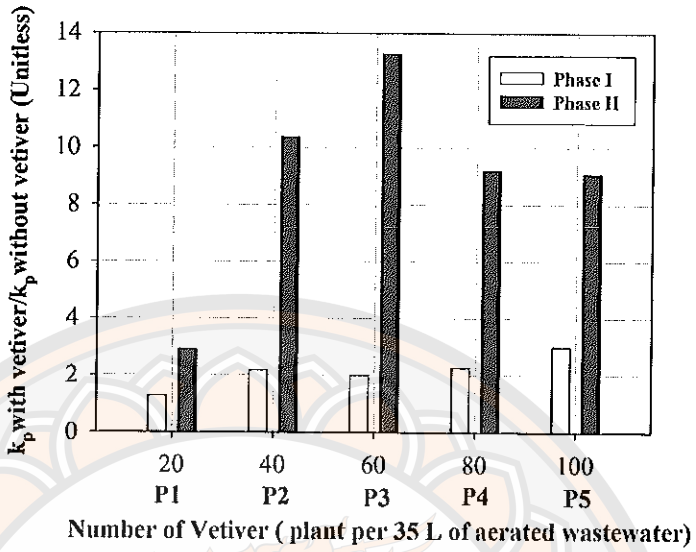
Table 8 COD removal kinetics fitting for P1 to P5 with aeration based on the two-phase hypothesis

Experiment	Phase 1			Phase 2			Enhance degradation rate (Time)
	R^2	Concentration Range (mg/L)	k ($x10^{-3}$/hr)	R^2	Concentration Range (mg/L)	k ($x10^{-3}$/hr)	
P1	0.6790	1384-666	0.9±0.2	0.9683	666-120	4.1±0.2	4.55
P2	0.9331	1538-518	2.2±0.2	0.9778	518-109	3.5±0.2	1.59
P3	0.4977	1153-814	1.0±0.2	0.9889	814-40	4.8±0.2	4.80
P4	0.9898	1307-814	1.5±0.2	0.9901	814-100	3.7±0.2	2.47
P5	0.8356	1307-666	1.9±0.2	0.9650	666-25	3.6±0.2	1.89

Each dataset (P1 to P5) was divided into two ranges for the two phases using the best overall R^2 values of the fittings as criteria. The R^2 values for both phases of all the cases are greater than 0.8685, suggesting a good fit with the pseudo first-order kinetics.

In general, the phenol degradation rate constants of phase I (k_{p1}) increased from P1 (k_{p1} = 1.40 ± 0.20 hr^{-1}) to P5 (k_{p1} = 3.30 ± 0.20 hr^{-1}) (Table 7). The phenol degradation rate constants of phase II (k_{p2}) are significantly higher (2.29 to 6.64 times) than those of phase I for the same number of vetiver plantlets on a floating platform. For example, the phenol removal rate constant (k_{p2}) in phase II of P1 = 3.20 ± 0.50 hr^{-1} while k_{p2} of P5 = 9.97 ± 0.50 hr^{-1}. Similarly, the COD removal rate constants of phase I increased from P1 (k_{COD1} = 0.90 ± 0.60 hr^{-1}) to P5 (k_{COD1} = 1.90 ± 1.00 hr^{-1}) (Table 8). The COD removal rate constants of phase II are significantly higher (1.59 to 4.80 times) than those of phase I for the same number of vetiver plantlets. For example, the COD removal rate constant (k_{COD2}) in phase II of P1 = 4.10 ± 0.70 hr^{-1} while k_{COD2} of P5 = 3.60 ± 0.50 hr^{-1}. This confirms that phenol and COD removal in IDIWW by vetiver plantlets is a two-phase process. Except P1 in phase II, the k_p values are larger than k_{COD} values for the same number of vetiver plantlets (P1 to P5) and the same phase (phase I or II). For phase I, k_{COD1} values are from 45.45 to 91.97 % of k_{p1} values, while for phase II, k_{COD2} values are from 30.70 to 36.63 % of k_{p2} values. This suggests that phenol removal was performed by both enzymatic polymerization (decreasing phenol but not substantially decreasing COD) and mineralization by meta cleavage and ortho cleavage pathways (decreasing both phenol and COD). Figure 29a summarizes enhanced phenol degradation rate constants via vetiver plantlets and aeration in comparison to phenol degradation rate constants by aeration alone.

(a)



(b)

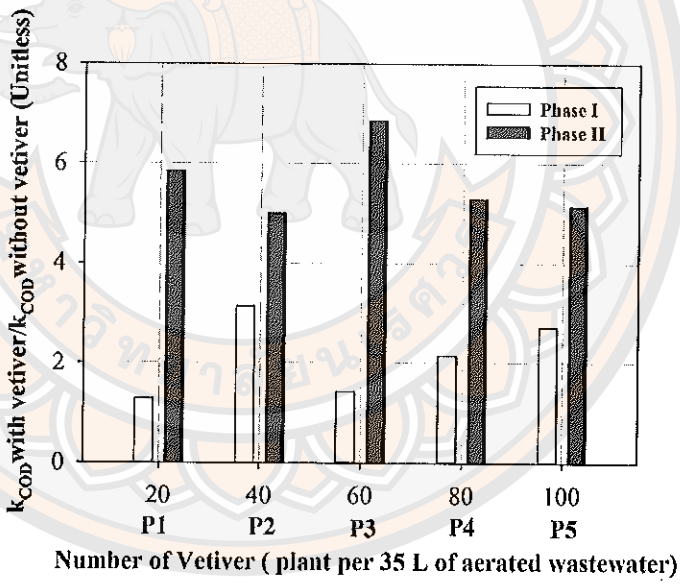


Figure 29 Enhanced a phenol transformation and COD removal rate constants

Note: (a) Enhanced a phenol transformation and (b) COD removal rate constants due to the presence of vetiver plantlets (P1 to P5) and aeration in phase I and II in comparison to that with aeration but without vetiver plantlets

The enhanced phenol removal in phase I is hypothesized to be from enzymatic polymerization promoted by inherent H_2O_2 and POD from vetiver root, while the enhanced phenol degradation in phase II is hypothesized to be from enzymatic polymerization via root produced H_2O_2 and POD coupled with enhanced rhizomicrobial degradation to be discussed next. The enhanced COD removal rate constants for both phases are similar to the enhanced phenol removal rate constants, as also shown Figure 29b.

3. Dynamics of microbes and rhizomicrobes in vetiver plantlets with aeration

As mentioned in the previous section, various phenol degrading microbes, including bacteria, such as *Rhodococcus* sp., *Pseudomonas* sp., *Bacillus* sp., *Enterobacter* sp., and *Alcaligenes* sp. as well as Y&Ms, such as *Trichosporon* sp., *Rhodotorula* sp., and *Fusarium* sp. were identified in the IDIWW. Table 9 summarizes characteristics of isolated colonies from the IDIWW (see micrographs of some selected microbes isolated from the IDIWW in Figure 30).

Table 9 Characteristics of isolated colonies from treated wastewater and vetiver roots

genera	morphology	gram	motility	oxidase	catalase	O/F	IMVIC
Bacteria							
Rhodococcus	Filamentous	+	-	-	+	+/-	--++
Pseudomonas	short rod	-	+	+	+	+/-	---+
Bacillus	rod	+	+	-	+	+/+	-++-
Micrococcus	rod	+	-	+	+	+/-	-++-
Enterobacter	spherical	-	+	-	-	+/+	--++
Alcaligenes	rod bacilli	-	+	+	+	+/+	--++

Fungi	Morphology	Gram Tube	Capsule	Spore Formation	Urease
Candida	Oval	+	-	Chlamydospore	ND
Trichosporon	Septate hyphae	-	-	Arthrospore	+
Rhodotorula	Oval	-	+	-	+
Fusarium	Septate hyphae	ND	ND	ND	ND

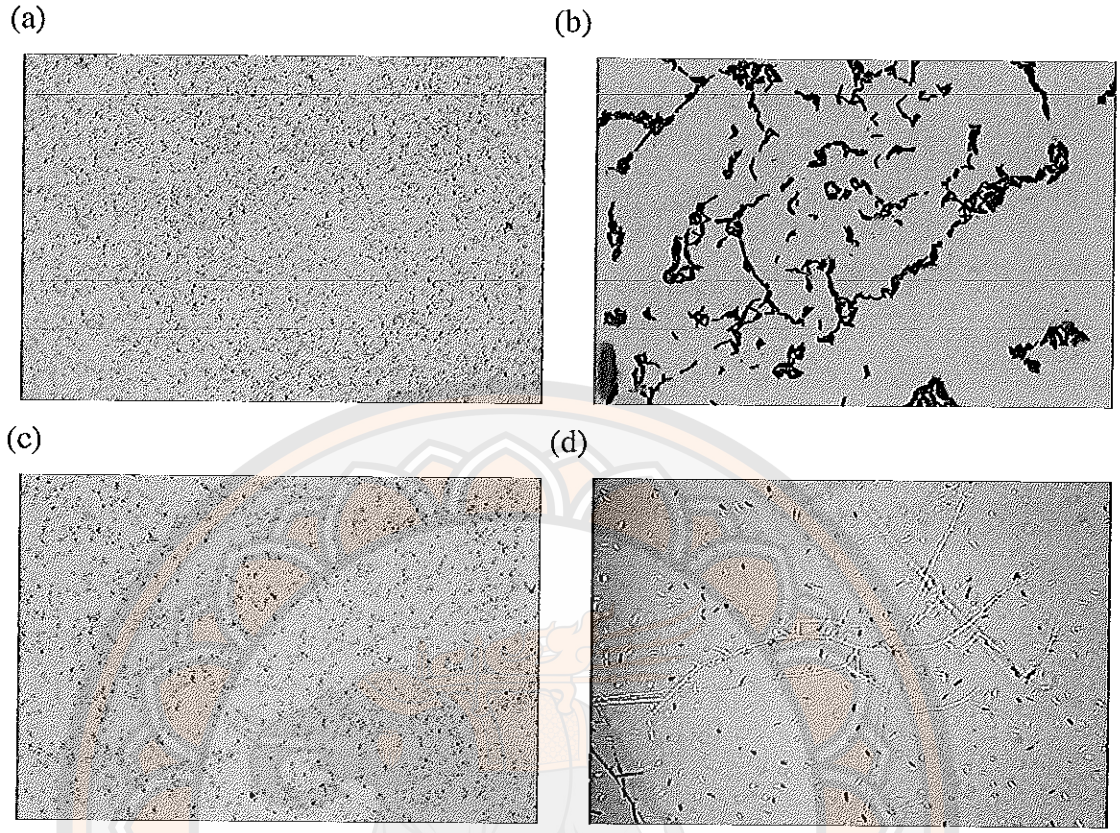


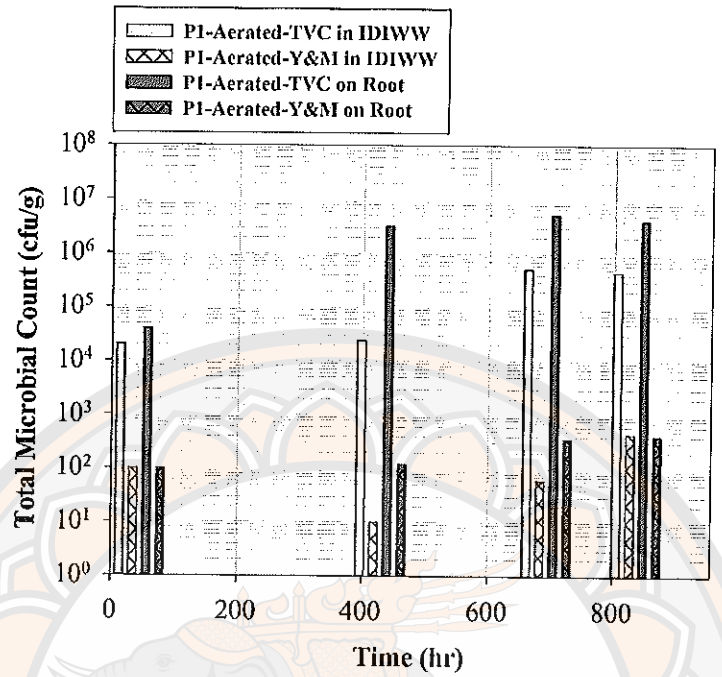
Figure 30 Micrographs of isolated colonies from IDIWW and vetiver roots

Note: including (a) *Pseudomonas* sp. *1000X*, (b) *Bacillus* sp. *1000X*, (c) *Rhodotorula* sp. *400X*, and (d) *Trichosporon* sp. *400X*.

The TVC of microbes as well as Y&Ms in IDIWW at 43 hr (phenol concentration = 460 and 473 mg/L for P1 and P5, respectively) were relatively low (i.e., TVC = 2×10^4 CFU/g and Y&M = 100 CFU/g for P1 with aeration and TVC < 10^3 CFU/ g and Y&M = 70 CFU/g for P5 with aeration) (Figure 31a and 31b). Even after some ongoing phenol degradation at 430 hr (phenol concentration = 285 and 78 mg/L for P1 and P5, respectively), the TVC and Y&M in IDIWW were slightly decreased (i.e., TVC = 2.4×10^4 CFU/g and Y&M = 10 CFU/g for P1 with aeration and TVC < 10^3 CFU/g and Y&M = 10 CFU/g for P5 with aeration) presumably due to phenol toxicity. Phenol is recognized as an inhibitory substrate at relatively low concentrations (100 mg/L) (Hill & Robinson, 1975). Nevertheless, microbes in the IDIWW were recovered toward the end of phenol transformation in phase I,

presumably due to enzymatic polymerization of phenol via root produced H_2O_2 and POD to yield non-toxic PPPs. For example, for P1 with aeration, after from 694 hr (phenol concentration = 201 mg/L), the TVC increased to 5.1×10^5 CFU/g, while the Y&M became 60 CFU/g; both TVC and Y&M increased by an order of magnitude from 430 hr (phenol concentration = 285 mg/L). At 838 hr (phenol concentration = 124 mg/L), the TVC remained at 4.5×10^5 CFU/g, while Y&M substantially increased to 4.4×10^2 CFU/g, 40 times greater than at 694 hr of aeration (Figure 31a and 31b). The same trend is even more pronounced for P5, for which phenol concentration was lower due to more extensive phenol transformation via the greater number of vetiver plantlets. For P5 with aeration, at 694 hr (phenol concentration = 2.3 mg/L), the TVC substantially increased to 4.7×10^5 CFU/g, while the Y&M became 2.6×10^3 CFU/g; both TVC and Y&M increased by two orders of magnitude from 430 h (phenol concentration = 78 mg/L). At 838 h (phenol concentration = 0.7 mg/L for P5), the TVC increased to 2.7×10^6 CFU/g, while Y&M substantially increased to 6.5×10^3 CFU/g, which is 3–5 times greater than at 694 hr (Figure 31a and 31b). Noticeably, even starting with less TVC and Y&M in the beginning, at 838 h, the TVC and Y&M of P5 were one order of magnitude greater than that of P1 in good agreement with around two orders of magnitude lower in phenol concentration of P5 in comparison to P1.

(a)



(b)

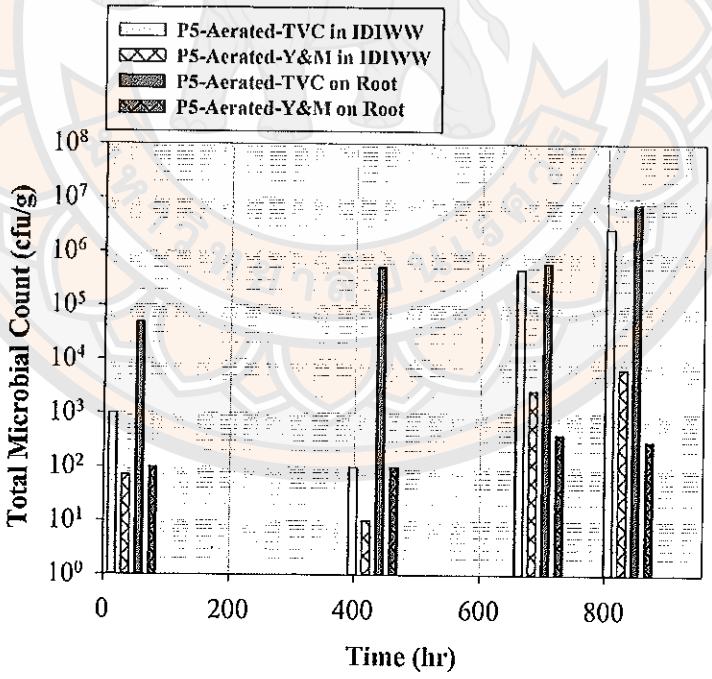


Figure 31 Total microbial count in IDIWW and vetiver roots as a function of time for (a) P1 and (b) P5 with aeration

The presence of vetiver plantlets substantially favored microbial vitality not only by phenol detoxification through the enzymatic polymerization but also through the special relationship between an endophyte and host (Danh et al., 2009; Kamran, Syed, Eqani, Munis, & Chaudhary, 2015; Zhang, L. et al., 2016). Microbes attached to the vetiver root will form biofilm, increasing biomass. Each biofilm microbium reacts to the environment fundamentally differently from a planktonic cell of the same species, as the complex microbial biofilm community has primitive homeostasis, a primitive circulatory system, and metabolic cooperativity (Valipour & Ahn, 2016). Besides providing physical attachment surfaces, plant roots also provide beneficial exudates, such as amino acids, simple sugars, complex carbohydrates, and oxygen, to plants (Da Silva, Kamath, & Alvarez, 2006; Kurzbaum, Kirzhner, Sela, Zimmels, & Armon, 2010; Stottmeister, U. et al., 2003; Valipour, & Ahn, 2016). All of these will make rhizomicrobes tolerate phenol and participate in phenol degradation more effectively. This agrees well with the observed bioaugmentation of microbes on the vetiver roots in the present study. As shown in Figure 31a and 31b for both P1 and P5 with aeration, the TVC and Y&M values on the vetiver roots were two times to two orders of magnitude greater than those in the IDIWW from 43 to 838 hr. The bioaugmentation of microbes on the vetiver root indicates the favorable condition for microbial survival and may be also responsible for the even more rapid phenol removal in phase II, the enhanced phenol degradation by rhizomicrobial activity proposed earlier.

4. Two-phase conceptual model of phenol degradation by vetiver plantlets with aeration

Considering all the evidence above, we proposed a new mechanistic understanding of phenol degradation by vetiver plantlets, as depicted in Figure 32a. The two phases are phase I, phytopolymerization and phyto-oxidation assisted by root produced H_2O_2 and POD, followed by phase II, a combination of phase I with enhanced rhizomicrobial degradation.

(a)

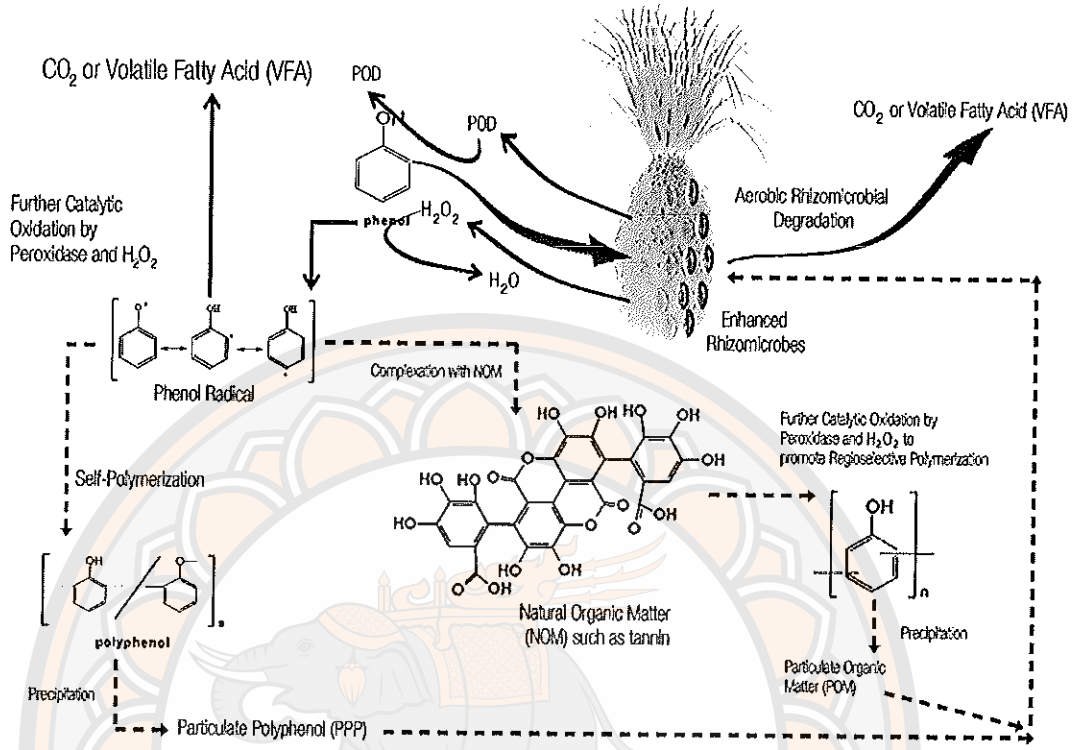


Figure 32 Conceptual model of two-phase phenol degradation by vetiver plantlets in a floating platform

(b)

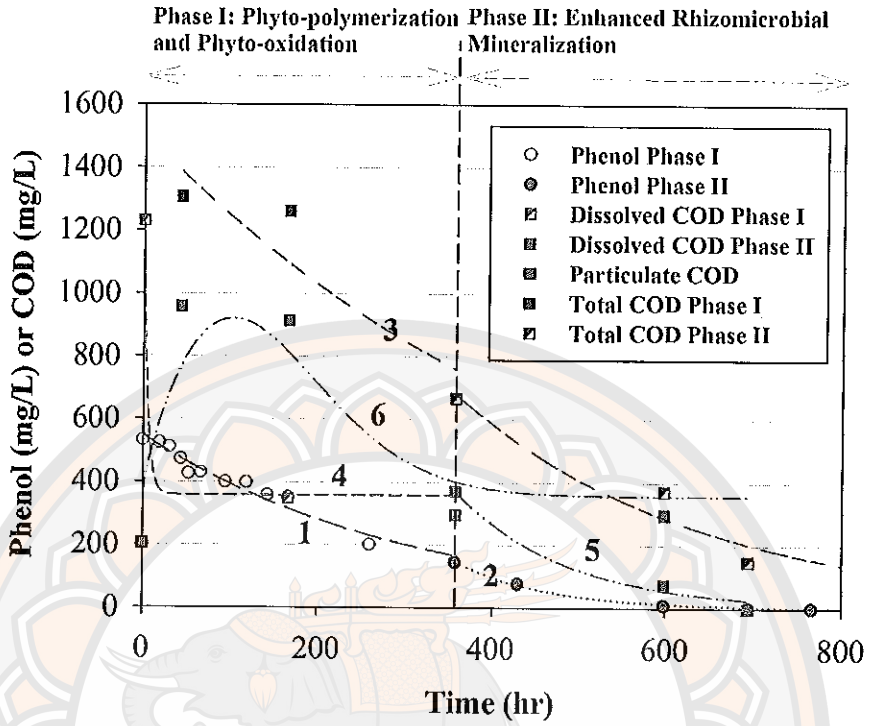


Figure 32 (cont.)

Note: (a) Conceptual model of two-phase phenol degradation by vetiver plantlets in a floating platform. The two phases are phase I, phytopolymerization and phyto-oxidation assisted by root produced H_2O_2 and POD, followed by phase II, a combination of phase I with enhanced rhizomicrobial degradation. (b) Phenol and COD removal kinetics as well as transformation of dissolved and particulate COD as specified to phase I and II for P5 with aeration.

To understand the proposed mechanism, let us consider the phenol degradation and by-product formation of P5 shown in Figure 32b as an example. According to this figure, the first 360 – 400 hr of phenol degradation (open red cycles) was dominated by phytopolymerization and phyto-oxidation at a rate constant of 3.30 ± 0.20×10^{-3} hr^{-1} (Table 7). This is three times faster than the phenol removal rate without vetiver grass but with aeration (1.10 ± 0.05×10^{-3} hr^{-1}). Further, H_2O_2 and POD, produced by vetiver roots assisted in the first phase of phenol transformation (Figure 32a and 32b), were responsible for this enhanced phenol transformation.

Phenol was detoxified via POD catalyzed transformation to phenol radicals, followed by polymerization to non-toxic polyphenols or region-selective polymerization with natural organic matters prior to precipitation as PPP or particulate organic matter (POM), respectively (Figure 32a).

In fact, the reactions of phenol with H_2O_2 catalyzed by POD involve radical formation, radical transfer reaction, and radical coupling reaction, resulting in the formation of much less toxic polyphenols (Figure 11). It should be noted that individual by-products of this enzyme-catalyzed oxidation process were reported to be even more toxic than the parent phenolic compounds. However, the cross-coupling and the co-polymerization of these products to polyphenols and their subsequent precipitation detoxify the transformation process (Ghioureliotis, & Nicell, 2000; González et al., 2012). This is evident by the rapid removal of dissolved COD because dissolved phenol contributes to dissolved COD (half-filled pink rectangles) and the rapid increase of particulate COD because PPP and POM contribute to particulate COD as discussed above (brown rectangles) (Figure 32b). In addition, because the total COD (blue rectangles) decreased exponentially in the first phase, phenol mineralization through *meta* cleavage and *ortho* cleavage pathways driven by microbes and rhizomicrobes also yielded more oxidizing VOA or CO_2 as by-products, which also occurred at the same time as the phytopolymerization of phenol and the precipitation of PPP, as suggested above. After the first phase, phenol decreased from 500 to around 145 mg/L, while PPP and POM, which are non-toxic to microbes, increased. Synergistically, rhizomicrobes intensively grew on the roots of vetiver plantlets (Figure 31b) and participated in microbial degradation of phenol at this lower concentration, increasing the phenol degradation rate (9.7×10^{-3} h^{-1}) by more than threefold in comparison to the phenol degradation rate in the first phase and by approximately ninefold compared with the phenol removal rate without vetiver grass (Figure 32b and Table 7). For P5, the combination of POD-assisted phytopolymerization and phyto-oxidation with rhizomicrobial degradation completely eliminated 500 mg/L of phenol in IDIWW within 766 hr (31.92 days), while the natural attenuation of phenol by microbes in wastewater alone would take as long as 235.4 days considering a degradation rate of 1.1×10^{-3} hr^{-1}. The same reaction mechanism was true for P1 to P4, but at different reaction rates (Table 7). Nevertheless, the phenol degradation rate

in this study is slower than that reported in a previous study. Singh et al. (2008) reported that after 4 days, vetiver grass in aseptic Murashige and Skoog liquid medium decreased phenol at an initial concentration of 500 mg/L by 76 %. Conversely, in our study of the same duration, vetiver grass degraded just 25%of the phenol at the same initial concentration. This is presumably because our study was conducted using real wastewater with COD, organic load (such as total petroleum hydrocarbon (TPH), and inorganic reducing species; the actual pollutant loads are not available for aseptic Murashige and Skoog liquid medium. These organic and inorganic reducing loads consume the oxidative capabilities of H_2O_2 and POD and may compete with phenol degradation.

Conclusion and engineering application

This study proposed a two-phase mechanism of phenol degradation in IDIWW by vetiver plantlets. Phase I is phyto-polymerization and phyto-oxidation assisted by root produced H_2O_2 and POD, while phase II is a combination of phase I with enhanced rhizomicrobial degradation. To our knowledge, this is the first that systematically reveals the complete phenol degradation mechanism by vetiver grass in real wastewater. Our proposed mechanism is different from that reported by Singh et al. (2008), who conducted their experiment in aseptic Murashige and Skoog liquid medium, in which rhizomicrobes might not play a substantial role in phenol degradation. In addition, Singh et al. (2008) did not conduct their phenol degradation study until phenol was completely degraded to meet the discharge standard. Thus, they might not have noticed phase II of the degradation mechanism in which vetiver rhizomicrobes substantially increase the rate of phenol detoxification.

CHAPTER IV

MATURE VETIVER ROOT SYSTEM RAPIDLY DEGRADES PHENOL IN ILLEGALLY DUMPED INDUSTRIAL WASTEWATER: ROLE OF RHIZOMICROBIAL BIOFILM PHENOL CONTAINING WASTEWATER

This Chapter is based on the following manuscript currently prepare for academic publication.

Pimpawat Teeratitayangkul, Siriwan Wichai, and Tanapon Phenrat

Introduction

Phenol is a raw material in several industrial processes including manufacturing of, for example, surfactants, explosive, paints, fertilizers, textiles, and rubber (Busca et al., 2008; Schmidt, 2005). Nevertheless, phenol is a toxic substance causing irritation and kidney inflammation, and disinfection of water-containing phenol using chlorine yields carcinogenic penta-chlorophenols, which can also affect the human reproductive system (Lee, G. F., & Morris, 1962; US.EPA, 2002; Veeramachaneni et al., 2001). To protect environmental and public health, the maximum contaminant level (MCL) of phenol in industrial effluent is 1 mg/L. Although large number of microorganisms including bacteria and fungi are capable of degrading phenol at low and moderate concentration range (Al-Khalid, & El-Naas, 2012), phenol at high concentration (>100 mg/L) is known to inhibit microbial activity due to its irritation (Hill, & Robinson, 1975), leading to failure of conventional biological wastewater treatment to detoxify wastewater contaminated with elevated phenol concentration. This might be a reason why phenol-concentrated wastewater was illegally dumped at Nong Nae village, in Panomsarakam District, Chachoengsao Province of the eastern part of Thailand causing the contamination of shallow groundwater with as much as 225 μg/L of phenol, while the acceptable maximum contamination level (MLC) of pentachlorophenol in drinking water is only 1 μg/L according to World Health Organization (US.EPA, 2010a; WHO, 2003). For this

particular case, we successfully applied phytoremediation using vetiver grass (*Vetiveria zizanioides* (L.) Nash) plantlets on a floating platform to treat illegally dumped wastewater contaminated with phenol as high as 500 mg/L (Phenrat et al., 2016). This phenol treatment involved two phases.

In phase I, to degrade phenol, vetiver roots produce the inherent phytochemicals in the root exudates, enzyme peroxidase (POD) and hydrogen peroxide (H_2O_2) as an oxidizing agent in the self-protection mechanism against xenobiotic stress (Das, & Roychoudhury, 2014; Halliwell, 2006). Thus, the first 360–400 hr of phenol degradation were dominated by phyto-polymerization and phyto-oxidation yielding particulate polyphenols (PPP) or particulate organic matter (POM) as by-products, while phenol decreased to around 145mg/L. In Phase II, rhizomicrobial growth was ~100-folds greater on and inside the roots of the vetiver plantlets than in the IDIWW at the initial condition and participated in the microbial degradation of phenol at this lower phenol concentration, increasing the phenol degradation rate by more than 3 folds. combination of phytochemical and rhizomicrobiological processes eliminated phenol in IDIWW in less than 766 hr (32 days) while without the vetiver plantlets, phenol degradation by aerated microbial degradation alone may require 235 days. The aerobic biodegradation pathways in Phase II including *ortho-* and *meta*-pathways start with using molecular oxygen to enzymatically hydroxylate phenol's aromatic ring to form catechol and then to open the ring through *ortho-* or *meta*-oxidation. These processes were intensively reviewed in a recent literature (Al-Khalid, & El-Naas, 2012). The presence of rhizosphere substantially favors microbial vitality and phenol degradation activity through the special relationship between an endophyte and host. Microbes attached to plant roots will form biofilm, increasing biomass.

Each biofilm reacts to the environment fundamentally differently from a planktonic cell of the same species, as the complex microbial biofilm community has primitive homeostasis, a primitive circulatory system, and metabolic cooperative (Valipour, & Ahn, 2016). Besides providing physical attachment surfaces, plant roots also provide beneficial exudates, such as amino acids, simple sugars, complex carbohydrates, and oxygen, to plants. All of these will make rhizomicrobes tolerate

phenol and participate in phenol degradation more effectively (Da Silva et al., 2006; Kurzbaum et al., 2010; Stottmeister, U. et al., 2003; Valipour, & Ahn, 2016)

Real wastewater which had capacity for microorganisms and complex characteristics was employed to the experiments. The feasibilities of using vetiver plantlets on the floating platforms with aeration and different densities were evaluated and found successful in the phenol degradation associated with rhizo-microorganisms. In addition, the two-phase conceptual model of phenol degradation by vetiver plantlets has been proposed. The PI was influenced by the oxidative stress, POD and H_2O_2 in phyto-oxidation and phyto-polymerization followed by the PII, a combination of PI with rhizo-microbial activities.

During the time, vetiver plantlets confronted a severe effects of phenol toxicity. The weights of vetiver plants were decreased and the same deterioration was observed on the leaves. The circumstance might delay the phenol degradation rates. To accelerate and advance the phenol degradation rate using vetiver grass, we continued working by developing the vetiver root systems. The longer duration of planting vetiver on the hydroponic systems progresses the growth of root system to be longer, denser and healthier. These benefits conduct the sufficient phytochemical exudates that overcome the external stress, phenol toxicity rapidly. The secretion of phytochemicals from roots is an important way for plants to respond to and alter their environment (Badri, & Vivanco, 2009). Furthermore, we also attempted to show that biofilm assists vetiver root on the phenol degradation (Kurzbaum et al., 2010). The indigenous as well as non-indigenous microorganisms in wastewater, multiply and adapt themselves using the vetiver root as habitations in biofilm (Chaparro, Badri, & Vivanco, 2014; Micallef, Channer, Shiaris, & Colón-Carmona, 2009).

Summary of Materials and Experimental Setup

1. Vetiver grass, Phenol, and Wastewater

All experiments were conducted in a greenhouse at room temperature (31±0.8 °C). Vetiver grass (*Vetiveria zizanioides* (L.) Nash), Song khla 3 ecotype, was used in this study. The vetiver grass was full-grown plant by age older than 3 weeks hydroponically. We cultivated the vetiver grass on the floating platforms in the wastewater from the illegal dumping pond but without phenol. The vetiver grass had a

physical root shape with the length of 15 to 20 cm. approximately and covered by thin membranes. The length of vetiver root was longer than vetiver plantlet used in our previous study by 2-3 times (Phenrat et al., 2016). Most of their leaves were greenish. Phenol (analytical reagent grade, 99% purified), was purchased from Panreac (Barcelona, Spain) and employed in all experiments. Wastewater-containing phenol was collected from an agriculture pond which was contaminated by illegal dumping. The wastewater for the experiments was prepared by adding phenol until the concentration reached 500 mg/L, the highest level of phenol concentration found at the illegal dumping incident.

2. Experimental Setup and phenol removal kinetic by Vetiver grass on a floating platform.

To start the experiments, 35 L of wastewater was introduced to glass tanks (40x20x60 cm) for 2 systems included aeration and non-aeration systems. Both of systems were set to evaluate the effect of aeration and the ability of microorganisms including of anaerobic, aerobic microorganism and fungi on phenol degradation. With aeration, air was supplied by pumping air 15 L/min via ceramic diffusers.

Each system was conducted by five different numbers of mature vetiver on platforms (20, 40, 60, 80 and 100 plants) and a platform without vetiver grass which defines as control experiments. Specifically, the symbol PFA was for aeration system while, PF was for non-aeration. Moreover, the performance of vetiver plantlets with aeration from previous study (Phenrat et al., 2016) were compared to the mature vetiver in this study, they were specified as the symbol PB.

The experiments were conducted until the phenol concentration was under the MLC of 1 mg/L for phenol contaminated in industrial wastewater or over 1,000 hr. Phenol, COD, volatile organic acid (VOA), polyphenol as lignin and tannin acid were examined by a function of time as well as H_2O_2. The analytical measurement is provided in Chapter 3, subtopic 3.2. Degradation rates were calculated by assuming first-order kinetics and fitted using non-linear regresssion as followed;

$$C_t = C_0 e^{-kt} \qquad \text{(Eq 4.1)}$$

Where, C_t and C_0 are phenol concentration by time 0 and t hr, k is the reaction rate coefficient (in units of 1/time) and t is the unit of time in hr.

The growths of vetiver grass were weighed in term of fresh weight weekly during the period of experiments to understand the effect of phenol to vetiver grass.

3. Enzyme Estimation and Assay of Activity

During the experiments, the root of vetiver grass was randomized at different times for enzyme estimation and microbial analysis. Vetiver samples were washed with DI water to clean up the root surface then, grinding and crashing in a mortar. Phosphate buffer pH 7.0 was used as enzyme diluent then mixed and equilibrated homogenous by inversion for 10 min. The examination of enzyme activity includes of Peroxidase (POD; EC.1.11.1.7), Superoxide dismutase (SOD; EC.1.15.1.1) and Catalase (CAT; EC.1.11.1.16) were suggested. The detailed description of the enzyme assays used in this experiment is presented in Appendix D.

4. Microbial Analysis

The number of indigenous microorganisms was measured by plate count technic after they were extracted from the wastewater and vetiver root preparation as mentioned above. The microbial and fungi colony was counted as CFU/mL. To identify the strains of phenol degraders, the biochemistry test and the Analytical Profile Index 20E (API 20E) (Nucera, Maddox, Hoien-Dalen, & Weigel, 2006; Truant, 2016) were used for this purpose. The detailed description of the microbial analysis used in this experiment is presented in in Appendix C.

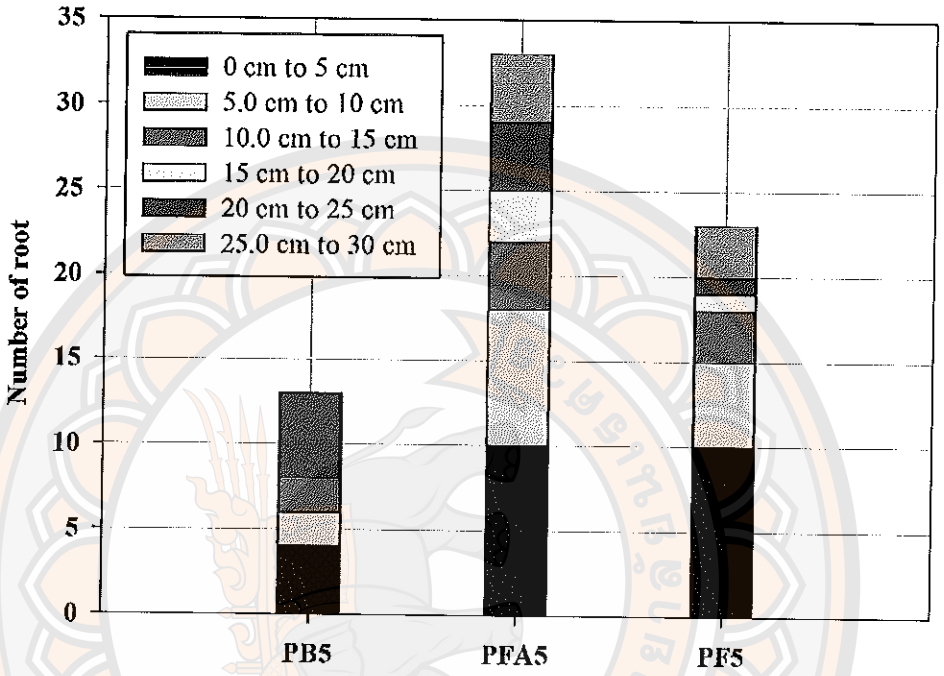
Results and Discussions

1. The Characteristics of Vetiver Grass.

According to the preparation of vetiver platforms prior to starting experiments, the 100 mature vetiver grass with aeration (PFA5), 100 mature vetiver grass without aeration (PF5) and 100 vetiver plantlets with aeration (PB5) were the representatives to indicate the characteristic included numbers of root, leaves and the content of microorganisms (Figure 33). The comparison of characteristics showed that PFA5 had the maximum number of the root, leaves and also the microorganisms followed by PF5 and PB5, respectively. These observed physical characteristics

mentioned that vetiver grass could develop their growths in real wastewater by increasing their roots and leaves.

(a)



(b)

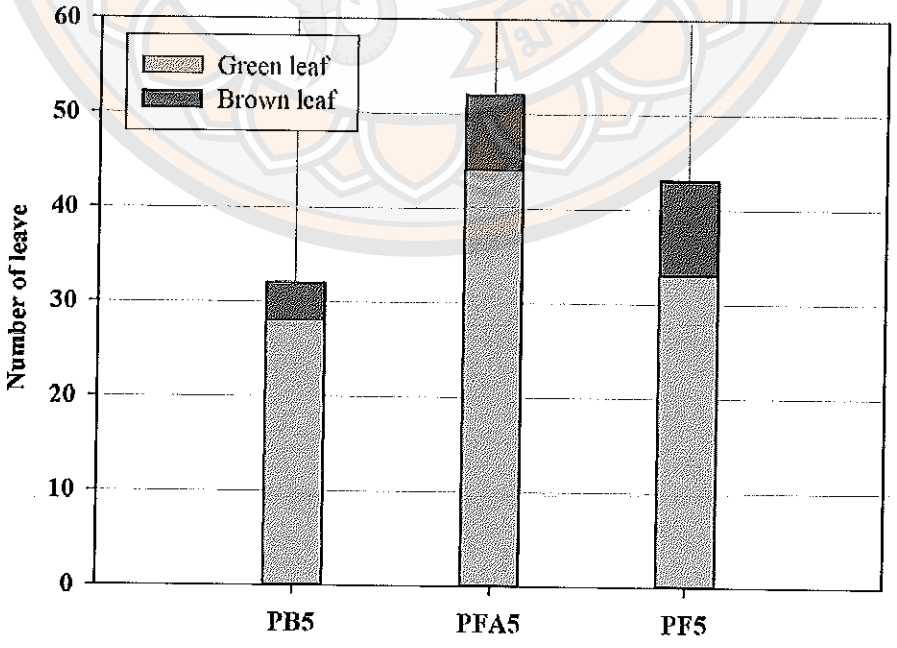


Figure 33 The characteristics of the vetiver grass used in the experiments

(c)

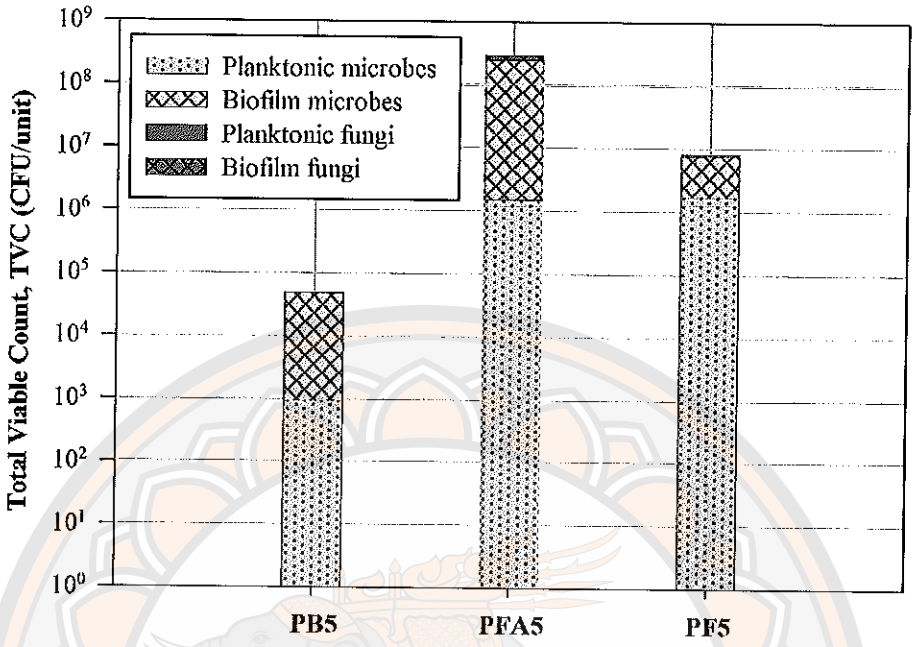


Figure 33 (cont.)

Note: (a) number of vetiver root, (b) the number of green and brown leaves and (c) the total viable count as planktonic microorganism and biofilms

As shown in Figure 33c, It was believed that the vetiver root was the suitable habitation supporting the formation of biofilm by providing the necessary root exudates as well as offering preferential colonization for planktonic microorganisms (Harsh P Bais, Weir, Perry, Gilroy, & Vivanco, 2006; Zhang, N. et al., 2014). Another point worth to consider is the air supply also promoted the growth of vetiver grass to develop the root system on remaining healthy (Chérif, Tirilly, & Bélanger, 1997). For microorganisms, growth and activity are stimulated. Biostimulation usually involves the addition of nutrients and oxygen to supporting their growth (Vidali, 2001).

During phytoremediation, the average fresh weigh reduction of vetiver platforms was examined for studying the effect of phenol toxicity. It was decreased exponentially. The biomass reduction rate was $0.2x10^{-4}$ hr^{-1} and $0.1x10^{-4}$ hr^{-1} for PFA5 and PF5 (Table 10 and 11) while, PB5 suffered more and showed the reduction rate of $10x10^{-4}$ hr^{-1} (Phenrat et al., 2016).

Table 10 The weight of vetiver plant and constant rate of their biomass changes in the experiment with aeration

Time (hours)	Weight/vetiver plant (grams)					
	PFA1 (20 plants)	PFA2 (40 plants)	PFA3 (60 plants)	PFA4 (80 plants)	PFA5 (100 plants)	Control (without phenol)
0	30	34.5	23.7	25.0	21.0	22.0
72	25	27.5	23.3	23.8	18.0	27.4
144	30	27.5	22.5	22.4	13.0	30.6
216	25	30.0	22.3	20.6	15.0	33.8
k ($x10^{-4}$/hr)	-5	-6	-3	-9	-0.2	19

Table 11 The weight of vetiver plant and constant rate of their biomass changes in the experiment without aeration

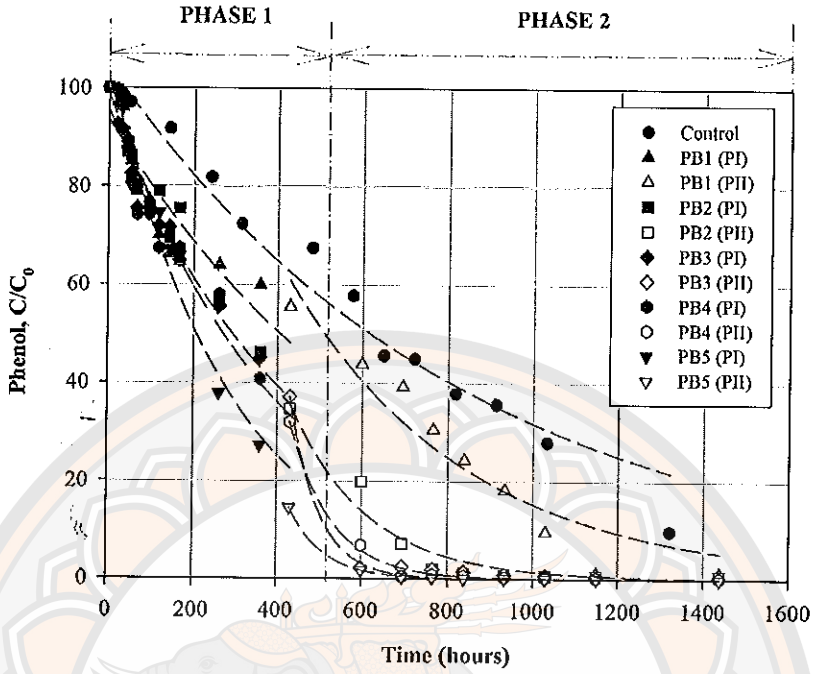
Time (hours)	Weight/vetiver plant (grams)					
	PF1 (20 plants)	PF2 (40 plants)	PF3 (60 plants)	PF4 (80 plants)	PF5 (100 plants)	Control (without phenol)
0	27	33.0	31.7	28.8	20.0	22.0
217	22.5	32.5	21.7	19.4	16.5	29.5
456	20	25.3	18.3	17.6	15.0	33.8
651	18	25.5	16.8	17.0	10.1	37.3
873	15	18.5	17.2	13.8	9.0	41.2
k ($x10^{-4}$/hr)	-6	-6	-7	-7	-0.1	7

It clarified that the phenol toxicity is more harmful to the vetiver plantlets than the mature vetiver grass. These physical and biological characteristics of mature vetiver grass have prompted to stress tolerance and lead to the enhanced phenol degradation. Supported by the biomass reduction with the passage of time indicated a change from toxic. At the first 100 hr (PI) of PFA5, the biomass decreased continuously together with phenol reduction. After 100 hr (PII), the biomass increased slightly. It could be assumed that the mature vetiver grass responded to the high toxicity of phenol by pausing its growth and acclimating itself to survive along with phenol degradation. However, with the same ages of plant, the biomass reduction rate of PF5 was lower than PFA5. It might be caused by the damage of phenol to the root cells in prolongation and affected to the biomass reduction and the growth rate (Hübner, Tischer, Tanneberg, & Kuschk, 2000; Wang, Cai, Feng, & Xie, 2014).

2. Kinetic of Phenol degradation.

To understand the mechanisms and evaluate the potential of phenol degradation by the mature vetiver platforms, first order reaction (Eq 4.1) was examined. Data for mature vetiver platforms from both aeration and non-aeration were well fitted to a first order kinetics model ($R^2 \geq 0.9$). The rate of phenol degradation in mature vetiver were identical as the two-phase mechanisms which described in our previous study (Phenrat et al., 2016) (Figure 34, Table 12, 13 and 14).

(a)



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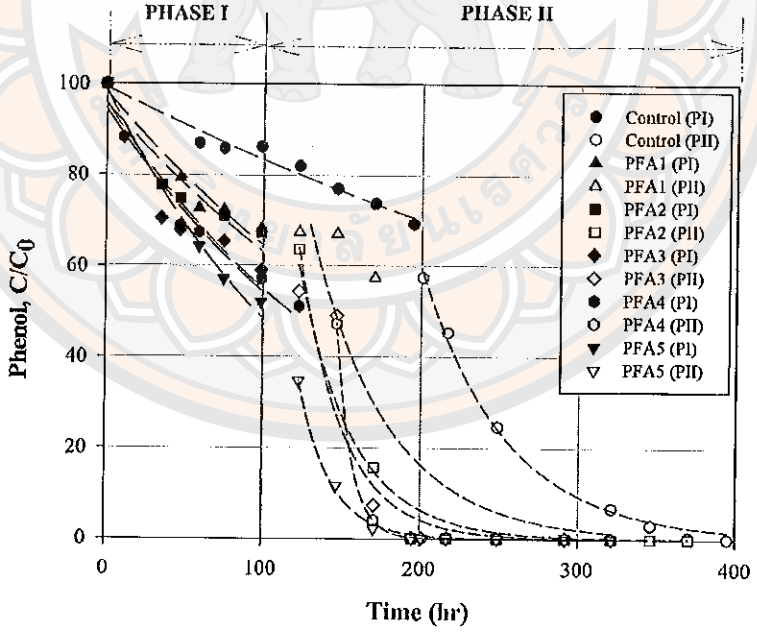


Figure 34 Phenol degradation kinetics of (a) the aerated experiments with vetiver plantlets (PB), (b) the aerated experiments with mature vetiver plants (PFA) and (c) the non-aerated experiments with mature vetiver plants (PF)

(c)

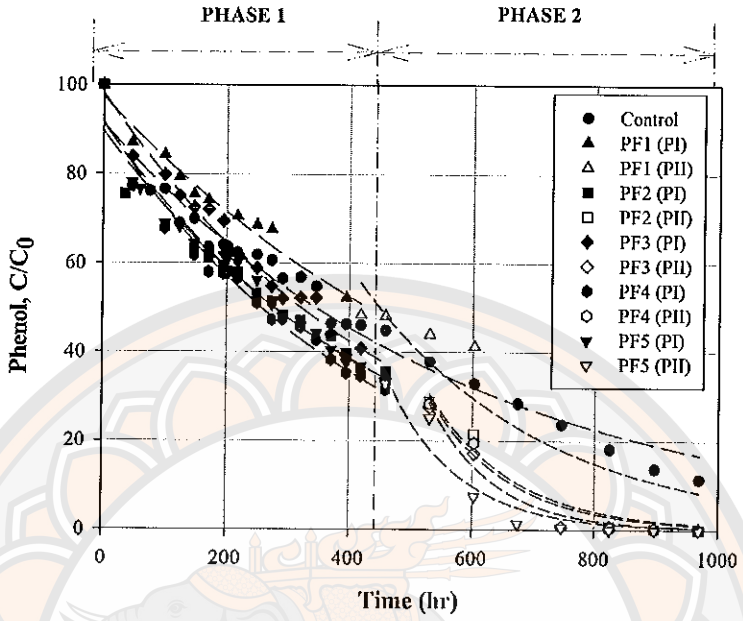


Figure 34 (cont.)

Table 12 Phenol degradation kinetics fitting for the vetiver plantlet on platforms with aeration base on the two-phase mechanisms

Experiment	Density of vetiver grass in platform	Phase 1			Phase 2			Enhance degradation rate (Time)
		R^2	Concentration Range (mg/L)	k ($x10^{-3}$/hr)	R^2	Concentration Range (mg/L)	k ($x10^{-3}$/hr)	
Control, PB	0	0.9180	565-0.1	1.00±0.10	-	-	-	
PB1	20	0.8685	550-120	1.40±0.20	0.9711	120-0.1	3.20±0.50	2.29
PB2	40	0.9855	530-90	2.40±0.20	0.9976	90-0.1	11.40±0.70	4.75
PB3	60	0.9745	533-117	2.20±0.10	0.9912	117-0.1	14.60±2.40	6.64
PB4	80	0.9462	537-104	2.50±0.20	0.995	104-0.1	10.10±0.80	4.04
PB5	100	0.9656	553-83	3.30±0.20	0.997	83-0.1	9.97±0.50	3.02

Table 13 Phenol degradation kinetics fitting for mature vetiver plant on platforms with aeration base on the two-phase mechanisms

Experiment	Density of vetiver grass in platform	Phase 1			Phase 2			Enhance degradation rate (Time)
		R^2	Concentration Range (mg/L)	k ($x10^{-3}$/hr)	R^2	Concentration Range (mg/L)	k ($x10^{-3}$/hr)	
Control, PFA	0	0.9571	520 - 385	2.15±0.21	0.911	385 - 1	26±0.28	13
PFA1	20	0.9460	540 - 390	4.00±0.00	0.7568	390 - 0.7	40.5±0.71	9.0
PFA2	40	0.8949	540 - 384	4.00±0.14	0.8149	384 - 0.7	40.5±0.98	8.2
PFA3	60	0.8329	562 - 368	5.50±0.70	0.7826	368 - 0.7	41.00±0.70	6.8
PFA4	80	0.9720	567 - 382	6.50±0.70	0.7744	364 - 0.7	40.00±0.00	5.7
PFA5	100	0.9950	588 - 336	6.50±0.70	0.9339	336 - 0.1	53.00±0.70	7.3

Table 14 Phenol degradation kinetics fitting for mature vetiver plant platforms without aeration base on the two-phase mechanism

Experiment	Density of vetiver grass in platform	Phase 1			Phase 2			Enhance degradation rate (Time)
		R^2	Concentration Range (mg/L)	k ($x10^{-3}$/hr)	R^2	Concentration Range (mg/L)	k ($x10^{-3}$/hr)	
Control, PF	0	0.9689	576 - 65.6	2.10±0.14	-	-	-	-
PF1	20	0.9816	519 - 250	1.50±0.70	0.9837	250 - 0.3	12.5±0.71	6.5
PF2	40	0.9702	585 - 208	2.00±0.00	0.9816	208 - 0.9	17.00±0.00	7.0
PF3	60	0.9729	544 - 185	2.05±0.70	0.9760	185 - 0.75	15.5±0.70	8.0
PF4	80	0.9804	593 - 187	2.10±0.14	0.9895	187 - 0.85	14.25±0.35	6.5
PF5	100	0.9805	543 - 178	2.15±0.14	0.9912	178 - 0.45	20.35±0.49	10.0

Briefly, PI was influenced by phytochemicals. The kinetic degradation rate of phenol which obtained from PI was less than those from PII. Due to PII was influenced by the combination of PI and rhizo-microbial activity.

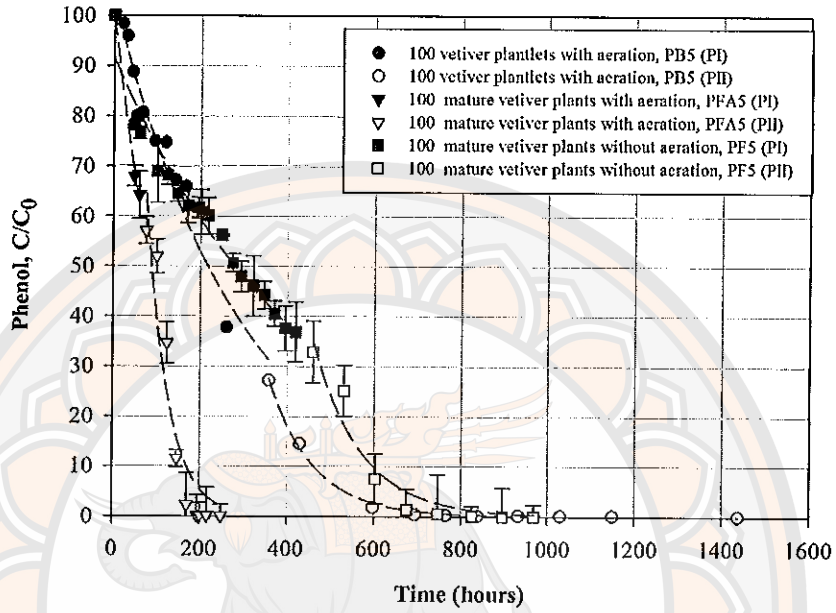


Figure 35 Phenol degradation kinetics of 100 vetiver plantlets and 100 mature vetiver plants with and without aeration base on the two-phase mechanism

Figure 35 summarizes the change in phenol concentration of PFA5 and PF5 bases on the two-phase mechanisms which agreed to our previous study PB. Phenol from PFA5 and PF5 were degraded to the MLC within 249 hr (PI=0-99 hr, PII= 100-249 hr) and 675 hr (PI=0-459 hr, PII= 460-675 hr) whereas, PB5 degraded phenol within 766 hr (PI=0-358 hr, PII= 359-766 hr). In the PI, $k_{PB5\text{-}PI}$, $k_{PFA5\text{-}PI}$ and $k_{PF5\text{-}PI}$ were $3.30\pm0.20\times10^{-3}$, $6.50\pm0.70\times10^{-3}$ and $2.15\pm0.14\times10^{-3}$ hr^{-1}, respectively and the PII, $k_{PB5\text{-}PII}$, $k_{PFA5\text{-}PII}$ and $k_{PF5\text{-}PII}$ were $9.97\pm0.50\times10^{-3}$, $53.00\pm0.70\times10^{-3}$ and $20.35\pm0.49\times10^{-3}$ hr^{-1}, respectively. At the same time, the overall k from PB5, PFA5 and PF5 were 13.27×10^{-3}, 59.50×10^{-3} and 22.50×10^{-3} hr^{-1}, respectively.

Here, we have shown that PFA5 was more successful compare with PF5 without aeration or PB5 with vetiver plantlets. These findings agreed to our experiments with difference number of vetiver grass (Figure 36 and 39).

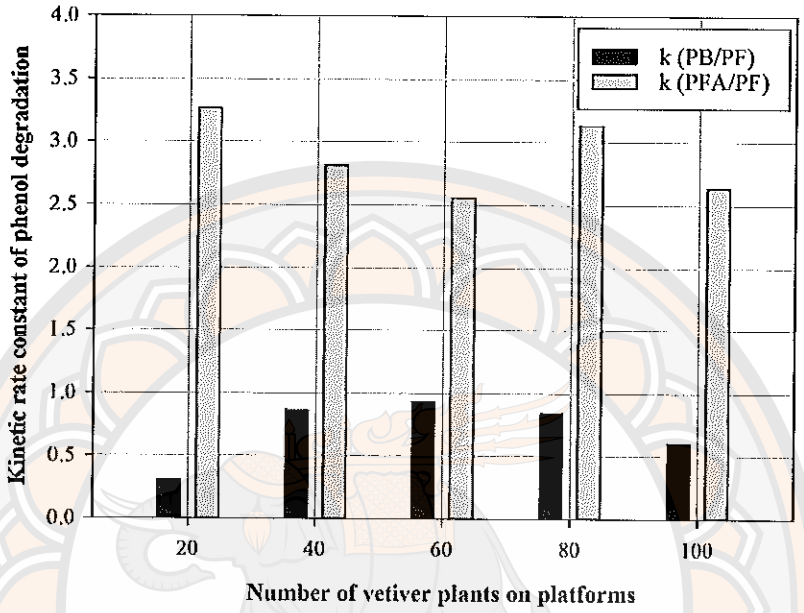


Figure 36 Enhancement of the overall phenol degradation rate constants due to the mature of vetiver plants and the air supply in the same number of vetiver plants (PB/PF and PFA/PF)

Table 15 Phenol degradation kinetics fitting comparison for PB5, PFA5 and PF5 base on the two-phase mechanisms

Experiment	Phase I			Phase II			Enhance degradation rate (Time)
	R^2	Concentration Range (mg/L)	k (x10^{-3}/hr.)	R^2	Concentration Range (mg/L)	k (x10^{-3}/hr.)	
PB5	0.9656	553 - 83	3.30±0.20	0.9970	83 - 0.1	9.97±0.50	3.02
PFA5	0.9950	588 - 336	6.50±0.70	0.9339	336 - 0.1	53.00±0.70	8.15
PF5	0.9805	543 - 178	2.15±0.14	0.9912	178 - 0.45	20.35±0.49	9.46

The maturity and air supplement were considered base on the two-phase mechanisms. We found that the kinetic rate constant of PB5 to PF5 (k_{PB5}/k_{PF5}) was less than 1 (0.6) hence, the maturity is more important despite, air was supplied for the vetiver plantlets. While, with the same maturity of vetiver root, air supply accelerated the kinetic rate constant of PFA5 to PF5 (k_{PFA5}/k_{PF5}) as the ratio of 2.6. These results present that the maturity and air supplement made a great effect on the phenol degradation. The assumptions established that the mature vetiver roots with aeration was capable to releasing root exudates and driving the phenol degradation through self-protective mechanism. Particularly, the stronger root provided sufficient nutrients and habitation for aerobic microorganisms to forming themselves as biofilm. Nutrients and plant defense compounds are also released into the local environment influenced planktonic bacterial colonization. Whereas, bacteria in biofilm matrices have been found to induce plant growth and to protect plants from phyto-pathogens (a process termed biocontrol), others might involve in pathogenesis. In addition, they have the ability to protect the cells from desiccation and other stresses (Bogino, Oliva, Sorroche, & Giordano, 2013; Danhorn, & Fuqua, 2007)

3. The maturity of vetiver grass leads to the phenol tolerance by releasing phytochemicals and essential enzymes.

The comparison between PFA5, PF5 and PB5 on phenol degradation, PFA5 and PF5 could degrade phenol beyond PB5 about 4.48 and 1.69 times greater indicated by kinetic rate constant. Therefore, with or without aeration, the physical strength of the root systems was concerned as the most important factor that affected the phenol degradation. These attentions of maturity are consistent with a study in wheat plant LU-26/*Ae.Cylindrical* with maturity tolerated to the salt stress at all salinity levels compared to others that were in early maturity (Farooq, & Azam, 2006). The study of the petroleum degradation using phytoremediation also showed that at maturity stage of *Sorghum bicolor L.*, the root systems were more fully developed and the rate of petroleum degradation paralleled the growth of plant roots (Banks, Kulakow, Schwab, Chen, & Rathbone, 2003). Even though, naphthalene, the recalcitrant hydrocarbon was adsorbed by alfalfa (*Medicago sativa*) root. It was found that adsorption increased by as much as a factor of four with later growth stage of the plants (Schwab, Al-Assi, & Banks, 1998).

The maturity of vetiver grass is important to the ROS generation in the root cells which involves with various enzymes in the response to environmental stresses. Normally, ROS, such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radicals (·OH), are an unavoidable consequence of aerobic metabolism. At low concentrations signaling effects of ROS appear to protect cellular survival and dominate over damage, and the reverse occurs at high ROS concentrations. Experimental data indicate that, in *Escherichia coli*, the respiratory chain can account for as much as 87% of the total H_2O_2 production (González-Flecha, & Demple, 1995). H_2O_2 is also generated intracellular and released under normal condition as well as wide range of stressful conditions (Leshem, & Levine, 2007; Tripathy, & Oelmüller, 2012). When exposure to exogenous oxidative stress such as toxic compounds in harsh environmental conditions, plants and bacteria induce the production of higher ROS than the normal condition and also accumulate ROS as the self-protective mechanism (Gambino, & Cappitelli, 2016; Kim, & Park, 2014; Liu et al., 2011; Yeom, Yeom, & Park, 2010). In plants, most of the H_2O_2 generated during the oxidative burst can be produced by peroxidases (Bestwick, Brown, Bennett, & Mansfield, 1997; Dat et al., 2000; Peng & Kuc, 1992). Several enzymes are found in the plant cells, together with small antioxidants help remove excessive ROS (Møller, 2001).

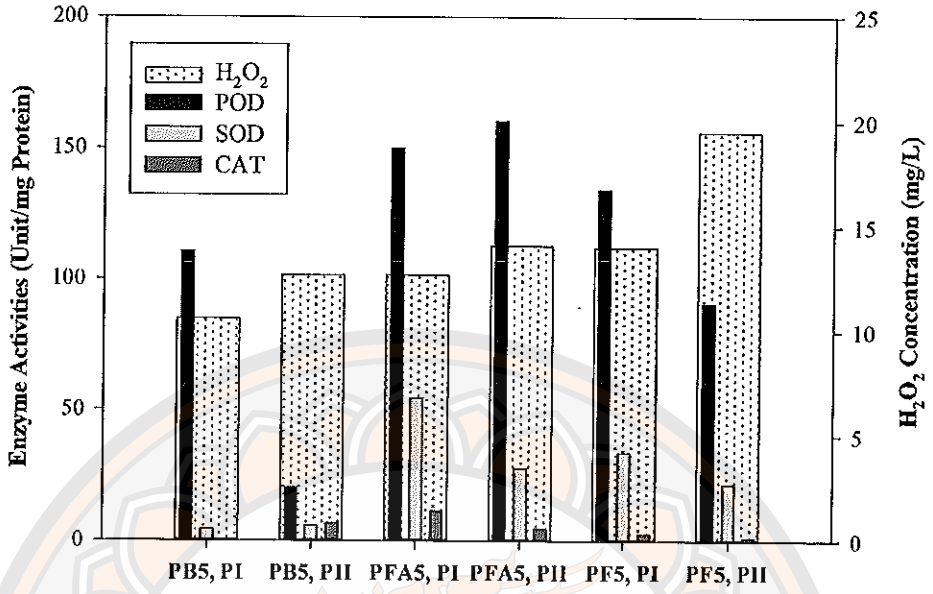


Figure 37 The H_2O_2 concentration and enzymes, POD, SOD and CAT in the root of the 100 vetiver plantlets (PB5), the 100 mature vetiver with aeration (PFA5) and the 100 mature vetiver root without aeration (PF5) as a function of phenol degradation phases (PI and PII)

As shown in Figure 37, PI of PFA5 and PF5, the finer root systems responded to the phenol toxicity and released the higher SOD of 54.6 and 33.6 U/mg protein and POD of 149.8 and 134.0 U/mg protein compared to PB5 that had SOD and POD of 4.2 and 110.4 U/mg protein, respectively. Superoxide ($O_2^{\bullet-}$) is the primary ROS formed in the cells which initiates a cascade of reactions to generate "secondary" ROS, either directly or prevalently through enzyme catalyzed processes. $O_2^{\bullet-}$ is a moderately reactive, short-lived ROS with a half-life. $O_2^{\bullet-}$ can transform to H_2O_2 either non-enzymatically or by SOD catalyzed reaction (Sharma et al., 2012). Maturity with and without aeration could influence to the phenol degradation as shown in the differences of SOD and POD levels, PI of PFA5 (0-99 hr) was shorter than PI of PF5 (0-419 hr) about 4 times. With the longer contact time to phenol toxicity, vetiver root had more suffer and responded by releasing low enzymes. The high level of $O_2^{\bullet-}$ elevated to the SOD level in PFA5 and PF5 which was transformed $O_2^{\bullet-}$ to H_2O_2, a

more stable ROS (Figure 37). could derive from both $_2O_2H$ $O_2^{\bullet-}$ transformation and the activities of microorganisms agreed to the fact that in PFA5 system had the highest microbial content compared to PB5 and PF5 (Figure 38).

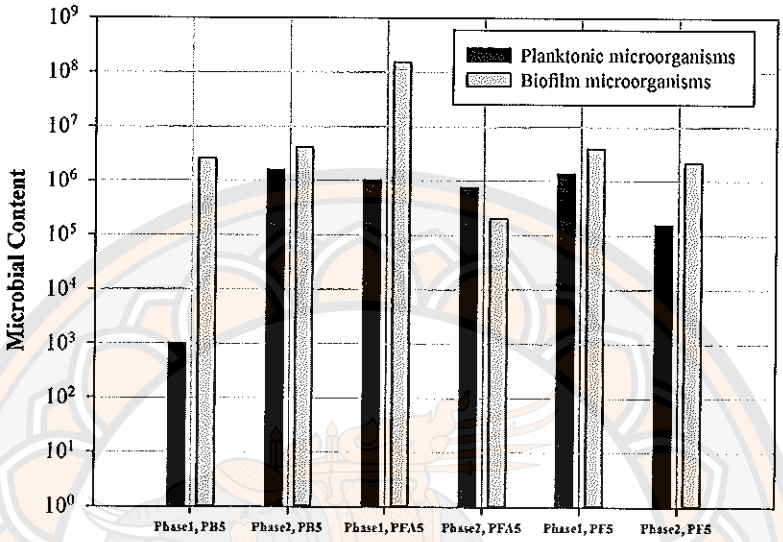


Figure 38 The total microbial count in wastewater and rhizosphere as a function of the degradation phases for the vetiver plantlets with aeration (PB5), the mature vetiver plants with aeration (PFA5) and the mature vetiver plants without aeration (PF5)

H_2O_2, the oxidant substrate of POD, reacts with phenol by breaking down phenol molecules to phenol radicals via phyto-oxidation then, phenol radicals couple themselves or other organic substrates to less harmful substances (Phenrat et al., 2016). The higher H_2O_2 and POD, the higher phenol degradation which could be proved by the rapid degradation rate of PFA5, PF5 and PB5. Although, PF5 had the similar level of H_2O_2 in PI but lower POD as co-enzyme lead to less reaction. So that, k_{PF5} was lower than k_{PFA5}.

In PII, the levels of SOD and POD could be seen on the similar pattern with PI ($SOD_{PFA5,\ PF5}$ and $POD_{PFA5,\ PF5}$ > SOD_{PB5} and POD_{PB5}) but SOD from PFA5 and PF5 dropped to 27.9 and 21.8 U/mg protein. Also, POD in PII of PF5 decreased to 90.6 U/mg protein. These attributed to the maturity of vetiver grass. In addition to the

increase of microbial contents, rhizo-microorganisms involved in phenol degradation in PII. The microbial involvement assisted the phenol degradation by consuming phenol as carbon source that was enhancing the greater degradation rate of PB5, PFA5 and PF5 to 3.02, 8.15 and 9.46 times, respectively. It is worth to consider that the POD of PFA5 in PII was increasing over those in PI. Due to the short time of degradation in PI, self-defense mechanisms could be firmly established on vetiver grass by inducible enzymes and ROS. It was possible that, vetiver root still imprinted with phenol toxicity which arose stress memory to deal with subsequent more severe stresses (Bruce, T. J. A., Matthes, Napier, & Pickett, 2007; Pandey, Irulappan, Bagavathiannan, & Senthil-Kumar, 2017). Hence, it was resulting in level of POD in PII. This assumption was also supported by Bin, & Jonathan (2000) who discovered that priming with sub-lethal oxidative stress (1.0 μM paraquat) induced increases in levels of six antioxidant related enzymes in the horseweed *Conyza bonariensis* which confer resistance to acute oxidant stress (Ye, & Gressel, 2000). Pre-treated plants exposed to 1.0 mM paraquat showed significantly enhanced recovery after 3 days.

CAT is critical for maintaining the redox balance during the oxidative stress (Sharma et al., 2012). While, the excessive production of H_2O_2 destroys the living cells. Enzyme CAT is the antioxidant enzyme that degrades H_2O_2 to water and oxygen. Environmental stresses can cause either enhancement or depletion of CAT activity, depending on the intensity, duration, and type of the stress. For example, in PI of PB5, the CAT level was hardly existing due to H_2O_2 was needed to be co-substrate in aggressive phyto-oxidation to degrade phenol. Shortly afterwards, H_2O_2 from the root of vetiver plantlet and the adaptable microorganisms, reached the excessive level and imbalanced with the decreasing of phenol in PII. It turned to be dangerous to the cells. The CAT was released slowly to balance the system by degrading H_2O_2. We also found this phenomenon from both PFA5 and PF5.

4. Aeration, by-product pathway and microbial characterization.

Generally, aerobic biological processes are conducted in the treatment of organic wastewaters for achieving high degree of treatment efficiency (Chan, Chong, Law, & Hassell, 2009). The expectation on phenol degradation using mature vetiver grass with aeration was initiated although, the inhibition on biological treatment by phenol and its derivatives were mentioned in many literatures (Ye Chen, Cheng, &

Creamer, 2008; Hill, & Robinson, 1975; Jones, Jansen, & McKay, 1973; Kurzbaum et al., 2010; Polcaro, Palmas, Renoldi, & Mascia, 1999).

The successful of aeration was presented by the kinetic degradation rate constant of PFA over PF. The ratio of PFA/PF indicated that aeration enhanced the degradation rate of 2.88 times averagely. The fluctuation of by-products from wastewater-containing phenol was indicated the difference between aeration and non-aeration systems.

In PFA5, the decrease of total COD(TCOD) from PI to PII was 2075.47 to 1,204.79 mg/L (41.95% efficiency removal) which agreed to the TCOD removal in aerobic treatment. However, the changes of the fractions of TCOD included dissolved COD(DCOD) which was presumably phenol and dissolved polyphenol (DPP) while, particulate COD(PCOD) was PPP and POM could imply to the pathways of by-products. Figure 39 proposed the pathways of by-products transformation.

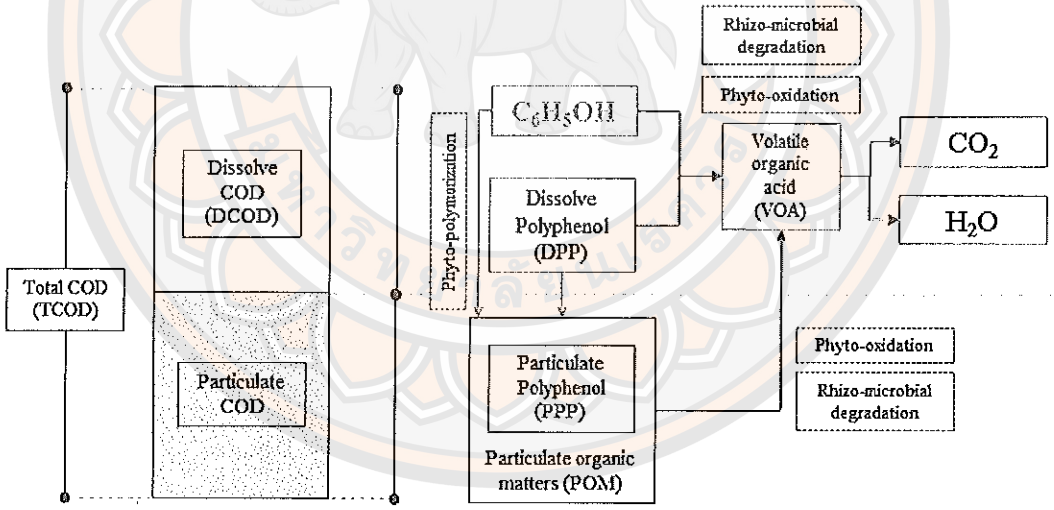


Figure 39 The proposed model of the by- products pathway via the transformation processes

The decrease of TCOD ($k_{TCOD\text{-}PFA5}$=0.004 hr^{-1}) in PI to PII was the result from the DCOD reduction (1,207.55 to 306.03 mg/L, k_{DPP}=0.004 hr^{-1}) (Table 16, Figure 40).

Table 16 The fraction of COD concentration of PB5, PFA5 and PF5 base on the two-phase mechanism

Experiment	Phase I			Phase II		
	PCOD (mg/L)	DCOD (mg/L)	TCOD (mg/L)	PCOD (mg/L)	DCOD (mg/L)	TCOD (mg/L)
PB5	1,284.28	289.30	1,573.58	315.94	85.56	401.49
PFA5	867.92	1,207.55	2,075.47	898.76	306.03	1,204.79
PF5	629.77	1,167.54	1,797.30	2,655.54	332.23	2,987.76

(a)

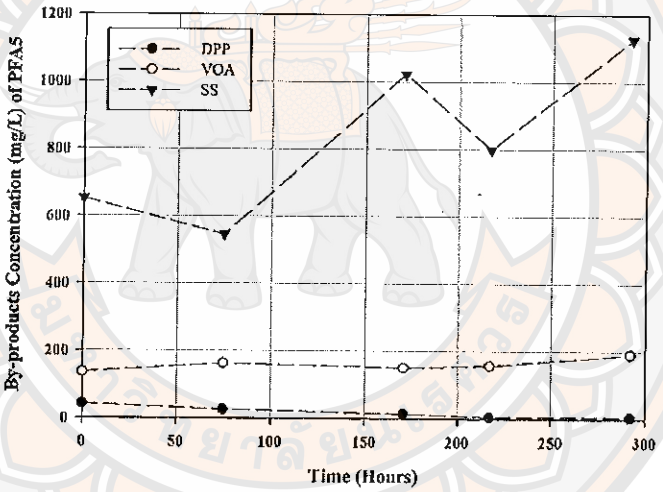


Figure 40 The transformation of by-products in the function of DPP, VOA and SS in (a) the system with aeration, PFA5 and (b) the system without aeration, PF5

(b)

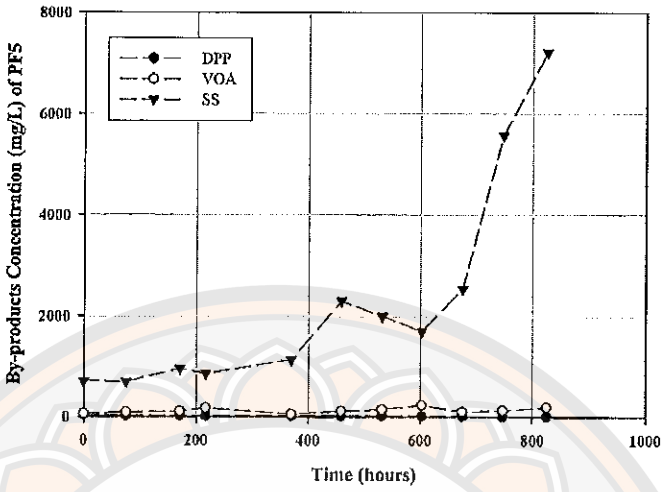


Figure 40 (cont.)

DCOD by phenol was degraded as the substrate in aerobic microbial degradation and phyto-oxidation. Then, the phenol radicals coupled with themselves to form DPP via enzymatic phyto-polymerization (Kobayashi, Uyama, & Kimura, 2001). At this time, some adaptable microorganisms also consumed DPP resulting in DCOD reduction. On the other hand, the combination of DCOD and DPP with others or themselves (hydrolysable state to condensed state) might be continued to the increase of PCOD. However, some of PCOD could be degraded via the pathways as mentioned above with the complicate bonding of PPP and POM which were unfavorable for microorganisms. Therefore, it slightly increased in the fraction of PCOD. Similarly, phenol radicals could also transform to VOA directly via the *ortho-* and *meta-* pathways through the oxidation process and bacteria activities (Farrell, & Quilty, 1999; Karlsson, Ejlertsson, Nezirevic, & Svensson, 1999; Wu, & Zhou, 2001). Finally, VOA could be oxidized to H_2O and CO_2 as the end-products (Arora, & Bae, 2014; Hassanshahian, & Cappello, 2013; Pimentel, Oturan, Dezotti, & Oturan, 2008).

PF5 without aeration showed that the TCOD from PI to PII increased from 1,797.30 to 2,987.76 mg/L. It was similar trend of DCOD fraction in PF5 to PFA5. While, DCOD decreased but PCOD increased. There were two possible pathways. First, DCOD might not be degraded via oxidation to VOA or consumed by microorganisms but combining to PCOD supported by SS accumulation (Figure 40b).

Although the microbial contents were similar to the aerated system in both planktonic and biofilm forming state but, molds as aerobe fungi were not found even in the low phenol concentration (Figure 41c and 41d). It pointed to the fact that some fungi for example, *Fusarium sp., Rhizopus sp., Aspagillus sp.* and *Penicilium sp.* played important role in phenol degradation.

Second, some of phenol radicals converted to VOA which accumulated and could not oxidize to H_2O and CO_2 supported by the low levels of enzymes in PII (Figure 40b). Besides, the accumulation of VOA might refer to ethanol, a slightly weak acid regards to the VOA group. Ethanol is the by-product of anaerobic respiration. Thus, the high accumulation of VOA could be verified to anaerobic process which was less advanced than aerobic process (van Schie, & Young, 2000).

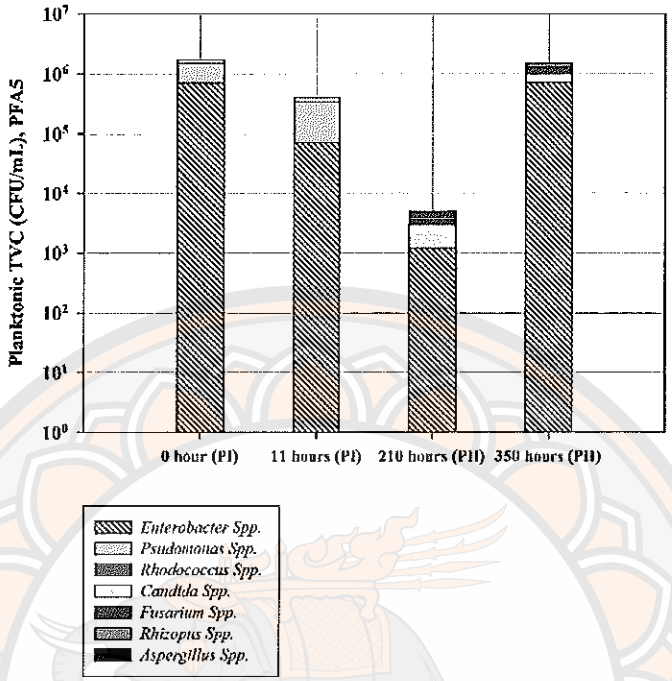
Both of possible pathways clearly refer to the important of aeration, oxygen from air supply involved in the generation of ROS as the oxidants to dealing with phenol toxicity (Bajaj, Gallert, & Winter, 2008; Collins, Foy, McHugh, Mahony, & O'Flaherty, 2005; Hussain et al., 2015; Nakhli et al., 2014). Particularly, the air circulation from diffusers allocated oxygen, nutrients and necessary supplements for vetiver hairy root cells to remain healthy and promote the growth of planktonic and biofilm microbes.

5. The microorganisms and their characteristics to the phenol degradation.

To consider the total viable count (TVC), we found that the PFA5 and PF5 had a greater number of TVC compared to the PB5 in the both PI and PII (Figure 48). It is reasonable to explain that microorganisms in PFA5 played their role since the PI of the phenol degradation.

At first, the root of mature vetiver had been covered by the biofilms which protected themselves, against the external stress. These biofilms were the first protective shelter from phenol toxicity damaging the cell of vetiver root (Doornbos, van Loon, & Bakker, 2012; Morikawa, 2006; Stottmeister, U. et al., 2003). While, PB5, the root of vetiver plantlets were not covered by the biofilms as much as PFA5 and PF5. The planktonic microorganisms were weaker, suffered and destroyed. Thereby, the root of vetiver plantlets were the first defender and took the responsibility to degrade phenol by releasing the phytochemicals without the assistance from microorganisms.

(a)



(b)

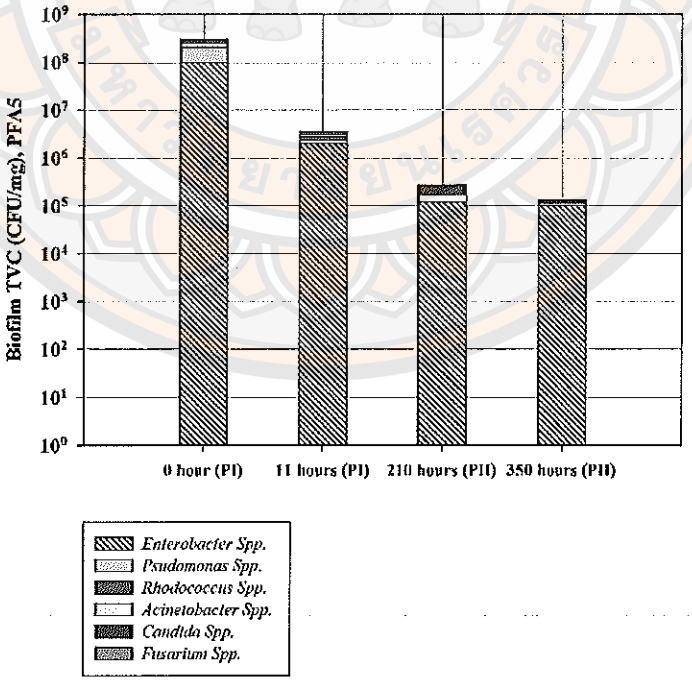
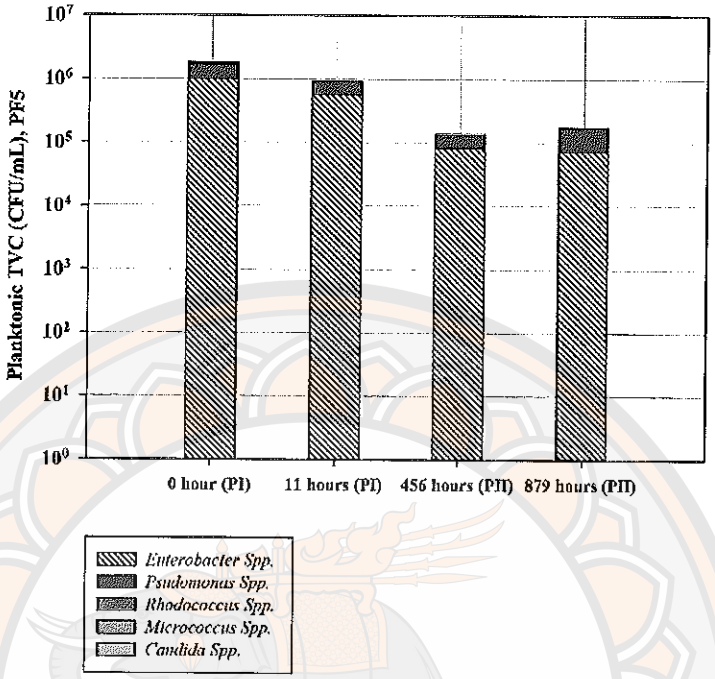


Figure 41 The acclimation and resistance of microbial communities to phenol toxicity in the experiments with the 100 mature vetiver plants

(c)



(d)

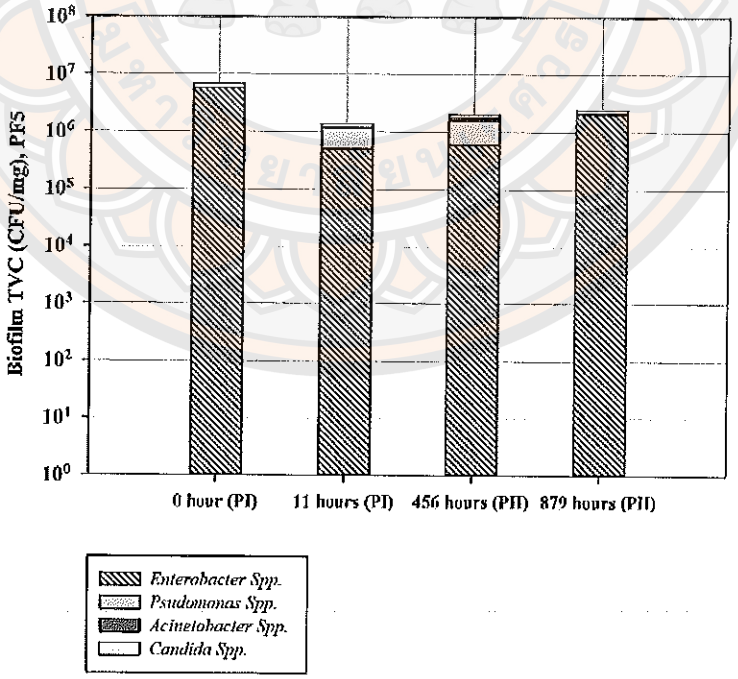


Figure 41 (cont.)

Note: (a) the planktonic state and (b) the adherent state as biofilms in the aeration systems, PFA5 and (c) the planktonic state and (d) the adherent state as biofilms in the non-aeration systems, PF5

In comparison between PFA5 and PB5 in case of the maturity, the planktonic microbes and biofilms were $1.71x10^6$ CFU/mL and $3.00x10^8$ CFU/g (Figure 41a and 41b) greater than those in PB5 more than 1,000 times since the early exposure to phenol. We also found that in PI, yeasts and molds (Y&M) of PFA5 were higher than the PB5 in 4 and 6 orders of magnitude for planktonic and biofilm forming states and it appeared 2 and 3 orders of magnitude for planktonic and biofilm forming states in PII. With the same aeration, mature vetiver root provided more root surface area which was habitation and nutrient sources for microorganisms compared to vetiver root plantlets.

To compare PFA5 and PF5 to evaluate the role of aeration, the planktonic microorganisms and biofilms of PF5 were $1.81x10^6$ CFU/mL and $6.72x10^6$ CFU/g which were less than PFA5 about 44.64 times in the term of biofilms (Figure 41c and 41d). This indicates that air supplement was a factor for the living indigenous microorganisms and encourages them to utilize more phenol as the carbon source. Especially, the aerated system provided sufficient oxygen to support the growth of plant root in the meantime, the root plants also enhanced the biofilms formation on and inside them by releasing the necessary supplements (U Stottmeister et al., 2003).

The various microbial strains were found as the phenol degraders in PFA5 included of *Candida* sp. ($1.8x10^3$ CFU/mL to $2.8x10^6$ CFU/mL as planktonic state), *Fusarium* sp. ($2.0x10^3$ CFU/mL to $3.5x10^6$ CFU/mL as planktonic state and up to $1.0x10^4$ CFU/g as biofilms), *Rhizopus* sp. (up to $1.3x10^6$ CFU/mL as planktonic state) and *Aspagillus* sp. (up to $3.0x10^4$ CFU/mL as planktonic state). Not only fungi could develop themselves but also some bacteria such as *Enterobactor* sp. ($1.2x10^4$ CFU/mL to $7.1x10^5$ CFU/mL as planktonic state and remained their living as biofilm on the roots) and *Psuedomonas* sp. (up to $3.0x10^3$ CFU/g as biofilms) (Figure 41a, 41b). It was implied that these indigenous bacteria and fungi had an effect on phenol degradation beyond others. They maintained defense mechanisms against oxidative stress throughout their evolution (Jang, I.-A., Kim, J., & Park, W., 2016). Agreed to

many researches who reported the potential of these microorganisms in phenol degradation (Adav, Chen, Lee, & Ren, 2007; Anselmo & Novais, 1984, 1992; Cai, Li, & Zhang, 2007; Knackmuss & Hellwig, 1978; Nawawi, 2014; Neumann et al., 2004; Powlowski, & Shingler, 1994; Sakai, Maeng, Tani, & Kato, 1994; V. L. Santos, & Linardi, 2004; Shourian et al., 2009; Spain, & Gibson, 1988; Thomas, Sarfaraz, Mishra, & Iyengar, 2002a; Yamaga, Washio, & Morikawa, 2010; Ying et al., 2007).

Interestingly, In PII of PFA5, planktonic microorganisms increased 3 orders of magnitude within 160 hr (at 210 hr to 370 hr, phenol concentration = 1.1 mg/L) while, biofilm on mature vetiver root surface had slightly decreased and remained stable. It might be caused by the decreasing of phenol toxicity therefore, they allow themselves to be suspended and form new colonies on other media (Morikawa, 2006). At the same time, some by-products from phenol degradation were PPP and POM, which suspended around. PPP and POM could be another carbon sources and habitations for the microorganisms. Planktonic microorganisms were attributed to the phenol degradation rather than biofilms in the low phenol concentration (Kurzbaum et al., 2010).

Conclusion

The mature vetiver plants in aeration system achieved the phenol degradation in illegal dumping wastewater with the rate of $58x10^{-3}$ hr^{-1} based on two-phase mechanisms. The factors enhanced the phenol degradation rate using vetiver grass were the maturity as well as the density of root system and aeration to promote the formation of biofilms. The mature vetiver root tolerated to the stress toxicity by releasing the phytochemicals included H_2O_2 and enzymes catalyzed reaction to degrade and transform phenol to other less harmful substrates via phyto-oxidation and phyto-polymerization. Also, indigenous microbial activities are important and concerned due to they have the high performance in phenol degradation by consuming phenol as carbon source. They formed themselves and covered on vetiver root surface as biofilms, the first defender exposed to phenol. These were resulting in the enhancement of rapid phenol degradation that can apply to the field-scale and other applications successfully.

CHAPTER V

PERMEABLE REACTIVE VETIVER ROOT BARRIER (PRVRB) FOR ABIOTIC AND BIOTIC PHENOL DEGRADATION: A LABORATORY-SCALED EVALUATION

This Chapter is based on the following manuscript currently prepare for academic publication.

Pimpawat Teeratitayangkul, Anukool Kietkwanboot, Oramas Suttinun and Tanapon Phenrat.

Introduction

The population increased rapidly coupled with fast industrialization leads to major environmental problems, including the production and improperly release of considerable amounts of toxic pollutants impact the environment (Danh et al., 2009; Max Roser, 2017). Studies indicate that a higher level of overall population and waste production are correlated with increased rates of illegal dumping of waste in developing countries (Henry, Yongsheng, & Jun, 2006; Khajuria, Yamamoto, & Morioka, 2010). Likewise, the illegal dumping waste was reported at Nong Nae Village, Chachoengsao province of Thailand in 2012, the dumping of phenol (C_6H_5OH)-contaminated wastewater was discovered. Phenol can transform to penta-chlorophenol which is pronouncing as carcinogenic by-product during drinking water production (Wetzel, Pan, Goodwin, & Wilkinson, 1983). Phenol and its derivatives pose the negative effect on environment and human health. As a result of the illegal dumping, shallow groundwater was contaminated with as much as 225 μg/L of phenol, while the acceptable maximum contamination level (MCL) of pentachlorophenol in drinking water is only 1 μg/L (US.EPA, 2009). The leakage of phenol form both accessible and inaccessible sources through groundwater and village's creek spread to agriculture ponds, groundwater wells and other water resources (Thaipublica, 2015a).

The sustainable solution was a challenge of public concern. Phytoremediation is proposed as one of the feasible, cost effective and environmentally friendly methods as well as it produces non-environmental disruption (Gerhardt et al., 2009; Ibáñez et al., 2012; Sheng et al., 2008). The use of vetiver grass as practical candidate plant for dealing with phenol contaminated shallow groundwater is an effective approach of remedial action. Accordingly, to eliminate phenol from abandoned water resources had been success by using vetiver plantlet platforms with the degradation rate of 9.97 $x10^{-3}$ hr^{-1} within 32 days. While, without the vetiver plantlets, phenol degradation by aerated microbial degradation alone may require 235 days. Removal mechanisms of phenol in wastewater by vetiver grass involves two phases: Phase I, phyto-polymerization and phyto-oxidation assisted by root-produced peroxide (H_2O_2) as oxidant to break the phenol bonds to phenol radicals and peroxidase (POD) as inducible enzyme. Phenol radicals which are not stable, couple with themselves or other substances to polyphenol. Followed by phase II, a combination of phase I with enhanced rhizomicrobial degradation involved in mechanism (Phenrat et al., 2016). In Murashige and Skoog's liquid medium under aseptic conditions, vetiver plantlets completely degraded phenol at the concentrations of 50 and 100 mg/L within 4 days moreover, they could degrade up to 70 and 89%, a high phenol concentration of 1000 and 200 mg/L without exogenous H_2O_2 (Singh et al., 2008).

Permeable reactive barrier (PRB) technology is regarded as the desirable low-cost technology providing on-site reactive treatment of contaminated groundwater and obtaining increased attention in the literatures (Yeh, Lin, & Wu, 2010). Many researchers reported the convincing combination of an underground permeable reactive barrier (PRB) and phytoremediation for example, PRB using *Panicum maximum* is distinctive features in remediation of municipal solid waste landfill leachate. The average organic removal was accomplished with efficiency of 68.5% COD 76.1% BOD and 73.5% total Kjeldahl nitrogen (Chiemchaisri, Chiemchaisri, & Witthayapirom, 2015). While, groundwater contaminated with TCE was treated with PRB constructed with composted plant mulch (shredded tree mulch, cotton gin trash, and river sand) as biowalls. The mean residence time of groundwater in the experiment was 17 days. Over 793 days of operation, TCE concentrations in the effluents varied from 0.1% to 2% of the influents. The results showed that both biotic

and abiotic processes played an important role in removing TCE in a biowall (Shen, & Wilson, 2007). Therefore, the performance of these barriers are expected to reduce the phenol concentration.

The underground permeable reactive vetiver root barriers (PRVRB) are applied to intercept phenol and its toxic derivatives from accessible (Figure 42a) and inaccessible (Figure 42b) source zones which are illegal dumping sources before moving through shallow groundwater and contaminated to consumption wells and other water resources. To our knowledge, this is the first that systematically presents the application of PRB and phytoremediation using vetiver grass in phenol degradation in shallow groundwater.

(a)

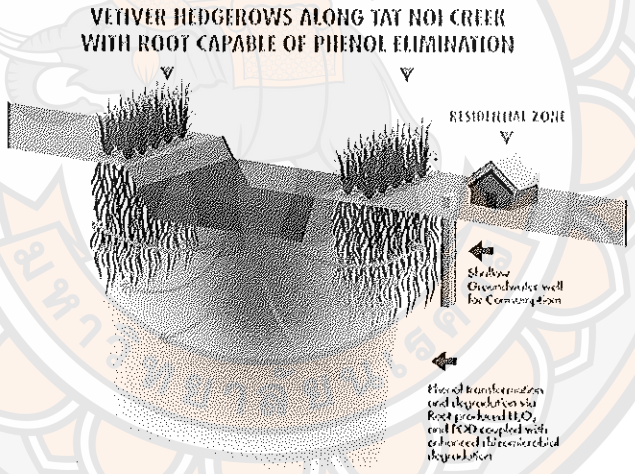


Figure 42 Conceptual model of permeable reactive vetiver root barrier (PRVRB) used for shallow groundwater treatment

(b)

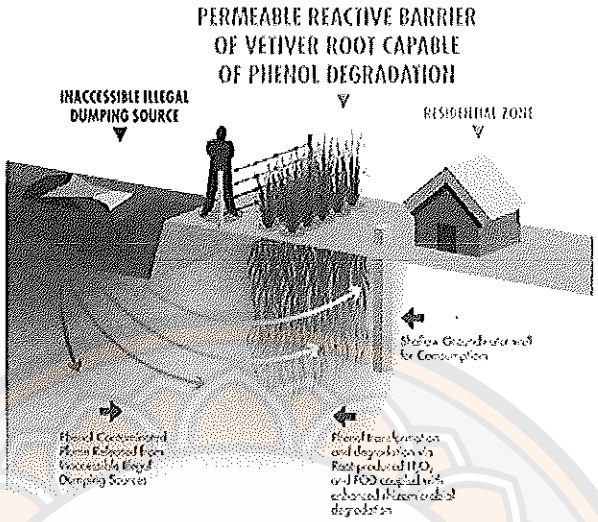


Figure 42 (cont.)

Note: (a) Vetiver hedgerows with PRB along contaminated natural creek to decrease contaminant migration to the shallow groundwater system, and (b) deep PRVRB to intercept contaminated shallow groundwater plume from the inaccessible sources

With PRVRB, root exudates released from vetiver root benefit to microorganisms at the root surface and in soil. The symbiotic relationship between bacteria and vetiver root is an interesting prospect to improve the efficiency of phytoremediation in phenol degradation. Naturally, the indigenous microbes might be delayed and nonspecific to phenol degradation due to the satisfying environment. On the contrary, some specifics can metabolize phenol as carbon source and show tolerance to unfavorable environmental conditions (Hussain et al., 2015; Kurzbaum et al., 2010; Phenrat et al., 2016; Shibata et al., 2006). To enhance the performance of PRVRB, the successful phenol degraders are isolated from our previous study. *Psuedomonas* sp., *Acinetobactor* sp. and *Enterobactor* sp. were pronounced in corresponding to enhances the phenol degradation in phenol-contaminated wastewater with the rate of 9.97 $x10^{-3}$ hr^{-1} and $58x10^{-3}$ hr^{-1} in the plantlet and biofilm state that were forming on the vetiver root surfaces (Phenrat et al., 2016). They were expected on bioaugmentation to form static

biofilms on PRVRB to increase the performance of systems. Thereby, this study was conducted to elucidate 1) the phenol degradation rate of contaminated groundwater in PRVRBs with and without bioaugmentation and 2) the optimal HRT of groundwater in phenol removal. Also, the performance of the selected phenol degraders was presented. The success of this study is applied for a sustainable in situ remediation of contaminated shallow groundwater with the combination of plant and its rhizosphere microorganisms.

Experimental Setup

1. Phenol and vetiver grass

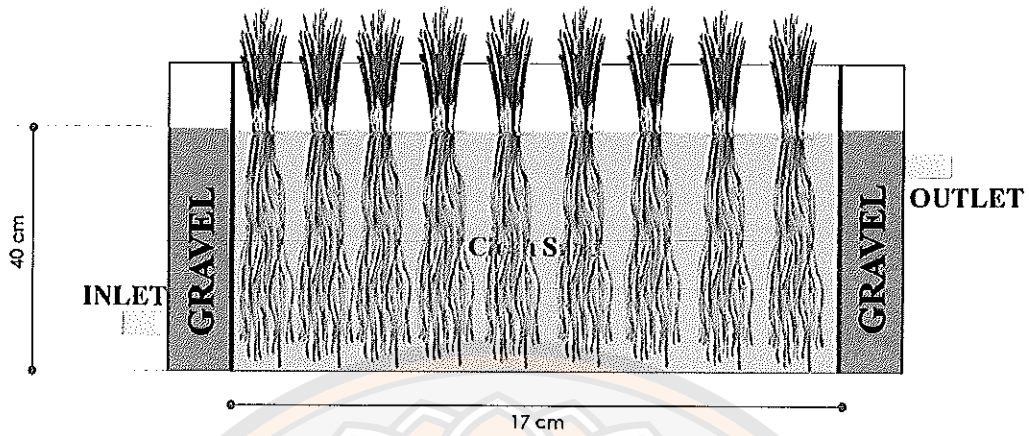
Phenol (analytical reagent grade, 99% purified) was purchased from Panreac (Barcelona, Spain). The phenol-contaminated water was purified 500 mg/ of phenol in DI water. This was the highest concentration of phenol first found at Nong Nae's illegal dumping incident.

Vetiver grass, Songkhla 3 ecotype (*Vetiveria zizanioides* (L.) Nash), from Chachoengsao Land development station was used in this study. Prior to the experiment, vetiver grass was fully-grown by the age of around 2 months with the root length of 20 cm.

2. Flow-through reactor for permeable reactive vetiver root barrier (PRVRB)

The two rectangular acrylic reactors with a size of 20 x 25 x28 cm (width x height x length) were packed with sand and used as flow-through reactors for the PRVRB. The clean sand had the median grain size of 0.075 mm. to 4.75 mm. The pore volume of the packed bed was 16.575 L The PRVRB reactor was planted by 80 of fully grown vetiver grass as shown in figure 43. In this PRVRB, all the vetiver grasses were planted using the massive root distribution as the barriers. On another hand, the unplanted reactor was set as control experiment consisted of only clean sand to evaluate the rate of phenol degradation without the vetiver grass.

(a)



(b)

Figure 43 Schematic of PRVRB experiment and an actual experimental setup

Note: (a) Schematic of PRVRB experiment and (b) an actual experimental setup.

3. Systems setup

After a week of planting the 80 vetiver grasses in PRVRB, the roots grew fast and longer to the bottom of reactor so, the phenol influent was fed into the inlet systems, both unplanted and PRVRB reactors, by the peristatic pump continuously. Phenol-contaminated water was introduced to the unplanted and the PRVRB systems to evaluate the treatment efficiency of unplanted and PRVRB systems with their indigenous microorganisms as well as to identify the optimal HRT. The seepage

velocities of 30 cm/d followed by 15 cm/d and 7 cm/d presented the hydraulic retention time (HRT) of 70, 140 and 300 hours, respectively. After flowing through the reactor, the effluent of phenol-contaminated water was collected at the outlet.

Phenol concentration in the effluent as a function of time was measured. An analytical solution of the advection-dispersion reaction (ADR) (e.g. Freeze and Cherry, 1979 (Freeze, 1979); Rungel, 1996 (Runkel, 1996)) transport phenomenon was employed to model the breakthrough curve and to determine the degradation rate constant of phenol.

$$C(x,t) = \left(\frac{C_0}{2}\right) \cdot erfc\left(\frac{x-vt}{2\sqrt{Dt}}\right) \cdot e^{-kt} \qquad \text{(Eq 5.1)}$$

Where C_0 is the concentration of chemical (mg/L), x is the distance from injection point (cm), v is the seepage velocity (cm/d), t is the time elapsed since injection (d), D is dispersion coefficient, erfc(x) is the complementary error function and k is a first-order decay rate ($time^{-1}$).

The phenol degradation rate constants were examined as a function of HRT for different experimental setup. The optimal HRT with the best treatment efficiency was identified and further used to study the role of bioaugmentation on PRVRB performance as to be discussed next. After all experiments achieved the steady state (%RSD less than 20), the samples of sand and vetiver root of all experiments were sampled for microbial analysis.

4. Microorganisms and inoculum preparation for bioaugmentation in PRVRB

As mentioned above, the optimal HRT with the best treatment efficiency was identified and further used to study the role of bioaugmentation on PRVRB performance The most effective microbial strains for phenol degradation were selected and isolated from our previous study (Phenrat et al., 2016). They included *Psuedomonas* sp., *Acinetobactor* sp.and *Enterobactor* sp. These isolates grew well in wastewater containing high concentration of phenol. They were capable of using phenol as carbon source and could adapt themselves to survive in toxic environments. These abilities had also been reported by many researchers (Neumann et al., 2004;

Shourian et al., 2009; Thomas, Sarfaraz, Mishra, & Iyengar, 2002b; Varma, & Gaikwad, 2008; Xin, Qu, Yuan, & Jing, 2011).

To prepare microbial inoculum, we prepared each strain by the method in Chapter 3, subtopic 3.3. Mixed microorganism cells were grown around $1.3x10^8$ CFU/mL in sterile saline diluent using the evaluation of 1.5 McFarland method (Washington, Ii, & Karlson, 1972) with an absorbance reading at 600 nm.

Sand media in an unplanted system was washed several time while, vetiver root was surface sterilized by repeated washes in sterile distilled water before culture on clean sand. After fresh preparation, 16 L of mix cultures consisted of *Psuedomonas* sp., *Acinetobactor* sp.and *Enterobactor* sp. were applied to both unplanted and PRVRB reactors. It was left for a static biofilm formation on the media surfaces around 3 weeks. To verify microbial biofilm formation, the sand and vetiver root samples were randomly harvested and counted for microbial attachment before experiments started.

5. Microbial analysis

The number of indigenous and augmented phenol degraders was determined by plate count technic after they were extracted from the sand and vetiver roots (Appendix C). The plates were incubated at 31 °C for 3 days. Subsequently, the microbial and fungi colony was counted as CFU/mL. Pure culture of some microbes were prepared for bioaugmentation.

5.1 Pure microbial harvesting

Cells suspended from a viable colony of bacteria cells cultured in nutrient broth at 30°C to an exponential bacteria growth stage were harvested, washed and batched before further use as inoculum for seeds/soil. To harvest bacteria biomass from broth media, the cultures are centrifuged at 3,000 rpm for 15 min in a Falcon 6/300 centrifuge. Harvested cells from each of the centrifuge tubes were batched together and washed 3 times. The first washing process involved re-suspending and stirring (with a sterile magnetic stirrer) the batched cells in sterile deionized water for 5 min. The bacteria-sterile water suspension is then centrifuged, batched and then washed once 0.85% NaCl electrolyte and one more time in deionized water to complete the 3 washing cycles. The washing process was done to ensure harvested

bacteria cells are free from contaminated minerals and other nutrients that are present in the media in which they were cultured.

McFarland Standards are used to standardize the approximate number of bacteria in a liquid suspension by comparing the turbidity of the suspension (McFarland, 1907; Washington et al., 1972). A chemical solution of barium chloride and sulfuric acid contribute the reaction between these two chemicals results in the production of a fine precipitate, barium sulfate. When shaken well, the turbidity of a McFarland Standard is visually comparable to a bacterial suspension of known concentration. The accuracy of the density of McFarland Standards can be checked using a spectrophotometer with a 1 cm light path cuvette; a McFarland Standard has an absorbance reading at 600 nm.

5.2 PCR – DGGE technique

The microbial community and diversity analysis was performed by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) technic. The microbial community and diversity analysis was performed by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) technique. DGGE is a widely used fingerprinting method for detection of the bacterial population and the diversity in a sample. Initially, DNA extraction from the soil was done using NucleoSpin™ DNA Trace kit (Macherey-Nagel, Germany) following the manufacturer instruction. PCR amplification of the bacterial 16S rRNA genes was performed with primer pairs, forward primer 341F 5'-CCT ACG GGA GGC AGC AG-3' and reverse primer 534R 5'-ATT ACC GCG GCT GCT GG-3' Meanwhile, the fungal and yeast 18S rRNA genes was determined with forward primer 5.8s 5'-CGC TGC GTT CTT CAT CG-3'and reverse primer ITS1f 5'-TCC GTA GGT GAA CCT GCG G-3' as previously described by Watanabe et al. (Watanabe, Kodama, & Harayama, 2001) and Fierer et al. (Fierer, Jackson, Vilgalys, & Jackson, 2005). The PCR reaction mixture contained each forward and reverse primer 1 μL at 25 nmol, DNA-template 3 μL, and finally 15 μL Taq polymerase.

The PCR conditions consisted of an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, the annealing temperature at 55°C for 30 seconds, and a final extension at 72°C for 1 minute. The PCR products were analyzed by 1.5% agar rose gel subjected to DGGE analysis. DGGE was

performed using polyacrylamide gel (8% of a 37: 1 acrylamide-bis-acrylamide mixture in a tris acetate EDTA (TAE) 1x buffer, 0.75 mm thick, and 16 × 10 cm) with a 30% to 70% denaturant gradient. Gels were pre-run 20 minutes at 60 V and run continuously 10 hours at 100 V in TAE 1x buffer at 60°C in DGGE System (Cleaver Scientific, United Kingdom). After 10 hours of electrophoresis, the gel was stained with ethylium bromide and identified under UV light. The DGGE bands were excised from the gels using a sterile scalpel and eluted in 30 µL water at 4°C for 36 hours. The DNA eluted from DGGE bands was amplified using primer pairs, 341F and 534R and 5.8S and ITS1f (without the GC-clamp). The 16S and 18S rRNA gene fragment clones were sequenced by the company U2Bio (Seoul, South Korea). The band sequences obtained were manually proof-read and corrected. Sequences were compared with the GenBank database by using the Nucleotide Blast (NCBI Resource, 2016). The BioEdit program (Hall, 1999) was applied to edit, assemble and align all of 16S rRNA gene sequences including the reference sequences obtained from GenBank database.

6. Analysis

Concentrations of phenols in soluble samples from all experiments were measured base on the 4-amino-antipyrine Standard Method with a Spectroquant® Move 100 mobile colorimeter (Merck KGaA, Darmstadt, Germany). Chemical oxygen demand (COD) was measured by the closed reflux method, Standard Method 5220C. The degradation of phenol using vetiver root in phenol-containing wastewater degraded phenol transform to other product include of polyphenol as lignin and tannin which are by-product from enzymatic polymerization (Anupama & Hampannavar, 2012; Uyama, & Kobayashi, 2002) were estimated by Standard Method 5550 and volatile organic acid (VOA) as acetic acid, the final product in the pathway of phenol oxidation (Santos, Yustos, Quintanilla, Rodríguez, & García-Ochoa, 2002) was carried out by Standard Methods 5560. In addition, existing of the H_2O_2 in wastewater was also measured by allowing it to react with a titanium sulfate reagent to produce pertitanic acid, which is yellow in color and using wavelength of spectrometer 400 nm (Lei Wang, & Xuanzhen Jiang, 2009). The descriptions were also described on Appendix B.

Results and discussions

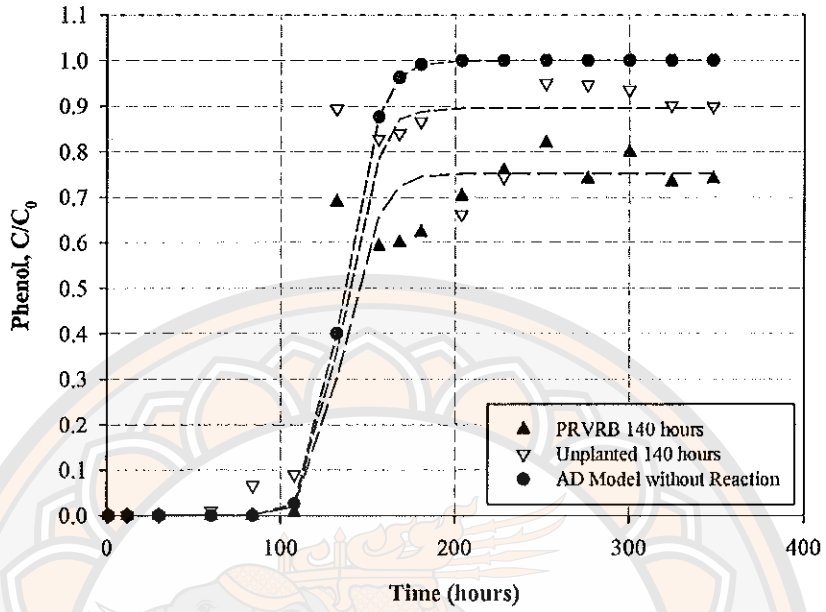
1. Phenol removal by Unplanted Systems

Figure 44 illustrates the breakthrough curves of the unplanted systems with HRT of 70, 140, and 300 hr. Phenol concentration in the effluent breakthrough at 150, 200 and 400 hr, respectively, for the experiments with HRT of 70, 140, 300 hr. Similarly, the conservative AD model suggests that phenol should be breakthrough at 150 and 100 with and without retardation (the retardation factor is 1.6). The increase of phenol concentration from 45 hr to 99 hr represents the dispersion front while the level of phenol concentration at plateau represents phenol removal efficiency. The unplanted experiments with HRT of 70 hr was incapable to degrade phenol as can be the effluent phenol concentration was similar to the conservative breakthrough. On the other hand, the breakthrough curves of unplanted systems with HRT of 140 and 300 hr show the phenol removal efficiency of around 10.41 and 36.20%, respectively. Using Eq.1, the degradation rate constants of phenol for HRT of 140 and 300 hr are 3.66×10^{-4} and 1.34×10^{-3} hr^{-1} (Table 17). Noticeably, the degradation rate constants of phenol at HRT of 300 hr is five times greater than that at the HRT of 140.

Table 17 Phenol degradation kinetics fitting and removal efficiency for the unplanted and PRVRB experiments with the flowrates of 70, 140, 300 hr and 300 hr with bioaugmentation

k (hr^{-1})	HRT (hours)		
	70	140	300
Unplanted	ND	3.66×10^{-4}	1.34×10^{-3}
PRVRB	ND	9.49×10^{-4}	3.07×10^{-3}
Bioaugmented Unplanted	ND	ND	2.12×10^{-3}
Bioaugmented PRVRB	ND	ND	5.04×10^{-3}
Efficiency (%)	HRT (hours)		
	70	140	300
Unplanted	ND	10.41	36.20
PRVRB	ND	24.79	64.31
Bioaugmented Unplanted	ND	ND	65.58
Bioaugmented PRVRB	ND	ND	91.08

(a)



(b)

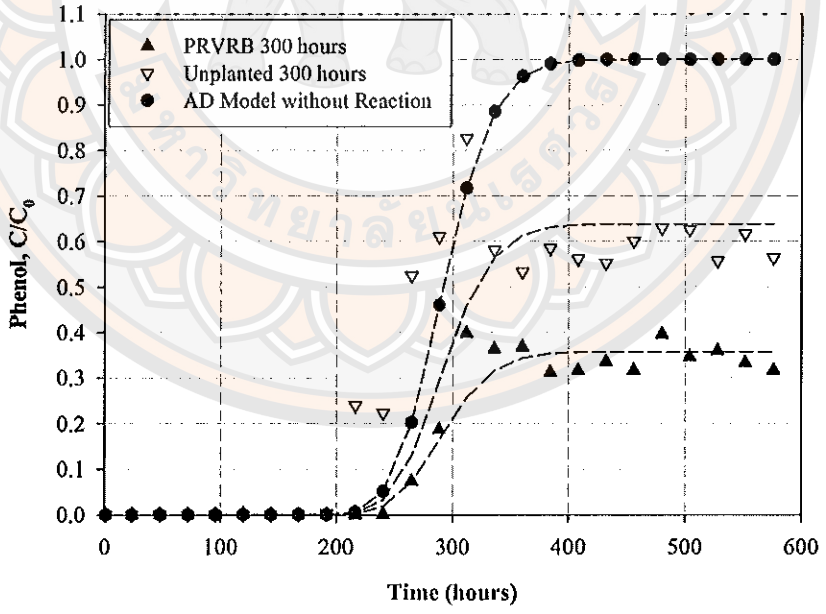


Figure 44 Advection-Dispersion reaction of continuous effluent of phenol-containing wastewater in the unplanted and the PRVRB systems

(c)

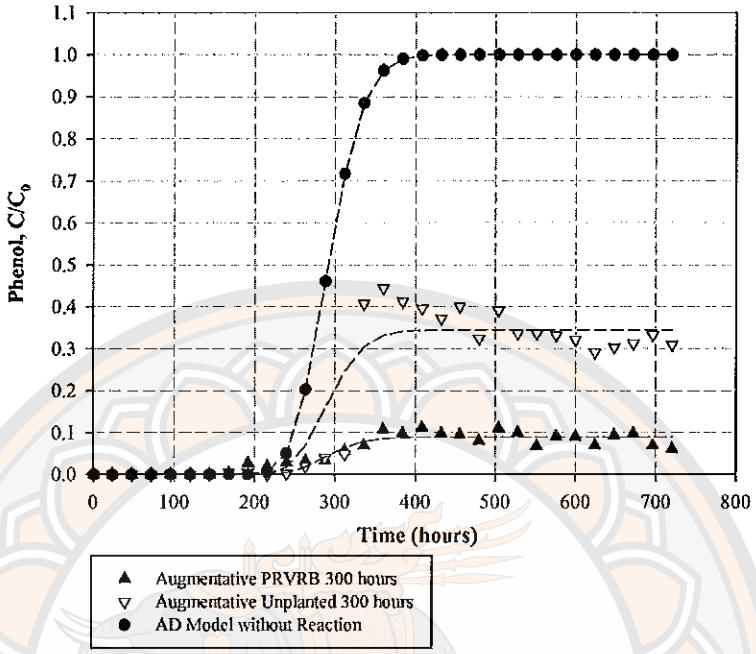


Figure 44 (cont.)

Note: (a) 140 hr, (b) 300 hr and (c) 300 hr with bioaugmentation

The significant decrease of phenol concentration even without vetiver mechanistically involves both abiotic and biotic processes. Abiotic removal by retardation (i.e. adsorption onto packed sand) was expected to play a substantial role (Dan, Fujii, Soda, Machimura, & Ike, 2017). By fitting the phenol breakthrough curve of the unplanted condition in this study, we yielded a retardation factor of 1.6. With this retardation factor, the sand-water partitioning coefficient (k_d) was calculated to be 0.096. This is similar to the study on Asti2 soil which consisted of 82% sand and 8% silt that presented the sorption of phenol, k_d of 0.0864 (Fiore & Zanetti, 2009). Considering Asti 2 soil, the organic carbon content is extremely low and adsorption becomes the main interaction mechanism.

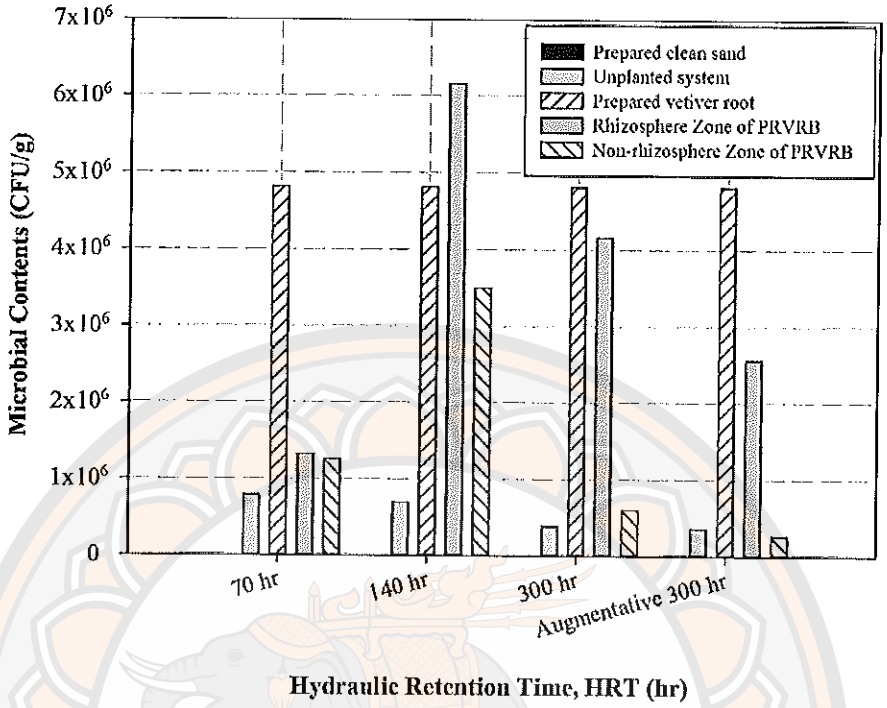


Figure 45 The microbial content of the samples from sand and vetiver root of the PRVRB systems with HRT of 70, 140, 300 hr and 300 hr with bioaugmentation

Nevertheless, the major phenol removal mechanism is supposed to be biotic degradation (Dan et al., 2017). Aerobic microorganisms may exist in sand or water but at their dormant state. Gradually, they will acclimate themselves to survive the in unplanted systems and participated in the phenol degradation process (Welz, Ramond, Cowan, & Burton, 2012). This hypothesis is supported by the number of bacterial count at the unplanted systems in Figure 45. For example, the clean sand collected before the experiments started had the microorganism amounts of 75 CFU/g. Then toward the end of the experiment, the microbial population was substantially increased, i.e. the microbial amount of$7.94x10^5$, $6.90x10^5$ and $3.80x10^5$ CFU/g for the HRT of 70, 140 and 300 hr, respectively (Figure 45).

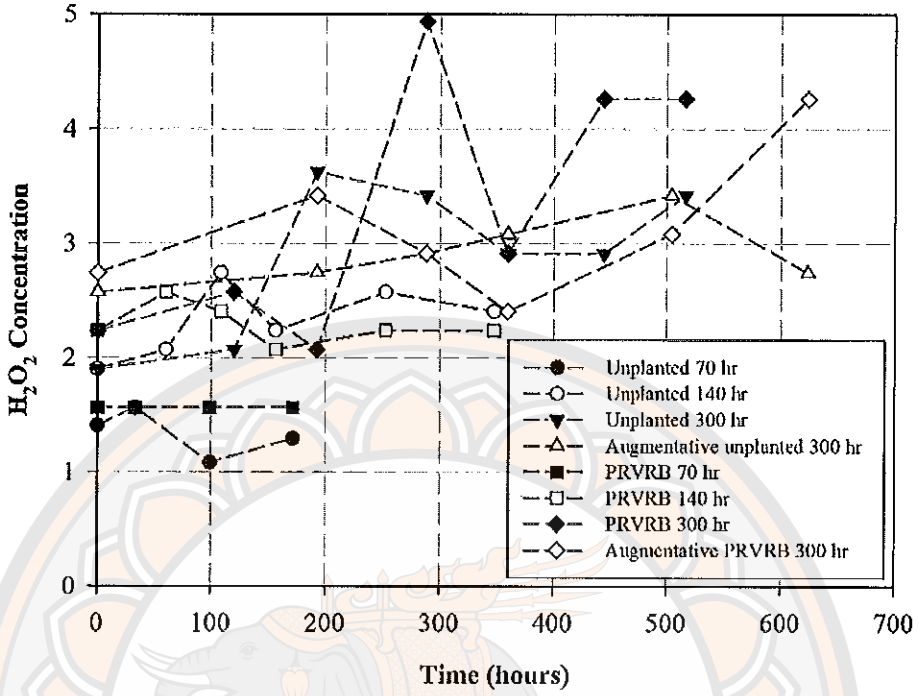


Figure 46 H_2O_2 concentration at the effluent port of the unplanted and PRVRB systems with HRT of 70, 140, 300 hr and 300 hr with bioaugmentation

When unplanted systems started, the H_2O_2 kept stable around 1.9 mg/L until the systems reach the breakthrough points at about 108 and 192 hr. The concentration of H_2O_2 move up to 2.74 and 3.62 mg/L then, decreased and remained stable again. These incidents indicated that microbial activities. These incidents indicated that microorganisms responded to phenol contaminated water by inducing the formation of H_2O_2 through the self-defense mechanism which emerged at the peak (Cabiscol, Tamarit, & Ros, 2010). Then, H_2O_2 was the oxidants reflecting on the decreasing of concentration until the systems achieved the stabilities (Figure 46). Therefore, the phenol removal efficiencies of 10.41% ($k_{up,\ 140hr} = 3.66x10^{-4}\ hr^{-1}$) and 36.20% ($k_{up,\ 300hr} = 1.34x10^{-3}\ hr^{-1}$) from HRT of 140 and 300 hr attributed to both abiotic and biotic mechanisms.

Noticeably, even without PRVRB, phenol can be abiotically and biotically removed by a sand filter (Dan et al., 2017). Indigenous microbe in sand can also degrade phenol once acclimated themselves to survive in toxic phenol concentration as

to be discussed next (Welz et al., 2012). On the other hand, for HRT of 70 hr, the water may flow through the sand filter so rapidly that the microbe did not have enough time for the degradation (Kurzbaum et al., 2010).

The images under microscopy revealed the bacterial colony which obtained from root and sand surfaces after all experiments reach the steady state with different HRT. The cocci and rod shapes with both gram positive and negative strains were seen on the Tryptic soy agar media (Figure 47).

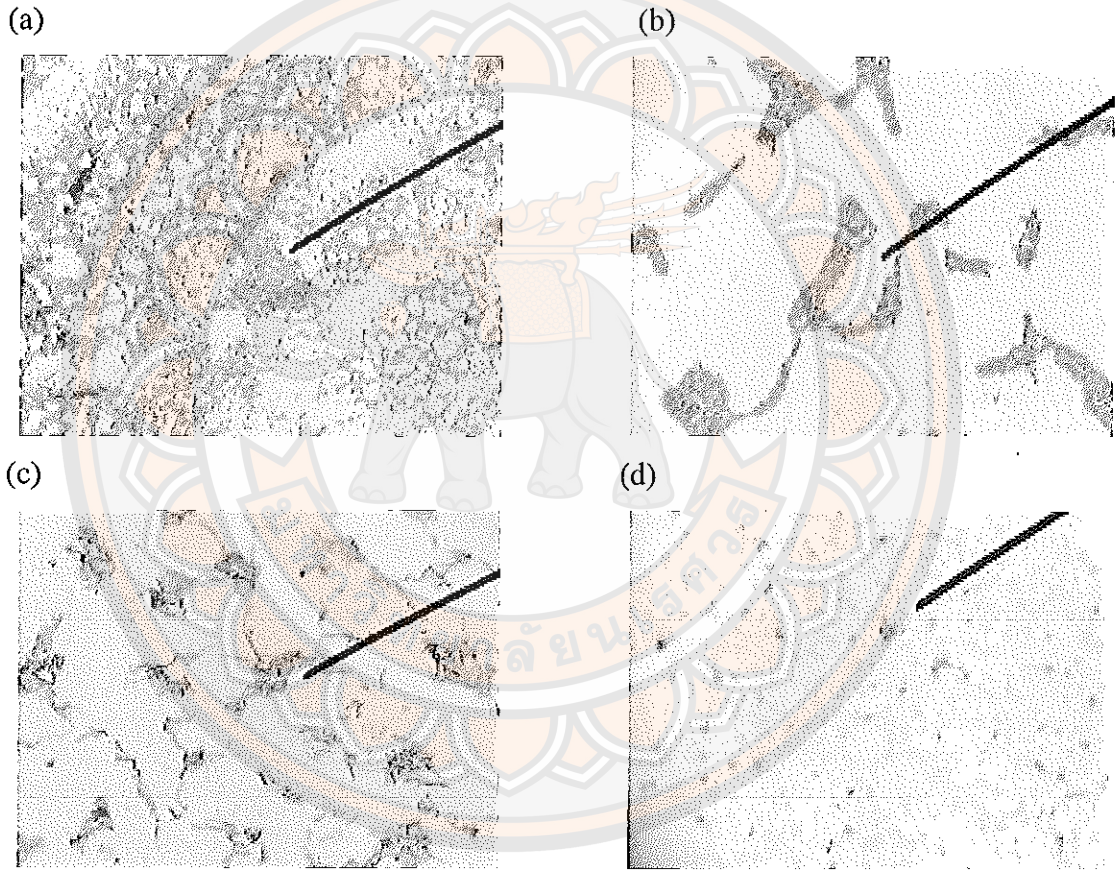


Figure 47 Isolated colonies under microscopy from sand surfaces and vetiver roots

(e) (f)

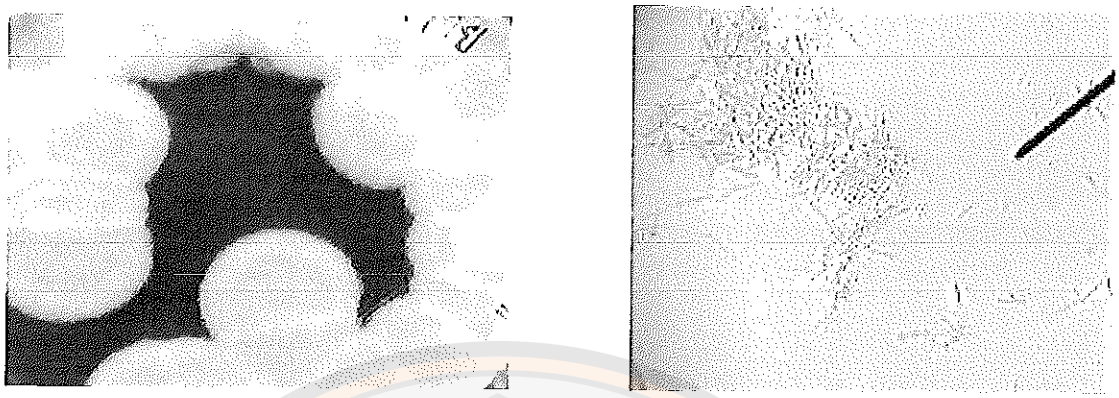


Figure 47 (cont.)

Note: (a) and (b) gram positive bacteria strains in the short rod shape 100X, (c) and (d) gram negative bacterial strains in the short rod shape 100X and (e) and (f) fungi strains 40X.

Microbial community structure in the systems were shown in Figure 48 as the profile of DGGE bands after PCR amplification. The bacterial 16S rRNA gene sequences from the samples with primers 341F and 534R yielded 14 lanes on the DGGE gel.

(a) (b)

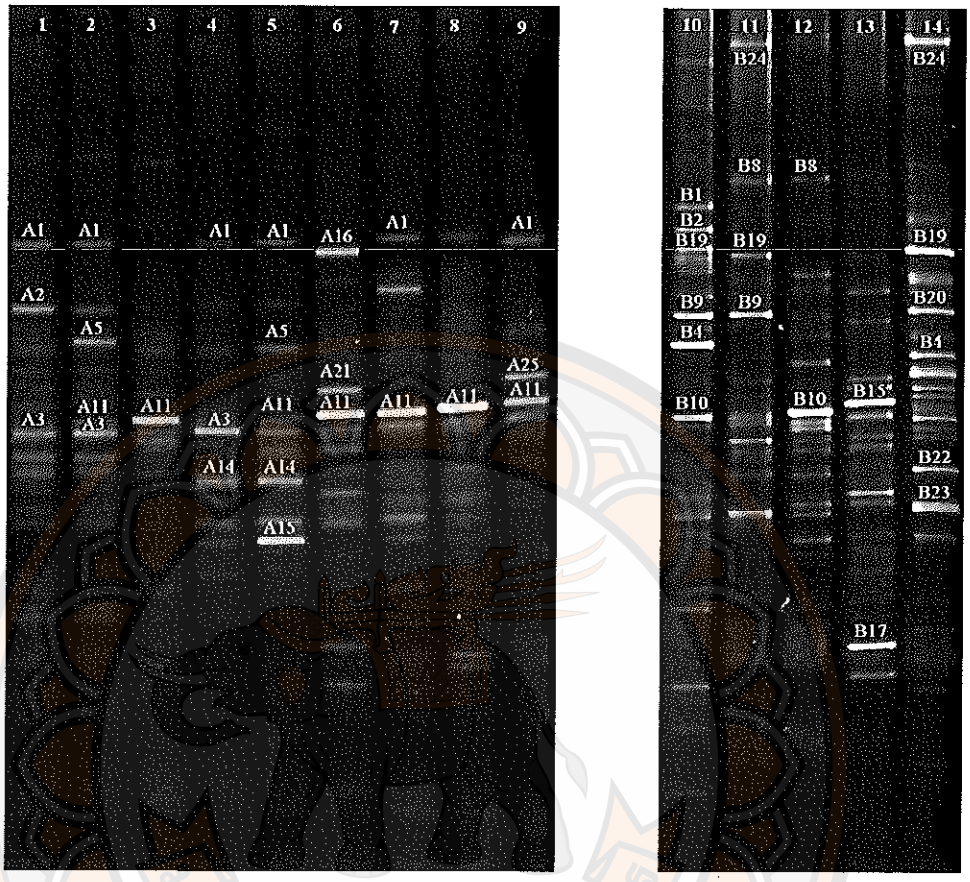


Figure 48 DGGE profiles of microbial 16S rRNA gene sequences

Note: (a) the unplanted systems (Lane 1-3, sand surfaces with the HRT of 140, 300 hr and 300 hr with bioaugmentation); the PRVRBs (Lane 4-6, sand surfaces at rhizosphere zone with the HRT of 140, 300 hr and 300 hr with bioaugmentation; Lane 7-9, sand surfaces at non-rhizosphere zone with the HRT of 140, 300 hr and 300 hr with bioaugmentation) and (b) the vetiver root from PRVRBs (Lane10, natural vetiver root; Lane 11-14, vetiver root with the HRT of 70, 140, 300 hr and 300 hr with bioaugmentation)

Each bands on DGGE gel refer to the identical fingerprint pattern of microbial strains (DNA). We could comprehend the emerged strains and their dynamic which responsed to phenol toxicity. Among all bands, the approximately dominant

microorganisms which was identified by the band intensity were 23 strains divideing to 10 strains from the sand surfaces and 13 strains from vetiver root. Each DNA sequences were compared to those found in the GenBank database by using NBCI BLAST program to identify the closest strains (Table 18).

Table 18 Sequence analysis of microbial 16S rRNA genes obtained from the DGGE gel bands

Description	System	Band	Closest match	Sequence similarity (%)	GenBank accession no.
Natural Root (Lane10)		B1	*Bacillus* sp.	95%	KU712052.1
		B2	*Pseudomonas* sp.	92%	EU876683.1
		B4	*Bacillus* sp.	96%	KP236211.1
		B9	*Pseudomonas* sp.	82%	HG330216.1
HRT of 70 hr	Unplanted	-	-	-	-
	PRVRB (Lane11)	B8	*Streptomyces* sp.	92%	KT025830.1
		B9	*Pseudomonas* sp.	82%	HG330216.1
		B19	*Pseudomonas* sp.	95%	AJ846284.1
		B23	*Acinetobacter* sp.	89%	KF008679.1
		B24	*Pectobacterium* sp.	91%	KF057994.1
HRT of 140 hr	Unplanted (Lane1)	A1	*Arthrobacter* sp.	90%	EU867321.1
		A2	*Pseudomonas* sp.	89%	KU131275.1
		A3	*Novosphingobium* sp.	90%	KT884900.1
	PRVRB (Lane4, 7 and 12)	A1	*Arthrobacter* sp.	90%	EU867321.1
		A3	*Novosphingobium* sp.	90%	KT884900.1
		A14	*Rhizobium* sp.	98%	AJ786078.1
		B10	*Flavobacterium* sp.	93%	LC158064.1
		B8	*Streptomyces* sp.	92%	KT025830.1
		A11	*Leptothrix* sp.	93%	JF345337.1
HRT of 300 hr	Unplanted (Lane2)	A1	*Arthrobacter* sp.	90%	EU867321.1
		A3	*Novosphingobium* sp.	90%	KT884900.1
		A5	*Flavobacterium* sp.	94%	KF591404.1
		A11	*Leptothrix* sp.	93%	JF345337.1

Table 18 (cont.)

Description	System	Band	Closest match	Sequence similarity (%)	GenBank accession no.
	PRVRB (Lane5, 8 and 13)	A1	*Arthrobacter* sp.	90%	EU867321.1
		A14	*Rhizobium* sp.	98%	AJ786078.1
		A15	*Lysobacter* sp.	99%	JQ349049.1
		A5	*Flavobacterium* sp.	94%	KF591404.1
		B15	*Flavobacterium* sp.	95%	KU991469.1
		B17	*Exiguobacterium* sp.	93%	KY471023.1
		A11	*Leptothrix* sp.	93%	JF345337.1
HRT of 300 hr with bioaugmentation	Unplanted (Lane3)	A11	*Leptothrix* sp.	93%	JF345337.1
	PRVRB (Lane6, 9 and 14)	A11	*Leptothrix* sp.	93%	JF345337.1
		A16	*Pseudomonas* sp.	95%	EU636659.1
		A21	*Candidatus* sp.	95%	JF815031.1
		B4	*Bacillus* sp.	96%	KP236211.1
		B19	*Pseudomonas* sp.	95%	AJ846284.1
		B20	*Actinobacterium* sp.	91%	KJ849465.1
		B22	*Enterobacter* sp.	88%	AB244436.1
		B23	*Acinetobacter* sp.	89%	KF008679.1
		A1	*Arthrobacter* sp.	90%	EU867321.1
		A11	*Leptothrix* sp.	93%	JF345337.1
		A25	*Bacillus* sp.	85%	KP992912.1

The microbial communities were well-known as the effective tool to deal with many toxic pollutants in bio-remediation due to their different complex activities (Farrell, & Quilty, 1999; Johnsen, Wick, & Harms, 2005; Karigar, & Rao, 2011; Shibata et al., 2006). Beyond the competition between each strains, the positive interaction among bacterial strains is essential for assessing the commonness and importance of cooperative evolution in natural communities for example first, synergism among strains in biofilm formation and second, growth promotion through secretion (Foster, & Bell, 2012).

To consider the indigenous bacterial strains from the unplanted system (Lane1 and 2) in Figure 48, the dominant strains similar to *Arthrobacter* sp., *Novosphingobium* sp., *Pseudomonas* sp., *Flavobacterium* sp. and *Leptothrix* sp. These microbes were known as phenol degrading bacteria. Many literatures suggested that, *Arthrobacter* sp., *Novosphingobium* sp. and *Flavobacterium* sp. were able to utilize phenol and chlorinated phenol, transform phenol to catechol, cresols and naphthol via *meta*-cleavage (Copley, 2000; del Castillo et al., 2012; Field, & Sierra-Alvarez, 2008; Hendriksen, Larsen, & Ahring, 1991; C. Karigar, Mahesh, Nagenahalli, & Yun, 2006; Tiirola, Männistö, Puhakka, & Kulomaa, 2002) thus, the biotic degradation of phenol by bacteria were assisted by these strains.

2. Phenol removal by PRVRB and Effects of the HRT on the removal efficiency

As for the experiments with PRVRB, although at the HRT of 70 hr, PRVRB failed to degrade phenol, the efficiencies of the PRVRB systems at the HRT of 140 and 300 were substantially enhanced in comparison to without PRVRB. As show in Figure 2, the breakthrough curve of PRVRB with HRT of 140 and 300 hr start with low initial concentration and rise significantly due to phenol dispersed to the effluent ports at 110 and 192 hr. Until 200 and 400 hr, phenol concentrations, C/C_0 stabilize at 0.75 and 0.36.

These numbers refer to the removal efficiency of PRVRB experiments with the HRT of 140 hr and 300 hr were 24.79% and 64.31%, respectively. Using Eq 6.1, the degradation rate constants of phenol for HRT of 140 hr and 300 hr were 9.49×10^{-4} hr^{-1} and $3.07 \times 10^{-3} hr^{-1}$, i.e. 2.6 times greater than without PRVRB. This enhanced removal efficiency and accelerated degradation rate constant was attributed to the capacity of vetiver root to release the inherent ROS and root exudates which are the oxidants in phyto-oxidation and beneficial to phenol microbial degrader (Chaparro et al., 2013; Chaparro et al., 2014; Senguttuvan, Paulsamy, & Karthika, 2014; S. Singh et al., 2008). To consider the phyto-oxidation and phyto-polymerization mechanisms which are driven by vetiver root, H_2O_2 was the representative of ROS which is an oxidant in phyto-oxidation to degrade phenol reflecting on the decreasing of phenol concentration until the systems achieved the stabilities. H_2O_2 was released from the vetiver root by phenol stress inducement and the metabolism of living cells which

could show the existing of phenol degraders (Mittler, Vanderauwera, Gollery, & Van Breusegem, 2004; Veal, Day, & Morgan, 2007).

In Figure 46, The H_2O_2 level of PRVRB system of 70 hours was low compared to other PRVRBs. It could approve the incapable degradation of the HRT 70 hours. While, PRVRB systems with the HRT of 140 and 300 hours showed the initial H_2O_2 similarly but H_2O_2 from 300 hours was higher than 140 hours.

The H_2O_2 levels were related to the enzymes which were measured from the vetiver root samples at the end of the experiments. At the end of experiments, the level of enzymes could appear because mild stress could still arouse stress memory in plants which are possible long-term stress imprint, leading to enhanced stress tolerance to subsequent more severe stresses (Bruce, T. J. A. et al., 2007; Pandey et al., 2017). The endogenous H_2O_2 level is tightly controlled by the coordinated action of H_2O_2-generating enzymes such as superoxide dismutase (SOD; EC 1.15.1.1) and H_2O_2-scavenging enzymes such as catalase (CAT; EC 1.11.1.6) and peroxidase (POD; EC 1.11.1.7) (Konieczny et al., 2014). SOD is the essential enzyme, its responsibility is to transform superoxide ($O_2^{\bullet-}$) to H_2O_2 which is more stable and less harmful to the living cells (Alscher, Erturk, & Heath, 2002; Nimse & Pal, 2015; Velikova, Yordanov, & Edreva, 2000). POD also appears to be a response to phenol toxicity by stimulated the ROS generation in detoxification (Sinsabaugh, 2010). We discovered that the higher enzyme activities came along with the longer HRT and H_2O_2 levels (Figure 49 and 46). Thus, the more SOD and POD, the more H_2O_2. The higher SOD and POD were resulting in the transformation of phenol to phenol radical by H_2O_2 as an electron acceptor which is catalyzed by POD. Then, phenol radicals coupled with itself or other organic matters to form polyphenols via oxidative polymerization (Kobayashi et al., 2001; Phenrat et al., 2016; Sinsabaugh, 2010; Uyama & Kobayashi, 2002). Therefore, the higher POD could refer to elevated phenol degradation. But sometimes, when it comes to be more dangerous due to the high ROS in the systems, CAT charges to mineralize excessive H_2O_2 to water and oxygen (Velikova et al., 2000). The results showed that PRVRBs with HRT of 140 and 300 hours had the higher level of POD, SOD and CAT that responded to phenol toxicity and leaded to the increasing of degrading efficiencies. While, with HRT of 70 hours, the enzymes were the lowest reflecting to the system failure (Figure 49).

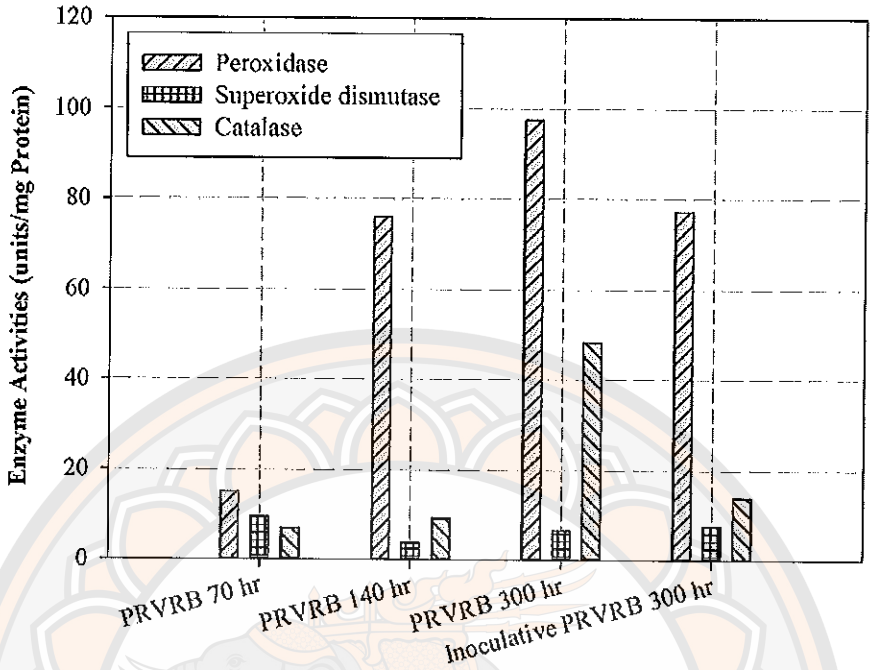


Figure 49 The levels of enzyme activities at the effluent port of the PRVRB systems with HRT of 70, 140, 300 hours and 300 hours with bioaugmentation

Also, indigenous microbial assistances were observed in PRVRBs. When experiments reach steady state at the HRT of 70, 140 and 300 hours, the PRVRBs presented the microorganisms of $2.58x10^6$, $9.66x10^6$ and $4.75x10^6$ CFU/g root and sand, respectively. These evidences showed that firstly, the contact time enabled the acclimation to these microorganisms to survive and consume phenol in toxic environment. The microbes formed themselves as the biofilms attached on the surface of vetiver root and sand around the rhizosphere. It is broadly accepted that the root surfaces provide a highly productive habitation increasing biomass on and in rhizosphere, roots also release some nutrients including substances that induce microbial genes involved in pollutant degradation (O'Toole, & Stewart, 2005; Tsao, 2003; A. Varma, Abbott, Werner, & Hampp, 2007). Secondly, when phenol degraded, the formation of some by-products were emerged. Phenol degradation caused the forming of catechol, the microbial growth's inhibitor (Dan et al., 2017; Schweigert,

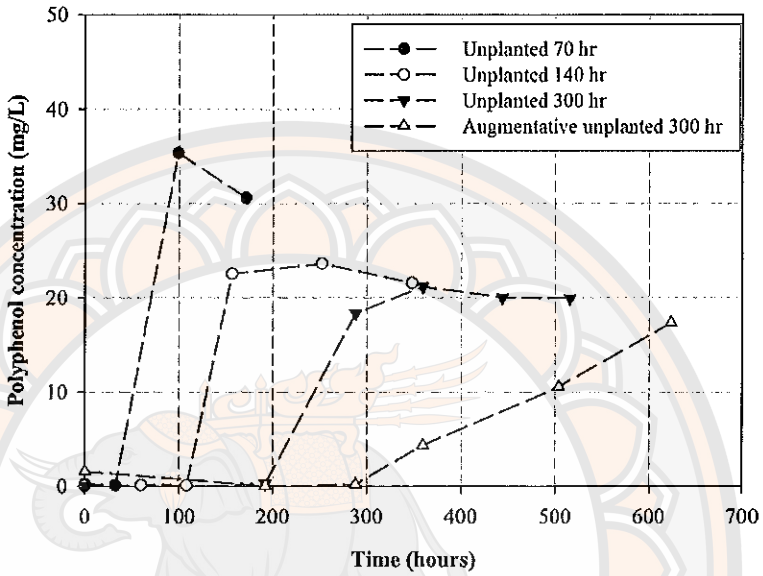
Zehnder, & Eggen, 2001). Catechol could impede the living of microorganisms. In the PRVRB system, there were more complex mechanisms included phytochemicals and rhizosphere microorganisms which associated together in phenol degradation. Despite, catechol is toxic to microorganisms but the vetiver root could decline some stresses by providing habitations and nutrients which assisted the acclimation to tolerate the stresses. The number of microbes decreased slightly. Conversely, it was not found this circumstance in unplanted systems. The microorganisms were weak and suffer after degrading phenol. Moreover, they could not acclimate themselves as much as we expected. It could be shown in the lower amount of microorganisms in the final state of unplanted system with HRT of 300 hours (Figure 45).

Thirdly, another reason that might cause the reduction of microorganisms in the systems with HRT of 300 hours was the competition among microbial strains. Although, extracellular polymeric substances (EPS) from biofilms are favorable protections from various stresses but within microbial community should not be supposed to cooperate peacefully. For limited space and nutrient resources, the competitive phenotypes by natural selection is emerged. Microorganisms compete each other commonly by the secretion of broad and narrow spectrum toxins and antibiotics (Boyd, Yoshida, Vereen, Cada, & Morrison, 1969; Nadell, Drescher, & Foster, 2016). Sometimes, ESP producers in mixed-species biofilm can suffocate other species and use their productions to push themselves into preferential resources (Hibbing, Fuqua, Parsek, & Peterson, 2010; Xavier, & Foster, 2007). The competition among strains and inappropriate environment could point to the decrease of microbial populations.

After the phenol ring cleavage by H_2O_2 and POD via phyto-oxidation, enzymatic polymerization was followed. The soluble polyphenols and VOA were the representatives of by-products from the phenol degradation ascribed to phytochemical exudates and the activities of microorganisms as phenol degraders. With the higher HRT, vetiver root suffered less than those with the lower HRT because the expansion of contact times. Vetiver root and their microorganisms could acclimate themselves (Figure 45 and 46) and react by releasing phytochemicals as H_2O_2 and enzymes to degraded phenol. Phenol decreased while, other by-products increased. Polyphenols

from PRVRB effluent ports tended to increase by the time and slowly decrease after the steady state (Figure 50).

(a)



(b)

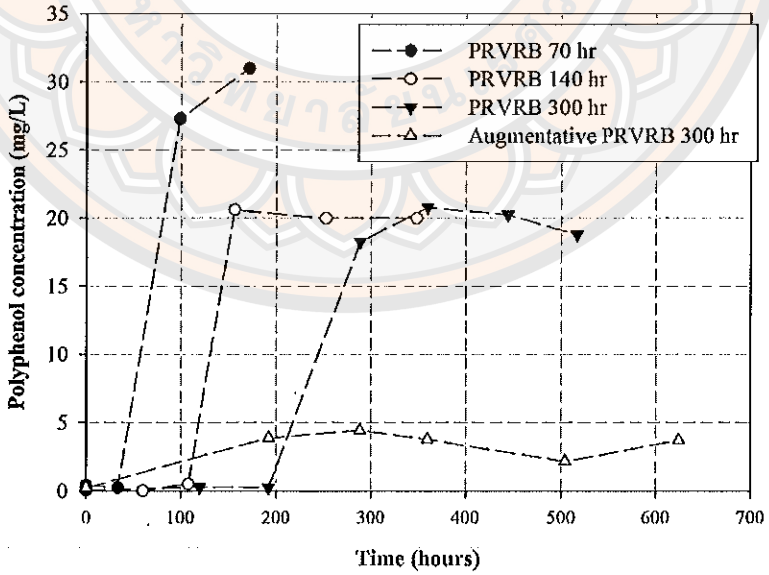


Figure 50 Polyphenols at the effluent port of (a) the unplanted and (b) the PRVRB systems with HRT of 70, 140, 300 hours and 300 hours with bioaugmentation

It was indicating that either existing phytochemicals could oxidize or some microorganisms consumed less toxic polyphenols to form a group of carboxylic acids which were the main products of polyphenol degradation via wet oxidation and microbial metabolisms (Achak, Mandi, & Ouazzani, 2009; Klinke, Ahring, Schmidt, & Thomsen, 2002; Sinsabaugh, 2010; Vymazal & Kröpfelová, 2009). Supported by the increasing of VOA after the PRVRBs accomplished the steady state from 26.89 mg/L as acetic acid to 156.88, 215.15 and 307.49 mg/L as acetic acid for PRVRB systems with HRT of 70, 140 and 300 hours, respectively (Figure 51).

(a)

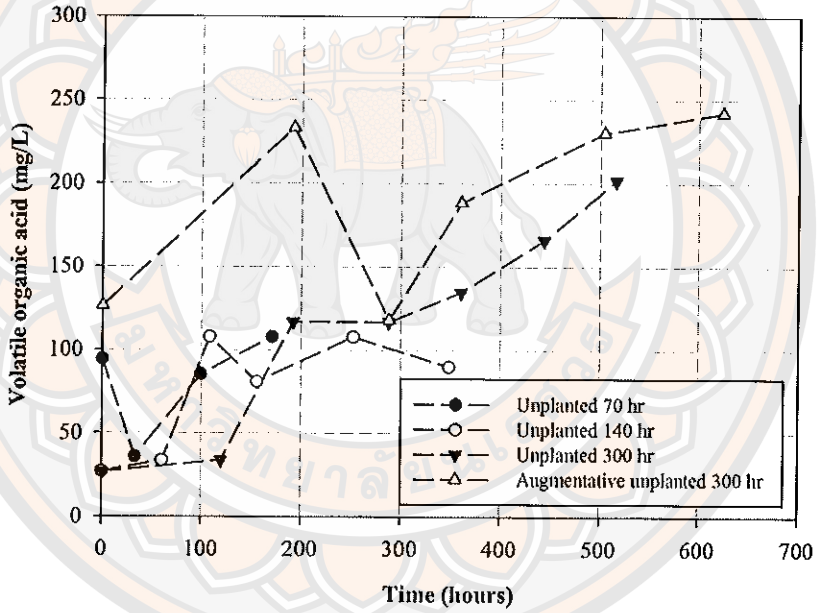


Figure 51 Volatile organic acid as acetic acid at the effluent port of the PRVRB systems with HRT of 70, 140, 300 hours and 300 hours with bioaugmentation

(b)

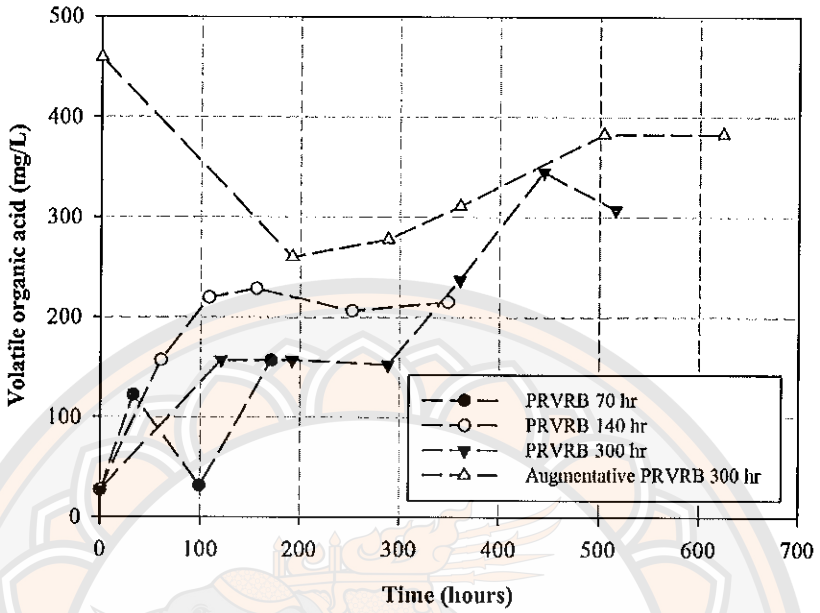


Figure 51 (cont.)

Besides, as shown in Figure 46, microbial content occurred rising up by the contact time but still less than the prior before approaching phenol due to its toxicity (excluded HRT of 140 hours). The highest microbial concentration was found in the rhizosphere zone agreed with many researchers that plants crucially enhance microbial activities as its rhizosphere provides the space for microorganisms to grow, its root exudates release nutrients for supporting growth including substances that induce microbial genes involved in pollutant degradation and also provide the oxygen needed (Ballesteros, Vuong, Secondes, & Tuan, 2016; O'Toole, & Stewart, 2005; Stottmeister, U. et al., 2003; Tanner, 2001; Tsao, 2003; Vymazal, & Kröpfelová, 2009).

It could be found that in high phenol concentrations, catechol is a major group of intermediate by-product from ring cleavage (Sophiphun et al., 2015; Welz et al., 2012) via phyto-oxidation before forming to polyphenol in phyto-polymerization. Catechol can be toxic to living cells, often exhibiting higher poisoning than other derivatives. It causes the damage to the cell membranes of both gram negative and

positive bacteria (Hernandez & Edyvean, 2008). Consequently, aggressive phenol degradation could be assumed to be the cause of catechol formation resulting in the lower microorganisms and efficiency of PRVRB with HRT of 300 hours as it could be. However, not only microbes but some fungi also appeared for the first time in the systems with the HRT of 300 hours (Figure 52) agreed by our previous study (Chapter 4).

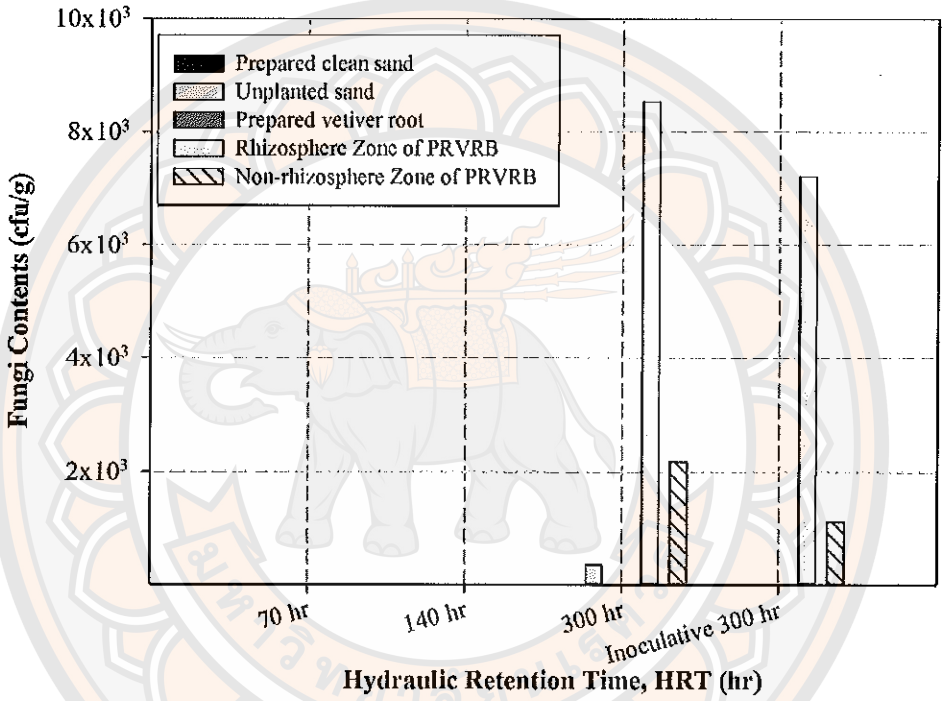
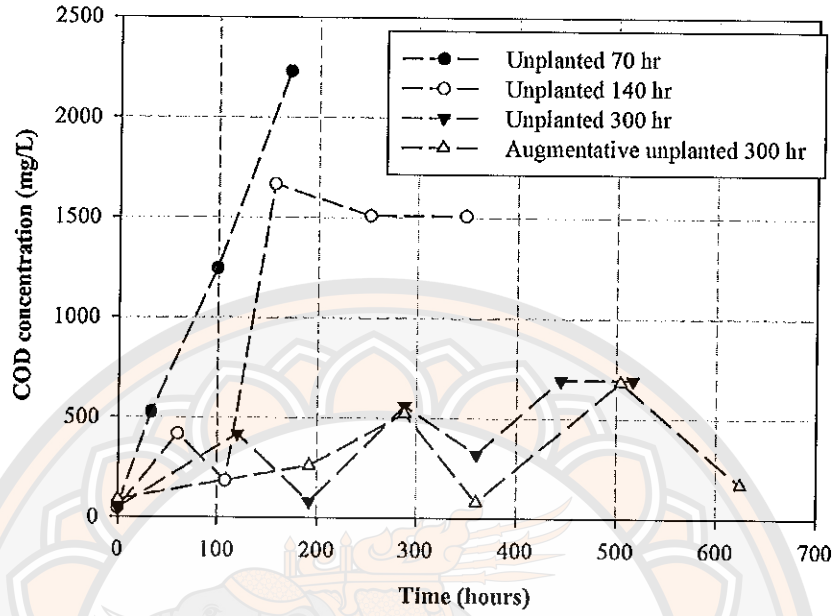


Figure 52 The fungi content of the samples from sand and vetiver root of the PRVRB systems with HRT of 70, 140, 300 hours and 300 hours with bioaugmentation

Rationally, the total COD removal, we found that the COD concentrations reduce variably more than 50% from influent to effluent and followed with the decrease of phenol (Figure 53). Phenol-containing wastewater allowed the biological degradation of either phenol and other organic matters in the systems by heterotrophic microorganisms which is biofilms on sands and vetiver roots.

(a)



(b)

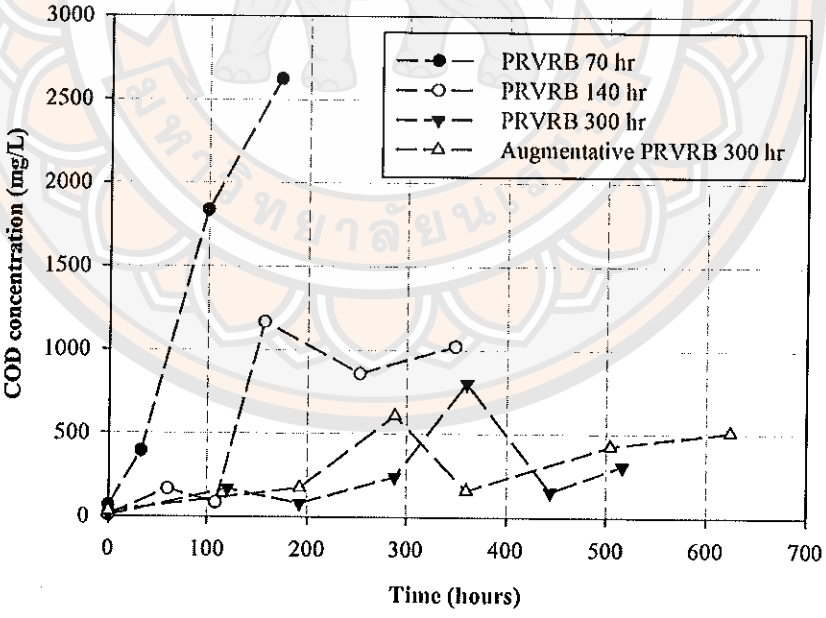


Figure 53 COD concentration at the effluent port of the PRVRB systems with HRT of 70, 140, 300 hours and 300 hours with bioaugmentation

To consider the indigenous bacterial strains at the surfaces of natural vetiver root, the most detected strains were *Psuedomonas* sp. and *Bacillus* sp. that protect plants from phytopathogens(Harsh Pal Bais, Fall, & Vivanco, 2004; Del Giudice et al., 2008; Santhanam, Weinhold, Goldberg, Oh, & Baldwin, 2015) .After the failure of phenol degradation from the systems with HRT of 70 hours, we could not detect the dominant strains in unplanted systems but some in PRVRB systems . Vetiver root was confirmed to be the suitable microbial habitation since the number of bands which presented on DGGE gel (Figure 48). Moreover, the band intensity of each strains which was the representative of the bacterial population showed that in PRVRB systems had the stronger intensity than unplanted systems. We noticed that with the extension of HRT, the number of bacterial strains also rose up by time. The higher HRT corresponded to the bacterial acclimation to the phenol toxicity. The microorganisms in the systems acclimated themselves and attached to the media especially to the vetiver root .PRVRB was more strengthen due to the association between this relationship .These evidences pointed out to the performance of PRVRB systems beyond unplanted systems at the higher HRT. However, the bacterial strains were considered as the important role in the phenol degradation in both unplanned and PRVRB systems.

As well, in Figure 48 and Table 18, the PRVRB systems also presented the existance of these microbes and some strains which could not discover in unplanted systems such as *Lysobacter* sp., *Exiguobacterium* sp., *Streptomyces* sp .and *Rhizobium* sp. *Lysobacter* sp .was reported as phenol degrader and biological control of plant disease .*Lysobacter* sp .and *Exiguobacterium* sp .can be found in normal environment including plant root and rhizosphere zone or even tolerance to biotic and abiotic stresses (Chauhan, Bagyaraj, Selvakumar, & Sundaram, 2015; Collavino, Sansberro, Mroginski, & Aguilar, 2010; del Castillo et al., 2012; Hayward, Fegan, Fegan, & Stirling, 2010). While, *Streptomyces* sp .could play an important role on producing POD as a lignin-induced enzyme to form precipitated polymeric lignin .Besides, some H_2O_2 was generated by this strain (Ramachandra, Crawford, & Pometto, 1987). Therefore, it was resulting in the higher phenol removal efficiencies of PRVRB systems compared to unplanted systems with indigenous bacteria.

3. Bioaugmentation on unplanted and PRVRB systems.

From both unplanted and PRVRB systems, it was found that the longer retention time allowed necessary contact time for abiotic and biotic removal processes. It allowed the adaptation of vetiver root by providing the toxic signaling and its microbes to acclimate themselves tolerate phenol toxicity in stress environment. The HRT of 300 hours was selected for the experiment of unplanted and PRVRB with bioaugmentation. Three selected bacterial strains including of *Enterobacter* sp., *Pseudomonas* sp. and *Acinetobacter* sp. were augmented and encouraged to form the biofilm into the systems as described above. The abilities of *Pseudomonas* sp., *Enterobacter* sp. and *Acinetobacter* sp .are mentioned and interested by many researchers that they are able to degrade phenol and other toxic pollutants (Whiteley, & Bailey, 2000) .This is illustrated by the degradation of phenol as single carbon source by *Psuedomonas* sp. SA01 achieved within 30 hours) up to 1 g/L((Shourian et al., 2009) .Planktonic and biofilm *Psuedomonas* sp. showed the high phenol removal rate, in constructed wetland systems and mainly in those with sub-surface flow (Kurzbaum et al., 2010) .*Enterobacter* sp. involved in the degradation of phenol and its derivatives for more than 3 years in anoxic treatment of industrial waste waters (Thomas et al., 2002a) .Also, *Acinetobacter* sp. was often determined as an effective phenol-degrader, consumed phenol up to 1g/L (Adav et al., 2007) because *Acinetobacter* sp. had a high inclination to produce EPS on the surface of media as biofilms which is the protective shelters and mineralized toxic stress (Jang, Kim, & Park, 2016; Yamaga et al., 2010) .

The results from the unplanted and PRVRB systems with bioaugmentation also show in Table 17 and Figure 49c. The initial of S-curves starts increasing at 192 hr, phenol concentration was first detected for both systems. The slopes were less compared to Figure 44a and 44b. the curves comes to levelling off at the steady state of 400 hr. The unplanted system degraded phenol with the efficiency of 65.58%) $k_{up,300\ hr\ with\ augmentation} = 2.12x10^{-3}\ hr^{-1}$ (while, PRVRB system performed the better degradation by degrading 91.08%) $k_{PRVRB,300\ hr\ with\ augmentation} = 5.04x10^{-3}\ hr^{-1}$(of phenol from initial state .Nevertheless, the effluent concentrations of phenol were 157 and 30 mg/L which are still greater than the MCL.

The high efficiency attributed to the combination of phyto- and bio-degradation. Although, the phytochemicals (enzymes and H_2O_2) and microorganisms were similar and seem to be lower than the non-augmented systems (Figure 45, 46 and 49) but achieve the most effective phenol degradation system. Besides, it pointed out to the activities of phenol degrading bacteria that were form themselves as biofilms before unplanted and PRVRB system started. These biofilm microorganisms were the first defender to degrade phenol. Some of them might be suffer and washed out of the systems resulting in the less amount at the end of experiments. During phenol degradation, it came to be the lower phenol concentration before the vetiver root reacted to the phenol stress thus, it was resulting in the lower H_2O_2 corresponded to the lower enzymes in comparison with the systems without augmentation. However, the decrease of POD contributed to the catalytic phenol degradation rapidly. As well, H_2O_2 could transform to water and O_2 which is necessary to augmented aerobic microorganisms by CAT stimulation in the function of symbiosis relationships resulting in the lower H_2O_2 and CAT (Figure 46 and 49).

Obviously, microbial communities arose with 10 predominant microbial strains which were increased from the PRVRB without bioaugmentation such as *Candidatus* sp., *Actinobacterium* sp., *Bacillus* sp., *Pseudomonas* sp., *Enterobacter* sp. and *Acinetobacter* sp (Figure 48 Lane 14 and Table 18) .The phenol removal efficiency of 91.08% was affirmed to be the successful phenol degrading bacteria augmentation especially, together with vetiver root. Due to, vetiver roots were beneficial for oxygen transfer to rhizosphere zone, promoting aerobic biodegradation (Dan et al., 2017; C. Tanner, 2001; Tanner, Kadlec, Gibbs, Sukias, & Nguyen, 2002). Especially for the longer contact time, biofilms often reduce phenol greatly by diffusion and mass transfer (Hsien, & Lin, 2005; Kurzbaum et al., 2010; Tang, & Fan, 1987). Whereas, there was opposite observation in unplanted system that *Leptothrix sp* .was the only dominant strain) Figure 48 Lane 3.(

Interestingly, among the several dominant strains, A11 was tentatively recognized as *Leptothrix* sp .It was usually found since the short HRT of 140 in PRVRB system to 300 hours in unplanned and PRVRB systems included with the bioaugmentation systems. Hence, the phenol removal efficiencies by the microbial activities seem to be caused by *Leptothrix* sp. According to the rapid degradation,

phenol is small monomers readily to transform by these degradation processes to derivatives .It was more possible to encounter with *Leptothrix* sp .which is defined as a thermophilic and aquatic Fe-oxidizing bacteria .*Leptothrix* sp .is also an organic pollutant degrader which could be inducible by polyesters and able to consume succinic acid and adipic acid .Succinic acid is the by-product from phenol and some aromatic compounds ring fission to catechol followed by β-ketoadipic acid and then break down to succinic acid through microbial metabolism (Kilby, 1951; Stanier, Sleeper, Tsuchida, & MacDonald, 1950; Wells & Ragauskas, 2012) .While, adipic acid could obtained after the transformation of cyclohexanol through phenol hydrogenation (Musser, 2005; Nakajima-Kambe et al., 2009) .

Conclusion

The phyto-utilization of PRVRB is an effective engineering application in the treatment of groundwater contaminated with phenol. The biotic and abiotic were attributed to the successful degradation in both unplanted and PRVRB systems. With the indigenous microorganisms and extended retention time to 300 hours, PRVRB degraded 64.31% of phenol with the rate of $1.34x10^{-3}$ hr^{-1}. While, enhancement with the selective microorganisms, the efficiency reach to 91.08% with the rate of $5.04x10^{-3}$ hr^{-1}. To consider microorganism's activities, bacteria was accredited to the important role meanwhile, fungi played the minor role. Identification and characterization of bacterial strains show that the successful bacterial augmentation of *Pseudomonas* sp., *Enterobacter* sp. and *Acinetobacter* sp. could perform the capable in phenol degradation. The indigenous strain, *Leptothrix* sp. was also corresponded on phenol degradation of unplanted systems. Nevertheless, more efforts are needed to measure the inhibitory compounds and specific by-products to benchmark the phenol degradation by PRVRB.

To achieve the rapid phenol degradation, vetiver root are a good candidate in phytoremediation that could release the essential phytochemicals, support the growth of microorganisms as the appropriate habitation and nutrients provider. We expect on the further study that the longer vetiver root could be formed the dense root systems as the network of rhizosphere manipulate and deal with the pollutants in the deeper zone of groundwater.

CHAPTER VI

SUMMARY OF RESEARCH RESULTS AND FUTURE STUDIES

The goal of this research is to evaluate the use of vetiver grass and its rhizo-microorganisms to degrade and enhance the remediating potential of phenol contamination in wastewater and shallow groundwater. The phenol degradation could be adequately described by first-order kinetics model both in the hydroponics and PRVRB studies. Phenol degradation efficiencies were strongly influenced by the ability of vetiver grass to release inherent phytochemicals such as ROS ($O_2^{\bullet-}$, H_2O_2) and enzymes (POD, SOD and CAT) and activities of rhizo-microorganisms. Phytochemicals played the important role in phenol break down to other less toxic derivatives while, adaptive rhizo-microorganisms utilized phenol as their carbon sources. Therefore, the experimental data obtained from the PhD research contribute to better understand the phenol degradation mechanisms in illegal dumping wastewater and contaminated shallow groundwater, which are crucial for the development of dedicated application of vetiver platforms and PRVRB for phenol treatment.

For the application of vetiver platform, it was documented for the first that systematically reveals the complete phenol degradation mechanism by vetiver grass and rhizo-microorganisms in real wastewater. Furthermore, we continued enhancing the rate of phenol degradation by supporting vetiver growth and the formation of biofilms. The mature vetiver grass with aeration enhanced the overall phenol degradation rate about 4.48 times greater than vetiver plantlets. Figure 54 shows the summary of results from the vetiver platforms.

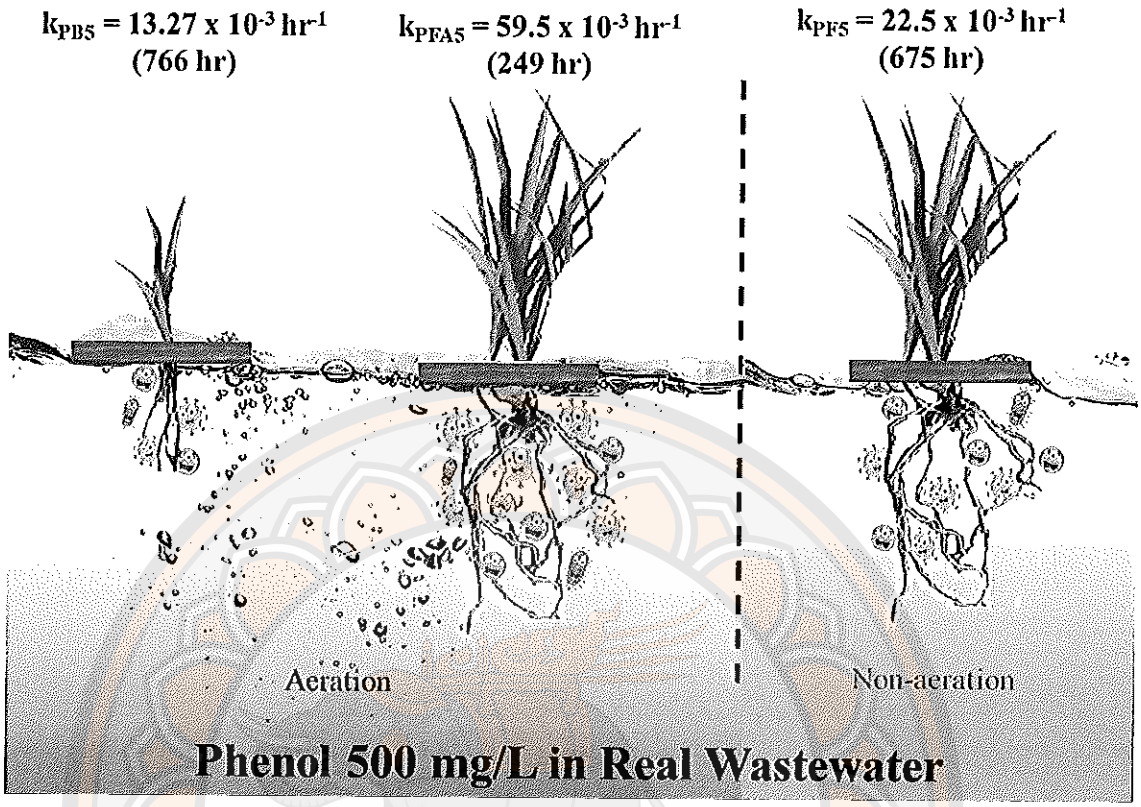


Figure 54 A summary of thesis results depicting the overall phenol degradation rate constant of (a) 100 vetiver plantlet on platform with aeration, (b) 100 mature vetiver grass on platform with aeration and (c) 100 mature vetiver grass without aeration

Also, the PRVRB could conduct the excellent phenol removal efficiencies in shallow groundwater with the appropriate HRT of 300 hr with the bioaugmentation as high as 91.08% within 14 days. While, with indigenous microbes, PRVRB could degraded 64.31% of phenol within the same time. Figure 54 presents the results of PRVRB concept.

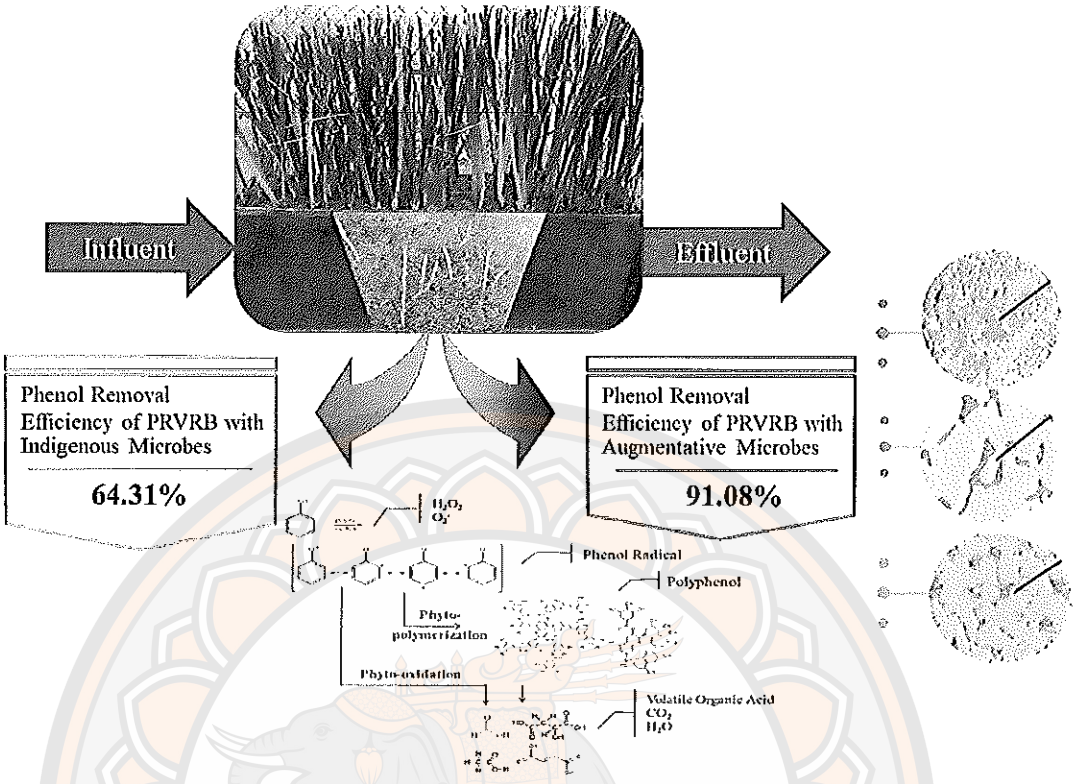


Figure 55 The results of PRVRB concept with indigenous and augmented microorganisms with the HRT of 300 hr.

Despite the achievements of this research, more work is still needed. For the future perspectives, vetiver grass is the high performance candidate for phytoremediation as well as assisting the bioremediation by microbial activities. It can apply to deal with various pollutants and the restrict area such as rural area and the deeper zone of contaminated area. There are also other scientific aspects to which the research results can contribute. These are highlighted as follows:

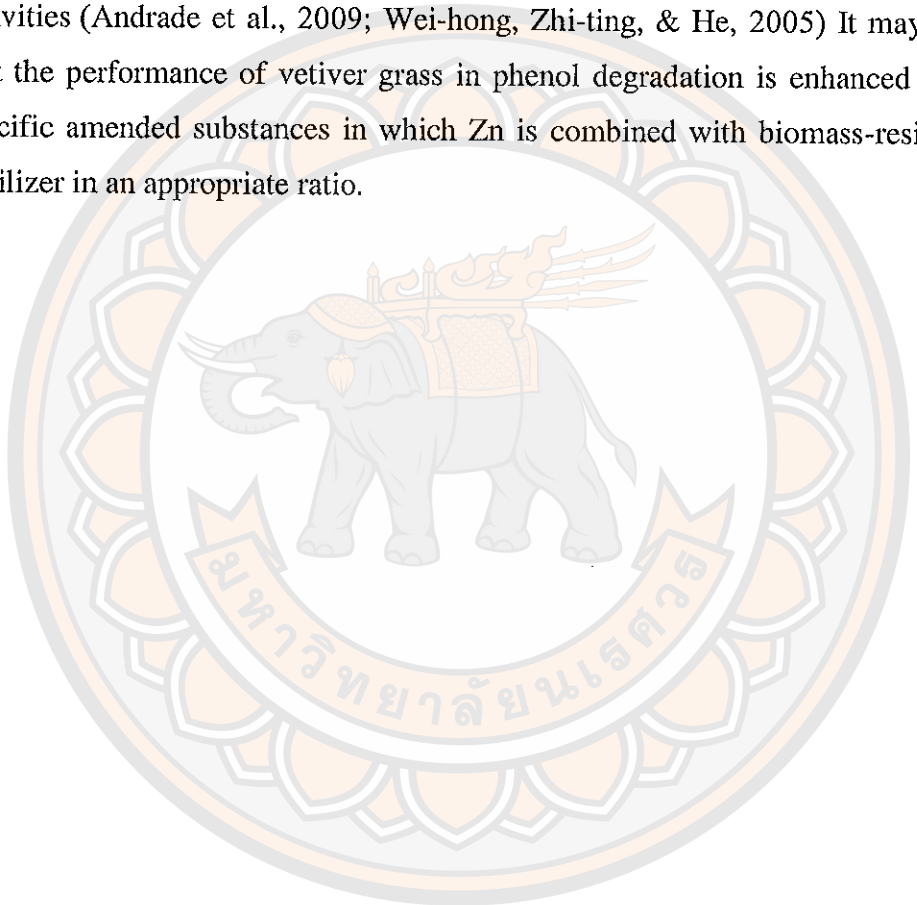
Other Selective Bioaugmentation

Bioaugmentation is the relevant key of success in this research. In our research, the mixed culture of microorganisms performed the great effect on phenol degradation. Although, we could not specify which strain was the best strain that tolerate to phenol toxicity. To specify the ability of utilizing phenol as carbon source, it can be done by inoculating each single strain to vetiver root with different phenol

concentration and evaluate their performance. After screening the microbial strains, selective strains will be involved and apply in other remediation.

Soil Amendments Increase Phytochemicals

Some soil amendments are expected to increase more potential of vetiver grass. We suggest that Zn has high possibility to induce oxidative stress which can be verified by increased lipid peroxidation rates and changes in CAT, POD and SOD activities (Andrade et al., 2009; Wei-hong, Zhi-ting, & He, 2005) It may be implied that the performance of vetiver grass in phenol degradation is enhanced when using specific amended substances in which Zn is combined with biomass-residue type of fertilizer in an appropriate ratio.



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APPENDIXES

APPENDIX A MATERIALS

The experiments in this study were conducted at Faculty of Engineering, Naresuan University included agricultural plots for planting vetiver grass, green house and chemical laboratory, Faculty of medical science, Naresuan University and Faculty of Faculty of Environmental Management, Prince of Songkhla University included biochemical laboratory.

A.1 Vetiver Grass Sampling

In this study, vetiver grass, Songkhla 3 ecotype is the traditional vetiver grass (Vetiveria zizanioides (L.) Nash) in Thailand, was obtained from Khon Kaen (Figure 3.1) and Chachoengsao (Figure 3.2) Land Development Station. Before the experiments started, vetiver grass was prepared and cultivated naturally at agricultural plots at Naresuan University, Phitsanulok Province.

Figure A1 Prepared vetiver grass from Khon Kaen Land development station

Figure A2 Prepared vetiver grass from Chachoengsao Land development station

Figure A3 Agricultural plots at Faculty of Engineering, Naresuan University

APPENDIX B CHEMICAL ANALYSIS

All chemical analyses were conducted base on Standard Methods for the Examination of Water and Wastewater (American Public Health, American Water Works, Water Pollution Control, & Water Environment, 1915). Samples for phenol, polyphenol, soluble COD, volatile organic acid and hydrogen peroxide determination were measure after filtrated through 1.2 μm filter (Whatman GF/C).

B.1 Phenol

Phenol concentrations in aqueous samples from the experiments were measured using the 4-amino-antipyrine colorimetric method with a Spectroquant® Move 100 mobile colorimeter (Merck KGaA, Darmstadt, Germany). The method is corresponding to EPA 420.1, APHA 5530 C+D, ISO 6439, and ASTM D1783-01. The detection limit was 0.5 mg/L.

B.2 Polyphenol

Polyphenol is the by-product from phenol degradation through phyto-oxidation and enzymatic polymerization (Everette et al., 2010). The determination of polyphenol as lignin and tannin was quantified using Standard Method Colorimetric 5550 (APHA, 2005). Polyphenols which contain aromatic hydroxyl groups react with Folin phenol reagent (tungstophosphoric and molybdophosphoric acids) to form a blue color detecting on the wavelength 700 nm.

B.3 Chemical Oxygen Demand (COD)

The chemical oxygen demand (COD) of the sample, a quantity of organic strength of wastewater, was determined by Method 5220C dichromate closed reflux, titrimetric method (APHA, 2005). 2.0 mL of the liquid sample, after appropriate dilution, was added to the digestion solution (0.01667 M potassium dichromate) followed by sulfuric acid reagent. The mixture was digested at 150°C for 2 hours. Ferroin indicator was add to the cooled mixture for titration with standardized 0.01 M ferrous ammonium sulfate (FAS). The end point is a reddish brown color change from green-blue color. Then, the COD value was calculated in the unit of mg O_2/L using the equation B1.

$$COD\ as\ mg\frac{O_2}{L} = \frac{(A-B)x\ M\ x\ 8{,}000}{Sample\ (mL)} - -Eq.B1$$

where:

A = mL FAS used for blank,

B = mL FAS used for sample,

M = molarity of FAS, and

8,000 = milliequivalent weight of oxygen x 1000 mL/L.

B.4 Volatile Organic Acid (VOA)

According to Standard Method 5560 and DiLallo et al. (APHA, 2005; DiLallo & Albertson, 1961), volatile organic acids (VOA) are classified as water-soluble fatty acids that can be distilled at atmospheric pressure. Volatile organic acids measurement by direct titration requires the reduction of the pH to 3.3 to destroy the bicarbonate ion and boiling the sample removes all of the carbonic acid and CO_2 remaining in solution. The back titration with phenolphthalein end point from pH 4.0 to 7.0 with standard 0.02N NaOH will include the organic acids and the other minor ions present (base alkalinity). After titration, equation B2 will be used to calculated the amount of total organic acid (as acetic acid mg/L).

$$VOA\ as\ acetic\ acid\ mg/L = \frac{(A-B)x\ N\ x\ 60{,}000}{Sample\ (mL)} - -Eq.B2$$

where:

A = mL NaOH used for sample,

B = mL NaOH used for blank, and

N = normality of NaOH.

B.5 Suspended Solid (SS)

Standard Method 2540D describes the method for determining total suspended solid (SS) (APHA, 2005). In this study, SS may represent the particulate complex polyphenol and insoluble organic compounds which are formed as the by-products of phenol degradation via phyto-oxidation and phyto-polymerization. Normally, SS

affects water or effluent quality adversely in a number of ways. A well-mixed sample is filtered through a weighed standard GF/C filter (pore size of 1.2 μm) and the residue retained on the filter is dried to a constant weight at 103 to 105°C for an hour and a half. The weight increasing of the filter shows the amount of SS. If the suspended material clogs the filter and prolongs filtration, it may be necessary to decrease the sample volume. To calculate SS, the equation B3 are conducted.

$$SS\ mg/L = \frac{(A-B)x\ 1{,}000}{Sample\ (mL)} - -Eq.B3$$

where:

A = weight of filter + dried remaining, mg, and

B = weight of filter, mg.

B.6 Hydrogen Peroxide (H_2O_2)

The existing of the H_2O_2 in wastewater was also measured by allowing it to react with a titanium sulfate reagent (titanyl ions) to produce pertitanic acid, which is yellow in color and using wavelength of spectrometer 400 nm (Eisenberg, 1943; L. Wang, & X. Jiang, 2009). A 1.00mL of the solution and 2.0 mL of titanium sulfate (30 g/L in sulfuric acid) were mixed together and diluted to 500 mL with distilled water in a volumetric flask. The absorbance of the mixture was measured at 400 nm with a 5-mm colorimetric cuvette, where the concentration of H_2O_2 was calibrated with known standard stock H_2O_2.

APPENDIX C MICROBIAL ANALYSIS

All bacterial handling (culturing and inoculation) were conducted under a sterile environment in Holten Horizontal Laminar Airflow HBB 2472. All materials used for microbial analysis were sterilized for 15 minutes at 120°C and 15 psi pressure using TOMY Portable Autoclave ES-315. Sterilized materials were dried under UV light in the Holten Horizontal Laminar Airflow HBB 2472.

C.1 Microbial enumeration and sample preparation

Colony forming units (CFU) were determined based on the surface-plate counting procedure. Vetiver roots and sand with biofilm of each experiment, being placed separately in sterile tubes with 10 mL of saline solution (0.15 mol/L) and shaken on a vortex mixer for 30 seconds at room temperature. Serial dilutions were made in duplicate and 0.1 mL of each dilution was spread onto nutrient agar (LABM, UK) Plates were incubated at 31 °C for 3 days after which CFU were counted.

C.2 Microbial culturing

Plate culturing is the fundamental protocol for obtaining single bacteria colony and the basis for evaluating biochemical responses in individual bacteria cells (Lederberg, & Lederberg, 1952). In this study, nutrient media were from Sigma-Aldrich. Nutrient media include nutrient agar (NA), tryptic soy broth (TSB), tryptic soy agar (TSA) for supporting general microbial colonies and sabouraud dextrose agar (SDA) for yeast and fungal growth in petri dishes was made by dissolving Nutrient media in deionized water. The Nutrient media was completely dissolved in deionized water and sterilized by autoclaving. Sterilized agar was poured into petri dishes and allowed to 45°C for pour plates technique under the Holten Horizontal Laminar Airflow HBB 2472 and left until became solid for spread plates technique (Koch, 2007).

The samples often must be diluted from their original dense concentration to a sparse concentration suitable for microbial culturing, measurement of cell numbers, analysis for genetic or metabolic properties, or washing preparatory to study. Distilled or tap water alone should usually not be used as diluent, because they are osmotically hypotonic to all bacterial cells and un-buffered against pH change. A common

general-purpose diluent is phosphate buffered saline and Physiological saline (0.85% NaCl).

For pour plate technique, 1 mL diluted sample places into the middle of the petri dish before adding 45°C nutrient media and mix the sample aliquot without causing bubbles to form. This technique allows the nutrient media set solid. Incubation the inverted plates for bacterial measurement but the plates for yeast and fungal measurement in a constant-temperature incubator with good temperature regulation at 30-40°C for 3 to 5 days.

For spread plate technique, Pipette 0.1 ml of the final dilution onto the agar surface of the petri plate. Sterilize a spreader by dipping it in 70% alcohol, shaking off the excess alcohol, and igniting. A spreader can be made quickly from a glass Pasteur pipette by using a Bunsen burner. The agar surface should be dry enough that the 0.1 mL diluted sample delivered from the pipette is absorbed in 15 to 20 seconds by the agar. Then, incubate the inverted plates for bacterial measurement but the plates for yeast and fungal measurement in a constant-temperature incubator with good temperature regulation at 30-40°C for 3 days. In case of isolation of pure culture, an inoculation loop was sterilized in Bunsen burner flame and used to transfer a scoop of the bacteria from the stock to the agar. The sterilized inoculation loop was repeatedly used to make streaks on the plate as appropriate, to ensure isolated growth of distinct colonies on the plates. The inoculated petri dishes were sealed with paraffin and incubated. To count microbial colonies after incubation, the equation C1 was applied.

$$Cells, CFU/mL = \frac{total\ bacteria\ counted\ x\ dilution\ factor}{Sample\ (mL)} --Eq.C1$$

To establish preserved storage of bacteria culture, glycerol stocks were prepared. 50% glycerol solution in distilled water was autoclaved and allowed to cool under Holten Horizontal Laminar Airflow HBB 2472. About 300 µl of the sterile glycerol was pipetted into 2 ml cryotubes placed on a block of ice. A sterile inoculation loop was used to harvest biomass of the bacteria into the glycerol solution in the appropriate amount (to an end bacteria concentration of 10 to 20% of the suspension). The mixture was gently stirred with sterile inoculation loop as it cools on ice. The stocks were kept frozen at -80°C (Lederberg, & Lederberg, 1952).

APPENDIX D ENZYME ASSAYS

To comprehend the phenol degraded mechanism and its effect; the root of vetiver grass was prepared at different time periods by washing with DI water to clean up the root surface then, grinding and crashing in a mortar. Phosphate buffer pH 7.0 was used as enzyme diluent and mixed and equilibrated homogenous by inversion for 10 min. The enzyme activities were determined by using spectrometer (Genesys 10S UV-Vis). The examination of enzyme activities suggests below.

D.1 Superoxide Dismutase

Inside a root cell, enzyme Superoxide dismutase (EC.1.15.1.1) is the enzyme against reactive oxygen species (ROS), Superoxide (O_2^-) which is produced in under stressed condition to protect living cell and catalyzed the dismutation of superoxide into O_2 and H_2O_2 (Alscher et al., 2002), Superoxide dismutase activity (SOD) was developed from the method of Kuthan H. and Werringloer (Kuthan, Haussmann, & Werringloer, 1986) by using Assay Test Kit from Bioassay system. Since one unit of SOD was defined as the concentration of 50% SOD that inhibits the reduction of ferricytochrome using the absorbance of 550 nm. The detection range of 0.05 - 3 U/mL was presented in this method.

D.2 Guaiacol Peroxidase

Enzyme Guaiacol Peroxidase (EC.1.11.1.7) has been identified as major contributors to reactive oxygen species (ROS) production in plant and also involved in callose deposition and expression of defense genes (Baxter, Mittler, & Suzuki, 2014). It might be responsible for about half of H_2O_2 production. Peroxidase (POD) activity was determined by spectrometer using 420 nm. following the appearance of their colored oxidation products includes tetraguaiacol (S. Singh et al., 2006). One unit of peroxidase will form by catalyzing oxidation of 1 μmol of guaiacol in 20 second at 25°C.

D.3 Catalase

Catalase enzyme (EC.1.11.1.6) is a pervasive enzyme which catalyzes the decomposition of hydrogen peroxide (H_2O_2) to water and oxygen. Catalase activity (CAT) in this study was quantified by using the Catalase Assay Kit from Sigma-

Aldrich. One unit of catalase will decompose 1.0 mM of H_2O_2 to oxygen and water in 20 second at pH 7.0 at 25 °C at a substrate concentration of 50 mM H_2O_2.

D.4 Protein Estimation

To measure the amount of protein in the given sample by Lowry's method is the objective to estimate the quality of enzymes (Waterborg, 2002). The principal to determine is based on both the Biuret reaction, in which the peptide bonds of proteins react with copper under alkaline conditions to produce Cu^+, which reacts with the Folin reagent, and the Folin–Ciocalteau reaction, which is poorly understood but in essence phosphomolybdotungstate is reduced to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic amino acids. The reactions result in a strong blue color, which depends partly on the tyrosine and tryptophan content. The method is sensitive down to about 0.01 mg of protein/mL, and is best used on solutions with concentrations in the range 0.01 - 1.0 mg/mL of protein.

The enzyme activities and protein in this study were calculated by using the following equation;

$$\frac{\text{Units}}{\text{mL}}\text{enzyme} = \frac{\Delta\text{Absorbance,time test sample} - \Delta\text{Absorbance,time test blank}}{\text{Extinction coefficient} \times \text{Volume of enzyme used}} - - Eq.D1$$

$$\frac{\text{Units}}{\text{mg}}\text{Protein} = \frac{\frac{\text{units}}{\text{mL}}\text{enzyme}}{\frac{\text{mg protein}}{\text{mL enzyme}}} - - Eq.D2$$

APPENDIX E LIST OF AWARD AND PRESENTATIONS

E.1 Awards

The Winners of the King of Thailand Vetiver Awards 2015: The Most Outstanding Research in the field of Non-Agriculture Application in Title of "Laboratory-scaled Developments and Field-scaled Implementations of Using Vetiver Grass to Remediate Water and Soil Contaminated with Phenol and Other Hazardous Substances from Illegal Dumping at Nong-Nea Subdistrict, Phanomsarakarm District, Chachoengsao Province,Thailand" by Tanapon Phenrat, **P. Teeratitayangkul**, T. Imthiang, Y. Sawasdee, S. Wichai, T. Piangpia, J. Naowaopas and W. Supanpaiboon.

รางวัลชนะเลิศการประกวดงานวิจัยนานาชาติด้านหญ้าแฝก THE KING OF THAILAND VETIVER AWARDS รับพระราชทานจากสมเด็จพระเทพรัตนราชสุดาฯ สยามบรมราชกุมารีจากงานวิจัยหัวข้อ "Remediation of Water and Soil Contaminatedwith Phenol" ณ เมืองดานัง ประเทศเวียดนาม

E.2 Publication 1

Phenrat, T., **Teeratitayangkul, P.**, Prasertsung, I., Parichatprecha, R., Jitsangiam, P., Chomchalow, N., & Wichai, S. (2016). Vetiver plantlets in aerated system degrade phenol in illegally dumped industrial wastewater by phytochemical and rhizomicrobial degradation. *Environmental Science and Pollution Research*, 1-12.

E.3 Presentation 1

Pimpawat Teeratitayangkul, Anukool Kietkwanboot, Oramas Suttinun and Tanapon Phenrat (2018). Efficiency of Phenol Degradation by Inoculation of Rhizo-microorganisms as Biofilms on Permeable Reactive Vetiver Root Barrier (PRVRB) in Horizontal Sand Filters. 2018 International Conference on Engineering and Natural Science (ICENS2018), 30 January – 1 February 2018, Sapporo, Japan.

E.4 Presentation 2

Pimpawat Teeratitayangkul, Anukool Kietkwanboot, Oramas Suttinun and Tanapon Phenrat (2017). Efficiency of Phenol Degradation by Phytochemicals and Indigenous Rhizo-Microorganisms on Permeabe Reactive Vetiver Root Barrier (PRVRB) in Horizontal Sand Filters. The 7th International TIChE Conference (ITIChE2017), 18 - 20 October 2017, Sgangri-La Hotel, Bangkok, Thailand.

E.5 Presetation 3

Pimpawat Teeratitayangkul and Tanapon Phenrat (2016). Oxidative Stress of Vetiver Grass together with Rhizomicrobial Activity Transformed Toxic Phenol in Illegal Dumping Wastewater to Benign By-products. Society of Environmental Toxicology and Chemistry Asia/Pacific 2016 Conference(SETAC), 16 – 19 September 2016, National University of Singapore, Singapore.

E.6 Presentation 4

พิมพวัฒน์ ธีรฐิตยางกูร และ ธนพล เพ็ญรัตน์ (2558). ประสิทธิภาพของหญ้าแฝกในการสลายสารฟีนอลในน้ำเสียปนเปื้อนจากอุตสหกรรม, การประชุมวิชาการสิ่งแวดล้อมแห่งชาติ ครั้งที่ 14, 27 – 29 พฤษภาคม 2558, เชียงใหม่, ประเทศไทย