Available online at:
http://journal.unila.ac.id/index.php/tropicalsoil
DOI: 10.5400/jts.2013.18.2.161

Synergism of Wild Grass and Hydrocarbonoclastic Bacteria in Petroleum Biodegradation

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Received 28 January 2012 / accepted 10 May 2013

ABSTRACT

The concept of plants and microbes utilization for remediation measure of pollutant contaminated soil is the newest development in term of petroleum waste management technique. The research objective was to obtain wild grass types and hydrocarbonoclastic bacteria which are capable to synergize in decreasing petroleum concentration within petroleum contaminated soil. This research was conducted in a factorial by using a randomized completely block design. The first factor was wild grass type which were without plant, *Tridax procumbens* grass and *Lepironia mucronata* grass. The second factor was hydrocarbonoclastic bacteria type which were without bacterium, single bacterium of *Alcaligenes faecalis*, single bacterium of *Pseudomonas alcaligenes*, and mixed bacteria of *Alcaligenes faecalis* with *P. alcaligenes*. The results showed that mixed bacteria (*A. faecalis* and *P. alcaligenes*) were capable to increase the crown and roots dry weights of these two grasses and bacteria population, decreased percentage of TPH (total petroleum hydrocarbon) and had better pH value than that of single bacterium. The highest TPH decrease with magnitude of 70.1% was obtained on the treatment of *L. mucronata* grass in combination with mixed bacteria.

Keywords: Biodegradation, hydrocarbonoclastics, wild grass

INTRODUCTION

Oil waste as the impact of petroleum industry can produce negative effect on living organisms and their ecology. This negative impact is due to the content of oil composition which is dominated by toxic hydrocarbons coumpounds. Oil waste can be originated from oil spill and scatter during the drilling activity, refinery production and transportation, oil seepage from its reservoir, oil spill and scatter during operation of loading or unloading at harbours, oil spill from leakage or waste from tanker/ship, used or rejected oil as well as wastes from other activities. The crude oil spill can be absorbed and accumulated within soil (Pezeshki *et al.* 2000).

As the pollution impact from petroleum is relatively significant, then the technology having characteristics of simple, cheap and not producing further impact is required. One of the recommended method to accelerate petroleum degradation process is called bioremediation technique. Petroleum biodegradation can be conducted by utilizing microbes such as bacteria, some yeast types, molds, cyanobacteria and blue algae (Singh and Ward 2004). Microbes can decompose petroleum compound because of their capability in oxydizing hydrocarbons and use these substances as one of their electron donor which involved in oil spill cleaning process through oxydizing petroleum into carbon dioxide gas (CO_2).

Petroleum-polluted area is the microbia isolate source that capable to degrade petroleum. Gofar (2011) had found 3 petroleum polluted isolates of indigenous hydrocarbonoclastic yeasts from mangrove forest of Sungsang, South Sumatra that capable to degrade petroleum *in vitro*. Widjajanti *et al.* (2010) had discovered 5 bacteria isolates having relatively high capability in petroleum degradation which consisted of *Bacillus aminovorans* (53.17%), *Pseudomonas alcaligenes* (53.04%), *Alcaligenes faecalis* (52.54%), *B. cereus* (51.68%) and *B. sphaericus var rotans* (51.30%) which was isolated from petroleum polluted area within mangrove area of South Sumatra.

In addition to petroleum biodegradation by using bacteria, plants are also capable to clean polluting substances from soil. Remediation by using plant at

petroleum contaminated soil is a process known as phytoremediation. Phytoremediation is an emerging green technology that can become a promising solution to the problem of decontaminating hydrocarbon-polluted soils (Shirdam *et al*. 2008). The principle of phytoremediation is based on certain plant natural ability to bioaccumulate, degrade, or render contaminants harmless in soils, water or air. Contaminants such as crude oil and its derivative have been mitigated in phytoremediation projects. Plants such as mustard plant, alpine pennycress and pigweed have proven to be successful at hyperaccumulation of crude oil and its derivatives in soil (Mendez and Maier 2008). Phytoremediation of polluted soil involves: uptake of crude oil from soil or water, accumulation or processing of these chemicals via lignifications, volatilization, metabolization, mineralization and the use of enzymes to break down complex organic molecules into simpler molecules (ultimately carbon dioxide and water) and increases the carbon and oxygen content of soil around the roots, which promote microbial/ fungal activity and decay of root tissues (Hong *et al*. 2001).

Organic pollutants such as *polyaromatic hydrocarbons* (PAHs) can be handled by using this technique. Sorghum (*Sorghum bicolor*) and common flax (*Linum usitatissumum*) showed promising remediation efficiency in highly contaminated soil, however, petroleum hydrocarbon contamination reduced the growth of the surveyed plants significantly. Sorghum and common flax reduced TPHs concentration by 9.500 and 18.500 mg kg^{-1}, respectively, compared with the control treatment (Shirdam *et al*. 2008). Results of study by Onwuka *et al*. (2012), the use of *Cynodon dactylon L.* in the remediation of crude oil polluted soil has been clean-up crude oil polluted soil. Mean total petroleum hydrocarbon (TPH) concentration after remediation with *C. dactylon* L. showed a significant decrease when compared to the mean TPH concentration before remediation. Rossiana (2004) showed that phytoremediation of mud waste containing 20 % of oil by using "sengon" plant (*Paraserianthes falcataria* L. Nielsen) in combination with bacteria of *Pseudomonas mallei*, *Bacillus alvei* and *Bacillus nigricans* has a potential to be developed as bioremediation agent. Phytoremediation using "sengon" plant in combination with these bacteria had decreased petroleum content by 18.76% and 23.6%, respectively. Other study done by Rossiana (2005) showed the decrease of petroleum content by 51.23% using treatment combination of "sengon" plant, bacteria and mycorrhiza. The concept of plants and microbia utilization for remediation of pollutant contaminated soil is the newest development in petroleum waste processing technology. The objective of this study was to obtain the wild grass types and hydrocarbonoclastic bacteria that were capable to synergize in decreasing petroleum concentration within petroleum polluted soil.

MATERIALS AND METHODS

Study Site

The survey had been conducted at Limau Village of Prabumulih City, South Sumatra in order to get plants which are naturally grow on petroleum contaminated soil. There were two dominant grasses which grew at petroleum contaminated soil having TPH concentrations of 11.84% and 33.14% that were identified respectively as *Tridax procumbens* (songgolangit) and *Lepironia mucronata* (purun). The hydrocarbonoclastic bacteria isolates were obtained from study by Gofar (2012) which consisted of *A. faecalis* and *Pseudomonas alcaligens* bacteria.

Experimental Setup

This research was conducted by using a randomized completely block design. The first factor was wild grass type treatments which were consisted of without plant, *Tridax procumbens* grass and *L. mucronata* grass. The second factor was hydrocarbonoclastic bacteria treatments which were consisted of without bacterium, single bacterium of *Alcaligenes faecalis*, single bacterium of *P. alcaligenes*, and mixed bacteria of *A. faecalis* with *P. alcaligenes*.

Ultisol soil samples for capability testing of selected plants and bacteria in bioremediation process were taken from 0-20 cm soil layer, air dried and sieved with 2 mm hole diameter siever. Soil samples had acid reaction (pH H_2O = 4.23) with high C-organic content (37.00 g kg^{-1}), medium total N content (2.80 g kg^{-1}), high available phosphorus content (11.55 mg kg^{-1}) polluted by petroleum with TPH content of 5% and subsequently incubated for 2 weeks. Hydrocarbonoclastics bacteria were applied on soil with population of 10^{10} cells L^{-1} at dose 10 mL per kg soil and further incubated for 1 week. The 2-week old seeds of *Tridax procumbens* and *L. mucronata* were planted according to treatments and subsequently nursed for 2 months. The observed parameters were the wet weight and dry weight of crown and roots of 6-week old tested plant, soil pH at 2, 4 and 6 weeks after planting, bacteria population at 6 weeks after planting, as well

as TPH concentrations at 4 and 6 weeks after planting.

Data Analysis

Means were compared by analysis of variance for significance difference ($P<0.05$). When Anova results indicated a significant treatment effect, honestly significant difference (HSD) at $P<0.05$ were used to separate treatment means for all properties using SPSS 16 program.

RESULTS AND DISCUSSION

Growth of Crown

The effects of hydrocarbonoclastic bacteria on crown dry weight, roots dry weight and crown/ root ratio of *L. mucronata* and *T. procumbens* are given in Table 1. Table 1 showed that bacterium types had produced no difference in term of crown dry weight and root dry weight of *T. Procumbens* as well as crown to root ratio for both of wild grass types. On the other hand, *P. alcaligens* bacterium and mixing of *P. alcaligens* with *A. Faecalis* produced higher crown dry weight and root dry weight for *L. Mucronata* and was significantly difference than that of without bacterium application. The crown/root ratio of *L. Mucronata* which was less than 1 (unity) showed that its root development was faster than its crown development. This was supposed due to rhizosphere as nutrients source for bacteria which results in faster development of bacteria population. Surtikanti and Surakusumah (2004) showed that roots of *T. Procumbens* innoculated with *Pseudomonas* sp. and *Bacillus* sp. on petroleum-contaminated soil had faster development than that of one without bacteria innoculation. Bacterium of *T. Procumbens* had high value of crown/root ratio which showed that its crown growth was faster than its root growth. It was assumed that phytostabilization process by plant had been occured in which certain contaminant subtances that was not absorbed into plant branches had attached to plant roots. These substances was tightly adhered to roots so that they would not carried by the flow into plant's tissue or into soil (Mackova *at al*. 2006).

TPH concentration

The wild grass and bacteria types had significant effect on the decrease of TPH concentrations at 4 and 6 weeks after planting, whereas their interaction had significant effect on the decrease of TPH concentrations at 6 weeks after planting such as shown in Table 2. Application of bacteria, plants and their interaction had produced significant effect on decrease of TPH concentration at 6 weeks after planting. Application of mixed bacteria (*A. faecalis* and *P. alcaligenes*) had produced lower TPH concentration and significantly difference than that of without isolates and single isolate of *A. Faecalis* or *P. alcaligenes*. Interaction of inter-species microorganisms within mix culture is paramount important for degradation process of hydrocarbon compounds. The complex reaction which involved many enzymes produced by microbes was occured at hydrocarbon degradation process. According to Mukred *et al*. (2008), lighter and simpler hydrocarbon compounds will firstly degraded by the bacteria culture and subsequently followed by more complex compounds. The use of mix culture of microbes in hydrocarbon degradation is better than that of single culture because the synergize work from microbes mix culture will affect hydrocarbon degradation process (Sathiskumar *et al*. 2008).

Table 1. Crown dry weight (g), root dry weight (g), and crown/root ratio of *L. mucronata* and *T. procumbens* grasses.

Grass types	Bacterium types			
	Without isolate	*A. faecalis*	*P. alcaligenes*	*A. faecalis* and *P. alcaligenes*
	Crown dry weight (g plant^{-1})			
L. mucronata	0.893 a	1.280 a	1.823 b	1.890 b
T. procumbens	0.433 a	0.643 a	0.480 a	0.770 a
	Root dry weight (g plant^{-1})			
L. mucronata	0.957 a	1.343 a	1.890 b	2.177 b
T. procumbens	0.130 a	0.177 a	0.117 a	0.207 a
	Crown/ root ratio			
L. mucronata	0.933 a	0.947 a	0.943 a	0.867 a
T. procumbens	3.613 a	3.807 a	3.683 a	3.530 a

Values followed by the same alphabet on the same row showed the insignificant difference.

Table 2. The effect of bacteria and plants on TPH concentration.

Bacteria types	TPH concentration (%) on treatments			Mean
	Without plant	*Lepironia mucronata*	*Tridax procumbens*	
	Observation at 4 weeks after planting			
Without bacterium	4.40	3.90	3.98	4.09 a
Alcaligenes faecalis	3.95	3.34	3.87	3.72 a
Pseudomonas alcaligenes	3.75	3.64	3.62	3.67 a
Alcaligenes faecalis and *Psedumonas alcaligenes*	3.61	2.31	2.87	2.93 b
Mean	3.93 a	3.30 b	3.58 ab	
	Observation at 6 weeks after planting			
Without bacterium	4.40 a	2.10 bc	2.02 bc	2.84 p
Alcaligenes faecalis	1.84 bc	1.82 c	1.91 bc	1.86 qr
Pseudomonas alcaligenes	2.67 b	1.82 c	1.94 bc	2.14 q
Alcaligenes faecalis dan *Pseudomonas alcaligenes*	1.65 c	1.32 c	1.51 c	1.50 r
Mean	2.64 x	1.77 y	1.84 y	

Honestly Significance Difference (HSD) for bacteria and plants are 0.674 and 0.582, respectively.

Plant of *L. Mucronata* or *T. Procumbens* can significantly decrease TPH at contaminated soil than that of without plant. Plant provides root exudates which consisted of carbon, energy, enzymes and oxygen that are required by microbes for their growth. The roots of plant can also release degradative enzymes into rhizosphere that capable to degrade carbon compounds from petroleum (Wenzel 2009). Moreover, due to direct release of degradative enzymes, plants are capable to stimulate the activity of degrading microbe organisms. Microbe population increment can increase organic contaminant degradation within rhizosphere. These enzymes are consisted of dehalogenase, nitroreductase, peroxidase, lactase and nitrilase. Moreover, alelopathy chemical substance released by roots of plant results in catabolic enzyme on degrading organisms which in turn increase the pollutant structural rhizodegradation. The main alelopathies on rhizodegradation are consisted of flavonoide and other chemical substances such as hirsutine, 2(3H)-benzoxazolinone or cyanide (Glick 2010).

The best treatment combination between bacteria and plants in decreasing TPH concentration was mixture of *A. faecalis* and *P. alcaligenes* in combination with *L. mucronata* plant that capable to decrease TPH concentration up to 1.32% compared to initial TPH concentration (5.00%) within 6 weeks after planting. The mixture of *A. faecalis* and *P. alcaligenes* bacteria had produced better hydrocarbon degradation process than that of single bacteria. Khashayar and Mahsa (2010) had reported that bacteria mix culture tested on several concentrations and petroleum mix were capable to decrease more than 80% of TPH concentration from its initial value. The use of *L. mucronata* in this study was indirectly capable to degrade petroleum hydrocarbon. The indirect role of plant that capable to degrade hydrocarbon is due to its capability to releases enzymes from roots such as nutrients, enzymes and sometimes oxygen for microbes within rhizosphere. In this case, plant induces bacteria population increment and increases the organic contaminants degradation within rhizosphere (Glick 2010).

Phytoremediation is environment-friendly as well as cost-effective, but may take more time than the conventional methods because it is a natural process (Ndimele 2010). One specific subset of phytoremediation, called rhizomediation, presumably occurs through the breakdown of organic contaminants in the root zone by soil microbes. Previous reviews of rhizosphere degradation of petroleum contaminants have discussed the complexity of rhizosphere controls on organic contaminant degradation (Newman and Reynolds, 2004; Gerhardt *et al*. 2009; Wenzel 2009), but have not yet compared the possible effect of different kinds of vegetation on rhizoremediation. Advancement and optimization of this technology

may depend on planting the most appropriate vegetation (Cook and Dean 2013).

In term of without bacterium treatment (Table 2), better TPH decreasing was found in the treatments by using *L.mucronata* and *T. procumbens* plants than that of without plant. This is due to the fact that plant capable to produce root exudates which can stimulate bacteria growth so that their activity in degrading hydrocarbon compounds will increase. Plant root exude a variety of chemicals that can act as biosurfactants, stimulate induction of microbial catabolic enzymes, and promote co-metabolisms. Co-metabolism occurs when a microbial enzymes produced for the metabolism of one substrate can also degrade a secondary substrate (the contaminant), with no additional energy expenditure or nutrition requirements (Singer *et al*. 2003).

Taxonomic characteristics of these isolates identified them as *Alcaligens faecalis* and *Pseudomonas alcaligens*. Sathishkumar *et al*. (2008), Lal and Khanna (1996) Rahman *et al*. (2002), and Malik and Ahmed (2012) had reported that *Bacillus* sp, *Staphylococcus* sp, *Micrococcus* sp, *Pseudomonas* sp, *Psychrobacter* sp, and *Alcaligens faecalis* are able to biodegradable some petroleum commercial products. *Micrococcus*, *Staphylococcus*, *Pseudomonas putida* and *Alcaligene*s were also reported to degrade diesel oil. The pattern of degradation showed that the microorganisms first attacked the lower and higher hydrocarbon chains and those of middle length were attacked later in the course of incubation (Bello 2007). Considerable information on the microbial degradation as defined, sole hydrocarbons is available in the literature, but less is known on the biodegradability of some petroleum commercial products such as kerosene. The dominant

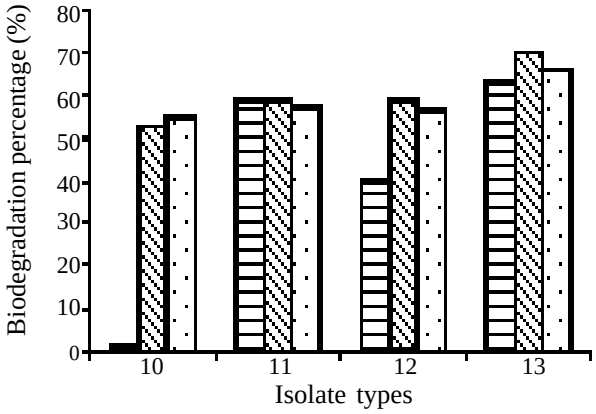

Figure 1. Biodegradation percentage of TPH (*Total Petroleum Hydrocarbon*) on the sixth week. ▭ = without plant, ▨ = *L. mucronata*, and ▩ = *T. procumbens*.

mechanism that breaks down these petroleum products is biodegradation, which is carried out by natural microbial populations (Malik and Ahmed 2012).

The effect of hydrocabonoclastic bacteria application at petroleum contaminated soil on the percentage of TPH decreasing was shown in Figure 1. This figure shows that TPH biodegradation percentage at contaminated soil which was inoculated with hydrocarbonoclastic bacteria was higher than that of control treatment (without inoculation and without plant). The control treatment had very slow hydrocarbon degradation with magnitude of 0.5% degraded TPH. This was in accordance with the study conducted by Surtikanti and Surakusumah (2004) which showed that control treatment had very slow hydrocarbon degradation. Although indigenous bacteria were available within soil, but they had no role in hydrocarbon decreasing process. The highest value of TPH degradation (70.1%) was found on treated soil with mix bacteria (*A. faecalis* and *P. alcaligenes*) and planted with *L. mucronata.* This was due to interaction between *L. mucronata* and mix bacteria in hydrocarbon degradation process.

Higher percentage of petroleum hydrocarbon biodegradation was obtained by using mix bacteria culture than that of single bacterium culture. This is due to the fact that bacteria applied in petroleum degradation process usually have higher capability if they are used as mix culture. Mix culture has more complete enzyme profile than single culture. According to Mukred *et al*. (2008), lighter and simpler hydrocarbon compounds can be firstly degraded at initial stage by bacteria culture and subsequently the undegradable compounds at initial stage are degraded by second culture. According to Glick (2010), complex compound structure such as petroleum hydrocarbon cannot be degraded completely by single bacterium species because each bacterium species requires certain substrates. Beneficial mutualism from bacteria interaction in form of mix culture has important role in petroleum degradation. Widjajanti (2012) had reported that petroleum bioremediation by using mix culture of hydrocarbonoclastic bacteria and yeast with mangrove used as testing plant can produce petroleum concentration of 1% within 16 days period.

The combination of *Alcaligenes* and *Pseudomonas* bacteria produced better biodegradations process than that of single culture. According to Malik and Ahmed (2012), these two genera are capable to degrade saturated hydrocarbon, monoaromatic hydrocarbon, and

polyaromatic hydrocarbon. It is predicted that due to their diverse capabilities in degrading hydrocarbon compounds, they can degrade hydrocarbon compounds either having low or high molecular weight. Milic *et al.* (2009) had explained that single microbe can only degrade specific oil compounds, whereas the mix culture can degrade higher level of oil compounds. The common life of some microbe types even will produce mutually benefit activities.

One of parameter which is used to monitor the occurrence of hydrocarbon compounds degradation by bacteria is the change of pH. Fluctuation of pH is an indicator to determine whether or not a process has been occurred. The decrease of pH values at the end of study either on treatment with plant and without plant (Figure 2) showed the existence of bacteria activities in hydrocarbon compounds degradation. According to Pikoli *et al.* (2000), pH is one of factor that determines bacteria life in degradation process of a substance. The activities of hydrocarbon compound degrading microorganisms are affected by environmental condition such as pH and temperature so that unsuitable environmental conditions cause microbes to be inactive in conducting degradation process.

Figure 2 showed relatively significant decrease of pH from the first to the sixth week of observation. This might due to accumulation of metabolism products from microbe itself. Degradation process is not only depend on chemical structures, but it is also affected by environmental factors such as pH, temperature and oxygen availability in the environment. This process may produce compounds such as CO_2 and organic acids which results in decreasing pH of medium. Pilon-Smith and Freeman (2006) had stated that microorganisms which conduct degradation process will produce acid compounds such as lactic acid, acetate acid and piruvate acid that may results in change of pH. The change of pH is also occurred if microorganisms use ammonium compounds as nitrogen source alternative for their growth. During the on going of biodegradation process, pH will also change if microbe utilize ammonium compound in form of NH_4^+. This microbe will cluster ammonium compound into cells as R-NH_3^+ with R as carbon sceleton. This process releases H^+ ions into environment so that accumulation of H^+ will occur during the on going degradation process resulting in decreasing pH value.

Hydrocarbonoclastic Bacteria Population

Hydrocarbonoclastic bacteria population within soil after 6 weeks application is given in Table 3.

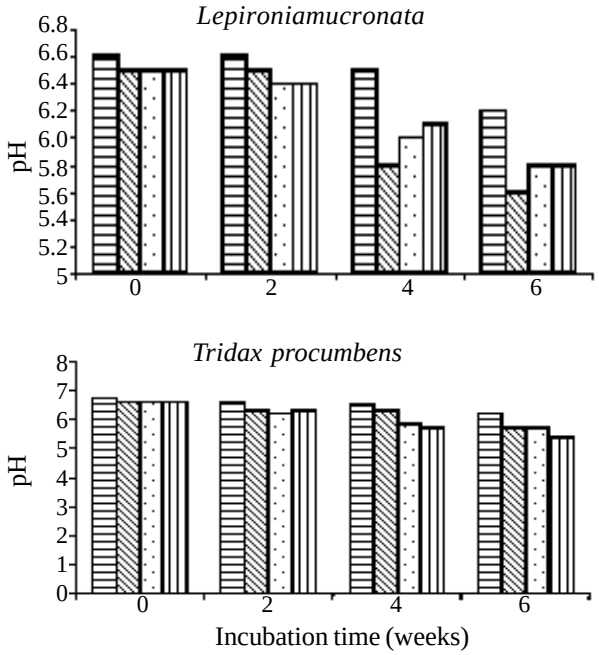

Figure 2. The weekly pH values due to application of several hydrocarbonoclastic bacteria and plant types. ■ = without bacteria, ■ = *A. faecalis*, ■ = *P. alcaligenes*, and ■ = *A. faecalis* and *P. alcaligenes*.

This results indicated that mix isolates application (*A. faecalis* and *P. alcaligenes*) produced higher hydrocarbonoclastic bacteria population and significantly difference than bacteria population within soil without isolate or having single isolate. The more bacteria colony is available in degradation process, the more optimum is the microbe activity available in degrading organic compound. The increasing trend of bacteria population cause increase in activity to utilize hydrocarbon compound within growth medium. Microorganisms growth are indicated by population increasing which in turn also increase microorganisms activity. Microorganisms will adapt to oil environment at initial stage and followed by growth stage in which bacteria cells will increase and available hyrdocarbon compound (substrate) will further decrease due to microorganisms activity. Results of this study showed that increasing population of hydrocarbon compounds degrading bacteria was followed by decrease of TPH. This was affected by the availability of organic exudates produced by roots of plant which can be used as nutrients source by bacteria. Nutrients availability for bacteria will results in growth and increasing population of bacteria within soil which in turn capable to increase hydrocarbon compounds degradation. The increasing degradation of hydrocarbon produce energy and carbondioxide which is used by bacteria and plants

Table 3. Hydrocarbonoclastic bacteria population within soil at 6 weeks after the treatment.

Treatment effect	Mean population of bacteria (log cfu g^{-1})
Bacteria:	
Without bacterium	4.918 a
Alcaligenes faecalis	5.269 a
P. alcaligenes	6.301 ab
Alcaligenes faecalis and *P. alcaligenes*	7.131 b
Plants:	
Without plant	5.348 a
Lepironia mucronata	6.135 a
Tridax procumbens	6.230 a

Values followed by the same alphabet on isolates or plants treatment showed insignificant difference with Honestly Significance Difference (HSD) for isolates and plants with magnitude of 1.259 and 1.091, respectively.

either directly or indirectly for photosynthesis process (Ndimele 2010).

Table 3 showed that bacteria population for *L. mucronata* and *T. Procumbens* plants treatment was higher than that of without plant treatment. This was assumed due to mutually benefit interaction between roots of plant and bacteria within rhizosphere zone. Bacteria are capable to provide energy and CO_2 which are produced from degradation process of hydrocarbon compounds. Energy and CO_2 are utilized by plants for photosynthesis process so that capable to produce root exudates. Exudates from roots of plants are used by bacteria as their nutrients source. Singh and Ward (2004) stated that bacteria and roots of plant within rhizosphere form a symbiosis. Bacteria population magnitude is affected by exudates in form of nutrients and other organic substances produced by plant's roots, whereas bacteria have a role to protect plants from toxic effect of petroleum. The higher the root exudates are produced by plants, the higher the bacteria population is available within rhizosphere zone.

CONCLUSIONS

The use of mixed hydrocarbonoclastic bacteria (*Alcaligenes faecalis* and *Pseudomonas alcaligenes*) was capable to increase the crown dry weight and root dry weight which had the role in phytoremediation, increased bacteria population as well as decreased TPH and pH in better way than that of single bacterium.

The *Lepironia mucronata* plant was capable to decrease TPH which was significantly difference than that of without plant at the fourth week after planting, whereas *Tridax procumbens* produced TPH concentration differences at the sixth week after planting. The highest decreasing TPH with magnitude of 70.1% was obtained from mixing of *Alcaligenes faecalis* and *Pseudomonas alcaligenes* bacteria with *Lepironia mucronata* grass.

ACKNOWLEDGEMENTS

We acknowledge our undergraduate and postgraduate students (Rodiah and Nasrul Harahap) which assisted in this study, Laboratory of Biological, Chemical and Soil Fertility of Agricultural Faculty, Sriwijaya University as well as Postgraduate School Program of Sriwijaya University that provided facilities for this study implementation.

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