Bioremediation of petroleum hydrocarbon contaminated soil by *Rhodobacter sphaeroides* biofertilizer and plants

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Abstract: Bio-augmentation is a promising technique for remediation of polluted soils. This study aimed to evaluate the bio-augmentation effect of *Rhodobacter sphaeroides* biofertilizer (RBF) on the bioremediation of total petroleum hydrocarbons (TPH) contaminated soil. A greenhouse pot experiment was conducted over a period of 120 days, three methods for enhancing bio-augmentation were tested on TPH contaminated soils, including single addition RBF, planting, and combining of RBF and three crop species, such as wheat (W), cabbage (C) and spinach (S), respectively. The results demonstrated that the best removal of TPH from contaminated soil in the RBF bio-augmentation rhizosphere soils was found to be 46.2%, 65.4%, 67.5% for W+RBF, C+RBF, S+RBF rhizosphere soils respectively. RBF supply impacted on the microbial community diversity (phospholipid fatty acids, PLFA) and the activity of soil enzymes, such as dehydrogenase (DH), alkaline phosphatase (AP) and urease (UR). There were significant difference among the soil only containing crude oil (CK), W, C and S rhizosphere soils and RBF bio-augmentation soils. Moreover, the changes were significantly distinct depended on crops species. It was concluded that the RBF is a valuable material for improving effect of remediation of TPH polluted soils.

Keywords: *Rhodobacter sphaeroides*; bioremediation; microbial communities; phospholipid fatty acid; petroleum hydrocarbon contaminated soil.

INTRODUCTION

Many microorganisms in the environment are capable to mineralize a large variety of petroleum hydrocarbon and/or to break down them to their less-toxic metabolites. Technique of bioremediation has been recognized as an attractive decontamination strategy for a variety of polluted environments. Bio-stimulation and bio-augmentation were two major bioremediation schemes that have been proposed. Bio-stimulation is defined as addition of various forms of rate-limiting nutrients and electron acceptors such as nitrogen, phosphorus, carbon, or oxygen in order to increase the population or activity of naturally occurring microorganisms available for bioremediation. Bio-augmentation is defined as the addition, to the polluted environment, of microorganisms that can biodegrade or bio-transform contaminants. The added microorganisms may functioned as the degrader for the contaminants and the stimulating factors which speed up the evolution of the local microbial communities (Yu *et al.*, 2005).

In general, microbial diversity and enzymes activity are primarily responsible for maintaining soil quality and degrading organic matter into suitable forms, e.g., for plant uptake in the cycling of nutrients. Moreover, they are very sensitive and rapidly response to the environmental perturbations. Therefore, microbial biomass and enzyme activities can provide useful information regarding soil and ecosystem health (Brohon *et al.*, 2001). Petroleum hydrocarbon (PH) present in soil may exhibit toxic activity towards different plants, soil microorganisms and invertebrates. Being in intimate contact with the soil environment, soil microorganisms are considered to be the best indicators of soil contaminants.

Using microbial biomarkers to identify microorganisms and characterize changes in microbial communities has been increasingly popular. One of the most useful biomarkers are phospholipid fatty acid (PLFA), which are membrane-bound cell components and have great structural diversity coupled with high biological specificity. Recently, PLFA method has been widely applied in bioremediation studies to estimate the responses of microbial biomass, community structure to the changes in soil microenvironment (Eibes *et al.*, 2006). Soil enzyme activities are sensitive to the soil management practices such as crops cultivated, application of fertilizers and herbicides. The effect of bio-augmentation on enzyme activities in soil has been reported widely (Bhattacharyya *et al.*, 2005). For examples, Han *et al*. reported that *Rhodobacter sphaeroides* could improve the degradation of dichlorvos, which was one of widely used organophosphate

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insecticides (Han *et al.*, 2010). It was reported that the *R. sphaeroides* could produce 5-aminolevulinic acid, which can not only be used as green herbicides, pesticides, plant growth promoters but also enhance the crops salt resistance and cold resistance. However, few works were studied on the effect of R. Sphaeroides biofertilizer on the degradation of petroleum hydrocarbon in contaminated soil.

The object of this study was to evaluate the impact of *R. sphaeroides* biofertilizer on bioremediation of petroleum hydrocarbon contaminated soil. PLFA profiles were used to reveal changes in the microbial community structure. Losses of total petroleum hydrocarbon (TPH) and the activities of soil enzyme were used to be the indicators for whether the microbial community functioned in TPH contaminated soil. The results of this study will be important to evaluate the potential of RBF in bioremediation to contaminated soil.

MATERIALS AND METHODS

Soil used in this experiment was collected from the top layer (0-25cm) of a farmland located in Fangshan County of Beijing, China. The soil properties were defined by the total organic carbon (TOC) 5.6 %, total nitrogen 0.14%, phosphorus 0.09%, pH8.3, electrical conductivity (EC) 0.3 (ms/m), sand 42.2%, silt 42.5%, clay 16.3%. The experiment was conducted in a greenhouse and the plants grew with natural light conditions. During the period of plant growth, the day temperature ranged from 18-30°C and the night temperature from 15-20°C. The pots (diameter and height 20cm × 30cm) used in the experiments contained 3kg of soil dry weight. The pots were sealed at the bottom to avoid chemical loss when watering. All the pots were watered at 450ml·kg^{-1} to reach saturation, and thereafter the soil was watered as needed, and the relative humidity was maintained at 70%. This experiment ran for 120 days.

This soil did not require nutrient supplements. Crude oil (Dagang oil field, China) was added to reagent grade acetone and mixed with the soil in 6800mg·kg^{-1} dry soil. The soil was sufficient mixed at room temperature to promote strong binding of the chemicals to the soil, and kept in cool and ventilated environments for 72h in the dark. The soil containing crude oil was then placed in pot (3kg) for the experiments, which were marked as CK-NO.

R. sphaeroides biofertilizer (RBF), obtained from Zhongke Huoli Biotechnology Company, LTD. Wuxi, China, with the amounts of activity bacterium 1 × 10^9 CFU/ml, was sprayed on the soil at 20ml·kg^{-1} of soil. The soils were mixed to achieve contact of the RBF with the soil, and were marked as CK+RBF.

Basing on their prevalence and potential usefulness three different crops wheat (*Triticumae sativum* L.) (W), cabbage (*Brassica oleracea* L. *var. capitata*) (C) and spinach (*Spinacia oleracea*) (S) were selected for indicator. Thirty grain of these plant seeds were sown in each pots soil, these were marked as crop-NO (include W-NO, C-NO, S-NO) in soil without RBF, and CK+RBF, crop+RBF (include W+RBF, C+RBF, S+RBF) in soils with RBF bio-augmentation, respectively. Three replicates were prepared for each treatment and three analysis samples from each replicate.

Standard chemicals of 19 fatty acid methyl esters were obtained from Matreya Inc. (Pleasant Gap, PA, USA). The approximately 40g of soil was collected from a depth of 0-25cm during the growing period. The soil samples were homogenized, visible plant residues were removed, and the soil samples were then passed through a 0.5-mm sieve. The soil was stored immediately at -20°C for PLFA analysis. The soil microbial PLFA was extracted based on the method from Bligh and Dyer (Bligh and Dyer, 1959) and analyzed with an Agilent 6890 Gas Chromatograph and 5973 Mass Spectrometer (GC-MS). The TPH content of the soil was measured with the weighted method (Rippen, 1999). Enzyme activity was measured using the method developed by Bao (Bao, 2005). Briefly, Dehydrogenase (DH) assays were performed using INT (2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazolium chloride) as an artificial acceptor. INTF (iodo-nitrotetrazolium formazan) was measured in a spectrophotometer at 490 nm. Urease (UR) activities were determined as the ammonia released in the hydrolysis reaction. The NH_3-N formed was determined in a spectrophotometer at 578nm. Phosphatase activity was determined using PNPP (p-nitrophenyl phosphate disodium) as substrate. The p-nitrophenol (PNP) formed was determined in a spectrophotometer at 398 nm. A unit (U) of enzyme activity was defined as the micrograms of substrate transformed at 37°C·1h by1g of dried soil.

One-way analysis of variance (ANOVA) was used to assess the total amount of PLFAs, microbial biomass in the pot soils. Correlation analysis was carried out using Spearman's rank correlation coefficients to assess the TPH remove, enzyme activity, distributions (%) of PLFA in the soils. Principal component analysis (PCA) of the mol% of PLFAs was used to compare the PLFA patterns of the sample soil with or without RBF. The factor loading scores for individual PLFAs were used to assess the relative importance of each PLFA. All statistical analyses were performed using Excel and SPSS 17.0.

RESULTS

The effect of RBF on the composition of PLFA was characterized in each sample soil (table 1). Fatty acids ranging from C13 to C19 were identified, including saturated (SAT), monounsaturated (MUFA), branched (BR), polyunsaturated (PUFA) and CYCLO species. The

majority of PLFAs in CK-NO bulk soil was terminal branched saturated fatty acids (TBSAT), which are considered to be indicative of gram-positive bacteria (G^+). The majority of PLFAs in W-NO, C-NO and S-NO rhizosphere soils were SAT, which is considered to be indicative of general bacteria. The majority of PLFAs in W+RBF, S+RBF bio-augmentation rhizosphere soils were SAT, but in C+RBF soil was TBSAT.

On the other hand, the changes in the numbers of soil PLFAs showed higher differences depended on crop species between the RBF bio-augmentation and no RBF soils. The average number of PLFAs was 9.5 in CK-NO soils, and was 10 in crop-NO rhizosphere soils, but was 14 in crop+RBF bio-augmentation rhizosphere soils.

There is a significant difference in the PLFA patterns between soils with and without RBF. The relative concentrations of some types of SATs, e.g., C15:0 and C16:0, compared to CK-NO soil, showed a significant increase, but the TBSAT, e.g., iC13:0, iC15:0 showed a decrease in CK+RBF bio-augmentation bulk soil. Compared to C-NO rhizosphere soil, the relative concentrations of some types of MBSAT, e.g., 10me14:0, and MUFAs, e.g., C16: 1ω7t, SATs, e.g., C16:0, were decreased, but TBSATs, e.g., i15:0 were increased in C+RBF bio-augmentation rhizosphere soils. Compared to S-NO rhizosphere soil, the SAT, e.g., C15:0, C16:0 were decreased, but TBSAT and MUFA, e.g., i15:0, 16:1ω7t, 18:1ω9t, were increased in S+RBF bio-augmentation rhizosphere soil (table.1).

The concentrations of middle branched saturated fatty acids (MBSAT) that represent actinobacteria, compared to CK bulk soil, have increased by 123%, 24.8% in W-NO, G-NO rhizosphere soils, respectively, but decreased by 11.9% in S-NO rhizosphere soil. The concentration of MBSAT compared to CK-NO soils, had increased by 33.5% in RBF bio-augmentation CK+RBF soil, but decreased by 99.3%, 64.6% in bio-augmentation C+RBF, S+RBF rhizosphere soils respectively.

The concentration of polyunsaturated fatty acids (PUSAT) that represents fungi, had not found in CK bulk soil, but had increased in rhizosphere soils, except S, such as W-NO (1.3nmol g^{-1}), C-NO (0.1nmol g^{-1}). It had increased in bio-augmentation soil, e.g. CK+RBF (0.72nmol g^{-1}), W+RBF (1.4nmol g^{-1}), C+RBF (1.7nmol g^{-1}), S+RBF (6.9nmol g^{-1}) soil, respectively.

The total PLFAs concentration, compared to CK bulk soil, had increased by 26.7% in CK-RBF soil, and had increased by 148%, 11.5%, 80.4%, in W, C, S rhizosphere soils, respectively; It compare to rhizosphere soils, increased by 2.0%, 28.6%, 273% in bio-augmentation W+RBF, C+RBF, S+RBF, respectively. The concentration of soil microbial total PLFA was maximum in S-RBF rhizosphere soil followed by W+RBF and C+RBF of it.

Changes in the microbial community structure determined by Principal Component Analysis (PCA) are presented in fig 1. PC1 (26.9%) and PC2 (22.9%) accounted for 49.8% of the variation within the data. Soil microbial PLFAs indicated a higher difference between rhizosphere soils with and without bio-augmentation. PCA showed that the distinctions were about 26.9% in microbial communities between C-RBF and C-NO, or S+RBF and S-NO, and the differences were 22.8% between W-NO and W+RBF soils (fig. 1).

The PLFA loading plot shows that for PC1, the variation observed among the samples was caused by the same 7 PLFAs (C17:0, C16:0, 18:1ω7t, 18:1ω9t, 18:1ω10, 18:2ω6, 9); for PC2, the variation observed among the samples was caused by the same 4 PLFAs (i15:0, 16:1ω7t, 10meC14:0, i14:0). Significant species differences were observed in 4 (C15:0, C16:0, i13:0, and i15:0) of the 11PLFAs between CK-NO and CK+RBF soils. The18: 1ω7t was significantly higher in W+RBF than in W-NO soil. The i15:0, 18:1ω7t, 18:2ω6, 9 were significantly higher in C+RBF than in C-NO soil. The proportions of 4 PLFAs (iC15:0, 16:1ω7t, 18:1ω9t, a14:0) were significantly higher in S+RBF than in S-NO soil.

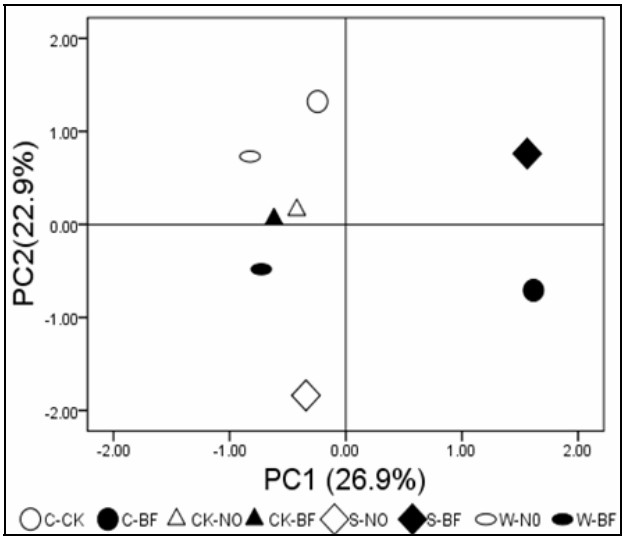

Fig. 1: Principle components analysis of PLFA profiles from soil microbial communities in CK, wheat (W), cabbage (C), and spinach (S) soils with (RBF) or without (NO) Bio-fertilizer

Activity of dehydrogenase (DH) increased in soils, which added bio-fertilizer and growth of plants. Such as, activity of DH, compared to CK bulk soil, had increased by 8.1% in RBF bio-augmentation CK soil, and increased 50.6%, 81.0%, and 83.8% in no RBF bio-augmentation W, C, S rhizosphere soils. The activity of DH compared to rhizosphere soils, had increased by 48.4%, 55.9%, 93.3% in RBF bio-augmentation W+RBF, C+RBF, S+RBF rhizosphere soils, respectively (fig. 2 A).

Table 1: Microbial community structure indicated by PLFA distribution, the concentration of PLFA (nmol·g^{-1}dry weight soil) in different treatments

PLFAs	CK		Wheat (W)		Cabbage (C)		Spinach (S)	
	NO	RBF	NO	RBF	NO	RBF	NO	RBF
C14:0	nd	nd	nd	nd	nd	0.53	0.26	1.96
C15:0	nd	2.99	nd	nd	nd	5.12	11.09	22.08
C16:0	1.38	14.50	21.87	21.46	8.45	nd	19.86	26.47
C17:0	0.07	nd	0.54	nd	nd	0.14	nd	3.02
C18:0	0.35	0.53	2.13	0.83	0.84	0.64	nd	2.63
i13:0	2.53	nd	5.92	7.07	0.37	nd	nd	8.48
i14:0	nd	nd	nd	nd	nd	nd	3.08	nd
i15:0	11.29	nd	3.69	1.89	1.45	13.08	2.28	31.23
i16:0	nd	nd	nd	0.82	1.27	nd	nd	5.07
a14:0	nd	nd	nd	nd	2.80	2.77	nd	12.14
a16:0	0.64	0.33	1.21	3.09	0.27	0.60	nd	4.89
10me14:0	4.69	6.27	10.47	10.66	4.83	nd	4.13	1.46
10me17:0	nd	nd	nd	nd	1.03	0.04	nd	nd
16:1ω7t	3.84	4.17	11.03	nd	4.81	0.43	nd	28.59
18:1ω9t	0.69	2.72	7.13	6.47	1.56	2.93	nd	8.37
18:1ω10	0.79	1.06	nd	nd	1.46	1.85	nd	11.84
18:1ω7t	nd	nd	nd	12.93	nd	7.80	6.68	1.66
18:2ω6,9	nd	0.72	1.30	0.94	0.14	1.21	nd	4.45
16:3ω3,6,9	nd	nd	nd	nd	nd	0.49	nd	nd
cy19	nd	nd	nd	0.45	nd	nd	nd	2.51
SAT (nmol·g^{-1})	1.80	18.02	24.55	22.29	9.29	6.43	31.21	56.16
TBSAT (nmol·g^{-1})	14.46	0.33	10.82	12.87	6.17	16.46	5.37	61.83
MBSAT (nmol·g^{-1})	4.69	6.27	10.47	10.66	5.86	0.04	4.13	1.46
MUSAT (nmol·g^{-1})	5.31	7.95	18.15	19.39	7.83	13.02	6.68	50.47
PUSAT (nmol·g^{-1})	0.00	0.72	1.30	0.94	0.14	1.70	0.00	4.45
CYCL (nmol·g^{-1})	0.00	0.00	0.00	0.45	0.00	0.00	0.00	2.51
Total (nmol·g^{-1})	26.27	33.28	65.29	66.61	29.29	37.65	47.39	176.87

Note: nd represent no detective in the soil

The general trend of alkaline phosphatase (AP) activity in response to various treatments was different to that of DH activity. The activity of AP, compared to CK bulk soil, had increased by 23.0% in RBF bio-augmentation CK+RBF soil, and increased by 22.8%, 22.7%, 22.7% in W,C, Srhizophere soils. But compared to rhizophere soils, it decreased by 20.9%, 12.7% and 12.5% in RBF bio-augmentation W+RBF, C+RBF, S+RBF rhizosphere soils (fig. 2 B).

The activity of urease, compared to CK bulk soil, had decreased by 14.0% in RBF bio-augmentation CK+RBF soil, and decreased by 37.2%, 22.4%, 44.1% in W, C, Srhizosphere soils, respectively. The activity of urease, compared to rhizosphere soil, had increased by 96.9%, 3.8% and 45.5% in RBF bio-augmentation W+RBF, C+RBF, S+RBF rhizosphere soils (fig. 2 C).

The TPH concentrations in initial soil was 6800mg·kg^{-1}, and not significantly different ($p>0.05$) among all pot soils. The losses of TPH, compared to initial soils, represented 26.1%, 46.2%, 54.7%, 59.3% of TPH in CK-NO, W-NO, C-NO, S-NO no bio-augmentation soils, and losses 32.7%, 46.2%, 65.4%, 67.5% in CK+RBF, W+RBF, C+RBF, S+RBF bio-augmentation soils after 4 months, respectively (fig. 3). The losses of TPH, compare to CK bulk soil, have increased by 25.1% in RBF bio-augmentation CK+RBF soil, and increased by 76.8%, 109.2%, and 127.0% in W, C and S rhizosphere soils. The remove of TPH, compared to rhizosphere soils, have increased by 0.01%, 19.65, 13.7% in RBF bio-augmentation W+RBF, C+RBF, S+RBF rhizosphere soils, respectively. The losses of TPH, compared to rhizosphere soils, had slight increased and the change was higher in C+RBF or S+RBF than W+RBF in the RBF bio-augmentation rhizosphere soils.

The correlation analysis between the losses of TPH and activity of three enzyme indicated that there was a significant positive correlation to DH, AP, which pearson's correlation coefficient was 0.99, 0.93, respectively, but negativity to urease, which pearson's correlation coefficient was -0.84 in soil without RBF. And had significant positive correlation toDH, correlation

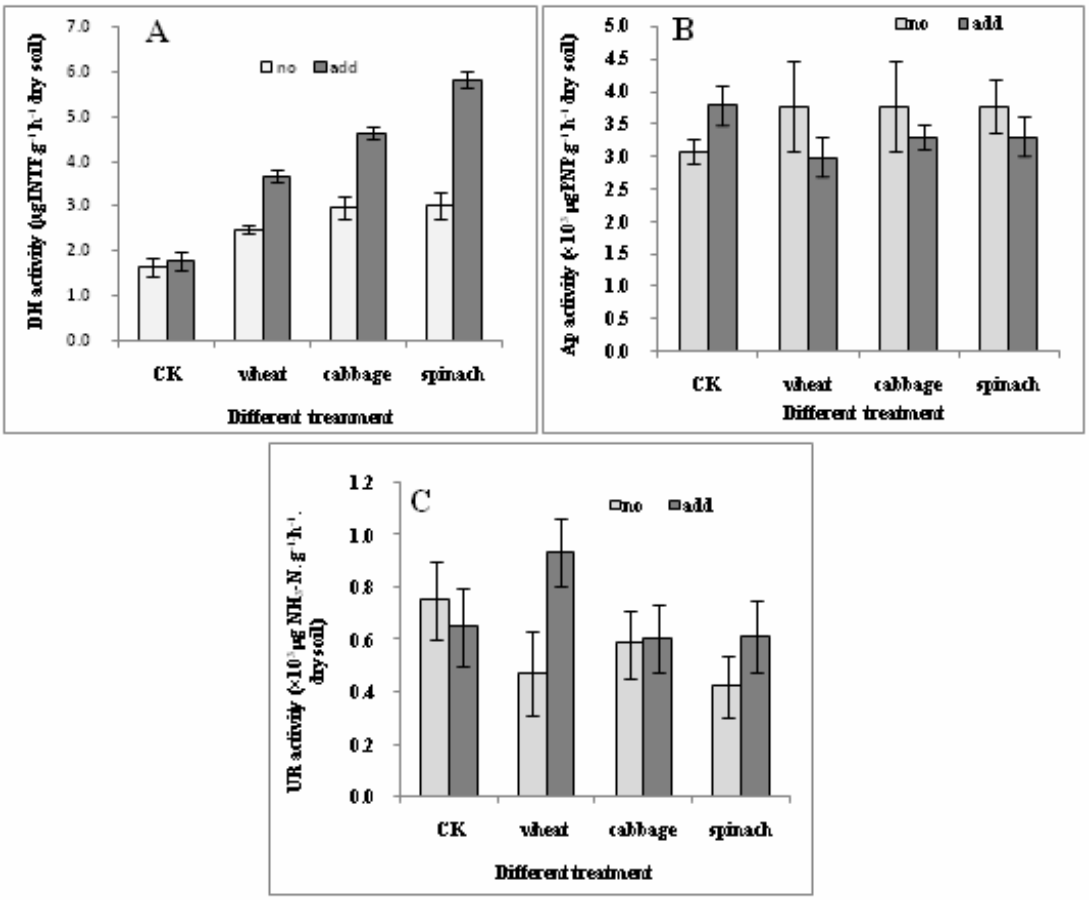

Fig. 2: Soil enzyme activity response to different treatments A. Dehydrogenase activity (DH); B. alkaline phosphomonoesterase (AP) activity; C. Urease (UR) activity; add present added *R. Sphaeroides* bio-fertilizer in soils, no present not added in soils.

coefficient was 0.96, but had low negativity to AP and urease activity, which pearson's correlation coefficient were -0.47 and -0.38 in the RBF bio-augmentation soils.

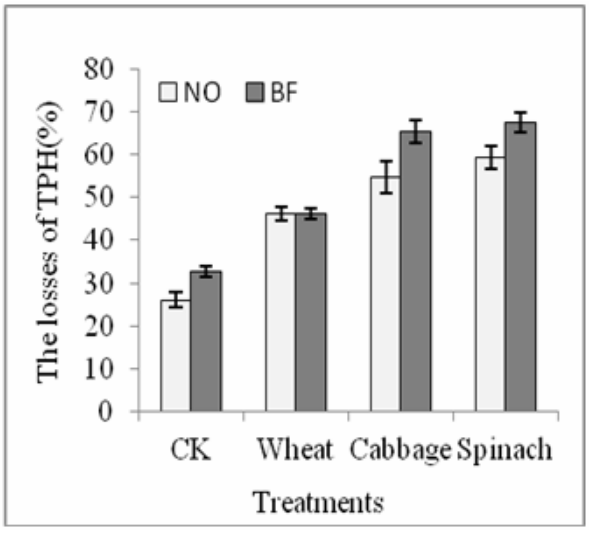

Fig. 3: The losses of total petroleum hydrocarbon in different pot soils (CK present the contrast had no plant, RBF present added *R. Sphaeroides* bio-fertilizer in soils)

DISCUSSION

The results suggest that crops and RBF have significant impact on soil microbial community composition and populations. The species and the concentration of soil microbial PLFAs had significant increased in rhizosphere soil and RBF bio-augmentation soils, moreover there were markedly distinction ($p<0.05$) depend on the species of crops.

ANOVA for the whole set of data on soil microbial PLFA concentration ($nmol \cdot g^{-1}$) revealed that there was a significant increase of PLFA in the RBF bio-augmentation soils, which were markedly different depending on the species of crops. Many studies have shown that the growth of plant in soil is the most important driving factor for changes of soil microbial community structure. The plant traits could influence soil community composition in many ways, e.g., soil carbon sequestration, root exudates, short-term C-cycling, etc, or roots physically alter the soil structure, thereby creating varying microhabitats suitable for different microbes. Bartelt-Ryser *et al.* reported that plants can affect the structures of

microbial communities through the release of root exudates, by providing surfaces for microbial colonization, or by stimulation of the microbial community in rhizosphere and believed that different plant species could influence the composition and number of soil microorganisms due to variations in the quantity and quality of root exudates (Bartelt-Ryser *et al.*, 2005).

The results indicated that activity of enzyme has been affect by adding RBF in soil, and had significant distinction depend on crop species. The TPH concentration could be increased in rhizosphere soil by the absorption of root, so the activity of DH increased and urease decreased in rhizosphere soils of three crops. Jiang *et al.* (2001) had reported that the activity of DH could be improved by the stimulation of TPH or their metabolic intermediate, but the activity of urease could be restrained by the TPH contamination in soil. The increase of the residues, exudates of root and soil microbe supported the observation of the increased activity of DH and urease in RBF bio-augmentation rhizosphere soils of three crops. Hu *et al*. reported that the activity of DH and urease could be improved by increasing the residues and the total content of microbe in soil (Hu *et al.*, 2005). The activity of AP was increased in RBF bio-augmentation bulk soil and the three crops rhizosphere soils but decreased in the RBF bio-augmented three crops rhizosphere soils. However, the reason is unclear currently and need to be investigated further.

The result showed that the degradation of TPH in soils had been improved by the growth of plants or adding of RBF in contaminated soil. There was highest degradation in RBF bio-augmentation rhizosphere soil, but there were significant difference depended on species of crops, and higher degradation in rhizosphere soil than in single RBF bio-augmentation bulk soil. Moreover, the losses of TPH correlated to the concentration and the profiles of the soil microbial PLFAs, as well. Diab (2008) regarded that plants could draw pollutants into the rhizosphere, and enhances the degradation of pollutant by microorganisms that extract energy from pollutants for growth within the soil and increased degradation of oil contamination in planted over non-planted soil systems. This observation emphasizes the fact that both the soil microorganisms and plants are the important factors in bioremediation reactions. The effect of *R. sphaeroides* based bioaugmentation was first reported and concluded that the RBF is a valuable microbial material for remediation of TPH contaminated soil. Further studies need to be conducted to investigate the impact of RBF on the cycling of soil microbial communities in oil-contaminated soils.

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