Effects of Different Surfactants to Petroleum Hydrocarbons Degradation of Mixed-bacteria

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Research

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Abstract

Surfactant-enhanced remediation (SER) had been widely applied to remove petroleum hydrocarbon (PH) pollutants, but the degradation mechanism that how to affect the hydrocarbon metabolite ability of microorganism under added surfactant is raring. In this work, the combination bacteria and surfactant were selected to remove PH, and the degradation bottleneck concentration of single-bacteria was 10000.00 mg/L according to the PH degradation rate. However, the degradation efficient of mixed-bacteria was further increased in adding surfactant condition.. Among them, the PH degradation rate of *Kocuria rosea* + *Bacillus odyssey* and *Microbacterium*. sp + *B. odyssey* had been respectively reached to 81.58±6.29% (added tween-80) and 88.35±7.58% (added rhamnolipid) under above bottleneck concentration. Compared with non-added surfactant, the relative abundances of global overmaps, amino acid metabolism, and carbohydrate metabolism were increased in adding tween-80 condition. However, when added rhamnolipid, the relative abundances of ABC transporters, two-component system, and bacteria chemotaxis had been exhibited enhancement obviously, and aimed to improve of transportation, absorption and degradation of PH. Additionally, the gene abundance of *alkB* and *nah* was also significantly increased with above condition. Meanwhile, the gene abundance of *alkB* and nah was also significantly enhanced by above condition. Sum up, this work offers an important information to insights into the changes of mixed-bacteria function during different systems to degrade PH.

1. Introduction

Petroleum and petroleum-based products were the important energy and resource for industry and daily life. However, widespread apply, improper disposal, incomplete combustion, and accidental leaks of organic hydrocarbons have become long-term persistent sources of contamination of fossil oils, coal, tar deposits and groundwater (Li et al., 2015). Among them, petroleum hydrocarbons (PHs) and particularly in polycyclic aromatic hydrocarbons (PAHs), has become a major environmental issue because of their adverse effect on human health (Lamichhane et al., 2017). With the increasing attention on PH remediation, several treatment strategies such as physical, chemical, and biological method have been applied to remediate PH pollution areas (Ghosal et al., 2016). Compared with physical and chemical method, biological method for contaminated soil and wastewater is known to have advantageous for both environmental and economic reasons (Maila and Cloete, 2005; Moscoso et al., 2012).

The metabolic processed naturally occurred, when biological method mainly utilized microorganisms, which including bacteria, fungi and algae, in order to degrade PH in the contaminated areas (Macaulay and Rees. 2014). In the extensive research has recently been conducted, people know that the advantage of the biological process over other methods. Among different kinds of microorganisms, the adaption and reproduction of bacteria was higher than any other microorganisms in the contaminated environment, meanwhile bacteria also have multiple metabolic pathways (both aerobic and anaerobic) with which they can degrade PH (Meckenstock et al., 2016). Hence, bacteria are seen as primary and most active degraders of petroleum pollution. Additionally, it was reported that the degradation level of mixed-microorganism is higher than these of single-microorganism for PH (Hasanuzzaman et al., 2007;

Cerqueira et al., 2011). When biological method was applied to remove PH, several factors (type and concentration of PH, bioavailability, and environmental index, etc.) could significantly impact the PH degradation efficient of microorganism in the condition of contaminated soil and groundwater (Kauppiand Sinkkonen. 2011; Boll et al., 2014; Varjani and Upasani. 2016). In different kind of factors, the bioavailability of PH is often limited by their low solubility and strong sorption to the adsorbents due to their complex chemical structures, thus its regarded as primary element in effecting PH degradation efficiency (Kavitha et al., 2014).

Nowadays, surfactant-enhanced bioremediation has been suggested as a promising method for the remediation of hydrophobic organic compounds in contaminated areas (Jain et al., 2011; Kavitha et al., 2014). Surfactants are amphiphilic compounds which can reduce surface and interfacial tensions by accumulating at the interface of immiscible fluids and increasing the solubility and mobility of hydrophobic or insoluble organic compounds (Li and Chen. 2009). Except solubilization of hydrocarbons surfactants, it also reported that it can alter cell surface hydrophobicity of microorganisms, the interaction with microbial cell surface may have both stimulatory and inhibitory effects on the biodegradation of hydrocarbons based on the mechanism of hydrocarbon uptake for a specific microorganism (Panda and Nazish. 2016). Moreover, it has been identified that some microorganisms could product surfactant, but the content of surfactant is not enough to reach the remediation of PH pollution (Blyth et al., 2015). Therefore, surfactant exogenous plus biological method could significantly enhance the degradation efficiency of PH.

Surfactant has been identified that it could increase the PH degradation efficiency of microorganism, but the enhance PH degradation mechanism of microorganism (especially mixed-microorganisms) by adding surfactant was still unknown. In this study, first of all, we selected single-bacteria as degrader to remove PH, at the same time identified the bottleneck concentration of PH for single-bacteria. Secondly, mixed-bacteria were utilized to degrade bottleneck concentration of PH, and the degradation conditions (includes surfactant and environmental factor) of mixed-bacteria were also optimized to increase the removal efficiency of PH. Finally, compared with the different degradation conditions of mixed-bacteria, and discussed the key element to further enhance the degradation level of mixed-bacteria. This work provides a new perspective to insight the degradation mechanism of mixed-microorganisms by surfactant plus biological method, meanwhile shows the key element of PH degradation by microorganism.

2 Materials And Methods

2.1 Strains and media

In this study, *Microbacterium* sp M-08 (D-1), *Kocuria marina* K-3 (D-2), *Kocuria rosea* k-24 (D-4), *Staphylococcus capitis* S-12 (D-6), and *Bacillus odyssey* B-17 (D-7) were selected as bacteria agent to degrade PH. An all the bacteria were sourced from petroleum contaminated soils and preserved in our laboratory.

The beef extract peptone (BEP) was used as plate medium in bacteria cultivation, which included beef paste (3 g/L), peptone (10.0 g/L), NaCl (5.0 g/L), and agar (20 g/L). The seed medium was tryptic soy broth (TSB), which composed by peptone (17 g/L), soybean peptone (3 g/L), NaCl (5 g/L), glucose (2.5 g/L), K_2HPO_4 (2.5 g/L). In addition, the degradation medium of bacteria was minimal medium (MM), and the concentrations of MM were as followed (g/L): $(NH_4)_2SO_4$ 1.0 g, NaCl 1.0 g, K_2HPO_4 1.0 g, KH_2PO_4 1.0 g, $MgSO_4$ 0.20 g, $CaCl_2$ 0.02 g, $FeSO_4$ 0.05 g. Meanwhile, 2 mL trace element stock solution composed of (mg/L): $FeCl_3.6H_2O$ 0.08, $ZnSO_4.H_2O$ 0.75, $CoCl_2.6H_2O$ 0.08, $CuSO_4.5H_2O$ 0.075, $MnSO_4.H_2O$ 0.75, $NaMoO_4.2H_2O$ 0.05, H_3BO_3 0.15. And the pH values of seed and degradation medium were adjusted to 7.2.

2.2 Experiment designed

This study mainly includes three aspects in researching: i) single-bacteria was applied to degrade wastewater with different concentrations of PH, which aimed to determine the degradation bottleneck of bacteria degrader; ii) mixed-bacteria was applied to solve the degradation bottleneck, meanwhile surfactant was added to increase the degradation efficiency of PH; iii) comparative metagenomics was used to indicate that the function difference of mixed-bacteria in different PH degradation conditions.

2.3 Single-and mixed-bacteria degrading PH experiment

In this study, single-bacteria were applied to degrade 5000.00 mg/L, 10000.00 mg/L, and 15000.00 mg/L PH, respectively. Bacteria were inoculated after culturing for 48 hr on the BEF agar, and these were transferred to seed medium in order to enhance bacteria growth. The cultures were incubated at 30°C for 36 hr, and 8 mL of the resulting seed culture was transferred to 80 mL of degradation medium in a 250 mL flask and incubated at 180 rpm/min for PH degradation.

Based on the above detection result, mixed-bacteria were applied to degrade PH in the concentration of degradation bottleneck of single-bacteria, and the degradation conditions of mixed-bacteria was same as bacteria. Meanwhile, the initial inoculation proportion of mixed-bacteria was 1:1.

2.4 Mixed-bacteria degradation PH optimization experiment

Two kinds of surfactant (tween-80 and rhamnolipid) were selected and added to improve the degradation efficiency of PH in this study. Also the addition concentrations of these surfactants were 50.00 mg/L, 100.00 mg/L, 150.00 mg/L, and 200.00 mg/L, respectively. Meanwhile, the degradation factors of the mixed-bacteria, such as temperature (20°C, 25°C, 30°C, and 35°C), initial pH (6.0, 6.5, 7.0, 7.5, and 8.0), rotation rate (150 r/min, 180 r/min, 200 r/min, and 220 r/min), and inoculated proportion of mixed-bacteria (3:1, 2:1, 1:1, 1:2, and 1:3) were also optimized in this work.

2.5 PH contents determination

After 14 days, took out 50 mL media and the Super Flash Alumina Neutral columns (SF 15–24 g, 20.8×112 mm. Agilent Technologies) was used to separate alkanes and PAHs, respectively (Wu et al., 2016). Alkanes and PAHs were acquired through eluting the columns by dichloromethane and n-hexane,

respectively. Moreover, the concentrations of alkanes and PAHs were determined by gas chromatography (GC) (Bruker, 430-GC, USA).

The parameters of GC were as followed: the flow ratio of carrier gas: nitrogen is 1:5; chromatographic column: BR-5 capillary column (30.0 m, 0.32-mm i.d, 0.25 μm df. Bruker); temperature programmed (alkanes and PAHs): an initial temperature in 5 min at 50 ℃, and then heating at a rate of 15 ℃/min to 290 ℃, and holding 290 ℃ for 5 min; injector temperature: 230 ℃;inject volume: 1μL; detector: hydrogen flame ion; detector temperature: 280 ℃. Additionally, all the samples were repeated three times in this experiment.

2.6 Metagenomic extraction, sequencing, and analysis

The samples of mixed-bacteria were collected, when different treatments were utilized to degrade hydrocarbons in 7 days, The samples were snap-frozen with liquid nitrogen, and then it was stored at -80 ℃. According to the manufacturer's instructions, the total DNA of mixed-bacteria was extracted by MoBioPowerSoil® DNA Isolation Kit (MoBio, USA). At the same time, the DNA contents of mixed-bacteria were detected by Nanodrop 1000 (Termo Fisher Scientific, Wilmington, DE, USA) and was storage in -20 ℃. Additionally, DNA library of mixed-bacteria was prepared according to the manufacturer's instructions (Illumina Inc., San Diego, CA, USA), and the metagenomic of these bacteria was sequenced by HiSeq 2000 Sequencing Platform (Illumina Inc., San Diego, CA, USA).

After removing adapters, low-quality reads and reads that belong to the host were discarded for raw data, the clean reads were assembled to contigs (length > 500 bp) by using IDBA-UD. QUAST and BWA were used to evaluate the level of assembly and coverage, respectively, meanwhile clean reads were blasted to RefSeq database. Additionally, the contigs (length > 500 bp) were utilized to predict the gene function by MetaGeneMark. The genes (length > 100 bp) of bacteria were clustered, and then constructed non-redundant gene sets through CD-HIT. Non-redundant gene sets of mixed strain were aligned and annotated to Kyoto Encyclopedia of Genes and Genomes (KEGG) database by BLAST-Like Alignment Tool Protein (BLATP). Additionally, non-redundant gene sets were also aligned and annotated to Carbohydrate-Active enZYmes (CAZy) database. Finally, trinity was used to screen the differences of expressing genes according to the gene abundance, and the search standard of difference in expression genes were fold change ≥ 4 and false discovery rate < 0.001.

2.7 AlkB and nah genes abundance quantitative PCR analysis

The *alkB* and *nah* genes abundance of mixed-bacteria in different degradation system was determined by q-PCR assays after 14 days, which the specific primers for *alkB* and *nah* were as follows: alkB-F (5'-AAYACIGCICAYGARCTIGGICAYAA-3'), alkB-R (5'-GCRTGRTGRTCIGARTGICGYTG-3'), NAH-F (5'-CAAAA(A/G)CACCTGATT(C/T)ATGG-3'), and NAH-R (5'-A(C/T)(A/G)CG(A/G)G(C/G)GACTTCTTTCAA-3') (Yang et al., 2015). Moreover, the PCR conditions and the standard curve construction were in accordance

with a previously publication (Perez-de-Mora et al., 2011). In this work, the amplification efficiency and coefficient (r^2) for *alkB* and *nah* were 97 %, 0.996 and 96 %, 0.995, respectively.

2.8 Data available

The raw datasets of metagenomic were stored into the National Center for Biotechnology Information (accession number: PRJNA693053).

3 Results And Discussion

3.1 Effects of PH concentration to the single- and mixed-bacteria degradation rate and biomass

Compared with physical and chemical method, biological remediation has become a low-cost and high-efficient method in removing hydrocarbons in different PH pollution environments (Santos Neto and de Oliveira et al., 2014). In this study, single- or mixed-bacteria was utilized to degrade PH of different concentrations. As shown in Fig. 1A, the degradation rate of single-bacteria was changed with the increase of PH concentration. When the concentration of PH was 5.00 g/L, the biodegradation rate was over 53.25 ± 2.15% (Fig. 1A). Among them, the PH degradation rate of D-2 was highest and it reached to 74.54 ± 3.26% (Fig. 1A). However, according to the degradation rate of PH (over 5.00 g/L), the biodegradation efficiency was significantly decreased (Fig. 1A), which shows that high concentration of hydrocarbons could limit the biodegradation and bioavailability in the PH pollution ecosystems. Under 10.00 g/L condition, the highest biodegradation rate was 53.41 ± 2.59% in D-6, meanwhile D-1 also had lowest hydrocarbon availability among bacteria (Fig. 1A). In addition, D-2 had the second higher biodegradation rate and it reached to 52.17 ± 2.47% (Fig. 1A). Compared with 10.00 g/L, the hydrocarbon biodegradation rate of 15.00 g/L was further declined, among which the highest (D-2) was only 37.49 ± 1.19%. Moreover, the biodegradation rate of D-1 was decreased to 16.57 ± 0.68% (Fig. 1A). Above all, the PH degradation bottleneck concentration of single-bacteria was 10.00 g/L in this work.

In oredr to enhance the biodegradation efficient of high concentration PH, mixed-bacteria were adopted to degrade 10.00 g/L PH. In this study, the combination of D-4 with D-6 had the highest PH degradation rate among different bacteria combinations, and the biodegradation rate has reached to 64.47 ± 2.36% in 10.00 g/L (Fig. 1B). Meanwhile, the biodegradation rate of PH in the combination of D-4 with D-7 was also over 60% and it reached to 60.54 ± 2.41% (Fig. 1B). When single-bacteria were used to degrade 10.00 g/L PH, the lowest of biodegradation rate was 38.74 ± 1.52% by D-1 (Fig. 1A). However, compared with single-bacteria, the biodegradation rate of D-1 plus D-4 has decreased to 24.25 ± 0.78% (Fig. 1B), which shows that the substrate competition of mixed-bacteria was higher than single-bacteria.

Meanwhile, by detecting the maximum biomass of single-bacteria in different PH concentrations (Fig. 1C), the biomass of bacteria might have a positive effect to biodegradation rate of PH. Between different treatments, the maximum value of single-bacteria biomass was obtained in 5.00 g/L, and that of D-2 has reached to 2.37 ± 0.06. However, with the increasing PH concentration, the maximum biomass

of single-bacteria was significantly declined (Fig. 1C). Among them, the maximum biomass of D-1 has decreased to 0.79 ± 0.02 in 15.00 g/L (Fig. 1C). Under the mixed-bacteria degradation condition, the maximum value of biomass was increased to 2.46 ± 0.08 and 2.32 ± 0.06 in the combinations of D-4 with D-6 and D-4 with D-7, respectively, but that of bacteria biomass in D-1 plus D-4 was decreased to 0.92 ± 0.03 (Fig. 1D).

3.2 Biodegradation characterized of PH under single- and mixed-bacterial conditions

The residual contents of PH under different biodegradation treatments were measured in this study, and the biodegradation result showed that bacteria has different degradation characteristics in different types of PH (Fig. 2). In the condition of low PH concentration (5.00 g/L), the residual contents of short-chain PH (tridecane to heptadecane) were lower than other types of PH (mid-long chain and PAHs), and the biodegradability of PAHs was lowest among different types of PH (Fig. 2A), which indicated that single-bacteria preferentially metabolism simple carbohydrate as substrate in different kinds of hydrocarbons (Moscoso et al., 2012). Moreover, the degradation efficiency of D-2 in different types of PH was highest among different bacteria (Fig. 2A). With enhancing of biodegradation strength (from 10.00 g/L to15.00 g/L), the residual contents of PH were obvious increased particularly in 15.00 g/L. At the same time, among PH, the residual contents of mid-long chain and PAHs were higher than short-chain in the high concentration PH condition (over 5.00 g/L). Additionally, the degradation ability of bacteria in different types of PH was also significantly decreased in the high PH concentration condition (Fig. 2A).

Among different groups, the residual contents of short-chain, mid-long chain, and PAHs were lowest in the combination of D-4 and D-6 (Fig. 2B). Meanwhile, compared with single-bacteria (D-2), the biodegradation contents of these types' PH were also increased in D-4 plus D-6 (Fig. 2B). However, the PH biodegradation contents of D-1 plus D-4 was lower than D-1 (Fig. 2B), which also indicated that the substrate competition of mixed-bacteria was higher than single-bacteria. Between different types of PH, the biodegradation rates of short-chain (particular in D-4 plus D-6) were higher than any others (Fig. 2B). Compared with short-chain, the biodegradation rates of mid-long chain and PAHs was low, especially PAHs (Fig. 2B). However, in the combination of D-4 plus D-6 and D-4 plus D-7, the biodegradation rates of PAHs were beyond 50%. Moreover, the mid-long chain biodegradation efficiency of mixed-bacteria was higher than single-bacteria (Fig. 2B).

3.3 Biodegradation characteristic of mixed-bacteria with surfactant

Due to the solubilization effect of surfactant, when the surfactant concentration is gradually close to critical micelle concentration (CMC), surfactant can boost the PH to dissolve in the hydrophobic center of micelles, which is conducive to microbial metabolism of hydrocarbons, and then improve the efficiency of bacteria degradation of hydrocarbons (Perez-de-Mora et al., 2011). When tween-80 was added into

petroleum hydrocarbon biodegradation system, and with the increasing of tween-80 concentration, the biodegradation rate of PH by mixed-bacteria was initially increased (50 mg/L to 150 mg/L), and then gradually decreased (150 mg/L to 200 mg/L) (Fig. 3A). Among different treatments, the highest biodegradation rate of mixed-bacteria was 71.26 ± 3.56% with 100 mg/L tween-80, and these results were obtained in the combination of D-4 and D-7 (Fig. 3A). Meanwhile, the biodegradation trend of PH by adding rhamnolipid was the same to tween-80 (Fig. 3B), but the group of D-5 plus D-7 was reached to the highest PH degradation rate under 150 mg/L rhamnolipid (Fig. 3B). Additionally, the biodegradation conditions of mixed-bacteria under with or without surfactant were optimized, and the result of biodegradation characteristic was shown in Table 1. The biodegradation rates of PH by tween-80 (100 mg/L) and rhamnolipid (150 mg/L) were increased to 76.09 ± 1.14% and 81.25 ± 4.27%, respectively (Table 1).

Table 1
PH degradation rate under different conditions by mixed-bacteria

Surfactant	Factor	Condition	Degradation rate (%)	Factor	Condition	Degradation rate (%)
Non-added	Temperature (°C)	20	72.39 ± 5.62	Speed (r/min)	150	73.25 ± 4.21
		25	75.33 ± 6.89		180	78.57 ± 5.62
		30	78.39 ± 5.82		200	80.41 ± 6.87
		35	79.36 ± 7.55		220	80.75 ± 4.55
	Initial pH	5	70.85 ± 6.48	Proportion of mixed-bacteria	3:1	69.15 ± 5.85
		6	72.08 ± 5.60		2:1	68.07 ± 6.67
		7	81.15 ± 6.75		1:1	72.09 ± 7.14
		8	80.52 ± 6.72		1:2	71.25 ± 6.76
		9	78.58 ± 5.84		1:3	70.85 ± 5.74
Tween-80	Temperature (°C)	20	72.39 ± 5.62	Speed (r/min)	150	73.25 ± 4.21
		25	75.33 ± 6.89		180	78.57 ± 5.62
		30	78.39 ± 5.82		200	80.41 ± 6.87
		35	79.36 ± 7.55		220	80.75 ± 4.55
	Initial pH	5	70.85 ± 6.48	Proportion of mixed-bacteria	3:1	73.69 ± 5.85
		6	72.08 ± 5.60		2:1	72.19 ± 6.67
		7	81.15 ± 6.75		1:1	81.58 ± 6.29
		8	80.52 ± 6.72		1:2	75.49 ± 6.76
		9	78.58 ± 5.84		1:3	76.28 ± 7.14
Rhamnolipid	Temperature (°C)	20	75.26 ± 5.74	Speed (r/min)	150	79.69 ± 5.49
		25	79.92 ± 7.22		180	83.96 ± 6.38
		30	84.65 ± 4.98		200	87.39 ± 6.84
		35	85.18 ± 5.37		220	87.69 ± 5.68
	Initial pH	5	73.56 ± 6.34	Proportion of mixed-bacteria	3:1	73.44 ± 7.65
		6	78.54 ± 5.28		2:1	81.39 ± 6.34
		7	88.05 ± 7.14		1:1	88.35 ± 7.58
		8	84.15 ± 6.34		1:2	79.69 ± 6.84

Surfactant	Factor	Condition	Degradation rate (%)	Factor	Condition	Degradation rate (%)
		9	76.29 ± 6.34		1:3	75.58 ± 7.22

Compared with non-surfactant added treatments, the biodegradation rate in different types of PH were further increased after optimizing degradation conditions. Among them, mixed-bacteria has the highest degradation rate for short-chain hydrocarbon, and the residual concentration was below 510 mg/L (Table 2). Meanwhile, the residual contents of mid-long chain were decreased to below 700 mg/L, particularly in D-5 plus D-7 (483.58 ± 39.48 mg/L) (Table 2). In this study, PAHs held the lowest biodegradation rate among different treatments, but the biodegradation rates of PAHs were beyond 35% after optimizing degradation conditions of mixed-bacteria (Table 2).

Table 2
Residual contents of PH under different degradation systems

Surfactant	Combinations	Short chain (mg/L)	Mid-long chain (mg/L)	PAHs (mg/L)
Non-added	D-4 + D-6	724.57 ± 69.58	893.74 ± 69.27	1172.69 ± 95.81
Tween-80	D-4 + D-7	500.22 ± 36.71	682.48 ± 59.47	659.3 ± 47.29
Rhamnolipid	D-5 + D-7	261.65 ± 26.57	483.58 ± 39.48	419.77 ± 36.49

3.4 Compared with the metagenomics difference of mixed-bacteria under different treatment conditions

In order to increase the degradation efficient of PH by mixed-bacteria, surfactants were added in degradation system of PH and proved that these could further enhance the degradation rate of mixed-bacteria. Meanwhile, the metagenomics of mixed-bacteria were measured which was aimed to compare the degradation mechanism of hydrocarbons under different treatments (with and without surfactants).

3.4.1 Metagenomics sequencing

In this work, the metagenomics of mixed-bacteria under different treatments were measured and shown in Table S1. After the raw data of metagenomics was filtered, the valid data of mixed-bacteria was beyond 6.70 Gb (Table S1). Meanwhile, the value of Q20 and Q30 in different treatments was beyond 97% and 93% (Table S1), respectively, which indicated that these data could prove the accuracy of metagenomics. Between different treatments, after valid data was assembled, the contigs number and map were over 1.250 and 99.70% (Table S2), respectively. Moreover, the numbers of coding gene were over 16.990 in each treatment, and these gene could construct a non-redundant gene set (containing 67,258 genes) (Table S2).

3.4.2 Annotation metabolic pathways of mixed-bacteria under different treatments

The non-redundant gene set constructed by different mixed-bacteria systems and was annotated to KEGG database, and the results were displayed in Fig. 4A. In the first level annotation of KEGG, which includes metabolism, genetic information processing, environmental information processing, and cellular processes have important role on the PH degradation of mixed-bacteria under different conditions (Fig. 4A). Among them, the metabolism of mixed-bacteria has the largest proportion in different functional modules (Fig. 4A).

In the second level annotation of KEGG database, the relative abundance of global and overview maps, carbohydrate metabolism, and amino acid metabolism under different treatments were higher than any other modules in the metabolism functional (Fig. 4A). In the different degradation systems of mixed-bacteria, strain could consider hydrocarbons or surfactant as carbon and utilized them to meet the demand for energy through glycolysis and tricarboxylic acid cycle (TCA), therefore these pathways played an important role on the carbon metabolism of mixed-bacteria (Wang and Shao. 2013). At the same time, when the mixed-bacteria were used to degrade PH under different conditions, the osmotic pressure of environmental may be changed. Therefore, as to adjust the degradation efficiency of mixed-bacteria to hydrocarbons, the amino acid metabolism could act as the balance role of osmotic pressure (Vieira et al., 2007). Additionally, the global and overview maps covered almost all functional pathways in microorganisms, so it played an important role in the process of microbial metabolism of hydrocarbons.

Surfactant not only could enhance the bioavailable of PH, but also has an advantage to improve of carbon metabolism, thus these processes promoted the genetic information expression of microorganisms (Congiu and Ortega-Calvo. 2014). Meanwhile, the compatibility of PH or surfactant with mixed-bacteria was different, which might be led the microorganism to response the environmental condition, so the the relative abundance of genetic information has changed. Therefore, the relative abundance of replication, repair and translation is higher than other functions between genetic information modules (Fig. 4A). Owing to the solubilization of surfactant, the contact areas between microorganisms and hydrocarbon were expanded, meanwhile greatly increasing the frequency of PH entering the cell through cell membrane (Banat et al., 2014), eventually it results in higher relative abundance of membrane transport in environmental information processing module (Fig. 4A). Additionally, different strategies were utilized to degrade hydrocarbons in this work, which led to different interaction modes between microorganisms under different conditions, so the relative abundance of cellular community in the module of cell process was higher than other functions.

3.4.3 Degradation functional difference of mixed-bacteria under different systems

Different strategies were used to degrade hydrocarbons in this work, and the degradation efficiency of mixed-bacteria was obvious increased under added surfactant condition, which indicated that the carbon metabolism function of microorganism might be affect by surfactant.

In the different degradation systems of mixed-bacteria, the relative abundance of global and overview maps, carbohydrate metabolism, and amino acid metabolism was higher than any other modules

through function difference analysis (Fig. 4B), meanwhile these results also showed that above functions had an important role in the process of microorganism hydrocarbons absorption, transportation, and metabolism. Moreover, in the functional module relative abundance of global and overview maps, carbohydrate metabolism, amino acid metabolism, membrane transportation, signal transduction, and cell motility with a difference between different degradation systems (Fig. 4B), which showed that the function of mixed-bacteria was adjusted and the ability of absorption and metabolism was enhanced in order to degrade hydrocarbon by adding surfactant. Additionally, compared with rhamnolipid, the relative abundance of each functional module was not significant different between tween-80 and non-added surfactant, which indicated that chemical surfactant only might enhance the bioavailable of hydrocarbons by solubilization and not adjust the inter functional modules of microorganism.

Further to analysis the hydrocarbon metabolism pathway of mixed-bacteria under different degradation conditions (Fig. 4C). In the functional module of global and overview maps, each pathway in tween-80 condition was higher than non-added surfactant and rhamnolipid system, especially biosynthesis of secondary metabolites, biosynthesis of antibiotic, biosynthesis of amino acid, and carbon metabolism. As chemical surfactant, tween-80 had a toxic with growth of microorganism. However, in order to maintain the stability of microorganism, the antibiotic and amino acid biosynthesis level of mixed-bacteria was increased to resist and adapt to current environment (Kempf and Bremer. 1998). Meanwhile, the PH also had an inhibition effect to the growing microorganism, thus the relative abundance of each functional module was enhanced to synthesized the needed energy of microorganism and to response to environmental stress under the simultaneous action of tween-80 and PH (Deng et al., 2016). Furthermore, in the condition of rhamnolipid, the relative abundance of whole functional module was lower than others system, which indicated that rhamnolipid might have the potential to increase the environmental adaptability of microorganisms.

In the functional module of amino acid metabolism, the glycine, serine, and threonine synthesis level of mixed-bacteria were enhanced in order to gain energy and maintain osmotic pressure, meanwhile these actions were response and adapt to the stress of PH and tween-80. Therefore, the relative abundance of above pathways in non-added surfactant and tween-80 was higher than rhamnolipid condition (Fig. 4C). In the functional module of carbohydrate metabolism, the relative abundance of glycolysis and TCA pathway in non-added surfactant and tween-80 was also higher than rhamnolipid condition (Fig. 4C), which indicated that the improvement of these pathways were aimed to increase the energy supply also adapt to current environment,

In the functional module of membrane transport and signal transduction, the relative abundance of ABC transporters and two-component system in rhamnolipid condition was higher than non-added surfactant and tween-80 system (Fig. 4C). Recently, it had been approved that ABC transporters and two-component system play an important role on the degradation of PAHs and improvement of microorganism stress resistance, respectively (Sierra-Garcia et al., 2003). Therefore, based on the above results showed that rhamnolipid might adjust the hydrocarbon transportation method of mixed-bacteria and enhance the PH

tolerance of microorganism, and eventually the potential of mixed-bacteria environmental adaptation was increased.

In the functional module of cell motility, the relative abundance of bacteria chemotaxis in rhamnolipid condition was higher than non-added surfactant and tween-80 system (Fig. 5). And the chemotaxis of microorganism had a close relationship with PH, thus above result indicated that the improvement of bacteria chemotaxis will provide an advantage to further to increase the degradation efficiency of hydrocarbons by mixed-bacteria (Wadhams and Armitage. 2004).

3.5 Annotation and difference analysis of carbohydrate enzyme under different mix-bacteria conditions

In the PH degradation process of microorganism, the carbohydrate enzyme had a vital role on the catalysis of hydrocarbon absorption, transformation, and energy acquisition (Varjani. 2017). Based on the annotation result of carbohydrate enzyme under different treatments (Fig. 5A), the relative abundance of glycosyl transferase (GT) was higher than any other enzymes in the degradation process of mixed-bacteria, and its relative abundance rate had been reached to 40.6%. Meanwhile, the relative abundance of glycoside hydrolase (GH) and carbohydrate esterase (CE) had a higher ratio in the mixed-bacteria hydrocarbon metabolism process (Fig. 5A). However, between these different carbohydrate enzymes, the relative abundance rate of PL had only 0.1% (Fig. 5A), which showed that PL could have a little role on the hydrocarbons degradation process of mixed-bacteria.

Among different treatment systems, the relative abundance of GT, GH, and CE had a higher level in the hydrocarbon metabolism process of mixed-bacteria (Fig. 5B). Under non-added surfactant condition, the relative abundance of GT was highest among different function enzymes, meanwhile these PL was also lower than any other enzymes (Fig. 5B). In treatment of tween-80 was added to the degradation system of mixed-bacteria, the relative abundance of GT was also highest, and these values were similar with non-added surfactant condition (Fig. 5B). However, compared with non-added surfactant system, the relative abundance of GH and carbohydrate-binding module (CBM) was increased in the condition of tween-80. And in the system of rhamnolipid, the relative abundance of GT and auxiliary activities (AA) was lower than non-added surfactant and Tween80 condition, yet these of CE, CBM, and PL was higher than another degradation system (Fig. 5B). To sum up, added surfactant, particular in rhamnolipid, could have an effect on the expressing level of carbohydrate enzyme and to regulate of microorganism functional modules, then it ultimately improve the potential of mixed-bacteria to metabolize hydrocarbons.

3.6 AlkB and nah gene abundance comparison of different degradation systems

In the hydrocarbon metabolism process of microorganism, the oxygenase expressed by *alkB* and *nah* genes played an important role in the degradation of aliphatic hydrocarbons and aromatic compounds (Choi et al., 2002). In this section, the gene abundance of *alkB* and *nah* was significant difference between different degradation conditions (Table 3), which indicated that increasing the solubility of

hydrocarbons and improving the conditions of microorganism degradation system could enhance the PHs utilization potential of mixed-bacteria.

Table 3
Abundance of the *alkB* and *nah* genes in different kinds of mixed-bacteria (copies per mL)

	Non-added	Tween-80	Rhamnolipid
alkB	$6.29 \pm 0.45\times10^{6}$a	$2.59 \pm 0.12\times10^{7}$b	$1.36 \pm 0.11\times10^{9}$c
nah	$3.57 \pm 0.27\times10^{5}$a	$6.82 \pm 0.51\times10^{6}$b	$4.82 \pm 0.36\times10^{8}$c
Lower-case letters in the same line indicate significant difference at $p < 0.05$.			

Under non-added surfactant condition, the gene abundance of *alkB* and *nah* was significantly lower than rhamnolipid and tween-80 (Table 3), which showed that enhancing bioavailable of hydrocarbons could promote the carbon metabolism of mixed-bacteria. Meanwhile, in the system of added surfactant, the above gene abundance also showed the significant difference (Table 3), which indicated that the toxic of surfactant had an inhibition effect to increase hydrocarbons degradation efficiency of mixed-bacteria.

Conclusion

In this work, combination added rhamnolipid with *Microbacterium*. sp + *B. odyssey* had the highest PH degradation rate (88.35±7.58%). Meanwhile, the degradation efficiency of mid-long chain PH and PAHs in above system was higher than non-added surfactant. Under added rhamnolipid system, the relative abundances of ABC transporters, two-component system, and bacteria chemotaxis had been showed the enhancement obviously, and it aimed to improve of transportation, absorption and degradation of PH. Also, the gene abundances of *alkB* and *nah* were also significantly enhanced by above condition. Therefore, the condition of surfactant-enhanced remediation plus biological method had the highest degradation PH efficiency.

Declarations

Availability of data and materials

All data analyzed during this study are included in this article

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Authors'contributions

Jia-qi Cui and Ya-qi Li performed the experiments and wrote this paper. Bing-zhi Li and Ying-jin Yuan gave helpful suggestions. Jian-ping Wen designed the experiments. All authors read and approved the final manuscript.

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Figures

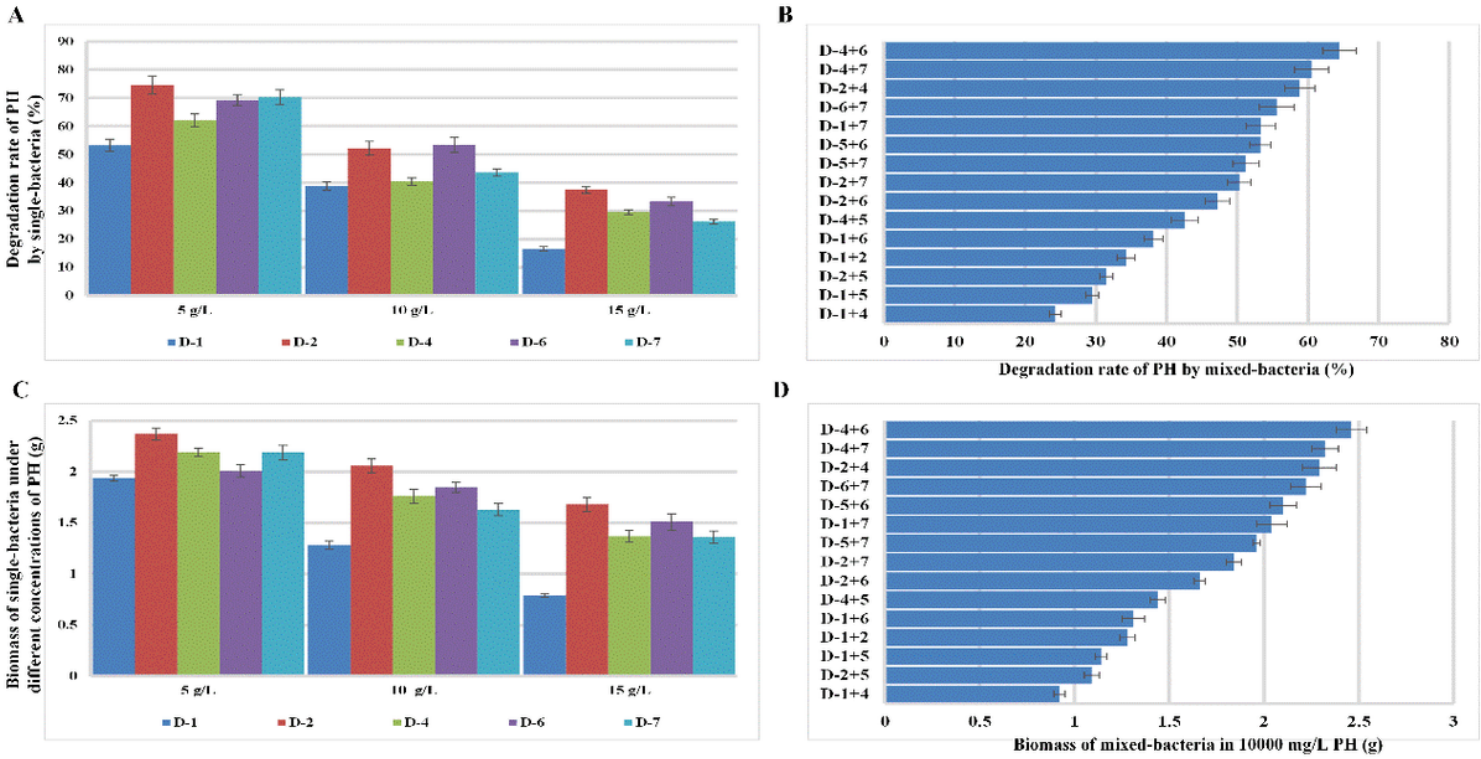

Figure 1

Degradation rate and biomass of bacteria under different treatment conditions A and B represented that the PH degradation rate of single- and mixed-bacteria, respectively; C and D represented that the biomass of single- and mixed-bacteria, respectively.

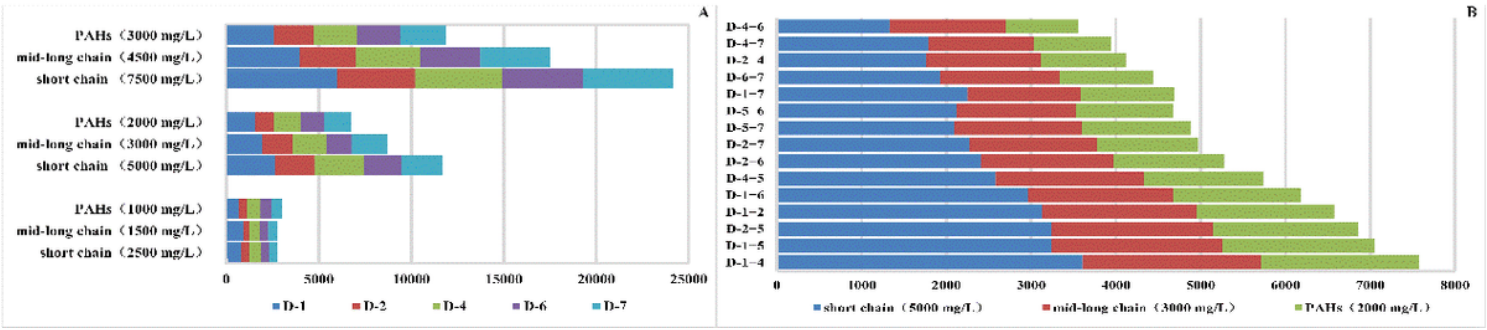

Figure 2

Degradation characteristics of different kinds of PH by single- and mixed-bacteria A and B represented that the degrade characteristics of single- and mixed-bacteria, respectively; Ax- and Ay-axis represented the different types and residual concentration, respectively of PH; Bx- and By-axis represented the combination of mixed-bacteria and PH residual concentration, respectively.

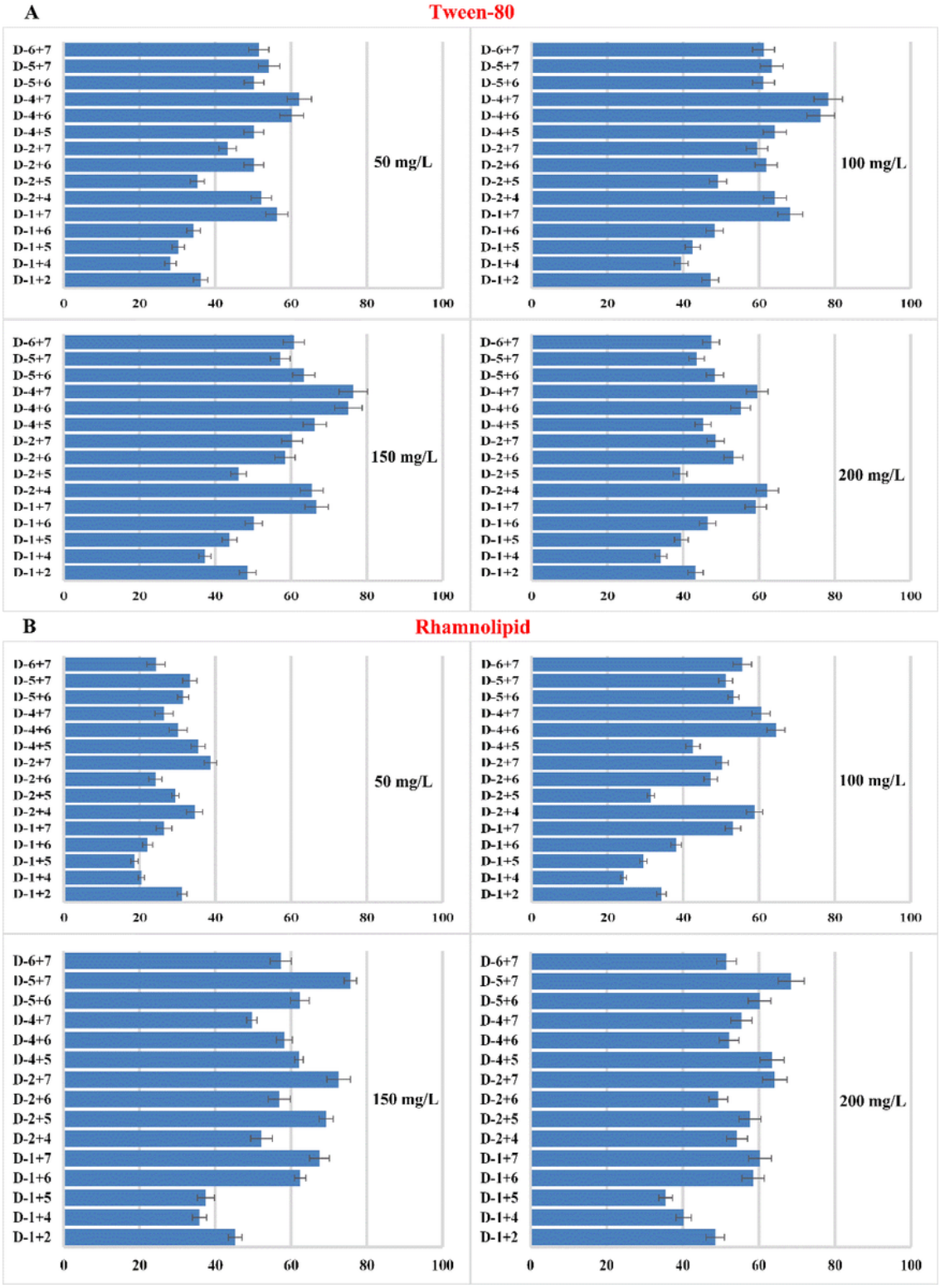

Figure 3

Degradation rates of mixed-bacteria under different surfactants added conditions. A and B represented that the PH degradation rate of mixed-bacteria under tween-80 and rhamnolipid added conditions, respectively; 50 mg/L, 100 mg/L, 150 mg/L, and 200 mg/L represented that the addition concentration of surfactant; x- and y-axis represented the combination of mixed-bacteria and PH degradation rate, respectively.

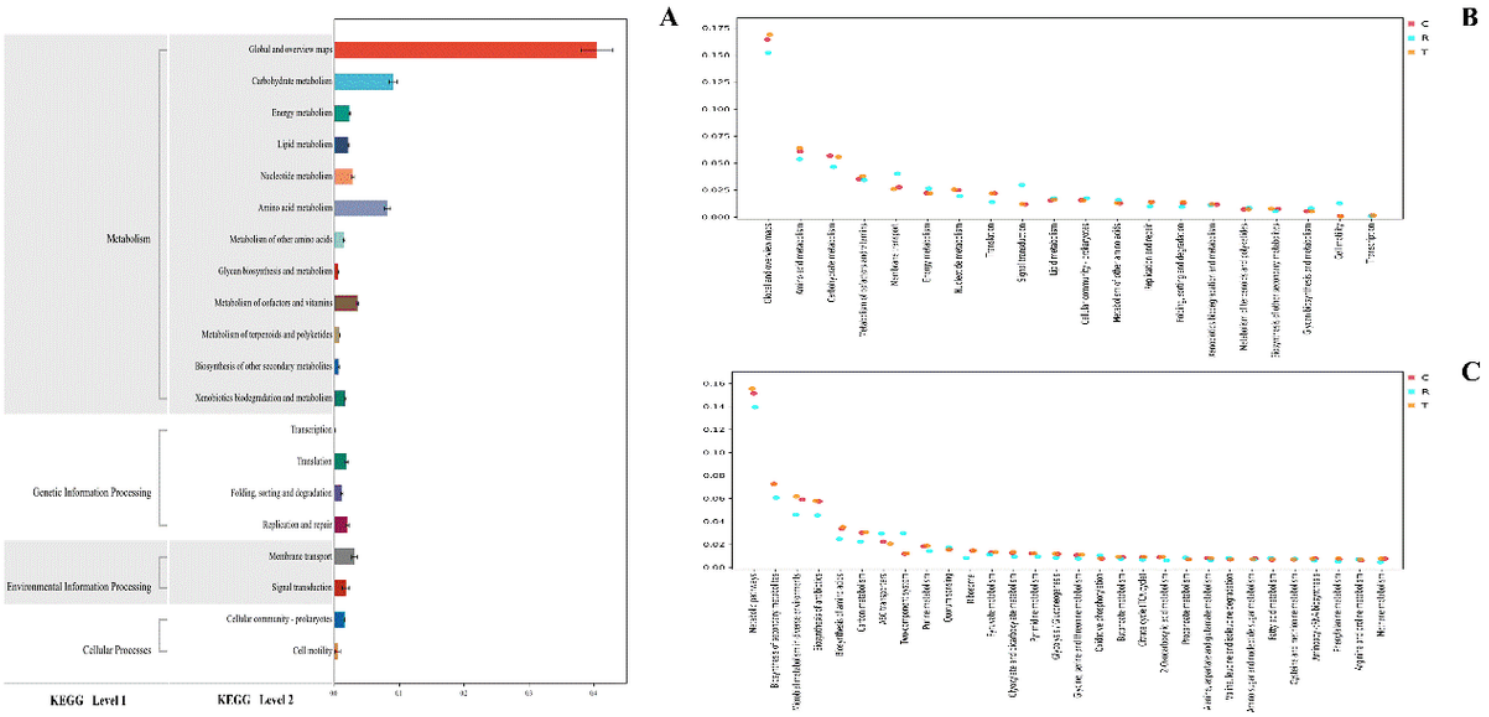

Figure 4

Function distributions and differences of mixed-bacteria in different degradation systems A represented that non-redundant gene set annotation results of mixed-bacteria under KEGG database; B and C represented that the function distributions and differences of mixed-bacteria under different treatment conditions, respectively; Bx-axis represented that the relative abundance; By-axis represented that the KEGG level 2 function; Cx-axis represented that the relative abundance; Cy-axis represented that the metabolic pathway; C represented that no-added surfactant condition; R represented that rhamnolipid added condition; T represented that tween-80 added surfactant condition.

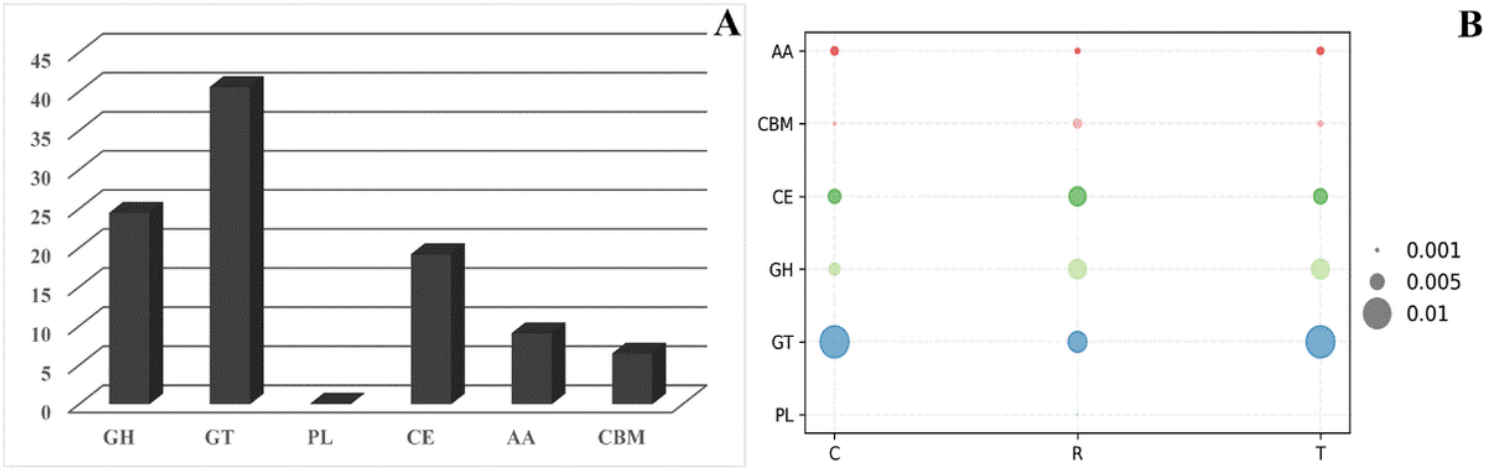

Figure 5

Carbohydrate enzyme relative abundance of mixed-bacteria in different degradation systems Ax-axis represented that the degradation conditions; Ay-axis represented that the types of carbohydrate enzyme;

Bx-axis represented that the types of carbohydrate enzyme; By-axis represented that the different PH treatment conditions of mixed-bacteria.

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