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Environmental impact statement

Petroleum hydrocarbons entering into the environment can seriously threat the human health and destroy the ecological balance which may take years or even decades to recover. The microbial consortium that we studied had high biodegradability on degrading a variety of the petroleum hydrocarbons (polycyclic aromatic hydrocarbons and crude oil). By immobilized, the ability of microbial adaptation to the environment can be improved which expand the application fields of microbial remediation.

Biodegradation of Different Petroleum Hydrocarbons by Free and immobilized Microbial Consortia

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Abstract

The efficiencies of free and immobilized microbial consortia to degrade different types of petroleum hydrocarbons were investigated. In this study, the biodegradation rates of naphthalene, phenanthrene, pyrene and crude oil reached about 80%, 30%, 56% and 48% under the optimum environmental conditions of free microbial consortium after 7d. We evaluated five unique co-metabolic substances with petroleum hydrocarbons, α-Lactose was the best co-metabolic substance among glucose, α-lactose, soluble starch, yeast powder and urea. The orthogonal biodegradation analysis results showed that semi-coke was the best immobilized carrier followed by walnut shell and activated carbon. Meanwhile, the significance of various factors that contribute to biodegradation of semi-coke immobilized microbial consortium followed an order of: α-lactose ＞semi-coke＞sodium alginate＞$CaCl_2$. Moreover, the degradation rate of

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immobilized microbial consortium (47%) was higher than that of a free microbial consortium (26%) under the environmental conditions of crude oil concentration 3 g/L, NaCl concentration 20 g/L, pH at 7.2~7.4 and temperature of 25 ^{o}C after 5 d. SEM and FTIR analysis revealed the structure of semi-coke became bigger porous and easier to adhered with microbial consortium and the functional groups (e.g., hydroxy and phosphate) were identified in microbial consortium and changed by immobilization. This study demonstrated that the ability of microbial adaptation to the environment can be improved by immobilized which expand the application fields of microbial remediation.

Keywords: immobilization, biodegradation, crude oil, PAHs, semi-coke

1 Introduction

Petroleum hydrocarbons entering into the environment can occur at any point in the "life cycle" of petroleum such as exploitation, production, transportation, refining, storage, industrial discharges and atmospheric fallout, especially accidental spills.[1] They widely distributed in atmosphere, terrestrial soil, marine waters and sediment; seriously threaten fishery, marine habitats of wildlife, halobios, the environment and human health; and destroyed the ecological balance which may take years or even decades to recover. The oil type is extremely important in determining the degree of environmental and socioeconomic damage.[1] Specially, polycyclic aromatic hydrocarbons (PAHs) are organic pollutants prevalent in the sediments of marine and freshwater environments.

These pollutants are carcinogenic, mutagenic and genotoxic to both aquatic and terrestrial organisms.[2] PAHs range from naphthalene (two aromatic rings) to compounds with six or more aromatic rings.[3] Naphthalene, a polycyclic aromatic hydrocarbon, is a ubiquitous environmental pollutant capable of causing illness.[4] Numerous studies have indicated that the respiratory system is a primary target for inhaled naphthalene. The majority of PAHs, and especially phenanthrene and pyrene those with three or four rings, are considered as semi-volatile and such compounds partition between the vapour and particle phases in the atmosphere. These compounds can deposit to surface water and soils where they have a long lifetime.[3] The removal and remediation of those compounds has always been an important task in the environmental protection.

Biodegradation is expected to be an economic and environmentally friendly alternative for removal of petroleum hydrocarbons.[5] At present, a large number of research interests are turning to the biodegradation of crude oil and PAHs by biostimulation and bioaugmentation.[6-8] As we know, environment conditions (such as temperature, pH, salinity, nutriment etc.) significantly affect the progress of biodegradation. Therefore, an increasing number of studies have focused on the bioremediation potential of pre-adapted microbes by changing the environment factors.[9-10] A lot of researchers only focus on biodegrading a single pollutant by specific microbe while contaminations will not exist in a single way in the environment.[10] Few researchers simultaneously focused on biodegrading efficiency of multiple contaminants by the same microorganism.[11-12] Furthermore, the application of bioremediation to

marine oil spills has traditionally thought to be limited by the ability of microbes to access nutrients such as nitrogen and phosphorus, as well as the dilution of nutrients and microorganism concentration, higher salinity and low temperature in the marine environment.[13]

Immobilization method has been proposed to overcome these problems, which was immobilized the cell (microorganism) or enzyme in a limited space by chemical or physical methods, kept them activity and could be reused.[14] Immobilized live cells can offer many advantages such as avoiding wash-out of cells, ensuring higher cell concentration in small volumes, embedding the nutrients and resisting the external disadvantages to microbe. At present, Immobilization of microorganism or enzyme in a variety of carriers such as alginate, mollusk shell and plant cells have been investigated for the advantageous utilization of immobilized biocatalysts comparing with free cells in various biotechnological processes.[15,16] The method of immobilization by cell entrapment in gel carriers have been well accepted in the treatment field of wastewater and polluted soil.[17-19] However, the application of these materials has generally precluded the marine environment and focused upon development for application in terrestrial environments.[15] Meanwhile, its application in marine environment was few and the selected carrier material was limited such as mussel shells, coir peat, mussel shell/agar complex, puffed foxtail millet and polyurethane foam etc.[15,17,20-23] Ideally, the carrier material would be biodegradable, available in large quantities, low cost, non-toxic and have appropriate physical properties to allow sufficient infusion with

bio-stimulants or aggregation of specific microorganisms used in bioremediation.[24] However, few researchers select efficient carriers basing on the idea of "using waste to treat waste".

Semi-coke is mainly produced from oil shale industry as wastes and one of the main problems of oil shale industry is how to treat semi-coke effectively. For example, in Estonia, 180-200 thousand tons of semi-coke were produced annually and discarded as wastes.[25] Semi-coke has high absorptive capacities to absorb Petroleum hydrocarbons, especially for PAHs. So, it can be used to prepare immobilized-bacteria and adsorption carriers.[26] Walnut shells are available in abundant supply as an agricultural byproduct of walnut-processing industry. It is a nontoxic and biodegradable material as a renewable resource. It is evident from the study that walnut shell media can be used to remove oil from wastewater and showed a higher recovery of oil in comparison to recovery in the case of oil on aqueous medium.[27] Thus, walnut shell can be applied in oil spill bioremediation. Activated carbon is one of the most widely employed adsorbents. It is mainly composed of carbonaceous material with various microporous structures. Its industrial usage can be found in the treatment process for purification, decolorization, and the removal of toxic organics and heavy metal ions.[28] Yet this study includes the application of activated carbon as a carrier.

Therefore, the main objective of this present work was to study the effect of environmental factors on biodegradation of petroleum hydrocarbons (crude oil and PAHs) by the free microbial consortium optimizing the degradation conditions; explore

the best preparation method and performance of immobilized microbial consortium formed using sodium alginate (SA) and different carriers (walnut shell, activated carbon and semi-coke); compare the effect of environmental factors on free and immobilized microbial consortia; and characterize the difference in immobilized and free microbial consortia by FTIR and SEM analysis.

2 Materials and methods

2.1 Chemicals

Phenanthrene (PHE) and pyrene (PYR) were obtained from Aladdin Chemistry Co. Ltd. Naphthalene (NAP), cyclohexane, acetone and petroleum ether (boiling point 60 ^{o}C~90 ^{o}C) were purchased from Sinopharm Chemical Reagent Co. Ltd. Other reagents were analytical grade and purchased from various commercial sources. Crude oil dehydrated and removed undissolved precipitate was obtained from Shengli oilfield. Walnut shell, semi-coke and actived carbon were crushed and screened by 60 mesh sieve.

2.2 Preparation of solution and media

The single substrate solutions were prepared in advance, respectively. The concentration of each PAHs (NAP, PHE and PYR) was 5 g/L in acetone. The mineral salt medium (MSM) was composed of (g/L distilled water) NaCl 0.5, $(NH_4)_2SO_4$ 0.1, $MgSO_4 \cdot 7H_2O$ 0.025, $NaNO_3$ 0.2, KH_2PO_4 0.4, $K_2HPO_4 \cdot 3H_2O$ 1. The degradation medium was composed of MSM and petroleum hydrocarbons (crude oil, NAP, PHE or PYR). After volatilizing the dissolved liquid of all stock solution, MSM was added into

an erlenmeyer flask containing PAHs or crude oil. Beef extract peptone medium was composed of (g/L distilled water) beef extracting 0.5, peptone 1, NaCl 0.5. The pH of media was adjusted to 7.2~7.4 with either HCl or NaOH solutions. All of media were sterilized in an autoclave at 121 ^{o}C for 20 min before used.[29]

2.3 Microorganisms and culture conditions

A microbial consortium isolated from Shengli oilfield polluted sludge was capable of degrading crude oil and PAHs (NAP, PHE and PYR) and used them as the sole carbon source. Four bacterial strains composing the utilized microbial consortium were named as PH-1, PH-2, PH-3 and PH-4. They were affiliated with *Pseudomonas* sp., *Bacillus* sp., *Ochrobactrum* sp. and *Pseudomonas* sp. by molecular biological identification, respectively.[29] The GenBank IDs of them were KF113575 (PH-1), KF113576 (PH-2), KF421109 (PH-3) and KF113577 (PH-4). The microbial consortium was the objective strains in the following experiments.

The microbial consortium was cultivated in beef extract peptone medium at 30 ^{o}C with rotary shaking at 120 rpm for 24 h. There were some pellets formed in the medium. Cells pellets were harvested by centrifugation (6000 rpm for 5 min at 4 ^{o}C) and washed 3 times with MSM for removal of impurities. Then pellets were resuspended in the same volume of MSM. After these procedures, the pellets could be used.[29]

2.4 Effect of different factors on petroleum hydrocarbons biodegradation by free microbial consortium

2.4.1 Effect of environmental factors on biodegradation.

The environment factors affected the petroleum hydrocarbons biodegradation. Due to the same microorganism behaves differently depending on the substrates and environmental conditions, the influences of temperature, pH, NaCl concentration and substrate concentration on petroleum hydrocarbons (NAP, PHE, PYR or crude oil) biodegradation were assessed. When temperature, pH and NaCl concentration were the variables, the initial concentrations of NAP, PHE, PYR and crude oil were 600 mg/L, 600 mg/L, 600 mg/L and 3 g/L in these experiments. The incubation temperatures were set at 25, 30, 35, 40 and 45 ^{o}C, respectively. The initial pH of MSM was adjusted to 5.0, 6.0, 7.0, 7.5, 8.0 and 9.0, respectively. The different NaCl concentrations were 3, 5, 7, 10 and 20 g/L, respectively. While the substrate concentration was a variable, the different concentrations of NAP, PHE and PYR were 10 mg/L, 20 mg/L, 25 mg/L, 30 mg/L, 40 mg/L, 50 mg/L, 80 mg/L, 100 mg/L, 500 mg/L, 800 mg/L and 1000 mg/L; and crude oil concentration was 1g/L, 2g/L, 3g/L, 4g/L, 5g/l and 7g/L, respectively. Constants were that 10% (*v*/*v*) of microbial consortium incubated at 25 ^{o}C, the initial pH of MSM 7.5 and NaCl concentration 5 g/L. Biodegradability of the microbial consortium for different substrates was explored under the optimum environment conditions with 10% of inoculation, shaking at 120 rpm. All samples were collected to measure the residual NAP, PHE, PYR and crude oil after incubating for 7d. The petroleum hydrocarbons was analyzed using the method of Xu *et al.*.[29] Briefly, the total petroleum hydrocarbons were extracted using the liquid – liquid extraction method by extractants (crude oil used petroleum ether and PAHs used cyclohexane) for three times.

The concentrations of NAP, PHE, PYR and crude oil were measured by Alpha-1860 ultraviolet spectrophotometer (Shanghai lab-spectrum instruments Co. Ltd., China) at the maximum wavelength of 221.5, 252, 241.5 and 225 nm, respectively. The corresponding extractant as the control was measured at the same time.

2.4.2 Effect of co-metabolism substrates on biodegradation.

The effects of co-metabolism substrates on biodegradation were investigated by adding 0.4% of glucose, α-lactose, soluble starch, yeast powder and urea in degradation media, respectively. After inoculation of the microbial consortium, all samples were incubated in a rotary shaker (CRYSTAL IS-RDS3, USA) at 25 ^{o}C, pH 7.5, NaCl concentration 5 g/L with different toxic substrates (NAP concentration 800 mg/L, PHE concentration 40 mg/L, PYR concentration 100 mg/L and crude oil concentration 3 g/L). The NAP, PHE, PYR and crude oil biodegradation rates were measured after 10 d, respectively. The assay for petroleum hydrocarbons was based on the method described by Xu *et al.*.[29]

2.5 Cell immobilized in alginate gel micro-spheres

Sodium alginate (SA) is a promising and extensive type of natural gels, recognized as cheap and nontoxic to microorganism. Carrier material was added to improve the mechanical strength, the durability of SA gel and provide the adsorption site for microorganism. Entrapment of the microbial consortium using SA and carrier material was performed as follows. SA was dissolved in sterile distilled water with different carrier materials and α-lactose. After sterilized at 121 ^{o}C during 20 min, the mixture was

cooled 30~40 °C, mixed thoroughly with bacterial suspension, then extruded through a syringe into $CaCl_2$ solution with a certain concentration (sterilised at 121 °C for 20 min and adjusted pH at 7.5) and kept it for 24 h to form micro-spheres. The formed particles were washed 3 times with physiological saline solution and then stored in sterile water at 4 °C before used.[30]

2.6 Optimization on the preparation of immobilized microbial consortium

Many factors restrict the preparation of immobilized microorganism and its biodegradation efficiency, such as gelata, carrier material and cross-linking agent. In order to prepare the suitable immobilized microbial consortium and furthest strengthen the efficiency of petroleum hydrocarbons bioremediation, gelata (SA), carrier material (walnut shell, semi-coke and activated carbon), nutriment (α-lactose), cross-linking agent ($CaCl_2$ solution) and the microbial consortium (20%, *v*/*v*) were used to prepare the immobilized microbial consortium. The factors were ensured in three different levels by single factor experiments, respectively. The factor levels of composition were showed in Table 1. The experiment was designed and conducted according to four factors and three levels orthogonal table.

The biodegradation experiments were conducted in the same degradation media which the crude oil concentration was 3 g/L shaking at 120 rpm, 25 °C. The crude oil biodegradation rates were measured after 7d in order.

2.7 Effect of environmental factors on crude oil biodegradation by immobilized microbial consortium

In order to investigate the ability of immobilized microbial consortium to adapt the environment, the influences of temperature, pH and NaCl concentration on crude oil biodegradation were assessed. The incubation temperatures were set at 20, 25 and 30 ^{o}C, respectively. The initial pH of MSM was ranged from 5.0 to 9.0, respectively. The different NaCl concentrations were ranged from 5 to 25 g/L, respectively. Constants were that 10% (*v*/*v*) of microbial consortium incubated at 25 ^{o}C, the initial pH of MSM 7.5, NaCl concentration 5 g/L and the initial crude oil concentrations 3 g/L. All samples were collected to measure the residual crude oil after shaking at 120 rpm for 5d.

2.8 Biodegradation of petroleum hydrocarbons by immobilized and free microbial consortia

To compare the biodegradation efficiency of petroleum hydrocarbons by immobilized and free microbial consortia, 10% (*v*/*v*) of corresponding biomass (immobilized and free microbial consortia) were inoculated into the degradation medium under the optimum environmental conditions of immobilized microbial consortium obtained from **section 2.7**, shaking at 120 rpm. The concentration of crude oil was measured after the medium incubated for 5 d.

2.9 FTIR analysis of immobilized and free microbial consortia

FTIR (Fourier transform infrared) spectrum study was carried out to explain the change in the functionalities and types of immobilized and free microbial consortia and the control. FTIR spectra were recorded in the 4000~400 cm^{-1} region for a KBr pellet by a FTIR spectrophotometer (TENSOR 27, Germany) with a resolution of 1.0 cm^{-1}. The

freeze drying treatment for FTIR study was performed in advance. The pellets of free microbial consortium (**section 2.3**), immobilized microbial consortium carried by semi-coke (**section 2.5**) and its control were pre-frozen at –79 ^{o}C in cryogenic refrigerator (Thermo scientific forma 700 series, USA) for 36 h and then were dried into powder by freezer dryer (SIM INTERNATIONAL FD5-5, USA).

2.10 SEM analysis of immobilized microbial consortium and its control

In order to assess whether the microbial consortium were embedded into the alginate gel micro-sphere and inspect the change in the micro structure of immobilized microbial consortium and its control, the samples carried by semi-coke before and after foaming were treated under the same conditions after incubated into beef extract peptone medium for 24 h shaking at 120 rpm, 25 ^{o}C. The procedure of SEM analysis was used as follows. Samples were fixed in 2.5% glutaraldehyde for 3 h and washed 3 times with phosphate buffer solution (0.1 M, pH 7.2~7.4) for 3 min once time. Then they were dehydrated in a graded series (30%, 50%, 70%, 80%, 90% and 100%) of alcohol and once time for 15 min. Finally, samples were dried at CO_2 critical point for over 60 min, immobilized and sprayed gold for 15 min. Samples were viewed in a Hitachi S-3400N SEM (scanning electron microscope) (Hitachi Ltd., Tokyo, Japan).

3 Results and discussion

3.1 Effect of environmental conditions on petroleum hydrocarbons biodegradation

3.1.1 Effect of temperature on biodegradation of free microbial consortium

Temperature plays very important roles in biodegradation of petroleum hydrocarbons, firstly by its direct effect on the chemistry of the pollutants, and secondly on its effect on the physiology and diversity of the microbial milieu.[31-33] To explore whether the optimum temperature for crude oil degraded by the specific microbial consortium was as same as PAHs, the experiments were conducted. The influence of temperature on petroleum hydrocarbons biodegradation was shown in Fig. 1a.

As shown in Fig. 1a, the degradation rates of crude oil, PHE and PYR by the microbial consortium reached the highest at 30 ^{o}C and they were 48%, 30% and 25%, respectively. The influence of temperature change on PHE was not great. However, temperature had significant influence on degradation of crude oil and PYR by the microbial consortium. The highest removal rate of NAP degraded by the microbial consortium was 46% at 35 ^{o}C but the degradation rate had a little change when temperature ranged from 25 to 35 ^{o}C. So the suitable temperature of microbial consortium degrading NAP was kept at 30 ^{o}C. Thus, 30 ^{o}C was the suitable temperature of the microbial consortium degrading NAP, PHE, PYR and crude oil. Besides, in the same concentrations, the degradation rate of PAHs followed an order NAP > PHE > PYR, which was consistent with the results reported by Zhou *et al.*.[34]

3.1.2 Effect of pH on biodegradation of free microbial consortium

Different pH values influence microorganisms on the utilization of nutrients, the microbial adsorption, the extracellular enzyme's production and secretion and so on. The optimum pH values for various microbe growths are multifarious, which lead to the

difference in biodegradation efficiency of the substrate and the microbe quantity. The influence of pH on petroleum hydrocarbons biodegradation by the microbial consortium was depicted in Fig. 1b. It suggested that pH value of MSM could affect petroleum hydrocarbons (NAP, PHE, PYR and crude oil) degradation. The effect of pH on the degradation rate of crude oil and PYR degraded by microbial consortium was slight. In addition, the most significant effect on crude oil and PYR degradation was observed at the pH range of 7.5~9.0 and 6.0~8.0. The highest degradation rate of crude oil and PYR reached 41% at pH 8.0 and 25% at pH 7.0. When pH value ranged from 7.0 to 8.0, the effect of pH on the microbial consortium degrading NAP and PHE was not great. The NAP and PHE biodegradation efficiency maintained above 60% and 17%. Besides they reached the highest point 68% at pH 7.5 and 26% at pH 7.0, respectively. This experiment demonstrated that the microbial consortium fit diverse pH to degrade different substrates and it was suitable for alkaline environment at pH 7.0~8.0 degrading petroleum hydrocarbons. Okoh[32] reported that the slight alkaline pH of seawater seems to be quite favorable for petroleum hydrocarbon degradation which was consistent with our results.

3.1.3 Effect of NaCl concentration on biodegradation of free microbial consortium

Inorganic salts are necessary nutrition elements in microbial proliferation. In the microorganism growth process, inorganic salts play a promoting role on enzyme reaction, maintaining cell membrane equilibrium and regulating osmotic pressure. The influence of NaCl concentration on petroleum hydrocarbons degraded by the microbial

consortium was depicted in Fig. 1c. It shows the degradability increased with NaCl concentration in the presence of low salinity. Nevertheless, the higher degradation efficiency could not be further achieved by continuing increase of NaCl concentration. The optimum NaCl concentration for the biodegradation of NAP, PHE, PYR and crude oil by the microbial consortium was 10 g/L, 5 g/L, 15 g/L and 7 g/L, respectively. The optimal degradation efficiencies of NAP, PHE, PYR and crude oil were 90%, 48%, 33% and 42% under these conditions, respectively. The effects of NaCl concentration on the microbial consortium degrading PHE and PYR were slight. Microbial consortium degrading PHE and crude oil were suitable for low NaCl concentration, while degrading NAP and PYR were suitable for high NaCl concentration. On the whole, this microbial consortium degrading NAP, PHE, PYR and crude oil were suitable for the NaCl concentration 5~10 g/L.

3.1.4 Effect of substrate concentration on biodegradation of free microbial consortium

A microbial consortium screened has different degradation abilities for different substrates such as NAP, PHE, PYR and crude oil. Meanwhile, the multifarious substrates concentrations produce different effects on microbial growth. Too high substrate concentrations are toxic to the microbes, while too low substrate concentrations cannot provide enough carbon sources for the microorganism growth. Therefore, it is necessary to explore the capability of microorganisms adapting to the environment with different substrate concentrations. The effect of substrate

concentration on the microbial consortium degrading petroleum hydrocarbon was shown in Fig. 2.

As shown in Fig. 2, the biodegrading capability increased with substrate concentrations when the concentrations were low. Nevertheless, higher degradation rates could not be further achieved by continuing the increase of the substrate concentrations. The degradation rate declined due to the different toxicity of substrate when the concentration was higher than the suitable concentration. Suitable substrate concentrations of the microbial consortium were NAP 800 mg/L, PHE 40 mg/L, PYR 100 mg/L and crude oil concentration 3 g/L and the highest biodegradation rate of NAP, PHE, PYR and crude oil reached 86%, 89%, 38% and 44%, individually. The degradation rate was similar to the results obtained by Chen *et al.*[35] whose reported that the removal percentages were 85-93% for PHE. The inherent biodegradability of these individual components is a reflection of their chemical structure, but is also strongly influenced by toxicity of the compounds.[32] It could be seen, for PAHs with 2, 3 and 4 benzene rings that the toxicity of PAHs was increased with ring number, the microbial consortium was more easily to degrade NAP with 2 rings than PHE (3 rings) and PYR (4 rings). Results were consistent with the paper that condensed polycyclic aromatics are degraded, one ring at a time, by a similar mechanism, but biodegradability tend to decline with the increasing number of rings and degree of condensation.[36] Biodegradability is inherently influenced by the composition of the oil pollutant. In crude oil occur in complex mixtures and influence each other's biodegradation. Some

isoalkanes are apparently spared as long as n-alkanes are available as substrates, while some aromatics are metabolised only in the presence of more easily utilisable petroleum hydrocarbons, a process referred to as co-metabolism.[32] In our results, the higher concentration of crude oil was degraded by this microbial consortium was probable due to the co-metabolism acted by some microorganisms and the high content of easier biodegradable substances such as nalkanes.

3.2 Biodegradability of free microbial consortium under the optimum conditions

To see from Fig. 1 to Fig. 2, the optimum environmental conditions of degrading petroleum hydrocarbons by the free microbial consortium were that NAP concentration 800 mg/L, pH 7.5, NaCl concentration 10 g/L; PHE concentration 40 mg/L, pH 7, NaCl concentration 5 g/L; PYR concentration 100 mg/L, pH 7, NaCl concentration 15 g/L; crude oil concentration 3 g/L, pH 8, NaCl concentration 7 g/L; all of temperatures at 30 ^{o}C. Biodegradation efficiency of the microbial consortium was explored under individual optimum conditions.

The degradation rate of NAP, PHE, PYR and crude oil reached 80%, 30%, 56% and 48% under the optimum environmental conditions, respectively. For comparison, it was reported that the individual removal percentages of PHE and PYR were 22-38 % and 39-40 % in the solution amended with free bacteria after a 21-day incubation in a solution system.[35] It showed that our free microbial consortium had higher biodegrading capability on degrading NAP, PHE, PYR and crude oil. So it can be used to degrade different types of petroleum hydrocarbons.

3.3 Co-metabolism of petroleum hydrocarbons

Petroleum hydrocarbons are a kind of complex mixture including alkane, aromatic hydrocarbon, colloid and asphaltene etc. They are toxic to microorganism and not the essential nutrients for microbial growth, especially for PAHs. Therefore, microorganisms require a period of time to adapt to the petroleum hydrocarbons contaminated environment. In order to shorten this adaption period and accelerate degradation of contaminants, some other carbon sources which microorganisms can easily utilize may be added. But there was another problem would exist that addition of other carbon sources may inhibit the biodegradation of contaminants and result in diauxic growth.[37] Hence, in order to enhance the biodegradation, it is necessary to explore the effects of different additional carbon sources as co-metabolic substances on petroleum hydrocarbons biodegradation. In this experiment, glucose, α-lactose, soluble starch, yeast powder and urea were added as co-metabolic substances, respectively.

Fig. 3 showed that the effects of different co-metabolic substances on biodegradation rates of NAP, PHE, PYR and crude oil. The biodegradation rate of NAP was much higher as adding soluble starch, glucose and α-lactose. α-Lactose was the best co-metabolic substrate which accelerated the biodegradation of NAP and the biodegradation rate of NAP was 96%. The biodegradation rates of PHE with soluble starch, glucose, yeast extract powder and urea were basically same, about 89%, 89%, 91% and 93%, respectively. But it with α-lactose was a little low, about 68%. Only α-lactose could improve the PYR biodegradation and the biodegradation rate of PYR

with it was about 61%. Soluble starch was much easier to accelerate the crude oil biodegradation than glucose, α-lactose and urea. Seen from above, co-metabolic substances acted on diverse substrates were slightly different. Besides, α-lactose was the most suitable co-metabolic substance and additional carbon source for biodegrading NAP, PHE, PYR and crude oil. This result was as same as that got by Qiang *et al.*[38] who researched the co-metabolic substance with anthracene.

3.4 The orthogonal results and analysis of immobilized microbial consortium biodegradation

To remediate spill oil in marine where present higher NaCl concentration, lack of nutrient, alkaline environment and low temperature, and explore the optimum biodegradation ways, immobilization method was employed as bioremediation technology to removal petroleum hydrocarbons. Because selected carrier material should be easy available, non-toxic and low-cost, walnut shell, semi-coke and activated carbon were chosen as the carrier materials to immobilize the microorganisms basing on the idea of "using waste to treat waste". Furthermore, α-lactose (**section 3.3**) as nutriment and co-metabolic substance was embedded into the micro-spheres. The orthogonal biodegradation analysis results of the microbial consortium immobilized by walnut shell, semi-coke and activated carbon were shown in Table 2, 3 and 4, respectively.

The removal rates of crude oil by the microbial consortium immobilized by walnut shell were about 20%~32% (Table 2), that by semi-coke were about 41%~65% (Table 3)

and that by activated carbon were about 26%~47% (Table 4). The highest biodegradation rates of crude oil by the immobilized microbial consortium were 32% (carrier was walnut shell), 65% (carrier was semi-coke) and 47% (carrier was activated carbon). Compared Table 2, 3 and 4, the removal rates of crude oil by the microbial consortium immobilized by semi-coke were the highest. Thus semi-coke was considered as the optimum carrier material. For walnut shell as carrier material (Table 2), the importance and the optimum conditions of various factors followed an order of walnut shell (20%)＞$CaCl_2$ (1%)＞α-lactose (0.1%)＞SA (6%); for semi-coke as carrier material (Table 3), that followed an order of α-lactose (0.3%)＞semi-coke (20%)＞SA (7%)＞$CaCl_2$ (3%); for activated carbon as carrier material (Table 4), that followed an order of α-lactose (0.5%)＞$CaCl_2$ (3%)＞activated carbon (20%)＞SA (4%). Therefore, the importance and the optimum conditions of various factors were different for various carrier materials. The optimum preparation condition of immobilization microbial consortium with semi-coke as carrier material followed an order of α-lactose (0.3%)＞ semi-coke (20%)＞SA (7%)＞$CaCl_2$ (3%).Thus, semi-coke was selected as the suitable carrier applied in the following experiments. These results may be depending on the absorbability of the carriers. In our early exploration experiments, adsorption capacity of inorganic carrier materials (semi-coke, activated carbon) for microbial consortium was higher than organic material (walnut shell), particularly the adsorption quantity of semi-coke was larger than other carriers. The inconsistent observations may be ascribed primarily to the different structural characteristics of three materials to stimulate

biodegradation.[35] It is feasible to use adsorption carriers with high absorptive capabilities to concentrate PAHs as well as microorganisms and thereby enhance dissipation of PAHs and crude oil.[35]

3.5 Effect of environment factors on crude oil biodegradation by immobilized microbial consortium

The microbial consortium immobilized by semi-coke was effected by temperature was shown in Fig. 4a. The degradation of crude oil by immobilized microbial was up to 48% at 25 ^{o}C. The removal rate of crude oil was at about 33% at 20 ^{o}C and 30 ^{o}C. For comparison, the best free microbe degradation of crude oil was 48% at 30 ^{o}C (Fig. 1a). When at 25 ^{o}C, the free microbe degradation rate was only 27% lower than the immobilized microbial. Thus, the microbial consortium can adapt to low temperature after immobilized, the optimum temperature changed from 30 ^{o}C to 25 ^{o}C, therefore, the immobilized microorganism was more advantageous to the low temperature marine environment in real oil spill bioremediation. However, the immobilized microbial degradation rate was as same as free bacteria that was probable due to the number of immobilized bacteria was less than the free bacteria during the degradation process. In addition, the degradation of the immobilized bacteria was measured after incubated 5 days which shorter than free bacteria (7 days).

Immobilized microorganism oil degradation rate was changed little during the pH ranged from 5 to 9 and the degradation rate was almost between 25% and 30% (Fig. 4b). The maximum degradation rate was 31% at the optimum pH of 7. Compare to the free

microbial consortium (Fig. 1b), free microorganism are greatly influenced by pH in alkaline conditions. It was easily to see that the microbial consortium could resist alkali and acid environment and was maintain a relatively stable degradation rate after immobilized. But compared with the crude oil degradation rate of free bacteria, immobilized microbial degradation rate is relatively low. It may be under the influence of the immobilized embedding number and a short degradation time of immobilized microbial.

The influence of NaCl concentration on crude oil biodegradation by the immobilized microbial consortium was depicted in Fig. 4c. The suitable NaCl concentration for immobilized microorganisms was in the range of 10 to 20 g/L and the crude oil degradation rate was about 43%. The optimum concentration of NaCl was 20 g/L. Relatively free microorganisms (Fig. 1c), 7 g/L was the optimal concentration of free microorganisms, the largest crude oil degradation rate was 41%, and greatly influenced by the concentration of NaCl. Apparently, the effect of the concentration of NaCl was relatively small and the range of suitable NaCl concentration has become wider after immobilization. Meanwhile, the crude oil degradation rate was slightly increased though the degradation time was shorter than free microorganisms.

3.6 Biodegradation of petroleum hydrocarbons by immobilized and free microbial consortia

The biodegradation efficiencies of crude oil by immobilized and free microbial consortia were investigated in the same degradation medium with crude oil

concentration 3 g/L, NaCl concentration 20 g/L, pH at 7 and temperature of 25 ^{o}C. Compared immobilized and free microbial consortium, the biodegradation rate of crude oil by immobilized microbial consortium (47%) was higher than that by free microbial consortium (26%) during 5d degradation. Thus, the microbial consortium subjected from immobilization, the biodegradation efficiency became significantly. Chen *et al.*[35] reported that using bacteria and adsorption carriers could enhance the removal of PAHs from water. The decreased amount of absorbed petroleum hydrocarbons suggests that the immobilized bacteria could directly degrade the carrier-associated PAHs and crude oil.

3.7 Surface functional groups on immobilized and free microbial consortia

FTIR analysis was used in order to determine the types and intensities of the surface functional groups in free and immobilized microbial consortia, as well as evaluate the effect of immobilization-treatment on these chemical groups. FTIR-spectra of immobilized microbial consortium, its control (the semi-coke carrier without microbial consortium) and free microbial consortium were shown in Fig.5.

The spectrums were characterized by appearance of the much sharper peaks in the wave regions 3864~630 cm^{-1}. The region of 3864 cm^{-1} and 3736 cm^{-1} were result from the shift of amino (–NH) and bonded hydroxyl (–OH) groups from –COOH. The peak at 2383 cm^{-1} and 2356 cm^{-1} presented carbon dioxide and 2310 cm^{-1} presented nitrile. They were appeared in free and immobilized microbial consortia spectra but absent in the immobilized control. The strong and slightly broad peaks observed in the region of

3451 cm^{-1} indicated both amino (N–H) and bonded hydroxyl groups (O–H stretching) that were observed in all of the samples. Peaks observed at 2930 cm^{-1} and 2851 cm^{-1} of immobilized control represented asymmetric and symmetric deformation of $–CH_2$ groups in aromatics, respectively Bayramoglu *et al.*[39] thought that 1654 cm^{-1} indicated the bending of N–H of both chitin and chitosan on the cell wall structure of fungal mycelia. Xu *et al.*[40] thought that 1652 cm^{-1} presented –C=O from amino. Therefore, bacterium probably has the part similar functional group and type such as –NH and –C=O. The strong peaks observed at around 1620 cm^{-1} was probably indicative of the bending of C=C in aromatic compound. Absorption peaks observed at about 1515 cm^{-1} in both free and immobilized microbial consortia represented N–H stretching of the primary and secondary amides.[41] Maciel *et al.*[42] thought the symmetric stretch for carboxylate ions was indicated by the peaks at 1408 cm^{-1}. Thus, 1387 cm^{-1} in three spectrums may be result from the shift of symmetric stretch for carboxylate ions. Bands were mainly due to phosphate stretching vibration in polysaccharides and nucleic acids in the range of 1200~900 cm^{-1} spectral region corresponding to C–O–P.[43] The phosphate group presented certain characteristic absorption peaks (P=O stretching at 1150 cm^{-1}; P–OH stretching at 1100~1030 cm^{-1}; P–O–C stretching at 1050~970 cm^{-1}) .[41,42,44] Therefore, the peaks observed at 1093 cm^{-1} and 992 cm^{-1} of free microbial consortium indicated the presence of the phosphate functional groups in bacteria. Rubio *et al.*[45] thought C–O–P stretching vibrations implied oligo– and polysaccharides appearing in the bacteria. 1110 cm^{-1} and 1029 cm^{-1} may present the vibration of mineral

crystal compounds (such as quartz, kaolinite, etc.).[46] Fan *et al.*[47] thought the absorption peak 1249 cm^{-1} and 873 cm^{-1} indicated a symmetrical C–O–S vibration associated to a C–O–SO_3 group in sodium alginate. However, these two frequencies were absent in the spectrum of immobilized microbial consortium and its control, which proved that the immobilized treatment made sodium alginate change into sodium alginate sulfate. The band 630 cm^{-1} represented the adsorption peak of Fe_3O_4.[48]

According to these spectrum, free microbial consortium contained functional groups such as –NH, C–N, P=O, –CH_2, P–OH, P–O–C and –COOH which indicated that bacterial cells contains polysaccharides, nucleotide and protein;[40] Immobilized control had –NH, –CH_2, C=C, –COOH, mineral crystal compounds and Fe_3O_4 which suggested that semi-coke, sodium alginate and lactose included mineral crystal and metallic compounds, and organism substance; Immobilized microbial consortium had –NH, –OH, C–N, C=C, –COOH, P=O, mineral crystal compounds and Fe_3O_4 which was not the merged one.[48] Compared them, though immobilization-treatment, asymmetric and symmetric deformation of –CH_2 groups was absent in the spectrum which probably proved that –CH_2 groups changed into carbonate; P–OH and P–O–C translated into P=O; –OH present in the spectrum. Xu *et al.*[40]thought immobilization prevents loss or damage of some molecular groups. Thus, all the results demonstrated that the functional groups (i.e. hydroxy and phosphate) were identified in microbial consortium biomass and changed by immobilization.

3.8 SEM analysis of immobilized microbial consortium and its control

In order to observe the microstructure of the surface and pore of immobilized microorganism and ensure whether the microorganism was embedded into the algae micro-sphere, immobilized microbial consortium and its control were studied using SEM at 6000× magnification. The SEM images were shown in Fig. 6.

The control possesses relatively strong and dense porous structure which was composed of small pores and had not bigger porous channels (Fig. 6a). However, the microbial consortium is most short rod-shape, punctured well into the semi-coke immobilization material and accommodated in its loose channel (Fig. 6b). Beside the immobilized microbial consortium had porous interior microstructure which was consists of large pores. It was reported by Wang *et al.*[49] that the carrier material had large porosity could permit excellent mass transport of oxygen, nutrients and degradation substrates. Our results showed that the semi-coke immobilization material with large pores had improving porosity and mass transfer property. In that case, the immobilized cells and the bioremediation had been improved. These results ensured that the microbial consortium were entrapped into the carrier by alginate gel and suggested that the structure of semi-coke became more porous and easier adhere with the microbial consortium after the carrier subjected from the immobilization. Further, the immobilized microbial consortium mainly attached in the surface and impaled into the pores of the carrier material. The distribution of cells on the semi-coke carrier was not uniform, some cells gathered and other dispersed, which was as same as the results got by Qiao *et al.*,[50] who thought there might be two reasons for the attachment of microbial

cells including physical effect between cells and the carrier, as well as adhesive effect between the carrier and the extracellular secretion of strain.

4 Conclusions

A microbial consortium isolated from Shengli oilfield polluted sludge was capable of degrading crude oil and PAHs (NAP, PHE and PYR). The degradation rate showed semi-coke was a better carrier than walnut shell and activated carbon. In addition, the structure of semi-coke became bigger porous and more easily adhered with the microbial consortium though immobilization-treatment. The degradation rate of crude oil by immobilized microbial consortium was 47%, higher than the free microbe. After immobilized, the microbial consortium was more able to adapt to the low temperature, alkali or acid and big span NaCl concentration environment. Thus, microbial consortium immobilized by semi-coke could be applied in real oil spill bioremediation.

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References

1 F. Mervin, Oil spill science and technology, *Gulf professional publishing*, USA, 2010.

2 Y. Wang, C. C. Shen, Z. Y Shen, D. Zhang and J . Crittenden, *Environ. Sci.: Processes Impacts*, 2015, DOI: 10.1039/c5em00077g

3 I. J. Keyte, R. M. Harrison and G. Lammel, *Chem. Soc. Rev*., 2013, **42**, 9333–9391

4 Y. S. Ling, H. J. Liang, M. H. Chung, M. H. Linc and C. Y. Lin, *Mol. BioSyst*., 2014, **10**, 1918–1931

5 Z. Z. Zhang , Z. W. Hou, C.Y. Yang, C. Q. Ma, F. Tao and P. Xu, *Bioresour. Technol*., 2011, **102(5)**, 4111–4116.

6 P. Arulazhagan and N.Vasudevan, *Mar. Pollut. Bull*., 2011, **62(2)**, 388–394.

7 B. Mortazavi, A. Horel, M. J. Beazley and P. A. Sobecky, *J. Hazard. Mater*., 2013, **244–245**, 537–544.

8 D. Sanscartier, T. Laing, K. Reimer and B .Zeeb, *Chemosphere*, 2009, **77(8)**, 1121–1126.

9 W. X. Xia, J. C. Li, Z. W. Song and Y. J. Sun, *J. Environ. Sci*., 2007, **19(12)**, 1491–1495.

10 J. S Ye, H. Yin, J. Qiang, H. Peng, H. M. Qin and N. Zhang, *J. Hazard. Mater*., 2011, **185(1)**, 174–181.

11 K. V. Sajna, R. K. Sukumaran, L. D. Gottumukkala and A.Pandey, *Bioresour. technol*., 2015, **191**, 133–139.

12 S. Xiong, X. Li, J. Chen, L. P. Zhao, H. Zhang and X. J. Zhang, Appl. *Microbiol. Biotechnol*., 2015, **99(3)**, 1451–1461.

13 B. A. McKew, F. Coulon, M. M. Yakimov, R. Denaro, M. Genovese, C. J. Smith, *et al.*, *Environ. Microbiol*., 2007, **9(6)**, 1562–1571.

14 H. Z. Wang and S. Y. Li, *Chem. Bull*., 1997, 22–27. (in chinese)

15 K. L. Simons, A. Ansar, K. Kadali, A. Bueti , E. M. Adetutu and A. S. Ball, *Bioresour. Technol*., 2012, **126**, 202–207.

16 S. He and G. Xue, *J. Hazard. Mater*., 2010, **178(1–3)**, 895–899.

17 K. L. Simons, P. J. Sheppard, E. M. Adetutu, K. Kadali, A. L. Juhasz , M. Manefield, *et al.*, *Bioresour. Technol*., 2013, **134**, 107–116.

18 S. X. Wang, X. J. Li, W. Liu, P. J. Li, L. X. Kong, W. J. Ren, *et al.*, *J. Environ. Sci*., 2012, **24(9)**, 1662–1669.

19 X. Zhao, Y. M. Wang, Z. Y. Ye, A. G. L. Borthwick and J. R. Ni. *Process. Biochem*., 2006, **41(7)**, 1475–1483.

20 D. Y. Hou, X. R. Shen, Q. Luo, Y. He, Q. R. Wang and Q. Liu, *Mar. Pollut. Bull*., 2013, **67(1–2)**, 146–151.

21 E. Quek, Y. P. Ting and H. M. Tan, *Bioresour. Technol*., 2006, **97(1)**, 32–38.

22 N. Massalha, A. Shaviv and I. Sabbah, *Water. Res*., 2010, **44(18)**, 5252–5259.

23 A. S. Y. Ting, N. H. A. Rahman, M. I. H. M. Isa and W. S. Tan, *Bioresour. Technol*., 2013, **147**, 636–639.

24 C. Elliott, Z. Ye, S. C. Mojumdar and M. T. Saleh, *J. Therm. Anal. Calorim*., 2007, **90(3)**, 707–711.

25 T. Pae, A. Luud, M. Sepp, *Oil Shale*, 2005, **22**, 333–344.

26 Q. Wang, H. Wang, B. Sun, *et al.*, *Fuel*., 2009, **88(8)**, 1520–1529.

27 A. Srinivasan, T. Viraraghavan, *Bioresour. Technol*., 2008, **99(17)**, 8217–8220.

28 J. W. Kim, M. H. Sohn, D. S. Kim, *et al.*, *J. Hazard. Mater*., 2001, **85(3)**, 301-315.

29 N. N. Xu, M. T. Bao, P. Y. Sun, Y. M. Li, *Bioresour. Technol*., 2013, **149**, 22–30.

30 K. Jézéquel and T. Lebeau. *Bioresour. Technol*., 2008, **99(4)**, 690–698.

31 M. D. Wallenstein, M. L. Haddix, D. D. Lee, R. T. Conant, E. A. Paul, *Soil. Biol. Biochem*., 2012, **47**, 18–26.

32 A. I. Okoh, *Biotechnology and molecular biology review*, 2006, **1(2)**, 38-50.

33 R. M. Atlas, *Microbiological reviews*, 1981, **45(1)**, 180.

34 H. W. Zhou, T. G. Luan, F. Zou, N. F. Y. Tam, *J. Hazard. Mater*., 2008, **152(3)**, 1179–1185.

35 B. Chen , M. Yuan and L. Qian, *J. Soil. Sediment*., 2012, **12(9)**, 1350–1359.

36 R. M. Atlas, R. Bartha, *Microbial ecology: fundamentals and applications*, Addison-wesley, USA. 1981.

37 K. Lee, J. W. Park and I. S. Ahn, *J. Hazard. Mater*., 2003, **105(1–3)**, 157–167.

38 J. Qiang, H. Yin, H. Peng, J. S. Ye, H. M. Qin , B. Y. He, *et al.*, J. *Environ. Sci*., 2009, **30(5)**, 1298–1305. (in Chinese)

39 G. Bayramoglu , G. Celik and M. Y. Arica, *J. Hazard. Mater*., 2006, **136(2)**, 345–353.

40 J. Xu, X. C. Song, Q. Zhang, H. Pan, Y. Liang, X. W. Fan, and Y. Z. Li, *J. Hazard. Mater*., 2011, **187(1–3)**, 450–458.

41 T. Mathialagan and T. Viraraghavan. *Bioresour. Technol*., 2009, **100(2)**, 549–558.

42 G. M. Maciel, C. G. M. Souza, C. A. V. Araújo, E. Bona, C. W. I. Haminiuk, R. Castoldi, *et al.*, *Chem. Eng. J*., 2013, **215–216**, 331–338.

43 R. Singh, A. Kumar, A. Kirrolia, R. Kumar, N. Yadav, N. R. Bishnoi, *et al.*, *Bioresour. Technol*., 2011, **102(2)**, 677–682.

44 M. X. Loukidou, K. A. Matis, A. I. Zouboulis and M. Liakopoulou-Kyriakidou, *Water. Res*., 2003, **37(18)**, 4544–4552.

45 C. Rubio, C. Ott, C. Amiel, I. Dupont-Moral, J. Travert and L. Mariey, *J. Microbiol. Methods*., 2006, **64(3)**, 287–296.

46 J. L. Zhou, S. Zhang, Y. G. Wang, X. C. Lin, C. H. Wang and D. P.Xu, *Coal. Sci. Technol*., 2012, **40(10)**, 116–119. (in chinese)

47 L. Fan, L. Jiang, Y. M. Xu, Y. Zhou, Y. Shen, W. G. Xie, *et al.*, *Carbohyd. Polym*., 2011, **83(4)**, 1797–1803.

48 M. K. Hong, B. J. Park and H. J. Choi, *J. Phys. Conf. Ser*., 2009, DOI: 10.1088/1742–6596/149/1/012055.

49 Z. Y. Wang, Y. Xu, H. Y. Wang, J. Zhao, D. M. Gao, F. M. Li and B. Xing, *Pedosphere*, 2012, **22(5)**, 717–725.

50 L. Qiao, D. H. Wen and J. L.Wang, *Bioresour. Technol*., 2010, **101(14)**, 5229–5234.

Table 1 Composition factors and levels of immobilized microbial consortium

Factor	Carrier material /%	SA /%	α-lactose /%	$CaCl_2$ /%
Level	5	4	0.1	1
	10	5	0.3	3
	20	6	0.5	5

Table 2 Orthogonal biodegradation analysis of microbial consortium immobilized by walnut shell

Experiment number	**Walnut shell /%**	**SA /%**	**α-Lactose /%**	**$CaCl_2$ /%**	**Removal rate of crude oil /%**
1	5	5	0.1	1	25
2	5	6	0.3	3	20
3	5	7	0.5	5	21
4	10	5	0.3	5	23
5	10	6	0.5	1	23
6	10	7	0.1	3	20
7	20	5	0.5	3	21
8	20	6	0.1	5	32
9	20	7	0.3	1	29
The average 1[a]	21.57	22.87	25.633	25.67	—
The average 2	22.30	25.03	23.97	20.17	—
The average 3	27.27	23.23	21.53	25.30	—
Range[b]	5.70	2.17	4.10	5.50	—

a：The average represents the arithmetic mean value of the individual factor's level. The maximum average of every factor represents the optimum level of individual factor.

b： Range represents the difference value between the maximum and the minimum among the four averages. The bigger range infers that the factor is more important.

Table 3 Orthogonal biodegradation analysis of microbial consortium immobilized by semi-coke

Experiment number	Semi-coke /%	SA /%	α-Lactose /%	$CaCl_2$ /%	Removal rate of crude oil /%
1	5	4	0.1	1	41
2	5	5	0.3	3	56
3	5	6	0.5	5	45
4	10	4	0.3	5	56
5	10	5	0.5	1	46
6	10	6	0.1	3	60
7	20	4	0.5	3	49
8	20	5	0.1	5	57
9	20	6	0.3	1	65
The average[a] 1	47.03	48.37	52.67	50.30	—
The average 2	53.80	52.93	58.57	54.93	—
The average 3	57.03	56.57	46.63	52.63	—
Range[b]	10.00	8.20	11.93	4.63	—

Table 4 Orthogonal biodegradation analysis of microbial consortium immobilized by activated carbon

Experiment number	Activated carbon /%	SA /%	α-Lactose /%	$CaCl_2$ /%	Removal rate of crude oil /%
1	5	4	0.1	1	29
2	5	5	0.3	3	37
3	5	6	0.5	5	37
4	10	4	0.3	5	31
5	10	5	0.5	1	33
6	10	6	0.1	3	32
7	20	4	0.5	3	47
8	20	5	0.1	5	26
9	20	6	0.3	1	37
The average[a] 1	33.93	35.37	28.80	32.70	—
The average 2	31.87	31.83	34.90	38.33	—
The average 3	36.50	35.10	38.60	31.27	—
Range[b]	4.63	3.53	9.80	7.07	—

List of the figure captions

Fig .1 Effect of environmental conditions on biodegradation by free microbial consortium: a) Temperature, b) pH, c) NaCl concentration

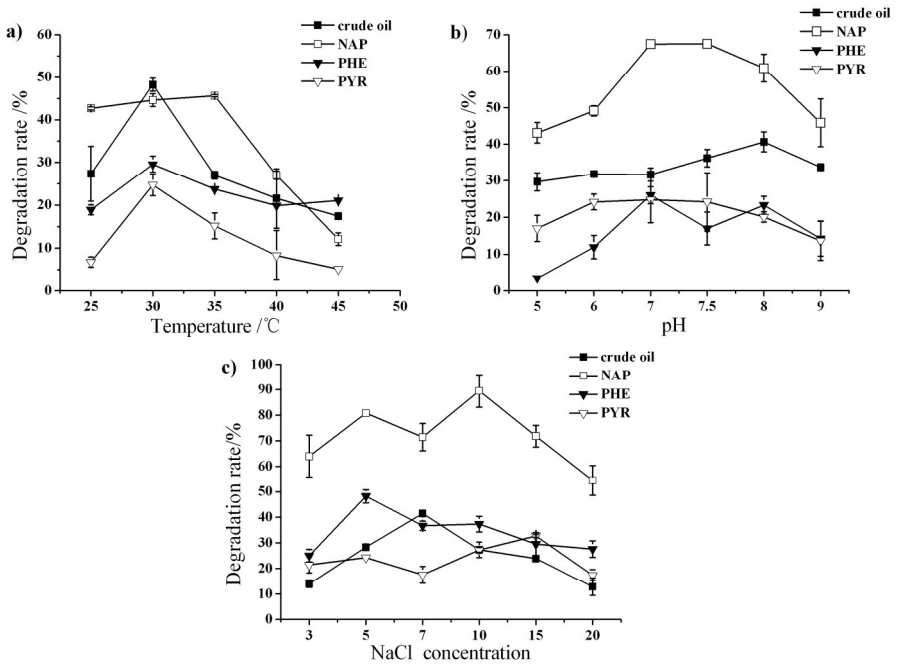
Fig. 2 Effect of substrate concentration on biodegradation: a) Crude oil b) NAP c) PHE d) PYR

Fig. 3 Effect of co-metabolic substances on biodegradation

Fig. 4 Influence of environment factors on crude oil degradation rate by immobilized microbial consortium: a) Temperature b) pH c) NaCl concentration

Fig. 5 FTIR analysis of immobilized and free microbial consortia

Fig. 6 SEM images of immobilized microbial consortium and control samples (bars represent 5 um). (a) Control samples. (b) Immobilized microbial consortium, and the microorganism was signed by red elliptical ring



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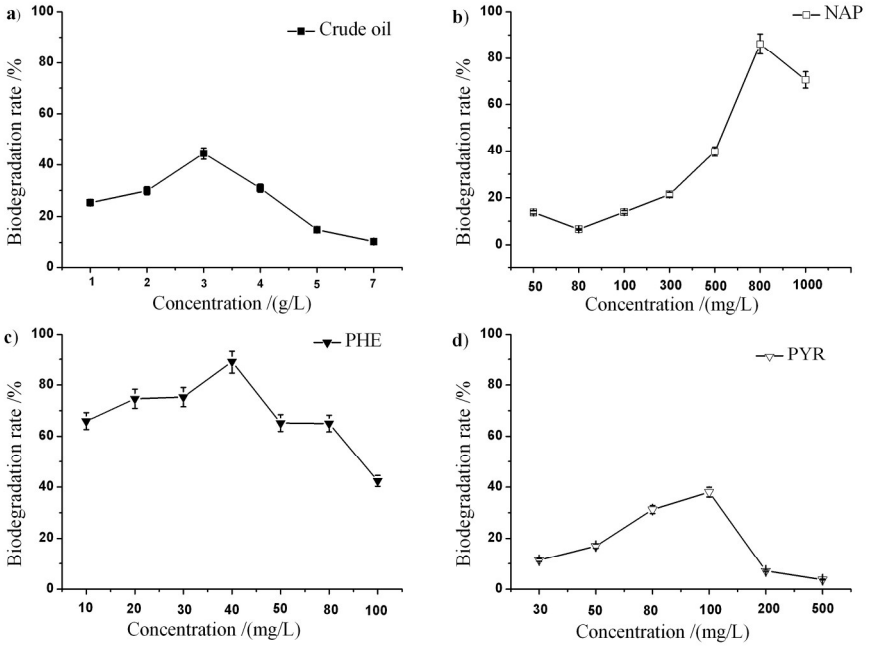

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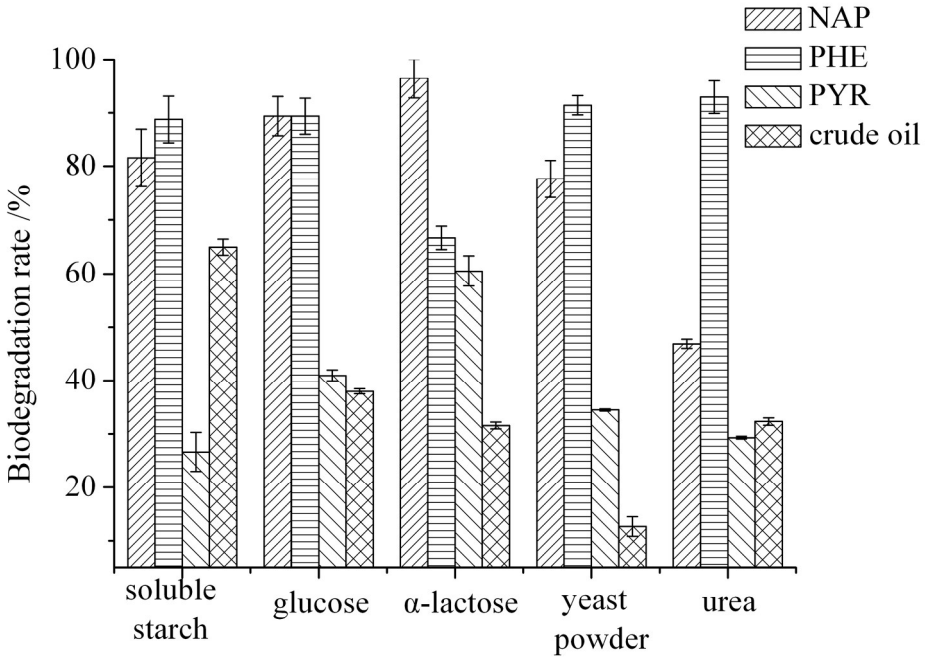

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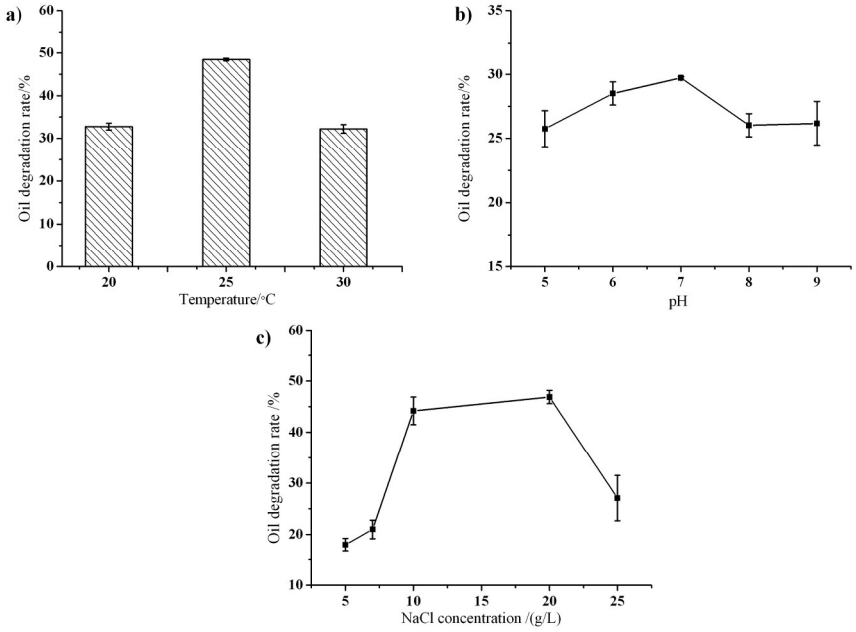

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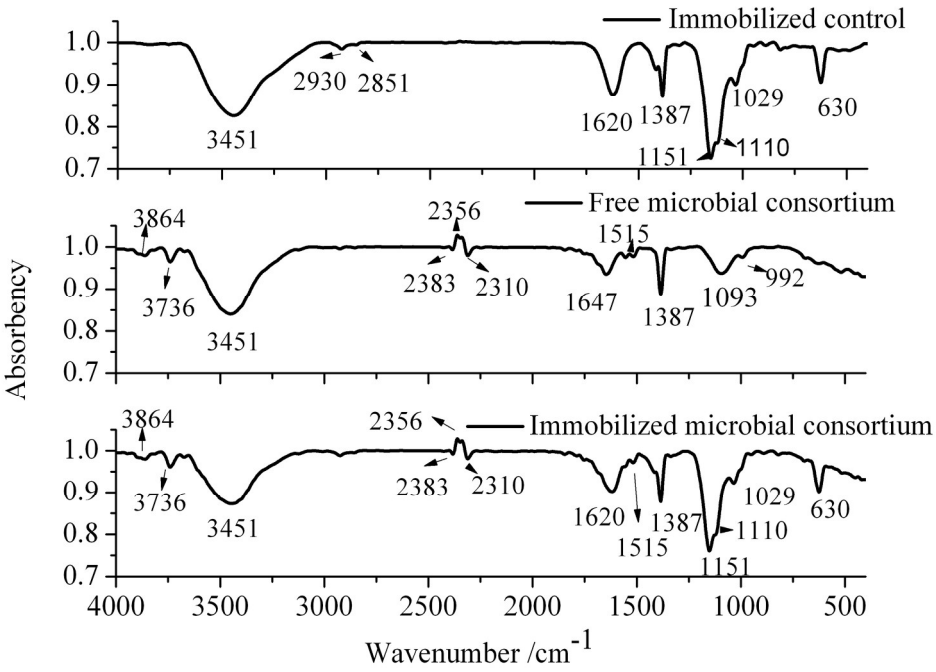

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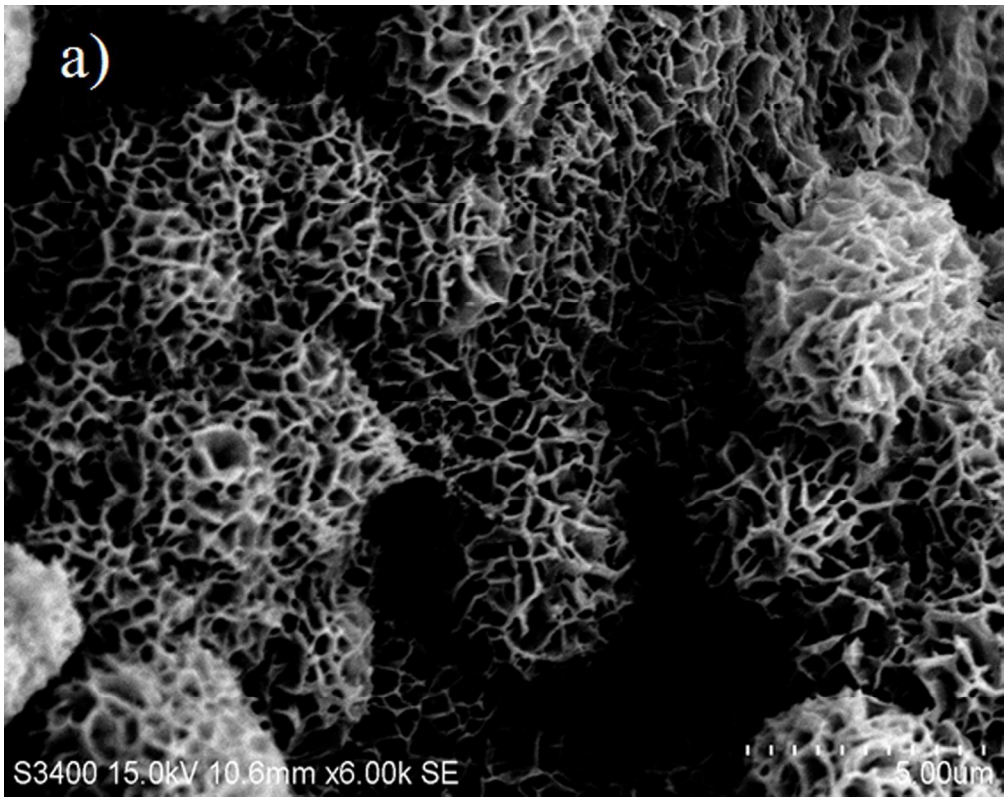

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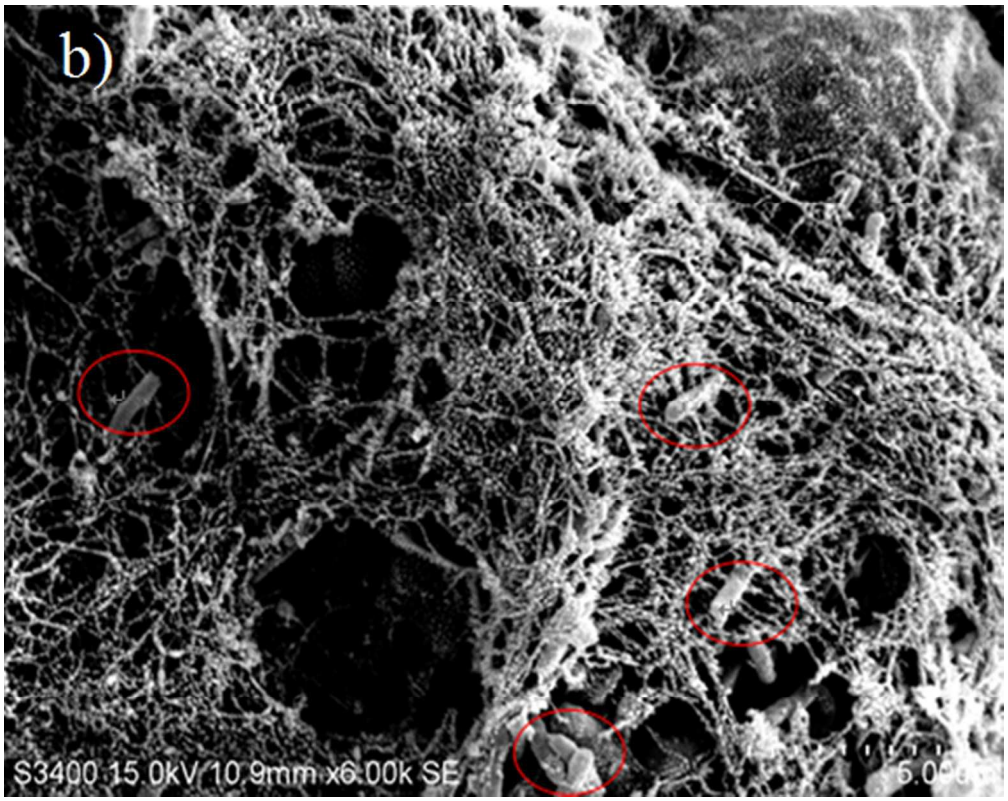

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