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Full Length Research Paper

Screening and identification of halotolerant yeast for hydrocarbon degrading and its properties studies

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A strain of hydrocarbon degrading yeast named YH-41 was isolated and purified from the soil polluted by crude oil from Shengli oil field, China. The strain was identified by morphological, biochemical characteristics and 18S rDNA sequence analysis, the results show that the strain was similar to the haploid nucleus of *Pichia ohmen*, which belong to *Candida.,* The GC analysis showed that the yeasts degradated diesel oil. Through bioremediation experiments, we can study the feasibility of bioremediation of oil-polluted soil by yeasts. The results show that the haploid nucleus of *P. ohmen* has a certain role on accelerating oil degradation in soil early in the test. The degradation rate of residual oil in the same period of time increased about 20% compared with other treatment without adding the yeasts.

Key words: 18S rDNA, *Candida, Pichia ohmen*, haploid, GC analysis and bioremediation.

INTRODUCTION

With the development of economy, human's energy demand also expands unceasingly, so all countries in the world are accelerating the exploration of oil and gas. Oil is a complex mixture which composed of thousands of different chemical property materials, which contains saturated hydrocarbons, Aromatics, asphaltene, Resin etc. (Ayotamuno et al., 2006). Pollution accidents caused by oil drilling, smelting, use and transportation have brought a series of environmental pollution problems: (1) the migration of light-weight oils will cause the pollution of groundwater and the atmosphere; (2) the heavy oil will abide existent in soil environment, which cause the reduction of the soil quality and destruction of agricultural production; (3) Polycyclic aromatic hydrocarbons (PAHs) have carcinogenic and teratogenic properties, so they are harmful to human health (Tsai et al., 2001), thus, it is imperative to manage oil pollution.

With the increasing in public awareness towards the conservation of the environment has led to the development of various physiochemical techniques for cleaning up oil contaminated sites. Although most of the physicochemical methods can be efficient for treating a wide range of pollutants, they are extremely expensive (Joo et al., 2008). Consequently, bioremediation has become a valuable alternative technology to many physiochemical methods as it is a cost-effective, cheap and environmentally friendly treatment (Saad, 2009). As bioremediation candidates, both bacteria and fungi are relatively plentiful in soil and both the groups contribute substantially to the biodegradation of hydrocarbons (Jones and Edington, 1968). A new oil pollution treatment technology which use yeast as additional agent has developed in Japan. Compared with other biological treatment technology, yeast processing technology has great advantages in many respects, such as the processing load, TOD, osmophilic, production of single cell protein and specific degrading enzymes different from the bacteria (Chigusa et al., 1989; Nawamura et al., 1992; Chigusa et al., 1996).

The present study is about the isolation of a novel yeast strain from Shengli Oil field, China which is capable of utilizing hydrocarbons. The strain was identified by morphological, biochemical tests and molecular technologies, while its degradation and bioremediation

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performance were also studied.

MATERIALS AND METHODS

Enrichment and isolation of oil-degrading yeasts

The yeasts were isolated by enrichment culture technique from soil samples contaminated by crude oil from Sheng Li oil field, Binzhou, north China. The concrete methods include the followings. For enrichment, 5 g of soil samples were inoculated into 100 ml YPD (1% glucose, 1% peptone, 0.5% yeast extract) medium and supplemented with 0.25% sodium propionate and 0.05% Chloramphenicol immediately after sampling and incubated at 30°C on a rotary shaker (180 rpm) for 2 days. 2 ml of the upper enrichment were added to 100 ml of minimal salts medium (MSM) which was modified from microbial experiment manual (Deqing, 1986). (NH_4NO_3 0.5%, KH_2PO_4 0.25%, $Na_2HPO_4{\cdot}12H_2O$ 0.05%, $MgSO_4{\cdot}7H_2O$ 0.1%, $FeSO_4{\cdot}7H_2O$ 0.001%, $CaCl_2{\cdot}2H_2O$ 0.01%, yeast extract 0.05%, pH 5.5 to 6.0). The medium was supplement with steam sterilized diesel oil (0.5% w/v) as carbon source, 0.25% sodium propionate and 0.05% Chloramphenicol, incubated at 30°C on a rotary shaker(180 rpm) for 5 days. After three cycles of enrichment, 1 ml of the culture was diluted in step, and 100 µL were plated on yeast peptone dextrose (YPD) agar plates. The yeasts colonies obtained were further purified on the YPD agar plates. The isolated pure yeasts culture was incubated in MSM supplement with diesel oil (0.5% w/v), respectively and pick out the oil degrading ones. The pure yeast culture which could degrade diesel oil were routinely subcultured in YPD and stored in 25% glycerol at -20°C.

Identification of the yeast strain by traditional and molecular biology methods

Traditional methods

The yeast colonies morphology were observed in both liquid and solid YPD medium, and the growth condition on the mineral salts medium (MSM) agar plate supplement with diesel oil were viewed. Whether pseudohyphaes grow on corn flour agar medium or ascospore grow on MacConkey medium were also needed to be observed. The physiological and biochemical characteristic were viewed following the protocol described by Deqing (1986).

Molecular biology methods

The total genomic deoxyribonucleic acid (DNA) of the yeast strain was isolated and purified by using the methods as described by Xufen (2006). The common primers for amplification of 18S rDNA in yeasts were used, the forward primer P1:5'-ATCTGGTTGATCCTGCCAGT-3' and the reverse primer P2:5'-GATCCTTCCGCAGGTTCACC-3' (Thanh et al., 2002). Polymerase chain reaction (PCR) products were separated by agarose gel electrophoresis and recovered by using column DNA gel recovery kits (TIANDZ, Beijing). The recovery PCR products were ligated into pMD-18T vector (Promega, America) and transformed into competent cells of *Eschrichia coli* DH5α. The transformants were selected on plates with ampicillin. The plasmids in the transformant cells were extracted by using the methods as described by Sambrook et al. (1989). In order to confirm that the PCR products had been ligated into the vector, the purified plamids were used as templates for amplification of 18S rDNA in yeast. The reaction system and the conditions for PCR amplification were the same as PCR of total genomic DNA. The 18S rDNA fragments inserted on the vector were sequenced by Shanghai Sangon Company.

GC-MS analyses

The diesel oil was used as the sole carbon source (0.5% w/v) in MSN at pH6 to determine the extent of degradation by the selected isolate. To study the degradation, flasks were inoculated with 3% (v/v) of the selected isolated (OD_{600}=2.0) in YPD (pH6) and incubated at 30°C under shaking conditions (180 rpm). Uninoculated control flasks were maintained to monitoring abiotic losses. After 8 days of incubation, the undegraded diesel oil residue was extracted thrice with equal volumes of petroleum ether and concentrated to 1.5 ml.

The concentrate was analyzed by GC-FID using a 30 m long DB5.625(0.25 mm × 0.25 µm film thickness) column for analysis with Helium (1 ml/min). The test conditions was modified from Tellez et al.,(2005): The injector and detector of GC were maintained at 300°C and the oven temperature was programmed to rise from 60 to 280°C with an increase of 10°C per minute and then held at 280°C for 5 min, a mass scan range of 40 to 800 with an EI of 70 ev. Various intermediate metabolites were identified by matching the retention times with authentic standards and by comparing the mass spectrum of the intermediates with those of the standard compounds of the mass spectral libraries.

Experimental design for oily soil remediation by yeast

Measurement of soil physical and chemical properties

The soil sample was come from Shengli Oil Field, discarded the 1 cm deep surface layer, got the 2 to 10 cm deep soil sampling. The basic physical and chemical properties of soil, which contains soil pH, soil moisture content, rapid available nitrogen and phosphorus content, and oil content, were determined by using the methods as described by Jiacheng (1988).

The preparation of yeast agent

One loop of the cells of purified strains were transferred to YPD medium and shaken overnight at 30°C. Cell culture (2 ml, OD600nm = 2.0) was transferred to 50 ml of Liquid Ferment Medium (glucose 1%, soya bean meal 1%, yeast extract 0.5%, KH_2PO_4 0.11%, $CaC1_2$0.01%, $MgSO_4{\cdot}7H_2O$ 0.15%, $MnSO_4$ 0.01%), and grown 16 h by shaking at 200 rpm and 28°C. The fermentation broth (5% inoculation) was transferred to steam sterilized Solid Ferment Medium (bran/turfysoil=1/2, water 20%) and grown for 2 days at 28°C, ventilate the solid ferment medium by stirring every 12 h. After fermentation, the yeast solid culture was air-dried in sterile room at room temperature, then the yeast agent was finished (Jianing et al., 2007).

Designing the experiment

The oil contaminated soil was divided into four groups and each contained 2 kg soil. Each group was treated with different processing scheme and different treatment agents were added to them respectively. The four processing schemes used in this study are as follows:

a) Oily soil;
b) Oily soil + $(NH_4)_2SO_4$ + KH_2PO_4;
c) Oily soil + $(NH_4)_2SO_4$ + KH_2PO_4 + sawdust and bran;
d) Oily soil + $(NH_4)_2SO_4$ + KH_2PO_4 + sawdust and bran + yeast agent.

In the final experiment conditions: the carbon:nitrogen:phosphorus ratio was 50:5:1 (w/w), sawdust/bran=4/1 (w/w), moisture content

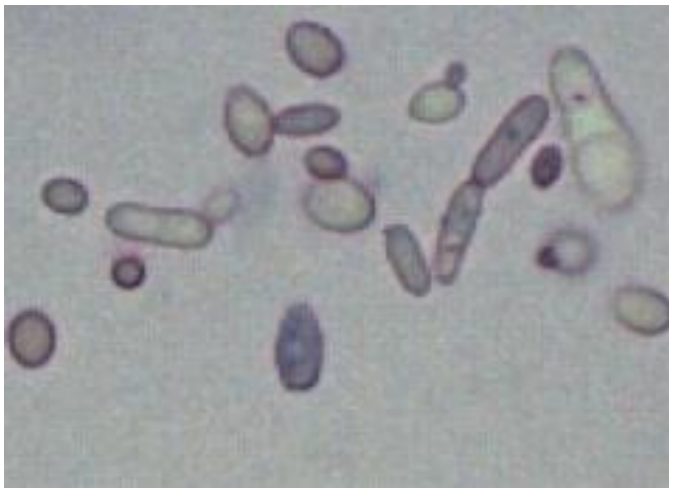

Figure 1. Cellular morphology of YH-41.

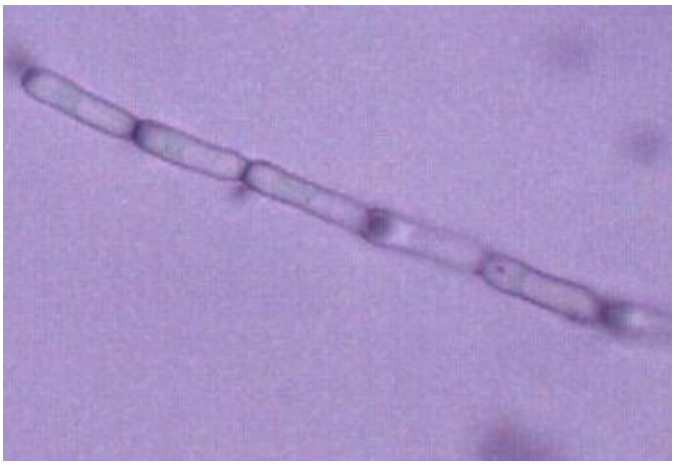

Figure 2. Pseudohyphae morphology of YH-41.

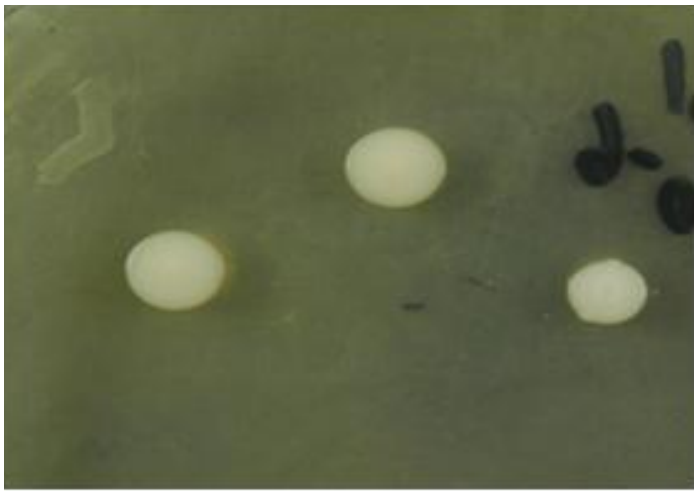

Figure 3. Colony morphology of YH-41.

Figure 4. Colony morphology of YH-41 on oil plate.

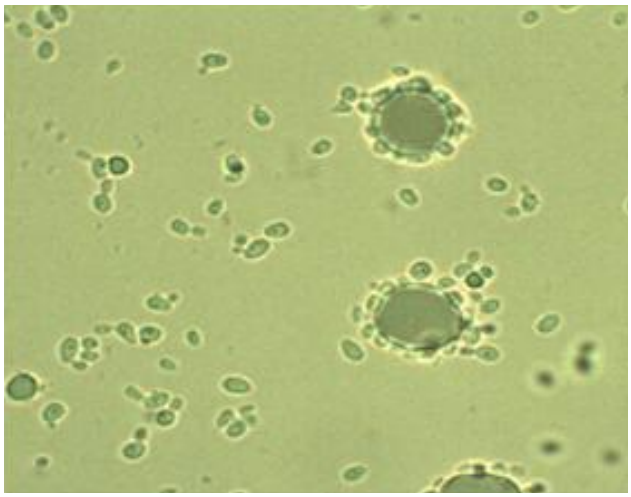

Figure 5. Yeasts YH-41 attached to the oil.

20%, stirring soil every 12 h. Every 5 days, the significant variations in soil were determined such as microorganism number, pH, soil dehydrogenase and residual oil, the experiment were lasting for 35 days.

RESULTS AND DISCUSSION

Isolation and identification of the oil-degrading yeast strain

A oil-degradation yeast named YH-41 was isolated from oil-polluted soil which was sampled from Shengli Oil Field, in north China. The basic morphological characteristics were shown in Figures 1 to 5. YH-41 formed white creamy colonies on YPD agar medium and oil agar plate, cells were divided by budding. No ascospore was grown on MacConkey medium and some pseudohyphaes were grown on corn flour agar medium.

Table 1. Morphological and biochemical characteristics of YH-41.

Characteristics		Characteristics	
Colony color	white and creamy	Growth on	
Reproduction	by budding	Ethanol	+
Ascospore	–	Glycerin	+
Pseudohyphae	+	Sorbitol	+
Sugar fermentation		Erythritol	–
Glucose	+	Mannitol	–
Lactose	–	Inositol	–
Galactose	+	Citric acid	+
Maltose	–	Lactic acid	–
Melibiose	+	Ammonium sulfate	+
Sucrose	+	Potassium nitrate	–
Raffinose	+	No vitamin	–
Growth on		Milk reaction	–
Lactose	–	Urealysis	–
Maltose	+	Gelatin hydrolysate	–
Cellobiose	+	37°C	+
L-arabinose	+	Hypertonicity	+
D-Xylose	+	Produce amyloid	–
Soluble starch	–		

"+": positive, "–": negative.

Table 2. Physical and chemical characteristics of test soils.

Soil samples	pH	moisture content (%)	Available N (mg/Kg soils)	Available P (mg/Kg soils)	Oil content (%)
test soils	8.0 ± 0.342	8.8 ± 0.546	17.3 ± 0.987	6.9 ± 0.586	1.58 ± 0.054

The yeasts were attached to oil drops in MSM medium supplement with diesel oil. Table 1 show the basic morphological and biochemical characteristics of YH-41.

Analyses of 18S rDNA gene sequence

The sequence of 18S rDNA gene of yeast YH-41 was aligned by using BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST). For comparison with currently available sequences, 25 sequences were retrieved with over 97% similarity belonging to 25 different species of *Candida* sp. (Table 2) from National Center for Biotechnology Information (NCBI) and performed multiple alignment by using DNAMAN 6.0, after format conversion by using DNASP4, the phylogenetic trees were constructed by using DNAMAN 6.0 (Figure 6). The sequence of 18S rDNA gene of yeast YH-41 has been submitted to GeneBank database under accession number GU570441.

When the data were analyzed, the yeast YH-41 was closely resembled *Candida* sp., but the exact species information could not be obtained from the phylogenetic trees. After analysis of the basic morphological and biochemical characteristics of YH-41, the strain YH-41 was identified as the haploid nucleus of *Pichia ohmen*, which belong to *Candida* sp.

Results of GC-MS analysis

The results of GC analysis shows that the strain of YH-41 was capable of utilizing most of diesel oil especially straight-chain alkane, but the alkane which conclude branched, benzene ring and double bond can be hardly utilized. The Figure 7 shows the GC analysis of uninoculated control of diesel oil. The number represent the carbon number of alkanes, intermediate peaks are branch alkanes and aromatic fractions. Figure 8 shows the degradation effects by the yeast YH-41,most of the alkanes were utilized by the yeast, the remainings were pristane, phytane, branch-chain alkanes and other compounds which difficult to degrade. Can be drawn from this analysis, the yeast obtained by this study have a

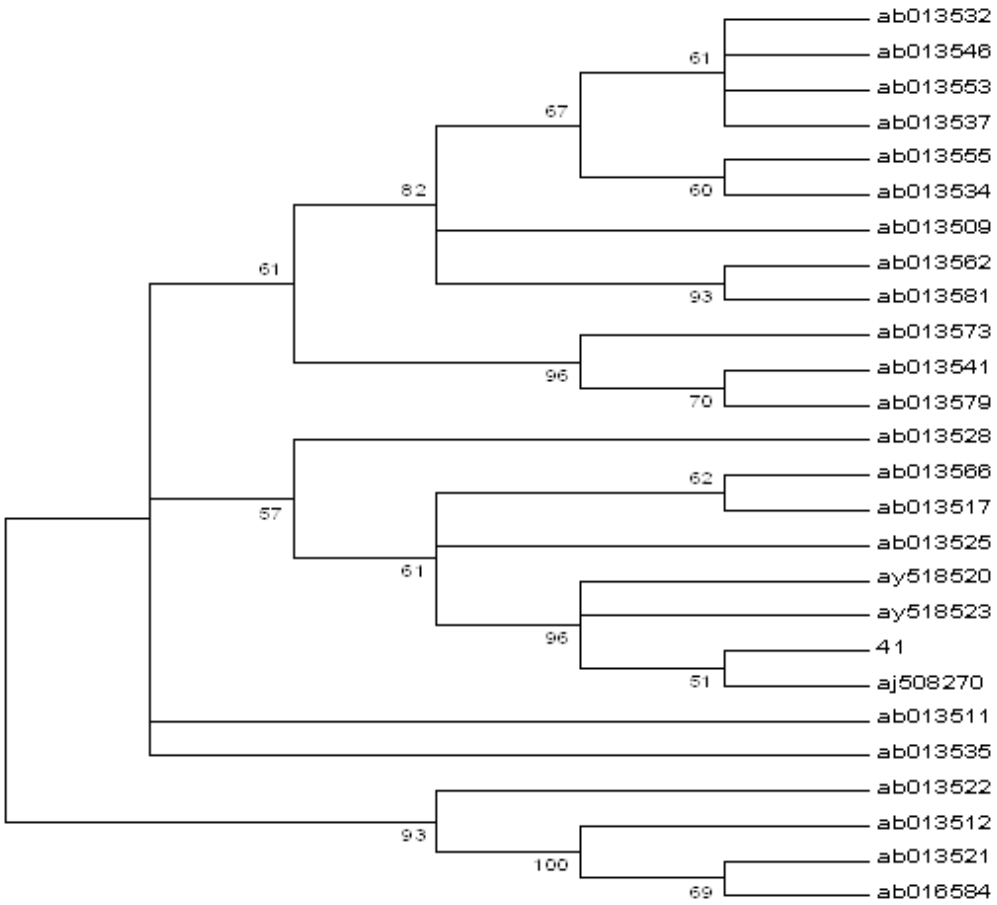

Figure 6. The phylogenetic tree for 18S rDNA system of strain YH-41.

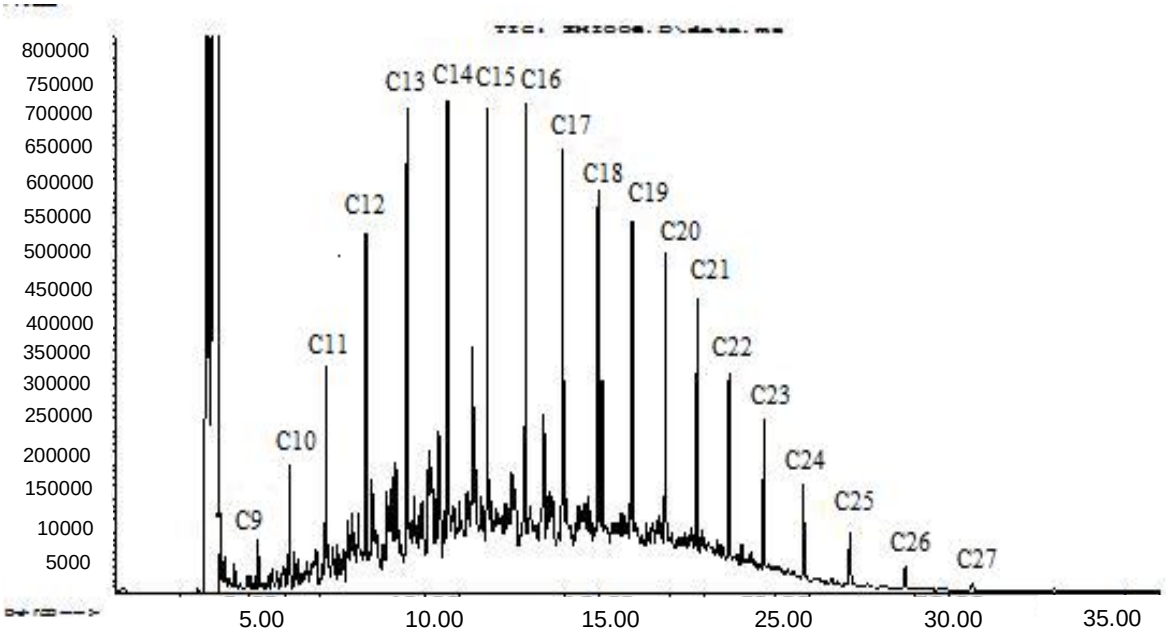

Figure 7. GC-MS of diesel oil in control treatment at the onset of the experiment.

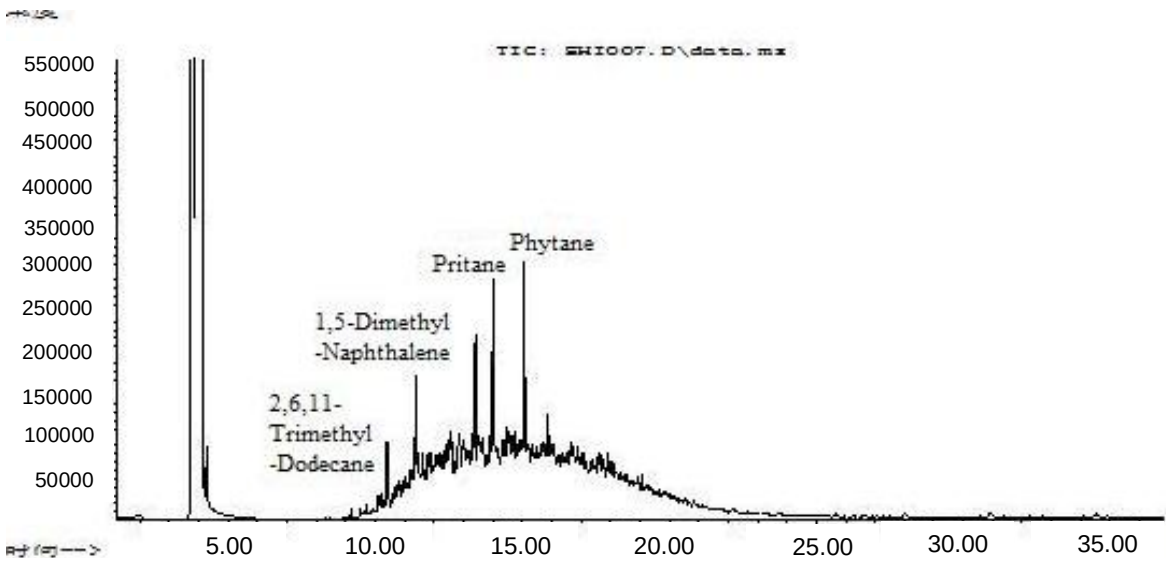

Figure 8. GC-MS of diesel oil by yeast degradation after 8 days.

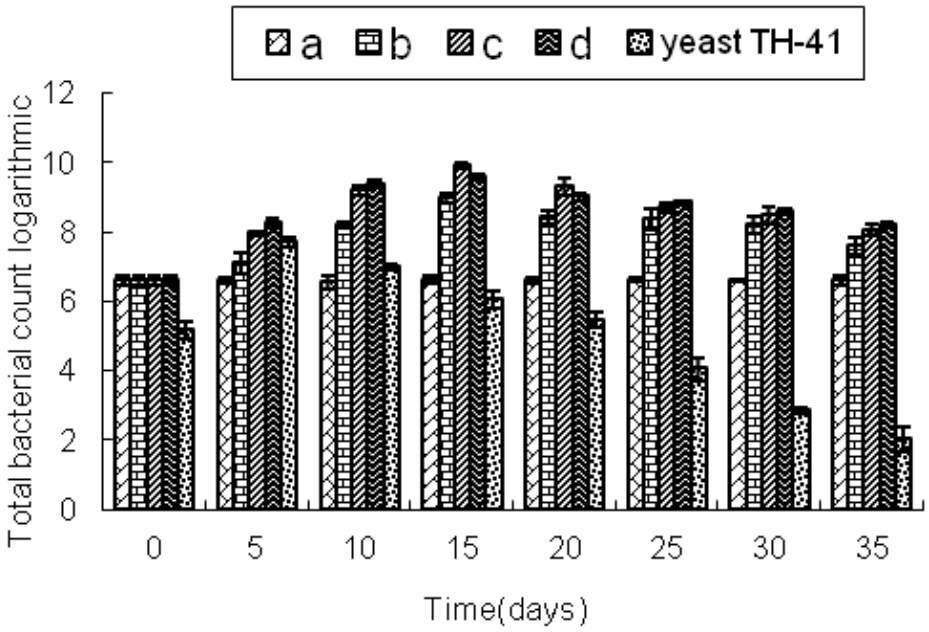

Figure 9. The number variation of bacteria colonies in the soil with remediation time.

great degradation ability to diesel oil, most of the alkanes especially straight-chain alkanes can be utilized, but the degradation ability to complex hydrocarbons was still limited.

Results of remediation of oily soil by YH-41

The Table 2 shows the basic physical and chemical characteristics of test soils. It contains soil pH, soil moisture content, rapid available nitrogen and phosphorus content, and oil content.

Figure 9 shows the variation of microorganism quantity of soil in the bioremediation tests. The yeasts YH-41 were inoculated into the soils, and after 5 days, their number reached the optimal value which was determined by previous trails, and the native microbes grew slowly. After 5 days, there was a downward trend for the number of yeasts YH-41, and then the native microbes began to grow fast. After 20 days, there was a great difference in

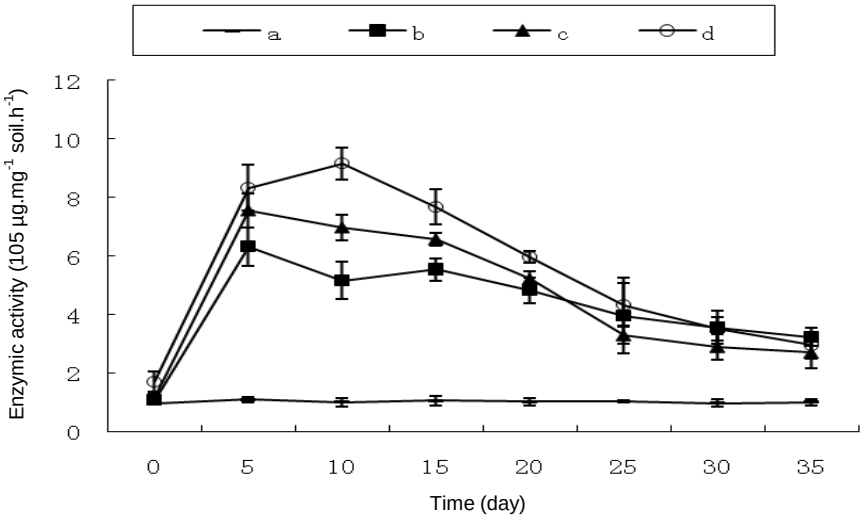

Figure 10. The activity variation of dehydrogenase in soil with remediation time.

quantities between inoculated yeasts and native microbes. The cause of these changes is analyzed as follows: in the initial stage of bioremedation tests, the inoculated yeasts as the dominant microbes grew fast and restrained the growthrate of other native microbes. After 5 days,the native microbes got out of the delay period and adapt to the new envronment, then they increased rapidly. It is possible that the injected yeasts could not compete successfully with microorganisms indigenous to the oily soil system for available carbon and nutrient resources. Another reason may be that some unkown substances generated and accumulated from the petroleum hydrocarbon metebolism process which could restrain the growth of injected yeasts, so the injected yeasts became weakness gradually and withdrew the polluted soil bioremediation system as inferior strains.

Figure 10 shows the variation of soil dehydrogenase activity. In group a and b the dehydrogenase activity were changing smoothly in the whole process, otherwise group c and d were both changing obviously. In the fifth day, enzyme activity of group b and c both reached the maximun value, after that enzyme activity began to decline. The enzyme activity of group d reached the maximun value in the tenth day, then began to decline. The reason maybe that with time increase the declination of petroleum hygrocarbon content, nutrient consumption and the accumulation of harmful metabolite. All the reason might cause the decline of metabolic activity and the degradation ability of petroleum. In the first 25 days, the enzyme activity kept d>c>b>a, the addition of yeasts YH-41 had positive influence on petroleum decomposition, simultaneously the addition of saw dust also improved the ability of the soil to hold moisture and oxygen. In group d, the injected yeasts as the dominant position in the first 5 days, after that the native microbe which could use oil began to increase rapidly, so the soil dehydrogenase could keep high activity and last for 10 days, which longer than other groups. So, from this experiment we can get that the injected yeasts YH-41 played a positive role in the oil-degradation process in the preliminary stage of bioremediation.

According to Figure 11, it shows that soil residual oil rate b, c, d three treatments, were decreased, but the decline in varying degrees, in which d> c> b> a. From this analysis we can see, N, P and other nutrients is the key factor in soil bioremediation restricting oil. Group a without any treatment for compare, showing weathering on the reduction of the role of petroleum hydrocarbons in soil is negligible. And adding handling b nutrients, the oil begins to decompose pollutants in the soil degradation rate of petroleum hydrocarbons 29.7%. Processing c: adding sawdust as the leavening agent, add the wheat bran as organic nutrients, which increase soil oxygen content, water capacity and fertility, and make the degradation of oil pollutants in soil in a substantial increased in capacity to deal with the degradation rate of c the final 60.7%. Processing d: on the basis of C dealing with added yeast YH-41, the degradation of pollutants, there has been improved to some extent, making processing the final degradation rate d 81.6%. Yeast makes oil bioremediation capacity of soils increased by 20% or so. So, the yeast YH-41 we isolated has a certain role in promoting on oil bioremediation of contaminated soil.

According to Figure 12, it shows that, with the conduct of bio-remediation process, soil pH decreased, partly because of the acid added nutrients, on the other hand, with the decomposition of petroleum hydrocarbon pollutants, which produced some acidic substances, all the reasons made soil pH changes. Later in the process of bioremediation, soil pH maintained between 5 and 6. In c, d post-treatment process, the soil pH in acid with a

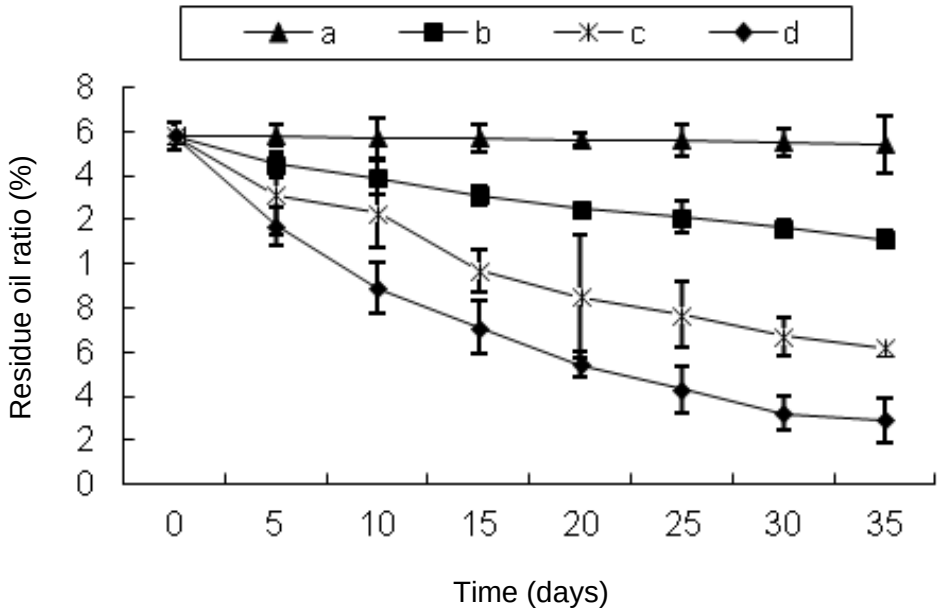

Figure 11. Ratio of residue oil in oil-contaminated soil with remediation time.

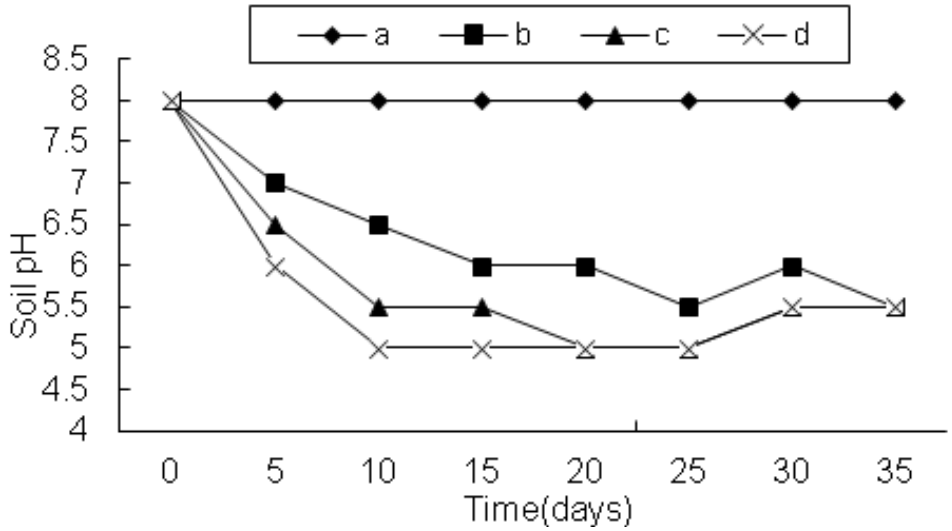

Figure 12. Variation of pH with remediation time.

small increase. That may due to the acid substances produced in the process of bioremediation were consumed in the post bioremediation stage. So it caused the soil pH changes.

DISCUSSION

Since hydrocarbon-degrading microorganisms are widespread in nature, bio-augmentation has been considered as a potential strategy for oil bioremediation since the 1970s. In this technique, oil-degrading microorganisms are added to supplement the existing microbial population, as the indigenous microbial population may not be capable of degrading the wide range of potential substrates, which are present in complex mixtures such as petroleum (Leahy and Colwell, 1990). Other conditions under which bio-augmentation may be considered are when the indigenous hydrocarbon degrading population is low, the speed of decontamination is the primary factor, and when seeding may reduce the lag period to start the bioremediation

process (Forsyth and Tsao, 1995).

Many studies showed that the bio-augmentation technology could significantly improve the efficiency of bioremediation (Pozdnyakova et al., 2008; Jacques et al., 2008). However, some other researchers pointed out those exogenous microorganisms were not always effective when used to repair the oil contaminated soil (Mariano and Kataoka, 2007; Ueno et al., 2006; Liu and Wang, 2008; Federici, 2007). In this case, the adding of exogenous microbial could significantly enhance the soil degradation, but greatly influence the composition of native microbial population. Meanwhile, the exogenous microorganism themself might be affected by the local environment and microbial restrictions, which might increase the difficulty of maintaining long term decontamination functions.

Indigenous microorganisms grow in oil-contaminated soil environment for long time, they have better adaptation ability to petroleum hydrocarbons and have high degradation ability, so the indigenous microbial which have oil degradation ability can be isolated, after enrichment then put them into indigenous petroleum contaminated soil, the effect of bioremediation should be even better theoretically. In 2007, the Japanese scientists (Ueno et al., 2007) based on this technology and proposed a new concept "Autochthonous bio-augmentation". His viewpoint was proved through experimental by many scientists (Atagana and Haynes, 2006; Das and Mukherjee, 2007), showed that indigenous microorganisms showed much better oil-degradation ability than exogenous microorganisms, and this technology will show better and more reliable bioremediation effect. Yeast, environmentally friendly microbe, can be used in single-cell protein production, so choosing yeast to conduct bioremediation research is also from the environmental protection point.

This research was based on the principle of "Autochthonous bio-augmentation". An oil-degradation yeast strain was isolated from the crude oil contaminated soil. As the GC analysis results showed, the yeast has a great ability of degrading straight-chain alkanes. After enrichment, the yeast agent was obtained, and supplied to indigenous oil-contaminated soil.

The results from the bioremediation can be analyzed: In oil-contaminated soils, N, P and oxygen are limited factors in bioremediation process. By adding the N, P salts to a certain extent improved the microbial decomposition of petroleum contaminants; through improving ventilation conditions could significantly increase the utilization of petroleum hydrocarbons by microorganisms. Another literature reported that the aerobic bacterias are the main microbes which could decompose the petroleum hydrocarbons in soil, and the degradation rate of anaerobes are very slow or negligible (Bailey et al., 1973; Ward et al., 1980). Therefore, the addition of leavening agent and nutrition could significantly increase the bioremediation rate.

The yeast YH-41 isolated in our laboratory shows a positive role on the bioremediation of oil contaminated soils. Especially early in the bioremediation of oil-contaminated soil, after pre-treatment of soil by the yeasts YH-41, the residue oil pollution more susceptible to be used by subsequent growth of indigenous bacteria, so the efficiency of bioremediation have been significantly improved. Later in the bioremediation process, due to species competition, lack of nutrients and accumulation of harmful metabolites, resulting in the injected yeasts population decreased and was gradually replaced by the rise of indigenous bacteria.

Conclusion

In this study, yeast (YH-41) that can degrade diesel oil was isolated from oil-contaminated soil from Shengli Oil Field, in north China. The yeast was identified by using the methods of basic morphological characteristics observation, physio-biochemical tests and molecular biology ways. Through analysis of the 18S rDNA gene sequence, the YH-41 could be classified as *Candida* sp., according to the physio-biochemical characteristics of the YH-41, the results show that the strain was very similar to the haploid nucleus of *P. ohmen,* so the yeast of YH-41 may well be the strain of the haploid nucleus of *P. ohmen,* which belong to *Candida* sp. From GC analysis results, the yeast showed great degradation ability to straight-chain alkane, but the complex compounds which contain branch and benzene ring can hardly be degraded. The results of bioremediation tests showed that after the yeast YH-41 added into the contaminated soil, the soil pollution degree reduced greatly. This shows that yeast addition accelerated the oil cleanup in soil.

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