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Molecular Detection of Petroleum Bacteria from Kaleybar Soils by PCR Method

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ABSTRACT

Microorganism's biotechnological potential is term less. New products could be found within new species from unknown ecosystems. Efficacy of microorganism's enzymes in petroleum bioremediation is one of strategic utility of them. Petroleum hydrocarbons biodegrading by microorganisms is an interesting study area for researcher. In this study Samples collected from Iran soils. By using PAF and 531R primers we can perform PCR (1) that corresponds to positions 8 and 531 on *Escherichia coli* 16S rDNA. An amplified DNA fragment of about 530 base pairs is the result of this.
With these primers multiple DNA fragment amplification was observed. The positive control, *Ps. oleovorans* ATCC 29347, exhibits two fragments after its PCR amplification. The 870 bp amplification is the first fragment , while the second fragment is about 280 bp. If amplification resulted from the multiple PCRs performed , Isolates were designated positive. The positive amplification did not have to be the same size as the *Ps. oleovorans* DNA fragment.
The investigation into the presence of the *alk*B gene had intriguing results. The positive control, *Ps. oleovorans*, consistently had two amplified fragments of DNA at 870 bp and ~280 bp. The isolates had a variety of DNA fragments amplified with this primer set. Figure 3 (S3 isolates) consist of a very good representation of this phenomenon. Almost all of the S3 isolates were suspected as positive for the *alk*B gene, yet none of these DNA fragments analyzed by sequencing were actually positive for the gene. In summary, it appears that some of the isolates from the Guadalupe sites consist of the alkane hydroxylase biodegradative enzyme. These organisms should thus be capable of
degrading short chain *n*-alkanes present in petroleum.
Keywords: petroleum bacteria,PCR,16srRNA.

INTRODUCTION

There is a plenty of ecosystems in our plant. Deserts, jungles, rivers and mountains roll out in all parts of earth like a color full vast carpet which contain various life cycles. Organisms can inhabit in specific environments according to their functional characteristics. Microorganisms are found in everywhere; environments diverged by microorganisms colonies within million of years. Microorganisms are the main component of biochemical cycles and world food webs in maintenance of Wight's by special relationships called symbiosis. In biogeochemical cycles several microorganisms may be Pull Together or they agents susceptive regeneration of elements which other organisms needed [1]. Principally greatest diversity in life kingdom belong to microorganisms and specially bacteria, which cause to them ubiquity. Microorganism's biotechnological potential is term less. Different microorganisms in various ecosystems produced plenty of metabolite. New products could be found within new species from unknown ecosystems. Metabolites which product by new species or biotechnological methods; could play important role in our life safety. Efficacy of microorganism's enzymes in petroleum bioremediation is one of strategic utility of them [1].Petroleum hydrocarbons biodegrading by microorganisms is an interesting study area for researcher [2]. Petroleum is heterogeneous mixtures of hydrocarbons are organic compounds containing carbon and hydrogen, which are highly insoluble in water. [3]. Degradation and production of hydrocarbons can provide with microorganism depending on each function in the environmental conditions [4]. Rate of microorganisms population in specify ecosystems play a key role in petroleum or other hydrocarbons removing from environment [3].An oil smear is an environmental hazard to all species of life. In fact, petroleum and derivatives have a strong ecological impact on contaminated ecosystems [6]. One of the methods of cleaning up oil spills that has been investigated is the use of oil-eating bacteria. Some of soil bacteria use oils in the environment as food. This ability depending on different bacterial strains found in the various natural environments. The use of microbial metabolic potential for eliminating environmental pollutants provides a safe and economic alternative to their disposal in waste dump sites and to commonly use physic-chemical strategies. Screening for enzymes for this purpose is necessary. In this article,

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we will discuss the advances in understanding the natural diversity and capabilities of microorganisms for degrading petroleum compounds for bioremediation purposes, and microbial activities. High enzymatic capacity prepares microbial communities to degrade complex hydrocarbons [9]. Microorganisms genetically diversity cause to the metabolic versatility for the contaminants into less-toxic final products, which are anent in natural biogeochemical cycles [9]. Main benefit of contaminant-degrading process is mineralization of compounds, in biomass from [10–12].

In this study we report the isolation and molecular detection of oil inhabited bacteria from the villatic environment of Kaleybar in north-west of Iran. Different habitats with high temperatures sulfur and iron concentrations (Hannert 2002) may contain interest bacterial biodiversity. AlkJ gene encodes a key aliphatic alcohol dehydrogenize, has been used to detect the presence of Pseudomonas hydrocarbon catabolism in the thermopile isolates which is necessary for growth of Pseudomonas on petroleum hydrocarbons [13]. Isolates detected by 16s rDNA gene sequencing and grown in liquid cultures with crude oil as carbon source in order to estimate their biodegradation potential.

MATERIALS AND METHODS

Isolation of the Petroleum Degrading Bacteria

Sampling areas

Samples collected from Arasbaran soil and villages from Kaleybar near the state of Sahand movntain at East Azarbayjan, Iran. samples were taken from the surface layer (0–5 cm) of five different parts in summer days. in the sampling areas Soil temperature range were between 18 and 25 C. Samples were collected in sterile plastic pockets .sterile plastic containers were filled with 5–10 gr of soil at 20 cm below surface. Soil samples were stored at room temperature and were analyzed within 72 h. All samples were collected at summer from July/2011 to September/2011. Soil samples isolated bacteria (50 g) were placed in sterile Erlenmeyer flasks mixed with 450 ml sterile Ringers solution (0.25 strength) and for 30 min they shaken on an orbital shaker at maximum speed (500 rev min). With Ringer solution Supernatant from soil samples mixed (400–500 ml) then filtered at room temperature by 0.22 lm GV filters. Collected particulates were resuspended in 10 ml of Ringers solution. Suspensions were spread on the surface of agar media (Table 1), and Duplicated plates were incubated aerobically at 60 _C and 80 _C for 3–4 days.

PCR Amplification

16S rDNA

By using PAF and 531R primers we can perform PCR (1) that corresponds to positions 8 and 531 on *Escherichia coli* 16S rDNA. An amplified DNA fragment of about 530 base pairs is the result of this. Final concentrations of the reaction mix were 10 mM Tris-HCl (pH 8.0), 1 mM dNTP, 20 μg/mL BSA, 2.5 mM MgCl2 and 2 U Amplitaq. PCR was performed ***for alk*B gene** with two different primer sets to determine the presence of the catabolic gene for alkane monooxygenase (*alk*B). Primers *alk*B-F (5′ TGGCCGGCTACTCCGATGATCGGAATCTGG 3′) and *alk*B-R (5′ CGCGTGGTGATCCGAGTGCCGCTGAAGGTG 3′) were used corresponding to positions 703 and 1572 on *Ps. oleovorans* ATCC 29347 16S rDNA, resulting in amplification of a DNA fragment of approximately 870 bp (9, 10). The second set of primers *alk*B-F477 (5′ AGGGTACNGTARATTCTTTATTGAGCAT 3′) and *alk*B R911(5′ AGRYTTARASACGATGRKRGCCGTATTCC 3′) were designed using Lasergene Software Suite (DNASTAR, Inc. Madison, WI), corresponding to positions 477 and 911 on *Ps. Oleovorans* ATCC 29347 16S rDNA. This resulted in an amplified DNA fragment of about 430 bp (7). All PCR products were visualized on a 1.4% agarose gel.

Nucleotide Sequencing

We performed Amplified 16S DNA fragments from isolated strains sequencing by using the Dye Terminator Cycle Sequencing Ready Reaction with polymerase FS..DNA amplified using the *alk*B fragments chosen for sequencing were then extracted from an agarose gel following the protocol of the QIA Gel Extraction Kit. Then all amplified sequences were used to conduct searches in non-redundant DNA databases (BLAST).

RESULTS

*alk*B primers corresponding to *Pseudomonas oleovorans* ATCC 29347

With these primers multiple DNA fragment amplification was observed. The positive control, *Ps. oleovorans* ATCC 29347, exhibits two fragments after its PCR amplification. The 870 bp amplification is the first fragment, while the second fragment is about 280 bp. If amplification resulted from the multiple PCRs performed, Isolates were designated positive. The positive amplification did not have to be the same size as the *Ps. oleovorans* DNA fragment. None of the suspected “positive” fragments were the same size as the one from

Ps. oleovorans. Figures 2, 3, and 4 illustrate the multiple band amplification found in *Ps. oleovorans* and the isolates. The bright amplifications were taken to be a positive result for *alk*B, and they were the DNA fragments that underwent gel extraction and sequencing to verify homology to *Ps. oleovorns alk*B.

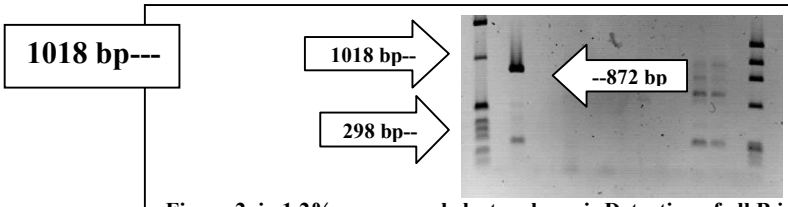

Figure 2. in 1.2% agarose gel electrophoresis Detection of alkB in S1 isolates by PCR was done

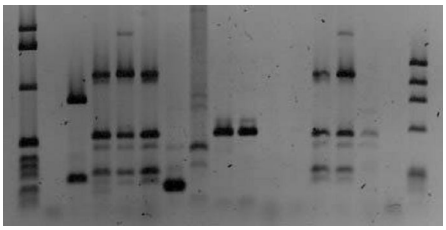

Figure 3. in 1.2% agarose gel electrophoresis by PCR using Ps. oleovorans primers Detection of alkB in S3 isolates was done12, S3RS6; 13, S3MD4A; 14, S3MD4B; 15, S3MD5; 16, open control; 17, ϕX174/Hae*III* Ladder.

Table 1 lists the isolates having DNA fragments that has successfully sequenced and isolated. No DNA fragment sequences were homologous to the *Ps. oleovorans alk*B gene. Attempts to sequence fragments from isolates S3EH1, S3EH3, and S3EH4 were unsuccessful. Due to the unexpected results of the initial round of sequencing, sequences were not obtained for the rest of the suspected positive PCR fragments.

Table 1. BLAST ID results of apparent positive *alk*B isolates

Isolate	BLAST ID	E value
***Ps. oleovorans*(870 bp)**	*P. oleovorans* TF4-1L (+OCT) plasmid *alk*B gene	0.0
***Ps. oleovorans* (280 bp)**	*Bordatella bronchioseptica ris*A and *ris*S genes	10
S1RS12 (280 bp)	*Streptomyces coelicolor* cosmid M11	3.7
S1RS16 (280 bp)	*Streptomyces coelicolor* cosmid M11	4.1
S3LS1	*Caenorhabditis elegans* cosmid F59A3	0.14
S3EH2	*Arabidopsis thaliana* DNA chromosome 4	0.85
S3EH7	*Streptomyces coelicolor* cosmid 3C3	0.007
S3EH8	*Streptomyces coelicolor* cosmid 3C3	0.007
S3MD4A	*Pseudomonas aeruginosa nir*C gene	0.53
S3MD4B	*Arabidopsis thaliana* DNA chromosome	4 0.86
S3MD5	*M. musculus Sry* locus	0.37
S5MM1	*Paracoccus denitrificans* NADH dehydrogenase	4E-47

DNASTAR®-designed *alk*B degenerate primers

using these primers amplification of multiple fragments did not occur. A single 430 bp DNA fragment amplified in all positive cases. Out of the thirty-nine isolates, six isolates produced DNA fragments of the same size as *Ps. oleovorans.* Table 2 lists these isolates and their 16S rDNA matches in the GenBank database.

Table 2. Identification of *alk*B positive isolates (degenerate primers).

Isolate	BLAST ID	E value
S1RS12	Rhodococcus sp.	0.0
S5RS3	Alcaligenes sp.	0.0
S5MM1	Unidentified alpha-proteobacterium	E-173
S5MM2	Phyllobacterium myrsinacearum	0.0
S5MM3	Bacillus halmapalus DSM 8723	0.0
S5MM5	Microbacterium terrae	0.0

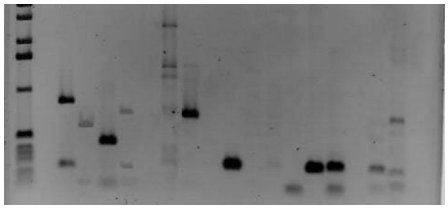

Figure 4. by PCR using Ps. oleovorans primers in 1.2% agarose gel electrophoresis Detection of alkB in S5 isolates was done

Figure 5 illustrates the single-band DNA fragment amplification with the six positive isolates and *Ps. oleovorans.* 20 µL of DNA, as opposed to 5 µL of DNA, was loaded into each lane of Figure 5 to achieve visualization of positive amplifications.

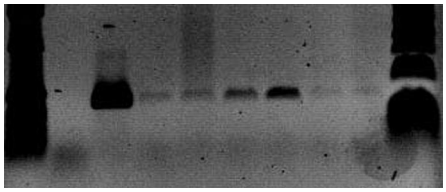

Figure 5. by PCR with degenerate primers designed to detect the presence of alkB. 1.2% agarose gel electrophoresis. Positive isolates amplified was done

DISCUSSION

*alk*B primers corresponding to *Ps. oleovorans* ATCC 29347

The investigation into the presence of the *alk*B gene had intriguing results. The positive control, *Ps. oleovorans*, consistently had two amplified fragments of DNA at 870 bp and ~280 bp. The isolates had a variety of DNA fragments amplified with this primer set. Figure 3 (S3 isolates) consist of a very good representation of this phenomenon. Almost all of the S3 isolates were suspected as positive for the *alk*B gene, yet none of these DNA fragments analyzed by sequencing were actually positive for the gene. None of the suspected positive isolates resulted in an amplification of DNA fragments with similar size to the *Ps. oleovorans alk*B gene. The wrong DNA fragment was selected for extraction and sequencing was the major problem with sequencing. The most abundant DNA fragment was selected for extraction and sequencing instead of choosing a fragment the same size as *Ps. oleovorans*. None of the DNA fragments analyzed contained part of the *Ps. oleovorans alk*B gene. Other DNA fragments that were not sequenced may have contained the *alk* B gene, but poor results from previous isolates made this seem improbable. This is the reason why the rest of the isolates from S5 soils were not sequenced. The initial assumption was that different organisms may contain *alk*B gene fragments of differing sizes. This was in error and further experiments should be conducted with DNA fragments of 870 bp (or 280 bp) selected for extraction and sequencing. These primers were possibly amplifying DNA fragments with no homology to *Ps. oleovorans alk*B. The *Ps. oleovorans alk*B primers were designed to detect the *alk*B gene on the OCT plasmid (5, 7, 8, 9, 10). They contain no degeneracies, making the PCR conditions more stringent. The annealing temperature was lowered to account for the stringency of the primers.

The result was multiple DNA fragment amplification, none of which appeared to be the actual *Ps. oleovorans alk*B gene after sequencing analysis. Only one DNA fragment, from S5MM1, may be related to the alkane degradation. It was homologous to a NADH dehydrogenase enzyme. Preliminary steps of alkane degradation require NADH to provide electrons for the hydroxylase enzyme. However, this is accomplished by *alk*T, not *alk*B. A second set of primers with degeneracies was therefore used to detect the presence of the *Ps. oleovorans alk*B gene.

DNASTAR® designed *alk*B degenerate primers

Six isolates each produced a single DNA fragment that was the same size as the amplified DNA fragment of *Ps. oleovorans.* None of the amplified DNA fragments were extracted or sequenced and this is important. Five of these isolates were found in the S5 soil sample, the higher *n*-alkane sample. This makes sense considering the likelihood that this gene may reside on a plasmid (3, 6, 8, 9, 10). Plasmid transfer would be likely to occur in a highly contaminated soil sample, conferring this gene to a variety of organisms. The DNA can be transferred within the microbial population via conjugation or transformation (3) If it is in this mobile form.

The low yield of DNA in amplification was the largest problem with these primers. Only one amplified DNA fragment from S5MM3 was positive in the repeated trials. After PCR amplification the other five amplified DNA fragments were not always visualized on the agarose gel.

Figure 5 reveals how faint some of the amplifications were, even with 20µL of sample loaded into each lane for purposes of visualization.

The 16S rDNA GenBank matches for the six isolates (Table 2) are all expected genera for soil microorganisms (3, 4, 6). Three of the isolates are common soil microorganisms (S1RS12, S5RS3, and S5MM3), and the other three are known soil microorganisms (S5MM1, S5MM2 and S5MM5). *Alcaligenes, Bacillus,* and *Rhodococcus* are common genera among soilmicroorganisms known for petroleum degradation (3, 6). *Proteobacteria, Phyllobacterium,* and *Microbacterium* are also soil microorganisms (4). The diversity of genera represented by the six isolates further supports the probability that the *alk*B gene resides on a plasmid.
In summary, it appears that some of the isolates from the Guadalupe sites consist of the alkane hydroxylase biodegradative enzyme. These organisms should thus be capable of degrading short chain *n*-alkanes present in petroleum.

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