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RESEARCH ARTICLE

A Quantitative PCR Approach for Recognizing the Functional Genes Involved in the Degradation of Naphthalene Dioxygenase and Polycyclic Aromatic Hydrocarbons in Contaminated Soils

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Abstract

Analysis of environmental pollutants is very important aspect; cheap and quick methods to clean-up the environment are required. Therefore, the polycyclic aromatic hydrocarbons (PAHs) and naphthalene dioxygenase (NAH-F/NAH-R) were separated from crude oil from north Al-Rumaila/ Iraq. These compounds were used in different concentrations as degraders that docking the functional genes of each of *Aspergillus Niger* strain AHBR3, *Cladosporium ramotenellum* and *Penicillium brevicompactum* isolate 66 small. The results were showed that the three microorganisms use these compounds as a source of food and energy to degrade the pollutants.

Keywords: *Pollutants, Polycyclic aromatic hydrocarbons, Aspergillus Niger, Cladosporium ramotenellum, Penicillium brevicompactum*

Introduction

In the last decades, several culture-independent, molecular DNA and phylogenetic techniques have been developed supplying many advantages over the traditional cultivation approach. [1]. The molecular techniques take advantage of the specificity provided by nucleic acid sequences for the identification of microorganisms and their independence of culturing microorganisms. Different, in the PCR-based, genotyping techniques have been developed and adapted for the finger printing of microbial communities on bio deteriorated cultural heritage.

These techniques have enabled a reliable study and monitoring of the microbial communities associated with different materials, such as stone, prehistoric caves, wall paintings, oil paintings, historical glasses, paper, parchment, human remains, etc [1].Numerous researchers have reported microorganisms having abilities to degrade oil,[1] isolated from natural habitats contaminated with oil. The driving force for petroleum biodegradation is the ability of microorganisms to utilize hydrocarbons to satisfy their cell growth and energy needs.

Hydrocarbon contaminated soil and marine sources have been the common choice for the isolation of such hydrocarbon combining and degrading bacteria [2].Polycyclic aromatic hydrocarbons (PAHs) are major pollutants globally and due to their carcinogenic and mutagenic properties their clean-up is paramount [2].

Bioremediation or using PAH degrading microorganisms (mainly bacteria) to degrade the pollutants represents cheap, effective methods. These PAH degraders harbour functional genes which help microorganisms use PAHs as source of food and energy [3].

Most probable number (MPN) and plate counting methods are widely used for counting PAHs degraders; however, as culture based methods only count a small fraction (<1%) of microorganisms capable of carrying out PAH degradation, the use of culture-independent methodologies is wanted [3].

Methods

Protocol

For optimal performance, add beta-mercaptoethanol (user supplied) to the fungal/ bacterial DNA binding buffer to a final dilution of 0.5% (v/v).

- Add 50-100 mg (wet weight) fungal or bacterial cells1 that have been resuspended in up to 200 µL of water or isotonic buffer (e.g., PBS) or up to 200 mg of tissue to a ZR bashing bead™ lysis tube (0.1 mm & 0.5 mm). Add 750 µL lysis solutions to the tube 2.
- Secure in a bead beater fitted with a 2 mL tube holder assembly and process at maximum speed for ≥ 5 minutes.
- Centrifuge the ZR bashing bead™ Lysis Tube in a micro centrifuge at 10,000 x *g* for 1 minute.
- Transfer up to 400 µL supernatant to a Zymo-Spin™ IV Spin filter (orange top) in a collection tube and centrifuge at 7,000 x *g* for 1 minute.
- Add 1,200 µL of fungal/ bacterial DNA binding buffer to the filtrate in the collection tube from step 4.
- Transfer 800 µL of the mixture from step 5 to a zymo-spin™ IIC Column 3 in a collection tube and centrifuge at 10,000 x *g* for 1 minute.
- Discard the flow through from the collection tube and repeat step 6.
- Add 200 µL DNA pre-wash buffer to the zymo-spin™ IIC column in a new collection tube and centrifuge at 10,000 x *g* for 1 minute.
- Add 500 µL fungal/ bacterial DNA wash buffer to the zymo-spin™ IIC Column and centrifuge at 10,000 x *g* for 1 minute.
- Transfer the zymo-spin™ IIC Column to a clean 1.5 mL micro centrifuge tube and add 100 µL (35 µL minimum) DNA elution buffer directly to the column matrix. Centrifuge at 10,000 x *g* for 30 seconds to elute the DNA.

The optimal condition has identified for (Initial denaturation and annealing) after a work several experiments to gain for this condition, the temperature has changed through the work of (Gradient PCR) for all samples to select the optimal condition, and also changed the concentration for DNA template between (1.5-2 µL) where is considered these two factors from important factors in primer annealing with complement.

Agarose Gel Electrophoresis of DNA

Electrophoresis has been done to determine DNA pieces after the process of extraction or to detect the result of the interaction of PCR during the presence of the standard DNA to distinguish the bundle size of the outcome of the interaction of PCR on the agarose gel.

Prepare of the Agarose gel

According to Sambrook *et al*, [4] the agarose gel has been made in 1.5% condensation by melting 1.5 g of agarose in 100 ml of previously made TBE buffer. Agarose has been heated to boil then left to cool down at (45-50°C).The gel has been poured in the pour plate in which the plate of agarose support has been prepared after fixing the comb to make holes that would hold the samples. The gel has been poured gently not to make air bubbles and left 30 minutes to cool down. The comb has been removed gently of the solid agarose. The plate has been fixed to its stand in the Electrophoresis horizontal unit represented by the tank used in the Electrophoresis. The tank has been filled with TBE buffer in which it covers the gel surface.

Preparation of Sample

The processor (3 µL) of loading buffer (Intron / Korea) has been mixed with 5 µL of the supposed DNA to be electrophoresis (loading dye), after the mixing process, the process of loading is now to the holes of the gel. An electric current of 7 v\c2 has been exposed for 1-2 h till the tincture has reached to the other side of the gel. The gel has been tested by a source of the UV with 336 nm after put the gel in pool contain on 3µl Red safe Nucleic acid staining solution and 500 ml from distilled water. Red Safe nucleic acid staining solution (20,000 xs) is anew and safe nucleic acid stain, an alternative to the traditional ethidium bromide (EtBr) stain for detecting nucleic acid in agarose gels.

It emits green fluorescence when bound to DNA or RNA. This new stain has two fluorescence excitation maxima when bound to nucleic acid, one centred at 309nm and another at 419 nm. In addition, it has one

visible excitation at 514nm. The fluorescence emission of Red Safe bound to DNA is centered at 537 nm. Red Safe Nucleic Acid Staining Solution (20,000x) is as sensitive as EtBr. The staining protocol for Red Safe Nucleic Acid Staining Solution (20,000x) is similar to that for EtBr. Compared to EtBr, known as a strong mutagen, Red Safe Nucleic Acid Staining Solution (20,000 xs) causes much fewer mutations in the Ames test. In addition, Res Safe Nucleic Acid Staining Solution (20,000x) has a negative result in mouse marrow chromophilous erythrocyte micronucleus test and mouse spermary spermatocyte chromosomal aberration test. So it is wise to choose Red Safe Nucleic acid Stainig Solution (20,000x) instead of EtBr for detecting nucleic acid in agarose gels. (Cat. No. 21141).

Gel Extraction (Sequencing) Protocol

Add absolute ethanol to the Wash Buffer prior to initial use. The step of Gel Extraction DNA is as the following: [5]

Gel Dissociation

- Excise the agarose gel slica containing relevant DNA fragments and remove any extra agarose to minimize the size of the gel slica.
- Transfer up to 300 mg of the gel slica to a 1.5 mL micro centrifuge tube.
- Add 500μL of DF Buffer to the sample and mix by vortex.
- Incubate at 55-60°C for 10-15 minutes or until the gel slica has been completely dissolved. During incubation, invert the tube every 2-3 minutes.
- Cool the dissolved sample mixture to room temperature.

DNA Binding

- Place the DF Column in a 2ml Collection tube.
- Transfer 800μl of the sample mixture from (step1) to the DF Column.
- Centrifuge at 14-16000×g for 30 seconds.
- Discard the flow-through and place the DF Column back in the 2 ml Collection tube.

Wash

- Add 600μl of Wash Buffer into the DF Column and let stand for 1 minute.
- Centrifuge at 14-16000×g for 30 seconds and then discard the flow-through.
- Place the DF Column back in the 2 ml Collection tube.
- Add 600μl of Wash buffer into the DF Column and let stand for 1 minute.
- Centrifuge at 14-16000×g for 30 seconds and then discard the flow-through.
- Place the DF Column back in the 2 mL Collection tube.
- Centrifuge 14-16000×g again for 3 minutes to dry the column matrix.

DNA Elution

- Transfer the dried DF Column to anew 1.5 ml micro centrifuge tube.
- Add 20-50μL of Elution Buffer into the centre of the column matrix.
- Let stand for 2 minutes the Elution Buffer is absorbed by the matrix.
- Centrifuge for 2 minutes at 14-16000×g to elude the purified DNA

Sequencing and Sequence Alignment

The PCR products were separated on a 2% agarose gel electrophoresis and visualized by exposure to ultra violate light (302 nm) after ethidium bromide or red stain staining. Sequencing of gene was performed by national instrumentation centre for environmental management (nicem) online at (http://nicem.snu.ac.kr/main/?en_skin=index.html), biotechnology lab, machine is DNA sequence 3730XL, Applied Bio system), Homology search was conducted using Basic Local Alignment Search Tool (BLAST) program which is available at the National Centre Biotechnology Information (NCBI) online at (http:// www.ncbi.nlm.nih.gov) and Bio Edit program.

Detection of Gene IT'S by Using PCR

Detection of *ITS* gene was conducted by using primers for amplification. A fragment of *ITS* was amplified using a forward primer (*ITS1* F: 5′- TCCGTAGGTGAACCTGCGG -3′) and a reverse primer (*ITS4* R: 5′ TCCTCCGCTTATTGATATGC-3′) (Primers set supplied by IDT (Integrated DNA Technologies company, Canada.). The PCR amplification was performed in a total volume of 25μL containing 1.5μL DNA, 5 μL Taq PCR Premix (Intron, Korea), 1μL of each primer (10 pmol) then distilled water was

added into tube to a total volume of 25µl.The thermal cycling conditions were done as follows: Denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 45s, 52°C for 1 min and 72 °C for 1min with final incubation at 72 °C for 7 min using a thermal Cycler (Gene Amp, PCR system 9700; Applied Bio system). The PCR products were separated by 1.5% agarose gel electrophoresis and visualized by exposure to ultraviolet light (302nm) after red stain staining (Intron Korea).

Result and Discussion

The *Aspergillus Niger* strain AHBR3 (Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Eurotiomycetes; Eurotiomycetidae; Eurotiales; Aspergillaceae; Aspergillus), *Cladosporium ramotenellum* culture personal: Jos: Houbraken: DTO: 323-D6 small (Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Dothideomycetidae; Capnodiales; Cladosporiaceae; Cladosporium) and *Penicillium brevicompactum* isolate 66 small (Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Eurotiomycetes; Eurotiomycetidae; Eurotiales; Aspergillaceae; Penicillium) were studied in different concentrations of aromatic compounds that separated from crude oil from north Al-Rumaila/ Iraq, in order to see its growth behaviour in different media using one day, three days and one week as seen in Table (1) below.

Table 1: The results of the *Aspergillus Niger* strain AHBR3, *Cladosporium ramotenellum* and *Penicillium brevicompactum* in different concentrations of each of PAHs and NAH-F/NAH-R, which separated from crude oil from north Al-Rumaila

Id	Growth behaviour		
Concentration ppm	one day	3 days	one week
1.88	+	++	+++
0.188	+	++	+++
0.0188	+	++	+++
0.00188	+	++	+++
0.000188	+	++	+++
0.376	+	++	+++
0.0376	+	++	+++
0.564	+	++	+++
0.0564	+	++	+++
0.00564	+	++	+++

- The label (+): natural growth behaviour
- The label (++): the growth behaviour slightly increases
- The label (+++): the growth behaviour rise up

The results of *Aspergillus Niger* strain AHBR3, *Cladosporium ramotenellum* and *Penicillium brevicompactum* were showed that the growth was well increased with period in each, which indicated that the crude oil was presented good source of food and energy to the three microorganisms. The KAPA Universal Ladder Kit is designed for determining the approximate size and quantity of double-stranded DNA on agarose gel. KAPA Universal Ladder kits contain eighteen DNA fragments (in base pairs): 100, 150, 200, 300, 400, 500, 600, 800, 1000, 1200, 1600, 2000, 3000, 4000, 5000, 6000, 8000, and 10000. The KAPA Universal Ladder contains four reference bands (500, 1000, 1600, and 4000) for orientation, see Figure (1) below.

and 4000) for orientation, see Figure (1) below.

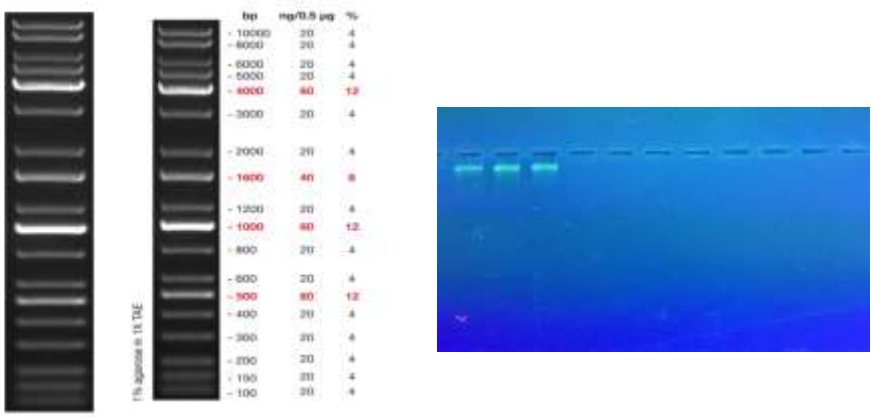

Figure 1: Gel electrophoresis of genomic DNA extraction from Fungi, 1% agarose gel at 5 vol /cm for 1:15 hour

Real-time PCR or quantitative PCR (q-PCR) is a very sensitive technique that allows the quantification of a specific nucleic acid sequence in real-time through PCR product detection [6].The detection of PCR products in real time is achieved by measuring the fluorescence produced during the extension step. Fluorescence is measured in each cycle and it is proportional to the amount of PCR product. Thus, the accurate quantification of the initial concentrations of DNA, cDNA and RNA can be achieved.

The SYBR green is a fluorescent dye that binds to ds DNA via intercalation between adjacent pairs and represents one of the most widely used fluorescent dyes. It emits a fluorescent signal while binding and can be used with any real-time cycler since its excitation and emission maxima are at 494 and 521 nm respectively. As mentioned above, the signal intensity is proportional to the accumulation of PCR product but primer-dimers and non-specific products will also add to the signal. Thus, primers need to be highly specific when using SYBR green to avoid overestimation of the target [6].A DNA ladder is a solution of DNA molecules of different lengths used in agarose or acryl amide gel electrophoresis. It is applied as a reference to estimate the size of unknown DNA molecules that were separated based on their mobility in an electrical field through the gel.

Therefore, the DNA ladders are essential molecules routinely used in every DNA dealing laboratory. Recently, many laboratory protocols describing the preparation of DNA ladders by employing the polymerase chain reaction (PCR) method have been reported.[6] Thus, The Components of the Maxime PCR Premix kit (i-Taq) was used, (Table 2).Followed by diagnosis of the gene, (Table 3).

Table 2: The components of the Maxime PCR PreMix kit (i-Taq)

Material	Volume
i-Taq DNA Polymerase	2.5U
DNTPs	2.5mM
Reaction buffer (10X)	1X
Gel loading buffer	1X

Table 3: Mixture of the specific interaction for diagnosis gene

Components	Concentration
Taq PCR PreMix	5µl
Forward primer	10 picomols/µ
Reverse primer	10 picomols/µ
DNA	1-1.5µl
Distill water	16.5 µl
Final volume	25µl

The specific primer of gene *ITS* was used with optimize conditions as seen in Table (4) below. The forward and reverse sequences of the primer were 5′- TCCG TAGG TGAA CCTGCGG -3′ and 5′ TCCT CCG CTTA TTGATATGC-3′ respectively. The Tm (°C) and GC (%) in each sequence were equal to (60.3 and 57.8) °C and (50 % and 41 %) respectively, with used product size equal to 650 base pair in each sequence.

Table 4: The optimum conditions

No.	Phase	Tm (°C)	Time	No. of cycle
1	Initial Denaturation	94°C	3 min.	35cycle
2	Denaturation -2	94°C	45 sec	
3	Annealing	52°C	1min	
4	Extension-1	72°C	1 min	
5	Extension -2	72°C	7 min.	

The PCR product (band size 650bp) was electrophoresis on 2% agarose at 5 volt/cm^2 using 1x TBE buffer for 1:30 hours. N: DNA ladder (100), which then was visualized under U.V light as seen in Figure (2).

Figure 2: The visualization of the PCR product electrophoresis under U.V light

The expectation value is defined to give an estimate of the number of times expected to get the same similarity coincidental and the lower the value of E. This indicates that the degree of similarity was high between sequences which give greater confidence. The value of a very close to zero means that these sequences are identical and the bit Score: statistical measure of the moral similarity and the higher value indicates that the high degree of similarity, and if dropped from the class of 50 points, the sense that there is no similarity mention. These results were utilized as below:

The internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence of *Aspergillus Niger* strain AHBR3, *Cladosporium ramotenellum* and *Penicillium brevicompactum* were presented as below:

Id		ORIGIN
Aspergillus niger strain *AHBR3*	LOCUS KF305742 657 bp DNA linear PLN 04-AUG-2013 misc_RNA <1..>657 /note="contains internal transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed spacer 2, and 28S ribosomal RNA"	1 tgcggaagga tcattaccga gtgcgggtcc tttgggccca acctcccatc cgtgtctatt 61 gtaccctgtt gcttcggcgg gcccgccgct tgtcggccgc cgggggggcg cctctgcccc 121 ccgggcccgt gcccgccgga gaccccaaca cgaacactgt ctgaaagcgt gcagtctgag 181 ttgattgaat gcaatcagtt aaaactttca acaatggatc tcttggttcc ggcatcgatg 241 aagaacgcag cgaaatgcga taactaatgt gaattgcaga attcagtgaa tcatcgagtc 301 tttgaacgca cattgcgccc cctggtattc cgggggggcat gcctgtccga gcgtcattgc 361 tgccctcaag cccggcttgt gtgttgggtc gccgtccccc tctccgggggg gacgggcccg 421 aaaggcagcg gcggcaccgc gtccgatcct cgagcgtatg gggctttgtc acatgctctg 481 taggattggc cggcgcctgc cgacgttttc caaccattct ttccaggttg acctcggatc 541 aggtagggat acccgctgaa cttaagcata tcataaggcg agaggaatca ttaccgagtg 601 cgggtccttt gggccaacct ccatccgtgt ctattgtacc ctgttgcttc ggcgggc
Cladosporium ramotenellum	LOCUS MF473250 835 bp DNA linear PLN 21-MAR-2018 misc_RNA <1..>835 /note="contains small subunit ribosomal RNA, internal transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed spacer 2, and large subunit ribosomal RNA"	1 tggctcggtg aggccttcgg actggcccag ggaggtcggc aacgaccacc cagggccgga 61 aagttggtca aacccggtca tttagaggaa gtaaaagtcg taacaaggtc tccgtaggtg 121 aacctgcgga gggatcatta caagtgaccc cggctacggc cgggatgttc ataaccctttt 181 gttgtccgac tctgttgcct ccggggcgac cctgccttcg ggcgggggct ccgggtggac 241 acttcaaact cttgcgtaac tttgcagtct gagtaaactt aattaataaa ttaaaacttt 301 taacaacgga tctcttggtt ctggcatcga tgaagaacgc agcgaaatgc gataagtaat 361 gtgaattgca gaattcagtg aatcatcgaa tctttgaacg cacattgcgc cccctggtat 421 tccgggggggc atgcctgttc gagcgtcatt tcaccactca agcctcgctt ggtattgggc 481 aacgcggtcc gccgcgtgcc tcaaatcgtc cggctgggtc ttctgtcccc taagcgttgt 541 ggaaactatt cgctaaaggg tgttcgggag gctacgccgt aaaacaaccc catttctaag 601 gttgacctcg gatcaggtag ggatacccgc tgaacttaag catatcaata agcggaggaa 661 aagaaaccaa cagggattgc tctagtaacg gcgagtgaag cagcaatagc tcaaatttga 721 aatctggcgt cttcgacgtc cgagttgtaa tttgtagagg atgcttctga gtggccaccg 781 acctaagttc cttggaacag gacgtcatag agggtgagaa

		tcccgtatgc ggtcg
Penicillium brevicompactum	LOCUS KY587318 687 bp DNA linear PLN 01-APR-2017 misc_RNA <1..>687 /note="contains small subunit ribosomal RNA, internal transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed spacer 2, and large subunit ribosomal RNA"	1 ggtgaacctg cggaaggatc attaccgagt gagggscctc tgggtccaac ctcccacccg 61 tgtttatttt accttgttgc ttcggcgagc ctgccttttg gctgccgggg gacgtctgtc 121 cccgggtccg cgctcgccga agacacctta gaactctgtc tgaagattgt agtctgagat 181 taaatataaa ttatttaaaa ctttcaacaa cggatctctt ggttccggca tcgatgaaga 241 acgcagcgaa atgcgatacg taatgtgaat tgcagaattc agtgaatcat cgagtctttg 301 aacgcacatt gcgccctctg gtattccgga gggcatgcct gtccgagcgt cattgctgcc 361 ctcaagcacg gcttgtgtgt tgggctccgt cctccttccg ggggacgggc ccgaaaggca 421 gcggcggcac cgcgtccggt cctcaagcgt atggggcttt gtcacccgct ttgtaggact 481 ggccggcgcc tgccgatcaa ccaaactttt ttccaggttg acctcggatc aggtagggat 541 acccgctgaa cttaagcata tcaataagcg gaggaaaatc attaccgagt gagggccctc 601 tgggtccaac ctcccaccgt gtttatttta ccttgttgct tcggcgagcc tgccttttgg 661 ctgccggggg acgtctgtcc cgggtcg

The results indicated that the method used a PAHs degrading *Aspergillus Niger* strain AHBR3, *Cladosporium ramotenellum* and *Penicillium brevicompactum* represents cheap and effective. These PAH degraders docked the functional genes which help microorganisms use PAHs as source of food and energy, which degrading the pollutants.

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