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TITLE:

Isolation, Propagation, and Identification of Bacterial Species with Hydrocarbon Metabolizing Properties from Aquatic Habitats

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SUMMARY:

We present the process of isolating, propagating, and characterizing hydrocarbon-degrading bacteria from aquatic habitats. The protocol outlines bacterial isolation, identification by the 16S rRNA method, and testing of their hydrocarbon-degrading potential. This article would help researchers in characterizing microbial biodiversity in environmental samples, and specifically screen for microbes with bioremediation potential.

ABSTRACT:

Hydrocarbon pollutants are recalcitrant to degradation and their accumulation in the environment is toxic to all life forms. Bacteria encode numerous catalytic enzymes and are naturally capable of metabolizing hydrocarbons. Scientists harness biodiversity in aquatic ecosystems to isolate bacteria with biodegradation and bioremediation potential. Such isolates from the environment provide a rich set of metabolic pathways and enzymes, which can be further utilized to scale up the degradation process at an industrial scale. In this article, we outline the general process of isolation, propagation, and identification of bacterial species from aquatic habitats and screen their ability to utilize hydrocarbon as the sole carbon source *in vitro* using simple techniques. The present protocol describes the isolation of various bacterial species and their subsequent identification using the 16S rRNA analysis. The protocol also presents steps for characterizing the hydrocarbon degrading potential of bacterial isolates. This protocol will be useful for researchers trying to isolate bacterial species from environmental habitats for their biotechnological applications.

INTRODUCTION:

Hydrocarbons (HC) are extensively used both as fuels and in chemical applications. Aromatic hydrocarbons such as benzene, toluene, and xylene are used widely as solvents[1]. Alkenes such as ethylene and propylene serve as precursors in the synthesis of polyethylene and

polypropylene polymers, respectively. Polymerization of another hydrocarbon, styrene forms polystyrene. This results in plastic pollution that is difficult to eradicate[2]. Anthropogenic activities introduce hydrocarbons into the environment during their production and transport. Hydrocarbon contamination of soil and water has serious concerns for the environment and human health. Microbes play a major role in maintaining the ecosystem by regulating the biogeochemical cycles and utilizing a wide range of substrates, which includes pollutants and xenobiotics as well, converting them into carbon and energy source. This process of detoxification of environmental contaminants by microorganisms is known as bioremediation[3–7].

Microorganisms with the capability to degrade hydrocarbons are found in aquatic and soil habitats[8–10]. Many bacteria with the potential to degrade alkanes and aromatic HCs have been identified, such as *Pseudomonas*, *Acinetobacter*, *Rhodococcus*, *Marinobacter,* and *Oleibacter*[11]. The development of technologically advanced culture-independent approaches has helped discover novel HC-degrading microbial communities[12]. Genomic material directly isolated from source samples is amplified and sequenced by high throughput methods such as Next Generation Sequencing (NGS) followed by analysis eliminating the need to cultivate microorganisms. NGS methods, such as metagenome analysis, are expensive and suffer from drawbacks related to the amplification process[13]. Cultivation techniques such as selective enrichment culture[14] that target isolation of hydrocarbon-degrading microbes are still useful as they allow researchers to probe and manipulate metabolic pathways in bacterial isolates.

Genomic DNA isolation and subsequent sequencing of the genomic material reveals valuable information about any organism. Whole-genome sequencing helps in the identification of genes that code for antibiotic resistance, potential drug targets, virulence factors, transporters, xenobiotic-metabolizing enzymes, etc[15–17]. Identification of bacteria by 16S rRNA sequencing is a common practice as the protein encoded by this gene is a component of the bacterial ribosome and is present in all the bacteria. Conservation of the gene sequence and function over the years makes it a reliable tool for identifying unknown bacteria and comparing an isolate with the closest species. In addition, the length of this gene is optimum for bioinformatics analysis[18]. All these features along with the ease of gene amplification using universal primers and improvement in gene sequencing technology make it a gold standard for the identification of microbes.

Here, we describe a procedure to recover cultivable microorganisms with HC-degrading potential from environmental samples. The method described below outlines the collection and identification of HC-degrading bacteria and is divided into five sections: (1) collection of bacteria from water samples, (2) isolation of pure cultures, (3) exploring HC-degrading capability of bacterial isolates (4) genomic DNA isolation, and (5) identification based on 16S rRNA gene sequencing and blast analysis. This procedure can be adapted to isolate bacteria for many different biotechnological applications.

PROTOCOL:

1. Sample collection, processing, and analysis

NOTE: Here, we present a protocol to isolate bacteria from aquatic habitats. Some of the isolates may be pathogenic, therefore, wear gloves and disinfect the work area before and after use.

1.1. Collect 500 mL of water sample in five sterile glass bottles from different sites of the water body. Measure the pH and temperature of each sample using a pH meter and thermometer, respectively.

NOTE: The protocol is not site-specific and can be easily adapted to isolate organisms from hydrocarbon-contaminated water bodies too.

1.2. Filter the sample in a batch of 100 mL through 0.22 µm-pore size filter sheets, in aseptic conditions.

NOTE: The diameter of the filter paper should not exceed the Petri dish diameter. For example, filter paper not exceeding 85 mm diameter is optimum for a 100–120 mm Petri dish.

1.3. Keep the filter papers over different nutrient media plates (PYE[19], R2A[20], M9, LB, NB, TSB, M63[21], and M2G[22]). The different types of growth media allow the selection and enrichment of different microorganisms. Compositions of various growth media are listed in **Table 1**. Use one paper for each media plate and peel off after 2 h using sterile forceps.

1.4. Serially dilute the unfiltered water samples (10^6 dilution) in sterile double distilled water by adding 100 µL of the collected water sample in 900 µL of sterile water. This results in a 1:10 dilution. From this sample, take 100 µL and add in 900 µL of sterile water to obtain 1:100 dilution. Repeat the dilution until the dilution fold is 1:1,000,000. Mix by pipetting. The final volume of each dilution will be 1 mL.

1.5. Spread 100 µL of the diluted water sample individually on all growth media plates mentioned in step 1.3 in triplicates.

1.6. Incubate the plates at 30 °C for 24 to 48 h depending on the growth of colonies.

NOTE: Most of the environmental isolates grow at an optimum temperature of 30 °C. If isolating the samples from an environment with extreme temperatures, incubate the plates at the same temperature as that of the collection site.

1.7. Next, pick the colonies using a sterile toothpick or pipette tip and perform quadrant streaking to get isolated colonies.

1.8. Incubate the plates overnight. Next day, screen the colonies based on their morphological features such as color, texture, shape, size, margin, elevation, etc. Restreak the colonies to obtain pure cultures.

1.9. Perform gram-staining of each pure culture[23] and proceed with glycerol stock preparation.

1.10. To prepare the glycerol stocks, inoculate a single colony in 3 mL of appropriate growth media and incubate at 30 °C. From the overnight culture, take 700 µL and add 300 µL of 100% glycerol (sterilized by autoclaving) in cryovials[24]. Freeze the vials at -80 °C for long-term storage.

2. Degradation of hydrocarbons

NOTE: The example below is to screen the isolates which can degrade styrene. It is a slight modification of the method adapted in a previous report[25]. Follow the steps under aseptic conditions.

2.1. From a freshly streaked plate, pick a colony and inoculate in 5 mL of Tryptic soy broth (TSB)/Nutrient broth (NB). Grow the culture overnight at 30 °C with shaking at 200 rpm till the absorbance reaches ~2.

NOTE: Other than TSB/NB, any growth medium can be chosen in which the bacteria reach high cell density.

2.2. The next day, pellet the cells at 2862 x *g* for 5 min at 4 °C and discard the supernatant.

2.3. Wash the pellet twice with 2 mL of autoclaved saline (0.9% NaCl) and spin at 2862 x *g* for 5 min at 4 °C.

NOTE: Saline is isotonic and, thus, it maintains the osmotic pressure inside bacterial cells.

2.4. Resuspend the pellet in 2 mL of liquid carbon-free basal medium (LCFBM). Measure the absorbance (OD_{600}).

2.5. Take two sterile Erlenmeyer flasks with 150 mL capacity for control and the experimental group. Label them as A and B.

2.6. In the uninoculated /control group, (flask A), add 40 mL of LCFBM and styrene (5 mM).

2.7. In flask B, add 35 mL of LCFBM and styrene (adjust the final concentration of styrene to 5 mM). Add the cell suspension with a final OD_{600} of cells ≈ 0.1 and make up the remaining volume with LCFBM up to 40 mL. Incubate the flasks at 30 °C with shaking at 200 rpm for 30 days.

NOTE: Hydrocarbons in excess can be toxic for the microbes, therefore, start with low concentration and gradually increase it.

2.8. Repeat the above for each additional strain that must be evaluated for hydrocarbon degradation.

2.9. Measure the OD_{600} of each flask every 5 days and plot a growth curve. Increase the incubation up to 45 days if the bacteria can utilize styrene. An increase in OD_{600} indicates that the bacterium can metabolize styrene.

3. Screening of catechol degradation by bacterial isolates

NOTE: The degradation of aromatic hydrocarbons such as styrene, benzene, xylene, naphthalene, phenols, etc. produce catechols as reaction intermediates. The catechols are further metabolized by bacteria with the help of catechol 1,2-dioxygenase and catechol 2,3-dioxygenase enzymes through the ortho- and meta-cleavage pathways, respectively[26]. These enzymes are also involved in the degradation of other hydrocarbons such as chlorobenzene[27]. The protocol mentioned below uses whole cell lysate for catechol 2, 3-dioxygenase enzyme assay[28]. The same lysis method can be used to screen the activity of catechol 1, 2-dioxygenase. However, the composition of the reaction mixture will vary. Both the enzymes are inducible in nature and can be induced by the addition of phenol to the growth media.

3.1. With the help of a sterile loop, inoculate the bacterial colony from a freshly streaked plate into mineral salts medium (MSM) supplemented with 1–4 mM phenol. Incubate the culture at 30 °C and 200 rpm. Harvest the culture at 4 °C when OD_{600} reaches between 1.4–1.6 (i.e., in late exponential phase) by spinning at 4500 x *g* for 20 min.

3.2. Wash the cell pellet with phosphate buffer (0.5 M, pH 7.5).

3.3. Resuspend the cells in the above-mentioned phosphate buffer and adjust the final OD_{600} ≈ 1.0.

3.4. Lyse the cells by pulsed sonication for 1.5 min, the duration of each pulse being 15 s. After this step, the suspension must be clear or less turbid. If not, increase the number of pulses and check whether the suspension is clear after each pulse to avoid protein degradation.

3.5. Remove the cell debris and unbroken cells by centrifugation at 9,000 x *g* for 30 min, maintaining the cold temperature (4 °C).

3.6. Carefully pipette the clear supernatant. This fraction has the crude extract for enzyme assay.

3.7. Determine the protein concentration of crude extract by either Bradford or Lowry's method[29,30].

3.8. To determine the activity of catechol 2,3-dioxygenase, measure the formation of the reaction end product (2-hydroxymuconic semialdehyde) by a spectrophotometer.

3.9. Prepare the reaction mixture by adding 20 µL of catechol (50 mM), 960 µL of phosphate buffer (50 mM, pH 7.5), and 20 µL of the crude extract.

3.10. For the negative control, replace the crude extract with phosphate buffer and adjust the final volume to 1 mL.

3.11. Incubate the reaction mixture for 30 min. At set time intervals, measure the absorbance at 375 nm. An increase in absorbance indicates the formation of the reaction end product, 2-hydroxymuconic acid semialdehyde (2-HMS). Perform the experiment in triplicates.

NOTE: Catechol is light-sensitive and oxygen-sensitive. Store the reaction mixture in dark and close the tubes tightly to prevent the natural degradation of catechol.

4. Genomic DNA isolation of the pure culture

NOTE: This is the general protocol for the isolation of genomic DNA. Gram staining was performed during the sample collection, processing, and analysis step. Due to the variation in cell wall thickness of gram-positive and gram-negative bacteria, the cell lysis method is modified accordingly. Wear gloves while isolating and sterilize the workbench with 70% ethanol to avoid the nucleases from degrading DNA. Some of the chemicals mentioned below can cause severe burns on the skin and proper care must be taken while handling them.

4.1. Isolation of genomic DNA from Gram-negative bacteria[31].

4.1.1. Pick a single colony and inoculate in a fresh growth medium in sterile test tubes.

4.1.2. Place the tubes in an incubator shaker at 200 rpm and allow the bacteria to grow overnight at 30 °C.

4.1.3. The next day, pellet 1.5 mL of overnight grown culture at 12,400 x *g* for 3 min.

4.1.4. Remove the supernatant and resuspend the pellet in 200 µL of lysis buffer (40 mM Tris-acetate, pH 7.8, 20 mM sodium acetate, 1 mM EDTA, 1% SDS).

4.1.5. Add 66 µL of NaCl solution (5 M) and mix well.

4.1.6. Pellet the resulting mixture at 12,400 x *g* for 10 min (4 °C).

4.1.7. Pipette the clear supernatant in a fresh microcentrifuge tube and add an equal volume of chloroform.

4.1.8. Invert mix the solution multiple times until a milky solution is observed.

4.1.9. Spin at 12,400 x *g* for 3 min and transfer the supernatant to a clean vial.

4.1.10. Add 1 mL of ice-cold 100% ethanol; mix by inversion till white strands of DNA precipitate out.

4.1.11. Centrifuge the precipitated DNA at 2,200 x *g* for 10 min at 4 ˚C and discard the supernatant.

4.1.12. Wash the DNA pellet with 1 mL of 70% ethanol and allow the DNA pellet to dry for 5 min at room temperature.

4.1.13. Once dried, resuspend the pellet in 100 µL of 1x Tris-EDTA(TE) buffer, and store the DNA at -20 ˚C.

4.1.14. Measure the concentration ($A_{260/280}$) using fluorimeter and run the DNA on agarose gel (1%) to assess the quality of DNA[24].

4.2. Isolation of genomic DNA from gram-positive strain[32]

4.2.1. Pick a single colony and inoculate in a fresh growth medium in sterile test tubes.

4.2.2. Place the tubes in an incubator shaker at 200 rpm and allow the bacteria to grow overnight at a suitable growth temperature.

4.2.3. Next day, take 1.5 mL of the grown culture and centrifuge at 8,600 x *g* for 5 min.

4.2.4. Remove the supernatant and resuspend the cells in TE buffer.

4.2.5. Adjust the OD_{600} = 1.0 with TE buffer and transfer 740 µL of the cell suspension to a clean microfuge tube.

4.2.6. Add 20 µL of lysozyme (100 mg/mL stock) and mix well by pipetting. Incubate at 37 °C for 30 min (in a dry bath).

4.2.7. Add 40 µL of 10% SDS and mix well.

4.2.8. Add 8 µL of Proteinase K (10 mg/mL). Mix well and incubate at 56 °C for 1–3 h (in a dry bath). The suspension should become clear now with increased viscosity, marking efficient cell lysis.

NOTE: The suspension can be left overnight if the cells are not lysed properly.

4.2.9. Preheat CTAB/NaCl mixture at 65 °C (in a dry bath) and add 100 µL of this mixture to the cell suspension. Mix well.

4.2.10. Incubate at 65 °C for 10 min (in a dry bath).

4.2.11. Add 500 µL of chloroform:isoamyl alcohol (24:1) and mix well. Spin at 16,900 x *g* for 10 min at 25 °C.

4.2.12. Transfer the aqueous phase to a fresh microcentrifuge tube avoiding the organic phase (viscous phase at the bottom).

4.2.13. Carefully add 500 µL of phenol:chloroform:isoamyl alcohol (25:24:1) and mix well. Spin at 16,900 x *g* for 10 min at 25 °C.

4.2.14. Take the aqueous phase in a fresh microcentrifuge tube. Add 500 µL of chloroform:isoamyl alcohol (24:1) and mix well.

4.2.15. Transfer the aqueous phase and add 0.6 volume isopropanol (prechilled at -20 °C).

4.2.16. The precipitated DNA strands must be visible in the threadlike form. Incubate at -20 °C for 2 h to overnight.

4.2.17. Centrifuge at 16,900 x *g* for 15 min at 4 °C to pellet the DNA.

4.2.18. Decant the isopropanol carefully and wash the pellet with 1 mL of cold 70% ethanol (prechilled at -20 °C) to remove any impurities.

4.2.19. Centrifuge at 16,900 x *g* for 5 min at 4 °C. Discard the supernatant.

4.2.20. Allow the pellet to dry at room temperature for 20 min or keep the tube at 37 °C. Make sure the pellet is not over-dried.

4.2.21. Resuspend in 100 µL of 1x TE buffer and store the DNA at -20 ˚C.

NOTE: If the pellet becomes over-dried and is difficult to resuspend, incubate the microcentrifuge tube with DNA pellet and nuclease-free water at 37 °C for 15–20 min and resuspend again by pipetting.

4.2.22. Measure the concentration ($A_{260/280}$) using a spectrophotometer after making 1:100 dilution in 1x TE buffer and run the DNA on agarose gel (1%) to assess the quality of DNA[24].

5. 16S rRNA sequencing

NOTE: The protocol outlined below is for amplification and sequencing of 16S rRNA for bacterial identification. Information derived from the 16S rRNA sequence is used for the identification of an unknown organism and to find the relatedness between different organisms.

5.1. To identify the strains, amplify the DNA isolated from the pure bacterial cultures by PCR with universal primers targeting 16S rRNA sequence for bacteria: **27F (5'-AGAGTTTGATCMTGGCTCAG-3')** and **1492R (5'- TACGGYTACCTTGTTACGACTT-3')**[33].

5.2. Prepare the PCR mix (25 µL reactions) on ice with 18 µL of autoclaved/nuclease-free water, 2.5 µL of 10x buffer, 0.5 µL of both forward and reverse primers (100 µM stock), 2 µL of the dNTPs mix (100 µM stock), 1 µL of DNA template (2–15 ng/µL) and 1 U of *Taq Polymerase.*

5.3. Use the following cycling conditions for 16S rRNA gene amplification: Initial denaturation at 94 ˚C for 10 min, (final denaturation at 94 ˚C for 40 s, primer annealing at 56 ˚C for 1 min, extension at 74 ˚C for 2 min) x 30 cycles, final extension at 74 ˚C for 10 min.

5.4. After the cycle ends, mix 5 µL of sample and 1 µL of 5x DNA loading dye. Run on 1% agarose gel to verify the amplification. Store the PCR products at 4 °C for the short term or freeze them at -20°C until further use.

5.5. For 16S rRNA gene sequencing, set up the same reaction as mentioned above for higher volume (100 µL).

5.6. Purify the amplicons for Sanger sequencing[24,34] using PCR product purification kit or mix the entire sample with DNA loading dye and load on an agarose gel to perform gel extraction method.

5.7. Once the sequencing is done, convert the results file in FASTA format and check the sequence similarity with the basic local alignment search tool (BLAST) on NCBI (http://www.ncbi.nlm.nih.gov/)[35].

REPRESENTATIVE RESULTS:

The schematic outlining the entire procedure for isolation and screening of bacteria from aquatic habitats and their subsequent identification by 16S rRNA analysis is represented in **Figure 1**. Water samples from a wetland in Dadri, India were collected in sterile glass bottles and immediately taken to the laboratory for processing. The samples were passed through filter sheets with 0.22 µm pore size, and the filter papers were kept in contact with different media plates. After 2 h, filter papers were removed, and the plates were incubated overnight at 30 °C for colonies formation (**Figure 2**). The next day, individual bacterial colonies were selected and streaked on fresh media plates (**Figure 2**). The pure culture generated was stored and subsequently used for further analysis. Using this method, we were able to create a library of more than 100 unique bacterial isolates. We aimed to identify bacterial isolates that can utilize hydrocarbons, especially styrene, which is the primary component of single-use plastic. The isolated bacteria were individually grown in respective media with the addition of liquid styrene as a sole source of carbon (**Figure 3**). We could identify four isolates that utilize styrene as a sole source of carbon. Two of the isolates were extensively characterized further for styrene degradation[25].

The bacterial isolates were then tested for the presence of enzymatic pathways for the degradation of hydrocarbon metabolism. Hydrocarbon metabolism in some bacteria results in the production of catechols as intermediates, which are further degraded by ortho-cleavage and meta-cleavage pathways. Catechol 1,2- dioxygenase and catechol 2,3–dioxygenase enzymes are responsible for ring-cleavage reaction[36]. Environmental bacteria possessing these enzymes have been shown to metabolize several aromatic compounds. Thus, a catechol degradation assay was performed to assess the HC-degrading potential of bacterial isolates (**Figure 4**). A representative assay for one of the isolates is shown in **Figure 4**.

To identify the bacterial isolates, 16S rRNA sequencing was performed. A preliminary gram staining was performed to characterize the bacteria, which helps identify and troubleshoot subsequent steps. Gram-positive bacteria are usually recalcitrant to cell lysis buffers leading

to low genomic DNA yield[37]. Thus, the results obtained from gram-staining[38] before genomic DNA isolation would help in choosing the protocol for genomic DNA isolation. After DNA isolation, the integrity of genomic DNA was confirmed by visualizing a small sample of DNA on agarose gel (**Figure 5A**) and quantified by UV absorbance method using a spectrophotometer. 16S rRNA gene was amplified using universal primers sequence (**Figure 5B**). While 500 bp are essential for sequencing, ideal results are obtained with 1,300–1,500 bp[39]. To obtain the degree of relatedness among isolated strains, a phylogenetic tree was constructed using the phylogeny.fr software[40] (**Figure 6**).

FIGURE LEGENDS:

Figure 1: Schematic workflow of the study

Figure 2: Images of bacterial colonies from water samples. The collected water sample was passed through 0.22 µm filter paper. The filter papers were kept over different media plates. The plates were incubated for 24–48 h until isolated colonies were observed. The single colonies were then streaked on fresh plates for pure culture isolation.

Figure 3: Representative results of microbial degradation of styrene and screening hydrocarbon degradation potential of bacteria. The cells were grown in LCFBM supplemented with 5 mM styrene as a sole carbon source for 40 days at 30 °C and 200 rpm. OD_{600} was measured every 5 days. The control flask had only LCFBM.

Figure 4: Representation of Catechol 2,3-dioxygenase enzyme assay to monitor the degradation of catechol. (**A**) The colorless substrate catechol is converted into a yellow-colored product by the action of catechol 2,3-dioxygenase. The reaction mixture contains catechol, phosphate buffer, and crude cell lysate. The formation of the product is detected by measuring absorbance at 375 nm. (**B**) Representative graph of catechol 2,3-dioxygenase enzyme assay with whole-cell lysate. The reaction mixture in negative control has buffer and catechol substrate without cell lysate. Absorbance was measured at 375 nm at an interval of 10 min.

Figure 5: Genomic DNA isolation and 16S rRNA PCR. (**A**) Gel electrophoresis of isolated genomic DNA. Lane M: DNA size marker, Lane 1–2: Genomic DNA. (**B**) Verification of 16S rRNA gene amplification by 1% gel electrophoresis. Gels were visualized by staining with ethidium bromide; Lane M: DNA size marker (1 kb), Lane 1–4: Amplified PCR products from different strains.

Figure 6: Analysis of the 16S rRNA gene sequencing results. Representative dendrogram construction using the phylogeny.fr program to depict the relatedness among *Exiguobacterium* strains isolated from a wetland (highlighted in red box) with the known *Exiguobacterium* sp. The 16S rRNA sequences of known *Exiguobacterium* sp. were obtained from NCBI. This figure has been taken from a previous paper (Chauhan et.al.) without any modification[25].

DISCUSSION:

It is well established that only approximately 1% of bacteria on Earth can be readily cultivated in laboratory[6]. Even among the cultivable bacteria, many remain uncharacterized. Improvements in molecular methods have given a new dimension to the analysis and evaluation of bacterial communities. However, such techniques do have limitations, but they do not make the culture analyses redundant. Pure culture techniques to isolate individual bacterial species remain the primary mechanism for the characterization of physiological properties. Soil and aquatic habitats harbor many bacteria with novel enzymes and pathways, which can be harnessed for biotechnological uses. This study describes a simple and inexpensive method for the isolation and characterization of bacteria from ecological samples.

Different bacteria have different nutritional requirements, hence various growth media were used to increase the probability of isolating diverse bacterial species. One major limitation of this method is that microbes with fastidious growth requirements may get excluded. Also, the main goal of this step is to maximize the number of bacterial species obtained from the sample. The number of bacterial species in the sample library would improve the chances of isolating microbes with bioremediation potential. Though we only varied growth media, varying growth temperature and oxygen concentration can also increase the chances of further expanding the sample library with unique species[41,42].

A critical step of the protocol is to check the utilization of the substrate being tested (styrene in our case). It is important to design the experiment for such investigation with care to avoid false-negative results. Depending on growth characteristics, the microbe may not immediately adapt to utilizing the substrate being tested and may require an enrichment process. In our case, the bacterial growth is slow in the LCFBM medium used to test the utilization of hydrocarbon as the sole carbon source[25]. To circumvent this problem, initial cultures can be started by adding (1% v/v) TSB or NB to the LCFBM medium to support bacterial growth. Identification of the cultivated microbe is accomplished through 16S rRNA sequencing[43]. This method offers a robust and cost-effective method for microbial identification. However, 16S sequencing can only provide higher-level taxonomical identification. For specific species-level identification, other family-specific primers have to be used combined with various biochemical tests[44,45].

Enzyme assay with whole-cell lysate requires using the efficient cell lysis method. Bacterial cell lysis is usually achieved by performing sonication. However, a freeze-thaw method is an alternative method for gentle cell lysis, which is believed to prevent denaturation of protein. The procedure consists of quickly freezing the cells at -80 °C and thawing at 4 °C in a sequential manner[46]. The addition of mild detergents such as NP-40 or triton-X-100 also aids in cell lysis and does not denature the proteins, due to their non-ionic nature[47]. However, bacteria with thick cell walls such as cyanobacteria[48] may not benefit from the gentle cell lysis method using detergents[49] and thus, the lysis method for enzyme assay must be chosen accordingly.

By focusing on the cultivable bacterial population from environmental samples, researchers can quickly perform many different experiments. The methods described here do not require the use of very sophisticated instruments and can be easily performed in a standard laboratory setup. Since hydrocarbons and hazardous chemicals are used, the laboratory should be equipped with proper handling and disposal standard operating procedures. The

approach described here can be easily adapted to study a variety of bacterial species for numerous biotechnological applications.

DISCLOSURES:

The authors declare no conflicts of interest.

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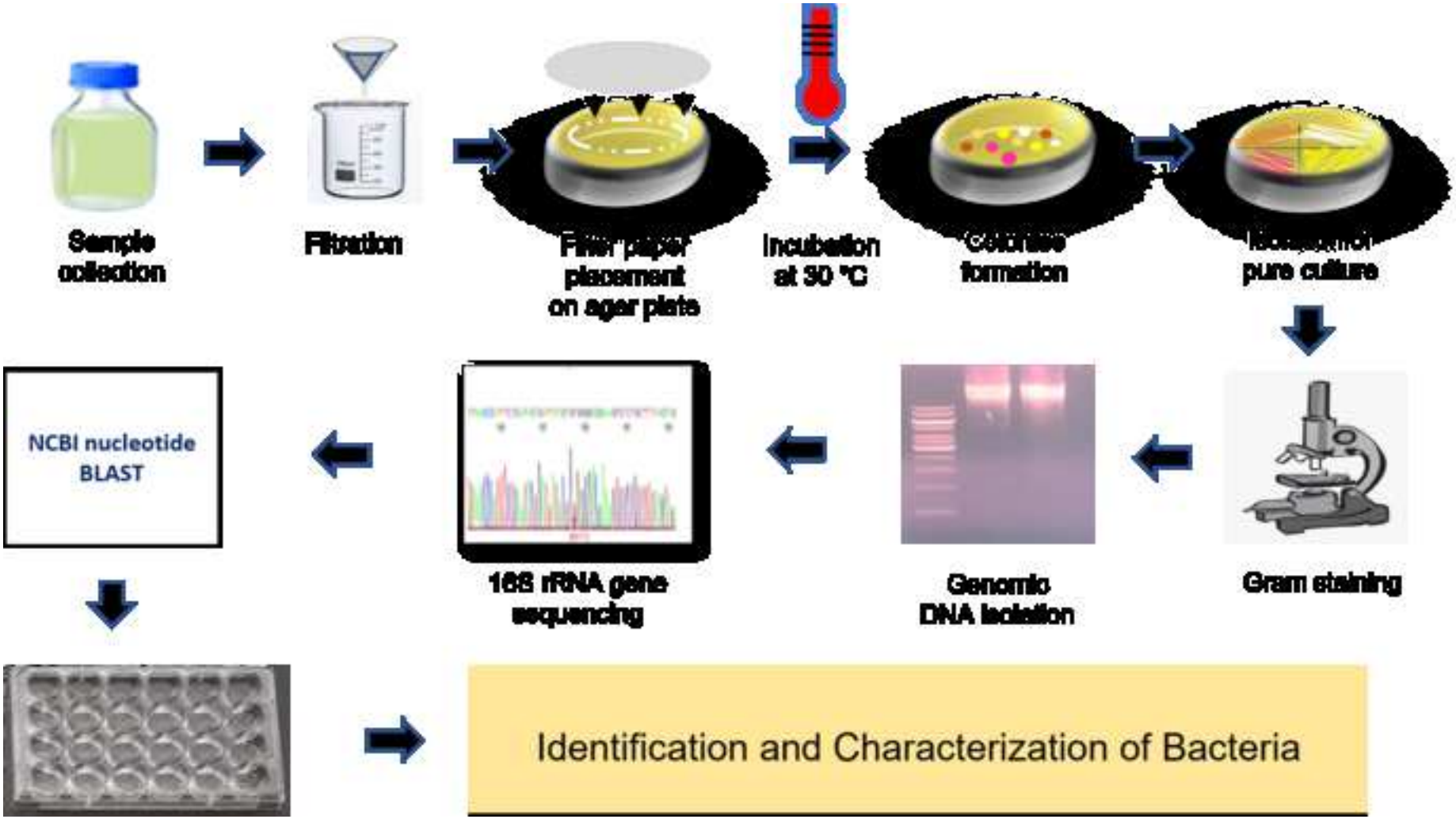
Figure



Figure

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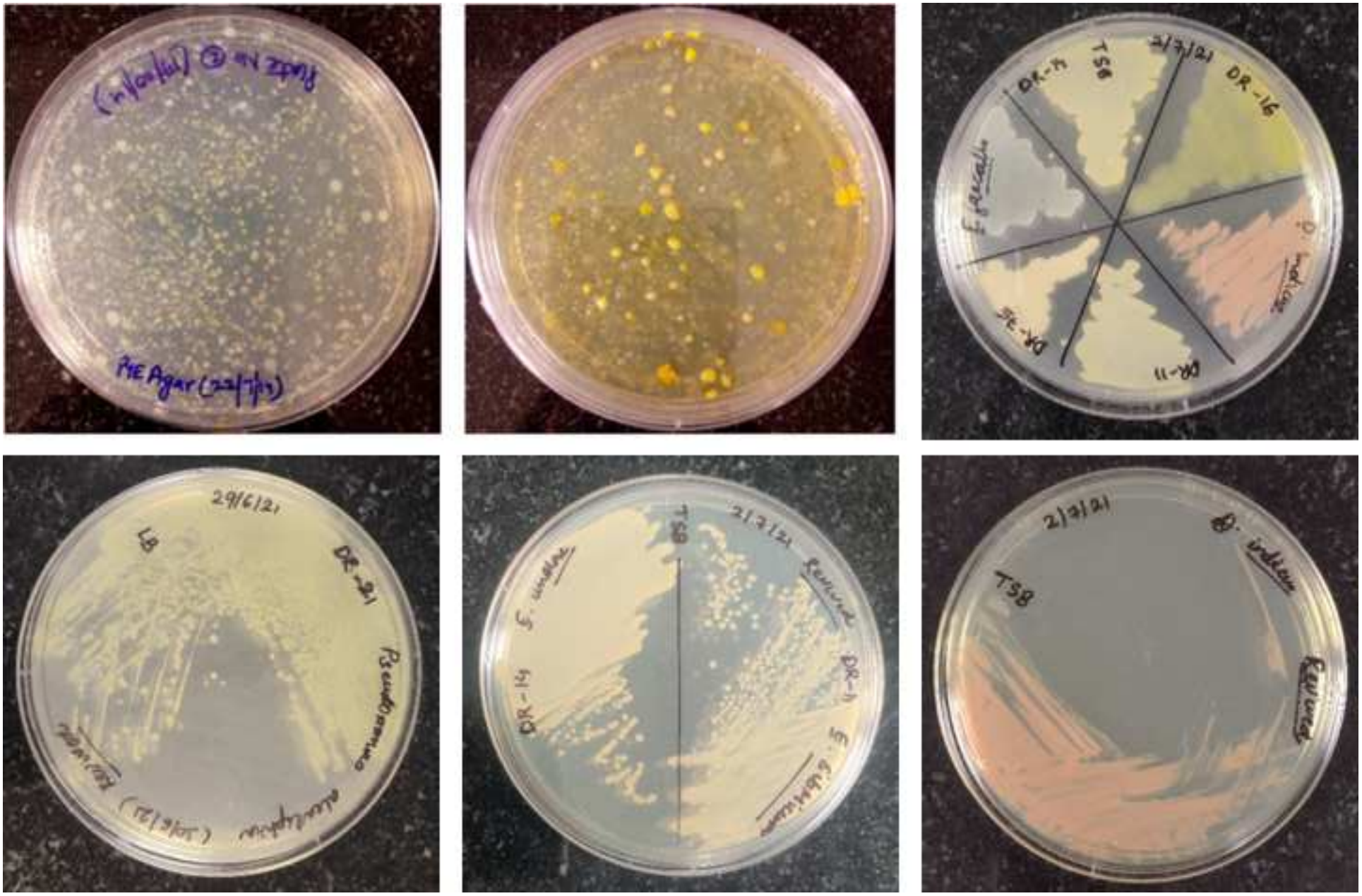

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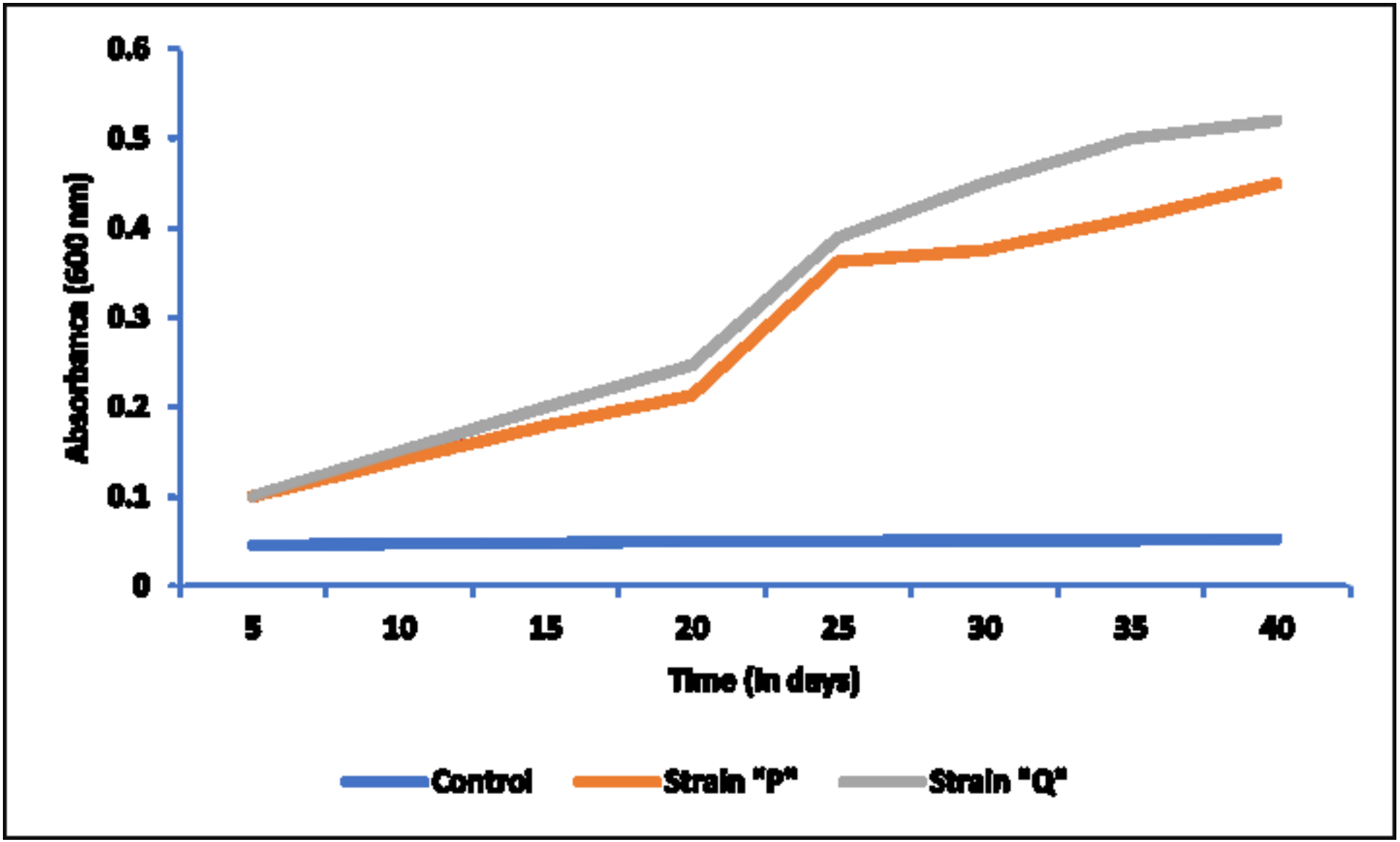

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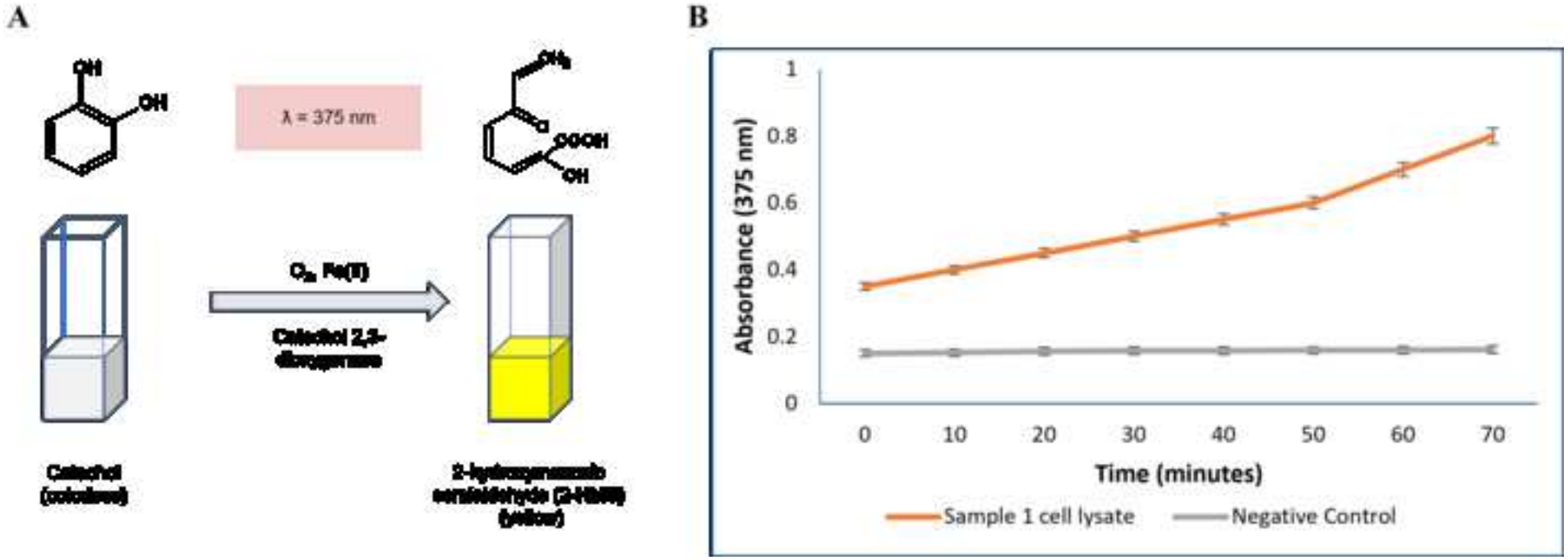

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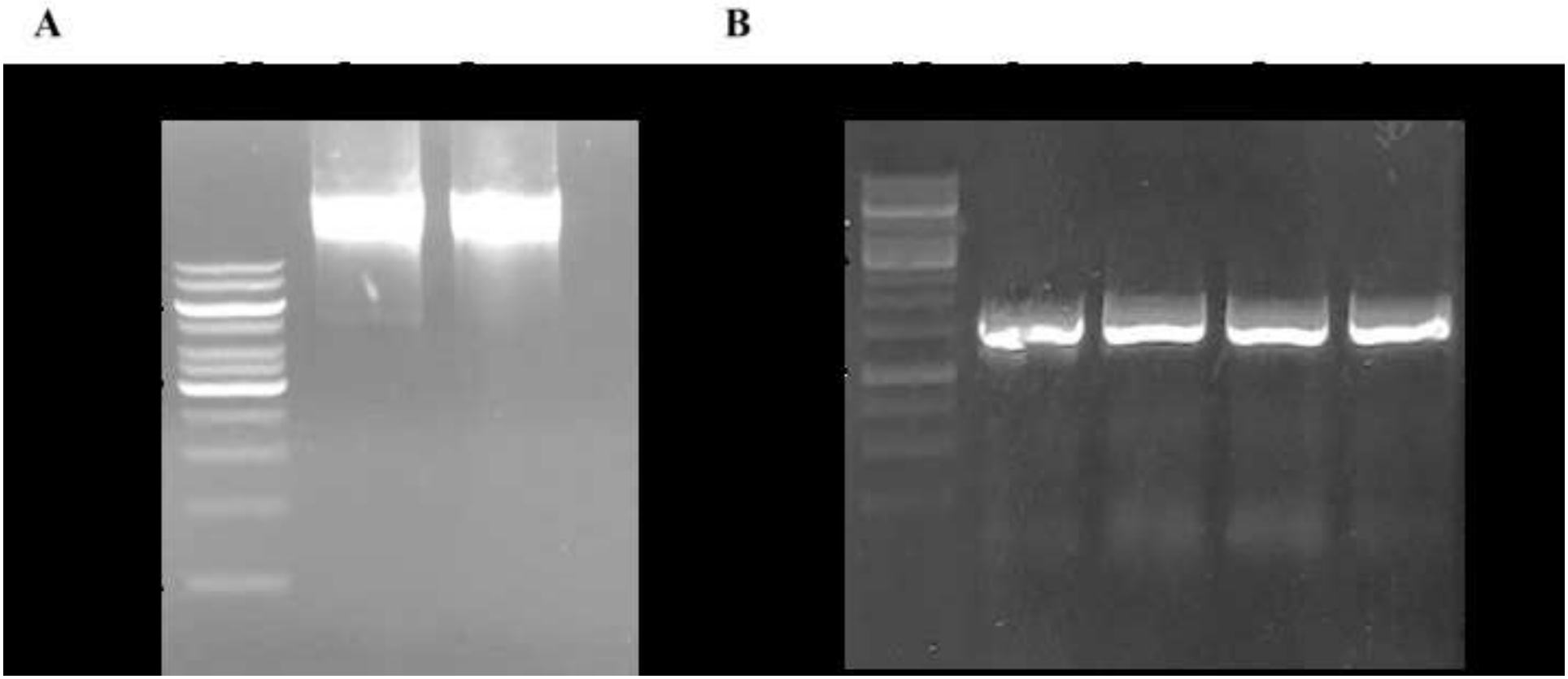

Figure

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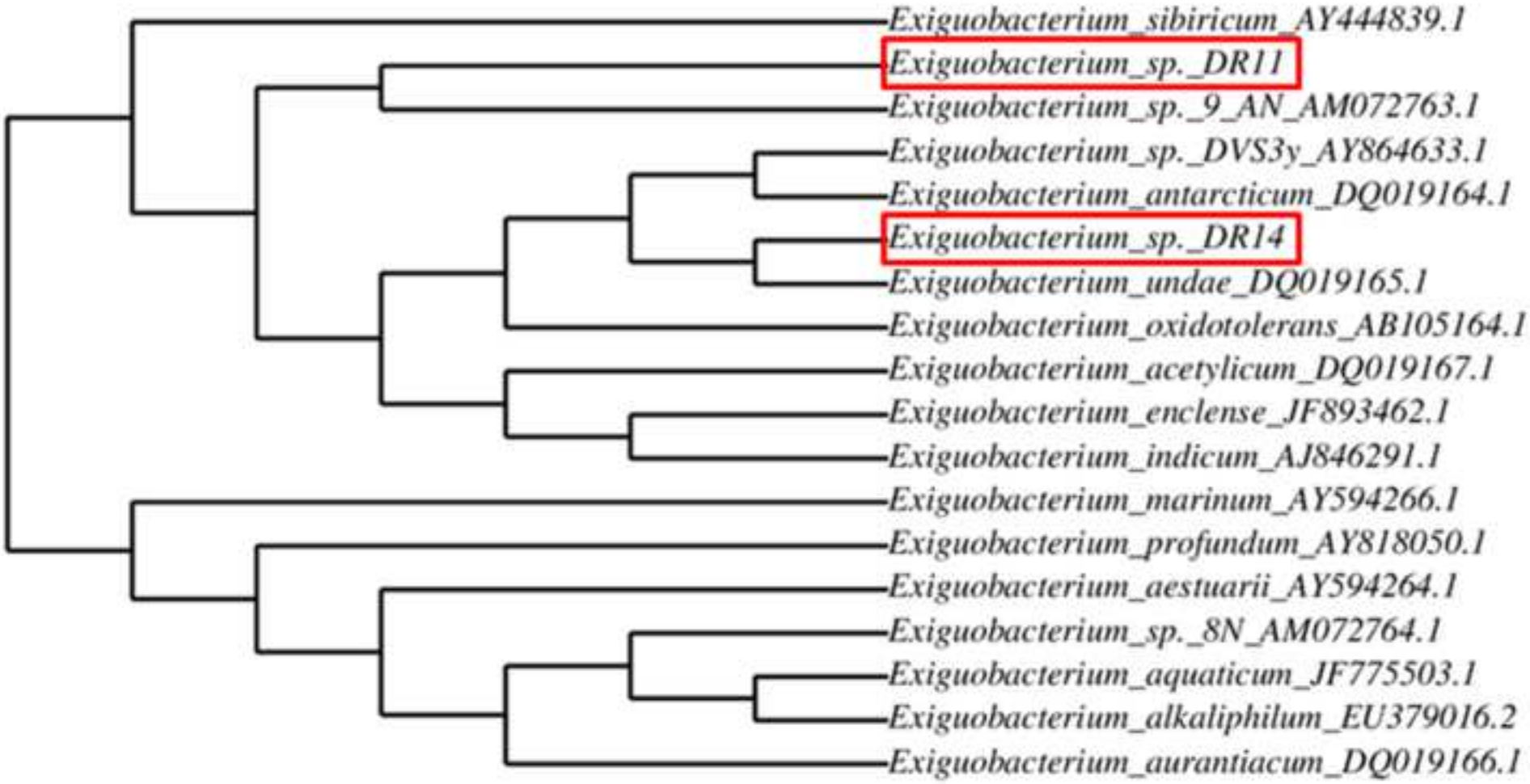

Peptone Yeast Extract (PYE)

Peptone
Yeast extract
1M $MgSO_4$
1M $CaCl_2$
Distilled water

Sterilize by autoclaving at 121°C and 15 PSI for 15 min.

Reasoner's 2A (R2A)

Casein acid hydrolysate
Yeast extract
Protease peptone
Dextrose
Starch, soluble
K_2HPO_4
Distilled water

Sterilize by autoclaving at 121°C and 15 PSI for 15 min.

M2G

10X M2 salts (1L) -

Na_2HPO_4
KH_2PO_4
NH_4Cl

Autoclave 10X M2 salts at 121 °C and 15 PSI for 15 min.

10X M2 salts
50mM $MgCl_2$
30% glucose (w/v)
1 mM $FeSO_4$ in 0.8 mM EDTA, pH 6.8
50 mM $CaCl_2$
Distilled water

Filter sterilize.

Lysogeny Broth (LB)

Casein enzymic hydrolysate
Yeast extract
NaCl
Distilled water

Sterilize by autoclaving at 121°C and 15 PSI for 15 min.

Nutrient Broth (NB)

Peptone
Yeast extract
NaCl
Glucose
Distilled water

Sterilize by autoclaving at 121°C and 15 PSI for 15 min.

Tryptic Soy Broth (TSB)

Pancreatic digest of casein
Papaic digest of soyabean meal
NaCl
K_2HPO_4
Dextrose
Distilled water

Sterilize by autoclaving at 121°C and 15 PSI for 15 min.

M63

NH_4Cl
KH_2PO_4
$FeSO_4.7H_2O$
20% glycerol
1M $MgSO_4$
Distilled water

M9 minimal media

5X M9 salts

$Na_2HPO_4.7H_20$
KH_2PO_4
NH_4Cl
NaCl
Distilled water

Autoclave 5X M9 salts at 121°C and 15 PSI for 15 min.

1X M9 media

5X M9 salts
20% glucose
1M $MgSO_4$
1M $CaCl_2$
Autoclaved water

NOTE – For solid media preparation, use 1.5% Bacto Agar (15 g/L)

2g
1g
1 ml
1 ml
Up to 1000 ml

0.5 g
0.5 g
0.5 g
0.5g
0.5 g
0.5 g
Up to 1000 ml

17.4 g
10.6 g
5.0 g

100 ml
10 ml
10 ml
10 ml
10 ml
Up to 1000 ml

10 g
5 g
10 g
Up to 1000 ml

15 g
3 g
6 g
1 g
Up to 1000 ml

17.0 g
3 g
5 g
2.5 g
2.5 g
Up to 1000 ml

2g
13.6 g
0.5 mg
10 ml
1 ml
Up to 1000 ml

12.8 g
3 g
1 g
0.5 g
Up to 200 ml

20 ml
2 ml
200 µl
10 µl
Up to 100 ml

Dear Dr. Mittal,

We thank you for handling the review of our manuscript entitled “Isolation, propagation and identification of bacterial species with hydrocarbon metabolizing properties from aquatic habitats.” We also thank the reviewers for their thorough consideration of our manuscript and for their helpful suggestions. We have considered all comments and suggestions, and have used them to enhance our manuscript. Please find our point-by-point responses to the reviewer’s comments in the letter below. The changes have been highlighted in the revised manuscript.

Sincerely,

Richa Priyadarshini

Response to the Editor

Editorial Changes

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have revised and proofread the manuscript to ensure that there are no spelling or grammar mistakes to the best of our ability.

2. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: QIAGEN, Millipore, Whatman, etc.

We thank the editor for pointing this out and all reference to commercial products have removed the revised manuscript.

3. Please provide a Summary clearly describing the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

Revised as suggested. We have provided a small summary describing the protocol at the beginning of each protocol in the revised manuscript.

4. Please provide an abstract between 150-300 word clearly stating the goal of the protocol.

Modified as suggested

5. Please provide references for line 53-62.

Reference has been provided

6. Please revise the Introduction to include the following as well along with citation(s):

a) A description of the context of the technique in the wider body of literature
b) Information to help readers to determine whether the method is appropriate for their application

Modified as suggested

7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary.

While we have taken editors suggestion, but have used our previous numbering as it is making the protocols more confusing. If necessary, we can make the required changes.

8. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

Modified as suggested

9. Protocol section only contains numbered action steps in imperative tense which describes how to perform the technique being presented. In this regard, please consider providing reaction set-ups and solution compositions as Tables in separate .xls or .xlsx files uploaded to your Editorial Manager account. These tables can then be referenced in the protocol text.

We have provided reference to the tables in the manuscript and have only one reaction set up which is mentioned in the text.

10. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

New references have been provided for the protocols used

11. Line 84: What was the volume of the water sample collected? How were pH and temperature measured? Please mention.

Volume of water and temperature and pH conditions are mentioned in line 103 in revised manuscript.

12. Line 85: What is the final volume of each serial dilution? Please specify.

Final volume of each serial dilution is added. Please see line 114-117.

13. Line 102: How were the glycerol stocks made? Please specify.

Steps of making glycerol stocks are mentioned in line 129-131 in revised manuscript

14. Line 110: How was the stationary phase determined? Please mention.

Using optical density method

15. Line 118: Is the final volume 40 mL for flask B as well? Please specify.

The final volume in flask B is 40 mL. Please refer to lines 170-173.

16. Line 143: Was 100% ethanol used? Please specify.

Yes 100% ethanol is used

17. Line 164, 169, 171: Were these steps carried out in a water bath? Please specify.

All the steps were carried out in dry bath at different temperatures as mentioned in revised manuscript.

18. Line 212: How was sequencing performed? Please provide all steps needed to perform this. Alternatively, provide citation(s) which describe how this could be done.

Sanger sequencing was performed and citations for the protocol have been provided

19. Line 219: Which 2 enzymes are being referred here. Above sentences only mention catechol 2, 3-dioxygenase.

Other enzyme is catechol 1, 2-dioxygenase. It is mentioned in line 335-336

20. Line 222: What were the cell growth conditions? Please mention.

21. Please include a single line space between all the steps. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Protocol steps for video have been highlighted in green.

22. Please move all the Figure Legends to the end of the Representative Results in the manuscript text.

Modified as suggested

23. Please discuss all result figures in representative result section.

Modified as suggested

24. As we are a methods journal, please revise the Discussion to also cover the following in detail with citations. The discussion currently has no citations.
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique

We have revised the discussion as suggested

25. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

As per RSC advances copyright guidelines no permission is required to reproduce figure as I am also the original author of the said figure.

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Response to the reviewers:

Reviewer #1:
Manuscript Summary:
This paper describes basic methods involved in the isolation of hydrocarbon-degrading bacteria and their characterization. It may be of interest to undergraduate students.

Major Concerns:
None. I am not familiar with JoVE but this presentation is at a basic level.

Minor Concerns:
Line 85 - Milli-Q water?

Replaced with autoclaved distilled water.

Line 88 - Diameter of filters

Added in revised manuscript in lines 105-108.

Line 90 - Why not perform initial enrichments on agar plates containing hydrocarbon of interest?

While enrichments on agar plates can be done but adjusting the concentration of hydrocarbons is difficult as high concentration of hydrocarbons is toxic to some bacteria.

Line 92. Not sure what the purpose of step 5 is visa vi step 4.

We thank the reviewer for pointing this and has been corrected in the revised manuscript.

Reviewer #2:
Manuscript Summary:
This research article looks fine. However, it is written in a report format and not a manuscript format.

Major Concerns:
1) Significant results should be mentioned in the abstract.

As per the format of JOVE we have only presented representative results in the manuscript

2) Protocols written in thesis format should be modified in concise format of a manuscript.

We thank the reviewer for the suggestions and have have tried to reduce the length of the revised manuscript.

3) Grammar errors should be checked throughout the manuscript.

Revised as suggested

4) Tables on medium composition are unnecessary.

We have considered the reviewer's suggestion and removed the media composition tables in the revised manuscript.

5) Figures 1 - 3 are unclear. Figure 5 seems to be taken from another publication. The accession number should be provided.

Modified as suggested

6) Table of materials is not necessary.

Table of materials is required by the JOVE format

7) Overall, it looks like a thesis and not a manuscript.

We have tried to shorten the length of the manuscript and removed extra details.

Reviewer #3:
Manuscript Summary:
This manuscript describes a protocol for the isolation and identification of bacterial species from aquatic habitats with the capacity to degrade hydrocarbons, very persistent pollutants in the environment. The subject is very interesting and of great environmental relevance due to the potential to use the isolated microorganisms to biodegrade and bioremediate contaminated areas exposed to episodes of hydrocarbon contamination (e.g.: petroleum accidental spills).

Major Concerns: Title:
1) What do you mean with "propagation"? Wouldn't it be more correct "culture"? 2) In the title, it is mentioned "aquatic habitats". However, steps for bacteria collection from "soil samples" are also described.

We thank the reviewer for pointing this out. Collection of soil samples has been removed from revised manuscript.

I have some doubts and questions about the methodology used: 1) What is the level of hydrocarbon contamination of the aquatic habitats used to take the water samples? 2) In my opinion, bacteria capable of metabolizing hydrocarbons should have selectively grown in contaminated areas due to a process of selection and adaptation. This would greatly increase the probability of success in subsequently identifying bacteria with potential metabolizing properties.

Many bacteria can pass through 0.22-µm membrane filters which can reduce the efficiency of the methodology by losing possible microorganisms with relevant hydrocarbon metabolizing capacity. This could been overcome by pelleting the cells from the water sample by centrifugation.

While we agree with the reviewer but aquatic habitats usually not high microbial load and centrifugation of small volumes does not lead to substantial cell pellet

Screening of hydrocarbon degradation potential of bacteria experiment in 24-well plates is described (lines 255-260) an illustrated (Fig. 3B). However, the methodological procedure is not described in the Protocol Section.

This section has now been removed to shorten the length of the manuscript.

Results of Fig. 3A are not described in the text (lines 255-260).

Figure 3A has now been descried in the modified manuscript in lines 390-392

Minor Concerns:

The sequencing methodology used should be indicated (line 212, Pag. 8).

Fig. 2 (line 252) is named before Fig. 1 (line 254) in the text.

Captions´ to the figures in the text are named differently along de MS: "Fig. 2" (line 252), "Fig 1" (line 254), Fig.4" (lines 266 and 267). Figure 3 is not cited in the text.

Modified as suggested

Figures resolution should be highly improved. For example, it is not possible to understand Fig. 4 since words are unreadable.

We have now modified figures improved the resolution in the revised manuscript

Please, revise the units' format for uniformity: e.g.: grams in the table of the "M9 minimal media" (line 371, "12.8 g") compared with that of "LCFBM" media (line 380, "0.7 g); concentration in table of line 356: "1 M" vs "10mM".

For greater consistency, use the same criteria for naming buffers: e.g.: "phosphate buffer (0.5 M and pH 7.5)" (line 226) versus "phosphate buffer, pH 7.5 (50 mM)" (line 242).

"CaCl2" should read "CaCl2" in the tables of line 346 and 356.

Modified as suggested

Lines 270-272: a reference could be included.

References have been revised

The Discussion Section should be improved by adding references to support what is mentioned in the text, i.e.: in paragraphs 1 and 2, and first sentence of the third paragraph (lines 279-298).
Lines 307-308: …by keeping over different "media plates and plates"?

Discussion has been revised and references have been added

References Section. Please, revise this section for uniformity. For example,
1) Sometimes all authors are named (e.g.: references 5-9), other times only the first author (e.g.: References 1-3).
2) Sometimes the name of the journals starts in capitals (e.g.: reference 9 and 11), other times no (e.g.: 5, 7 and 8).
3) The complete name of the Journals is written but in reference 4.
4) References 9 and 11: The name of the microorganisms, genera and specie, should be in italic.
A list with the "Name of Material/Equipment" at the end of the MS. For greater detail and precision, the reference of the products (code) could be included.

References have been revised as per JOVE format