Psychrobacter: Antarctic Cooperation on Bioremediation of Oil-Contaminated Waters

By

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Contents

Statement by Scientific Advisor

I assisted in this project as follows:

- Facilitated use of University of Chile Biochemistry Lab facilities.
- Provided orientation on lab methodology.
- Reviewed written report, poster, and demonstration stand setup.

Executive Summary

Around the world, concern is growing about hydrocarbon pollution. In Antarctica, research and military bases routinely use, store and distribute hydrocarbons. The result are over twelve major oil spills in the past six years alone.

This issue is an attractive focus for research, as the search for decontamination options could well provide solutions for both Antarctica and other contaminated zones.

This experimental research was intended to search and select Antarctic psychrophiles capable of metabolizing phenanthrene, a polycyclic aromatic hydrocarbon (PAH), as a sole carbon source. The intent was to use these psychrophiles in bioremediation of contaminated soil and water under low-temperature conditions.

The working hypothesis was that Antarctic psychrophiles capable of degrading phenanthrene can be used in bioremediation of contaminated soil and water.

The initial stage included an individual review of five Antarctic strains isolated in the University of Chile Microbiology Lab. One was selected due to significant growth in the presence of phenanthrene as a sole carbon source.

To expand the range of candidate bacterial isolates, phenanthrene growth trials were conducted in cultures inoculated with bacterial consortia (ten strains each). This procedure resulted in the selection and isolation of eleven Antarctic strains with degradation potential.

One particular strain exhibited a strong capacity for using phenanthrene as a carbon source and could be used in bioremediation processes.

The hypothesis and the stated objectives were met in the allotted time, creating new opportunities for further study and analysis of candidate strains.

Introduction

Human activity in Antarctica has increased significantly in the past century. More than thirty nations that conduct Antarctic research bring with them associated logistics (Brander J. M., 2001). Each year Chile sends an expedition to gather new data about Antarctica. Chile's 47th Scientific Antarctic Expedition (ECA 47) reported finding some 230 new microorganisms with significant biotechnology potential. (Blamey J. 2011, Nature).

The downside of a rising human presence are increased chances of negative environmental impacts.

While Antarctica is the world's most pristine region, contamination near permanent bases, specifically around fuel storage tanks, is on the rise. At Chile's O'Higgins Base, total polycyclic aromatic and aliphatic hydrocarbons in soil range from 2.63 to 49.43 g/kg. A recent study warns that contamination may linger for decades unless an emergency cleanup plan is implemented. (Astorga M., et al, 2011).

The above readings were taken in 2006 in an area noted for constant fuel flows and sketchy spill records. Actual hydrocarbon pollution is thought to be much greater, as readings do not exist for Antarctic bases and areas hit by larger oil spills, such as the 1,000 liters a Russian base discharged into Fildes Bay in 2005. (Infobae, 2005).

The Chilean Antarctic Institute (INACH) notes that annual diesel fuel consumption in the area of Chilean influence, which includes 19 permanent bases, stands at 5,013,500 liters shipped in by air and sea. Twelve large oil spills have occurred in this area alone in the six-year period ending in 2008. (Vidal P., 2008).

Addressing these issues requires assessing the viability of a cleanup strategy in Antarctica and other low-temperature zones. Considerations include installation, energy consumption and environmental factors, not to mention that interventions in Antarctica and other areas of vast natural wealth are restricted under international treaties.

In addition, financial and environmental costs render the use of special machinery or chemicals on soil and water impracticable.

As such, the solution may lie in bioremediation, a technique that uses native microorganisms to break down or degrade hazardous compounds into substances that are less toxic or even harmless to the environment and human health. (Arroyo M, et al., N.A.).

As the Madrid Protocol bans introduction of exogenous microorganisms into the Antarctic, bioremediation must of necessity rely on native species.

Identifying native microorganisms capable of degrading hydrocarbons under environmental conditions as extreme as those prevailing in Antarctica would significantly reduce damages and the time required to eliminate pollutants in ecosystems where low temperatures limit bacterial growth. (W. Mac Cormack, 2011).

To address this pressing issue, our research focused on identifying Antarctic psychrophiles capable of using organic pollutants (such as polycyclic aromatic hydrocarbons) as a carbon source for growth.

Available bacteria –microscopic unicellular organisms- included 55 strains isolated from soil, water, and ice samples collected in the South Shetland Islands during ECA 47 by a research group led by Chilean scientist José Manuel Pérez-Donoso.

These bacteria were added to a liquid medium containing phenanthrene ($C_{14}H_{10}$), a polycyclic aromatic hydrocarbon prevailing in samples collected near O'Higgins Base. Phenanthrene is considered highly toxic by the U.S. Environmental Protection Agency (EPA). While a highly complex persistent organic pollutant, phenanthrene may be turned by bacteria into harmless substances.

Our experimental research intends to be a first step toward the use of bioremediation in addressing hydrocarbon pollution in extremely low-temperature zones worldwide. Bacteria exhibiting significant PAH degradation potential should prove a valuable contribution to the design of appropriate bioremediation strategies.

Formulating the Problem

Question

Are psychrotolerant Antarctic bacteria capable of using phenanthrene as a sole carbon source?

Can psychrotolerant Antarctic microorganisms be used in bioremediation of hydrocarbon-polluted waters?

Hypothesis

Psychrotolerant Antarctic bacteria are indeed capable of degrading phenanthrene and can be used in bioremediation of hydrocarbon-polluted waters in extreme low-temperature zones.

General Objective

Identify candidate psychrotolerant bacteria capable of metabolizing phenanthrene in a liquid medium.

Specific Objectives

1. Identify Antarctic isolates capable of using phenanthrene as a sole carbon source.

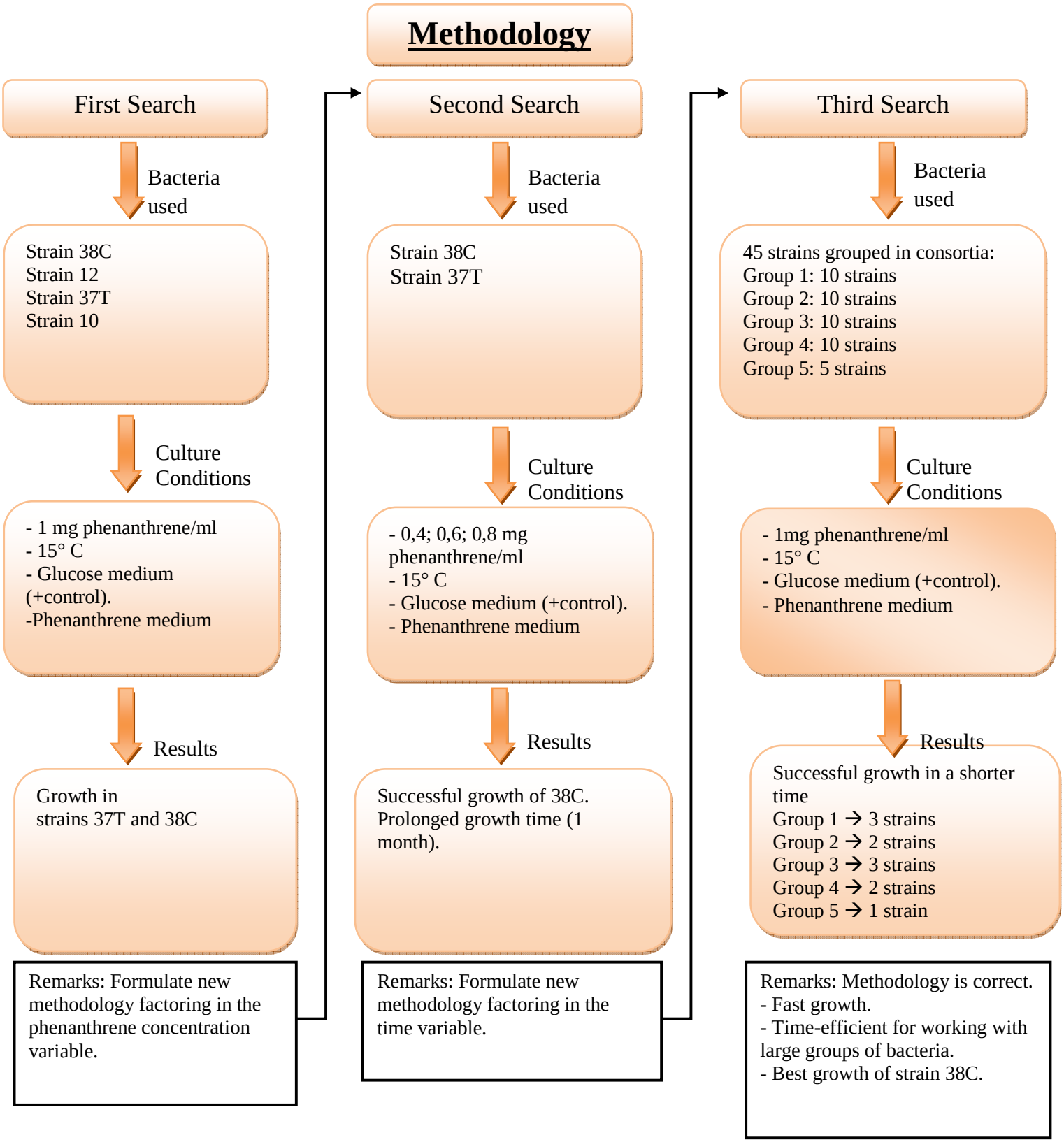
2. Identify and characterize bacteria exhibiting suitable growth and phenanthrene degradation properties.

3. Determine phenanthrene degradation potential at various pollutant concentrations.

Activity Schedule

Activity	July 2012				August 2012				September 2012				October 2012				March 2013				April 2013			
Week	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	
Information search																								
Topic selection																								
Formulate hypothesis and objectives																								
Lab work																								
Reformulate objectives																								
Write report																								
Collect results																								
Review report for submission to national competition																								
Proof and edit final report																								

Materials and Research Methodology



Area of Study

This experimental research was conducted at the Microbiology and Bionanotechnology Research Group of the Biochemistry Laboratory, Faculty of Chemistry and Pharmacy of the University of Chile.

The study used strains of bacteria recently isolated from ice, water, and soil samples collected by the 47th Scientific Antarctic Expedition (ECA 47).

Materials

Micropipettes (P10, P20, P200, P1000), beaker, Petri dishes, inoculation loop, Falcon tubes (15 & 50 ml), Eppendorf tubes (1.6 ml), 15° C incubator with agitator, vortex, laminar flow hood, pH meter, spectrophotometer UV (optical density reader), DNA electrophoresis chamber, power supply.

I. Search for Antarctic Bacteria Capable of Degrading Phenanthrene into a Sole Carbon Source

- Preparation of Culture Media

M9 glucose (200 ml) medium	Minimal aqueous medium for phenanthrene (200 ml)
- M9 salts -->40 ml - $MgSO_4$ (1M) --> 400 µl - Glucose 20% --> 20 ml - CaCl (1M) --> 20 µl - H_2O 138,58 µl	- NaCl --> 5 g - K_2HPO_4--> 1 g - $H_2(NH_4)PO_4$ --> 1g - $(NH_4)_2 SO_4$ --> 1 gr - KNO_3 --> 3g

- An M9 glucose medium was prepared in a beaker and measured with a pH meter until attaining pH 7.
- The solution was topped up with sterile distilled water to 150 ml.
- The solution was then passed through a sterile 0.2 µm filter under a laminar flow hood.
- The minimal culture medium for bacteria was prepared concurrently.
- Samples of phenanthrene for use (1 mg/ml) in the minimal bacterial medium were irradiated and sterilized with UV light (10 min).

- Seeding and Growth of Antarctic Bacteria

- "Mother" Petri dishes in an LB medium were labelled for seeding of Antarctic psychrophiles strains.
- An inoculation loop was used to seed bacteria on Petri dishes using the streak plate technique.
- Samples were incubated at 15º C (optimal temperature).

- First Search

- Four strains with a prior record of resistance to heavy metals (10, 12, 37T and 38C) were selected.
- Strains were seeded in glass and Falcon tubes, complemented with phenanthrene as sole carbon source (5 mg), and topped up to 5 ml with minimal phenanthrene medium.
- Bacteria were then inoculated using an inoculation loop.
- The same procedure was used to seed the same four strains in test tubes containing an M9 aqueous medium and a negative control with no bacteria added.
- Samples were incubated at 15º C with agitation.
- After 10 days samples were taken from tubes with incubated strains. Using a loop, a small amount was deposited in Petri dishes with LB medium to verify the presence of bacteria.
- Low strain growth (excepting 38C and 37T) required trying lower pollutant concentrations to rule out phenanthrene volume as growth inhibitor.

- Second Search

- Strains 37T, 38C and *E. coli* (negative control) were seeded in glass test tubes with various phenanthrene concentrations (0.4 mg; 0.6 mg; 0.8 mg) in 5 ml.
- One inoculum of each strain was diluted in 200 µl H_2Od. 20 µl were used to inoculate tubes containing the culture medium.
- To quantify colonies added, inocula of each strain were diluted in 1000 µl H_2Od. Optical density at 600 nm was evaluated in a spectrophotometer in order to obtain a more exact reading of bacterial volume in the culture medium.
- Once samples were inoculated in tubes containing phenanthrene, the process was repeated in M9 glucose media.
- This was followed by incubation at 15° C.
- Growth time was overly long (1 month), requiring a faster methodology encompassing a wider range of bacteria. **Strain 38C did exhibit significant growth under specific tests.***

- Third Search

- Forty-five new strains of previously isolated Antarctic psychrophiles were chosen at random.
- Strains were segmented into five groups. Four contained ten strains, one five strains, and one was a negative control.
- Strain groups were seeded in Falcon tubes with minimal phenanthrene medium, supplemented by 1 mg/ml hydrocarbon, topped up to 5 ml. The process was repeated in M9 glucose media.

- A 10 µl inoculum of each strain was added to an Eppendorf tube with 200 µl H_2Od for each group.
- The solution was taken to a vortex in order to homogenize the inoculum.
- 10 µl from each prepared inocula were added to Falcon tubes containing the various groups.

- After 12 days of incubation, we obtained **11 strains** capable of metabolizing phenanthrene and use it as a sole carbon source. **These strains were subjected to specific tests.***

II. Specific Tests with Selected Bacteria

* A number of characterization experiments were conducted with strains selected for their ability to use phenanthrene as a carbon source.

- Extraction of Plasmid DNA

- A sample of bacteria was deposited in a 40 µl lysis buffer.
- Tubes were incubated at 50° C for 5 minutes and then in ice for another 5 minutes.
- These were then centrifuged at 12,000 rpm for 10 minutes.
- 7 µl of the supernatant were added to agarose gel.
- Positive control for plasmid analysis was strain *E. coli gshA* (it has the pBAD-TOPO Invitrogen plasmid).*
- To prepare agarose gel for DNA electrophoresis, we used 50 ml 1x TAE buffer and 0.5 mg agarose (1%). The solution was heated to dissolve the agarose.
- Comb was withdrawn from the gel.
- Gel was run at 100 V for one hour and a half.

- Microdrop Count of Strain 38c Colonies

- To quantify bacterial growth in the presence of phenanthrene, we determined colony-forming units (CFUs) of strain 38C grown in the presence of phenanthrene at various times relative to the number of CFUs added at the onset (time zero).
- Eppendorf tubes with 90 µl of sterile H_2Od were used to do eight serial dilutions, adding a 10 µl sample of strain 38C from each Falcon tube to the first dilution, then transferring this to the next dilution until covering all.
- This procedure was also performed at days 3 and 6 of incubation at 15° C.

- Microdrop Count of Colonies for Groups

- A microdrop count of colonies was performed at three different times in Petri dishes containing an LB medium, keeping in mind that a minimal amount of diluted inoculum was added at the onset.

- Eppendorf tubes with 90 µl of sterile H2Od were used to perform eight dilutions, adding a 10 µl sample from each group to the first dilution.
- This procedure was also performed at days 3 and 7 of incubation at 15° C.

- Isolation of Degrading Strains Grown in Phenanthrene-Exposed Groups
  Strains selected for their ability to grow in the presence of phenanthrene in each group were isolated for subsequent analysis in Petri dishes containing an LB medium.

- Identification of Degrading Strains
  Degrading strains were identified by sequencing PCR-amplified 16S ribosomal DNA (Macrogen Korea) using adequate primers.
  Strain 38C was found to belong to the genus *Psychrobacter sp.* and is so named hereafter.

Results Obtained

Chart 1: *Psychrobacter sp.* (38C) Growth in Various Media (1-Month Incubation)

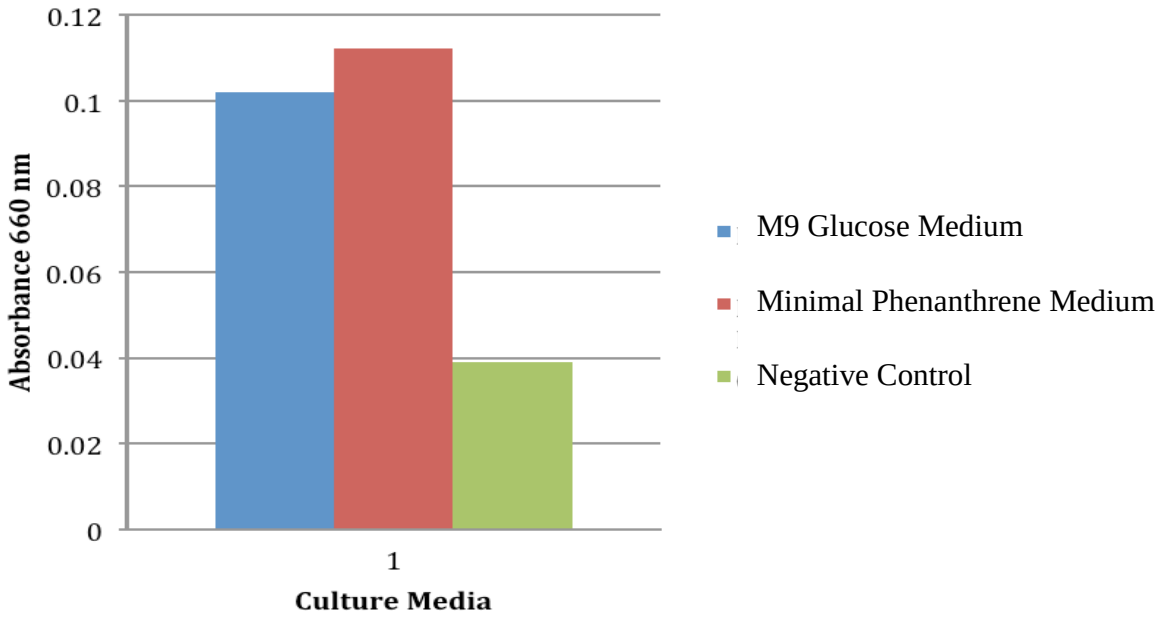


Chart 2: *Psychrobacter sp.* (38C) Growth at Various Phenanthrene Concentrations

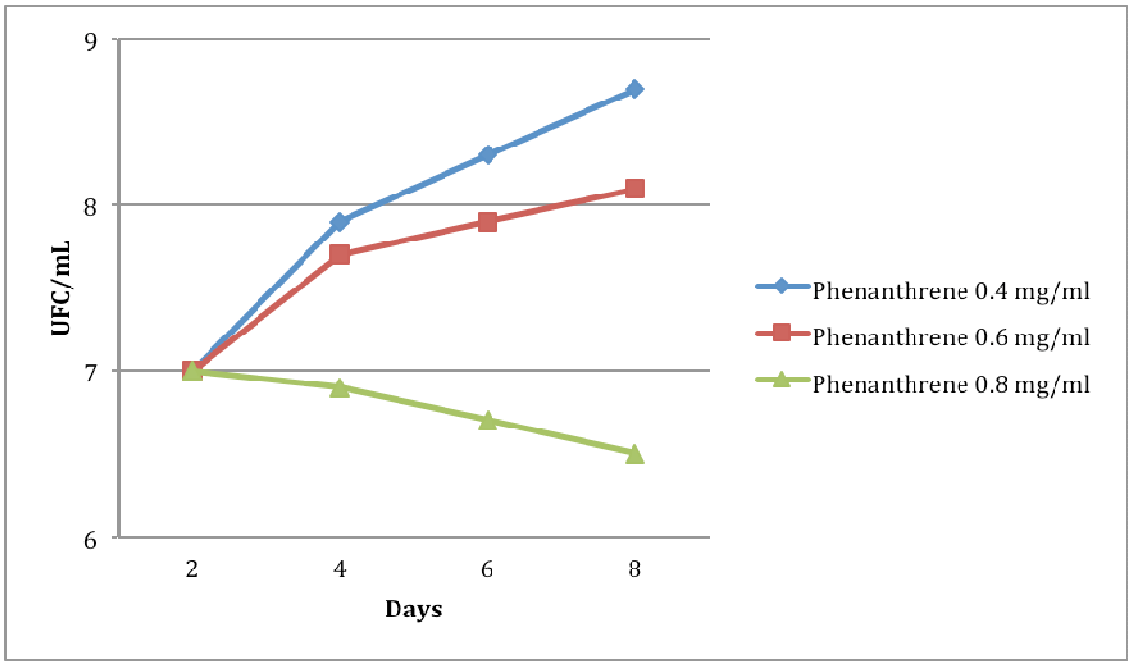


Chart 3: Metabolization of Bacterial Consortia in a Phenanthrene Medium

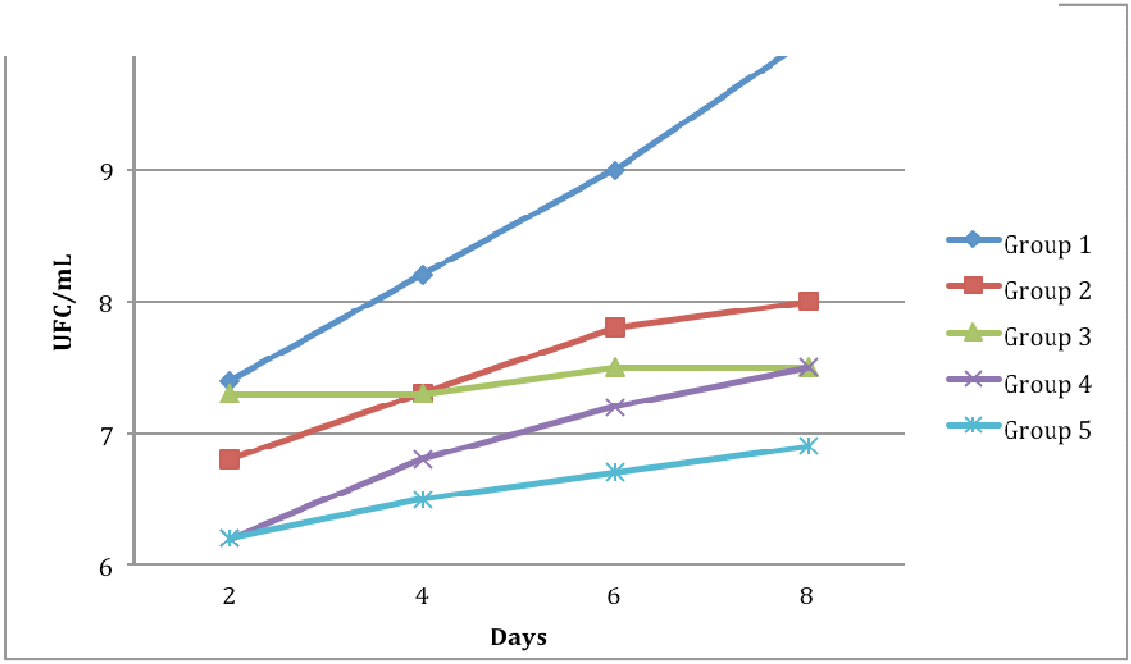


Image 1: *Psychrobacter sp.* (38C) Turbidity in Phenanthrene and Glucose Media

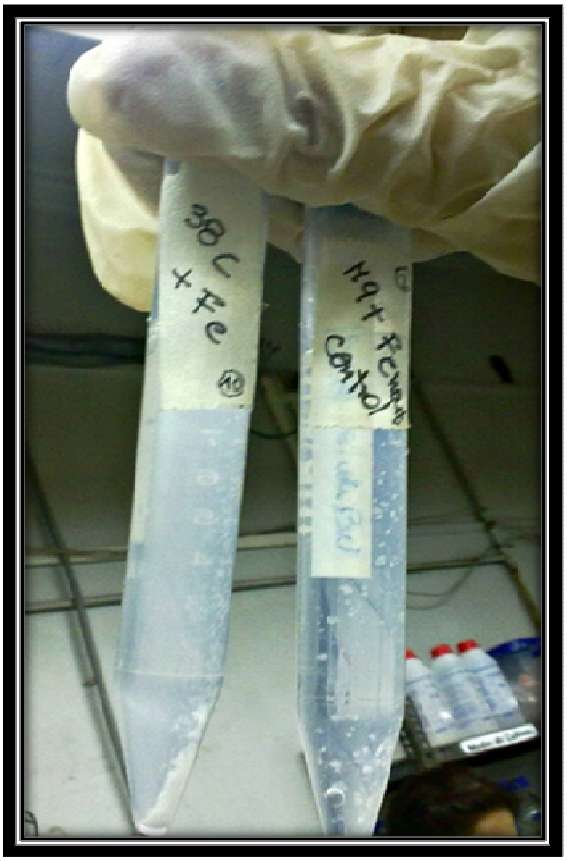


Image 2: View of Plasmids

Lanes
C. Positive control
1. Strain Group 1
2. Strain Group 1.2
3. Strain Group 1.3
4. Strain Group 2
5. Strain Group 2.1
6. Strain Group 3
7. Strain Group 3.1
8. Strain Group 3.2
9. Strain Group 4
10. Strain Group 4.1
11. Strain Group 5
12. Strain *Psychrobacter sp.* (38C)

Discussion

Chart 1: *Psychrobacter sp.* (38C) Growth in Various Media (1-Month Incubation)

Analysis of Chart 1 is based on a wavelength of 600 nm, as sample turbidity can be determined.
This reveals similar growth for strain 38C in a phenanthrene medium (blue) and in a glucose medium (red), as well as absence of growth in the control (green).
The culture in the glucose medium was expected to grow efficiently. Surprisingly, strain 38C exhibited similar growth in a phenanthrene medium.
The experiment clearly showed that strain 38C is able to metabolize phenanthrene as a sole carbon source. As the measurement was taken after 1-month incubation, this also shows that it does so efficiently over an extended period of time.

Chart 2: *Psychrobacter sp.* (38C) Growth at Various Phenanthrene Concentrations

To test if strain 38C is capable of degrading phenanthrene, three different hydrocarbon concentrations were prepared in 5 ml of medium.
The chart shows unexpected behavior at low phenanthrene concentrations relative to high concentrations, where colonies increased considerably over time.
Growth at low concentrations can be attributed to low energy and carbon sources for bacterial growth, and possibly to a medium leak during shaker incubation of samples.

Chart 3: Metabolization of Bacterial Consortia in a Phenanthrene Medium

***Considering that degrading strain growth was evident over an extended period of time and that data encompass bacterial growth over nine days, the Chart shows the following**:

- Group 1: Sudden increase of colony-forming units over time. Given that analysis was conducted over a nine-day period, **growth was successful**. Complementing numerical data, images of the container tube for the group show significant turbidity relative to the control tube. The sudden increase between days four and six may be due to an initial capacity for assimilating hydrocarbon, then use it as a source of growth energy.

- Group 2: Given that this group exhibited the least number of colonies through the first few days of incubation, growth results are good, with colonies per ml of sample **increasing progressively over time**.

- Group 4: Colonies increased gradually over time. **This shows that strains did metabolize hydrocarbon in order to grow and may continue to increase if exposed to pollution for longer periods of time.**

- Groups 3 and 5: **It is probable that bacteria remain in a state of latency** during exposure to phenanthrene, which may account for minimal grow in these groups. Significantly, only one strain in each group survived after a week's incubation.

Image 1: *Psychrobacter sp.* (38C) Turbidity in Phenanthrene and Glucose Media

This image clearly shows turbidity of a sample in a phenanthrene medium relative to a glucose medium.
Just as it was capable of metabolizing glucose in order to grow, the sample can be expected to do so using a highly toxic component such as phenanthrene.

Image 2: View of Plasmids
The electrophoresis image shows the twelve strains used in the search for degrading strains and one positive control corresponding to *E. Coli* GSH, a mutant plasmid strain (Second column, left to right).
The result shows that no analyzed strain exhibits plasmids.

Conclusions

- Results obtained validated the hypothesis and met stated objectives.
- Twelve psychrotolerant Antarctic strains were found to be capable of short-term metabolization of phenanthrene as sole carbon source.
- Strain 38C exhibited the best growth and phenanthrene degradation properties.
- Over prolonged periods of time, *Psychrobacter sp. (*strain 38C) efficiently uses phenanthrene as a source of energy and may be applied to bioremediation of waters in low-temperature zones.

Prospects

Twelve of 53 non-described strains of psychrotolerant Antarctic bacteria were found to be capable of metabolizing phenanthrene and increase colonies over time using phenanthrene as a sole carbon source.

The most promising of these was *Psychrobacter sp.* (38C) a bacterial strain with no plasmids that grows efficiently in high phenanthrene concentrations in an aqueous medium. As tests show that best results are obtained over the long term, the potential for use in bioremediation of polluted waters is high.

We intend to further explore strains with biotechnology potential, as follows:

- Analyze phenanthrene metabolization genes by PCR-amplified sequencing using degrading metabolic pathways described in mesophiles. The intent is to confirm the presence of these pathways in selected bacteria. In addition, we hope to understand the characteristics of phenanthrene metabolization proteins acting at low temperatures in selected strains by comparing with proteins of mesophiles growing at higher temperatures.
- Determine the degradation potential of isolated strains under various stress, medium pH and nutrient, temperature, and hydrocarbon concentration criteria.
- Apply the third methodology in future cooperative searches for psychrotolerant bacteria suitable for use in bioremediation of extreme low-temperature zones.
- Design polluted water bioremediation projects based on strains isolated during research.
- Apply identified psychrotolerant Antarctic bacteria in bioremediation of polluted waters in areas of the world noted for adverse conditions, based on strain ability to act in nutrient-poor media at extreme temperatures.

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