

Bioremediation Approaches in a Laboratory Activity for the Industrial Biotechnology and Applied Microbiology (IBAM) Course †

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INTRODUCTION

Industrial Biotechnology and Applied Microbiology (IBAM) is an optional 128-hour course for Chemistry and Biology students at the Faculty of Sciences, University of Buenos Aires. It is attended by 25 students, working in teams of two. The curriculum, with eight lab exercises, demonstrates bioremediation processes.

Bioremediation is a biological mechanism that allows the recovery of contaminated soils and waters. Focusing on bacteria, different genera are able to degrade oil industry by-products. The presence of these pollutants in pristine environments creates an acute contamination that modifies autochthonous microbiota, causing selection of decomposing species (1).

Hydrophobic pollutants are hard to degrade, so microorganisms have to synthesize substances to increase their bioavailability, such as biosurfactants. Biosurfactants are amphipathic molecules that diminish the surface tension (σ) between two liquids with different polarity, making the emulsification of one liquid in the other easier. σ is the contractive tendency of the surface of a liquid that allows it to resist an external force caused by the cohesion of similar molecules. Hydrophobic compounds suspended in pure water will spontaneously assemble themselves into larger masses. But the presence of a surfactant—decreasing σ —permits the stability of minute droplets in the bulk of the water. The Emulsification Index (EI) evaluates the capacity of a liquid to stabilize emulsions, so σ and EI are useful to characterize surfactant solutions.

This laboratory tip is an exercise in oil bioremediation that demonstrates the influence of pollutants on

autochthonous microbiota, biodegrader isolation, and biosurfactant production (L. Raiger Iustman et al., *Bioremediation Approaches in a Laboratory Activity for the Industrial Biotechnology and Applied Microbiology Course*, presented at the 19th Annual ASM Conference for Undergraduate Educators, San Mateo, CA, 2012). The experimental steps are: (A) evaluation of microbial tolerance in pristine soil microcosms contaminated with hydrocarbons; and (B) isolation of degraders and biosurfactant production analysis by σ and EI determinations in culture supernatants.

PROCEDURE

The activity is planned for five 4-hour work days, and is divided into two parts:

Part A: Evolution of microbial communities in hydrocarbon-contaminated soil

Objective: To analyze the effect of acute oil pollution on the indigenous microbiota over time.

Day 1: Microcosm preparation and initial count

1. Weigh 20 g of pristine soil without any history of contamination, and put it in each of 6 closed jars.
2. If necessary, sterilized water should be added to moisten the soil.
3. Add 2 ml of diesel (10% v/w) to 2 jars and 1 ml of xylene (5% v/w) to 2 others. Leave the remaining 2 jars without any contamination.
4. Homogenize soil to promote aeration by shaking jars vigorously.
5. Take 1 g sample at 0 hours and 4 hours of exposure at 25–28°C, suspend each sample in 10 ml 150mM NaCl, homogenize and perform 1:10 serial dilutions up to 10⁻⁴. Inoculate 100 μ l of each dilution in Nutrient Agar (Appendix 1), for total aerobe counting.
6. Incubate plates and microcosms at 25–28°C until next class.

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†Supplemental materials available at <http://jmbe.asm.org>

Day 2 (2 days after day 1): Count

1. Observe colonies and record their morphological diversity. Count colonies of both 0- and 4-hour samples. Calculate CFU/g soil.
2. Prepare 48-hour sample as detailed above.

Day 3 (7 days after day 1): Count and degrader selection

1. Repeat Day 2, step 1, for the 48-hour sample.
2. Prepare 168-hour sample as detailed above.
3. To isolate biodegraders, select 4 colonies from plates and inoculate on M9-agar plates (Appendix 1) with diesel as sole source of carbon (Fig. 1). Incubate plates at 28°C until next class.

Day 4: Count and observation of degrader colonies

1. Repeat Day 2, step 1, for the 168-hour sample.
2. Observe growth on M9-diesel plates.

Part B: Biosurfactant synthesis study

Objective: To analyze biosurfactant production from diesel-degrading bacteria.

Day 4: Inoculation of degrading microorganisms

1. Inoculate 10 ml M9-Broth-10% v/v diesel (Appendix 1) with:
 - Control strain (*Pseudomonas putida* KA-08) (2)
 - Isolated biodegraders
2. Incubate at 28°C, 300 rpm, until next class.

Day 5: Culture supernatant recovery

1. Harvest 8 ml of each culture in a conical tube, avoiding diesel.
2. Centrifuge at 5000 × g for 10 min.
3. Remove the supernatant with a clean glass pipette (Appendix 2) and transfer to another 15-ml plastic tube.

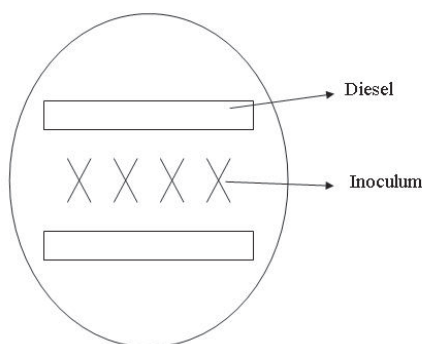


FIGURE 1. Diagram of the inoculation pattern for diesel degrader selection in plate with small channels.

Culture supernatant tensoactive properties study

Determine tensoactive properties, EI and σ , in cell-free supernatants of:

- Control strain (*Pseudomonas putida* KA-08) (2)
 - Diesel-degraders obtained on Day 5
 - Sterile distilled water
- a. EI determination:
1. Mix 2 ml of supernatant with 2 ml of *n*-hexane in a glass tube.
 2. Shake in vortex for 5 min.
 3. Measure both liquids' total height and height of the emulsion (cm).
 4. Express the EI as:
EI = (emulsion height/total height) × 100
 5. Interpret the obtained results.

b. σ measurement:

The σ is measured with a Du Nouy tensiometer (Fig. 2) as follows:

1. Submerge the ring into sterile distilled water helped by the scale handle and adjust at 0°.
2. Rotate the handle carefully to the point of ring detachment from liquid surface.
3. Register the torsion angle value at this point. Repeat 5 times.
4. Repeat the procedure with the culture supernatants.
5. The torsion angle corresponding to the tearing strength can be changed into force (Appendix 3). To calculate σ :

$$\sigma = \frac{(0.0048 \times \text{angle} + 0.0007) \times 981 \text{ dyne}}{2 \times 5.966 \text{ cm}}$$

CONCLUSION

An initial decrease of total CFU/g related to toxicity is noticed in both contaminated microcosms. At the end of

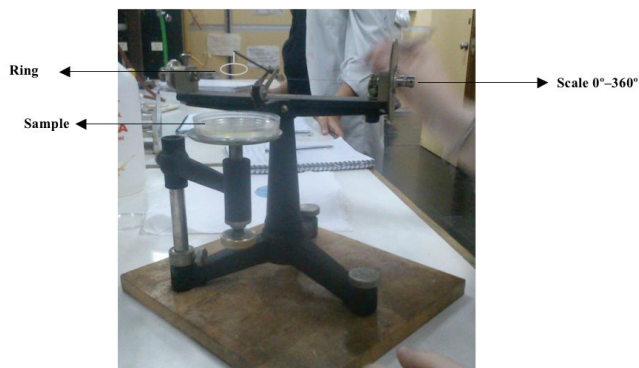


FIGURE 2. Du Nouy tensiometer.

the experiment, a recovery of CFU/g is observed, revealing enrichment in biodegraders (Fig. 3(A)). Homogeneity in the morphology of colonies can be recorded at 168 hours of exposure to hydrocarbons as a result of the selection process (Fig. 3(B)).

An EI higher than 50% means that the compound is a good emulsifier and is clearly related to σ decrease and to biosurfactant secretion: diesel degraders are isolated, a lower σ and higher EI are detected in their culture supernatants compared to water (Table I, Fig. 4).

Besides the improvement in good microbiological practices, the students showed enthusiasm for different aspects of the experiment: while biology students explored and learned new concepts on solubility, emulsions, and bioavailability, chemistry students showed curiosity about bacterial behavior and manipulation of microorganisms for environmental benefits.

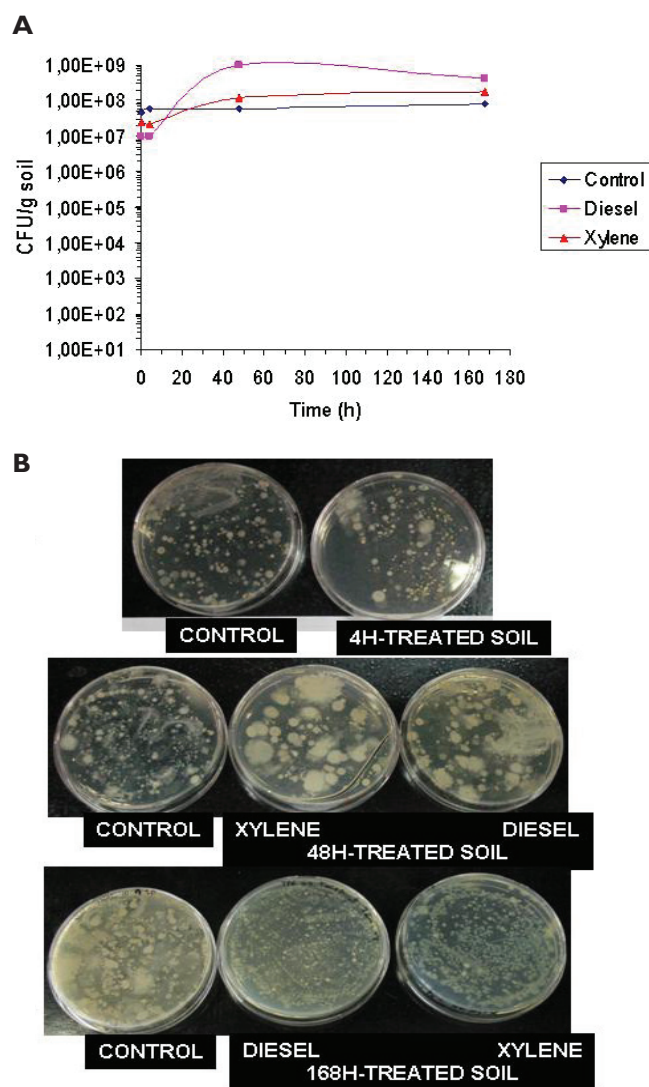


FIGURE 3. (A) CFU/g soil in microcosms along incubation time at room temperature. (B) Increase of the morphological homogeneity of the colonies obtained after long exposures to contaminants.

SUPPLEMENTAL MATERIALS

- Appendix 1: Culture media
- Appendix 2: Special treatment for glass material for surfactants' experimental work
- Appendix 3: Du nouy tensiometer – principles of torsion balance for surface tension (σ) measurements
- Appendix 4: Safety guidelines

ACKNOWLEDGMENTS

The ASM advocates that students must successfully demonstrate the ability to explain and practice safe laboratory techniques. For more information, read the laboratory safety section of the ASM Curriculum Recommendations: Introductory Course in Microbiology and

TABLE I.
Surface tension and emulsification index as examples of the data obtained from supernatants from isolated diesel-degrading bacteria.

Sample	Torsion Angle (°)	σ (dyne/cm)	EI ^a (%)
Water	202	79,8	0
M9 Broth	160	63,2	0
Control strain	156	61,6	34,5
G1	151	59,6	0
G3	157	62,0	42
G4	130	51,4	21
G5	132	52,1	0
G6	134	52,9	0
G8	84	33,2	17

^aEI, emulsification index.

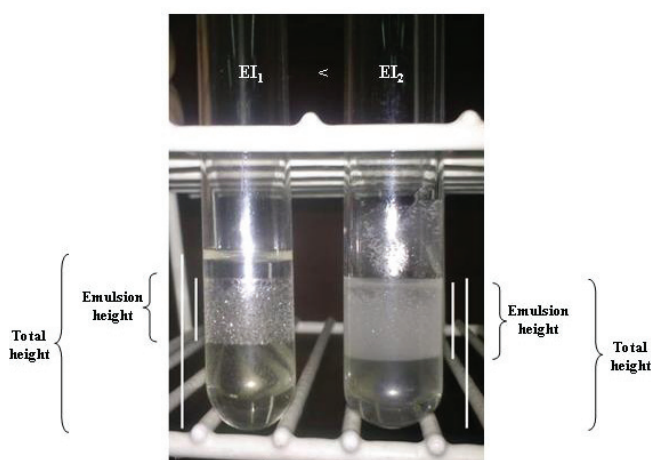


FIGURE 4. Biosurfactant production of selected strains: EI analysis by calculating the quotient between emulsion height of liquid phases and the total height.

the Guidelines for Biosafety in Teaching Laboratories, available at www.asm.org.

This activity was supported by the Faculty of Sciences, University of Buenos Aires, Argentina. The authors declare that there are no conflicts of interest.

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