SOP Isolation of PAH-degrading microorganisms

Protocol designed by Muratova et al. (2003) on the basis of (Kiyohara et al., 1982)
Revised and updated (October 2013) by Anna Muratova.

1. Methods

1.1. Rhizosphere soil sampling

- Rhizosphere soil is sampled from an industrial area contaminated with oil hydrocarbons.
- Plants are removed from the contaminated site with a titanium spade; the bulk soil is shaken off the roots, and the plants with rhizosphere soil are placed in plastic bags with labels indicating the plant species, the date, time and place of sampling.

1.2. Isolation of microorganisms degrading polycyclic aromatic hydrocarbons (PAH)

- In the laboratory, the roots are separated from the plants and samples of thin roots with the rhizosphere soil (approximately 1 g) are aseptically collected.
- Root sample is placed into an Erlenmeyer flask with 100 mL of sterile tap water and is shaken for 30 min. The roots are taken off, and the suspension is kept to let the soil particles settle out, after which a range of dilutions in physiological water are made for the isolation of rhizosphere microorganisms.
- For isolation of PAH-degrading microorganisms, a plate test (Kiyohara et al., 1982) is used.
- Soil suspension dilutions are plated on agar medium for PAH degraders.
- Plates are incubated at 30°C for 3-5 days.
- The surface of the plates is sprayed with a 3% solution of a PAH (e.g., phenanthrene) in ether.
- After 3-5 days, clearing zones (1–3 mm) are observed around some colonies. These colonies are termed PAH degraders.
- The selected strains are purity-checked.
- The degradative activity of the isolated PAH degraders is studied in liquid medium under batch cultivation conditions.

1.3. Identification

- For identification, physiological and biochemical tests, immunochemical analysis, and 16S rRNA gene sequence analysis are used.

1.4. Storage

The cultures are maintained by cryopreservation (at -70ºC) and overlaying with mineral oil.

1.5. Cryopreservation

- A tube with 5 mL of liquid LB medium is inoculated with an 18-48-h culture.
- The tubes are incubated at 30°C till late exponential growth.
- The cell suspension is diluted with fresh medium containing 40% glycerol.
- The cell suspension is distributed into 0.5-1.0 mL sterile Eppendorf tubes. The tubes are labeled with strain name, collection number, and preservation date.
- The tubes are frozen in liquid nitrogen.
- The frozen samples are maintained at -70ºC.

1.6. Overlaying with paraffinic oil

- A tube with 9 mL of semi-liquid LB medium is inoculated with an 18-48-h culture.
- The tubes are incubated at 30°C.
- The agar columns with the inoculants are overlaid with 1-2 mL of paraffinic oil.
- The tubes with the inoculants are stored at 4ºC.

2. Media

Physiological water
Prepare a solution of 0.85% NaCl and sterilize.

2.1. Medium for PAH degraders (Muratova et al., 2003)

K_2HPO_4	0.5 g
NH_4Cl	1.0 g
Na_2SO_4	2.0 g
KNO_3	2.0 g
$MgSO_4$	0.5 g
$FeCl_3$	traces
Micronutrient solution (after autoclave)	1.0 mL
Distilled or deionized water	1 L
Agar	2.0 g

Autoclave at 120ºC for 30 min.

Micronutrient solution

H_3BO_3	0.5 g
$CuSO_4 \cdot 5H_2O$	0.04 g
KI	0.1 g
$FeCl_3$	0.2 g
$MnSO_4 \cdot H_2O$,	0.4 g
$(NH_4)6Mo_7O_{24} \cdot 4H_2O$	0.2 g
$ZnSO_4 \cdot 7H_2O$	0.4 g
Distilled or deionized water	1 L

Autoclave at 120ºC for 30 min.

2.2. LB medium

Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Distilled or deionized water	1 L
Agar	0.5 or 2.0 g

Autoclave at 120ºC for 30 min.

3. References

Muratova A., Hübner Th., Tisher S., Turkovskaya O., Möder M., Kuschk P. Plant – rhizosphere-microflora association during phytoremediation of PAH-contaminated soil // Int. J. Phytorem. – 2003. – V.5, № 2. – P. 137-151.

Kiyohara H., Nagao K. and Yano K. Rapid screen for bacteria degrading water-insoluble, soil hydrocarbons on agar plates // Appl. Environ. Microbiol. – 1982. – Vol. 43. – P. 454-457.