SOP Isolation of plant-growth-promoting rhizobacteria

Protocol designed by Ekaterina Dubrovskaya, Anna Muratova and Sergey Golubev (October 2013)

1. Methods

1.1. Rhizosphere soil sampling

- Plants are removed from soil 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 plant-growth-promoting rhizobacteria (PGPR)

- In the laboratory, the roots are separated from the plants and thoroughly washed in running tap water. Then, they are transferred into 1 L flasks containing 0.5 L of sterile tap water and shaken for 30 min. The procedure is repeated three times, after which the same procedure is repeated with distilled water three times.
- The washed roots are moved to a sterile Petri dish and are cut into minuscule pieces with scissor pretreated with alcohol and burnt in the flame of an alcohol lamp. The root pieces are transferred into tubes containing nitrogen-free medium (2.1). The tubes are incubated at 30^{o}C for 3-5 days.
- 0.1 mL of culture liquid is transferred to the tubes containing fresh medium and incubated for 5-7 days.
- Dilutions of the inoculum are plated on the same agar medium and incubated for 3-5 days.
- The isolated colonies are selected.
- The selected strains are checked for their ability to solubilize inorganic phosphate by using Muromtsev's medium (2.2). Phosphate-solubilizing colonies surrounded with clearing zones on unclear agar medium are selected.
- Synthesis of the phytohormone indole-3-acetic acid (IAA) by the isolated strains is examined with Salkowski colorimetric method (Glickmann and Dessaux, 1995). IAA producers form colored indolic compounds on tryptophane-containing medium (2.3).
- The PGP ability of the isolated strains is tested with plants in pot or *in vitro* experiments.
- The strains with PGP abilities are selected.

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. Nitrogen-free medium (Tarrand et al., 1978)

K_2HPO_4	0.25 g
$MgSO_4 \cdot 7H_2O$	0.2 g
NaCl	0,1 g
$Na_2MoO_4 \cdot 2H_2O$	1.0 mg
$MnSO_4 \cdot H_2O$	2.0 mg
$FeSO_4 \cdot 7H_2O$	0.01 g
$CaCl_2 \cdot 2H_2O$	0.02 g
$(NH_4)_2SO_4$	1.0 g
Na malate	5.0 g
Glucose	5.0 g
Yeast Extract	0.05 g
Biotin	0.1 mg
Distilled water	1.0 L
Agar	0.5 or 1.5 g

Adjust pH to 7.2-7.4; autoclave at 105ºC for 30 min.

2.2. Muromtsev's medium (Muromtsev, 1957)

Glucose	10.0 g
K_2SO_4	0.2 g
$MnSO_4$	0.4 g
$CaCl_2$	2.2 g

Na_3PO_4	3.8 g
KNO_3	0.5 g
Asparagine	0.1 g
Distilled water	1.0 L
Agar	2.0 g

Adjust pH to 7.2-7.4. Add agar; autoclave at 105ºC for 30 min.

2.3. Medium with tryptophane

K_2HPO_4	0.5 g
KNO_3	2.0 g
NH_4Cl	1.0 g
Na_2SO_4	2.0 g
$MgSO_4 \cdot 7H_2O$	0.2 g
Na succinate	1.0 g
DL-tryptophane	0.4 g
$FeSO_4$	traces
Micronutrient solution (after autoclave)	1.0 ml
Distilled water	1.0 L

Autoclave at 105ºC 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
$(NH_4)_6Mo_7O_{24}. 4H_2O$	0.2 g
$ZnSO_4 \cdot 7H_2O$	0.4 g
Distilled or deionized water	1 L

3. References

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