SOP Isolation of *Azospirillum* bacteria

Protocol first designed by Fedorova et al. (1985) on the basis of (Döbereiner and Day, 1976) and (Caceres, 1982)
Revised and updated (October 2013) by Ekaterina Dubrovskaya.

1. Methods

1.1. Rhizosphere soil sampling

- Most often, *Azospirillum* bacteria are isolated from the rhizosphere of cereals.
- 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 azospirilla

- 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 scissors pretreated with alcohol and burnt in the flame of an alcohol lamp. The root pieces are transferred into tubes containing 6 mL of semiliquid selective media 2.1 (for most of *Azosirillum* spp.), 2.2 (for *Azospirillum amazonense*) or 2.3 (for *Azospirillum halopreferance*). The tubes are incubated at 30^{o}C (for medium 2.1) or at 37 ^{o}C (for media 2.2 and 2.3) for 3-5 days.
- 0.1 mL of culture liquid is transferred to the tubes containing fresh medium and incubated for 5-7 days.
- On semiliquid medium, azopirilla form a special subsurface growth ring. The tubes with special microaerophilic growth are inspected, and the microbial growth ring is observed under the microscope. The samples with special helical movement are checked and selected.
- Dilutions of the inoculum are plated on the same agar medium and incubated for 3-5 days.
- The isolated colonies are selected.

1.3. Identification

- Preliminary identification is made with the immunodiffusion method.
- The strains forming precipitation bands are selected. Physiological and biochemical tests, immunochemical analysis, and 16S rRNA gene sequence analysis are used for the identification of isolated cultures.

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 medium 2.1 (2.2 or 2.3) 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 medium 2.1 (2.2. or 2.3) 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. Azospirillum 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 minutes.

2.2. Azospirillum amazonense medium (Magalhäes et al., 1983)

K_2HPO_4	0.2 g
KH_2PO_4	0.6 g
$CaCl_2 \cdot 2H2O$	0.02 g
$MgSO_4 \cdot 7H2O$	0.2 g
$Na_2MoO_4 \cdot 2H_2O$	2.0 mg

$FeCl_3$	0.01 g
Bromothymol blue (0.5% in 0.2N KOH)	5.0 mL
Sucrose	5.0 g
Agar	0.5 or 1.5 g
Distilled water	1.0 L

Adjust pH to 6.0; autoclave at 105ºC for 30 min.

2.3. Malate medium with 0.25% NaCl (Reinhold et al., 1985)

DL-Malic acid	5.0 g
KOH	4.5 g
KH_2PO_4	0.6 g
K_2HPO_4	0.4 g
$MgSO_4 \cdot 7H_2O$	0.2 g
NaCl	2.5 g
$CaCl_2 \cdot 2H_2O$	0.02 g
$MnSO_4$	0.01 g
$Na_2MoO_4 \cdot 2H_2O$	0.002 g
Fe(III) EDTA (0.66% w/v in water)	10.0 mL
Biotin	0.1 mg
NH_4Cl	0.5 g
Yeast Extract	0.1 g
Agar	0.5 or 1.5 g
Distilled water	1.0 L

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

3. References

Döbereiner J. and Day J. Associative symbioses in tropical grasses: characterization of microorganisms and dinitrogen-fixing sites. In: Proceedings of the First International Symposium on Nitrogen Fixation Vol. 2 / Eds Newton W.E., Nyman C.J. // Washington State Univ. Press, Pullman, Washington. – 1976. – P. 518-538.

Caceres E.A.R. Improved Medium for Isolation of *Azospirillum* spp. // Appl. and Environm. Microbiol. – 1982. – Vol. 44, N 4. – P. 990-991.

Reinhold B., Hurek T., Fendrik J. Strain-specific chemotaxis of *Azospirillum* spp. // J. Bacteriology. – 1985. – Vol. 162. – P.190-195.

Magalhäes F.M., Baldani J.I., Souto S.M., Kuykendaee J.R., Döbereiner J. A new acid-tolerant *Azospirillum* species // An. Acad. Bras. Cienc. – 1983. – Vol. 55, N 4. – P. 417-430.

Tarrand J.J., Krieg N.R., Döbereiner J. A taxonomic study of the *Spirillum lipoferum* group, with descriptions of a new genus, *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov. // Can. J. Microbiol. – 1978. – Vol. 24, N 8. – P. 967-980.