Resuscitation of Extreme Halophiles from Ground Water.

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Background:

Of all the microbes that exist on earth only 1% percent of the total are culturable. Of this 1% there are special groups of microbes called extremophiles. **Extremophiles** are organisms that grow optimally under one or more chemical or physical extremes, such as pH or unorthodox temperatures. Organisms living in saline solutions more concentrated than that of sea water are called Halophiles. Sea water is about a 3% NaCl. Halophilic organisms have the ability to live in salt solutions that are more concentrated. The optimal growing conditions for some, called **Extreme Halophiles**, are in nearly saturated salt solutions that is about 35% NaCl. Microbes of this nature are usually found in extreme environment such as Great Salt Lake in Utah.

Halophiles are highly adaptable both externally and internally to deal which the osmotic pressure of concentrated saline solutions. Halophiles maintain a highly concentrated internal environment by producing and storing high amounts of polymers (sugars), alcohols and amino acids in their cytoplasm. Other groups use an ion pump to maintain a high potassium ion concentration in their cytoplasm. While some produce special enzymes that are only activity in the presence of salt.

Externally, microbes can alter the composition of their cell wall. Such as, the proteins found on the cell wall tend to be negatively charged. This negative charge aids in stabilizing the cell by attracting positively charge sodium ions (Na^+) in saline solution. Thus, the higher the Na^+ concentration, the more stable the cell wall. Consequently, placing halophiles in environments with low Na+ concentrations will undermine the integrity on the cell wall.

Material:

- Ground water sample
- 3 M Incubation buffer / with trace elements (see table) [a, b]
- 3 M R2A Molten Agar and Agar Plates (see table) [c]
- 3 M R2A Broth Tubes (see table) [c]
- 55^{o} C Water Bath
- Micropipette (P-1000 & P-200/ with plastic tips)
- Manual Pipette pump w/ 10 ml plastic pipettes
- Sterile glass test tubes (one per sample)
- Sterile microcentrifuge tubes. (one per sample)
- 4^{o}C incubator or Ice and 42^{o}C incubator
- Vortex, Bunsen Burner, 70% ethanol
- Parafilm, open top dish filled with water.

Methods:

Procedure A: Resuscitation (Day 1)

1.) Obtain ground water sample(s).
2.) Vortex samples to resuspend particles.
3.) In a 1:1 ratio, pipette 500µl of incubation buffer into a microcentrifuge tube. Next pipette 500µl of sample into the same microcentrifuge tube. *(resuspend several time with micropipette.)*
4.) Incubate samples with resuscitation buffer at 4^{o}C for 24 hours.

Procedure B: Pour Plating (Day 2 and 3)

1.) Pipette 10 ml of molten agar in sterile glass test tubes.
2.) Place molten agar test tube in 55^{o}C water bath to retard solidification.
3.) Obtain incubation buffered sample(s) from 4^{o}C. Let sample(s) stand at room temperature for 30 minutes to avoid heat shock.
4.) Pipette sample from microcentrifuge tube to molten agar tube in water bath.
5.) Immediately pour molten agar into empty sterile culture dish. Rotate dish gently until molten agar has completely covered the surface.
6.) Let plates cool and solidify overnight in a sterile environment (i.e. flow hood) at room temperature.
7.) The next day, parafilm each pour plate to retard dehydration. Then, place plates into a 42^{o}C incubator with an open top dish filled with water. (*this will give the incubator a moist atmosphere)* Allow 7 to 10 days for growth.

Procedure C: Broth enrichment and stock cultures.(Day 7)

1.) Survey plates for unique colonies. (*note: plate may not have any growth)*
(Salt concentration maybe adjusted to accelerate growth)
2.) Prepare one R2A broth tube for each selected colony. *(Make sure broth is room temperature to avoid heat shock).*
3.) Using Flame sterilization with ethanol, excavate each colony and place it into the R2A broth test tube. *(Caution: make sure proper aseptic techniques are used.)*
4.) Place R2A broth tubes into a shaking 42^{o}C incubator for 5 to 7 days.

Procedure D: Streaking plates. (Day 12)

1.) Obtain one R2A 3M agar plate for each R2A 3M broth tube incubated.
2.) Using a micropipette, pipette 10-15µl of enrichment broth to agar plate.
3.) Using Flame sterilization, streak agar plates in a corner to corner fashion.
4.) After streaking, parafilm plates and incubate at 42^{o}C for 5 to 7 days.
(Some organism may growth faster at slightly lower salt concentrations. i.e. 2.5M)

Table A: Reagents for preparing resuscitation buffers for Halophile

Salts	Molecular weight	Final Concentration	g/100 ml
$MgSO_4$ * $7H_2O$	246.48	150 mM	3.7
KCl	74.56	50 mM	0.37
$CaCl_2$* $2H_2O$	147.02	2 mM	0.03
MOPS (7.2 pH)	209.3	100 mM	2.09
NaCl (2M)	58.44	0.87 M	5.08
NaCl (3M)	58.44	1.87 M	10.93
NaCl (4M)	58.44	2.87 M	16.77
Trace Elements	see table b	see table b	100uL

Table B: Preparing Trace elements for resuscitation buffer.

Trace elements	Molecular Weight	g/ml
$CuSO_4$*$5H_2O$	159.61	50 ng/ml
$Fe(NH_4)_2(SO_4)_2$*$6H_2O$	392.14	4.55 ug/ml
$MnSO_4$*H_2O	150.99	300 ng/ml
$ZnSO_4$*$7H_2O$	161.44	440 ng/ml

Table C: Preparing Agar and Broth Medium

Ingredients	g/500 ml	Mol. Weight	[Final Conc.]
Distilled Water[1]	417ml	18.016	-------
Yeast Extract	0.083	-------	0.0166% (w/v)
Tryptone	0.083	-------	0.0166% (w/v)
Casamino Acids	0.083	-------	0.0166% (w/v)
D-glucose	0.25	180.16	2.78 mM (0.05%)
Souble Starch	0.25		0.05% (w/v)
K_2HPO_4	0.15	174.18	1.72 mM
Na Pyruvate	0.15	110	2.73 mM
$MgSO_4$*7H2O	10	246.48	81 mM
Na_3Citrate	1.5	294.1	10.2 mM
KCl	1	74.56	26.8 mM
$CaCl_2$*$2H_2O$	0.1	147.02	26.8 mM
1M Tris-HCl	25 ml	157.6	50 mM
Trace Elements	500 uL	see table b	see table b
2 M NaCl	58.44	58.44	2.0 M
3 M NaCl	87.66	58.44	3.0 M
4 M NaCl	116.88	58.44	4.0 M
Agar[2]	7.5	-------	1.5% (w/v)
LB Broth[2]	12.5	-------	-------

1: When adding distilled water subtract the amount of Tris-HCl and subtract 1 ml per gram of NaCl used.
2: When preparing medium boil and stir while mixing. In addition, boil for a half hour after all components are added.
*From optimal growth, after isolation, adjust salt concentration with respect to the organism.