Enrichment for Phototrophic "Non-sulfur" and "Sulfur" Bacteria

Content

1. Media Preparation

1a. Medium for Phototrophic "non-sulfur" Bacteria from Habitats with Brackish Water

spatially separated enrichment in agar shakes (culture vials 18 x150 mm, stopper # 1)

- ***Basic Salt Medium (BSM)***

Compound	Amount per liter	Final Concentration [mM]
H_2O d.	960 ml	
$(NH_4)_2SO_4$	0.33 g	5 mM NH_4^+
$MgSO_4 \cdot 7\ H_2O$	0.493 g	2 mM Mg^{2+} (7 mM SO_4^{2-})
$CaCl_2 \cdot 2H_2O$	0.147 g	1 mM Ca^{2+}
NaCl	17.53 g	300 mM Na^+
KCl	0.37 g	5 mM K^+ (307 mM Cl^-)

- ***Electron Donor***

Compound	Molecular Weight	Amount per liter g	Final conc. mM
Succinic acid $C_4H_6O_4$	118.1	0.59	5
Glutaric acid $C_5H_8O_4$	132.12	0.66	5
Malic acid $C_4H_6O_5$	134.1	0.67	5
Na-Acetate $\cdot 3H_2O$	136.1	0.68	5

- ***Procedure***
  - ***NaOH*** add as pellets until pH is approx. 6.8
  - ***Agar (washed): 0.9% final concentration*** Autoclave, let cool to 45°C under an N_2/CO_2 (9:1) stream, keep at this temperature in the waterbath (or liquify if necessary in microwave oven), add the following ingredients aseptically from sterile stock solutions and mix well after each addition:
  - ***Trace elements (acidic, 1000 x)*** add 1 ml
  - ***$NaHCO_3$ (1M)*** add 6 ml (6 mM, final concentration)
  - ***K-phosphate (1M, pH 7)*** add 4 ml (4 mM, final concentration)
  - ***Vitamins (1000 x)*** add 1 ml (1x, final concentration)
  - ***Yeast extract (10%)*** add 5 ml (0.05 %, final concentration)
  - ***$Na_2S \cdot 9H_2O$ (100 mM, pH 8)*** add 0.4 ml (0.1 mM, final concentration) as reductant (not assimilative electron source).
  - ***pH final = 6.8-6.9 check before dispensing into tubes***
  - Dispense under N_2/CO_2 into 18x150 mm tubes 9 ml into each tube
  - keep liquid at 43°C in a waterbath, add inoculum, mix well, dilute and let solidify.

Hints

For enrichments from natural samples we use the combination of several organic and/or inorganic electron donors; for pure cultures we use only one.

For dilution series use BSM without agar.

1b Medium for Phototrophic "Sulfur" Bacteria from Habitats with Brackish Water

spatially separated enrichment in agar shakes.
Liquid medium is made up 1.5x concentrated, which will be diluted to 1x when adding the agar. Add a magnetic stirring bar to the medium dispensing bottle before autoclaving.

- ***Basic Salt Medium (BSM)***

Component	Amounts per liter for 1.5x	Final concentration in 1x final medium
H_2O_d	940 ml	
HCl (2N)	3.5 ml	
$(NH_4)_2SO_4$	0.495 g	5 mM NH_4^+
$MgSO_4 \cdot 7H_2O$	0.74 g	2 mM Mg^{2+} (7 mM SO_4^{2-})
$CaCl_2 \cdot 2H_2O$	0.22 g	1 mM Ca^{2+}
NaCl	26.3 g	300 mM Na^+
KCl	0.55 g	5 mM (>307 mM Cl^-)
KH_2PO_4	0.204 g	1 mM (PO_4-P) (6 mM K^+)

BSM may be autoclaved as acidic solution as long as it does not contain agar (agar will hydrolize in acid)

- ***Procedure & Additions***
  let cool to room temperature under an N_2/CO_2 (9:1) stream after autoclaving and add the following ingredients from concentrated stock solutions aseptically to the cooled medium:

Stock solution	Volume added per liter medium 1.5x	Final concentration in medium 1x
NaOH (2N)	2 ml	
Trace elements, acidic (1000x)	1.5 ml	1x
$NaHCO_3$ (1M, O_2-free)	30 ml	20 mM
Na-Acetate (0.75 M)	3 ml	1.5 mM
Na-pyruvate (0.5 M)	4.5 ml	1.5 mM
pH approx. 7; check and adjust if necessary		
Vitamins 1000x	1.5 ml	1x
Vitamin B_{12} supplement (1000x)	1.5 ml	1x
DCMU (20 mM) if required only	0.15 ml	2 mM
$Na_2S \cdot 9H_2O$ (100 mM, pH 8) for Chromatiaceae	15 ml	1 mM
or		
$Na_2S \cdot 9H_2O$ (100 mM, pH 8) for Chlorobiaceae and at beginning of enrichment	40 ml	2.5 mM
$Na_2S_2O_3 \cdot 5H_2O$ (200 mM)	40 ml	5 mM

pH_{final} = 6.8 - 6.9 for Chlorobiaceae
pH_{final} = 7.2 - 7.4 for Chromatiaceae
check and adjust before dispensing into tubes !

Fill aseptically and anoxically into small tubes and close with butyl rubber stopper.

2. Stock Solutions and Media Supplements

- **$NaHCO_3$ (MW 84.01, 1 M)**
  dissolve 42 g in 500 ml $H_2O_{d.}$ while bubbling with CO_2, divide into working portions (100 ml) and autoclave in tightly closed screw-cap bottles; use CO_2 as gas phase
- **$Na_2CO_3 \cdot H_2O$ (MW 124, 100 mM)**
  dissolve 6.2 g in 500 ml O_2-free (boiled) water
- **Na-acetate $\cdot 3H_2O$ (MW 136.08, 0.75 M)**
  dissolve 10.2 g in $H_2O_{d.}$ to 100 ml, pH adjusted to approx. 7, autoclave in closed screw-cap bottle, let cool under N_2/CO_2 (9:1) stream and close tightly
- **Na-pyruvate (MW 110, 0.5 M)**
  dissolve 1.1 g in 15 ml $H_2O_{d.}$, adjust pH to approx. 7, add $H_2O_{d.}$ to 20 ml, sterilize by filtration (0.2µm), store under O_2-free conditions in an air-tight vial
- **K-phosphate (KH_2PO_4 MW 136.09, 1 M)**
  dissolve 13.61 g in 100 ml distilled water, adjust pH with KOH pellets to pH 7, store as sterile stock solution
- **Resazurin (MW 229.18, E_h^0 -45 mV, 100x)**
  dissolve 11.5 mg in 98 ml $H_2O_{d.}$, add 2 ml K-phosphate (1M, pH 7)

  Resazurin will decolorize at HS^- concentrations of 2 mM and higher, when added 100-fold diluted to the medium (Resazurin:Medium = 1:100)
- **Bromothymolblue (pH-Indicator)**
  dissolve 40 mg in 6 ml NaOH (0.01N), add $H_2O_{d.}$ to 100 ml.
  Assay: mix with medium 1:1 (one drop each on Parafilm); blue pH ≥ 7.3, green pH ≤ 6.8, yellow pH ≤ 6
- **$Na_2S \cdot 9H_2O$ (MW 240.18, 100 Mm)**
  wash cristals with distilled water, dry them on paper towel, dissolve 12 g in 500 ml Na_2CO_3 (100 mM) under N_2/CO_2 (9:1) atmosphere, dispense as 50 ml portions into glass vials and close air-tight with mushroom stopper, autoclave. This stock solution is alkaline; it needs to be acidified to about pH 8 before adding it to the medium
- **Na-thiosulfate (MW 248.19, 200 mM)**
  $Na_2S_2O_3 \cdot 5H_2O$: 9.94 g, dissolve in O_2-free $H_2O_{d.}$ to 200 ml, sterilize by filtration
- **Polysulfide-sulfur**
  Elemental sulfur: 0.64 g, NaOH 2N: 165.5 ml. This corresponds to 0.1 M sulfur-S after neutralization. Autoclave twice and store as alkaline solution. Neutralize before use with sterile HCl to the desired pH. Allow to aggregate for several hours to become colloidal sulfur.
  There is no need to sterilize the HCl if concentrated HCl (= 11.6 normal) is used for neutralization. You will need 34.5 ml HCl conc. to adjust to pH 7
- **HCl neutralizing solution (sterile) 2N**
  $H_2O_{d.}$ 82.8 ml, HCl conc (11.6N) 17.2 ml, make the dilutions in the hood, add HCl slowly, protect your eyes ! gives HCl 2N100 ml
  diluted HCl: 0.2N and 0.02N sterile
- **NaOH neutralizing solution (sterile) 2N**
  $H_2O_{d.}$: 80 ml, NaOH, (MW 40.01): 8 g, add in portions in the hood on ice, protect your eyes ! add water to100 ml
- **diluted NaOH, 0.2N and 0.02N**
- **Copper reagent for qualitative sulfide test**
  $CuSO_4 \cdot 5H_2O$: 0.63 g (5 mM), carefully add HCl conc. (11,6N) 2.15 ml (final concentration 50 mM), $H_2O_{d.}$ add to 500 ml, add the water carefully in the hood, protect your eyes!
  Assay: on Parafilm

Cu-reagent: 0.2 ml + culture aliquot: 0.05 ml (or similar proportions with larger volume aliquots).
Will turn brown if sulfide is present

- **Yeast extract 10%**
  dissolve 1 g in 10 ml $H_2O_{d.}$, sterilize by filtration (0.2µm pore size filter)
- **Glass beads (5 mm) for agar shakes**
  wash in alkaline ethanol, rinse well with $H_2O_{d.}$, add 1 bead per glass tube before autoclaving
- **DCMU (inhibits photosystem II of cyanobacteria) 20 mM**
  DCMU (MW 233.1): 233 mg, Ethanol abs. (MW 46, d_{20} 0.79, 17.17 M): 50 ml
  Be aware: by adding 0.1 ml of this stock solution to 1 l of medium, one also supplements 1.72 mM of ethanol
- **Trace element solution 1000-fold concentrated**
  acidic stock solution, without complexing agent:
  6.03 ml HCl conc. (11.6 N); 70 mM final conc. + 0.85 g $FeSO_4.7H_2O$; 7.5 mM final conc.
  Dissolve the ferrous sulfate completely in the acidic solution before adding distilled water carefully (protect your eyes !) to a final volume of 990 ml.
  Then add the salts listed below in the sequence given and make sure each salt has dissolved completely before you add the next one.

Compound	Amout per liter [mg]	Concentration in Stock Solution [mM]
$ZnCl_2$	68.1	0.5
$MnCl_2{\cdot}4H_2O$	89.1	0.45
H_3BO_3	6.18	0.1
$CoCl_2.6H_2O$	190.34	0.8
$CuCl_2.2H_2O$	1.7	0.01
$NiCl_2.6H_2O$	23.8	0.1
$Na_2MoO_4{\cdot}2H_2O$	48.4	0.2

Add distilled water up to a final volume of 1000 ml, divide into “working aliquotes” (e.g. 25 ml) and autoclave. This acidic stock solution remains clear and slightly yellowish. No precipitation of insoluble components should occur.
Use 1 ml per liter of medium without prior neutralization.

- ***Vitamin Solution (Stock Solution 1000-fold concentrated)***
  Dissolve the following ingredients in 1 liter of an aqueous solution made up of $NaHCO_3$ (16.8 mg, 0.2 mM) and $(NH_4)_2SO_4$ (4 mg, 0.03 mM), pH adjusted to 6.6. Make sure each vitamin has dissolved completely before you add the next one

Compound	Amout per liter [mg]	Concentration in Stock Solution [mM]
Biotin	10	41
p-Aminobenzoic acid	10	73
Folic acid	10	23
Pyridoxin HCl	20	97
Riboflavin	30	80
Nicotinic acid	50	406
D(+)Ca panthothenate	30	63
Cyanocobalamine	20	15*
α-Lipoic acid	20	97**

  *additional Vitamin B_{12} will be added to media for phototrophs (see Vitamin B_{12} supplement)
  ** will have to be dissolve in 0.5 ml $NaHCO_3$ (1 M) before adding it to the stock solution.
  Sterilize by filtration (0.2µm pore size filter) and store frozen in "working portions" of 2 ml in sterile plastic tubes.

- ***Vitamin B_{12} supplement 1000-fold concentrated stock solution***
  Dissolve 5 mg vitamin B_{12} in 50 ml aqueous ammoniumsulfate solution (1 mg $(NH_4)_2SO_4$ in 50 ml distilled water). Sterilize by filtration. Store frozen in "working portions" of 2 ml in sterile plastic tubes.

- ***Dilution medium: H_2S-reduced half concentrated sea water.***
  Water for dilutions of media components:
  - distilled water autoclaved
  - half concentrated sea water autoclaved

3. Procedures

- ***Flasks and Tubes for Slants, Shakes, liquid Enrichments and Dilutions***
  - ***Small Tubes for Shakes***
    (13x100 mm, butyl rubber stopper #00): + glass bead + 3ml medium 1.5x strength + 1.5 ml agar 2.7%
  - ***Intermediate Tubes for Shakes***
    (16x125 mm, butyl rubber stopper #0): 6 ml medium 1.5x strength + 3 ml agar, 2.7%
  - ***Large Ttubes for Shakes***
    (18x150 mm, butyl rubber stopper #1): 9 ml medium complete 1x strength, containing agar 0.9%
  - ***Hungate Tubes***
    with butyl rubber septum: 11 ml medium 1.5x strength + 5 ml sterile H_2O_d. After adding the inoculum a small gas head space (approx. 0.3 - 0.4 ml) should remain. In these tubes the concentration of sulfide can be varied by adding at any time different amounts of sulfide from the stock solution. These liquid enrichments are suited to obtain pure cultures by means of dilution series.
  - ***Pfennig Bottles***
    with aluminum srew caps: narrow bottles in which all the cells obtain enough light at all times. Fill medium to the top leaving space for inoculum and gas space (≤1ml). Gently mix the cultures once a day.

- ***Isolation on Agar Slants***
  Add a 0.02 ml aliquote, likely to contain phototrophic bacteria and spread the liquid by turning the tube gently. Place it on a slightly slanted surface and let the water of the film diffuse into the slant. Add 0.1 ml neutralized dihydrogensulfide solution (creates reducing conditons via the gas phase) to the lowest part of the slant and incubate the tubes vertically.

- ***Isolation on flat Bottle Agar Plates***
  Add a 0.05 ml aliquote, likely to contain phototrophic bacteria and spread the liquid by turning the bottle gently. Place it on a flat surface and let the water of the film diffuse into the plate. Add 1 ml neutralized dihydrogensulfide solution (creates reducing conditons via the gas phase) to the lower compartment of the bottle and incubate the bottle horizontally in the light.

- ***Agar for Shakes (washed)***
  3 g Agar, washed three times with H_2O_d.. H_2O_d. added to 100 ml
  Since some of the agar will be lost during the washing procedure, we will end up with a final agar concentration of approximately 2.7% which will give a final concentration in the shakes of 0.9%. The agar stock solution is distributed into 200 ml screw cap bottles and closed with a cotton plug for autoclaving. Let the agar cool under an N_2/CO_2 (9:1) stream, close with the sterilized screw cap and store the solidified agar in the cold room. For use, melt the agar in the microwave oven and keep at 60°C in waterbath.

- ***Dilution Series in Shakes***
  Six tubes, closed with butyl rubber stoppers*, containing a glass bead and 3 ml of the supplemented liquid medium are used for each enrichment. Add 0.5 ml of the "physically enriched**" inoculum to the first tube, turn once and transfer 0.5 ml aseptically to the second tube (7-fold dilution). Repeat the dilution step four more times (final dilution after the addition of the agar is about $2 \cdot 10^5$ times).
  The transfer steps should be carried out under the exclusion of O_2. Check the pH of one of the tubes (7.2-7.4 for Chromatiaceae, 6.8-6.9 for Chlorobiaceae) with the liquid Bromothymol indicator; check the sulfide content with the copper reagent. If necessary, add some more sulfide from a 10 mM "feeding solution", but do not exceed the optimal sulfide concentrations of 1 mM and 2.5 mM for Chromatiaceae and Chlorobiaceae, respectively.
  Later, we will pick single colonies with thin glass capillaries and transfer them into either a new shake or into a minituarized liquid culture. We would like to obtain well separated single colonies.

*Butyl rubber stoppers and butyl rubber membranes need to be boiled in water from time to time since they have the tendency to accumulate organic substances from the air and from the culture gasphase.
**Bacteria can be released from sand grains by gentle shaking. Enrich by centrifugation if needed. Motile organisms can be trapped in a light gradient capillary tube.

- ***Liquid Cultures***
  Due to its toxicity at low pH-values, only a limited amount of sulfide can be fed at the beginning of batch enrichments. Therefore, sulfide needs to be added from time to time which can only be done in liquid cultures. Cultures with a whitish turbidity contain a lot of sulfur. The disappearance of the white turbidity is a clear sign that sulfur has been used up and that sulfide needs to be re-amended.

- ***Preconditoning of Media***
  In order to establish proper anoxic conditions, it is recommended, to let freshly prepared medium sit in the dark for at least 6 hours or up to several days before inoculation. If it is not possible to follow this step, it becomes even more essential to work as anoxically as possible.

4. Variability of Enrichment Conditions

1. Light intensity = distance from the light source.
2. Optimal light intensities are 50 to 300 lux for *Amoebobacter sp.* and Chlorobiaceae and less than 2000 lux for the small Chromatiaeceae. Continuous irradiation at the higher light intensities and sulfide concentrations >2mM favors the growth of small-celled Chromatiaceae (*C. minus, C.vinosum*)
3. Light-dark cycles (12 h light/ 12 h dark or 6/6 or 4/8) and low light intensities (50-300 lux) support growth of the large Chromatiaceae (*C.okenii, T.jenense*) and the gas vacuolated Chromatiaceae (e.g. *A.purpureus*)
4. Light quality = incubation behind cutoff RG-filters (see transmittance characteristics of filters)
5. Physical micro-enrichment using phototactic swimming through capillary tubes into enrichment medium.
6. Electron sources: replace Na_2S by $Na_2S_2O_3$, by polysulfide or by FeS.
7. Enrich in long gradient agar shakes (auxanographic method) in order to obtain organisms with different O_2 sensitivities.
8. If one is interested in obtaining cells with a lot of storage polymer, one reduces the nitrogen in the BSM to 1/10 of its original strength, maintaining the osmotic balance with NaCl and incubates photosynthetically in the presence of an ample supply of electron donor

Enrichments

During the Woods Hole Course enrichments were carried out with different inocula enriched and diluted from salt marsh samples

- ***Phototrophic Non-sulfur Bacteria:***

  In "Large tubes for shakes" (18 x150 mm, butyl rubber stopper #1): 9 ml medium complete 1x strength, containing agar 0.9%. Keep molten agar medium in 43°C water bath; add 0.45 ml inoculum suspension to the first tube; dilute in steps of 10x to a final dilution of 10'000.

- ***Phototrophic, Purple Sulfur Bacteria (Chromatiaceae):***

  In "Intermediate tubes for shakes" (16x125 mm, butyl rubber stopper #0) containing 6 ml medium 1.5x strength. Make dilutions in the liquid medium down to 10000-fold. Once the dilution series are made in liquid medium at room temperature, put the tubes into the 35°C waterbath. Add 1.5 ml agar (2.7%, kept at 60°C under an N_2/CO_2 gas phase), turn the tube twice immediately and let solidify at room temperature. Add 1 drop of $Na_2S \cdot 9H_2O$ solution (1 mM) of the appropriate pH, flush once more with N_2/CO_2, stopper tightly and keep the shakes in the dark over night (in order to give time to equilibrate to the proper redox potential and to scavenge any O_2 remaining in the tube). Then incubate under the desired light conditions. In order to keep air away from the agar surface one might want to seal the shake with a paraffin/paraffinoil (1:1) plug, which can be melted away before colonies are picked later.

- ***Phototrophic, Green Sulfur Bacteria (Chlorobiaceae):***

  In "Small tubes for shakes" (13x100 mm, butyl rubber stopper #00): + glass bead + 3ml medium 1.5x strength + 1.5 ml agar 2.7% after dilutions down to 10^{-4} have been made.