NCMA Medium 2: Artificial Sea Water-FeS/Gradient Tubes

Medium appropriate for cultivating marine iron-oxidizing bacteria (FeOB).

Artificial Sea Water (ASW) per liter distilled H_2O:

NaCl	27.5 g
$MgCl_2$	5.38 g
$MgSO_4.7H_2O$	6.78 g
KCl	0.72 g
NH_4Cl	1.00 g
$CaCl_2.2H_2O$	1.40 g
K_2HPO_4	0.05 g (add after other constituents have dissolved)

This can be kept as an ASW stock solution.

The following are added when the assembling the final medium:

$NaHCO_3$	10 mM (0.84 g/L)
Mineral Solution	0.1% (See Recipe: NCMA Medium 7: Mineral solution)
Wolfe's vitamin solution	0.1% (See Recipe: NCMA Medium 6: Wolfe's Vitamin solution)

Preparation of FeS Stock Solution

FeS is prepared by heating 300 ml of dH_2O to 50ºC in a 500‑ml beaker with a stir bar present. Separately preweigh 46.2 g of ferrous sulfate and 39.6 g of sodium sulfide. While stirring the water rapidly, add the ferrous sulfate followed immediately by the sodium sulfide. A thick black precipitate will form instantly. This mixture is stirred continuously for 2 to 3 min to ensure complete dissolution and mixing of the ferrous sulfate and sodium sulfide. The black FeS sludge is decanted into a narrow-mouthed glass bottle (500 ml) that can be stoppered tightly. The bottle is filled to the top with dH_2O and capped. The FeS is allowed to settle for several hours and then the overlaying water is decanted and replaced. The resulting FeS precipitate must be washed extensively using deionized water (decanting of the supernatant and its replacement with deionized water at 50°C), removing Na^+, NH_4^+, and, above all, S_2^- ions until the precipitate reacts neutrally (pH measurement in FeS, not in the supernatant).This procedure is repeated at least five times to wash the FeS. After washing, the pH of the FeS solution should be close to neutrality.

After removing FeS for use, it is important to top the bottle up with dH_2O and keep it stoppered tightly to limit the influx of oxygen. With limited oxygen exposure, the FeS can be maintained at room temperature for up to 3 months. Even under these conditions the FeS does age, however, and slowly loses its ability to release Fe(II). Each batch of FeS is slightly

different. If the FeS smells strongly of sulfide following the washing steps or has a strongly alkaline pH, it should be discarded. Remember that in the presence of acid, sodium sulfide will immediately release hydrogen sulfide, an extremely toxic gas. Preparing FeS in a chemical fume hood is strongly recommended.

Preparation of Gel‐Stabilized Gradient Tubes

Typically 17 x 60 mm (o.d. x length) borosilicate glass vials are used as vessels for gradient tube cultures. The top layer, consisting of semisolid mineral media, and the bottom layer, which serves as the iron source, are prepared separately.

Bottom Layer
The bottom layer contains 1% (w/v) *high melt* agarose and equal volumes of ASW and FeS stock solution and is autoclaved

Top Layer
In a separate container, the top layer is prepared by adding 0.15% *low melt* agarose to ASW along with 2.4 mM sodium bicarbonate and 1 µl of Mineral Solution per milliliter of medium and is autoclaved.

Assembly

Shortly after autoclaving, 0.75 ml of the bottom layer is pipetted into each culture tube. One‐milliliter pipette tips that have been cut to enlarge the opening at the tip to prevent clogging are recommended. The bottom layer is allowed to cool for at least 30 min to ensure it is well set; meanwhile, the top layer is cooled to between 35 and 40º C.

One µl per milliliter of Wolfe's vitamin solution is added to the top layer and the pH is adjusted to within the range of 6.1 to 6.4 by bubbling aseptically with sterile CO_2 gas. The gassing time is dependent on the volume of medium and the flow rate of the CO_2 and can be determined empirically.

To make the top layer, 3.75 ml of medium is pipetted over the bottom layer of each tube. The tubes may be capped with either a butyl rubber stopper or a screw cap with a septum to maintain an aerobic headspace. The top layer should be allowed to solidify a minimum of 3 h to a maximum of overnight. Allowing the tubes to sit uninoculated for longer periods will lead to undesirable amounts of abiotic iron oxidation. To inoculate, 10 µl of the desired inoculum is drawn into a pipette tip and inserted just above the FeS layer; the pipette tip is drawn upward as the inoculum is dispensed. Each tube should be stoppered and incubated at the appropriate temperature. Growth is visible as the line of the inoculum spreads into a rust‐colored band at the oxic–anoxic interface; however it is essential to confirm the presence of the bacteria by

epifluorescence microscopy. It is recommended that the cultures be transferred every 3 to 4 weeks. Refrigerated storage will prolong the useful life of a culture.

Notes:
The most common reason for lack of growth of FeOB is a lack of iron, followed by lack of pH control. Lack of iron is generally a result of FeS that either does not release adequate Fe(II) because it was made improperly, or is too old. An alternative to FeS is ferrous carbonate, which works well, but has a short shelf-life, e.g. 1 -2 weeks. If pH is an issue the top layer medium may be amended with 10 mM MES buffer and adjusted to pH 6.3 – 6.4 prior to autoclaving. A 500 mM solution of sodium bicarbonate can be used to adjust the pH.

Washing of FeS while continually checking pH is indispensable, since hydrolysis of any residual sulfide ions ($S^{2-} + H_2O \rightarrow HS^- + OH^-$) raises the pH to 7.7 directly over the FeS sediment. The elevated pH prevents bivalent iron from dissolving. The separation of the adsorbed sulfide ions from the FeS precipitate during the washing procedure is a slow process and takes approximately 5 days (5–9 washings at intervals of at least 4 h). Delayed FeS sedimentation caused by formation of FeS-hydrosol can easily be eliminated by including a few drops of a saturated $FeCl_3$ solution or by once washing with tap water.

References:

Emerson D & Merrill Floyd M (2005) Enrichment and Isolation of Iron-Oxidizing Bacteria at Neutral pH. pp 112-123. Chap. In: Jared RL (Ed) Environmental Microbiology. Abelson JN & Simon MI (Eds), Methods in Enzymology. Vol 397. Academic Press / Elsevier

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