

NCMA Medium 3: Modified Wolfe's Mineral Medium FeS/Gradient Plates

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

MWMM per liter distilled H₂O:

NH ₄ Cl	1.0 g
MgSO ₄ ·7H ₂ O	0.2 g
CaCl ₂ ·2H ₂ O	0.1 g
K ₂ HPO ₄	0.05 g (add after other constituents have dissolved)

This can be kept as an MWMM stock solution.

The following are added when assembling the final medium:

NaHCO ₃	5 mM (0.42g/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 dH₂O to 50°C 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 dH₂O 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⁺, NH₄⁺, and, above all, S₂⁻ 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 dH₂O 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). If the FeS smells strongly of sulfide following the washing steps or has a strongly alkaline pH, it should be discarded. 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 Plates

The top layer, consisting of 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 MWMM and FeS and is autoclaved.

Top Layer

In a separate container, prepare the top layer of MWMM along with 5 mM sodium bicarbonate and 1 μ l of Mineral Solution per milliliter of medium and is autoclaved.

Assembly

After autoclaving, the FeS/agarose layer is added to petri plates to a depth of 3 to 4 mm; approximately 8 ml per standard 100 x 15-mm petri plate or 5 ml for small 60 x 15-mm petri plates. After the FeS/agarose layer has initially solidified, it is allowed to set for approximately 30 min. It is best to do this under anoxic conditions, e.g., by storing the plates in an anaerobic chamber or anaerobe jar that has been flushed with N₂ to prevent chemical oxidation of Fe at the surface.

Once the top layer has reached room temperature, one μ l per milliliter of Wolfe's vitamin mix 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 CO₂ gas. The gassing time is dependent on the volume of medium and the flow rate of the CO₂ and should be determined empirically.

Aliquot 15 ml of the top layer onto each plate (8 ml for the smaller plates). The plates are incubated in a sealed anaerobic jar (www.bd.com; GasPak 150 jars) with a GasPak EZ Campy Container System microaerophilic pouch (www.bd.com; cat # 260680). This system produces an atmosphere of approximately 5–10% O₂ and 5 to 12% CO₂ in the headspace. The entire top layer is inoculated with 2 to 3 ml of the desired organism per 100 ml media. Growth is indicated by accumulated Fe floc, as well as the appearance of an opalescent metallic film on the liquid surface. It is always necessary, however, to confirm the presence of FeOB by epifluorescence microscopy.

Notes:

The difficulties of culturing iron-oxidizing bacteria (FeOB) generally are due to overlooking interactions in the medium among the following factors: phosphate, ferrous and ferric iron,

oxygen, pH, and redox potential. The most important growth-inhibiting chemical processes in the medium are: (1) iron phosphate precipitation caused by too high a concentration of phosphate; (2) chemical autoxidation of ferrous iron caused by too high a concentration of oxygen or by the redox potential being too high; and (3) the pH becoming more acidic when elevated O₂ concentration causes hydrolysis of ferric iron. All three processes eventually reduce the Fe(II) content of the mineral medium of the FeS cultures below the lower limit of 5 ppm. In order to retain the required Fe(II) concentration, it is absolutely necessary to have the following initial conditions in the medium: oxygen content 1 ± 0.2% (0.42 ppm), pH 6.0 (values from CO₂ bubbling, border values between 5.5 and 6.3), Eh +330 mV to +350 mV (Eh border values from - 55 to +395 mV), phosphate (as K₂HPO₄) 0.05 g/ liter. They lead, following interactions between mineral medium and FeS-sediment, to an iron(II) concentration of 10–20 ppm which guarantees good growth of FeOB. The conditions of gassing must be varied according to the volume in each vessel, so that common standardized instructions may not be given for all sizes of flasks.

Identical for all cultures, though, is the production and storage of ferrous sulfide as well as the production of ASW medium. During the first process, essential mistakes may be made that inhibit the growth of FeOB

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 FeCl₃ 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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