**Background**

Obligately anaerobic, Slow growing, Fastidious (1)

Dissimilatory reduction 🡪 hydrogen sulfide

Require free sulfate for growth. (2)

SRB require electron accepter for growth, usually sulfate. Sources for anion may be dietary or endogenous. (3)

Metabolic end product is sulfide, converted to H2S with external H+

Sulfide is corrosive/binds rapidly to metals. (4)

SO4 2- reduction to produce H2S requires 8 e.

Step 1: SO4 2- activated using ATP and ATP sulfurylase so that it binds 🡪adenoside phophosulfate (APS) and elemental PPi

Step 2: Dissimilative: SO4 2- in APS reduced to sulfite (SO3 2-) using APS reductase. (2e in, AMP out)

Step 3: SO3 2- reduced to H2S via sulfite reductase. (6e in)

Provides H2S and free energy.

Electron transport chain generates proton motive force.

H+ moves across membrane/down gradient through ATPase 🡪 ATP synthesis

Capable of energy conservation by reducing elemental S and (source of electrons) 🡪 H2S.

Sulfur reducers cannot activate sulfate to APS.

Use elemental S instead. (Book pg 421-423)

Sulfur reducers also reduce e acceptors such as nitrate, ferrous iron, thiosulfate as alternative to elemental S. (Book pg 658)

Any sulfur compound with ox state above sulfide (-2) can be e acceptor

Dissimilatory: sulfate ion used as oxidant for degradation of organic material

H2S significant able to inhibit the growth of aerobic org.

Requires inorganic e acceptor.

Type of Carbon source used for reduction of acceptor varies by genus.

Acetate and Hydrogen are essential substrates.

Obligate anaerobes lack catalase. (Lab manual pg 41)

Play important role in sulfur cycle (5)

**Materials/Methods**

**Sources** !MM

Sewage, industrial effluents, water, soil (1)

Found in sediment. (2)

sediments, sewage sludge, and colons. (3)

Found in marine/estuarine sediments.

Saline ponds.

Especially where habitat contaminated by alternative nutrients from sewage effluent.

Anaerobic, but found in low conc in oxic conditions.

polluted environments such as spoiled food, sewage plants (4)

all kinds of soils; river, sea, pond

and tap waters; water from gas-holders and oil-storage tanks; oil-well waters; sewage; marine sediments. (5)

periodontal pockets, gastrointestinal tract, normal microflora of human oral cavity

sea water, water sediments mud (6)

**Isolation/Incubation/Media !MM**

Chocolate agar

5 days, 37 degrees C

Not good in broth medium, better to use solid.

May take 5-7 days for a colony to appear. (1)

“A semisynthetic basal medium with a pH of 7.2 and a redox potential below -100 mV was used for enrichment of SRB. The medium was made by sterilizing separate solutions that were aseptically combined under anaerobic conditions.

Solution 1 contained CaCl2-21^20, 3 mg; K2HPO4-3H2O, 0.65 g; NH4CI, 1.0 g; Na2SO4, 1.0 g; yeast extract 1.0 g and resazurin 0.0003 mmol/1 in 800 mf of deionized water.

Solution 2 contained the following electron donors (27): sodium acetate, 2.5 g; sodium pyruvate, 2.0 g; sodium propionate, 2.0 g; sodium citrate, 0.5 g; sodium lactate, 2.0 g in 100 ml of deionized water.

Solutions 1 and 2 were autoclaved for 20 min at 120°C in screw-cap bottles that were closed immediately after sterilization.

Solution 3 contained MgSO4-71120, 2.0 g; FeSO4-7H2O, 0.5 g; 0.5 ml of 6 N HCl in 50 ml of water.

Solution 4 contained NaHCOj, 2.0 g in 50 ml of water.

Solution 5 contained 30 mg of Na2S2O4 (dithionite) in 1 ml of water.

For preparation of solutions 3 to 5, oxygen-free deionized water was used and the solutions were filter-sterilized. Solutions 1 to 5 were mixed in an anaerobic chamber (Braun, Garching, Ger- many) in an atmosphere of 91% N2, 4% H2 and 5% CO2, and an oxygen concentration below 1 ppm. Agar plates were prepared with the same medium supplemented with 15 g/1 of agar.” (2)

All incubation in anaerobic chamber.

Use enrichment/mixed inocula or chemostat methods.

Batch culture: closed. No need to add. Simpler.

SRB are slow growing. Unable to compete in enrichment system?

Anaerobic plating does not distinguish bw different genera

(2)

“A modified version of Postgate’s liquid growth medium ‘E’ was used for the enumeration and isolation of SRB. The composition was as follows (g 1-l distilled water): KH,PO,, 0.75; NaSO,, 1.0; NH&I, 1.0; MgSO, - 7H,O, 2.0; CaCl, .2H,O, 1.0; NaCl, 3.0; yeast extract, 0.1; sodium ascorbate, 0.2; sodium thioglycollate, 0.05; FeSO, \* 7H,O, 0.5.

A combination of electron donors (acetate, lactate, pro- pionate and butyrate), suitable for the growth of the major SRB genera, were added as sodium salts to give a final concentration of 5 g 1-l.

The medium was autoclaved at 121“ C for 15 min and stored under anaerobic conditions (oxygen-free nitrogen headspace) until use… SRB counts in the sonicated samples were determined in duplicate using the agar shake dilution method” (3)

DRCA agar, TSN agar, Reduced-growth medium for SRB

Iron indicator for sulfide production

Samples incubated in anoxic chamber, 37 degrees C

3-7 days until black colonies

(6)

Anaerobic chemostat enrichments. Chemostat = continuously add medium.

Sodium acetate as carbon source

Inoculated with 50g freshly collected sediment.

2 day intervals, 10ml samples aseptically removed.

Aliquot volumes transferred to agar shake dilution tubes (same medium)

25 degrees C, up to 7 days

Chomostat enrichments using acetate as carbon source were successful, target species becoming dominant after 12-14 days

(7)

Samples diluted in sterile 0.6% MgCL2 solution, homogenized by a rotary shaker, and immediately deposited on Millipore filters. Each filter places in tube containing medium 1, inclubated at 35C until FeS ppt on filter.

Measurement/process by spectrophotometer. Non-ppt sulfides measured. (8)

“ A basal bicarbonate-buffered and sulfide-reduced medium was used. The mineral medium contained per litre of distilled water: 0.2 g KH2PO4,0.25gNH4Cl,0.5gKCl,0.1gCaCl2·2H2O, 0.4 g MgCl2·6H2O, 1.0 g NaCl and 0.5 ml of a resazurin solution (0.5 mg ml−1) as a redox indicator. The medium was supplemented with (per litre) the following: 30 ml 1 M NaHCO3 solution, 1 ml of a vitamin solution, 1 ml of trace element solution with ethylenediamine tetraacetic acid (EDTA; Widdel and Bak 1992) and 0.1 g of yeast extract. As a reducing agent, 7.5 ml l−1 of 0.2 M Na2S·9H20was added. Either lactate or ethanol (20 mM) was used as an electron donor and sulfate (10 mM) as electron acceptor”

Growth assessed photometrically.

Determined by substrate consumption, or product formation, or checking for increase in optical density”

“The tubes with growth in the highest dilution were used for isolation in agar shake tubes”

(9)

Media for both halophiles and non-halophiles.

“The general method used consists essentially in making enrichment cultures and then preparing deep agar (‘ shake ’) cultures or plates in various dilutions. A solid medium containing sulphate, lactate and a trace of ferrous salt is used; the sulphate-reducers form black colonies which can readily be picked off and placed in liquid medium. This apparently simple procedure rarely meets with immediate success except sometimes with thermophilic strains. It is difficult to obtain colonies free from contaminants, and it is frequently necessary to undertake a tedious succession of deep agar dilution cultures and platings out before pure cultures are obtained. We have found that the inclusion of 3 yo Na,SO,. 7H,O in the media eliminates most of the contaminating organisms in mixed cultures and greatly facilitates subsequent purification of the sulphate-reducers.”

“Most soils incubated with medium A (below) reduce sulphate even if no anaerobic precautions are taken, presumably because the aerobic organisms also present deoxygenate the medium. Nevertheless, growth is much better when oxygen is rigorously excluded.”

“the McIntosh & Fildes anaerobic jar, with an atmosphere of hydrogen containing 5% carbon dioxide, was found to be the most satisfactory and efficient for general use.”

“Medium A (Baars, 1930): K,HPO,, 0-5 g.; NH,CI, 1 g.; CaSO,, 1 g.;

MgSO, .7H,O, 2 g. ; sodiumlactate (70 yo solution), 5 g. ; FeSO, . (NH,),SO, .6H,O, 0.5 g. ; tap water, 1 1. ; pH 7.0-7.5, which should be confirmed after sterilization.

This medium gives a considerable precipitate on sterilization for 20 min. at 20 lb./sq.in. which is no disadvantage in crude cultures, but may be with pure cultures when, for example, growth is determined by turbidity measurements.

Medium B (Starkey, 1938): K,HPO,, 0.5 g.; NH,Cl, 1 g.; Na,SO,, 1 g.; CaC1,. 2H,O, 0.1 g. ; MgSO,. 7H,O, 2 g. ; sodium lactate (70 yo solution), 5 g. ; FeSO, . (NH,),SO, . 6H,O, 0.5 g. ; distilled water, 1 1. (Starkey used tap water) ; pH 7.0-7.5. This medium gives only a slight precipitate on autoclaving and may be filtered clear without significant loss and resterilized. It is preferable not to incorporate the ferrous salt in the stock media, but to sterilize a 1 yo solution by steaming 1 hr. on 3 successive days, and to add 5 ml. supernatant liquid to each 100 ml. medium immediately before use.

Media A and B are suitable for growing obligate and facultative non-halophilic strains in crude cultures. For halophilic strains 1-3 "/o NaCl is added or tap or distilled water replaced by sea water.”

“Baars (1930) considerably diminished the number of contaminating organisms by adding 4 ml. of 0.1 N-H,S to 60 ml. medium, but Starkey (1938) did not find this method useful for isolation purposes”

“Strains from a saline environment are best isolated on medium A or B with the addition of 2-3 yo NaC1, though many halophilic strains can reduce sulphate in its absence. Most soils and sewages readily yield flourishing crude cultures of sulphate-reducers when 1-2 g. are incubated anaerobically with media A and B, with and without 3 yo Na,SO,. 7H,O. For these cultures it is convenient to employ stoppered bottles containing 30-50 ml., but cultivation in ordinary culture test-tubes in anaerobic jars is equally effective. Waters, on the other hand, vary enormously in the volume necessary to yield positive cultures. With a heavily contaminated soil water we have obtained sulphate reduction from inocula of 0.00001 ml. ; with other waters 50 or 100 ml. must be incubated with the medium. Hence, with waters obviously heavily infected, e.g. smelling of H,S and showing vibrios under the microscope, only 1-2 ml. need be used, but normally our practice with waters is to incubate 1, 10, 50 and 100 ml. samples. Bunker (unpublished observation) grew a strain from 25 ml. tap water. For the larger quantities we use stoppered bottles of appropriate size or flasks containing alkaline pyrogallol plugs. To prevent undue dilution by the larger inocula, stronger media are used.”

“When considerable blackening of the medium has occurred, which may take

2-3 days, and sometimes a month, the culture is examined for vibrios. If vibrios predominate, which occurs sometimes in sulphite but rarely in non-sulphite medium, the culture is ready for the isolation of pure strains. If not, transfers are made into fresh medium, preferably using portions of the black sludge, which usually contains a greater concentration of vibrios than the supernatant liquid. Subculturing is continued until microscopical examination reveals a flourishing culture of vibrios ; usually three or four transfers are necessary in the absence of sulphite.”

(5)

Anaerobic Methods.

Candle Jar. Burning convert oxygen to CO2 and Water.

Gas-Pak system. Chemical packet consumes oxygen when water is added. (Lab Manual pg 41)

!MM E. coli, P. aeruginosa, C. beijerinckii for controls

**Identification**

Tiny, pinpoint, round colonies

Gram negative !MM E. coli, S. aureus for controls

Curved/rod-shaped

Single polar flagellum (1) !MM P. fluorescens for control

Presence indicated by strong blackening due to precipitation of FeS.

Morphological criteria and substrate utilization tests to identify (2)

2 categories for those that use lactate for carbon/energy:

spore-forming straight OR curved rods

Activity can be detected to odour of H2S

Identify using motility tests, Gram reaction, cell size, morphology (4)

Identify using respiratory type, morphology, cultural aspects, stain affinity, gram stain (6)

evidence of motility, production of only H2S, catalase test, sensitivity to kanamycin, colistin, vancomycin. (6)

motile, curved, gram-neg

Motile vibrios

Lost motility and became spirilloid in old cultures

Gram negative

No sporulation (8)

Incomplete oxidizers of lactate and ethanol to acetate with sulfate as e acceptor.

Used H2 and formate as carbon source when acetate present.

Presence of desulfoviridin

Vibro-shaped morphology

Gram negative

“The cell pellets were re- suspended on glass slides with a drop of 3% (v/v) H2O2, bubbles indicated the presence of catalase. Detection of desulfoviridin was performed according to Postgate” (1959).

(9)

Thioglcolate broth (Lab Manual pg 41)

Gas production visualized in Durham tubes.

Look for insoluble precipitate when combined with metal salts. (Lab Manual pg 57)

Catalase Activity.

3% H2O2 on slide. Bubbles🡪catalase. Else 🡪obligate anaerobe (Lab Manual pg 61)

!MM same controls as gram stain

Media for sulfate reducers should include FeSO4, to see FeS precipitate.

SIM (Sulfide Indole Motility) Medium. Dark tube🡪presence of H2S. (Lab Manual pg 63)

!MM 5 SIM deeps, E. Coli, E. aerogenes, P. vulgaris for controls

TSI (Triple Sugar Iron Agar) to test for H2S production. Lighter colors 🡪presence of H2S (Lab Manual pg 67)

!MM 5 TSI slants, E. Coli, A. faecalis, P. aeruginosa for controls

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