CULTIVATION AND PRESERVATION OF MEMBERS OF THE FAMILY HALOBACTERIACEAE

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Members of the family *Halobacteriaceae* may be described as aerobic, extremely halophilic archaeobacteria. They share a number of features common to other archaeobacteria, but may be distinguished rapidly from other archaeobacterial phenotypes on the basis of their ability to grow aerobically at salt concentrations ranging from 1.5 molar (about 9% w/v) up to saturation (about 30% w/v).

The natural habitat of these organisms are highly saline lakes such as Great Salt Lake (USA), the Dead Sea (Israel) and Lake Magadi (Kenya), or man made highly saline environments such as solar salterns (salinas).

The members of the family *Halobacteriaceae* may be currently divided into the following six genera, *Halobacterium*, *Halococcus*, *Haloferax*, *Haloarcula*, *Natronobacterium*, and *Natronococcus*. These genera may be divided into a numberr of groups on the basis of morphological and physiological properties.

The classical rod shaped organisms are to be found within the genus *Halobacterium*, while the classical sarcinas are placed in the genus *Halococcus*. Members of the genus *Haloferax* are recognised by their relatively short generation times, their higher magnesium requirements, their lower salt optima, and their flattened cell shape. The genus *Haloarcula* contains those strains often described as " box-shaped bacteria". Alkaliphilic members of this family may be divided on the basis of their coccoid or rod shaped morphology into the genera *Natronococcus* and *Natronobacterium*, respectively.

Isolation of members of the family *Halobacteriaceae* may be carried out in media containing appropriate concentrations of salt. By varying the salt

concentration between 1.5 molar and 4.5 molar various kinds of strains and species can be enriched. Other studies have shown that apart from the use of complex media for isolation, the nature of the carbon source plays an important role in the enrichment of different strains, and it is possible to obtain selective enrichment by including single carbon sources in defined media. Reducing the levels of or excluding complex nutrients and adding sugars, polyols, or other simple organic compounds may lead to the selective enrichment of specific strains under aerobic conditions.

In addition to isolation under aerobic conditions it has also proved possible to isolate a variety of strains growing either microaerophilically (in the light when bacteriorhodopsin is produced) or anaerobically (on arginine or in the presence of nitrate). Variations in the levels of magnesium and calcium as well as the final pH of the medium also play a significant role in the selection of certain isolates, such as the members of the alkaliphilic genera.

The first authentic records of the isolation of members of the family *Halobacteriaceae* may be traced back to Klebahn in 1919. However, the only strains which have survived from the early studies are those of Lochhead, 1934 and Petter 1933. Currently divided into six genera and sixteen species the members of the *Halobacteriaceae* held in the DSM are routinely maintained on one of the following media:

Medium for neutral pH requiring strains:

Medium 1

NaCl	200.0 g
KCl	2.0 g
$MgSO_4$. $7H_2O$	20.0 g
tri-Na citrate	3.0 g
Yeast extract (Oxoid)	10.0 g
Casein hydrolysate (Oxoid)	7.5 g
$FeCl_2$. $4H_2O$	36.0 mg
$MnCl_2$.$4H_2O$	0.36 mg
Distilled water	1000.0 ml

Adjust pH to 7.2 - 7.4

Medium 2

NaCl	200.0 g
KCl	2.0 g
$MgSO_4.7H_2O$	20.0 g
tri-Na citrate	3.0 g
Yeast extract (Oxoid)	5.0 g
Casein hydrolysate (Oxoid)	5.0 g
Na-glutamate	1.0g
$FeCl_2.4H_2O$	36.0 mg
$MnCl_2.4H_2O$	0.36 mg
Distilled water	1000.0 ml

Adjust pH to 7.2 - 7.4

Medium for alkaliphilic strains:

Medium 3

NaCl	200.0 g
KCl	2.0 g
tri-Na citrate	3.0 g
Yeast extract	10.0 g
Casein hydrolysate (Oxoid)	7.5 g
Na_2CO_3	5.0 g
$FeCl_2.4H_2O$	36.0 mg
$MnCl_2.4H_2O$	0.36 mg
Distilled water	1000.0 ml

Adjust pH to 9.5 - 10.0

Medium 4

NaCl	200.0 g
KCl	1.0 g
KH_2PO_4	1.0 g
NH_4Cl	1.0 g
Na glutamate	1.0 g

Yeast extract	5.0 g
Casein hydrolysate (Oxoid)	5.0 g
Na_2CO_3	5.0 g
$FeCl_2.4H_2O$	36.0 mg
$MnCl_2.4H_2O$	0.36 mg
Distilled water	1000.0 ml

Adjust pH to 9.5 - 10.0

If necessary, add agar 20.0 g/l to medium (1 to 4) for solid media.

In the case of both the neutrophilic and alkaliphilic strains liquid cultures may be prepared without any special precautions, since neither salt nor the high pH of the alkaliphilic medium appears to have an adverse affect on the constituents of liquid media. When preparing solid media using agar it may be advisable to autoclave the salt separately from the agar, and allowances should be made in the pH of the NaCl free medium so that the final pH lies between 7.0 to 7.2 when NaCl is added.

In the case of the alkaliphilic media it is essential that the Na_2CO_3 is not autoclaved with the agar, otherwise the agar will darken and not solidify properly. In preparing the alkaline media without Na_2CO_3 care should be taken that the pH of the carbonate free medium is not too acid before autoclaving the agar, otherwise the agar is also adversely affected. While the source of the yeast extract or casein hydrolysate is not critical, care should be taken that low grade preparations are not used since it has been shown that they may contain compounds which cause lysis of the cells and prevent cell growth. Other media have been described in the literature and may be more suitable for certain experimental conditions than these media, which are primarily used for the preservation and reactivation of the strains in the culture collection. In general, cultures are incubated at 37C, although there are some strains which require a lower growth temperature, and the optimum of the majority of the other strains is above 40C.

In the laboratory, members of the family *Halobacteriaceae* may be sub-cultured in either liquid or on solid media. In both cases prolonged incubation can lead to the cultures drying out. In the case of agar plates, which often require 2-4 weeks, incubation, drying out may be prevented by placing the petri dishes in sealed, clean plastic bags. When handling cultures it is important to remember that they should not be suspended in dilute saline solutions or in distilled water, as the cells of all but the members of the genus *Halococcus* will lyse. Similarly cells should not be subjected to excessive mechanical stress as this

will also lead to cell lysis. Growth in large batches, such as in a fermenter, can cause unexpected problems. In particular the limited solubility of oxygen in large culture volumes may result in less than optimal cell yields unless the cultures are adequately aerated. A second effect of reduced oxygen tension in the highly saline culture media is that this promotes corrosion of metal parts, making some fermenters unsuitable vessels for the mass cultivation of these microorganisms. In many cases a simple all glass vessel serves as an adequate alternative.

Members of the family *Halobacteriaceae* may be maintained in the laboratory using a number of methods. Routine sub-culturing at intervals of 2 months has proven a simple method for the maintenance of small collections, although the fast growing members of the genus *Haloferax* may require more frequent transfer. Storage at sub-zero temperatures (-20C or -70C) using elevated concentrations of glycerol (10-15% w/v) has been used in some laboratories, as has storage in stab cultures kept under liquid paraffin at 4-8C. All strains held in the DSM are stored in liquid nitrogen using the capillary method described previously (Hippe, 1984). Fresh growth medium containing dimethyl sulphoxide (5% v/v final concentration) is used to suspend cells harvested under sterile conditions from liquid cultures.

Some culture collections (ATCC and NCIMB for example) have introduced freeze drying methods for the storage and distribution of members of the family *Halobacteriaceae*. This method is not used routinely in the DSM at present, although it appears that the method of drying these bacteria using the pre-dried milk method used in the DSM is suitable for these organisms (Malik.,1988). The critical point in the use of such methods appears to be preventing the cell suspension from freezing. The harvesting of cells for the freezing or drying of a strain appears to be best carried out from liquid cultures (10-20ml) centrifuged at 3,500-4,000 rpm for 15-20 min. Cells are then resuspended in fresh growth medium (containing DMSO or glycerol to give the appropriate and concentration).

Agar grown cultures may also prove suitable, although the cells are subjected to unnecessary mechanical stress (because of a lack of a normal eubacterio cell wall) unless great care is taken. Investigations on the biochemistry, physiology, taxonomy, genetics, and ecology of this group of archaeobacteria are continuing to provide us with more interesting information on this group of extremophiles, which may be easily handled in most microbiological laboratories without specialised equipment.

Selected references for further reading

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