

### Cultivation of Hyperthermophiles

Hyperthermophiles are defined by a temperature optimum for growth around or above 80°C. Most representatives of this group belong to the Archaea, whereas only a few are found among the Bacteria (e. g., *Aquifex pyrophilus*). Hyperthermophiles are distributed in several physiological groups. Many of them are strictly anaerobic and chemolithoautotrophic, but heterotrophic and microaerophilic or obligately aerobic representatives also exist.

**Actively growing strains** of hyperthermophiles are delivered from the DSMZ as cultures that have reached late logarithmic or **stationary growth phase**. Hence, please do not try to increase the cell density of these cultures by further incubation. If, in exceptional cases, a freshly inoculated culture is shipped it is explicitly stated on the culture vial.

In order to exemplify the handling of hyperthermophiles the cultivation of two distinct strains is described in detail: *Pyrolobus fumarii* DSM 11204<sup>T</sup> grows under anaerobic conditions and *Sulfolobus solfataricus* DSM 1616<sup>T</sup> under aerobic conditions.

#### ***Pyrolobus fumarii* DSM 11204<sup>T</sup>**

*Pyrolobus fumarii* belongs to the *Crenarchaeota* and represents the most extreme thermophilic organism known to date available in pure culture. It was isolated from material of a black smoker (hydrothermal vent on the sea floor) at the Mid Atlantic Ridge and extends the upper temperature limit for life to 113°C (Blöchl et al., 1997).

You will receive from the DSMZ an actively growing culture of DSM 11204<sup>T</sup> in a serum bottle which contains 20 ml of medium 792 under 2 bar overpressure of a 80% H<sub>2</sub> and 20% CO<sub>2</sub> gas mixture.

**Overpressure** is applied to culture vials of most anaerobic hyperthermophiles in order to prevent the medium from boiling during incubation and to keep the pH value stable. In addition, numerous anaerobic hyperthermophiles have an autotrophic metabolism and hence H<sub>2</sub> and/or CO<sub>2</sub> represent important nutrients which have to be supplied in excess to avoid growth limitation.

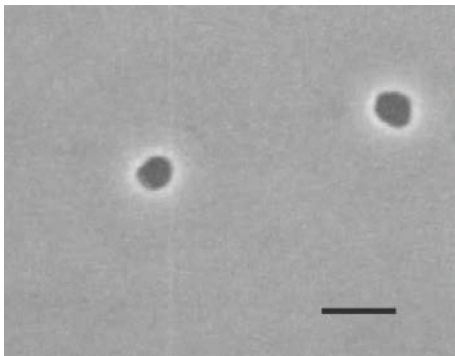
**Caution: Please wear safety goggles during handling of overpressurized cultivation vessels and place vials during incubation in containers that protect against broken fragments in case of explosion. Use culture vessels (e.g. Balch tubes) that resist substantial pressure and are filled only up to 25% with liquid medium. Serum bottles are normally not pressure rated and should be handled with special care.**

In general, the maximal cell densities reached by hyperthermophiles in laboratory batch cultures are rather low resulting in a faint or almost non-visible turbidity at the end of the growth phase. In addition, some media tend to form precipitates after incubation leading to an increase of turbidity even without growth of the culture. Hence, proliferation of cells should be always checked microscopically.

After receipt of a culture of DSM 11204<sup>T</sup> first remove overpressure by puncturing the septum (after sterilization with 70% ethanol) with a sterile injection needle. Remove a sample with a sterile anoxic syringe (see DSMZ Technical Information [Cultivation of Anaerobes](#)) and check the culture for cells by phase-contrast microscopy. Then pressurize again the vial to 2 bar overpressure with H<sub>2</sub>/CO<sub>2</sub> gas mixture. Normally, under the microscope only a few nonmotile, irregular coccoid-shaped or lobate cells can be found per field of view (Fig. 1).

Prepare medium 792 recommended for strain DSM 11204<sup>T</sup> according to the instructions given on the DSMZ web site ([http://www.dsmz.de/microorganisms/medium/pdf/DSMZ\\_Medium792.pdf](http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium792.pdf))

Please note that *Pyrolobus fumarii* is an obligate chemolithoautotrophic microorganism and even traces of organic substances (as impurities of used chemicals or remainings on glass ware) may significantly inhibit growth. Therefore, only use ultrapure chemicals for medium preparation and thoroughly cleaned glass vials rinsed with distilled water as containers for the medium.



**Fig. 1** Phase-contrast micrograph of *Pyrolobus fumarii* DSM 11204<sup>T</sup>. Bar, 5  $\mu$ m.

Vials with freshly prepared medium are inoculated with 5-10% of a culture grown to the late logarithmic phase. It is advisable to prepare a **dilution series** of all hyperthermophiles sensitive to lysis before incubation. Incubate at 103°C for 16 to 74 h. After a variable lag phase growth of this strain can be very fast with **rapid lysis** after stationary phase, thus we do not recommend incubation overnight. Rather, we suggest to check growth at regular intervals during the day. *Pyrolobus fumarii* couples growth with the reduction of nitrate to ammonium. Hence, proliferation of cells can be checked either by microscopy or monitoring of  $\text{NH}_4^+$  production with Nessler's or OPA reagent (Holmes et al. 1999). Often ammonium production starts before a significant number of cells can be found. At the beginning of the stationary phase a white precipitate is formed and the medium can turn slightly pink due to the accumulation of nitrite. That is because nitrite increases the redox potential in the medium which causes the redox indicator resazurin to turn from colorless to pink.

At this phase the culture is not very stable even at room temperature and should be transferred immediately in fresh medium. Only young cultures (early logarithmic growth phase) are relatively stable and can be stored several weeks at 20°C

### ***Sulfolobus solfataricus* DSM 1616<sup>T</sup>**

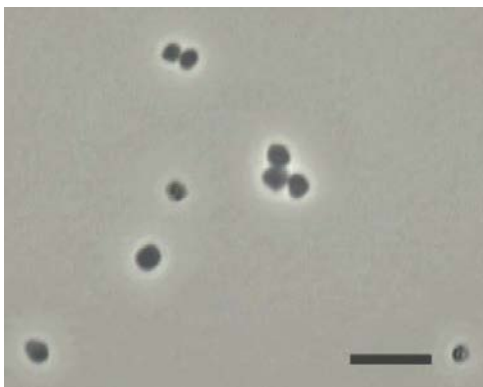
The archaeon *Sulfolobus solfataricus* DSM 1616<sup>T</sup> belongs phylogenetically to the *Crenarchaeota* and was isolated from a volcanic hot spring in Italy (Zillig et al., 1980). All members of the genus *Sulfolobus* are **obligately aerobic**, acidophilic, and thermophilic to hyperthermophilic. The preferred substrates for organotrophic growth are complex organic materials, like peptone or yeast extract. Most strains can grow also facultatively lithotrophic by the oxidation of reduced sulfur species (e. g., sulfidic ores or elemental sulfur) to sulfuric acid. The strain *Sulfolobus solfataricus* DSM 1616<sup>T</sup> has lost however the ability to oxidize elemental sulfur ( $\text{S}^0$ ). Members of this genus are adapted to acidic environments and generally do not grow above a pH of about 6.0. At pH values above 7.5 the cells of most strains lyse. Hence, care has to be taken to adjust the pH of the medium to the correct value. The **pH optimum** for growth of most strains is between 3.0 and 4.5.

You will receive from the DSMZ an ampoule with a freeze-dried sample of strain DSM 1616<sup>T</sup>. Prepare medium 182 recommended for this strain according to the instructions given on the DSMZ web site ([http://www.dsmz.de/microorganisms/medium/pdf/DSMZ\\_Medium182.pdf](http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium182.pdf)).

Open the ampoule carefully as described in [Handling of cultures and opening of ampoules with freeze-dried material](#). Add approx. 0.5 ml of the freshly prepared medium to the freeze-dried pellet and resuspend it. Transfer the cell suspension by using a sterile Pasteur pipette to 5 ml medium in **screw cap tubes** (18 x 100 mm, borosilicate glass).

Prepare 1:10 and 1:100 dilutions in two other tubes and incubate all tubes in a slanted position at 70°C. Growth should become evident after 2-5 days. However, sometimes cultures need a longer time for recovery and should be given at least two weeks incubation time before regarding them as non-viable. For the cultivation of larger volumes prepare medium in screw cap Erlenmeyer flasks (e. g., 30 ml medium in a 200 ml flask) and incubate without shaking. Use 10% (v/v) of a culture grown to the late logarithmic phase as inoculum.

Growing cultures are sensitive to lysis and should be checked by phase-contrast microscopy in regularly intervals. Cells of *Sulfolobus* species have a highly irregular, spherical shape with diameters ranging from 0.7 to 2 µm. They occur usually as single cells and stain Gram-negative. A phase-contrast micrograph of cells of DSM 1616<sup>T</sup> is shown in Fig. 2.



**Fig. 2** Phase-contrast micrograph of *Sulfolobus solfataricus* DSM 1616<sup>T</sup>. Bar, 5 µm.

Several methods are possible for the **maintainance** of grown cultures of *Sulfolobus* and *Acidianus* species:

1. Storage at 60°C: Cultures generally are viable for about 2-4 weeks. Use for transfer to fresh medium at least an inoculum size of 20% (v/v).
2. Storage at room temperature: Cultures have to be transferred weekly to fresh medium. Use at least 10% inoculum.
3. Storage under N<sub>2</sub> gas atmosphere: According to Grogan (1989) the viability of *Sulfolobus* species could be significantly increased when cultures were stored anaerobically (under nitrogen) at a pH of about 5.5. Similarly, the facultative aerobic strains of *Acidianus* survive better anaerobically.
4. Storage after neutralization: After incubation keep the culture for 1 h at room temperature. Transfer 4.5 ml of the grown culture to another tube containing 20 mg CaCO<sub>3</sub>, sterilized together with some drops of water. Store neutralized cultures at 4-8°C. Cultures treated in this way have been found viable after 1 month of storage.
5. Preservation in liquid nitrogen:  
Incubate 50 ml culture in a 300 ml screw cap Erlenmeyer flask. Keep the grown culture for 2 h at room temperature and add sterile CaCO<sub>3</sub> in excess. After a short time allowed for settling of CaCO<sub>3</sub> and CaSO<sub>4</sub>, the supernatant is transferred to sterile tubes and centrifuged. The cell sediment is suspended in a small amount of fresh sterile medium, which has been neutralized with CaCO<sub>3</sub> and supplemented with 10% glycerol (sterilized separately). Ampoules with 0.1 ml of the suspension are frozen and stored in the vapour phase of a liquid nitrogen container. For reviving, ampoules are thawed quickly in a water bath at 37°C and transferred into about 20 ml medium, which has not been neutralized.

### Literature

Blöchl, E., Rachel, R., Burggraf, S., Hafenbradl, D., Jannasch, H. W., and Stetter, K. O. 1997. *Pyrolobus fumarii*, gen. nov. and sp. nov., represents a novel group of archaea, extending the upper temperature limit for life to 113°C. *Extremophiles* **1**, 14-21

Grogan, D. W. 1989. Phenotypic characterization of the archaeobacterial genus *Sulfolobus*: comparison of five wild-type strains. *J. Bacteriol.* **171**, 6710-6719

Holmes, R. M., Aminot, A., Kerouel, R., Hooker, B. A., Peterson, B. J. 1999. A simple and precise method for measuring ammonium in marine and freshwater ecosystems. *Can. J. Fish. Aqua. Sci.* **56**, 1801-1808.

Zillig, W., Stetter, K. O., Wunderl, S., Schulz, W., Priess, H., and Scholz, J. 1980. The *Sulfolobus*-*Caldariella* group: taxonomy on the basis of the structure of DNA-dependent RNA polymerases. *Arch. Microbiol.* **125**, 259-269

### Notes

#### 1. Abbreviations

approx.= approximately

fig = figure

2. Red colored information indicates an important subject regarding to the content given herein.

3. The information contained herein is offered for informational purposes only and is based on the present state of our knowledge. Recipients of our microorganisms must take responsibility for observing existing laws and regulations. DSMZ does accept no responsibility for the accuracy, sufficiency, reliability or for any loss or injury resulting from the use of the information.

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