Culturing of *Chlorella vulgaris* - Standard Operating Procedure

**Introduction**

*Chlorella vulgaris* is a planktonic unicellular green alga used as a food source for *Daphnia magna* (may not be suitable for *D. pulex* as cells are very large – pers com with a number of people over the years). Thus, this SOP describes the culture methods used for *C. vulgaris* intended for *D. magna*.

*Chlorella vulgaris* is cultured in Bold’s Basal Medium, prepared as described in SOP BBM at end of this document. Cultures are maintained in 5 L glass fermenter bottles and aerated (0.2 µm filtered) to aid gas exchange and to keep algal cells in suspension.

Precautions must be taken at all times when preparing the inoculum, fermenter vessels and removing the algae to prevent microbial contamination. This involves use of sterile techniques at all times.

The culture is maintained on a semi-continuous basis. This means that once a week, when the cultures are established and sufficiently green, at least 500 ml of the algal suspension is removed and the fermenter contents replenished with fresh BBM medium. This method of maintenance results in the density of algal cells in the culture fluctuating with time. The removed algal suspension is then used to prepare the *D. magna* food. Take algae from alternate fermenters (keep at least two cultures running simultaneously) every week or when necessary so as to allow enough time for re-growth. Record volumes taken from each vessel and volume of food prepared (at OD 1/10 dilution 440nm – 10mm pathway) in logbook.

NB! Do not keep fermenters active for more than three months as dead cells accumulate at the bottom of the vessel and will, if fed to the daphnia cause gastric blockages. I.e. the food quality declines. If there is the slightest suspicion that a fermenter may be contaminated, discard without hesitation.

**Algal Slopes**

*Chlorella vulgaris* is stored on agar slopes as a reserve and to inoculate future fermenters. Nutrient agar is prepared by dissolving 1.5 g in 100 ml RO water, aliquoting 4 ml volumes into as many culture tubes as possible and sterilising by autoclaving. After they have been autoclaved but whilst still liquid, place tubes on their sides so that agar sets on a slope.

Always inoculate slopes in duplicate, at least once a month. One is used to create further slopes and the second one to inoculate a fermenter. Incubate inoculated slopes under illumination at 20°C for two weeks and store at 4°C in dark. Label tube with *C. vulgaris*, inoculation date and operator’s initials.

**Aseptic techniques for algal slope preparation**

- Loosen the top of a stock algal slope tube
- Flame a wire loop to redness, also heat the chuck of the handle initially
- With the loop in one hand and the slope in the other, remove the top of the slope tube with the crook of the little finger of the loop hand (retain the top, do not put it down)
- Flame the neck of the slope tube containing the algae on a Bunsen burner
- Cool the loop briefly, on a patch of agar with no algae before collecting some algae by rasping the growth area.
- Re-cap algal slope
- Whilst still holding the loop, remove the top of a fresh agar slope tube as described above
- Flame the neck of the fresh tube and inoculate with algae on loop using a gentle zig-zag motion
- Re-cap tube and incubate under illumination at 20°C for up to two weeks
- Store at 4°C in darkness

**Inoculating the fermenter**

Prepare fermenter with 4 L BBM solution (see below). Bung with non-absorbent cotton wool and cover top with aluminium foil. Take down to Central Services as routine autoclave, no need for pre-booking as small enough to fit in system. Allow to cool to 20°C before inoculating.

Prepare a small stock of BBM solution (~100 ml) and autoclave. Then aliquot 1ml into 1.5 ml microcentrifuge tubes and store in freezer. These will be used to inoculate the fermenters.

Algal fermenters are inoculated directly from a stock algal slope on nutrient agar. Pipette 1 ml of sterile BBM from aliquots into a slope tube, mix by pipetting up and down and transfer contents to fermenter. At all times using aseptic techniques (see below).

Alternatively fermenters can be started from a pre-prepared inoculum culture. Inoculum cultures are prepared in 100ml Duran bottles containing 50 ml of sterile BBM, using aseptic techniques and incubated under illumination at 20°C until sufficiently green. It is ideal for speed starting new fermenters, but requires forward planning! It is a good idea to have sterile stocks of 50 ml BBM in the fridge for this purpose.
Fermenter vessel setup

Refer to above diagram for assemblage details.

Notes: The fermenters consist of a 5 L glass bottle, a rubber bung with four glass tubes; two short and two long.

The air supply must be filtered through a 0.22 µm syringe-driven filter unit (Millipore). Keep maximum aeration so that cells are kept in suspension and there is sufficient air exchange. Sterile tubing used (TWT-200-064A, Nalgene) must be autoclaved along with glass tubes in bung, connectors and non-return valve (Nalgene). Connect glass tubes and Nalgene tubes where possible before autoclaving. To autoclave wrap in aluminium foil. Place a 200 µl filter tip in air vent to avoid bacterial contamination (although air is blow out through this vent it is possible for microorganisms to enter, especially is there is a power cut, or pump stops working. It is somewhat frustrating to risk a culture in this manner).
Label fermenter as culture A, B or C, BBM preparation date, pH, inoculation date and operators initials.
Aseptic techniques for fermenter inoculation

- Loosen the non-absorbent cotton wool bung in the neck of the fermenter but keep covered with the aluminium foil
- Loosen the top of an algal slope tube
- Flame the neck of algal slope tube on a Bunsen burner
- Transfer 1 ml BBM from aliquot to algal slope tube
- Cap algal slope and suspend algae by briefly shaking tube
- Flame the neck of the fermenter
- Remove the cotton wool bung inside the aluminium foil
- Pour contents of algal slope and recap fermenter with cotton wool
- Flame the neck of the fermenter again and assemble with glass tubes, tubing and bung near the Bunsen burner
- Connect fermenter to the 20 L BBM stock solution using Bunsen burner in culture room.

Removing algal suspensions from the fermenters

To remove algae from the fermenter first close the vent pipe (c on diagram above) then immediately open the siphon out tube (b). Discard the first 50 ml as this may contain dead cells accumulated in the exit tube. Collect remainder for food preparation. To stop algal flow close siphon out tube (b) first and then open air vent again (c).

Top up fermenter with fresh BBM, equal to the volume removed, by loosening clamp on 20 L BBM stock (d). Fresh solution should flow freely into fermenter by siphon action. When newly assembled, to prime 20L BBM stock, it is necessary to use a sterile 50 ml syringe with a sterile fine needle. Close clamp to fermenter and puncture tube with syringe in between clamp and non-return valve, suction to fill tube with solution, remove needle and seal hole with insulating tape or similar to stop leakage/possible contamination. The replacement solution is now ready to use as instructed above.

Always set up a new fermenter when preparing a fresh 20 L BBM stock and assemble together.

Measure the optic density of a 1/10 dilution of the algal suspension before centrifuging and record in log book. This will allow to monitor algal growth as well as give and indication of the volume of water to resuspend the algae in (see below).

Centrifuge collected algal suspension, in 1 L Sorval containers, at 3,000 rpm for 30 min at room temperature. Discard supernatant and resuspend in deionised water to an optical density of 0.800 at 440 nm of a 1/10 dilution using a spectrophotometer.

Log Book must contain at least the following information:
- Fermenter reference (i.e. A, B or C),
- Algal suspension removal date
- Algal suspension removal volume
- Algal suspension OD (1/10)
- Volume of BBM replaced (if not equal to that removed)
- Volume of food prepared
- OD (1/10) of food prepared
- Operators Initials
SOP on how to prepare Bold’s Basal Medium (BBM)

Introduction

Bold’s Basal Medium (BBM) is an inorganic salts medium widely used for the culture of free-living planktonic freshwater algae and is used at the University of Reading for culturing *Chlorella vulgaris* as food for *Daphnia magna*. It is recommended for culturing such organisms by the Culture Centre of Algae and Protozoa, Cambridge (CCAP 1992)

Preparation of Stock Solutions

When the medium is prepared in aqueous solution from a single stock solution containing all chemicals some constituents precipitate, because of this stock solutions of each chemical should be prepared separately as indicated in Table 1. All solutions must be prepared using distilled (RO) water.

<table>
<thead>
<tr>
<th>Stock Solution No.</th>
<th>Chemical name</th>
<th>Formula</th>
<th>Weight (g)</th>
<th>Distilled Water (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>di-potassium hydrogen orthophosphate</td>
<td>K₂HPO₄</td>
<td>1.875</td>
<td>250</td>
</tr>
<tr>
<td>2</td>
<td>Potassium di-hydrogen orthophosphate</td>
<td>KH₂PO₄</td>
<td>4.375</td>
<td>250</td>
</tr>
<tr>
<td>3</td>
<td>Magnesium sulphate</td>
<td>MgSO₄·7H₂O</td>
<td>1.875</td>
<td>250</td>
</tr>
<tr>
<td>4</td>
<td>Sodium Nitrate</td>
<td>NaNO₃</td>
<td>6.250</td>
<td>250</td>
</tr>
<tr>
<td>5</td>
<td>Calcium chloride</td>
<td>CaCl₂·2H₂O</td>
<td>0.625</td>
<td>250</td>
</tr>
<tr>
<td>6</td>
<td>Sodium Chloride</td>
<td>NaCl</td>
<td>0.625</td>
<td>250</td>
</tr>
<tr>
<td>7</td>
<td>EDTA tetrasodium salt</td>
<td>EDTA - Na₄</td>
<td>5.000</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
<td>Potassium hydroxide</td>
<td>KOH</td>
<td>3.100</td>
<td></td>
</tr>
<tr>
<td>9*</td>
<td>Ferrous sulphate</td>
<td>FeSO₄·7H₂O</td>
<td>0.498</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Sulphuric acid conc.</td>
<td>H₂SO₄</td>
<td>0.1mL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(wt per mL = 1.84g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Zinc sulphate</td>
<td>ZnSO₄·7H₂O</td>
<td>0.353</td>
<td>25</td>
</tr>
<tr>
<td>11</td>
<td>Manganese chloride</td>
<td>MnCl₂·4H₂O</td>
<td>0.058</td>
<td>25</td>
</tr>
<tr>
<td>12</td>
<td>Cupric sulphate</td>
<td>CuSO₄·5H₂O</td>
<td>0.063</td>
<td>25</td>
</tr>
<tr>
<td>13</td>
<td>Cobaltous nitrate</td>
<td>Co(NO₃)₂·6H₂O</td>
<td>0.020</td>
<td>25</td>
</tr>
<tr>
<td>14</td>
<td>Sodium molybdate</td>
<td>Na₃MoO₄·2H₂O</td>
<td>0.048</td>
<td>25</td>
</tr>
</tbody>
</table>

*Needs heating to ~50-60°C to dissolve. NB. All chemicals should be weighed on aluminium foil.
Storage conditions and expiry dates of stock solutions

Stock solutions 1 - 6 may be stored up to 3 months and solutions 7 - 14 may be stored up to 12 months from preparation, or all may be stored indefinitely if autoclaved. All solutions can be stored at room temperature in the dark. If precipitation occurs in any stock solution it should be renewed regardless of expiry date.

Each stock solution should be labelled with:

- Name of chemical:
- Solution number:
- Date of preparation:
- Operator’s initials:
- Expiry date:

Preparation of BBM

The following volumes of appropriate stock solutions are based on preparing 1L of BBM media should be added in numerical order per, as given in Table 1, to a conical graduated flask containing 900mL distilled water. Once all chemicals have been added, distilled water is added to complete 1L volume of medium.

- 10mL of each stock solutions 1-6
- 1mL of each stock solutions 7-9
- 0.1mL of each stock solutions 10-14

The flask may be placed on an auto-stirrer for 30 min to ensure thorough mixing of the medium.

After stirring, the pH of the medium is measured. This should be in the range of 6.7 ± 0.3 (i.e. 6.4 - 7.0). NB! If the pH is outside this range, the batch of medium should be discarded and another batch prepared.

BBM containers should be labelled with:

- Date of preparation:
- Operator’s initials:
- pH:

Batches of BBM prepared in this way are not sterile and should be autoclaved following preparation. Autoclaved medium can be stored indefinitely.

When preparing 20 L in glass bottle for semi-continuous algal culture, bung air inlets with non-absorbent cotton wool or filter tip, to avoid stock BBM contamination.