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HEPES-Phosphate Medium, Suitable for Studies of Trace Element Nutrition in Photoautotrophic and Heterotrophic Auxenochlorella protothecoides.

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Disclaimer

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Abstract

This protocol describes a method for preparing a defined medium for phototrophic and heterotrophic *Auxenochlorella protothecoides* (UTEX 250). It is adapted from the commonly used TAP medium for *Chlamydomonas reinhardtii* (Goodenough, 2023, Chapter 11), with special attention to trace element supplementation (Kropat, Hong-Hermesdorf, et al., 2011) and buffer composition. The Tris-acetate buffer is replaced with a HEPES buffer to control acidification of the growth medium as cells consume glucose. As a starting point, we determined the elemental composition of laboratory-grown *Auxenochlorella protothecoides* cells by ICP-MS/MS (Hui et al., 2022) and calculated how much of each essential element would be required in the growth medium to support growth of a culture with an initial density of 10^5 cells / mL to stationary phase (2.4 x 10^8 cells / mL). The elemental composition of the medium before and after growth of cells to stationary phase was also measured to ensure that each element was provided in excess (2- to 5-fold) to accommodate variation in elemental quotas in response to the external conditions. All elements provided in the medium were measured except for H, C, N, O, and Cl. High purity chemicals ($\geq$99.999% pure with certificate of analysis indicating the level of contamination with other salts) are required for successful elemental deficiency and all glassware and plasticware are freshly washed with 6 M HCl (Quinn & Merchant, 1998).

Guidelines

Download all Safety Data Sheets (SDS) for each chemical you will purchase. Review the proper handling and disposal guidelines from your institution for each chemical. Review your municipal wastewater drain disposal guidelines before disposing any chemicals down the drain. Wear approved personal protective equipment (PPE) such as a lab coat, closed toe shoes, eye protection, and gloves.

To avoid precipitation in the stock solutions and the final medium, it is important to add the chemicals in the specified order. Various methods can be used for mixing solutions. To mix macronutrient stock solutions, we recommend slowly adding chemicals to the acid washed 1 L bottles that are prefilled with the specified volumes of Milli-Q H_2O and mixing by shaking the tightly capped bottle vigorously. Alternatively, you may slowly add solutes to 1 L bottles containing an acid washed PTFE coated 55 mm magnetic stir bar (VWR 76264-442), placed on a magnetic stirrer at 450 rpm. Minimize exposure of solutions to dust particles by closing bottles while mixing. For micronutrient solutions, we recommend adding chemicals slowly to the 250 mL bottle filled with the specified volume of Milli-Q H_2O and placing the tightly capped bottle on a rocker for 00:30:00. Some chemicals may not dissolve completely unless titrated to the proper pH. To avoid metal contamination from a pH probe, we recommend using metal free pipette tips to transfer 30 µL of the solution onto an MQuant pH-indicator strip (non-bleeding) pH 5.0 – 10.0 (Supleco 1.09533.001). Do not insert the pH strip into the solutions.

For hygroscopic chemicals, we recommend ordering the smallest amount that will meet your needs and reconstituting the entire bottle into solution immediately after opening. Chemicals labeled as anhydrous, ultra-dry, dehydrated, or hygroscopic will have different molecular weights than chemicals containing hydrates. Most ultra-pure chemicals are only available in the hygroscopic form, so be sure to verify the molecular weights and the masses required to achieve the concentrations in this protocol. When hygroscopic chemicals are exposed to atmospheric moisture, they will gain mass, so it is important to process the entire vial to generate a solution of known concentration immediately after opening. Search for the chemical's solubility in water and obtain an appropriately sized acid washed HDPE bottle with a pre-drawn line indicating the fill level. Fill the HDPE bottle halfway with Milli-Q H_2O. Add the chemical to the HDPE bottle containing water. Rinse the vial with water and pour the water from the rinse into the HPDE stock bottle. Repeat rinse three times, transferring each rinse to the HDPE bottle. Fill the bottle to the fill line. Mix the solution well. Label the solution with the estimated concentration and store at 4 °C. The actual concentration can be verified by ICP-MS/MS measurement.

Materials

(see guidelines and before start section before ordering materials)

Consumables

Trace metal grade ammonium chloride, MW 53.49
Trace metal grade calcium chloride, MW 110.98
Trace metal grade magnesium sulfate heptahydrate, MW 120.37
Trace metal grade HEPES, MW 238.30
Trace metal grade potassium dihydrogen phosphate, MW 136.08
Trace metal grade sodium hydroxide, MW 40.00
Trace metal grade thiamine hydrochloride, MW 337.27
Trace metal grade dextrose, MW 180.6
Trace metal grade EDTA, MW 292.24
Trace metal grade ammonium molybdate tetrahydrate, MW 1235.86
Trace metal grade sodium selenite anhydrous, MW 172.94
Trace metal grade zinc sulfate, MW 287.56
Trace metal grade manganese(II) chloride tetrahydrate, MW 197.9
Trace metal grade ferric chloride, MW 162.2
Trace metal grade sodium carbonate anhydrous, MW 105.99
Trace metal grade copper(II) chloride, MW 134.45
Trace metal grade 12 M HCl
Trace metal grade potassium hydroxide, MW 56.11
Trace metal grade Milli-Q water (see guidelines and before start section)
Pure baking Soda
1 L HDPE Bottles x 5 (Nalgene 2104-0032)
250 mL HDPE Bottles x 10 (Nalgene 2104-0008)
Plastic spatulas
pH strips, pH 5 - 10
0.22 micron pore size syringe filter
Autoclave tape
Aluminum foil
RAININ P1000, P200, P20 pipettes

Equipement

Magnetic stir plate
PTFE coated 55 mm magnetic stir bars
4 L beaker
1 L HDPE graduated cylinder
100 mL HPDE graduated cylinder
Fume hood
Analytical balance
Sterile hood

Autoclave
Autoclave bin
Refrigerator
RAININ P1000, P200, P20 pipette tips

Safety warnings

HCl is highly corrosive and toxic. Wear a lab coat, gloves, and eye protection when handling HCl. Perform all work that involves HCl in a fume hood to avoid inhalation.

Before start

Table 1. Nutrient composition of the HEPES phosphate medium.

Species	Concentration (mM)	Species	Concentration (µM)
C	111	Mn	12
N	7.5	Fe	20
Na	*6.28	Cu	2
Mg	2.4	Zn	10
P	11.3	Se	0.1
S	0.4	Mo	0.2
K	18.1		
Ca	0.34		

*Na concentration includes Na from NaOH, Na_2SeO_3, and Na_2EDTA present in some trace element solutions.

Obtain only high purity chemicals which are usually labelled as “metals basis,” “extra pure,” or “≥99.999%.” More importantly, check the “Specifications” tab of each chemical to verify that it has been assayed for the elements that you are interested in. Choose brands that have the lowest allowance for the elements that your lab is interested in. Contact the chemical’s manufacturers to get the certificate of analysis (CoA) for each chemical. Specify the lot number you have chosen so that the correct item is shipped.

When chemicals arrive, record each lot number and label the bottles with the date received, date opened, and quantity (e.g. 1 of 5). High purity trace metal grade chemicals typically have a higher cost than normal chemicals, so it is recommended that they be labeled and their use restricted for trace metal work. Avoid inserting spatulas into containers; instead, carefully shake or pour chemicals out. Chemicals brought out of the original container should not be returned into the container. Use plastic, non-metal, spatulas to handle chemicals.

Navigate to the supplier’s website and enter the lot number in the “Certificates” search tab. Download and review the certificate of analysis (CoA) of the specific batch. For example, you may find the certificates search tab for Millipore Sigma at this link: https://www.sigmaaldrich.com/US/en/documents-search?tab=coa You can view an example CoA for potassium dihydrogen phosphate by entering 1.05108 as the product number and B1642708 as the batch number. Record the values of each element you are interested in studying and calculate the potential concentration of contaminating metals expected in the final medium. See an example of this calculation for potentially up to 0.005 ppm of Cu contamination from potassium dihydrogen phosphate in equation 1. These estimates will provide insight into potential contamination, but ultimately, you should measure the final medium.

Equation 1. Calculation of potential Cu contamination in the final medium from KH_2PO_4 alone.

$$\frac{[KH_2PO_4] \text{ in final medium}}{1.13 \times 10^{-2} mol / L} \times \frac{\text{M.W. of } KH_2PO_4}{136.09\ g/mol} \times \frac{[Cu] \text{ in } KH_2PO_4}{0.005 / 10^6} \times \frac{\text{M.W. of Cu}}{1\ mol / 65.55\ g} = \frac{[Cu] \text{ in final medium}}{1.17 \times 10^{-10} mol / L}$$

The final medium may have as much as [M] 117 picomolar (pM) of Cu from KH_2PO_4 alone. Total potential Cu contamination in the medium will be the sum of potential Cu contamination from all chemical components. See table 2 for potential contamination from macronutrient stocks if 1 ppm of each metal is present in each chemical.

Table 2. Concentrations of Fe, Cu, and Zn in the final medium if 1 ppm level in macronutrient ingredients.

Component	M.W. (g / mol)	Potential contamination (nM) Fe	Cu	Zn
Ammonium chloride	53	7	6	6
Calcium chloride	111	1	1	1
Magnesium sulfate	120	5	5	4
HEPES	238	43	37	36
Potassium dihydrogen phosphate	136	28	24	24
Potassium hydroxide	56	7	6	6
Sodium hydroxide	40	5	4	4
D-glucose	180	358	315	306

Use only ICP-MS grade ultrapure Milli-Q H2O, which can be sourced for example, from the Milli-Q® Advantage A10 Water Purification System (Model: Z00Q0V0T0). This system is internally outfitted with an A10 UV lamp (Cat# ZFA10UVM1), a Q-GARD® T2 pack filter (REF QGARDT2X1), and a Quantum® TIX Ultrapure Cartridge (REF QTUM0TIX1). An additional filtration step using a Q-POD® Element with a Quantum® ICP filter (REF QTUM00ICP) ensures ppt (parts per trillion) to sub ppt levels of trace elements. Before using the Milli-Q H_2O, verify that the resistivity is 18.2 MΩ.cm at 25 °C and the total organic carbon (TOC) reading is <10 ppb.

Wash all culture flasks, stock solution containers, and graduated cylinders with 6 M HCl

1 Dilute pure (certified ACS plus) [M] 12 Molarity (M) HCl to [M] 6 Molarity (M) . To dilute 1 L of [M] 12 Molarity (M) HCl to 2 L of [M] 6 Molarity (M) HCl, add 1 L of Milli-Q H_2O to an empty [M] 12 Molarity (M) HCl bottle that has only previously contained unused [M] 12 Molarity (M) HCl (or use a dedicated mixing bottle). Slowly add 1 L of [M] 12 Molarity (M) HCl to 1 L of Milli-Q H_2O. Always add acid to water and never add water to acid. The bottle may heat up as the acid is diluted, so let it cool before capping and mixing.

2 Do not use glass containers to store stock solutions as glass will leach metal contaminants. Use new (previously unused) wide-mouth polypropylene / translucent high-density polyethylene (HDPE) bottles. For macronutrient stock solutions, prepare five 1 L bottles (Nalgene 2104-0032). For micronutrient stock solutions, prepare ten 250 mL bottles (Nalgene 2104-0008) and one bottle for [M] 2 Molarity (M) NaOH. Keep the bottles capped as much as possible to avoid dust from entering the bottles.

3 For each bottle, fill with 100 mL , 250 mL , or 1 L , of Milli-Q H2O to mark the fill line. This can be achieved by weighing the bottle with the appropriate amount of water. Repeat for all bottles.

4 Discard the Milli-Q H_2O used in step 3 and add 250 mL of fresh (unused) [M] 6 Molarity (M) HCl to one bottle. Cap bottle and swirl to wash the interior. Pour into the next bottle and repeat for all stock solution bottles. Rinse bottles with Milli-Q H_2O at least 7 times.

5 To clean culture flasks, perform an initial [M] 6 Molarity (M) HCl wash by filling a 250 mL culture flask with 100 mL [M] 6 Molarity (M) HCl and swirl. Pour used HCl into the next flask and repeat for all flasks.

6 For the secondary wash, add 200 mL of [M] 6 Molarity (M) HCl to each flask. Cover each flask with parafilm and leave in the fume hood for 24:00:00 or more. Ensure that the parafilm is properly adhered to the flasks so that they do not get sucked into the fume hood

1d

intake vents. HCl should never be left in the fume hoods uncovered. It is corrosive and may destroy the hoods.

7 Rinse each flask with Milli-Q H_2O seven times (Quinn & Merchant, 1998). Rinse the exterior of the flasks as well so that there is no HCl residue. [M] 6 Molarity (M) HCl will corrode work surfaces. Leave cleaned flasks covered with parafilm so that no dust particles in the air (potentially with contaminating metals) enter the flasks.

8 Re-use or discard used HCl. The [M] 6 Molarity (M) HCl from the secondary rinse of culture flasks can be re-used up to 5 times or until a color change is observed (whichever comes first). HCl used for the first round of culture flask and stock solution bottle washing should not be reused and should be neutralized safely before discarding.

9 To neutralize 1 L of [M] 6 Molarity (M) HCl, fill a 4 L beaker with 1 L water and put it in a secondary container within a fume hood. Slowly add 1 L used [M] 6 Molarity (M) HCl to dilute to [M] 3 Molarity (M) HCl. Neutralize 2 L of [M] 3 Molarity (M) HCl by adding $NaHCO_3$ (Arm & Hammer pure baking soda) slowly, scoop by scoop (< 25 g) until no foam is formed. Use a pH indicator strip to verify that the acid is safely neutralized (pH 7). Please refer to your institutional and municipal guidelines for disposing liquids with high concentrations of NaCl.

Make the macronutrient, thiamine, and buffer stock solutions on a bench near balances, rockers, and/or stirrers. Perform the filter sterilization (steps 13.4 and 14.3) in a sterile hood. Record the actual mass of chemicals added to each solution.

10 Make [M] 1 Molarity (M) HEPES Stock (1 L)

10.1 Dissolve 238.3 g HEPES in approximately 500 mL Milli-Q H2O and fill to 1 L . Mix well until dissolved.

10.2 Do not adjust pH.

10.3 Store at 4 °C .

11 Make [M] 2 Molarity (M) NaOH Stock (100 mL)

11.1 Add 8 g of NaOH and fill to 100 mL with Milli-Q H_2O. Mix well until dissolved.

11.2 Store at Room temperature in a properly designated cabinet for strong bases.

12 Make 100× Phosphate Solution (1 L).

12.1 Dissolve 153.77 g of KH_2PO_4 in approximately 600 mL of Milli-Q H_2O. Then add 38.15 g of KOH and fill to 1 L using Milli-Q H_2O. Do not adjust the pH. Mix well until dissolved

12.2 Store at 4 °C .

13 Make 40× Macronutrient Solution (N, Ca, Cl, Mg, and S) (1 L).

13.1 Dissolve 1.5 g of fresh anhydrous $CaCl_2$ in approximately 300 mL of Milli-Q H_2O. Alternatively, use a liquid stock of $CaCl_2$.

13.2 In a separate acid washed bottle, dissolve 11.55 g $MgSO_4 \cdot 7H_2O$ and 16.05 g NH_4Cl in approximately 500 mL of Milli-Q H_2O completely.

13.3 Mix slowly with $CaCl_2$ solution and fill to 1 L with Milli-Q H_2O. Mix well until dissolved.

13.4 Filter-sterilize the solution, using a 0.22 µm pore syringe filter with proper sterile technique in a sterile hood.

13.5 Store at 4 °C and only open the bottle in the sterile hood.

Note

Macronutrient solution will precipitate in the final medium if autoclaved.

14 Make [M] 2 millimolar (mM) (1000×) thiamine stock solution.

14.1 Dissolve 675 mg of thiamine hydrochloride in approximately 800 mL of Milli-Q H_2O.

14.2 Fill to 1 L. Mix well until dissolved.

14.3 Filter-sterilize the solution using a 0.22 µm pore syringe filter in a sterile hood using proper sterile technique.

14.4 Store at 4 °C. Open bottle in the sterile hood only.

Make the preliminary concentrated stock solutions of trace elements.

15 Make Pre 1 - [M] 125 millimolar (mM) Na_2EDTA concentrate (250 mL)

15.1 Dissolve 11.63 g of $Na_2EDTA{\cdot}2H_2O$ in 180 mL of Milli-Q H_2O. Note pH needs to be adjusted to completely dissolve Na_2EDTA. See next step.

15.2 Titrate to pH 8 with trace element grade [M] 2 Molarity (M) NaOH solution (from step 11). Use 43 mL – 47 mL of [M] 2 Molarity (M) NaOH. Measure the pH by pipetting 30 µL of solution onto a pH-indicator strip. Record the volume of [M] 2 Molarity (M) NaOH used.

15.3 Fill to 250 mL with Milli-Q H_2O. Mix well until dissolved.

15.4 Store at 4 °C

16 Make Pre 2 - [M] 285 micromolar (μM) $(NH_4)_6Mo_7O_{24}$ (250 mL)

16.1 Dissolve 88 mg of $(NH_4)_6Mo_7O_{24}$·4H2O in Milli-Q H_2O and fill to 250 mL . Mix well until dissolved.

16.2 Store at 4 °C

17 Make Pre 3 - 1 mM Na_2SeO_3 (250 mL)

17.1 Dissolve 43 mg of Na_2SeO_3 in Milli-Q H_2O and fill to 250 mL . Mix well until dissolved.

17.2 Store at 4 °C .

Make individual metal · EDTA stock solutions (1000×)

18

Note

The micronutrient stocks are the same as those described for Chlamydomonas (Kropat, Hong-Hermesdorf, et al., 2011), except that Zn and Mn are increased four-fold and two-fold, respectively. The Zn·EDTA and Mn·EDTA stock concentrations are increased in this protocol.

Make [M] 25 millimolar (mM) Na_2EDTA (250 mL).

18.1 Add 50 mL of Pre 1 ([M] 125 millimolar (mM) Na_2EDTA concentrate from step 15).

18.2 Fill to 250 mL with Milli-Q H_2O. Mix well until dissolved.

18.3 Store at 4 °C .

19 Make [M] 28.5 micromolar (µM) $(NH_4)_6Mo_7O_{24}$ (250 mL).

19.1 Add 25 mL of Pre 2 ([M] 285 micromolar (µM) $(NH_4)_6Mo_7O_{24}$ from step 16).

19.2 Fill to 250 mL with Milli-Q H_2O. Mix well until dissolved.

19.3 Store at 4 °C .

20 Make [M] 0.1 millimolar (mM) Na_2SeO_3 (250 mL).

20.1 Add 25 mL of Pre 3 ([M] 1 millimolar (mM) Na_2SeO_3 from step 17).

20.2 Fill to 250 mL with Milli-Q H_2O. Mix well until dissolved.

20.3 Store at 4 °C .

21 Make Zn·EDTA stock solution (250 mL).

21.1 Add 22 mL of Pre 1 ([M] 125 millimolar (mM) Na_2EDTA concentrate from step 15)

21.2 Add 720 mg of $ZnSO_4·7H_2O$.

21.3 Fill to 250 mL with Milli-Q H_2O. Mix well until dissolved.

21.4 Store at 4 °C .

22 Make Mn·EDTA stock solution (250 mL).

22.1 Add 24 mL of Pre 1 ([M] 125 millimolar (mM) Na_2EDTA concentrate from step 15).

22.2 Add 594 mg of $MnCl_2 \cdot 4H_2O$.

22.3 Fill to 250 mL with Milli-Q H_2O. Mix well until dissolved.

22.4 Store at 4 °C .

23 Make Fe·EDTA stock solution (250 mL) (does not use Pre1).

23.1 Combine 2.05 g $Na_2EDTA \cdot 2H_2O$ with 580 mg of Na_2CO_3 and dissolve completely in 100 mL of Milli-Q H_2O

23.2 If using fresh anhydrous $FeCl_3$ powder, add 811 mg of anhydrous $FeCl_3$ to the solution only after the previous two components are completely dissolved. Alternatively add trace metal grade $FeCl_3$ liquid stock to a final concentration of [M] 20 millimolar (mM) .

23.3 Fill to 250 mL with Milli-Q H_2O. Mix well until dissolved.

23.4 Store at 4 °C .

24 Make Cu·EDTA stock solution (250 mL).

24.1 Add 4 mL of Pre 1 ([M] 25 millimolar (mM) Na_2EDTA concentrate from step 15).

24.2 Add 67 mg of fresh anhydrous $CuCl_2$ or add liquid stock solution (see note above) to a final concentration of [M] 2 millimolar (mM) .

24.3 Fill to 250 mL with Milli-Q H_2O. Mix well until dissolved.

24.4 Store at 4 °C .

Note

Optional: Measure the elemental content of all stocks using an ICP-MS. Determine if stock solutions contain contaminants and if concentrations are correct.

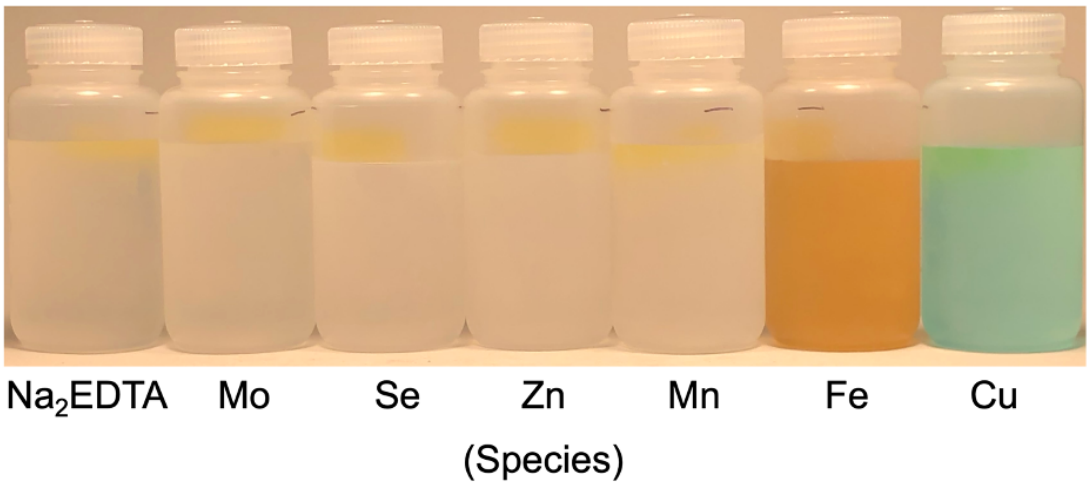

Figure 1. Trace metal stock solutions.
All solutions should be free of precipitates and colorless, except for the Fe stock (brown) and the Cu stock (blue).

Making 1 L of HP medium

25 Close all stock bottles tightly and invert several times to mix.

26 Fill an acid washed 1 L graduated cylinder to 700 mL with ICP-MS grade Milli-Q H_2O.

27 Add 10 mL of [M] 1 Molarity (M) HEPES stock (from step 10).

28 Add 10 mL of 100x phosphate buffer (from step 12).

29 Add 1 mL of each trace element solution (from steps 18 to 24).

> **Note**
>
> If you are using Chlamydomonas micronutrient stocks from (Kropat, Hong-Hermesdorf, et al., 2011), increase Zn and Mn by adding 4 mL of Zn·EDTA and 2 mL Mn·EDTA stocks.

30 Use parafilm to tightly cover the graduated cylinder. Hold the parafilm in place with one hand and mix well by inverting the graduated cylinder at least 10 times. Alternatively, use an acid washed stir bar and an acid washed 1 L bottle to mix.

31 Add [M] 2 Molarity (M) trace metal grade NaOH (from step 11) to bring the pH to pH 7 – pH 7.5. Record the volume of 2 M NaOH used.

31.1 Measure the pH by pipetting 30 µL onto the MQuant pH-indicator strips (non-bleeding pH 5.0 – 10.0, Supleco 1.09533.001).

32 For photoautotrophic growth, fill to 975 mL. For growth with 2% glucose, fill with Milli-Q H_2O to 935 mL.

33 Use parafilm to tightly cover the graduated cylinder. Hold the parafilm in place with one hand and mix well by inverting the graduated cylinder at least 10 times. Alternatively, you may mix in an acid washed 1 L bottle with a pre-labeled 975 mL or 935 mL fill line.

34 For photoautotrophic growth, aliquot 97.5 mL into 250 mL flasks using an acid washed 100 mL graduated cylinder. For mixotrophic or heterotrophic growth, add 93.5 mL.

35 Cover flasks with a double layer of 3 in. x 3 in. aluminum foil (Kirkland Signature Reynolds foodservice foil RK611 item 31680).

36 Put flasks in an autoclave bin and fill the bin with 200 mL of H_2O. Autoclave using the liquid setting. Cool to Room temperature .

37 Add 2.5 mL of filter sterilized 40 x macronutrient (N, Ca, Cl, Mg, and S) solution (from step 13) to each 100 mL flask or 25 mL to 1 L bottle using sterile technique in the sterile hood.

38 Add 100 µL of filter sterilized 1000 x ([M] 2 millimolar (mM) stock) thiamine (from step 14) to each 100 mL flask in a sterile hood

39 For mixotrophic or heterotrophic growth, add 4 mL of filter sterilized 50% glucose to each flask. For photoautotrophic growth, skip this step.

40 Store at Room temperature and use culture flasks within two weeks.

41 Please refer to your institutional and local rules and guidelines for proper and safe disposal of media and stock solutions.

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