Research

Microalgae as a Source of Extracellular Vesicles: Laboratory Cultivation within the Nanostructurome Methods Pipeline

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Abstract:
Extracellular vesicles (EVs) are cell-derived, membrane-bound structures that play vital roles in intercellular communication, biomolecule transport, and various physiological processes. Recent studies have shown that, in microalgal cells, EVs act as key facilitators of intercellular communication via transfer of bioactive compounds to convey regulatory signals and instructions. In aquatic ecosystems, this is most evident in the influence EVs have over community structure, trophic interactions, and cell fate. Microalgae-derived EVs have promising applications in biotechnology, environmental monitoring, and sustainable bioengineering. However, for large-scale production of these microalgae-derived EVs to be feasible and cost-effective, several challenges are yet to be solved, including development of standardized isolation methods, further characterization of EVs biochemical composition, as well as a more comprehensive understanding of their function. Cultivating microalgae in a controlled laboratory setting, with standardized protocols for culture maintenance, is crucial in order to address and solve these challenges, efficiently and reliably.

Keywords: Microalgae; Extracellular vesicles; Laboratory cultivation; Good laboratory practice

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1. Definitions

EVs: Extracellular vesicles

2. Background

Extracellular vesicles (EVs) are membrane-bound structures released by cells into the extracellular space. They play crucial roles in intercellular communication, biomolecule transport, and various physiological and pathological processes (Petrovčíková et al., 2018). EVs carry a diverse array of bioactive compounds, including lipid mediators, proteins, and nucleic acids, which can modify the recipient cell's phenotype (Record et al., 2018). Microalgae, as sustainable sources of bioactive compounds, are also known to produce EVs (Picciotto et al., 2021). EVs-mediated intercellular communication in aquatic ecosystems can influence community structure and trophic-level interactions, and modulate cell fate, morphology, and susceptibility to viruses (Schatz & Vardi, 2018). In recent years, the sustainability, scalability, and renewability of the EVs derived from microalgae were investigated and appeared promising compared to EVs from other sources (Paterna et al, 2022). For example, EVs from microalgae *Tetraselmis chuii* are taken up by human and *Caenorhabditis elegans* cells, recognized and internalized via energy-dependent mechanisms, localizing in the cytoplasm of specific cells and persisting for several days (Picciotto et al., 2021).

Microalgae-derived EVs can be used as nanocarriers for bioactive compounds in health supplements, cosmetics, and food ingredients, and have potential in therapeutic applications due to their ability to deliver drugs or other therapeutic molecules (Picciotto et al., 2021). Up to this point, the existing literature on this topic remains relatively limited, and further research is needed to fully confirm and explore the potential of EVs in these applications. For cost-effective production and utilization of microalgae-derived EVs to be attainable, there are a few recognized challenges that have yet to be overcome:

a) Standardized isolation methods - Developing robust and standardized methods for isolating and purifying microalgae EVs is crucial for reproducible research and applications.
b) Characterizing EVs content - Thoroughly characterizing the composition of microalgae EVs is needed to understand their functions and potential applications. Unlike mammalian exosomes, where there are many known proteins that are used as biomarkers for the presence of these unique EVs [http://www.exocarta.org/], to date,

there are no known candidates to serve as biomarkers for EVs in aquatic environments

c) Understanding EVs' functions - Further research is needed to elucidate the specific roles of microalgal EVs in both microalgal physiology and potential interactions with other organisms.

Overall, microalgae represent a promising source of EVs with potential applications in various fields. Continued research focusing on overcoming the current challenges will unlock the full potential of these vesicles.

In this review, the cultivation methods and proper maintenance of microalgae cultures will be exemplified using several common species (*Chlorella sorokiniana, Chlamydomonas reinhardtii, Chlamidomonas acidophila, Haematococcus pluvialis*).

3. Purpose, Scope, and Applicability

In recent years, the interest for the concept of employing and optimizing microalgal cultivation techniques for various biotechnological and industrial applications, has been steadily growing. One of the key research angles has been the cultivation of microalgae as a means for large-scale production of EVs (Picciotto et al., 2021). The advantage behind the utilization of microalgae for these purposes lies, at least in part, in their ability to thrive in various unfavorable environments. This has biotechnological implications as an opportunity to exploit underutilized resources such as industrial byproducts, wastewater and acid mine drainage ponds (Liberti et al, 2024). Different microalgae strains can be screened and selected for their EV production capabilities. Cultivation in laboratory settings is needed to provide a controlled environment for optimization of microalgal growth conditions (Paterna et al., 2022), as well as allow for development of scalable approaches to EV production and isolation (Picciotto et al., 2021). For example, modifying culture conditions, such as media composition or growing microalgae in co-culture with bacteria, can enhance the culture growth rate or EVs production (Martin et al., 2021). Research in controlled laboratory conditions can also enhance our understanding of the EV-mediated communication in aquatic environments, as a major challenge in the development of EV-based environmental monitoring (Schatz & Vardi, 2018).

4. Health and Safety Warning

Before disposal, sterilization of previously used cultures is performed by autoclaving (125 °C, 60 minutes), to ensure that the risk of contamination is kept to a minimum.

5. Cautions

There is no risk regarding the use of hazardous chemicals in microalgae culture maintenance. Chemicals used for culture media comprise of inorganic salts that should be disposed of according to regulations for disposal of non-hazardous chemicals. To minimize the risk of potential contamination spreading from personnel or the laboratory environment to the microalgae cultures (and vice versa), all work surrounding microalgal culture must be performed in a properly functioning laminar flow hood. All personnel should follow sterile technique procedures when working with microalgae, and should wear protective gear such as lab coats, protective gloves, and goggles, in accordance with the experiments conducted. Microbiological waste generated during the handling of microalgae cultures should be managed according to strict safety and disposal guidelines. It should be treated with appropriate care to avoid contamination and the spread of pathogens. Key steps include:

a) segregation, microbiological waste should be separated from general waste and placed in designated biohazard containers;

b) disinfection, disinfectants or sterilization methods (such as autoclaving) should be used to decontaminate waste before disposal;

d) disposal, dispose of the waste following local environmental and health regulations, often through licensed waste disposal services for biohazardous materials.

Handling microalgal cultures involves the use of glass laboratory equipment (e.g., flasks), requiring careful handling to prevent potential injuries, including cuts. The use of centri-

fuges requires following safety guidelines to prevent mechanical issues and sample contamination. Regular equipment maintenance must be performed to ensure safe and efficient operation.

6. Personnel Qualifications / Responsibilities

Staff handling microalgae cultures should have qualifications in microbiology and/or biotechnology, with training in laboratory safety, biohazard management, and proper handling and disposal of biological materials (Brand et al, 2013).

7. Materials, Equipment, and Supplies

a) Materials:

Nutrients - Inorganic salts, vitamins for medium preparation

Culture vessels (glass or plastic bottles, Erlenmeyer flasks, or tubes for closed systems)

b) Equipment:

Phytotron - chamber for controlled growth conditions of plants and algae, equiped with a light source – LED lamps or fluorescent lamps with an appropriate light spectrum (usually 400–700 nm, PAR spectrum) and temperature controll mechanism.

Orbital shakers - provide continuous circular motion to the liquid culture vessels to maintain cells in suspension, enhance gas exchange and ensure uniform nutrient distribution. They are placed inside the fitotron.

Optical microscope – to monitor cell morphology, track growth parameters such as cell number and viability. Should be equiped with a mountable microscope camera for imaging.

Temperature regulation - incubator with shaking platform for temperature control, thermostatic water bath, phytotron with temperature control

Sterilization equipment – autoclave, sterile filters (0.2 μm), and UV lamps to prevent contamination

Aeration and mixing – aerators or magnetic stirrers to enrich the culture with CO_2 and prevent sedimentation

Centrifuges or membrane filtration systems – for biomass separation

Monitoring equipment (pH sensors, CO_2 dosers)

c) Other equipment: microcentrifuge tubes (0,2 ml, 0,5 ml, 1,5 ml, 2 ml), micropipettes, micropipette tips, sterile deionized water

8. Computer Hardware & Software

Optika PROView, a professional image analysis software for light microscope (Optika, Italy). As part of the essay for assessing cell viability, after obtaining images on the light microscope, the analysis is performed using the Image J software (NIH, USA).

9. Overview of the microalgae cultivation and maintanence

9.1. Media preparation

Cultivation of microalgae in the appropriate cultivation medium is of key importance because it provides all the necessary nutrients and optimal conditions for their growth and development. Microalgae require specific nutrients, such as nitrogen, phosphorus, iron and trace elements, which enable maximum growth and productivity. Maintaining proper pH and salinity is important, as improper conditions can slow growth or even cause cell death. A properly formulated medium also prevents the growth of competing microorganisms, such as bacteria and fungi, which can contaminate the culture and reduce its yield. Many established recipes exist for preparing microalgae culture media, such as CCAP procedure (Culture Collection of Algae and Protozoa, 2022; https://www.ccap.ac.uk/) and Andersen's Algal Culturing Techniques (Andersen, 2005). These recipes specify the amounts of nutrients, trace metals, and vitamins needed for optimal growth. To prepare the medium, the individual components must be accurately weighed and mixed. All prepared media must be sterilized. Commercial microalgae culture media are also available from various suppliers (e.g.,

https://www.variconaqua.com/). These media provide convenience and consistency as they are pre-mixed and quality-controlled. However, they may be more costly and are typically restricted to commercially available strains. It's worth noting that some studies have explored the use of agricultural fertilizers as a cost-effective alternative for large-scale microalgae production (Novoveská et al, 2023).

Culture maintenance can be carried out through the use of liquid medium or agar plates, according to current needs. The difference between the two types of cultivation medium is reflected in their composition and application. Liquid medium is most often used because it allows for even distribution of nutrients and better absorption of light, which is crucial for photosynthesis. Microalgae in a liquid medium grow in suspension, which enables continuous mixing and enrichment of the medium with gases such as CO_2. On the other hand, a solid medium, usually prepared with the addition of agar (w/v 1.5%), is used for the isolation and preservation of pure cultures of microalgae (Sánchez-Bayo et al, 2020). On the surface of agar plates, microalgae form colonies, which facilitates the recognition and selection of specific morphologies. This type of medium is not suitable for mass production, but is useful in laboratory research and culture preservation.

9.2. Contamination Prevention

Sterilization of media and equipment is essential to prevent contamination. Autoclaves are generally used for this. Axenic (pure) cultures are often desired, and aseptic techniques are practiced during inoculation and sub-culturing to prevent contamination from other microorganisms (Vu et, 2018). Media should not be stored for more than two weeks and stock solutions for media preparation should be prepared in small quantities. Particular care should taken with phosphates and NaCl, as they are the highly susceptible to contamination and mold growth. This recommendation is based on laboratory experience.

9.3. Cultivation conditions

Although the conditions vary between species in certain subtleties (detailed examples for some common species will be provided as a supplement), maintenance and cultivation of microalgae in laboratory conditions require the following:

Culture vessels/devices: Various containers used for microalgae cultivation: a) flasks, laboratory-scale, with aeration or agitation, b) suitable rubber stoppers ili silicone stoppers, or cotton plugs , c) glass tubes or petri dishes, for strain maintanance on agar media and d) phytotron, a controlled environment chamber for precise growth conditions, d) laminar flow hood, for work with microalgae provides a sterile environment by directing filtered air in a smooth, uniform flow to prevent contamination during cultivation and handling.

Lighting: Controlled light intensity and spectrum are crucial for the optimal growth of microalgae. Different species may require specific light conditions, such as a particular intensity (measured in lux or μmol photons m^{-2}/s^{-1}) and wavelength range (spectrum), to ensure efficient photosynthesis. Artificial lighting systems like LEDs or fluorescent lamps are commonly used to provide consistent light conditions in laboratory settings. The growth of microalgae is significantly influenced by light quality, with blue light (450-495 nm) being the most efficient for photosynthesis, while red light (620-750 nm) and white light also play important roles in biomass production (Sánchez-Bayo et al., 2020).

Shaking: The shaking of microalgae in the lab involves agitating the culture medium to ensure uniform distribution of nutrients, gases, and light while preventing sedimentation of the microalgae. This is typically achieved through mechanical stirrers, air bubbling, or rotary shakers. Proper mixing ensures that microalgae receive adequate nutrients and exposure to light, which are essential for their growth and productivity. For most commonly cultivated species, the average velocity ranges from 50 to 120 rpm.

Aeration: Aeration of microalgae in the lab refers to the process of introducing air or oxygen into the culture medium to provide the necessary oxygen for algal growth and maintain proper gas exchange. This is typically done using air pumps, sterile filters, or bubbling systems to ensure that the microalgae receive sufficient oxygen and carbon dioxide, promoting optimal growth conditions.

Monitoring: Equipment to track pH, temperature, and cell density is essential for ensuring optimal growth conditions for microalgae. pH can be monitored using a pH probe and adjusted if necessary. Temperature is monitored with a thermometer or temperature sensor (typically between 20°C to 25°C, depending on the species). Cell density is established using a spectrophotometer or cell count chambers (e.g., Sedgewick-Rafter, Neubaer, etc.; see Andersen et al, 2005). Common viability assays are also recommended for this purpose, and they include live/dead staining (FDA-PI) and membrane integrity tests (Evans Blue) (Elder et al, 2021). Regular monitoring allows for adjustments to the culture conditions as needed, ensuring healthy and productive microalgal growth.
Harvesting (if needed): Centrifuges or filtration systems are used to collect microalgae from the culture medium. Centrifugation separates cells from the medium based on their density, while filtration removes the algal cells using mesh or membrane filters.

9.4. Growth curve parameters

Monitoring optical density (OD), biomass, viability, and cell count are common methods for characterizing microalgae growth (Humphrey et al., 2019; Ling et al., 2021). These parameters provide a comprehensive understanding of the growth curve.
OD is a quick and easy way to estimate the cell density in a culture (Humphrey et al., 2019) (Nielsen & Hansen, 2019). It measures the opacity of the culture, which increases as the number of cells increases. To avoid interference from chlorophyll absorption, it is more accurate to estimate the cell density of photosynthetic microorganisms using a wavelength outside the pigment absorption range, such as 750 nm.
Biomass is a direct measure of the total mass of cells in a culture. It provides a more accurate representation of the amount of biological material produced during growth than OD alone (Bellinger, 1974).
Viability indicates the proportion of live cells in a culture. It is important for understanding the overall health and productivity of a culture. A decline in viability can indicate stress or nutrient limitation.
Cell count provides a direct measure of the number of cells in a culture (Sarrafzadeh et al., 2015) and is useful for determining the growth rate and generation time of a culture. Still, it can be time-consuming, especially when done manually. Also, cell numbers alone may not accurately reflect the biomass of the population if the cells differ in size (Bellinger, 1974).
The growth of a microalgae culture occurs in five phases: lag phase, exponential phase, declining relative growth phase, stationary phase, and lysis/death phase (Price & Farag, 2013). The two of them are the key phases of microalgae culture growth. The exponential phase is characterized by rapid cell division and a high metabolic rate. During this phase, microalgae are actively growing and multiplying, leading to a steep increase in the biomass.
The stationary phase is characterized by the growth rate slowing down significantly as resources become limited or waste products accumulate (Zhao et al., 2018). The number of new cells produced is roughly equal to the number of cells dying, resulting in a stable population size. The metabolism of the cells also slows down as they adapt to the less-favorable conditions. The duration of these phases and the overall growth time can vary depending on the microalgae species, culture conditions (e.g., temperature, light, nutrients), and the specific goals of the cultivation (Krzemińska et al., 2013; Bernard & Lu, 2022). While 15-20 days is a reasonable average, some species may grow faster or slower (Ra et al., 2016).

9.5. Culture maintenance

The transfer of the microalgae culture into a fresh medium is carried out under a laminar flow hood under sterile conditions. Before starting, the hood is irradiated with UV-C light for 1 hour to minimize the risk of biological contamination. Vessels for culture maintenance are sterilized at 160ºC for 3 hours (dry sterilization), while freshly prepared media, deionized water, lids for Erlenmeyer flasks, and pipette tips are subjected to wet sterilization (114ºC, 25 minutes) and consequential drying in the case of stoppers. All items, including vessels containing old cultures need to be sprayed with 70% ethanol

before being placed in the laminar hood. When working in the laminar hood, it is mandatory to wear a clean coat, gloves disinfected with 70% ethanol, and ensure the ventilation system is operating. After completing the inoculation, the work surface is wiped with 70% ethanol, and the hood is again exposed to UV-C radiation.

The culture is directly transferred from one liquid medium to another. Still, in the case of some species such as *C. acidophila*, a specific volume of the old culture is firstly centrifuged at 5000×g for 5 minutes, the pellet is then washed with deionized water (dH_2O), centrifuged again and resuspended to the initial volume. Further, it is filtered through standard filter paper to remove potential fungi and bacteria. The filtrate is inoculated into a fresh medium.

The transfer from agar to liquid medium follows a similar procedure as for a liquid ones. It is directly applied on the cooled, previously sterilized agar in a Petri dish or test tube, or firtsly washed without filtration in case of *C.acidophila*. The transfer is performed using a sterile loop, previously sterilized on the flame of flame burner. It is essential that the agar is firmly set and well-cooled before use.

These procedures are repeated continuously, depending on the species-specific time (Supplement, Table 2), to prevent contamination and maintain the culture in a mature state. To reduce the growth of present fungi and bacteria, the cultures of these microalgae are treated with specific antimycotics and antibiotics every two to three months, or as needed.

10. Data management

Irrelevant.

11. Waste management

The waste should be disposed of according to the manufacturer's instructions and according to applicable laws. The gloves used by the staff should be disposed of according to good laboratory practices.

12. Related Protocols or SOPs

Supplement – examples of some common microalgae species cultivation and maintanence conditions.

13. Quality Control and Quality Assurance

Instrument maintanence

All instruments are regularly maintained and calibrated by trained service technicians and personnel.

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References

1. Andersen RA, editor. Algal culturing techniques. Amsterdam: Academic Press; 2005.
2. Bellinger EG. A note on the use of algal sizes in estimates of population standing crops. British Phycological Journal. 1974; 9:157-161. https://doi.org/10.1080/00071617400650201
3. Bernard O, Lu LD. Optimal optical conditions for microalgal production in photobioreactors. *J Process Control.* 2022; 112:69-77. DOI: 10.1016/j.jprocont.2022.01.007.
4. Brand JJ, Andersen RA, Nobles Jr DR. Maintenance of microalgae in culture collections. In: *Handbook of microalgal culture: applied phycology and biotechnology.* 2013. pp. 80-89.
5. Elder CE, Dreux G, Burja AM, et al. Advances in microalgal research: A review of recent developments and future directions. *Crit Rev Biotechnol*. 2021; 41:357-375. DOI:10.1080/07388551.2020.1854672.
6. Humphrey I, Chendo MAC, Njah AN, Nwankwo DI. Optimization of microalgae growth for biofuel production using a new empirical dynamic model. Biofuels. 2019;12:1209-1224. doi:10.1080/17597269.2019.1608012

7. Krzemińska I, Pawlik-Skowrońska B, Trzcińska M, Tys J. Influence of photoperiods on the growth rate and biomass productivity of green microalgae. *Bioprocess Biosyst Eng.* 2014; 37:735-41. DOI: 10.1007/s00449-013-1096-0.
8. Liberti D, Pinheiro F, Simões B, Varela J, Barreira L. Beyond bioremediation: the untapped potential of microalgae in wastewater treatment. Water. 2024; 16:2710. https://doi.org/10.3390/w16192710
9. Ling KS, Jamaian SS, Zulkifli FH. Estimation of the microalgae growth using the optimization approach. In: *Journal of Physics: Conference Series.* 2021; 1988:012017. DOI: 10.1088/1742-6596/1988/1/012017.
10. Martin N, Bernat T, Dinasquet J, Stofko A, Damon A, Deheyn DD, et al. Synthetic algal-bacteria consortia for space-efficient microalgal growth in a simple hydrogel system. *J Appl Phycol.* 2021; 33:2805-2815. DOI: 10.1007/s10811-021-02355-6.
11. Masojídek J, Ranglová K, Lakatos GE, Silva Benavides AM, Torzillo G. Variables governing photosynthesis and growth in microalgae mass cultures. Processes. 2021; 9:820. https://doi.org/10.3390/pr9050820
12. Nielsen SL, & Hansen BW. Evaluation of the robustness of optical density as a tool for estimation of biomass in microalgal cultivation: the effects of growth conditions and physiological state. Aquaculture Research. 2019; 50: 2698-2706. https://doi.org/10.1111/are.14227
13. Novoveská L, Nielsen SL, Eroldoğan OT, Haznedaroglu BZ, Rinkevich B, Fazi S, et al. Overview and challenges of large-scale cultivation of photosynthetic microalgae and cyanobacteria. Mar Drugs. 2023 ;21:445. DOI: 10.3390/md21080445.
14. Olaueson MM, Stokes PM. Responses of the acidophilic alga Euglena mutabilis (Euglenophyceae) to carbon enrichment at pH 3.1. J Phycol. 1989; 25:529-539. DOI:10.1111/j.1529-8817.1989.tb00259.x
15. Paterna A, Rao E, Adamo G, Raccosta S, Picciotto S, Romancino D, et al. Isolation of extracellular vesicles from microalgae: a renewable and scalable bioprocess. *Front Bioeng Biotechnol.* 2022; 10:836747. DOI: 10.3389/fbioe.2022.836747.
16. Petrovčíková E, Vičíková K, Leksa V. Extracellular vesicles–biogenesis, composition, function, uptake and therapeutic applications. *Biologia.* 2018; 73:437-448. DOI: 10.2478/s11756-018-0049-z.
17. Picciotto S, Barone ME, Fierli D, Aranyos A, Adamo G, Božič D, et al. Isolation of extracellular vesicles from microalgae: towards the production of sustainable and natural nanocarriers of bioactive compounds. *Biomater Sci.* 2021; 9:2917-2930. DOI: 10.1039/D0BM02154A.
18. Price K, Farag IH. Resources conservation in microalgae biodiesel production. *Int J Eng Technol Res*. 2013;1:49-56.
19. Ra CH, Kang CH, Jung JH, Jeong GT, Kim SK. Enhanced biomass production and lipid accumulation of *Picochlorum atomus* using light-emitting diodes (LEDs). *Bioresour Technol.* 2016; 218:1279-83. DOI: 10.1016/j.biortech.2016.07.078.
20. Ramírez-López C, Perales-Vela HV, Fernández-Linares L. Biomass and lipid production from *Chlorella vulgaris* UTEX 26 cultivated in 2 m^3 raceway ponds under semicontinuous mode during the spring season. *Bioresour Technol.* 2019; 274:252-260. DOI: 10.1016/j.biortech.2018.12.105.
21. Record M, Silvente-Poirot S, Poirot M, Wakelam MO. Extracellular vesicles: lipids as key components of their biogenesis and functions. *J Lipid Res.* 2018; 59:1316-1324. DOI: 10.1194/jlr.R085443.
22. Sánchez-Bayo A, Morales V, Rodríguez R, Vicente G, Bautista LF. Cultivation of microalgae and cyanobacteria: Effect of operating conditions on growth and biomass composition. Molecules. 2020; 25:2834.
23. Sarrafzadeh MH, La HJ, Seo SH, Asgharnejad H, Oh HM. Evaluation of various techniques for microalgal biomass quantification. *J Biotechnol.* 2015; 216:90-7. DOI: 10.1016/j.jbiotec.2015.01.001.
24. Schatz D, Vardi A. Extracellular vesicles—new players in cell–cell communication in aquatic environments. *Curr Opin Microbiol.* 2018; 43:148-154. DOI: 10.1016/j.mib.2018.01.012.
25. Vu CHT, Lee HG, Chang YK, Oh HM. Axenic cultures for microalgal biotechnology: establishment, assessment, maintenance, and applications. *Biotechnol Adv.* 2018; 36:380-396. DOI: 10.1016/j.biotechadv.2018.01.004.
26. Zhao, JM, Ma CY, Liu LH. Temporal scaling of the growth dependent optical properties of microalgae. Journal of Quantitative Spectroscopy and Radiative Transfer. 2018; 214, 61-70. https://doi.org/10.1016/j.jqsrt.2018.04.024

Supplement

Chlorella sorokininana is a green freshwater mixed algae species from the *Chlorophyta* division. The strain of this microalga, CCAP 211/8K, was obtained from the Culture Collection of Algae and Protozoa (CCAP), UK. The optimal medium for maintaining this culture is 3N-BBM + V, whose initial pH is ~ 7.5. The medium is prepared according to the CCAP procedure (https://www.ccap.ac.uk/wp-content/uploads/MR_3N_BBM_V.pdf). The culture is maintained on liquid and solid medium (1.5% w/v agar in medium). These microalgae are grown at 22ºC in a phytotron on orbital shakers (120 rpm) with a continuous photon flux density of 120 μmol m–2s–1. The early stationary phase in case of *C. sorokiniana* is reached after 20 days of growth.

Hematococcus pluvialis is a freshwater microalga species in the *Chlorophyta* division. The culture is usually grown in a liquid medium 3N-BBM + V, with an initial pH of ~7.5. The microalga is grown at 25ºC (room temperature), without shaking, under a 16:8 light-dark cycle and photon flux density of 50-80 μmol m–2s–1. Cultures are maintained only in a liquid medium. The growth curve is determined by monitoring the following parameters: optical density, biomass, viability, and cell count. The growth period for *H. pluvialis* is approximately 30 days, after which the culture needs to be transferred to a fresh medium. This alga is characterized by the complex life cycle consisting of four distinct phases/cell types. Microzooids are small motile green cells, <10 μm, and macrozooids are green, ovoid cells with a prominent gelatinous extracellular matrix and two flagella, ranging from 10 to 20 μm in diameter. These two types are dominant in the exponential phase. Palmella are green, spherical cells (average diameter 20-40 μm) and typically appear during the 'green' phase of culture growth. Hematocysts are red, spherical cysts (30-60 μm) and represent a metabolically dormant cell type characteristic of the 'red' phase of culture growth, which is triggered by the depletion of essential resources or specific stress conditions. The palmella stage contains the red pigment astaxanthin, which is widely exploited in the food and cosmetics industries.

Chlamydomonas reinhardtii is a green unicellular alga with an approximate diameter of 10 μm, moving using two flagella. It is used as a model organism in cell and molecular biology research. This alga is an extensively studied biological model organism, appreciated for its ease of cultivation and highly tractable genetics, which allows precise manipulation for research purposes. The cells of *Chlamydomonas reinhardtii* are mostly spherical but can also have an ellipsoidal shape, with a very thin cell wall. The optimal medium for maintaining this culture is TAP (TRIS-acetate-phosphate) (CCAP, https://www.ccap.ac.uk/wp-content/uploads/MR_TAP.pdf). The culture is maintained in both liquid and solid media (1.5% w/v agar in medium). This microalga is cultivated at 22ºC in a phytotron, on orbital shakers (120 rpm), under a continuous photon flux density of 120 μmol $m^{-2}s^{-1}$. The growth period is approximately 28 days, after which the culture needs to be transferred to a fresh medium.

Chlamydomonas acidophila 136 and 137 are green extremophilic microalgae. The strains of these microalgae, CCAP11/136 and CCAP11/137, were obtained from the Collection of Algae and Protozoa (CCAP), UK. *Chlamydomonas acidophila* PM01 is a green extremophilic microalga species isolated from acidic mining lakes. This strain was collected from Parys Mountain, Anglesey, North Wales, UK.

Maintenance is carried out in MAM medium at pH 3 (Table 1). The medium is prepared following the recipe from Olaveson & Stokes (1989), with certain modifications. The cultures are maintained in a liquid medium.These microalgae are cultivated at 22ºC, in a phytotron, on orbital shakers (120 rpm), with a continuous photon flux density of 120 μmol m–2s–1. The growth curve is determined by monitoring the following parameters: optical density, biomass, viability, and cell count. The growth period of these microalgae is approximately 20 days, after which the culture must be transferred to a fresh medium.

Table 1. MAM medium recipe

Component	50 ml	100 ml	800 ml	400 ml
$(NH_4)2SO_4$	2.5 g	5 g	8 ml	4 ml
$CaCl_2 \cdot 2H_2O$	0.05 g	0.1 g	8 ml	4 ml
$MgSO_4 \cdot 7H_2O$	2.5 g	5 g	8 ml	4 ml
KH_2PO_4	1.5 g	3 g	8 ml	4 ml
NaCl	0.15 g	0.3 g	8 ml	4 ml
$Na_2EDTA \cdot 2H_2O$	1 g	2 g	0.4 ml	0.2 ml
Trace Metals	See below	0.8 ml	0.4 ml	
$FeSO_4 \cdot 7H_2O + H_2SO_4$	0.249 g + 0.05 ml	0.498 g + 0.1 ml	0.8 ml	0.4 ml
Vitamin B12	1 mg/ml	1 mg/ml	0.8 ml	0.4 ml
Trace Metals Mix (1 ml)				
Component	Amount			
H_3BO_3	2.86 g			
$MnCl_2 \cdot 4H_2O$	1.81 g			
$ZnSO_4 \cdot 7H_2O$	0.222 g			
$Na_2MoO_4 \cdot 2H_2O$	0.390 g			
$CuSO4 \cdot 5H_2O$	0.079 g			
$Co(NO_3)2 \cdot 6H_2O$	0.0494 g			

Table 2. Overview of the time points for reaching of the early stationary phase of growth and transfer to media in days for each of microalgae species

Species	Time to early stationary phase (days)	Transfer point to fresh medium (days)
Chlorella sorokiniana	20	30
Chlamydomonas reinhardtii	28	40
Chlamydomonas acidophila, strains 136 and 137	15	27
Chlamydomonas acidophila, strain PM01	15	20
Haematococcus pluvialis	27	30