

Microalgae Culture Techniques

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Microalgae (i.e. single-celled algae or phytoplankton) represent the largest, yet one of the most poorly understood groups of microorganisms on Earth. As happens with plants relative to terrestrial animals, microalgae serve as the natural nutritional base and primary source of bulk nutrients in the aquatic food chain. Microalgal use by indigenous populations has occurred for centuries. Cultivated microalgae have long been integral to the hatchery production of many farmed finfish, shellfish, and other commercially important aquaculture species. By contrast, macroalgae are less widely used in aquaculture, although they do provide an important source of nutrition for certain farmed invertebrates, such as sea urchins and abalone. However, the cultivation of microalgae is only a few decades old. Commercial large-scale culture started in the early 1960's in Japan with the culture of *Chlorella* by Nihon Chlorella (Taipei, Taiwan).

Microalgae serve as live food for commercially important molluscs, fish and crustaceans. Consumers are grown directly in the presence of their food, produced *in situ*. Depending on consumer requirements (Which often depend on life stages), the microalgae are consumed either directly (e.g. by herbivorous fish, bivalve molluscs, larval shrimp and prawns, zooplankton), or indirectly via the 'algae-zooplankton' food chain (e.g. most fish). Microalgae not only play an important role in aquaculture as a food source but, together with bacteria, also help maintain the oxygen and carbon dioxide balance in cultures.

Major Classes and Species of Cultured Algae Used in Aquaculture

- The major classes of cultured algae used as live feed include:
 - **Diatoms**
 - **Flagellated algae**
 - **Chlorococcalean green algae**
 - **Filamentous blue-green algae (cyanobacteria)**
- These algae range in size from **a few micrometres (µm) to over 100 µm**.

Common Marine Algae Used in Commercial Mariculture

Diatoms:

- *Skeletonema costatum*
- *Thalassiosira pseudonana*
- *Phaeodactylum tricornutum*
- *Chaetoceros gracilis*
- *Chaetoceros calcitrans*

Flagellates:

- *Isochrysis galbana*
- *Isochrysis tahiti*
- *Monochrysis lutheri* (also known as *Pavlova lutheri*)
- *Tetraselmis suecica*
- *Dunaliella* spp.

Green algae (Chlorococcalean): *Chlorella* spp.

Common Freshwater Algae Used in Aquaculture

- *Chlorella*
- *Scenedesmus*
- *Chlamydomonas*
- *Spirulina* (a blue-green alga commonly found in alkaline waters)

The role of microalgae in aquaculture hatcheries

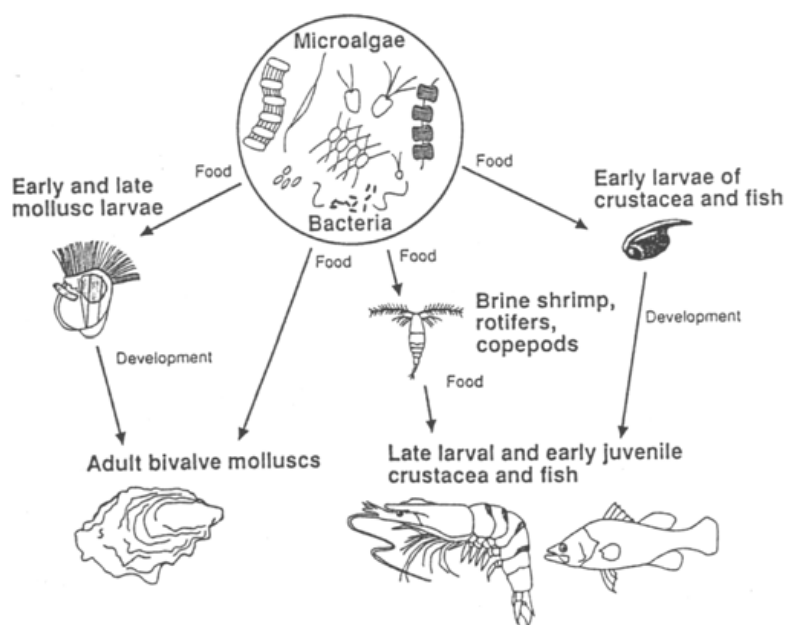


Fig.1 The central role of microalgae in mariculture (Brown et al., 1989)

The role of microalgae in aquaculture hatcheries may be summarized as follows:

- All developmental stages of bivalve molluscs are directly reliant on microalgae as a feed source. Bivalve hatcheries therefore cultivate a range of microalgal strains for broodstock conditioning, larval rearing and feeding of newly settled spat.

- Farmed gastropod molluscs (e.g., abalone) and sea urchins require a diet of benthic diatoms when they first settle out from the plankton, before transferring to their juvenile diet of macroalgae.
- The planktonic larval stages of commercially important crustaceans (e.g., penaeid shrimps) are initially fed on microalgae, followed by zooplanktonic live prey.
- The small larvae of most marine finfish species and some freshwater fish species also initially receive live prey, usually in the presence of a background of microalgae. Depending on whether these microalgae are allowed to bloom within the fish larval rearing tanks, or are added from external cultures, this is referred to as the “green water” or “pseudo-green water” rearing technique.
- The zooplanktonic live prey referred to above are microscopic filter-feeders that are themselves commonly fed on microalgae, although inert formulated feeds have been developed as a more convenient diet form for use by hatcheries.

NUTRITIVE VALUE OF MICRO - ALGAE

✚ More than **40 species of algae** are presently used as live food for aquatic invertebrates and vertebrates.

✚ An ideal algal food should:

- Be **non-toxic**.
- Be **small enough to be ingested**.
- Have a **digestible cell wall**.
- Contain adequate **essential nutrients**.

✚ Different aquatic animals digest algae differently:

- **Bivalve molluscs** (such as oysters and clams) cannot digest thick cell walls.
- **Rotifers** (e.g., *Brachionus*) can digest thick-walled algae using their **mastax**.
- Therefore, **Chlorella** is suitable for **Brachionus** but not for oysters or clams.

✚ The **amino acid content and balance** in algae are important for the growth and development of aquatic organisms.

✚ **Lipids**, especially **polyunsaturated fatty acids (PUFAs)**, are essential for the healthy development of fish larvae.

✚ The nutritional composition of algae changes with **culture conditions**:

✚ During the **exponential growth phase**, algae contain **more protein**.

✚ During the **stationary phase**, they contain **more carbohydrates**.

Algae	Protein	Lipids	Carbohydrates	Fibre	Ash	Nucleic acid	Moisture

<i>Spirulina</i>	62-68	2"3	15-20	5-8	10-12	6	5-6
<i>Chlorella</i>	40-50	10-15	12-16	6-8	8-10	6	5-8
<i>Scenedesmus</i>	50-55	12-19	10-15	10-12	6-8	4-6	5-7

Different Algal culture techniques

Algae can be produced using a wide variety of methods, ranging from closely controlled laboratory methods to less predictable methods in outdoor tanks. The terminology used to describe the type of algal culture includes:

Indoor/Outdoor. Indoor culture allows control over illumination, temperature, nutrient level, contamination with predators, and competing algae, whereas outdoor algal systems make it very difficult to grow specific algal cultures for extended periods.

Open/Closed. Open cultures such as uncovered ponds and tanks (indoors or outdoors) are more readily contaminated than closed culture vessels such as tubes, flasks, carboys, bags, etc.

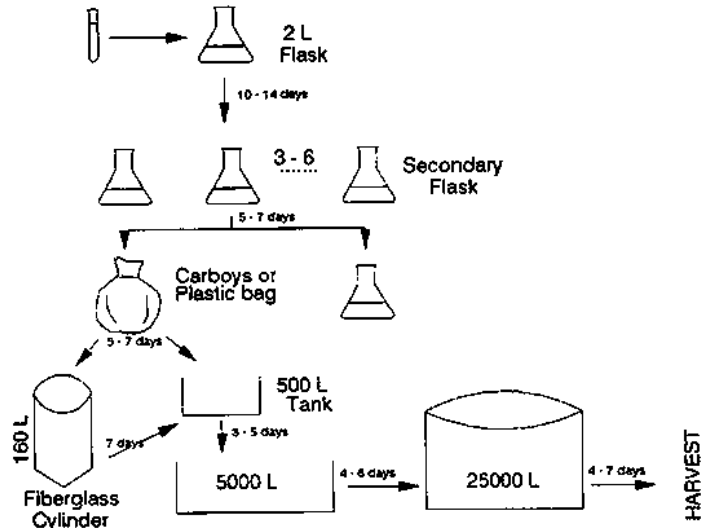
Axenic (=sterile)/Xenic. Axenic cultures are free of any foreign organisms such as bacteria and require a strict sterilisation of all glassware, culture media, and vessels to avoid contamination. The latter makes it impractical for commercial operations.

Batch, Continuous, and Semi-Continuous.

These are the three basic types of phytoplankton culture which will be described in the following sections.

Batch culture: The batch culture consists of a single inoculation of cells into a container of fertilized seawater followed by a growing period of several days and finally harvesting when the algal population reaches its maximum or near-maximum density. In practice, algae are transferred to larger culture volumes prior to reaching the stationary phase, and the larger culture volumes are then brought to a maximum density and harvested. The following consecutive stages might be utilized: test tubes, 2 L flasks, 5 and 20 L carboys, 160 L cylinders, 500 L indoor tanks, 5,000 L to 25,000 L outdoor tanks.

Batch culture systems are widely applied because of their simplicity and flexibility, allowing them to change species and remedy defects in the system rapidly. Although often considered the most reliable method, batch culture is not necessarily the most efficient method. Batch cultures are harvested just before the initiation of the stationary phase and must thus always be maintained for a substantial period of time past the maximum specific growth rate. Also, the quality of the harvested cells may be less predictable than that in continuous systems and for example, vary with the timing of the harvest (time of the day, exact growth phase).



Another disadvantage is the need to prevent contamination during the initial inoculation and early growth period. Because the density of the desired phytoplankton is low and the concentration of nutrients is high, any contaminant with a faster growth rate is capable of outgrowing the culture. Batch cultures also require a lot of labour to harvest, clean, sterilise, refill, and inoculate the containers.

Continuous Culture: A continuous culture is a culture system in which fertilised seawater is continuously supplied to the growth chamber while an equal amount of algal culture is simultaneously removed, allowing the algae to remain close to their maximum growth rate.

Types of Continuous Culture

1. Turbidostat Culture

- Algal concentration (cell density) is maintained at a **pre-set level**.
- Fresh culture medium is added **automatically** whenever the culture becomes too dense.
- **Cell density remains constant**, while the dilution rate varies.

2. Chemostat Culture

- Fresh culture medium is supplied at a **constant, predetermined rate**.
- The medium contains a **limiting nutrient** (e.g., nitrate).
- **Growth rate remains constant**, while cell density is determined by the nutrient supply.

Advantages of Continuous Culture

- Maintains algae near the **maximum growth rate**.
- Produces algae of **uniform and predictable quality**.
- Suitable for **automation and technological control**.
- Improves **system reliability**.
- **Reduces labour requirements**.

Disadvantages of Continuous Culture

- **High installation and operating cost**.
- **Complex** to manage.
- Requires **constant light and temperature**.
- Generally limited to **indoor, small-scale production**.

Semi-Continuous Culture: In the **semi-continuous culture** method, only a **portion of the algal culture is harvested periodically**. The harvested volume is immediately replaced with **fresh enriched medium** to restore the original culture volume, allowing growth to continue.

Procedure

1. Grow the algal culture.
2. Harvest part of the culture.
3. Refill the tank with fresh nutrient-enriched medium.
4. Allow the culture to regrow.
5. Repeat the harvesting and replenishment cycle.

Advantages

- Produces **more algae than the batch culture** using the same tank size.
- Extends the productive life of the culture.

- More economical than repeatedly starting new batch cultures.

Disadvantages

- Over time, **competitors, predators, contaminants, and metabolic wastes** accumulate.
- Eventually, the culture becomes unsuitable and must be replaced with a fresh culture.

Comparison of Continuous and Semi-Continuous Culture

Feature	Continuous Culture	Semi-Continuous Culture
Medium addition	Continuous	Periodic
Harvesting	Continuous	Partial and periodic
Growth	Near maximum, constant	Repeated growth cycles
Control	Highly controlled and automated	Moderately controlled
Cost	High	Lower than continuous
Productivity	High-quality, consistent algae	Higher yield than batch culture
Main limitation	Expensive and complex	Contamination and metabolite buildup

Isolation of Pure Algal Strains

Isolation of the required species of microalgae can be done by one of the following methods.

1. Pipette method: Large organisms can be pipetted out using a micro-pipette under the microscope and transferred to culture tubes having suitable culture media.

2. Centrifuge or washing method: By repeated centrifuging of the samples at different revolutions and by inoculating the deposits, it is possible to get different organisms.

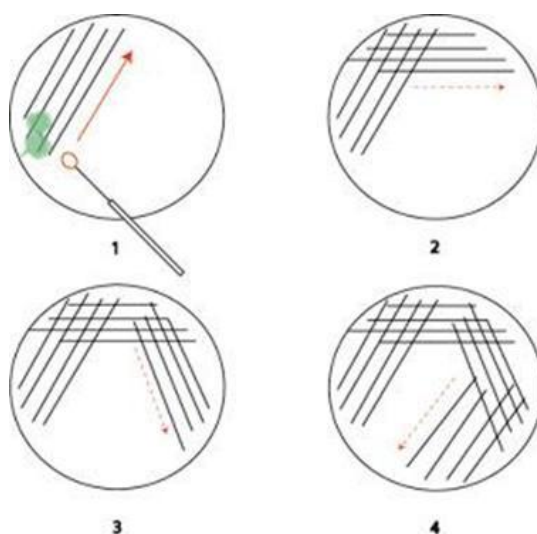
3. By exploiting the phototactic movements: By this method, most of the phytoflagellates can be isolated. Make a dark chamber with a small hole on one side and keep the sample in a beaker nearer to the hole. Place a candle near the hole outside. Since the flagellates have a tendency to move towards the light, it is visible after some time that these organisms crowd near the candlelight. By pipetting, these organisms can be separated, and by tube culture methods, it can be raised to a pure culture.

4. Agar plating method: For preparing the agar medium, 1.5 gm of agar is added to 1 litre of suitable culture medium, e.g. Schreiber's medium, Miquel's medium, TMRL medium, and Conway medium, or even natural seawater.

This agar solution is sterilized in an autoclave for 15 minutes at 60 kg pressure and 100°C temperature. Now this medium is poured into sterilised 15 cm diameter Petri dishes and kept for 24 hours. For isolation, the required species can be picked up by platinum needle or loop under a microscope and streaked on the surface of the agar plate.

After inoculation, these Petri dishes are placed in an incubator for 7-8 days, providing light (1000 lux) and constant temperature (25°C). Within this time, the required species, if it has grown into a colony, is removed by a platinum loop under the microscope and transferred to culture tubes. Further, from culture tubes to small conical flasks and larger flasks, the algae can be grown on a mass scale.

Agar Plating Technique



Agar plates with algal streaks

The following agar plating technique can be used to isolate algal strains from raw seawater and for the maintenance of existing algal strains.

- Prepare a 0.9% agar medium by weighing out 9 g of agar powder and placing it into a 2 l conical flask to which 1 l of seawater is added
- Heat the flask on a Bunsen flame and let it boil twice, i.e. heat until it boils, let it Cool and let it boil a second time
- Add nutrients (see Tables 2.3 & 2.4) before autoclaving
- Cover the flask with aluminium foil
- Autoclave at 125 °C for 30 minutes at 1 atm
- Sterilise Petri dishes by incubation for 30 minutes at 150°C
- Agar plates are prepared aseptically by pouring the warm autoclaved agar into the sterile Petri dishes near a Bunsen flame or in a laminar flow, cover up the Petri dishes and leave them to cool for about 2 h
- Streak the algal sample onto the agar surface with a sterile platinum loop (previously heated to red-hot and cooled)
- Place the Petri dishes upside-down on an illuminated glass rack
- Depending on the density of the inoculum, cell colonies can be observed to grow on the surface after 5 - 21 days
- Select the best colonies and transfer them with a sterile platinum loop into a test tube filled with 5-10 ml of culture medium and shaken regularly during incubation on an illuminated glass rack; when a colour change is observed in the tube, check the isolated algal strain under the microscope

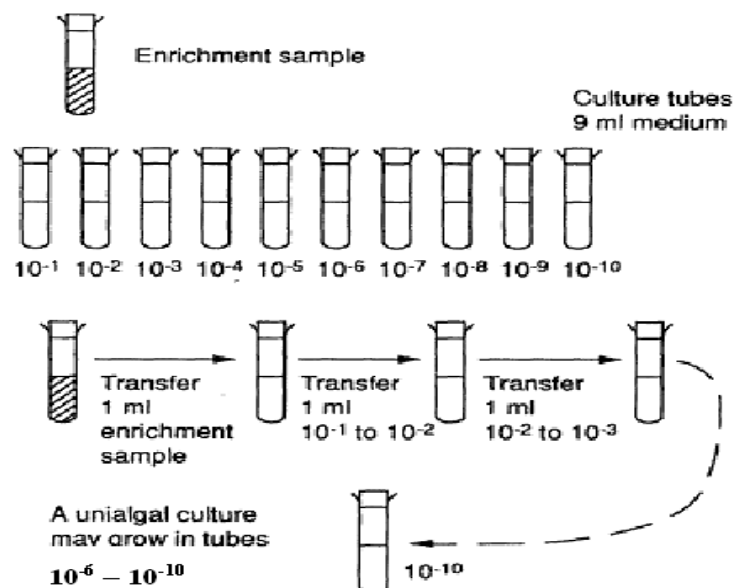
Serial dilution culture technique: This method is used mainly for the isolation of phytoflagellates (Sournia, 1971). In this method, mainly 5 dilution steps (the inocula corresponding to 1, 10^{-1} , 10^{-2} , 10^{-3} and 10^{-4} or 4 steps – 0.001, 0.01, 0.1, and 1 ml) are involved for the isolation of the required species. For the serial dilution technique, nearly 25 culture tubes (15 ml) are required. After filtering the seawater through a 10-20 micron sieve, the filtrate has to be inoculated into five series of culture tubes in various concentrations. This has to be kept under sufficient light (1000 lux) with uniform temperature (25°C) conditions. After 15 days, some discolouration can be seen in the culture tubes, due to the growth of microalgae. Further purification of this culture can be done by sub-culturing it in 500 ml or one-litre conical culture flasks. Once the culture is fully purified, it can be transferred into 3- or 4-litre Haufkin culture flasks and maintained as stock culture. After the isolation of the required organisms in culture tubes, they may be sub-cultured again in a few 50 ml test tubes. These test tubes are the base from which

the algal food starts producing and from where the continuous supply of non-contaminated algal feed is obtained for the operation of the large-scale culture systems. Once the system is started, the test tube culture can be transferred to small culture flasks and to bigger flasks by adding 3-5 ml of the stock culture. Therefore, every two weeks a new set of 10 test tubes for each species should be inoculated from the previous set. The filtration of water and medium enrichment should be done no earlier than 3 days before inoculation.

Procedure

Using an aseptic technique, dispense 9 ml of medium into each of ten test tubes with a sterile automatic dispenser or sterile 10 ml pipettes. Label tubes 10-1 to 10-10, indicating dilution factor.

- Aseptically add 1 ml of enrichment sample to the first tube (10^{-1}) and mix gently.
- Take 1 ml of this dilution and add to the next tube (10^{-2}), mix gently.
- Repeat this procedure for the remaining tubes (10^{-3} to 10^{-10}).
- Incubate test tubes under controlled temperature and light conditions:
 - (a) Temperature and photoperiod — as close to the natural environment as possible
 - (b) Light intensity — slightly lower than the natural environment
- Examine cultures microscopically after 2—4 weeks by withdrawing a small sample aseptically from each dilution tube. A unialgal culture may grow in one of the higher dilution tubes, e.g. 10^{-6} to 10^{-10} . If tubes contain two or three different species, then micromanipulation can be used to obtain unialgal cultures



MAINTENANCE OF STOCK AND STARTER CULTURES

Stock cultures, otherwise known as master cultures, of the preferred species are the basic foundation of culture. They are normally supplied as monospecific (uni-algal) cultures from reputable culture collections maintained by national institutions or research laboratories. Since they are valuable, they are normally kept in specialized maintenance media, for example, Erdschreiber, or in F/2 media, or on nutrient-enriched agar plates or slopes, under closely controlled conditions of temperature and illumination. A special area or room of the algal culture room is usually allocated for this purpose.

Stock cultures are used **only** to provide lines of starter cultures (also known as inocula) when required. Every effort should be made to minimize the risk of contaminating the stock and starter cultures with competing microorganisms. The sterile procedures described below should be followed to ensure that contamination does not occur. Stock cultures are kept in small, transparent, autoclavable containers. For example, 500 ml borosilicate glass, flat-bottomed boiling or conical flasks fitted with a cotton wool plug at the neck, suitable for containing 250 ml of sterile, autoclaved medium, are ideal.

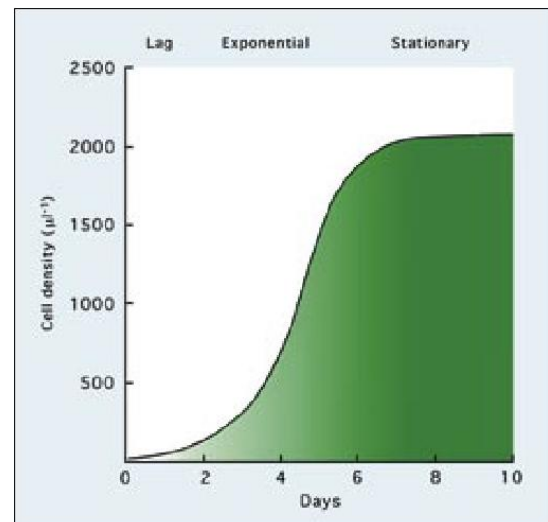
Starter culture management

Procedures for the maintenance of starter cultures (inocula) are almost identical to those described above. These cultures are specifically grown to provide inocula to start larger volume cultures needed to produce food. A line of starter cultures is originally set up from the stock culture of the required species. Starter cultures, like the stocks, can be grown in 500 ml boiling flasks in 250 ml of culture medium. With larger volume starters it is advantageous to increase the level of illumination and to aerate it with an air/carbon dioxide mixture.

Growth phases of cultures

Harvesting takes place in a semi-continuous culture during the exponential phase of growth. Batch harvests are made generally at the peak of exponential growth as the cultures enter the stationary phase. An illustration of the meaning of these terms is given in Figure 19. In this case the species cultured is the large, green flagellate, *Tetraselmis*. At inoculation from the starter culture, the starting cell density in the culture is 25 to 50 cells per ml (cells per microlitre).

Figure: Phases in the growth of algal cultures illustrated by a typical growth curve for a green flagellate.



After inoculation, these cells grow and divide increasingly rapidly as they acclimatize to the culture conditions. This acclimatization period, which lasts for 2 to 3 days, is called the **lag phase**. Once adapted to the conditions, the rate of cell division accelerates and an increase in the number of cells in the culture is logarithmic. This period lasts for 4 to 6 days and is called the **exponential growth phase**. Cell division rate then slows as light penetration through the culture and/or nutrients become limiting. The culture then enters the **stationary phase**, which can last for many days in the case of flagellates or only for a short time for diatoms. Cultures of flagellates remain in this phase by the recycling of nutrients from dead and decaying cells, but in the case of diatoms, which may produce self-inhibiting metabolites, that attract bacterial growth, the culture collapses.

Extensive outdoor culture

Commercial hatcheries need to produce large volumes of good quality, high-food-value algae daily to support economic-scale seed production. Outdoor tank culture makes use of natural light. Culture in rectangular or circular tanks with overhead illumination is used in shrimp hatcheries in India. This involves the fertilization of a large volume of seawater with the basic nutrients necessary for production, namely nitrogen, phosphorus, and silica in one form or another. It is possible to induce mono-specific blooms by prior fine (<2 µ particle retention) filtration of the impounded seawater and the introduction of an inoculum of the required species, as long as it is hardy and vigorous. However, it is difficult to maintain such blooms for long periods because they rapidly become contaminated with other microorganisms.