Isolation and Culture of Microalgae

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Introduction

Marine microalgae or phytoplankton are the floating microscopic unicellular plants of the seawater which are generally free living, pelagic with the size range of 2–20 μm. The important components of microalgae are the diatoms, dinoflagellates, green algae, blue-green algae, and coccolithophores. Most microalgae have got immense value as they are rich sources of essential fatty acids, pigments, amino acids, and vitamins. They play a critical role in the coastal aquaculture of fish, molluscs, shrimps, and oysters, especially to meet the nutritional requirement of the larvae as well as for bioencapsulation. It is an established fact that the success of any hatchery operation mainly depends on the availability of the basic food, the phytoplankton. The maintenance and supply of the required species at appropriate time form a major problem being encountered by the algal culturists. The procedure for the phytoplankton culture involves aspects such as the isolation of the required species, preparation of the suitable culture media, and maintenance of the culture in the laboratory scale, as well as large scale under controlled conditions of light, temperature, and aeration, and their constant supply to the aqua farmers in different phases of growth. A culture may be defined as an artificial environment in which the microalgae grow. The culture of phytoplankton is an important aspect of planktonology, and the mass culture of phytoplankton is achieved under laboratory-controlled conditions and under field/outdoor conditions. Under laboratory conditions, sterilized or thoroughly cleaned containers are filled with filtered/sterilized seawater (28–34‰) and enriched with the addition of fertilizers, i.e., Guillard and Ryther's F medium, Walne's medium, or TMRL medium. The culture containers are inoculated with pure strains of the desired phytoplankton previously cultured in the laboratory. They are provided with heavy aeration and light using aerators and fluorescent bulbs respectively in a controlled laboratory with temperature of 25 ± 2 °C. The exponential growth phase is generally observed in 36 h to 3 days after inoculation. Cell density of 1.5–4.5 million cells per ml could be recorded. As a sufficient quantity of phytoplankton inoculums usually is present in the coarsely filtered seawater when the nutrients are added, a phytoplankton bloom develops in a course of few days under substantial sunlight. However, it happens sometimes that diatom bloom is inhibited by lack of sunlight or due to the nature of seawater in the tank. In such cases, the addition of new seawater and/or addition of ferric chloride in small amounts may stimulate instant resumption of the diatom in culture.

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History of Microalgae Culture

Microalgae culture is one of the methods of modern biotechnologies. The first unialgal culture was achieved by Beijerinck in 1890 with *Chlorella vulgaris*, and the use of such cultures for studying plant physiology was developed by Warburg in the early 1900s (Warburg 1919). Mass culture of microalgae really began to be a focus of research after 1948 at Stanford (USA), Essen (Germany), and Tokyo, and the classic book edited by Burlew (1953) summarizes many of these early studies. Interest in applied algal culture continued, especially with studies on the use of algae as photosynthetic gas exchangers for space travel and as microbial protein sources. Commercial large-scale culture of microalgae commenced in the early 1960s in Japan with the culture of *Chlorella* followed in the early 1970s with the establishment of a *Spirulina* harvesting and culturing facility in Lake Texcoco, Mexico, by Sosa Texcoco S. A. In 1977, Dainippon Ink and Chemicals Inc. established a commercial *Spirulina* plant in Thailand, and by 1980 there were 46 large-scale factories in Asia producing more than 1,000 kg of microalgae (mainly *Chlorella*) per month (Kawaguchi 1980), and in 1996 about 2,000 t of *Chlorella* were traded in Japan alone. Other *Spirulina* plants were established in the USA (e.g., Microbio in California and Cyanotech in Hawaii). Commercial production of *Dunaliella salina*, as a source of β-carotene, became the third major microalgae industry when production facilities were established by Western Biotechnology Ltd and Betatene Ltd (now Cognis Nutrition & Health) in Australia in 1986. These were soon followed by other commercial plants in Israel and the USA. As well as these algae, the large-scale production of cyanobacteria (blue-green algae) commenced in India at about the same time. More recently, several plants producing *Haematococcus pluvialis* as a source of astaxanthin have been established in the USA and India. Thus, in a short period of about 30 years, the industry of microalgal biotechnology has grown and diversified significantly.

Role of Microalgae in Aquaculture

Microalga is an important source of nutrition and is used widely in the aquaculture of other organisms, either directly or as an added source of basic nutrients. Aquaculture farms rearing larvae of molluscs, echinoderms, crustaceans, and fish use microalgae as a source of nutrition. Low-bacterial and high-microalgal biomass is a crucial food source for shellfish aquaculture.

Microalgae can form the start of a chain of further aquaculture processes. For example, a microalga is an important food source in the aquaculture of brine shrimp. Brine shrimp produces dormant eggs, called cysts, which can be stored for long periods and then hatched on demand to provide a convenient form of live feed for the aquaculture of larval fish and crustaceans.

Other applications of microalgae within aquaculture include increasing the aesthetic appeal of finfish bred in captivity. One such example can be noted in the aquaculture of salmon, where a microalga is used to make salmon flesh pinker. This is achieved by the addition of natural pigments containing carotenoids such as astaxanthin produced from the microalga *Haematococcus* to the diet of farmed fish.

Methods of Isolation of Microalgae Single Species

The isolation of the required species can be done by one of the following methods:

Washing method or centrifugation: Repeated washing or centrifuging the water samples results in the isolation of larger organisms.

By exploiting the phototactic movement: By this method, the phytoflagellates will move to one direction and with a micropipette can be isolated.

By agar plating method: For preparing the agar medium, 1.5 % agar is added to 1 L of suitable medium or even natural seawater, and this agar solution is sterilized in an autoclave for 15 min under 150 lbs pressure and 120 °C temperature. Then this medium is poured in

sterilized Petri dishes and left for 24 h. In case of culture tubes, the medium is poured in 1/3 part in tubes and properly plugged with cotton before autoclaving.

Micromanipulation: The algal cell is to be isolated in drop of enrichment sample. While observing, the cell is sucked up into micropipette. The cell is transferred to a drop of sterile medium on agar plate. This process is repeated to "wash" the cell. The more times a cell is washed, the less likely is bacterial contamination. However, the risk of cell damage increases with the number of times a cell is handled. The optimum number of washes will depend on the type of algae. Then transfer the cell to dilute medium in a tissue culture plate, Petri dish, or culture tube. Culture vessel is placed under low light at appropriate constant temperature. Growth is checked under the microscope, or we have to wait until macroscopic growth can be detected (3–4 weeks after transfer). A colonial unialgal culture results from this method.

Serial dilution: Tubes have to be labeled as 10^{-1}–10^{-10} to indicate dilution factor. Aseptically 1 ml of enrichment sample is to be added to the test tube (10^{-1}) and mixed gently. 1 ml of this dilution is taken and added to the next tube (10^{-2}) and then mixed gently. This procedure is repeated for the remaining tubes (10^{-3}–10^{-10}). Test tubes are incubated under controlled temperature and light conditions: The cultures are examined 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}–10^{-10}. If the tubes contain two or three different species, then micromanipulation can be used to obtain unialgal cultures.

Growth Dynamics

The growth of an axenic culture of microalgae is characterized by five phases. Growth usually refers to changes in the culture rather than changes in an individual organism. Growth denotes the increase in number beyond that present in the original inoculums. Five distinct phases of growth are described (Fig. 1):

The lag phase – After the addition of inoculums to a culture medium, the population remains temporarily unchanged. The cells at this point increase in size beyond their normal dimensions. Physiologically, they are very active and are synthesizing new protoplasm. The organisms are actually metabolizing, but there is a lag in cell division.

The logarithmic or exponential phase – The cells begin to divide steadily at a constant rate. Given optimum culture conditions, growth rate is maximal at this stage.

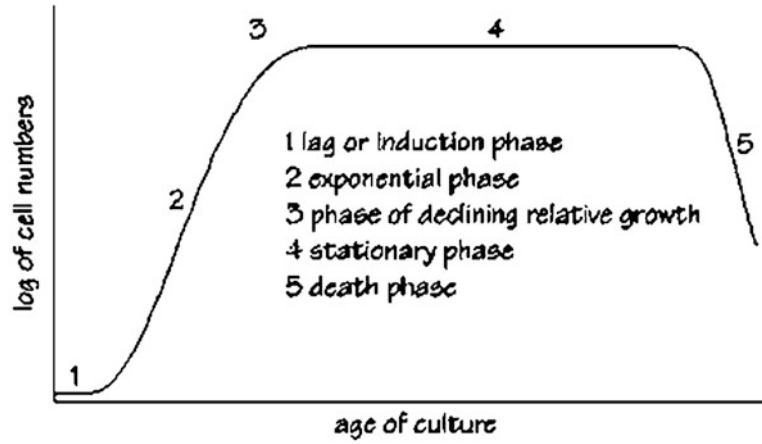

Fig. 1 Schematic diagram of algal growth stages

Phase of declining growth rate – Cell division slows down when nutrients, light, pH, carbon dioxide, or other physical and chemical factors begin to limit growth.

The stationary phase – At this point, the logarithmic phase of growth begins to taper off after several hours (or days) in a gradual fashion. The population more or less remains constant for a time, perhaps as a result of complete cessation of division or the balancing of reproduction rate by an equivalent death rate.

The phase of decline or death – The rate at which some cells die is faster than the rate of reproduction of new cells. The number of viable cells decreases geometrically.

Materials Required for Culture of Marine Microalgae

Culture Apparatus

Vessels made up of "Pyrex" or "Corning" glass are usually used for phytoplankton culturing. But from test tube to concrete tanks may be used, depending on the quantity of the culture required. For small-scale experiments, Erlenmeyer flasks equipped with inlet and outlet tubes for aeration are used. Glass tubes or a flask plugged with cotton provides enough aeration. The vessels should be cleaned well and sterilized in a hot air oven.

Light

As with all plants, microalgae photosynthesize, i.e., they assimilate inorganic carbon for conversion into organic matter. Light is the source of energy which drives this reaction, and in this regard intensity, spectral quality, and photoperiod need to be considered. Light intensity plays an important role, but the requirements vary greatly with the culture depth and the density of the algal culture: at higher depths and cell concentrations, the light intensity must be increased to penetrate through the culture (e.g., 1,000 lux is suitable for Erlenmeyer flasks; 5,000–10,000 is required for larger volumes). Light may be natural or supplied by fluorescent tubes. Too high light intensity (e.g., direct sunlight, small container close to artificial light) may result in photoinhibition. Also, overheating due to both natural and artificial illumination should be avoided. The duration of artificial illumination should be minimum 18 h of light per day, although cultivated phytoplankton develop normally under constant illumination. In controlled rooms, white daylight fluorescent lamps may be used. Outdoor cultures rely on sunlight for illumination.

Temperature

Temperature usually affects an organism's metabolic rate. Low temperatures are usually maintained in controlled rooms (18–23 °C). Transfer of algal starters or inoculate previously cultured in controlled rooms when scaled up for mass production should be done early morning to avoid stress brought about by sudden temperature increase. The optimal temperature for phytoplankton cultures is generally between 20 and 24 °C, although this may vary with the composition of the culture medium, the species, and the strain cultured. Most commonly cultured species of microalgae tolerate temperatures between 16 and 27 °C. Temperatures lower than 16 °C will slow down growth, whereas those higher than 35 °C are lethal for a number of species. If necessary, algal cultures can be cooled by a flow of cold water over the surface of the culture vessel or by controlling the air temperature with refrigerated air-conditioning units.

pH

The pH range for most cultured algal species is between 7 and 9, with the optimum range being 8.2–8.7. Complete culture collapse due to the disruption of many cellular processes can result from a failure to maintain an acceptable pH. The latter is accomplished by aerating the culture. In

the case of high-density algal culture, the addition of carbon dioxide allows to correct for increased pH, which may reach limiting values of up to pH 9 during algal growth.

Aeration/Mixing

Mixing is necessary to prevent sedimentation of the algae, to ensure that all the cells of the population are equally exposed to the light and nutrients, to avoid thermal stratification (e.g., in outdoor cultures), and to improve gas exchange between the culture medium and the air. The latter is of primary importance as the air contains the carbon source for photosynthesis in the form of carbon dioxide. For very dense cultures, the CO_2 originating from the air (containing 0.03 % CO_2) bubbled through the culture is limiting the algal growth, and pure carbon dioxide may be supplemented to the air supply (e.g., at a rate of 1 % of the volume of air). CO_2 addition furthermore buffers the water against pH changes as a result of the CO_2/HCO_3^- balance. Depending on the scale of the culture system, mixing is achieved by stirring daily by hand (test tubes, Erlenmeyer), aerating (bags, tanks), or using paddle wheels and jet pumps (ponds). However, it should be noted that not all algal species can tolerate vigorous mixing.

Carbon Dioxide

Providing the algae with extra carbon, in the form of the gas carbon dioxide (CO_2), would facilitate much faster growth. CO_2 is supplied from compressed gas cylinders, and only very little is needed (about half of one percent) in the air supplied to the culture. The CO_2 has to be passed through a flowmeter to ensure that the amount used will keep the pH of the culture between 7.8 and 8.0. The pH can be checked with indicator papers, which change color with a change in pH, or with a pH meter. Both the air and the CO_2 should be filtered through an in-line filter unit of 0.3–0.5 μm before entering the culture, as this helps to prevent other, possibly contaminating, organisms from getting into the cultures.

Salinity

Marine phytoplankton are extremely tolerant to changes in salinity. Most species grow best at a salinity that is slightly lower than that of their native habitat, which is obtained by diluting seawater with tap water. Salinities of 20–24 $g.l^{-1}$ have been found to be optimal.

Nutrient Medium

In laboratory cultures, however, natural waters themselves are unsatisfactory for sustained algal growth mainly because some essential nutrients are usually present only in trace amounts. The concentration of these elements largely depends on dynamic equilibrium which is disturbed as soon as water is collected. Miquel (1890–93) observed that the waters of lakes, ponds, and the sea could not support in the laboratory continued and luxuriant growth of algae. Natural waters has to be enriched by the addition of some mineral salts that compounded in the famous solutions A and B. This marked the beginning of the use of enriched culture media where specific conditions are imposed to encourage growth of particular organisms. After the work of Allen and Nelson (1910) on Miquel's solutions, the newly formulated Allen-Nelson "Miquel seawater" medium became the standard for enriching seawater. Synthetic or artificial media were later developed for studies related to algal physiology and nutrition. Provasoli et al. (1957) have observed that artificial media show the most constant results for algal culture in contrast to enriched seawater media which may show varying results depending upon the time and place of collection of the seawater base. Some observations (Chu 1942), however, showed that although the heaviest cultures obtained have been in defined media, frequently growth fails

in it and more often stops at a much earlier stage than in media containing natural materials. In the years that followed, the general trend was toward the improvement of the currently existing synthetic media and the introduction of new ones. Concentrations of cells in phytoplankton cultures are generally higher than those found in nature. Algal cultures must therefore be enriched with nutrients to make up for the deficiencies in the seawater. Macronutrients include nitrate, phosphate (in an approximate ratio of 6:1), and silicate. Silicate is specifically used for the growth of diatoms which utilize this compound for production of an external shell. Micronutrients consist of various trace metals and the vitamins thiamin (B_1), cyanocobalamin (B_{12}), and sometimes biotin. Several enrichment media that have been used extensively and are suitable for the growth of most algae are the Walne's medium and the Guillard's F/2 medium. Various specific recipes for algal culture media are described by Vonshak (1986). These recent advances in algal culture somehow present probable answers to the many problems in aquaculture.

Selection of the Culture Medium

On securing the desired organism, microalgal sample to be transfer into a series of Petri dishes, each containing different enriched media. They have to be exposed to sunlight or artificial light. This preparatory culture is used to select the suitable medium for the particular species. During this time, the organism multiplies in one of the media and provides enough material for further process of culturing. Pure cultures are sometime obtained only after several attempts. The preparatory cultures may be maintained till pure cultures are obtained.

Culture Media

The following are some of the culture media found suitable to most planktonic algae:
TMRL medium (Tung Kang Marine Res. Lab.)

Potassium nitrate	10 g/100 ml of DW
Sodium orthophosphate	1 g/100 ml of DW
Ferric chloride	0.3 g/100 ml of DW
Sodium silicate	0.1 g/100 ml of DW

The chemicals are kept separately in 100 ml reagent bottle. 1 ml each to 1 l of sterilized seawater is added. This medium can be used for the mass culture of diatom.

Schreiber's medium

Potassium nitrate	0.1 g
Sodium orthophosphate	0.02 g
Soil extract	50 ml
Filtered and sterilized seawater	1

Soil extract is prepared by boiling 1 kg of garden soil in 1 l of fresh water for 1 h. After 24 h, clear water is decanted and kept in a bottle. 50 ml of this soil extract can be added to each liter of sterilized seawater. This can be used as a medium while isolating the nanoplankton.

F/2 medium

$NaNO_3$ (75.0 g/L dH_2O)	1.0 ml
$NaH_2PO_4 \cdot H_2O$ (5.0 g/L dH_2O)	1.0 ml
$Na_2SiO_3 \cdot 9H_2O$ (30.0 g/L dH_2O)	1.0 ml
F/2 trace metal solution	1.0 ml
F/2 vitamin solution	0.5 ml
Filtered seawater to	1.0 L

After all additions, the medium will be autoclaved.

F/2 trace metal solution

$FeCl_3 \cdot 6H_2O$	3.15 g
$Na_2EDTA \cdot 2H_2O$	4.36 g
$CuSO_4 \cdot 5H_2O$ (9.8 g/L dH_2O)	1.0 ml
$Na_2MoO_4 \cdot 2H_2O$ (6.3 g/L dH_2O)	1.0 ml
$ZnSO_4 \cdot 7H_2O$ (22.0 g/L dH_2O)	1.0 ml
$CoCl_2 \cdot 6H_2O$ (10.0 g/L dH_2O)	1.0 ml
$MnCl_2 \cdot 4H_2O$ (180.0 g/L dH_2O)	1.0 ml
Distilled water to	1.0 L

F/2 vitamin solution

Vitamin B_{12} (1.0 g/L dH_2O)	1.0 ml
Biotin (0.1 g/L dH_2O)	10.0 ml
Thiamine HCl	200.0 mg
Distilled water to	1.0 L

Filter sterilizes into plastic vials, and stored in the refrigerator.

Note: F/2 medium contains extensive silica precipitate and should be used only when growing diatoms. For other algal groups, use F/2-Si medium.

Conway's or Walne's Medium

1. *Nutrient solution A per liter of DW*

FeCl3.6H2O	1.3 g
MnCl2.4H2O	0.36 g
H_3BO3	33.6 g
EDTA (disodium salt)	45.0 g
NaH2PO4.2H2O	20.0 g
NaNO3	100.0 g
TMS stock	1.0 ml

2. *Trace metal solution B (TMS) per 100 ml DW*

ZnCl2	2.1 g
CoCl2.6H2O	2.0 g
(NH4)6Mo7O24.4H2O	0.9 g
CuSO4.5H2O	2.0 g

This solution is normally cloudy. Acidify with a few drops of conc. HCl to give a clear solution.

3. *Vitamin solution C per 100 ml*

Cyanocobalamin	10.0 mg
Thiamine	10.0 mg
Biotin	200.0 μg (micrograms)

Medium per liter

Nutrient solution **(A)**	1.0 ml
Trace metal solution (B)	0.5 ml
Vitamin solution **(C)**	0.1 ml
Sterilized seawater	1.0 l

Besides the above mentioned laboratory-prepared chemical which serves as nutrients, commercial fertilizers can be used for the mass culture of diatoms and nanoplankters, in open tanks for economic purpose. The media used for the open culture are:

Urea 46	10 mg/l
Super phosphate	10 mg/l
Ammonium sulfate	100 mg/l

Stock Culture Maintenance

Stock cultures are kept on hand for initial start-up and as a source of "clean" algae when the mass tank cultures or the smaller intermediate cultures become contaminated, die, or are harvested. Stock cultures are usually kept in heat-resistant, transparent test tubes or conical flasks. All algae culture systems require a set of stock cultures, usually of about 250 ml in volume, to provide the reservoir of algal cells from which to start the larger-scale cultures which will be used for feeding. Stock cultures are kept in small flasks, such as 500 ml borosilicate glass, flat-bottomed boiling flasks fitted with cotton-wool bungs. Flasks are available in sizes ranging from 1.7 fl. oz. to 4 quarts (50–4,000 ml). The culturist can choose the size that is most convenient and practical for his/her operation. An adequate volume of stock culture must be available for the next step in vessel size. Air is not bubbled into stock flasks, thus eliminating one potential source of contamination. Each flask is half filled (or less) with media. Partially filled flasks provide a larger surface area for gas exchange than full flasks. Good gas exchange increases the prospect of stable, long-lived, and healthy stock cultures. Many hatcheries keep backup stock cultures. Some are kept on-site in duplicate culture chambers. Others are kept at locations remote from the hatchery. In this way, problems with equipment, power failures, unexplained crashes, and other unforeseen difficulties can be minimized. By following a strict transfer regime, pure algal monocultures can be kept healthy and used indefinitely to seed new cultures.

Subculture

Stock cultures must be subcultured frequently (preferably weekly). Subculturing involves inoculating some cells from an old stock culture into fresh culture medium, so that the cells can continue to grow and divide and remain healthy. If subculturing is not carried out, the algal cells in the stock culture will eventually die. It is

important to take precautions to prevent contaminants from the air entering the stock cultures when subculturing. To start a new stock culture, about 20 ml of algae are taken from a stock culture which has been growing for 6–7 days and poured into a flask containing 250 ml of fresh culture medium. After removing the cotton-wool bungs, and before and after pouring, the necks of both flasks should be passed through a gas flame, such as that from a Bunsen burner or spirit lamp, to kill any surface and airborne contaminants, such as bacteria, that might enter the culture. To grow, the new stock culture should be put about 20 cm from a fluorescent lamp that is lit continually. After subculturing, the remainder of the old stock culture can be used to start a batch culture of up to 10 l. This method is described more fully in stock culture maintenance. If the stock culture is not required immediately, it may be kept for up to 3 weeks on a shelf in a north-facing window (away from direct sunlight), but after this time, it should be discarded and the culture should be less than 25 °C. It is noted that stock cultures do not require an air/CO_2 supply.

Mass Cultures

Stock cultures are used to inoculate intermediate-sized cultures. Many hatcheries use five gallon bottle (19 L) cultures as a step between flask stock cultures and large mass tank cultures. These bottles are available in either plastic (polycarbonate) or glass. Glass bottles are heavy and breakable but can be autoclaved without ill effects. Lightweight plastic bottles have seams that could develop leaks, particularly if autoclaved. Other intermediate-sized vessels such as translucent 2.5 gal bottles, plastic one gallon milk jugs, or polyethylene bags can be used. Polyethylene material for constructing bags can be purchased as tubing (diameters from 1.5–58 in.). The ends can be heat sealed to any convenient length. They require external support but are disposable and do not need to be sterilized when first used. Many hatcheries are using this technique to mass culture algae. For a small, laboratory-sized hatchery, these containers may provide enough algae to satisfy production needs. Commercial-sized bivalve hatcheries require mass tank cultures containing 25–5,000 gal (100–20,000 L) or more of quality algae. In these operations, mass tank cultures are typically started from healthy bottle cultures. Tank and bottle cultures should be provided with the most favorable environment possible. Deviating from ideal conditions will reduce algal growth rate. Lower light levels or reliance on natural sunlight could be used to reduce electrical costs but with a concurrent decrease in production. Some hatcheries eliminate aeration to reduce the spread of biological contaminants. Departures from optimal conditions may be necessary for other reasons. For example, if several algal species are cultured in one room, a compromise in environmental conditions may be necessary. A cooler temperature, such as 65 ° F, would increase the time needed to attain peak density for some species but may eliminate a biological contaminant. Inoculum sufficient for a bottle produces a visible tint in the water. For example, one or two pints (500–1,000 ml) would be adequate inoculums for a 5 gal bottle. Under favorable conditions, a newly inoculated *Isochrysis galbana* culture in a 5 gal (19 L) bottle takes 10–14 days to reach peak density (10–12 million cells/ml). The faster-growing *Thalassiosira pseudonana* reaches its peak (four million/ml) in about 3 days. Once maximum density is attained, healthy *I. galbana* bottle cultures are stable for two additional weeks. Cultures of *T. pseudonana* will deteriorate within 5 days once reaching maximum density. Growth rate and culture stability of most other algae fall between these two. Using a larger volume of inoculum will decrease the time for a culture to reach peak density. For example, a half gallon (2 L) inoculum instead of one pint (500 mL) would shorten by 30–50 % the time a 5 gal (19 L) bottle needs to reach peak density. Algal growth could be increased by bubbling in a mixture of air and 10 % carbon dioxide instead of

air alone. Fast-growing cultures could be enhanced with carbon dioxide pulsed in several times a day, keeping the pH below 10. Carbon dioxide is available in pressurized bottles from bottled air suppliers in most communities. A dense (10 million cells/ml), healthy 5 gal (19 L) bottle culture contains enough inoculum to seed a 240 gal (900 L) mass tank culture. At lower densities (five million cells/ml, for example), two or more bottles would be necessary to produce the same amount of algal inoculum. Larger tank cultures would benefit from a proportionally larger inoculum. Mass cultures in 240 gal tanks ($8' \times 4' \times 1'$) should reach peak density in 2–3 days when configured as follows:

Isolating/Obtaining and Maintaining of Cultures

Sterile cultures of microalgae used for aquaculture purposes may be obtained from specialized culture collections. A list of culture collections is provided by Vonshak (1986) and Smith et al. (1993a). Alternatively, the isolation of endemic strains could be considered because of their ability to grow under the local environmental conditions. Isolation of algal species is not simple because of the small cell size and the association with other epiphytic species. Several laboratory techniques are available for isolating individual cells, such as serial dilution culture, successive plating on agar media, and separation using capillary pipettes. Bacteria can be eliminated from the phytoplankton culture by washing or plating in the presence of antibiotics. The sterility of the culture can be checked with a test tube containing seawater with 1 $g.l^{-1}$ Bacto™ Peptone. After sterilization, a drop of the culture to be tested is added, and any residual bacteria will turn the Bacto™ Peptone solution turbid. The collection of algal strains should be carefully protected against contamination during handling and poor temperature regulation. To reduce risks, two series of stocks are often retained, one which supplies the starter cultures for the production system and the other which is only subjected to the handling necessary for maintenance. Stock cultures are kept in test tubes at a light intensity of about 1,000 lux and a temperature of 16–19 °C. Constant illumination is suitable for the maintenance of flagellates but may result in decreased cell size in diatom stock cultures. Stock cultures are maintained for about a month and then transferred to create a new culture line.

Some Types of Culture

Batch Culture

Batch culture is a system where the total culture is harvested and used as food. If required, another culture can be set up to replace it. 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, and 5,000–25,000 l outdoor tanks. According to the algal concentration, the volume of the inoculum which generally corresponds with the volume of the preceding stage in the upscaling process amounts to 2–10 % of the final culture volume. Where small amounts of algae are required, one of the simplest types of indoor culture employs 10–20 l glass or plastic carboys which may be kept on shelves backit with fluorescent tubes. Batch culture systems are widely applied because of their simplicity and flexibility, allowing to change species and to remedy defects in the system rapidly. Although often considered as the most reliable method, batch culture is not necessarily the most efficient method. Batch cultures are harvested just prior to 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, varies 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 labor to harvest, clean, sterilize, refill, and inoculate the containers.

Semicontinuous Culture

Semicontinuous culture is a system where part of the culture is harvested and used as food and the amount taken is replaced with fresh culture medium (clean seawater and nutrient salts). After allowing 2–3 days for the remaining cells to grow and divide, the process is repeated. Semicontinuous cultures may be operated for up to 7–8 weeks. The semicontinuous technique prolongs the use of large tank cultures by partial periodic harvesting followed immediately by topping up to the original volume and supplementing with nutrients to achieve the original level of enrichment. The culture is grown up again, partially harvested, etc. Semicontinuous cultures may be indoors or outdoors, but usually their duration is unpredictable. Competitors, predators, and/or contaminants and metabolites eventually build up, rendering the culture unsuitable for further use. Since the culture is not harvested completely, the semicontinuous method yields more algae than the batch method for a given tank size

Continuous Culture

The continuous culture method (i.e., a culture in which a supply of fertilized seawater is continuously pumped into a growth chamber and the excess culture is simultaneously washed out) permits the maintenance of cultures very close to the maximum growth rate. Two categories of continuous cultures can be distinguished:

Turbidostat culture, in which fresh medium is delivered only when the cell density of the culture reaches some predetermined point, as measured by the extinction of light passing through the culture. At this point, fresh medium is added to the culture, and an equal volume of culture is removed. The diluted culture increases in cell density until the process is repeated.

Chemostat culture, in which a flow of fresh medium is introduced into the culture at a steady, predetermined rate. The latter adds a limiting vital nutrient (e.g., nitrate) at a fixed rate, and in this way, the growth rate and not the cell density is kept constant. In a chemostat, the medium addition ultimately determines growth rate and cell density.

Laing (1991) described the construction and operation of a 40 l continuous system suitable for the culture of flagellates, e.g., *Tetraselmis suecica* and *Isochrysis galbana*. The culture vessels consist of internally illuminated polyethylene tubing supported by a metal framework. This turbidostat system produces 30–40 l per day at cell densities giving optimal yield for each flagellate species. A chemostat system that is relatively easy and cheap to construct is utilized by Seasalter Shellfish Co. Ltd, UK. The latter employs vertical 400 l capacity polyethylene bags supported by a frame to grow *Pavlova lutheri*, *Isochrysis galbana*, *Tetraselmis suecica*, *Phaeodactylum tricornutum*, *Dunaliella tertiolecta*, and *Skeletonema costatum*. One drawback of the system is the large diameter of the bags (60 cm) which results in self-shading and hence relatively low algal densities. The disadvantages of the continuous system are its relatively high cost and complexity. The requirements for constant illumination and temperature mostly restrict continuous systems too indoors, and this is only feasible for relatively small production scales. However, continuous cultures have the advantage of producing algae of more predictable quality. Furthermore, they are amenable to technological control and automation, which in turn increases the reliability of the system and reduces the need for labor. In many chemostat continuous culture systems, the nutrient medium is delivered to the culture

at a constant rate by a peristaltic pump or solenoid gate system. The rate of media flow can be adjusted and is often set at approximately 20 % of culture volume per day. Air is pumped into the culture vessel through a sterile filter. This bubbling air has three effects: it supplies CO_2 and O_2 to the culture, aids in circulation and agitation of the cultures, and pressurizes the head space of the culture vessel so as to provide the force to "remove" an amount of media (and cells) equal to the volume of inflowing media. The culture may be aseptically sampled by opening the clamp on a sample port. The magnetic stirrer and aeration help to prevent the cells from collecting in the bottom of the culture vessel. A truly continuous culture will have the medium delivered at a constant volume per unit time. However, delivery systems such as peristaltic pumps or solenoid gates are inherently unreliable. In order to deliver exactly the same amounts of medium to several cultures growing at once, a "semicontinuous" approach can be taken. The rate of flow of medium into a continuous culture system is known as the "dilution rate." When the number of cells in the culture vessel remains constant over time, the dilution rate is said to equal the rate of cell division in the culture, as the cells being removed by the outflow of medium are being replaced by an equal number through cell division in the culture. The principal advantage of continuous culture is that the rate of dilution controls the rate of microbial growth via the concentration of the growth-limiting nutrient in the medium. As long as the dilution rate is lower than the maximum growth rate attainable by the algal species, the cell density will increase to a point at which the cell division rate ("birth rate") exactly balances the cell washout rate ("death rate"). This steady-state cell density is also characterized by a constancy of all metabolic and growth parameters. On the other hand, if the dilution rate exceeds the maximum cell division rate, then cells are removed faster than they are produced and total washout of the entire cell population occurs.

Culture of Diatoms

Diatoms are important members of phytoplankton community, and they are sometimes called golden algae, because the characteristic yellow-brown pigment masks the green chlorophyll. Diatoms have radiosymmetry they are round and shaped like pill boxes, while others have bilateral symmetry and are elongated. Round diatoms float better than the elongate forms, therefore the elongate diatoms are often found in the shallow seafloor attached to floating objects, while the round diatoms are more truly planktonic and are found in areas of cold, nutrient-rich water. A hard, rigid transparent cell wall impregnated with silica surrounds each diatom. Pores connect the living position of the cell inside to its outside environment; buoyancy of the diatoms is increased by low density of the interior of the cell and the production of oil as a storage product. The small volume and accompanying relatively large surface area also provide them with greater exposure to sunlight, and water contains the gases and nutrients for photosynthesis and growth. In addition, some diatoms have spines or other projections that increase their ability to float. Diatoms reproduce very rapidly by cell division; when their populations discolor the water, it is known as bloom. Culture of this group of phytoplankton requires specialized media like TMRL medium, PM solution, Takeda medium, etc.

Culture of Dinoflagellates

Dinoflagellates are single celled protists that contain photosynthetic pigment vary in colour from green to brown to red and can exist at lower light levels. Their external walls do not contain silica; some are smooth and flexible, but others are armored with plates of cellulose. Dinoflagellates usually have two whiplike appendages or flagella that beat within grooves in the cell wall, giving the cells limited motility. Under favorable conditions, they multiply even more rapidly than diatoms to form blooms, but they are not

as important as the diatoms as a primary ocean food source. Some dinoflagellates are called fire algae, because they glow with bioluminescence at night. Dinoflagellates are hard to grow; certain species such as *Prorocentrum micans* grow better in medium FE, which is prepared by mixing equal volumes of Guillard's medium F with Foyn's Schreiber medium E (Subba Rao 1980) and autoclaved at 125 °C (2 atm pressure) for 20 min; some dinoflagellates such as *Ceratium* grow well in Chan's medium (1978) or MET 44 (Schöne and Schöne 1982) and the toxigenic *Protogonyaulax tamarensis* or *Pyrophacus steinii* in T1 medium (Ogata et al. 1987; Pholpunthin et al. 1999). Oceanic ultraplankton is growing successfully in medium K (Keller et al. 1987).

Determining Algal Densities

Monitoring algal growth in mass tank cultures is essential for successful production; a more accurate measure of growth can be made by comparing actual cell numbers with the previous day's count. At present, there are many kinds of counting procedures available for enumerating phytoplankton, two of the procedures are used when counting algae in mixtures, as from field sampling while the other two are used when counting unialgal samples, such as in growth or bioassay experiments in a laboratory. Some techniques are relatively "low-tech" and can be used also in remote locations; others are "high-tech" and require an expensive instrument. The Sedgewick-Rafter counting chamber (Fig. 2) is a low-tech device routinely used for counting algae in mixed assemblages. This cell limits the volume and area of the sample to enable easier counting and calculation of phytoplankton numbers. It consists of a brass or polystyrene rectangular frame mounted on a heavy glass slide from which a precise internal chamber has been cut; its dimensions are $50 \times 20 \times 1$ mm^3, with an area of 1,000 mm^2 and a volume of 1.0 ml. The base is ruled in a 1 mm grid. When the liquid sample is held in the cell by its large, rectangular glass cover slips, the grid subdivides 1 ml volume into microliters.

The phytoplankton sample placed into the Sedgwick-Rafter counting chamber is allowed to stand on a flat surface for 20 min to enable the phytoplankton to settle. It is then transferred to the stage of an upright light microscope and securely positioned, ready for counting. Counts are done with the 4× or (more usually) the 10× objectives of the compound microscope. A Whipple disk is inserted into one of the ocular lenses in order to provide a sample grid. It is necessary to first determine the area (A) of the Whipple field for each set of ocular and objective lenses used. This is accomplished with a stage micrometer. There are 50 fields in the length and 20 fields in the width of the chamber (comprising a total of 1,000 fields). A horizontal strip corresponds to 50 fields. All cells within randomly selected fields are counted.

"High-tech" methods for counting unialgal samples are the electronic particle counter (e.g., Coulter® counter) and the digital microscopy. In spite of relatively high cost, an

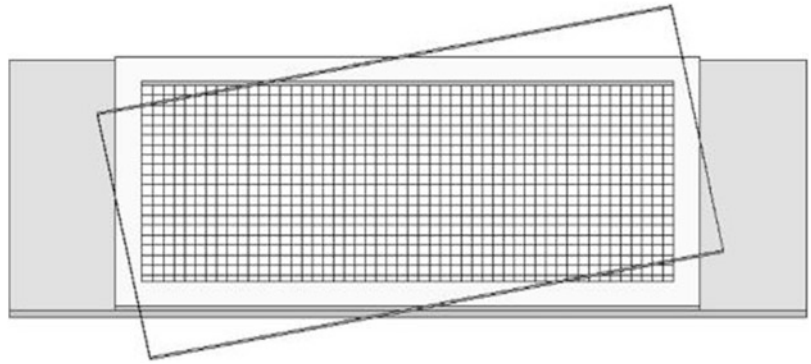

Fig. 2 The Sedgewick-Rafter counting chamber

electronic particle counter is highly recommended for performing growth or bioassay studies that require many counts and high accuracy. In addition, the instrument will provide particle size biovolume distributions. The principle of operation is that particles, suspended in an electrolyte solution, are sized and counted by passing them through an aperture having a particular path of current flow for a given length of time. As the particles displace an equal volume of electrolyte in the aperture, they place resistance in the path of the current, resulting in current and voltage changes. The magnitude of the change is directly proportional to the volume of the particle; the number of changes per unit time is proportional to the number of particles in the sample. When opened, the stopcock introduces vacuum into the system, draws sample through the aperture, and unbalances the mercury in the manometer. The mercury flows past the "start" contact and resets the counter to zero. When the stopcock is closed, the mercury starts to return to its balanced position and draws sample through the aperture. Variously sized aperture tubes are available for use in counting variously sized particles; the aperture size is chosen to match that of particles.

Digital microscopy necessitates of a microscope equipped with a digital TV camera connected to a personal computer running dedicated application software for cell recognition and cell counting. It represents an automatic, reliable, and very fast approach to growth determination.

The number of cells can be determined by studying a sample from a culture under a 400× microscope. The sample is placed on a special slide called a hemacytometer. These slides are available from scientific or medical supply stores and come with instructions. To use the hemacytometer, place a pipette filled with a sample from the culture tank against the "V" groove of the slide (a cover slip must be in place). Withdraw the pipette before the sample runs off the flat grid area, but not before the grid area is completely covered with the algal suspension. Wait about 5 min for the cells to drift onto the grid. The grid area resembles the pound symbol (#) inside a square border so that there are nine equal squares. Each of the four corner squares is further divided into 16 smaller squares. The total number of algal cells in all 16 small squares in the 4 corner areas (64 total small sqs.) is counted. Divide this number by 4, for an average, and multiply by 10,000 (hemacytometer volume factor). The product is equal to the number of cells per ml in the sample. Motile algae like *I. galbana* must be killed before counting. A weak toxin such as acetic acid or ethanol may be used (one drop into 20–30 ml of algal sample). Dense cultures (>3 million) should be diluted 1:10 to facilitate counting and to reduce error. Remember to multiply by 10 (for a 10:l dilution) or by the appropriate correction factor if a different dilution is made. There are numerous other methods to quantify algal densities; however, using a microscope and hemacytometer will reveal the presence of biological contaminants. Peak or maximum density (for existing conditions) is attained when, on consecutive days, the cell count does not increase by a factor of two. With practice, approximate cell counts can be estimated by observing the gross color of each culture. These estimates are based on the tint of the water caused by the algal cells and comparing it to the actual cell count. Tedious and time-consuming cell counts (or other growth measurements) can be eliminated from the daily routine. Cell counts should be made periodically as a check on the gross estimate. In practice, culture crashes can be caused by a variety of reasons, including the depletion of a nutrient, oxygen deficiency, overheating, pH disturbance, or contamination. The key to the success of algal production is maintaining all cultures in the exponential phase of growth. Moreover, the nutritional value of the produced algae is inferior once the culture is beyond phase 3 due to reduced digestibility, deficient composition, and possible production of toxic metabolites. Cells can be counted either with an electronic particle counter or directly under a microscope, using a hemacytometer. The Coulter® counter and similar instruments need appropriate calibration for each algal species to be counted. Detailed instructions on operation of electronic cell

counting can be found in Sheldon and Parsons (1967). The presence of contaminating particles in the same size range as the algae and failure of cells to separate after cell division may be possible sources of erroneous counts. Counting with a microscope has the advantage of allowing control of the quality of the cultures. The major difficulty in microscopic counts is reproducibility, which is a function of the sampling, diluting, and filling of the counting chamber, as well as the choice of the right type of counting chamber and range of cell concentration. A relationship between optical density and cellular concentration, can be established using a spectrometer. However, variations may occur due to the fact that the chlorophyll concentration in the algal cell varies according to the culture conditions and therefore affects this relationship. In this way, a culture under low lighting conditions will be comparatively more pigmented and will eventually result in higher readings for optical density.

Harvesting and Preserving Microalgae

In most cases, it is unnecessary to separate microalgae from the culture fluid. Excess and off-season production may, however, be concentrated and preserved. The various techniques employed to harvest microalgae have been reviewed by Fox (1983) and Barnab (1990). High-density algal cultures can be concentrated by either chemical flocculation or centrifugation. Products such as aluminum sulfate and ferric chloride cause cells to coagulate and precipitate to the bottom or float to the surface. Recovery of the algal biomass is then accomplished by, respectively, siphoning off the supernatant or skimming cells off the surface. Due to the increased particle size, coagulated algae are no longer suitable as food for filter feeders. Centrifugation of large volumes of algal culture is usually performed using a cream separator; the flow rate being adjusted according to the algal species and the centrifugation rate of the separator. Cells are deposited on the walls of the centrifuge head as a thick algal paste, which is then resuspended in a limited volume of water. The resulting slurry may be stored for 1–2 weeks in the refrigerator or frozen. In the latter case, cryoprotective agents (glucose, dimethyl sulfoxide) are added to maintain cell integrity during freezing. However, cell disruption and limited shelf life remain the major disadvantages of long-term preserved algal biomass. Concentrated cultures of *Tetraselmis suecica* kept in darkness at 4 °C maintain their viability, whereas the latter is completely lost upon freezing. Furthermore, cultures stored in hermetically sealed vials lose their viability more rapidly than those kept in cotton-plugged vials.

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