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CHAPTER 07

ALGAL CULTURE METHODS

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Stock Culture

All algae culture systems require a set of 'stock' cultures, usually of about 250ml 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 500ml borosilicate glass, flat-bottomed boiling flasks fitted with cotton-wool bungs. Two types of culture medium can be used:

a) Erdschreiber culture medium, which is difficult to prepare but very reliable; and

b) simpler, but less reliable culture medium

Sub culture

Stock cultures must be sub-cultured frequently. Sub-culturing 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 sub-culturing 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 sub-culturing.

To start a new stock culture, about 20ml of algae are taken from a stock culture which has been growing for 6 to 7 days and poured into a flask containing 250ml 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 the new stock culture should be put about 20cm from a fluorescent lamp that is lit continually. The air temperature around the culture should be less than 25°C. Note that stock cultures do not require an air/CO_2 supply.

After sub-culturing, the remainder of the old stock culture can be used to start a batch culture of up to 10 litres. 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.

Types of Culture

There are many different ways of culturing algae. These range from closely-controlled methods on the laboratory bench top, with a few litres algae, to less predictable methods in

outdoor tanks, containing thousands of litres, in which production relies on natural conditions. Several methods have been developed at Conwy, for the production of algae for use as food for various marine animals.

There are certain requirements for all methods. A culture must be inoculated, and the algae left to grow and divide. The rate of growth and division varies with different types of algae left to grow and divide. The rate of growth and division varies with different types of algae and also depends on how well the various culture conditions necessary for growth have been met. When there are sufficient algal cells in the container for feeding, one of the three culture methods below may be followed:

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.

Semi-continuous culture

Semi-continuous 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 sea water and nutrient salts). After allowing 2-3 days for the remaining cells to grow and divide, the process is repeated. Semi-continuous cultures may be operated for up to 7 to 8 weeks.

Continuous culture

This falls into two categories:

i) *turbidostat* culture, in which the number of algal cells in the culture is monitored and, as the cells divide and grow, an automatic system keeps the culture density at a pre-set level by diluting the culture with fresh medium; and

ii) *chemostat* culture, in which a flow of fresh medium is introduced into the culture at a steady, pre-determined rate.

With both types, the surplus culture overflows into a collecting container, from which it can be taken and used as food.

With semi-continuous and continuous culture methods, the number of food cells produced (the yield) varies with the density of the culture. For each type of algae, the greatest yield is obtained by maintaining the culture at an optimum density. This optimum density can be determined experimentally and is given for each of the culture systems as described in the following section.

Some culture methods

Batch culture (small volumes of up to 10 litres)

Where small volumes of algae culture are required, for example, of 2 litres to 10 litres per day, production is most conveniently achieved in flasks. An example of a batch culture system for producing 3 litres of food per day from a culture of *Chaetoceros calcitrans* is given below and is described as daily procedures.

A set of three, 250 ml stock cultures is started by inoculating from one existing 250 ml stock culture on each of 3 successive days. The new stock cultures are grown at a temperature of about 21°C, and at a distance of 15 to 20 cm from a 65 watt fluorescent tube. After 3 days, and then daily, each of these stock cultures is used in turn to inoculate a new 250 ml stock culture then daily, each of these stock cultures is used in turn to inoculate a new 250 ml stock culture and the remainder is added to the 3 litre sea-water culture medium in the flasks, which have been prepared as follows:

Three-litre borosilicate glass flasks with cotton wool plugs are filled with sea water. The contents are either autoclaved at 1.06kg per square centimeter (15 psi) for 20 minutes, boiled for 30-45 minutes, or pasteurized. Whichever method is used, the sea water in the flasks should be allowed to cool before adding nutrient salts, Alternatively, sea water that has been filtered through a half micron filter may be used. To the 3 litres of sea water in the flask, 6 ml solution A, 0.6 ml of solution C and 6 ml of solution D are added.

A fresh 3 litre culture is started daily from a 3-day-old stock culture and aerated with a mixture of air and CO_2 at about 2 to 3 litres per minute. The gas mixture is filtered through an in-line catridge unit containing a 0.3-0.45 micron filter to reduce the risk of airborne contamination. When grown at about 21°C next to a continually lit, double flurescent lamp unit, a density of 45000 to 60000 cells per microlitre is reached in 3 to 4 days in culture medium prepared from heat treated sea water. In medium prepared from filtered sea water, growth of *Chaetoceros* is not as rapid and the density will only reach 20000 to 30000 cells per microlitre in this time. The culture should immediately be used for feeding, as if kept it will enter a declining phase, collapse, and become unsuitable.

Algae other than *Chaetoceros* usually take longer to grow under similar culture conditions. For these algae, stock cultures should be routinely sub-cultured every 6 to 7 days. The batch cultures, which are started from 6 or 7 day old stock cultures, will take 7 to 8 days to grow to a density suitable for feeding. Larger containers, of up to 10 litres, may be used for this method of culture. Nutrient salt solutions are used a s follows:

Solution A- 1ml per litre
Solution C- 0.1 ml per litre; and

Solution D (diatoms only) – 2ml per litre.

The batch cultures may be used directly as food or as inocula to start larger volume batch, semi-continuous or continuous food cultures.

Semi-continuous culture

Two-hundred-litre vessels

A method for large scale production in 200 litre, internally illuminated, glass reinforced plastic (grp) vessels, using semi-continuous culture is described below.

The vessels are 150 cm high, 40-45 cm in diameter and each has a central lighting unit into which are fitted six fluorescent lamps. A glass- fibre cooling pipe is moulded onto the outer jacket. These vessels are most useful for growing diatoms, but they may also be used for flagellates. The vessels are sterilized by filling with a solution of sodium hypochlorite (50 parts per million (ppm) free-chlorine concentrations). Note that domestic bleach contains about 100000 to 150000 ppm chlorine, so a dilution of 1 ml per 2-3 litres would give the required concentration. They are allowed to stand for 2-4 hours and the drained, and flushed with filtered air for 24 hours to drive off residual chlorine.

The vessels are filled (200 litres come to about 15 cm from the top) with filtered sea water at 20 psu to 25 psu salinity for diatiom cultures or 25 psu to 30 psu for flagellate cultures. For diatom cultures, filtration to 2 microns is usually sufficient, while for culture of flagellates, filtration to half a micron is preferable. Two hundred milliliters of solution A 20 ml of Solution C and, for diatom cultures, 1200 ml of Solution D are added to the vessels. The culture is inoculated with 2 to 5 litres of a 4 to 8 day-old batch culture, grown as described in the previous section and aerated with a filtered air/CO_2 supply at about 15 litres per minute.

Cultures should reach densities suitable for harvesting after 4 to 7 days. The cultures should be subsequently be diluted for the maximum yield. The amount of harvest which achieves this yield can be calculated from the following equation:

$$\text{Volume to harvest in liters} = \frac{\text{200 x densities to which culture needs to be diluted}}{\text{Actual culture density of algae harvested}}$$

After harvesting, the vessels are topped-up to 200 litres with filtered sea water of the correct salinity. For each litre harvested, 1 ml of solution A, 0.1 ml of solution C and, for diatom cultures, 6 ml of solution D are added. It is usually more convenient to harvest the culture every 2 to 3 days (e.g Mondays, Wednesdays and Fridays). That part of the harvest which is required for feeding on the intermediate days can be aerated in a plastic container away from bright light and in a cool place.

The length of time during which the culture is able to produce food will vary with the type of algae. Production of algae from 200 litre vessels should average the equivalent of 60-80 litres per day at the cell densities. When the culture is no longer required, or it has come to the end of its production period, the vessel can be drained and cleaned with a stiff brush, to remove any algae adhering to the sides. The vessel can now be sterilized for preparation for a new culture.

Continuous culture

This method is suitable for the culture of flagellates. The internally-illuminated, continuous culture vessels are made from polyethylene tubing supported by a metal framework. They consist of 160 cm lengths cut from 71 cm wide polyethylene, 'layflat' tubing. The tubing is free of potential contaminants due to the heat used in the manufacturing process and no further sterilization is necessary. The cut length is heat sealed across the width of one end and positioned around the acrylic cylinder containing the lamps. The six nuts and bolts securing the outer supporting mesh jacket are fastened and the outer reflective sheet of white, corrugated plastic is held in place by 12.7mm nylon power belting, which also supports the sensor housing unit against the outer surface of the culture.

The polyethylene tubing is filled with sea water at 25 psu to 30 psu salinity that has been filtered through a sterile, 0.45 micron, particle retention catridge filter. If the water has a high silt load, it should first be passed through a 2 micron filter. Solution A (100ml) and Solution C (10ml) are added to the sea water in the vessel. This is 2.5 times the usual amount, and is added to ensure that nutrient levels do not become limiting at the high cell densities at which the cultures are maintained. A 2.5 cm diameter circle is cut from the tubing, with its centre about 7 cm above the water level. Into this is fitted a 1.9 cm rigid, PVC tank connector, from which a 150 cm length of 1.5 cm bore flexible PVC tubing is run into a 125 litre collecting vessel. The overflow allows for automatic harvesting of the culture into the reservoir.

A supply of filtered air, enriched with sufficient carbon dioxide to maintain culture pH at 7.6-7.8 is introduced through a 0.4 cm bore, 150 cm long, acrylic tube inserted into the top of the culture. A flow rate of about 15 litres per minute ensures efficient mixing of the culture.

Cooling water, at a flow rate of about 0.35 litre per minute, is allowed to run down over the outer culture surface in order to maintain the culture temperature at about 21°C. The 40 litre culture should be inoculated with a 2 litre batch culture that has grown for 7 to 8 days.

Automatic harvesting of the culture is controlled by the following method. A cadmium sulphide photo-conductive cell is enclosed in a light-proof housing against the outer surface

of the culture. The housing is placed about 50 cm from the base of the vessel and positioned so that the stream of air bubbles rising through the culture does not interfere with its operation. The resistance of the photo-conductive cell will increase as the light intensity reaching it from the lamps falls, when density of the culture increases, due to growth and division of the algal cells. A circuit switching the peristaltic pump will be energized when the resistance of the cell becomes greater than a present value on a relay sensitive to input resistance in the range 50-5000 ohm. Sea water, at 25 psu to 30 psu and enriched with 2.5 ml of solution A per litre and 0.25 ml of solution C per litre, is then pumped from the culture medium reservoir through the filter into the vessel and the volume is maintained by an overflow. The outflow of algae culture from the vessel is collected in an aerated container. As the culture is diluted, the decrease in resistance of the photo-conductive cell, caused by the higher light intensity now reaching it, is sensed by the relay and the pump circuit is switched off.

The relay should be set so that automatic harvesting of the culture occurs at the density that gives the most yield. When the yield begins to fall appreciably, all of the culture should be harvested for feeding and the bag discarded. A new, clean bag should be fitted to the vessel and the above operating procedure repeated.

Photobioreactors

Design Considerations

Many configurations of photobioreactors have been devised and built. These range from tubular and cylindrical systems to conical systems (Watanabe and Hall 1996, Morita *et al.*, 2000) to flat-sided vessels (Delente *et al.*, 1992, Iqbal *et al.*, 1993, Hu *et al.*, 1998), and these systems employ a wide variety of light sources (Takano *et al.*, 1992, Lee and Palsson 1994, An and Kim 2000). As such, each is usually a highly customized system and the availability of commercial photobioreactors is limited.

Despite various configurations, several basic design features must be considered when building a photobioreactor: how to provide light, how to circulate the algae, which materials to use for construction, how to provide CO_2 and remove O_2, and how to control pH and temperature. Sophistication is driven by purpose, and if whole algal biomass is the desired final product, a relatively unsophisticated system will suffice. Glass and acrylic are widely used in the construction of photobioreactors, and ultraviolet (UV)-stabilized acrylic is superior because it is lighter, more flexible, stronger, and easier to machine, cut, bond, and so on. Construction requires some minimal construction skills, but certainly not professional skills. Assembly of the photobioreactor system is merely the integration of various monitor and control subsystems (e.g., pH control and temperature control).

Light

Light is the most important parameter in the design and construction of a photobioreactor. Despite its importance, light can be a very difficult input to measure for efficient use (Pirt *et al.,* 1983, Kirk 1994, Ogbonna *et al.,* 1995, Janssen *et al.,* 2002). Light can be supplied continually or in light-dark cycles. As cell concentration changes, the light requirements change. Algal growth is limited by too little light, but too much light can be as deleterious. Phototrophs must receive sufficient light to exceed their light compensation point for their net growth; insufficient growth detracts from the net growth of the culture because of respiratory loss (Radmer *et al.,* 1987). Increasing light beyond the compensation point results in an increase in the growth rate until the culture becomes light saturated, and higher light intensities can lead to photoinhibition (AcienFernandez *et al.* 1998, Csögör *et al.,* 2001, Yun and Park 2001, Wu and Merchuk 2002, Barbosa *et al.,* 2003, Suh and Lee 2003). Incandescent lamps are inferior to the other lamps and are seldom used for photobioreactors (Table 13.1). Both fluorescent and high-intensity discharge lamps have very good electrical efficacy and substantial lifetimes. Fluorescent lamps distribute light more or less uniformly along the length of the lamp; light emanates from a point source in high-intensity discharge lamps, requiring distance between the lamp and algae for proper light dispersion. Although high-intensity discharge lamps are slightly more efficient than fluorescent lamps, however, fluorescent lamps offer the best all-around choice (Radmer *et al.,* 1987, Radmer 1990, Delente *et al.,* 1992).

Circulation

Circulation is important to ensure optimum illumination of the algae, adequate gas exchange, and temperature and pH control within the culture. The “airlift” principle in which circulation is accomplished by creating differences in water density in different regions of the photobioreactor is the one in commonly using (Manfredini and Molina Grima *et al.,* 1995, Blanch and Clark 1997). This principle is common in fermentors (see later discussion) and has been adapted for use in photobioreactors.

Photobioreactors can be bubbled with air, but the low CO_2 concentration in air (0.033%) will often limit phototrophic growth. With an airflow of 1 L min^{-1}, assuming all carbon dioxide is used and the biomass is 50% carbon, there is enough carbon to support 3.54 *10^{-4} grams biomass · min^{-1}; this is a very low productivity. The simplest approach is to blend CO_2 with air, for example, 0.2 to 5.0% of the total gas flow (Lee and Pirt 1984, Merchuk *et al.,* 2000, Morita *et al.,* 2001, Babcock *et al.,* 2002). Care must be taken to ensure that the CO_2 input does not adversely lower the pH level of the culture. Also, with an open system, most of the CO_2 will exit the vessel unused. Small bubble size helps facilitate diffusion.

Temperature and pH

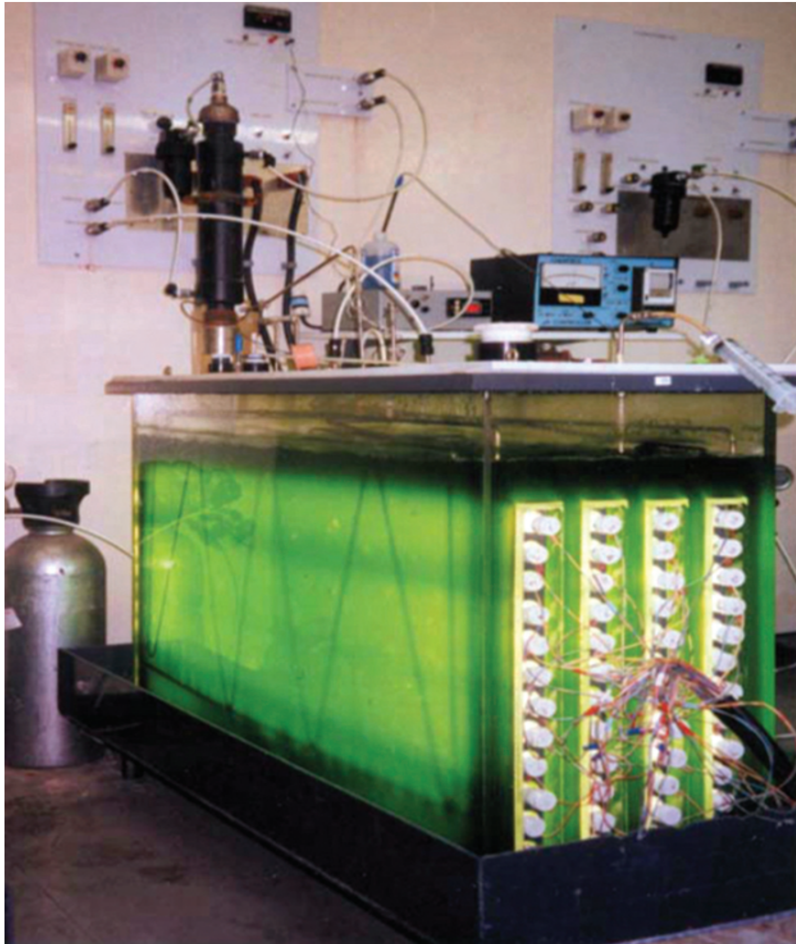
After light, CO_2, and O_2, pH and temperature are the next most important parameters to measure and control. Fortunately, these are very simple parameters to control using existing off-the-shelf technology (Sonnleitner 1999). Commercially available pH controllers (Omega Engineering) are recommended. Photosynthetic systems will always generate heat because of the inefficiency of photosynthesis in converting light energy into chemical energy (Pirt, 1983, Morita *et al.,* 2001). The theoretical conversion of red light into chemical energy (NADPH) is only 31%; 69% is lost as heat. The amount of cooling depends on the incident light intensity and the cell concentration (*i.e.*, how much light is absorbed), but regardless, cooling will be necessary. In principal, it is quite easy to control the temperature using commercially available temperature controllers (Omega Engineering). Cooling is achieved with a heat exchange system; external cool water is circulated through a good heat conducting material, which then draws heat from the photobioreactor. The more significant challenge is how to install a cooling system on a photobioreactor. The most preferred system is the one uses stainless steel cooling coils submerged in the culture medium. There is excellent contact between the cooling coil and water, which provides good cooling, but the cooling coil sometimes interferes with the circulation within the vessel. A refrigerated water source (or tap water, depending on the cooling demands of the culture) is used, and a temperature probe, connected to a controller, operates a solenoid to regulate cooling water flow. The best strategy is to use a "normally open" solenoid so that if there is a failure of the controller, the photobioreactor will overcool, which is preferred to overheating.

Sterilization

For many phototrophs, gross contamination by bacteria and fungi is not a significant problem because there is generally very little free organic carbon to support their growth (see Chapter 5 on sterility). A higher concern is to prevent contamination of the photobioreactor by other phototrophs. Photobioreactors are made of optically clear materials (e.g., glass or acrylic) which do not lend themselves to steam sterilization. Furthermore, the size of most photobioreactors exceeds what can be accommodated in an autoclave. Systems exist for sterilization with ozone (Quesnel 1987), but these systems are expensive and difficult to use. A more practical solution is sanitization rather than sterilization. Photobioreactor sanitization can be easily accomplished with bleach. Air pumps, analyzers, and other equipment can be kept free of algae and other microorganisms by the use of the appropriate prefilters.

Operational Strategies

With adequate measurement and control of the basic culture parameters of light, CO_2, O_2, pH, and temperature, it is possible to optimize these parameters to achieve the desired



Martek 120-liter photobioreactor

end product from the photobioreactor. The end product dictates the alga, which in turn dictates the general growth conditions. *Yield* and *productivity* are terms that require careful definition. Yield is the production of mass per unit volume, and it is often expressed in terms of g L^{-1}. Productivity is yield per unit time and is often expressed in terms of g L^{-1} $hour^{-1}$ or g L^{-1} day^{-1}. The growth conditions that produce maximal yield are rarely the same conditions that produce maximal productivity. For a given alga and/or a given product, it is possible to methodically test and optimize each culture parameter to determine the best value for maximizing either yield or productivity.

Although this is certainly a straight forward approach, a more efficient means to optimizing culture parameters is to employ a statistically based factorial or fractional factorial

approach to experimental design (Anderson and Whitcomb 2000).This method uses statistics to identify the culture parameters and the interactions between culture parameters that are most important for achieving maximal yield and/or productivity. Perhaps more importantly, this approach identifies those parameters that are not important for maximum yield and/or productivity and thus eliminates the need for much unnecessary experimentation.

Harvesting Methods

The algal culture is still relatively dilute in a fully optimized photobioreactor. A dry weight concentration of even 5 to 10 g L^{-1} is far below that of a fermentor system (>50 g L^{-1}). The two most common harvesting approaches for photobioreactors are flocculation and centrifugation (Sukenik and Shelef 1984, Becker 1994). Although flocculents enable a concentration of the algal biomass, centrifugation is usually still necessary to achieve a suitable volume reduction. Small-scale recovery can be easily done in large centrifuge bottles, but larger scale cultures are best done with a continuous flow centrifuge such as a Sharples (Sharples Penwalt).

Other methods

There are other, similar, methods for growing marine algae that may be more appropriate in certain circumstances. For example, externally-illuminated 20 litre glass jars can be operated as batch cultures or semi-continuous cultures for a variety of algae. Another, widely used, type of culture container includes a mesh framework to support a 200-400 litre externally-illuminated polyethylene bag. Both of these methods have the disadvantage of being less efficient, as the light energy cannot penetrate the culture as well as the internally- illuminated unit, but they do have the advantage of being easier and cheaper to construct. Polyethylene bag cultures may also be operated as continuous, chemostat, cultures by using small pumps to introduce fresh filtered medium to the culture at a pre-determined rate.

Extensive methods may be employed to culture marine algae. Outdoor tanks are nutrient-enriched with agricultural fertilizers. Adding 1.5 g of urea, 1.6 g of triple superphosphate and 10.6 g of sodium metasilicate will provide the required amounts of nitrogen, phosphorous and silica to stimulate growth and division of algal cells and, depending on the temperature of the sea water and the amount of sunshine on the tanks, blooms of algae will develop. This system can be operated as a batch culture or managed as a semi-continuous or continuous culture with an in-flow of nutrient-enriched sea water to provide fresh medium as an impetus to further algal growth. The method is especially suitable for providing algae for feeding to bivalave mollusk spat held in nursery systems, or for mass 'grow-out' of brine shrimps (*Artemia*).

Conclusions

Whatever method of large-scale algae culture is adopted, it is liable to be expensive and technically difficult to operate. For this reason, a great deal of research is now being directed towards a suitable algae replacement diet for use in commercial aquaculture. Successful development of such a diet will perhaps eventually remove the need for large-scale production of algae for food.