ALGAL CELL CULTURE

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Contents

Summary

Our ability to culture microalgae has advanced rapidly over the past 50 years. We now have sufficient understanding of their physiological requirements to culture hundreds, probably thousands, of different species. The fundamental techniques used to culture algal cells are reviewed in this section. Use of these relatively simple techniques has led to an improved understanding of the factors that limit algal growth. Understanding these limitations has allowed insightful pioneers to design more efficient culturing systems. In some experimental systems, efficiencies have reached the theoretical maximum for photosynthesis providing a highly efficient means of converting light energy into biomass.

Microalgae produce more protein per unit dry weight than almost any terrestrial plant. They are also the main source of some organic molecules important in human nutrition and physiology such as long chain polyunsaturated "omega three fatty acids. Researchers are currently surveying the vast range of microalgal species for novel compounds of economic value such as anti-tumor and anti-bacterial compounds. Some have already been discovered and more will be found. The advent of genetic engineering makes it possible for the energy required to synthesize many organic molecules to be supplied free from sunlight, captured by microalgal photosynthesis and converted into a range of useful products.

Only a few companies culture a handful of species on a large scale. Microalgae remain a source of great, but mostly unrealized, potential. There is still a need for technological solutions to improve the efficiency of culture and harvest systems. It would be hard to believe that these problems will not be overcome by the many small groups of researchers working on them around the world. There should not be any doubt that

microalgal culture has an exciting future. In comparison with terrestrial agriculture we are just beginning. There has been a rapid expansion in capability over the last 50 years and every reason to think it will continue to expand. There are many compounds of commercial potential in microalgae and finding economically viable methods of producing them will challenge future generations.

1. Introduction

Microalgae are the basis of food webs in bodies of water. Microalgal cell culture involves growing these same organisms but under more controlled conditions. A great deal of effort in the last 100 years has been directed at achieving intensification of the culturing process to yield greater cell densities in less time. In many ways the intensification process is similar to that which occurred in terrestrial agriculture over the last 10,000 years.

A number of fundamental steps have been taken to allow successful microalgal culture. Foremost has been greater understanding of the requirements of microalgae. These requirements can be simplified to water, light and inorganic solutes (solution of mostly inorganic ions). The optimal growth requirements have been determined for a few species while relatively simple media recipes have been developed that will support many different species. In this section a well tested recipe of enrichments for seawater is provided and discussed in detail. Water and the procedures used to prepare water for use as media are also presented. The growth characteristics of a typical microalgal batch culture in enriched seawater are described.

Another important, and still ongoing, step in the domestication of microalgae is to determine the best species to grow. Currently microalgae are grown for a variety of uses including: food for humans, fodder, food for animals in aquaculture, chemicals, drugs and treatment of wastewater. We are still in the process of determining which wild species are best suited to these roles. There are collections of microalgae in culture in many countries around the world. Most of these collections will provide a pure strain of microalgae in a small volume of medium for a modest fee. In some laboratories the selection of strains with enhanced performance is also underway.

The first goal in the domestication process is the successful isolation of a pure strain of microalgae. The procedures that can be used to achieve isolation and the subsequent steps necessary to culture microalgae are described along with a range of techniques that have been developed to facilitate algal culture. Some of these techniques are adapted from other fields, but some of them are unique to microalgae.

The major types of culturing systems are used for microalgae and their relative strengths and weaknesses are presented. Some details of a few species currently mass cultured are included but most of the emphasis is upon understanding how the fundamental issue of maintaining optimal growth conditions is affected by the various possible culture techniques. Irrespective of whether the culture system is open or closed, tubular, spherical or flat; the fundamentals of a successful algal culture system are the same. Understanding the fundamentals should make it possible to culture microalgae on any scale.

2. Cell Culture Characteristics

2.1. General Considerations

It would be a mistake to underestimate the diversity of life that can be defined as microalgae. The range of diversity in the Class Mammalia (from giant cetacean to marsupial mouse) is small by comparison. Although estimates vary, there are probably 15 to 30,000 species of microalgae. Due to space limitations a considerable effort has been made to present the most generally applicable information about microalgae. In few cases, where it was deemed appropriate, a more detailed discussion of the range of possible variations in the techniques used to culture microalgae are presented. Also some specific examples relevant to a limited number of species are provided particularly where these species are important in worldwide microalgal production. In most cases any reader seeking more detailed information will be able to find a suitable reference in the bibliography.

Although it is not possible to justify on a systematic basis, the cyanobacteria are included in this article even though they are not algae, not plants, not eukaryotes. They are prokaryotes (no nuclear membrane, no internal organelles). In terms of similarity of the culturing techniques used, however, they are rightfully included with other photosynthetic, oxygen evolving, mostly single-celled, aquatic organisms.

2.1.1. Morphology

As a group the microalgae consist largely of single celled forms, but some are "multicellular" in the sense that the cells may be physically connected. "Multicellular" microalgae are commonly chains or filaments. The size of individual cells ranges from less than one micron to more than 2000 microns in length. Cell shapes can be very complex but typical shapes are round, oval and cylindrical. External cell coverings range from ubiquitous polysaccharides to silica and calcium carbonate. The silica cell coverings (frustules) form complex and intricate (even beautiful) structures on most members of the *Bacillariophyta* (diatoms). Those species that form calcium carbonate external coverings (coccolithophorids) make the calcium carbonate into ornate scales with a huge variety of shapes and sizes. These scales have a tendency to fall off the cell and due to their unique reflective qualities blooms of these species are highly visible to satellites in space. Many species of microalgae are flagellated and some have other flagella-like structures (e.g. haptonema). The immense diversity of morphology has fascinated naturalists for hundred of years (since the first microscopes) and needs to be seen to be appreciated.

2.1.2. Physiology

Microalgae are largely photosynthetic but there are many exceptions. For example, up to 50 percent of the *Dinophyta* are nonphotosynthetic. Most microalgae form O_2 as their primary waste product, whereas a few form H_2S. Storage products are largely simple sugars {α-(1-4) or β-(1-3) -linked polysaccharides}or lipids (mostly triacylglycerols). Although microalgae are a much better source of protein than most terrestrial crops, even after the latter have been processed (Table 1), they are largely not grown for

biomass, carbohydrate or protein production. A few species are grown for the human health food market as a source of micronutrient dietary supplements.

	Protein	Carbohydrate	Lipid	Pigment	ash	total
	%	%	%	%	%	
Microalgal average	**39**	**23**	**8.3**	**1.7**	**21.7***	**94**
Grain	11	83	2.2		2.2	
Bran	18	66	3.5		10.0	
Flour	12	71	12			

* the high ash content is largely due to the silica found in diatoms

Table 1: Average proximate composition (as a percentage of dry weight) of 11 species of microalgae in five Classes (after Parsons et al. 1961) in comparison with some common foodstuffs.

Many unique biomolecules are found within this large and diverse group of organisms. Major components that are currently extracted on a relatively large scale include various lipids and pigments. In particular, the long chain polyunsaturated "omega three fatty acids that seem to be highly beneficial to human health largely originate from microalgae. Along with fatty acids, pigments such as β-carotene (and other carotenoids that have a wide range of uses) can be harvested from algae grown in large scale culture at a cost that is competitive with a synthetic product. Other new products are being developed. The range of products and their potential value is very exciting. One interesting new development is the adaptation of toxins for use in medicine. A wide range of toxins are found in microalgae that currently cause environmental problems. These same biomolecules may have medicinal uses.

2.1.3. Reproduction

In microalgae most reproduction is *via* binary fission yielding two daughter cells. A few species form four or eight cells when dividing. Typically a species will produce many generations *via* binary fission (vegetative growth). Most commercial operations rely upon this fact to produce a harvestable biomass. For most species the physiological trigger into another life history phase is not well understood but often involves environmental conditions becoming less suitable for growth. The next stage in a typical life history often involves sexual recombination of DNA sometimes before and sometimes after the formation of a cyst. Cysts can have a very different physiology than the vegetative cell, but it is not known whether their biochemistry is fundamentally different. The investigation of cysts for unique biomolecules has hardly begun, but it seems likely that these cell types should yield potent anti-bacterial compounds. Cysts are frequently found in sediments where they can survive for many years prior to germination fulfilling a similar ecological function as bacterial cysts or seeds from terrestrial plants. In some microalgal species sexual reproduction can be relatively simple with populations apparently consisting of only two mating types, but populations can be composed of eight or more mating types that do not all interbreed. Most species are likely to have a sexual reproduction stage in their life cycle, but life cycles and their

stages are described for only a few species. It is possible that many small flagellates that appear sporadically in natural communities are life history stages of some other form of microalgae. For one fish killing species of microalgae (*Pfiesteria piscimorte*) the life history cycle has been described to contain a number of stages that span a great range of morphologies from vegetative cell to amoeboid to cyst.

2.2. Habitats

Microalgae can be free living (phytoplankton), epibenthic, or benthic (microphytobenthos), or even intracellular symbionts. Most of the commercially grown species are free-living forms that are either single celled or "multicellular" (in the sense that they form filaments made of cells joined end to end). The fact that most commercial products are derived from free-living forms means that there has been more emphasis upon developing culture techniques for cells suspended in water. Perhaps the largest exception to the use of free-living forms is the rapidly growing field of abalone rearing on surfaces (plates) with a microalgal film of benthic phytoplankton (largely diatoms). The plates are used to raise abalone in their recently settled post-larval phase until they can be transferred to other diets.

2.3. Isolation

Bringing a new algal species into culture or saving a cross-contaminated algal culture both require the same techniques. The most widely used methods of isolation are capillary pipet removal and serial (multiple) dilution into new containers. In some cases, particularly for algae less than 10 μm, it may be easier to isolate them using streaking onto seawater-based agar. Unless otherwise indicated all equipment, supplies and media used should be sterile.

An inverted microscope is best for isolating small cells, but a very high power dissection scope or compound scope can be used. First it may be necessary to make a capillary pipette. These are normally made inside a laminar flow hood from sterile Pasteur pipets (with cotton plug in the wide end) and kept sterile until used. Heat the thin end of the pipette, two cm from the end, in a low flame (could be the pilot flame on burners so equipped). If you heat it too fast it will not stretch out to a suitable shape, so it must be warmed slowly. As the glass softens and reddens use a *smooth* action to both remove the pipette from the flame while simultaneously pulling the tip with sterile forceps. The heated glass should stretch (approximately two to six cm) and become thin. Break off the tip near where it bends under its own weight. This should yield a new tip with a 75 − 150 μm bore. Place a few drops of sterile medium in three to seven "pools" clockwise around the edge of a sterile petri dish or in the wells of multiple compartment dishes. Place the drop of sample material in the center (petri dish) or in one well. Observe it under the microscope. Locate one cell (or as few as possible) of the desired type, insert pipette tip into the liquid and capillary action will draw up some liquid into pipette. Place this liquid into a nearby "pool" of medium. For those that practice this regularly the use of a bulb or some flexible tube on the end of the Pasteur pipette with the other end in mouth and the application of very small amounts of positive or negative pressure can facilitate cell isolation. Observe this "pool" and try to locate some of the desired cells. Using the capillary pipette (a new one if the sample was heavily

contaminated with other species), try to remove one cell (or as few as possible) and place into a second "pool". Observe again under the microscope and repeat as necessary until there is only one cell or only one species present. Make the last isolation a transfer into a new well or small test tube. Repeat the whole process five to 10 times. Incubate the five to 10 new cultures for one to two weeks. Check to see if a pure culture has resulted.

Alternative methods of isolation (especially useful for smaller cells) include streaking cells in sterile petri dishes half full of media solidified with 1 − 1.5 percent agar. Place one or two drops of the mixed cells onto the agar. Streak with a flame-sterilized wire loop several parallel lines across the agar. Cover the petri dish and incubate under suitable conditions for four to eight days. Observe the petri dish under the microscope and select suitable colony(ies). Remove using sterile wire or capillary pipette and place on a slide under a cover slip, and observe under a high power (400 to 1000 times) compound or inverted microscope. If the desired cells are present, repeat the streaking and then isolate the new colonies into liquid medium.

2. 4. Culture Purification

Note that all materials used in these procedures must be sterile. Cultures contaminated with bacteria can be "cleaned-up" by various techniques including those given above under isolation. If re-isolation does not free the culture from bacterial contamination the following steps may be successful:

- Washing: Purification can sometimes be accomplished by multiple washings associated with the pipette isolations, or by picking clean colonies off the agar plates. If further washing is necessary then these techniques can be supplemented with centrifugation. Algal cells are placed in centrifuge tubes half full of media and centrifuged until loosely near bottom. As bacteria are less dense they are mostly in the supernatant and so can be removed by pouring it off. Repeat five times. Transfer single cells to new medium or streak plate as in isolation techniques (above).
- Sonication: similar to washing, but cells are sonicated in centrifuge tubes prior to centrifuging. The length of time they should be sonicated depends upon the sonicator power' and the cell's characteristics and needs to be tested. When using cultures try sonicating for five seconds to several minutes, place the cultures back into growth conditions and check for viability (growth). Use the longest sonication period which yields suitable growth. The theory is that strongly adhered bacteria can be sonicated off the algal cells, and then separated by centrifugation. Once centrifuged, discard the bacteria-rich supernatant and proceed as for isolation techniques (above).
- Antibiotics: Prepare antibiotic stock as follows: dissolve 100 mg penicillin G (K or Na salt) and 50 mg streptomycin-SO_4, add 10 mg chloramphenicol dissolved in one mL 95 percent ethanol. Filter through a 0.45 µm membrane filter.
- Dilute your contaminated algal culture into six new containers with media. You can use test tubes or Erlenmeyer flasks or other suitable containers (all sterile; use sterile techniques). You will need to select an appropriate dilution for the algal species (range 0.25 to 10 percent) but the greater the dilution the less

bacteria are transferred. Add antibiotic solutions at 15:1, 25:1, 50:1, 100:1, 200:1 times ratio (i.e. if you have 50 mL of medium then add ~ 3.0, 2.0, 1.0, 0.5, 0.25 and 0.125 mL antibiotic solution, respectively). These six treatments give a range of penicillin of ~ 20 - 500 mg L^{-1} (experience may lead to deletion of some of these treatments for some algal species under specific conditions).

- Incubate the dilutions under conditions suitable for growth of your algal species.
- After 24 and 48 hours aseptically transfer some individual cells from each dilution treatment to new, antibiotic-free medium. Start three tubes from each treatment. The highest probability of success (axenic cultures) is with the strongest, but non-toxic, antibiotic treatment.
- Incubate under conditions suitable for growth of your algal culture.
- Test for bacterial contamination after three weeks.

Another technique for controlling bacterial contamination is treatment with potassium tellurite (see bibliography). Eukaryotes, including most microalgae and fungal contaminants, can be reduced in cultures of cyanobacteria by adding 50 ppm cycloheximide.

Other algal species contaminating a culture can be eliminated as follows:

1. Cyanobacteria can be eliminated by treatment with antibiotics. Use 25 mg L-1 streptomycin and the general treatment as given above.
2. Diatoms can be reduced by eliminating silica from their culture medium. They will also stop growing if 5 mg L-1 of germanium dioxide is added to the medium.
3. Motile species may migrate to light and away from nonmotile contaminant algal cells. They can be isolated by illuminating one end of a pipette.

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Biographical Sketch

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