

AN ABSTRACT FOR THE THESIS OF

Jason G. Modrell for the degree of Master of Science in
Chemical Engineering presented on August 10, 1993.

Title: Bioreactor Development and Cell Culture of the
Marine Macroalgae *Porphyra* (sp.) and *Laminaria*
saccharina

Redacted for Privacy

Abstract approved: _____
Dr. Gregory L. Rorrer

Two approaches to liquid cell culture of marine macroalgae were used: protoplast isolation of *Porphyra* and gametophyte culture of *Laminaria saccharina*. A bubble column bioreactor was designed to cultivate photosynthetic macroalgal liquid cell cultures. Bubble column bioreactor cultivation was compared to static flask and immobilized bead systems in terms of growth rates of *Laminaria saccharina* gametophytes. Also, three levels of an inorganic carbon source (0 g/L, 0.34 g/L, 0.64 g/L bicarbonate) were compared on the basis of growth rate in the bubble column bioreactor system.

Protoplast isolation of *Porphyra* was achieved with a yield of $1.96 \pm 0.81 \times 10^6$ viable cells/ml-gram fresh weight (1s, n=5). Ideal digestion conditions were obtained by using *Aplysia* enzyme extract, 23 °C, diffuse light, 2 wt% Cellulysin, and 70 rpm agitation. Cell culture of the protoplasts did not sustain growth at 15 °C, 12L:12D photoperiod, 70 rpm agitation, PES medium, and 2500 lux light intensity.

Growth rates of *Laminaria saccharina* female gametophytes in the static flask cultures appeared zero order with a rate of 0.079 $\mu\text{g Chl } a/\text{ml-day}$. The bubble column bioreactor showed first order growth, in an exponential region, with a specific rate of $0.282 \pm 0.013 \text{ day}^{-1}$ (1s, n=2) and a final dry cell density of $822.5 \pm 38.9 \text{ mg/L}$. Gametophytes in alginate beads showed no growth. Each of the comparison systems was run at 340 mg/L bicarbonate carbon source, 16L:8D photoperiod, 2500 Lux light intensity, in GP2 artificial seawater medium.

Bicarbonate levels of 0, 340 and 680 mg/L gave doubling times of 2.77, 2.47 and 3.40 days respectively in a bubble column bioreactor. Initial bicarbonate level was deemed non-limiting to growth because of adequate carbon dioxide transfer to the growth medium by the sparged gas.

Bioreactor Development and Cell Culture of the
Marine Macroalgae *Porphyra* (sp.) and *Laminaria saccharina*

by

Jason G. Modrell

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed August 10, 1993

Commencement June 1994

APPROVED:

Redacted for Privacy

Assistant Professor of Chemical Engineering in charge of major

Redacted for Privacy

Head of Department of Chemical Engineering

Redacted for Privacy

Dean of Graduate School

Date thesis is presented August 10, 1993

Typed by researcher for Jason G. Modrell

ACKNOWLEDGMENTS:

I would like to say thank you to the following for their financial, emotional and artistic support throughout the trying years this thesis took to put together.

Ron Modrell

Teresa Albin

Greg Rorrer

Kristin Rorrer

Richard Meier

Richard Steele

Financial support in the form of research assistantship was provided by Sea Grant project R/AQ65.

TABLE OF CONTENTS

Introduction	1
Literature Review	4
<i>Porphyra</i> Protoplast Isolation	4
<i>Laminaria saccharina</i> Gametophyte Culture	6
Cell Immobilization	8
Summary	9
Cell Culture Development	11
<i>Porphyra perforata</i> Culture	11
Collection	11
Enzyme Preparation	11
<i>Porphyra</i> Tissue Preparation for Digestion	16
<i>Porphyra</i> Tissue Digestion	18
Protoplast Isolation	20
Protoplast Culture	21
<i>Laminaria saccharina</i> Culture	22
Collection	22
Sub-Culture Procedure	22
Growth Measurements	24
Cultivation Studies	27
Static Flask	27
Setup	27
Experimental Conditions	27
Bubble Column Reactor	28
General Set-up	28
Sparger Design	31

TABLE OF CONTENTS (CONTINUED)

Light Stage	34
Experimental Conditions	34
Immobilized Cell Culture	39
Alginate Bead Encapsulation	39
Bead Forming Solutions	39
Casting Apparatus	40
Experimental Conditions	42
Results and Discussion	44
<i>Porphyra perforata</i> Culture	44
Protoplast Isolation and Culture	44
<i>Laminaria saccharina</i> Gametophyte Culture	47
Maintenance Cultures	47
Static Flask	51
Cell Immobilization	53
Bead Characteristics	53
Immobilized Cell Cultivation	54
Bubble Column Bioreactor	54
Comparison of Cultivation Systems	58
Effect of Bicarbonate Concentration on Growth	62
Calibration Curve	65
Conclusions	67
Future Work	69
Bibliography	70

TABLE OF CONTENTS (CONTINUED)

Appendices

Appendix A: Experimental Protocols	73
Appendix B: Raw Data	100

LIST OF FIGURES

<u>Figure</u>	<u>Page</u>
1. Life Cycle of <i>Laminaria saccharina</i>	7
2. <i>Porphyra perforata</i> Culture Flowchart	12
3. Dimensions of Bubble Column Bioreactor	30
4. Bubble Column Bioreactor General Set-up	32
5. Sparger Configurations	33
6. Light Stage Dimensions	35
7. Light Stage Calibration	36
8. Alginate Bead Casting Apparatus	41
9. Viable Protoplasts	46
10. Tissue Digestion in Progress	48
11. Gametophytes 100x	49
12. Gametophytes 470x	50
13. Static Flask Growth Curve	52
14. Alginate Bead Growth Curve	55
15. Bubble Column Bioreactor Growth Curve (340mg/L)	56
16. Flowrate Variation	57
17. Bubble Column Bioreactor Growth Curve (340mg/L)	60
18. Effect of Bicarbonate Concentration on Growth Rate	63
19. Calibration Curve	66

LIST OF TABLES

<u>Table</u>	<u>Page</u>
1. Enzyme Medium Compositions	15
2. PES Enriched Seawater Medium Composition	17
3. Antibiotic Composition (1 Liter PES Medium)	19
4. GP2 Medium Composition	23
5. Static Flask Experimental Conditions	29
6. Bubble Column Bioreactor Experimental Conditions	38
7. Alginate Bead Experimental Conditions	43
8. Protoplast Isolation Results	45
9. Bioreactor Comparison Results	59
10. Cultivation Comparison	61
11. Effect of Bicarbonate Concentration	64

Bioreactor Development and Cell Culture of the Marine Macroalgae *Porphyra* (sp.) and *Laminaria saccharina*

Introduction

Terrestrial plant cell cultures today produce many products of value. These products include flavorings, fragrances and pharmaceuticals (Kargi and Rosenberg, 1987). A potential source of new products is marine macroalgae. There has been much work in the areas of cell culture and bioreactor development for terrestrial plants (Kargi and Rosenberg, 1987). These studies are of use in determining general guidelines in development, but relatively little work has been done with marine algae (Butler and Evans, 1990).

Recently, a metabolic pathway known as the arachidonic acid cascade has been discovered in some red and brown macroalgae on the Oregon coast (Gerwick et. al., 1990). Although the arachidonic acid cascade is essential to mammals it had never been found in marine plants until recently. In the arachidonic acid cascade, arachidonic acid is enzymatically oxidized to form important bioactive compounds known as eicosanoids. These eicosanoids are a family of molecules that perform important bioregulatory functions in humans. They include the HETES, prostaglandins, thromboxanes, hepoxilins, and leukotrienes. In fact many commercial drugs are manufactured from the eicosanoids (Nelson et. al., 1982).

In addition to the known eicosanoids many new eicosanoids have been discovered in these red and brown seaweeds. The effect of these new eicosanoids has yet to be determined. One eicosanoid, 12S-HETE, is a

potent immunohormone. Its functions include inhibition of platelet aggregation and inflammation moderation. It is found in human blood and is valued at \$2000-\$4000/mg.

In order to produce these pharmaceuticals or other compounds commercially a method of approach must be worked out. Currently there are three ways of approaching the manufacture of eicosanoid natural products. The first is chemical synthesis. This approach is very difficult. The stereochemistry of these products is very important and hard to control without the aid of enzymes. Low yields are typical and therefore usually make the approach economically prohibitive. The advantage of this approach however is complete control of the process and ease of scale-up.

A second approach is to farm the plant that manufactures the natural product. The difficulties arise when seasonal variations and other uncontrollable environmental factors are considered. Even if these uncertainties are acceptable, the concentrations of these species are usually low. Typically, the eicosanoids make up 5% of the total extractable lipids (Gerwick et. al., 1990). This would mean huge quantities would have to be harvested and put through an expensive extraction process.

The last option is to culture the algal species in a bioreactor. Bioreactors provide a system of controlled cultivation. Many parameters can be manipulated to optimize production of the compound, such as, light, food source, photoperiod, etc. Although this approach is very attractive there are some drawbacks. Marine macroalgae has never been studied in bioreactor culture. Cell culture techniques of macroalgae lag far behind that of terrestrial plants. The hormonal mechanisms in terrestrial plants that make cell culture attainable (cytokinins and auxins) are not well understood in marine macroalgae.

As a result, this study attempts to form the basis from which future studies of macroalgae cultivation in bioreactors may be launched. Establishment of a liquid cell culture of macroalgae is the first step followed by bioreactor design for photosynthetic operation and the manipulation of a carbon source and its effects on growth are studied.

The specific objectives for this project are to use two approaches to liquid culture establishment. A protoplast isolation approach will be used with the algal species *Porphyra*. In addition to this, gametophytes, a filamentous life cycle stage of the species *Laminaria saccharina*, will be put into liquid culture. Another objective is to design a low shear bioreactor capable of supporting photosynthetic cells. Once these objectives are accomplished the liquid cultures will be cultivated and compared in three systems. The systems are static flask, immobilized bead, and bubble column bioreactor. Additional emphasis will be put on the bioreactor by comparing the growth rates of cultures with varying initial concentrations of an inorganic carbon source (bicarbonate).

Once these initial studies of cell culture and bioreactor development are performed, this work can be used as a base for future work in this area of biotechnology. An understanding of macroalgal cell culture performance in a bioreactor will be exceedingly important in future studies of these systems. A literature review follows, outlining the state of the art of culturing *Porphyra* and *Laminaria* species.

Literature Review

The following literature review looks at the current state of the art with respect to cell cultures from marine macroalgae. The review focuses on protoplast isolation from *Porphyra* and tissue culture of the gametophytic life phase of *Laminaria saccharina* as techniques for establishing cell cultures. Cell immobilization is also reviewed as a cultivation technique for macroalgal cells.

Porphyra Protoplast Isolation

Marine plant protoplasts have cell membranes but lack a polysaccharide cell wall. The cell walls are removed through enzymic attack. Although protoplast isolation in terrestrial plants was begun in the 1960s (Butler et. al., 1990) only recently has it been shown in macroalgae.

To release the protoplasts from the main body of the target algae it is necessary to include enzymes that attack both the microfibrillar and polysaccharide matrix components of the cell. Protoplasts have been obtained from the green algae *Enteromorpha intestinalis* using 4 wt% driselase combined with 0.4 wt% pectinase (Millner et. al. , 1979). It was found that a viability of 85-90% was obtainable using only these commercial enzymes. Saga obtained a yield of 1×10^6 viable cells per gram of fresh tissue weight by digesting *Enteromorpha linza* with only 2 wt% Onozuka R-10 cellulase (Saga, 1984). Plant regeneration was also observed in PES medium.

These successes with green algae using only commercial enzymes are more difficult with the red and brown algae. To achieve protoplast release in the red and brown algal systems other enzymes must be included that are more specific to the polysaccharides found in the algae.

The source of these specific digestive enzymes is often marine animals which feed on the target algae. Extracts from the digestive parts of these animals contain crude enzyme mixtures suitable for cell wall digestion (Butler et. al., 1990). Saga and Sakai (1984) used the gut extract from *Strongylocentrotus intermedius* (sea urchin) to digest *Laminaria japonica*. They obtained a 5×10^3 cells per gram fresh weight yield with 10% viability. Butler et. al. (1989) used an extract from *Haliotis tuberculata* (abalone) to digest *Laminaria digitata*. They found a protoplast yield of 1×10^8 cells per gram of fresh weight digested.

Porphyra, a red algae, was the focus of this study. Tang (1982) used an extract from *Turbo coronatus* followed by cellulase R10 (2 wt%) to digest *Porphyra suborbiculata*. The protoplasts were then cultured in PES medium with an initial 2M glucose osmoticum. Cell wall regeneration and 1-2 cell divisions were reported. Polne-Fuller and Gibor (1990) used the extract from a *Haliotis* species at pH 6.0 with a 0.6M sorbitol osmoticum on *Porphyra perforata*. A viability of 75% was reported. Tait et. al. (1990) used a limpet acetone powder extract on *Porphyra umbilicalis* and achieved regeneration of the protoplasts.

Cell suspension cultures via protoplast isolation of *Porphyra* species have also been reported. Tait et. al. (1990) was able to achieve final dry cell densities (1 month culture), with *Porphyra*, of ~1000 mg/L by varying sodium nitrate concentrations in IS3 liquid medium. Chen (1989) reported 1-2 cell divisions per day in microwell plates for *Porphyra linearis*. The above

indicates that cell suspensions can be established via protoplast isolation, but does not give any detailed measurements typically associated with bioreactor development.

***Laminaria saccharina* Gametophyte Culture**

The life history of *Laminaria saccharina* is shown in Figure 1. The algae alternates between a large sporophyte plant and microscopic gametophyte stage (Bold and Wynne, 1985). The sporophyte is highly differentiated, but the gametophyte grows in a filamentous manner and remains relatively undifferentiated. In the central region of the sporophyte blade meiosis occurs releasing spores. These spores then develop into either male or female gametophytes. The male gametophytes release sperm in response to chemical signal sent by developed eggs in the female gametophytes. After fertilization the eggs remain attached to the female gametophytes and grow into sporophytes once again.

Isolation of the haploid gametophytes from diploid sporophytes is discussed in Steele and Thursby (1992, 1988). A sporophyte blade is surface sterilized. Spore release is induced by placing the blade on moist filter paper overnight. The spores are then collected and diluted sterilely. After 4-8 weeks male and female gametophytes are separated under a microscope and placed in culture.

Once isolated the gametophytes were grown in GP2 artificial seawater (Steele and Thursby, 1988). For vegetative growth, iron is removed from the medium. This lack of iron inhibits gametogenesis and egg formation, and thus

Life Cycle of Laminaria

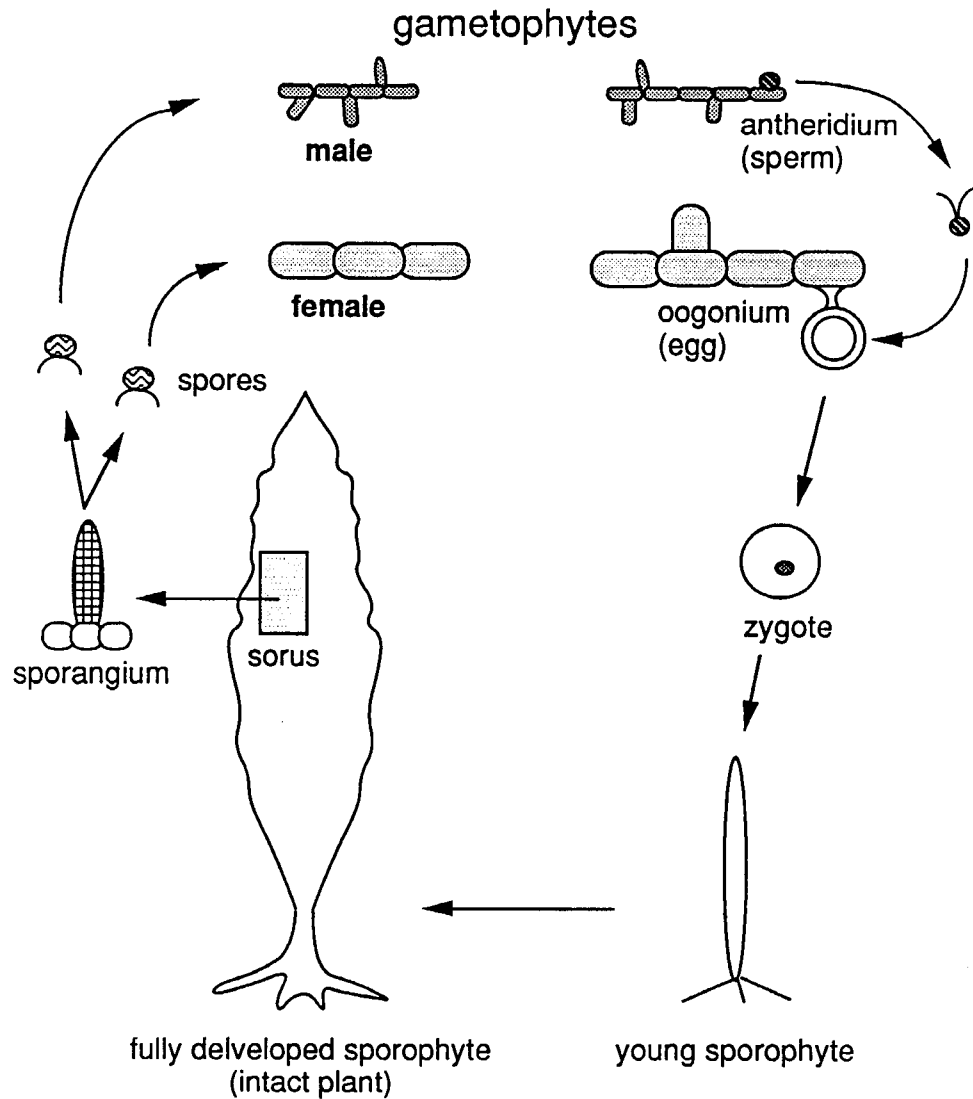


Figure 1: Life Cycle of *Laminaria saccharina*

provides faster vegetative growth (Montomura and Sakai, 1984). If iron is important the cultures can be grown under red light (Luning and Dring, 1975) to inhibit gametogenesis.

The gametophytes are maintained at 12 °C (Luning and Neushul, 1978). It has been shown that a temperature exceeding 22 °C kills the cultures (Bolton and Luning, 1982). The cultures should not be placed in illumination higher than 4000 lux white light to avoid gametogenesis (Luning, 1980). No reports were given in regards to growth rates of the cultures.

Cell Immobilization

Much of the interest in immobilized algae is relatively recent (Robinson et. al. , 1986). Algae immobilization has focused around the production of primary metabolic by-products, such as, ammonia (Kerby et. al. , 1983) and hydrogen (Smith and Lambert, 1981). Immobilization of the algae facilitates a greater degree of control over experimental conditions. By immobilizing the algae it can be placed into packed beds and recovered for analysis easily. Free floating algae is much harder to characterize.

Two methods of immobilization are available. The first is to allow the algae to penetrate a highly porous immobilizing matrix during growth. Polyurethane foams are typically sliced into small (0.125 cm³) cubes and placed in the growing culture (Muallem et. al. , 1983). The algae invades the matrix and the foam cubes are later removed for placement in the process line.

The other method is to entrap the algae in a polysaccharide matrix. The algae cells are first grown then they are transferred to the matrix. Several natural polysaccharides have been used for this, such as, agar,

kappa-carrageenan, and alginate (Robinson et. al., 1986). One way to transfer the cells to the matrix is accomplished by mixing the viable cells in the polymer matrix while it is in liquid form. Then, small beads are cast by dropping the algae laced polymer into a solidifying solution. The solution may solidify the polymer by temperature reduction or chemical reaction.

The beads produced by active entrapment can be redissolved chemically. Alginate beads formed with calcium can be redissolved in a number of solutions. Their effectiveness depends upon the calcium chelating agent and its concentration (Dainty et. al., 1986). Typical solvents are sodium dihydrogen phosphate (NaH_2PO_4) and sodium citrate.

The growth of immobilized cells is generally lower than that of free cells (Robinson et. al., 1986). This is probably due to restricted nutrient diffusion through the matrix and physical resistance of the matrix itself. It has also been found (Robinson et. al., 1985) that chlorophyll concentrations are somewhat higher in immobilized cells than in free cells. This is probably due to reduced light transmittance.

Summary

The current literature suggests that marine macroalgal cell cultures are attainable. The methods of protoplast isolation and gametophyte culture are both viable options for initiating cultures of marine macroalgae. It should be noted here that gametophyte culture is a limited approach with respect to liquid cell culture establishment. It was chosen because of the filamentous and undifferentiated characteristics of the *Laminarian* gametophytes. These characteristics are species specific and apply only to a few species.

Cell immobilization as a method of cultivation of cell suspensions has

been well documented. The method of entrapment was chosen over the penetration method. This choice was made because of the small growth rates associated with macroalgal cell cultures. A small growth rate means a longer incubation time in the presence of the material to be penetrated.

Cell Culture Development

***Porphyra perforata* Culture**

Collection

Fresh tissue of *Porphyra perforata* was collected at low tide at Seal Rock, Oregon. All samples were collected between the months of June and August 1992. The samples were picked by hand and placed in seawater collected at the site. The samples were then placed in a cooler with ice for transportation. Upon arrival at the lab individual fronds were patted dry with paper towels and placed in a sealable plastic container. The container was then placed in a freezer kept at - 20 °C. The fronds remained viable under these conditions for up to 3 months. The overall culture procedure is outlined in Figure 2.

Enzyme Preparation

Two methods of enzyme preparation were tried. The first method was the direct extraction of digestive gut juices from marine herbivores. Live *Aplysia californica* sea hares were obtained from Marine Specimens Unlimited, Pacific Palisades, California. The animals were shipped overnight air shortly after capture in the wild. The animals came individually wrapped in their own bags containing native seawater with a pure oxygen atmosphere overlay. Bags of ice wrapped in newspaper were also included in the packages for temperature control during the trip.

PORPHYRA CULTURE ACQUISITION

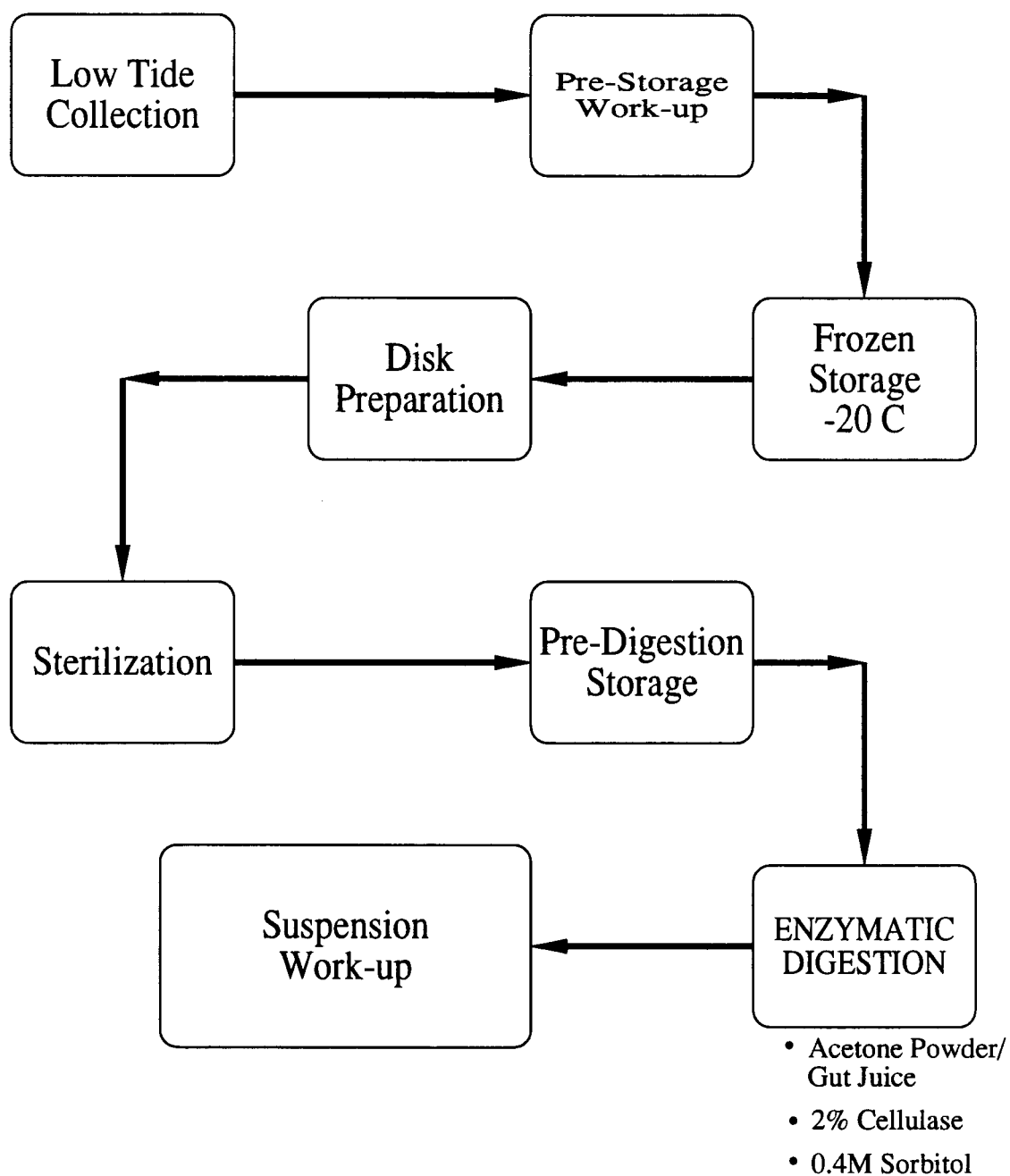


Figure 2: *Porphyra perforata* culture flowchart

Immediately upon arrival, the animals (in plastic bags) were transferred to a natural seawater tank kept at 12 °C with constant filtration in a cold room. After a 1 hour temperature equilibration soak, the bags were opened and the animals were released into a section of the tank. The sections measured 1ft x 2ft and 7in deep. Each section held one animal. The animals were fed fresh *Porphyra perforata* for at least one week prior to enzyme acquisition. The animals were monitored daily and kept well fed.

After the one week food acclimation period, the sea hares were ready for harvest. Each animal was first anesthetized by injecting with 50 ml of 7% $MgCl_2$ in sterile, natural seawater. The injection was made in the underside of the animal. Once the animal was relaxed it was placed into a dissection tray filled with seawater and surrounded by ice.

The dissection procedure started by pinning the animal to the tray with steel dissection pins at the head and tail such that the body was stretched and taught, lying on its back. Next the animal was cut open along the foot with scissors pinning back the foot as it was cut open. The gut and hepatopancreas was located and tied off at either end with surgical thread. The stomach assembly was then excised from the animal and the gut cut open over a clean, sterile, polypropylene centrifuge tube. The contents of the stomach were drained into the tube and the tube was placed on ice. The tube was then immediately transferred to a -70 °C freezer for storage until use.

The actual *Porphyra* digestion medium contained stomach juice, cellulase, sorbitol, and buffer solution. The buffer solution consisted of 25 mM Trizma Base (Sigma, Cat# T-1503) and 25 mM MES (monohydrate [2-(N-morpholino)-ethanesulfonic acid], Calbiochem, Cat# 475893) in PES artificial seawater. The buffer was then adjusted to a pH of 6.5 . The enzyme solution was prepared in 50 ml batches. To 45 ml of chilled (0 °C) buffer

solution, 3.64 grams of sorbitol (0.4 M) was added as an osmoticum for the protoplasts. The digestion medium also contained 2 wt% (1g to 50 ml final solution volume) Cellulysin cellulase (Calbiochem) and 5.0 ml of the raw *Aplysia* gut juice (1:10 v/v).

The solution was allowed to mix for 1 hour in a cold room at 4 °C. After mixing was complete the solution was transferred to sterile, 50 ml, polypropylene centrifuge tubes and centrifuged for 15 minutes at 5000 rpm to pellet any debris from the gut juice. The solution was then transferred to a 115 ml sterilizing filter assemblage (Nalgene, 0.45 µm pore size) via Pasture pipet so as not to disturb the pellet. The filter assemblage also contained just enough glass wool to cover the filter, as a pre-filter, to catch any large pieces of debris that might clog the filter. The filter assembly was immersed in an ice water bath during the entire operation.

After sterile filtering, the 50 ml of enzyme solution was transferred, using sterile technique, to sterile centrifuge tubes in 5 ml aliquots. The tubes were then stored in a freezer at -20 °C until ready for use. A summary of components is listed in Table 1.

The second method of enzyme preparation employed the use of acetone powders made from the guts of marine herbivores, specifically limpets (Sigma, Cat# L-1251) and abalones (Sigma, Cat# A-7514). The powders were stored in a freezer at -15 °C until used. A 100 ml beaker was placed on ice and 5.0 grams of the acetone powder was added to it. Then 50 ml of sterile PES artificial seawater was added and the solution was allowed to mix, covered, for 1 hour (1g acetone powder per 10ml seawater). Then the solution was decanted into sterile centrifuge tubes and centrifuged for 20 minutes at 2000 rpm to pellet any large particles.

Table 1: Enzyme Medium Compositions

Enzyme Type	Quantity of Crude Enzyme	Volume of PES Medium	Mass of Cellulysin	Mass of Sorbitol
<i>Aplysia</i> Medium	5.0 ml	45 ml buffer*	1 gram	3.64 grams
Limpet Medium	5.0 grams	50 ml seawater	1 gram	3.64 grams
Abalone Medium	5.0 grams	50 ml seawater	1 gram	3.64 grams

* Tris/MES Buffer

The centrifuged solution was then transferred via Pasture pipet to sterile dialysis membrane tubing stored at 4 °C in sterile deionized water. The tubing was pre-treated out of the box by boiling in 2.5 mM EDTA at pH 8.0 for 30 minutes twice. It was then boiled in deionized water three times to remove any trace of the EDTA. This helped soften the tubing for easier use. The filled dialysis tubing was immersed in sterile Instant Ocean seawater at 4 °C for 16 hours under constant stirring to remove low molecular weight compounds as a first pass at purification. The seawater was replaced after 12 hours to increase the diffusion driving force.

After dialysis, the enzyme mixture was removed from the tubing and placed in a sterile 100 ml beaker on ice. To the enzyme solution, 3.64 grams of sorbitol (0.4M) was added as an osmoticum along with 2 wt% (1g to 50 ml final volume) Cellulysin cellulase (Calbiochem). The solution was then brought to a pH of 6.5 after 20 minutes of mixing. Next the solution was transferred to sterile centrifuge tubes and centrifuged for 20 minutes at 2000 rpm to pellet any large particles. The solution was then transferred to a 115 ml filter sterilizing assemblage (Nalgene, 0.45 µm pore size) containing glass wool as a pre-filter. The solution was filter sterilized on ice and transferred to sterile centrifuge tubes in 5 ml aliquots using sterile technique. A summary of components is listed in Table 1.

Porphyra Tissue Preparation for Digestion

Frozen fronds were taken from the freezer and immediately placed in sterile PES enriched seawater medium (Table 2) at 4 °C to thaw. The frond was then checked for viability by the use of 0.33 wt% phenosafarin dye and microscopic investigation. Viable cells do not stain and remain green in color,

Table 2: PES Enriched Seawater Medium Composition*

Component	Formula	mg/L
Sodium nitrate	NaNO_3	56.0
Boric acid	H_3BO_3	4.56
Cobalt (II) sulfate heptahydrate	$\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$	0.019
Iron (III) chloride hexahydrate	$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	0.196
Chelated iron	$\text{Fe}(\text{NH}_4)_2(\text{SO}_4) \cdot 6\text{H}_2\text{O}$	2.808
Manganese sulfate tetrahydrate	$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	0.656
Sodium EDTA	Na_2EDTA	6.4
Sodium glycerophosphate hexahydrate	$\text{Na}_2\text{glycerophosphat} \cdot 6\text{H}_2\text{O}$	8.000
Zinc sulfate heptahydrate	$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.088
Biotin		0.0008
B12		0.0016
Thiamine-HCl		0.080

* These components are added to 1 liter of natural seawater

while dead cells stain red. The frond was then placed on a clean plexiglass surface and 5 mm discs were cut from it using a sterile cork borer. The discs were placed in sterile PES medium on ice as soon as they were cut. Ten discs were set aside for a fresh weight measurement. Thirty discs were cut for each digestion (57.0 mg fresh weight).

The sterile medium was then decanted off the disks and replaced with deionized water. The disks were sonicated in a 100 ml sterile beaker containing sterile deionized water for 30 seconds. The water was decanted off and replaced with sterile PES medium and sonicated for 30 seconds. The seawater was decanted off and replaced with fresh, sterile PES medium and the disks were sonicated again for 30 seconds and the seawater decanted off. This procedure had the effect of removing most of the surface ephytes.

The disks were then incubated in a sterile hood for 3 minutes in ~25 ml of a 1:10 v/v dilution of Betadine (Perdue Fredrick Company, Norwalk, CT) in sterile PES medium. After incubation the disks were washed several times with sterile PES medium to remove all traces of the Betadine solution. The disks were placed in an antibiotic solution with the composition shown in Table 3 for 2 days to complete the tissue sterilization procedure.

Porphyra Tissue Digestion

After sterilization in the antibiotic solution, 30 discs (57.0 mg fresh weight) were removed and washed with sterile PES medium. The discs were placed on a sterile plastic 100x15 mm petri dish and chopped into fine pieces ($<1 \text{ mm}^2$) using a sterile utility knife fitted with a flame sterilized safety razor blade. The chopped *Porphyra* was then checked for viability using a 0.33 wt% phenosafarin stain and microscopic observation.

Table 3: Antibiotic Composition (1 Liter PES Medium)

Component	(mg/L)
Penicillin G	1000
Streptomycin Sulphate	2000
Kanamycin	1000
Neomycin	200
Nystatin	25

A 5.0 ml aliquot of previously mixed enzyme solution was thawed and placed in a 60x15 mm sterile plastic petri dish. The chopped pieces of *Porphyra* were then placed in the enzyme solution, using sterile technique with the aid of a utility knife, to give a final concentration of 11.4 mg fresh weight per ml of enzyme solution. The petri dish was sealed using Parafilm and placed at the appropriate digestion temperature on an orbital shaker at 70 rpm for 16 hours.

Protoplast Isolation

After digestion, the enzyme mixture containing the protoplasts was filtered through a sterile 60 µm nylon mesh filter to remove any undigested material. The undigested material was washed on the filter with sterile PES medium containing 0.4 M sorbitol to wash through any held up protoplasts. The filtrate was then transferred to a sterile centrifuge tube and centrifuged for 15 minutes at 1350 rpm to pellet the protoplasts. The enzyme solution was then removed with a sterile Pasteur pipet and the pellet was resuspended in sterile PES medium containing 0.4 M sorbitol. This washing procedure was repeated 3 times.

The washed protoplasts were then placed in 60x15 mm sterile plastic petri dishes with 5 ml of sterile PES medium containing 0.4 M sorbitol. A small sample was then taken to assess initial viability and protoplast density using a Fuchs Rosenthal hemocytometer.

Hemocytometer counts were performed at 100x magnification using phenosafranin as a viability indicator. A small sample of protoplasts, in culture, was taken with a sterile Pasteur pipet. Two drops were placed on a small piece of Parafilm, which aided in sample handling due to its hydrophobic

properties. Next a 1/8TH drop of 0.33 wt% phenosafarin in deionized water was also placed on the Parafilm. The dye and culture sample were mixed on the Parafilm by allowing the dye to enter the pipet through capillary action and then injecting the dye into the droplet of culture. Bubbles were introduced into the combined droplets and the two liquids were mixed by swirling the bubbles with the tip of the pipet.

About 0.25 ml of the dye-culture mix was then transferred to the hemocytometer via the Pasture pipet and the total number of protoplasts were counted under 100x magnification. The counting procedure was accomplished by counting the total number and viable number of protoplasts in five 1 mm² ruled sections on the Hemocytometer. Each section has a known depth of 0.2 mm. From this a value for the number of protoplasts per unit volume was calculated. The percent viability was calculated by dividing the viable number by the total number and multiplying by 100. The protoplast yield was calculated as the total number of viable cells divided by the initial fresh weight digested.

After counting, the protoplast cultures were then immediately placed on an orbital shaker at 70 rpm under 2500 lux light intensity with a photoperiod of 12 hours light 12 hours dark (12L:12D) at a temperature of 15 °C.

Protoplast Culture

The osmoticum concentration of the culture medium was stepped down every 24 hours in 0.2 M increments to prevent growth inhibition by sorbitol. This was done by centrifugation at 1350 rpm for 15 minutes and then resuspension with fresh PES medium. Viability counts by hemocytometer

were made every other day to follow cell growth. The cultures were maintained at 15 °C, 12 hours light 12 hours dark, 70 rpm on an orbital shaker, and 2500 lux light intensity.

***Laminaria saccharina* Culture**

Collection

Laminaria saccharina female gametophyte cultures were obtained from Dr. Richard Steele, Environmental Protection Agency, Newport, Oregon in 355 ml sealed bottles. The cultures were transferred to Oregon State University in an ice chilled cooler. Upon arrival the cultures were immediately placed in a low temperature incubator kept at 12 °C. The cultures were subcultured every three weeks into a GP2 artificial seawater medium. The exact composition of this medium can be found in Table 4. Cultures were kept in 250 ml Erlenmeyer flasks under 2500 lux light intensity with a photoperiod of 16 hours light: 8 hours dark. The cultures were not agitated.

Sub-Culture Procedure

Every three weeks, four of the best looking cultures from a culture line of ten were selected for subculture. Selection was based on a deep brown color and no sign of contamination under 100x magnification. Another important characteristic of culture selection was that the gametophytes should not appear to adhere to the flask walls.

The culture flasks selected for subculturing were removed from the incubator and the culture was filtered through a sterile 60 µm nylon mesh filter. The filter cake was then washed with sterile GP2 medium. The filter cake was back washed into a 250 ml blending vessel. The blending vessel

Table 4: GP2 Medium Composition

	Component	Formula	mg/L
Base Solution	Sodium chloride	NaCl	21,030
	Sodium sulfate	Na ₂ SO ₄	3,520
	Potassium chloride	KCl	610
	Postassium bromide	KBr	88
	Sodium tetraborate decahydrate	Na ₂ B ₄ O ₇ · 10H ₂ O	34
	Magnesium chloride hexahydrate	MgCl ₂ · 6H ₂ O	9,500
	Calcium chloride dihydrate	CaCl ₂ · 2H ₂ O	1,320
	Strontium chloride hexahydrate	SrCl ₂ · 6H ₂ O	20
Nutrients	Sodium nitrate	NaNO ₃	63.5
	Sodium phosphate	NaH ₂ PO ₄ · H ₂ O	6.4
	Sodium citrate dihydrate	Na ₃ C ₆ H ₅ O ₇ · 2H ₂ O	0.52
Trace Metals	Sodium molybdate (VI) dihydrate	Na ₂ MoO ₄ · 2H ₂ O	0.012
	Potassium iodide	KI	0.042
	Zinc sulfate heptahydrate	ZnSO ₄ · 7H ₂ O	0.0112
	Sodium orthovanadate	Na ₃ VO ₄	0.0048
	Manganese chloride tetrahydrate	MnCl ₂ · 4H ₂ O	0.0034
Vitamins	Thiamine-HCl		0.25
	B ₁₂		0.000125
	Biotin		0.000125

was previously cleaned under sterile conditions with sterile deionized water. Sterile fresh GP2 medium was then added to a volume of 100 ml and the vessel was sealed.

The culture was then blended on the highest setting, "liquefy", for 30 seconds. This blending procedure does not harm the majority of the culture, but serves to break the long, tough, filamentous chains that are characteristic of the female gametophytes. After blending, the sealed blending vessel containing the blended culture is returned to the laminar flow hood for subculture.

To ten sterile 250 ml flasks, 90 ml of sterile GP2 medium was added. Then, 10 ml of freshly blended three week old culture was added to the subculture flasks to a total volume of 100 ml. Of the four flasks originally chosen for subculture, two are chosen to inoculate three flasks and the remaining two inoculated two flasks. The newly inoculated flasks were then placed in an incubator at 12 °C, 2500 lux, 12:12 L:D photoperiod.

Growth Measurements

Two methods of growth measurement were utilized. One measured the amount of chlorophyll *a* present in a known volume and the other measured the dry cell mass in a known volume. The chlorophyll method was chosen because of its low sample volume requirements and reproducibility. Because chlorophyll content in a cell can change due to light fluctuations, light intensity was always kept constant in all cultivation experiments. This was also done so that chlorophyll content could later be correlated with dry cell density.

Chlorophyll content was assayed using a spectrophotometric method relying on an absorbance peak occurring at a wavelength at 665 nm. For

each assay a 5.0 ml sample was obtained, using a graduated pipet, from the culture and vacuum filtered through a 20 μ m pore size nylon mesh filter (Spectra Mesh) using an aspirator. The filter was washed with deionized water on the filter stage prior to culture addition. Then the filter was folded into eighths to form a wedge shape. Next the filter, with cells, was placed in a 50 ml polypropylene centrifuge tube with the "point" of the wedge touching the bottom of the centrifuge tube. With each assay a blank was also prepared by washing a nylon filter with deionized water and following the same procedure without filtering culture.

Once all samples were obtained and placed in clean dry centrifuge tubes, 5.0 ml of HPLC grade methanol was added to each tube via a volumetric pipet. The tubes were then capped using tight fitting polypropylene caps provided by the manufacturer of the centrifuge tubes. Each sample was then mixed on a 'Vortex' (VWR) machine at speed setting 4 for 30 seconds. The samples were then immediately placed in a dark refrigerator at 4 °C, overnight to allow complete extraction of the chlorophyll.

After complete extraction, overnight, the samples were removed from the refrigerator and vortexed once again for 30 seconds at speed 4. The spectrophotometer (Hitachi, model 100-10) was warmed up prior to sample analysis (see appendix A for warm up procedure). The spectrophotometer was then set to a reading of +0.000 absorbance using the blank sample. A voltmeter was used as a readout to prevent any error arising from parallax viewing of the analog display.

Before each sample was added to the cuvette (NSG, Precision Cells, 10 mm light path), the cuvette was washed four times with pure HPLC grade methanol and allowed to drain upside down into a clean Kimiwipe. The optical surfaces of the cuvette were then wiped visibly clean using a Kimiwipe. The

samples were withdrawn from the centrifuge tubes using disposable Pasteur pipets, such that the tip of the pipet was blocked by the 20 μm mesh filter. In this way, any cell matter in the liquid sample was filtered out upon entering the pipet. Once the sample was transferred to the cuvette, the cuvette was placed in the spectrophotometer and the absorbance was read at 665 nm from the digital voltmeter. This base corrected absorbance was then multiplied by 16.29 μg Chl *a*/ abs unit (Geider and Osborne, 1992) to find the chlorophyll *a* content.

The cell density of a sample, based on dry cell weight per liter of culture, was determined by filtering a known volume of culture through an oven dried (70 $^{\circ}\text{C}$, 24 hours), pre-weighed, 47mm diameter, 0.45 μm pore size, Millipore filter (Cat# HAWP-047-00) under aspiration. After filtration the cells were washed with deionized water to remove any traces of saltwater medium in the filter. The filters were then removed from the filter stage and placed in a clean dry glass petri dish. The petri dish was placed in an oven at 70 $^{\circ}\text{C}$ overnight to dry.

The dried filter and cells were then weighed on an analytical balance (0.1 mg resolution) to determine the dry weight of cells contained in the filtered volume. Typical initial dry cell densities of cultures were in the 60-100 mg/L range so large volumes had to be filtered for accurate results. Washing of the cells on the filter prior to drying was essential to remove salts that could have affected the weight measurement.

Cultivation Studies

Static Flask

Setup

Static flask cultures were maintained in 250 ml Erlenmeyer flasks. Originally the cultures were agitated on an orbital shaker, but the filamentous cells coagulated into large spheres. Therefore, the cultures were not agitated and the filamentous cells were allowed to grow as mats. The flasks were placed in a low temperature incubator at 12 °C and were illuminated overhead by three disposable Gro-lux and/or cool white fluorescent lights to an intensity of 2500 lux. The photoperiod was controlled by a light timer set for 16 hours light and 8 hours dark (16L:8D).

Aluminum foil was placed under each culture flask to minimize light loss to the surroundings. Each flask was sealed with aluminum foil to maintain sterility and to reduce moisture loss.

Experimental Conditions

For each static flask run 10, 250 ml Erlenmeyer flasks were cleaned and autoclaved (20 min, 121 °C, 15 psig). To each flask 90 ml of sterile GP2 medium was added. One three week old maintenance culture was chosen as an inoculum source. The culture was blended for 30 seconds on "liquefy" speed and 10 ml was added to each static flask. The flasks were then sealed with aluminum foil and numbered.

The first flask was taken as the initial point at zero days and four 5.0 ml samples were taken for the chlorophyll absorbance analysis and three 20 ml samples were taken for dry weight analysis. After the first point, the above

analysis was performed every three days. Only one experimental condition was examined with this cultivation system. The conditions were 2500 lux light intensity, 12 °C temperature, 16L:8D photoperiod, 340 mg/L bicarbonate (HCO_3^-) carbon source, and no aeration. The experimental conditions are summarized in Table 5.

Bubble Column Reactor

General Set-up

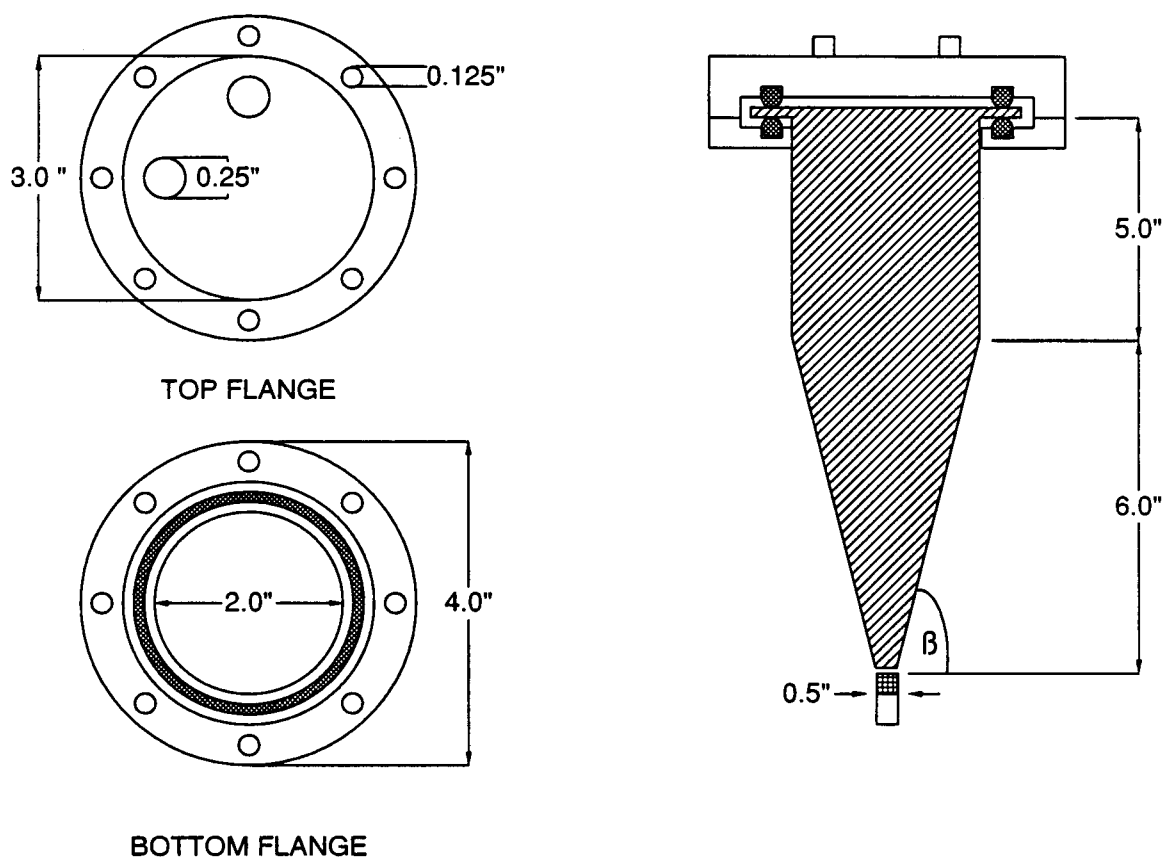
The glass reactor vessel and stainless steel head plate are shown in Figure 3. The glass reactor vessel consists of a 5.0 in. straight section and a 6.0 in. slanted section at an angle of 89 degrees from the bottom horizontal. This gives the vessel an effective culture volume of 280 ml. The body is sealed to the headplate using two Viton O rings, one above and below the top lip of the glass body. The head plate has two stainless steel Swage-lok bored-through connectors. One is the air outlet fitted with a 1/4 in. hose connection. The other port is for culture sampling.

The sampling assembly consists of a 1/4 in. stainless steel tube that penetrates the culture solution on one end and is reduced to a 1/8 in. hose connection at the other end. A 2 in. section of 1/8 in. diameter autoclavable tubing is attached to the sampling hose connection and fitted with a lure-lok connector on one end and clamped in the middle. The air outlet port is connected to 6 in. of 1/4 in. diameter autoclavable tubing with a 0.2 μm Gelman sterilizing filter at one end. This prevents contamination from the atmosphere.

Table 5: Static Flask Experimental Conditions

Condition	Setting
Temperature	12-15 °C
Light Intensity	2500 Lux
Photoperiod	16L:8D
Medium	GP2 (340mg/l bicarb)
Length of Cultivation	~30 Days
Culture Volume	100 ml
Agitation	None

BIOREACTOR DETAIL



TOTAL VOLUME = 280 ML

LENGTH/DIAMETER = 5.5

ANGLE OF CONE = 83 DEGREES

Figure 3: Dimensions of Bubble Column Bioreactor

The overall bubble column reactor set-up is shown in Figure 4. The entire assemblage is contained in a temperature controlled incubator. Air flowed into the reactor from a Whisper 300 air pump through a calibrated flow meter. The air then traveled through a 0.2 μm pore size Gelman filter to filter out any bacteria that might contaminate the culture.

The air was then humidified by bubbling through sterile deionized water and distributed through a 1/2 in. diameter glass frit sparger. The frit was graded as "coarse" giving it an average pore size of 40-60 μm . The air then bubbled up through the liquid culture providing enough mixing to keep the filamentous gametophyte cells suspended. Air exited the reactor through a 0.2 μm Gelman filter. Air tubes lead out of the incubator to prevent the icing of cooling coils.

Sparger Design

To keep the culture suspended, sparger design became the most important factor. Four designs are listed here (Figure 5).

The basket, teardrop, and annular dispersion spargers were all removable. This was done to facilitate cleaning and testing of the spargers. Of the removable spargers the teardrop design functioned the best. The major problem with the removable spargers was the ability of the female gametophytes to attach themselves to any ledge or crevice. The best result was the teardrop design in which the sparging takes place close to the reactor vessel wall leaving little space or sharp angle for the gametophytes to attach themselves.

The long cultivation times of the gametophytes outweighed the convenience of a removable sparger. A fixed sparger was used in the final

BUBBLE COLUMN BIOREACTOR

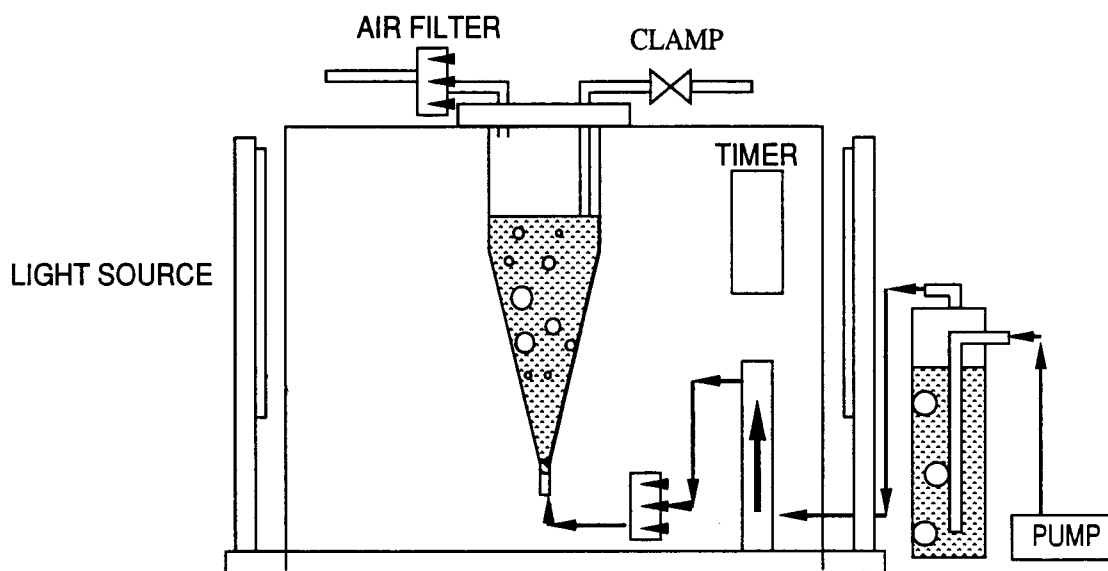
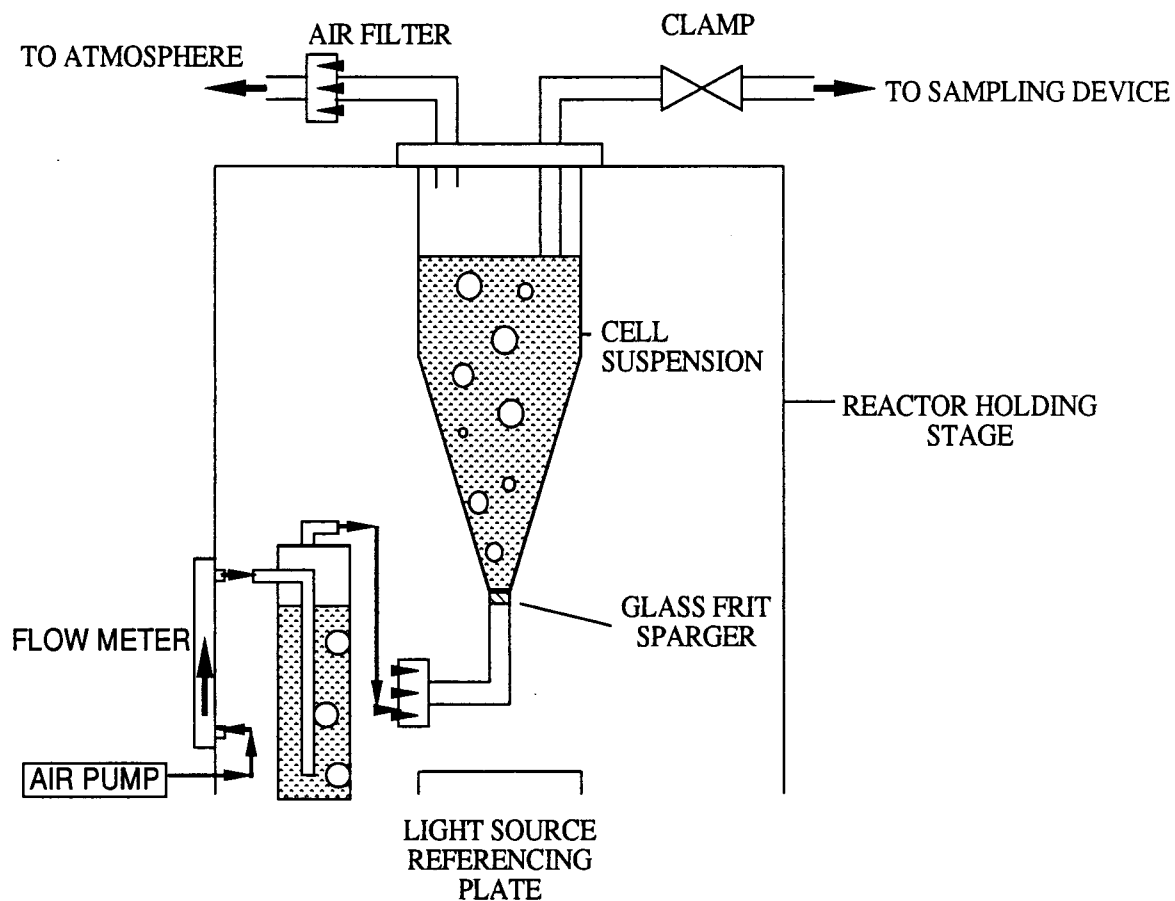


Figure 4: Bubble Column Bioreactor General Set-up

SPARGER DESIGN

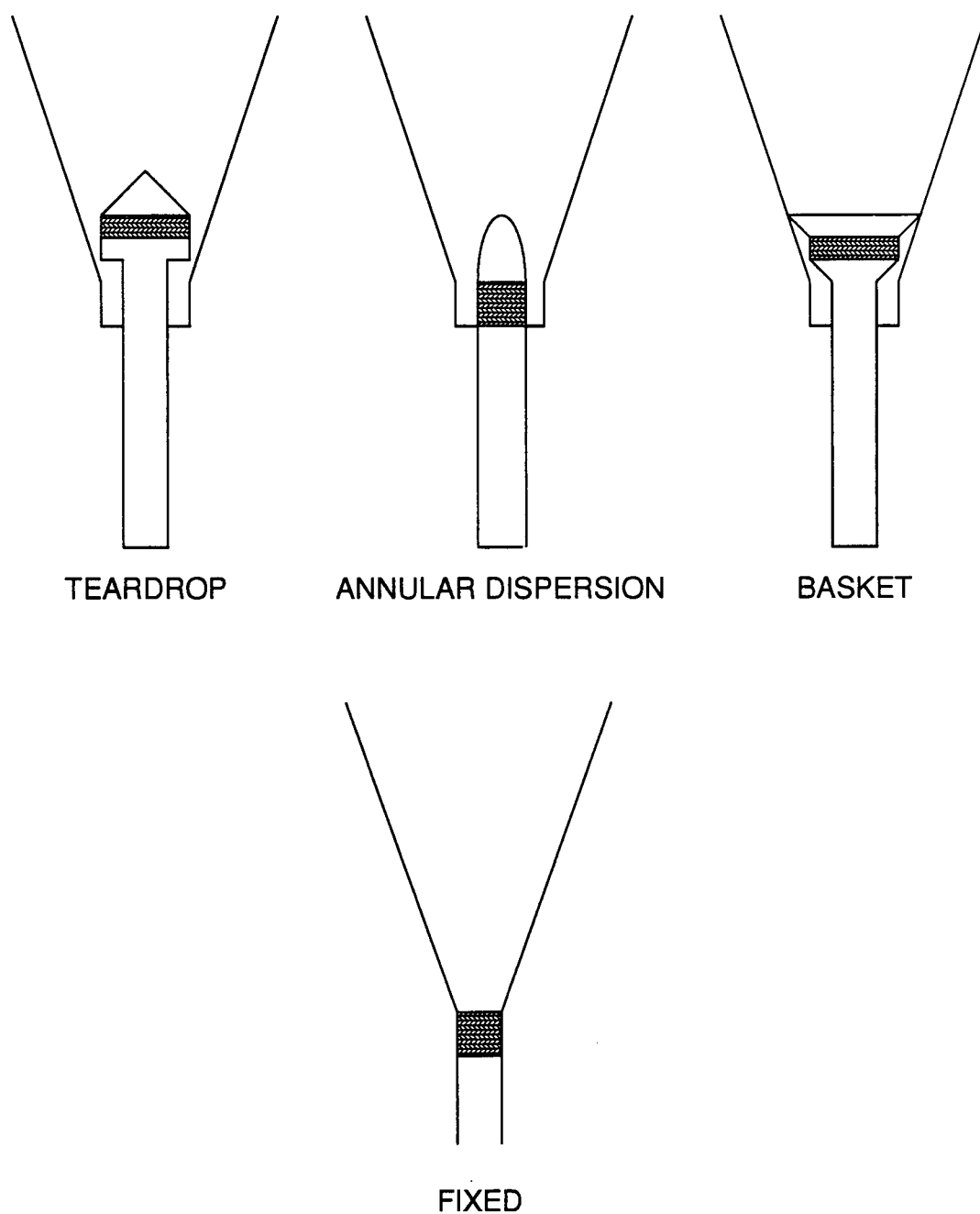


Figure 5: Sparger Configurations

design and gave excellent results. The vessel was extruded to a 1/2 in. diameter point and the sparger was sealed in here. This gave a small sparging area that resulted in more complete bubble distribution and higher bubble velocity. The end result was more effective suspension.

Light Stage

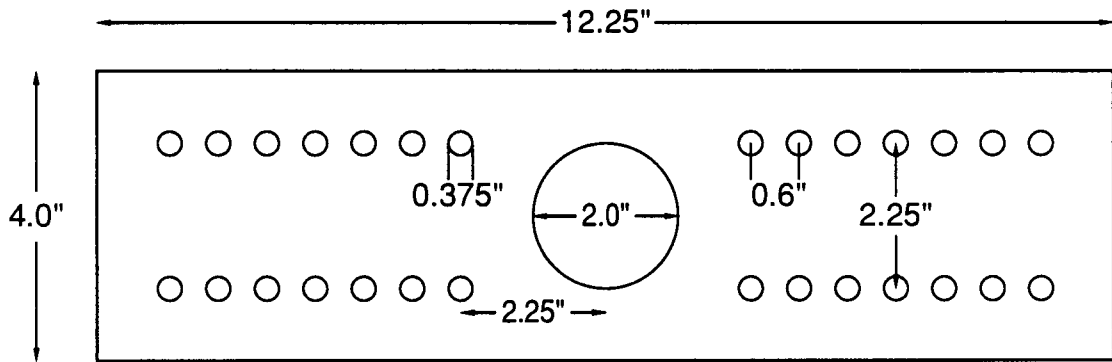
A light stage (Figure 6) was built to provide illumination for photosynthesis. It consisted of two 6 watt fluorescent tubes, 8 in. long mounted on plexiglass blocks. The plexiglass blocks were fitted with referencing pegs 2.25 in. apart. To control light intensity, peg holes (0.375 in. diameter) were drilled 0.6 in. apart in a referencing plate to hold the blocks. Figure 7 shows light intensity as a function of position on the referencing plate.

Experimental Conditions

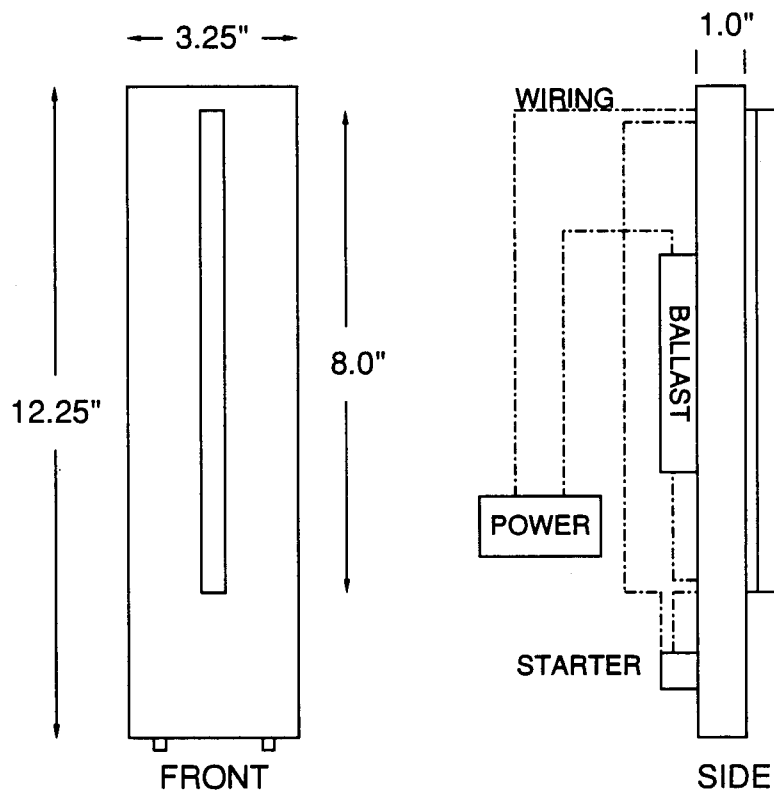
The reactor vessel and headplate assembly was washed with phosphate-free soap (Liquinox) and rinsed 10 times with deionized water. The vessel and headplate was then dried in an oven for 2 hours. To prevent the gametophytes from sticking to the walls the vessel was silanized using Sigmacote (Sigma, Cat# SL-2), which provides a inert hydrophobic film of silicone. About 10 ml of the silanizing agent was poured into the clean dry vessel and all glass surfaces were wetted with the agent.

The vessel was allowed to air dry for 24 hours to cure the silanizing agent. Next the vessel was rinsed 10 times with deionized water to remove

LIGHT STAGE DESIGN



LIGHT SOURCE REFERENCE PLATE



LIGHT FIXTURES

Figure 6: Light Stage Dimensions

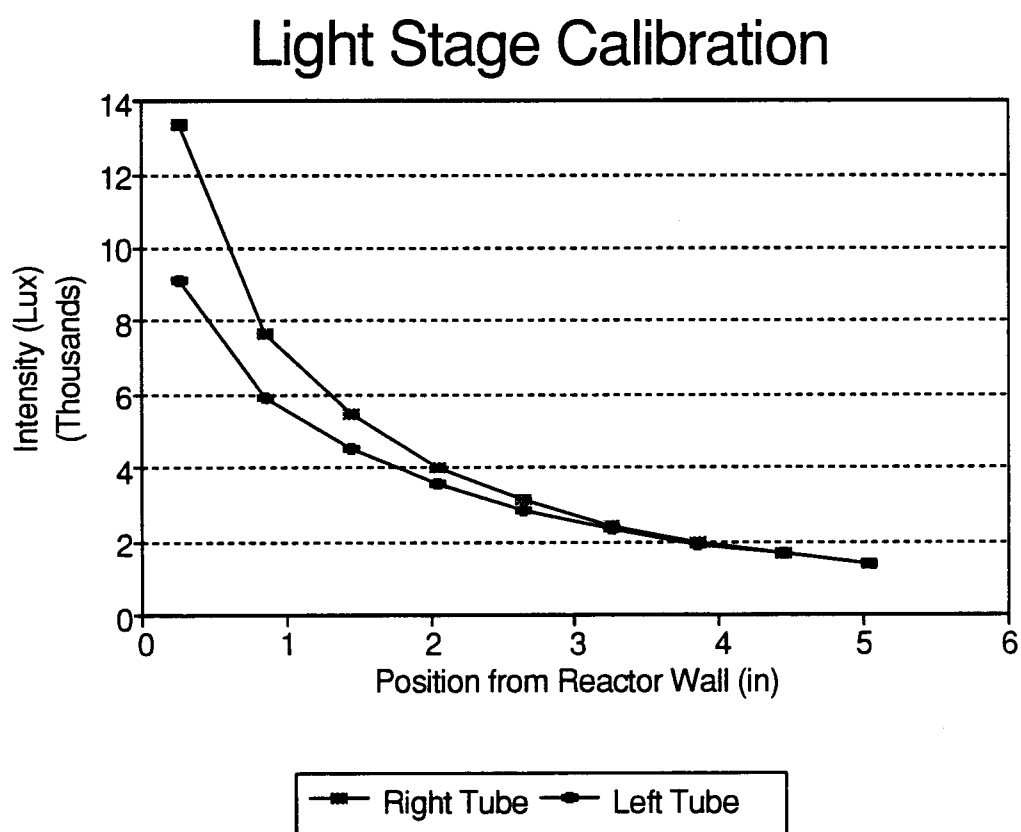


Figure 7: Light Stage Calibration

any traces of the silanizing agent not cured to the walls or frit. The entire vessel was then assembled (headplate, hoses, filters, clamps) and autoclaved at 121 °C under 15 psig for 20 minutes.

After sterilization the reactor was allowed to cool in a laminar flow hood. Using a sterile graduated cylinder, 250 ml of sterile GP2 medium was measured out and added to the reactor with the headplate removed. At this point the appropriate nutrients were added to the medium as determined by experimental design.

An inoculum source was chosen from existing maintenance cultures using the same criteria as used in subculturing (see above). The inoculum was blended for 20 seconds on "liquefy" speed. Using a 10 ml graduated pipet, 30 ml of inoculum was added to the reactor giving an initial dry cell density of 29.4 mg/L. The reactor was then sealed placed in a low temperature incubator at 13 °C, and connected to the air pump. The light intensity was set at 2500 lux with a photoperiod of 16L:8D.

The experimental design was to examine the effect of carbonate concentration on growth rate and overall growth. Three levels of concentration were examined , 0 g/L, 0.34 g/L, and 0.68 g/L. Two 5 ml samples were taken initially for chlorophyll analysis and three 20 ml samples were taken from the inoculum to determine starting dry weight conditions. Two 5.0 ml samples were taken for chlorophyll analysis at two day intervals during the nominal 20 day cultivation period. The samples were filtered through a 20 µm nylon mesh and the mesh was placed in clean 50 ml polypropylene centrifuge tube. To this 5 ml of HPLC grade methanol was added and the same procedure was followed as that outlined above for chlorophyll absorbance. The experimental conditions are summarized in Table 6.

Table 6: Bubble Column Bioreactor Experimental Conditions

Condition	Setting
Temperature	12-13 °C
Light Intensity	2500 Lux
Photoperiod	16L:8D
Medium	GP2 (0-680 mg/l bicarb)
Length of Cultivation	~20 Days
Culture Volume	280 ml
Agitation	97.1 ml/min

Immobilized Cell Culture

Alginate Bead Encapsulation

Female *Laminaria saccharina* gametophytes were encapsulated in an alginate gel matrix. This is accomplished by mixing the gametophytes into an alginic acid solution. The solution is then added dropwise to another solution containing ions capable of causing the alginic acid to gel, forming a bead. The benefit of this culture scheme is the ease with which the beads can be handled and the ability to control reaction configurations.

Bead Forming Solutions

The beads were formed from an alginic acid solution. Four grams of powdered alginic acid (Sigma, 3500 cps, Cat# A-2033) was weighed into a clean 250 ml beaker. The beaker was sealed with aluminium foil and autoclaved for 15 minutes at 15 psi. 200 ml of sterile GP2 base medium containing no multivalent cations was then mixed with the alginic acid on a hot plate at 70 °C for 30 minutes in a sterile flow hood. Multivalent cations form the links between alginic species that allows the casting to alginate to take place. After the alginate had dissolved completely the solution was allowed to cool to room temperature still under stirring.

As previously outlined a gametophyte inoculum source was chosen from existing maintenance cultures. Two, three week old maintenance cultures were first blended then filtered through a 20 µm nylon mesh filter. The gametophytes were then removed from the filter using a sterile spatula

and placed in the mixing, cooled alginic acid solution. The final dry cell density was ~60-100 mg/L.

The casting solution consisted of sterile GP2 medium containing all nutrients and ions with the addition of 0.3M CaCl_2 . The divalent calcium ions form the backbone of the newly ionically crosslinked alginate matrix. The calcium chloride was weighed out into a clean 500 ml beaker. The beaker was then sealed and autoclaved for 15 minutes at 15 psi. To the sterilized calcium chloride, complete GP2 medium was added using a sterile graduated cylinder.

Casting Apparatus

The bead casting apparatus is shown in Figure 8. The alginic acid and gametophyte solution remained under constant mixing on a magnetic stir plate. A 1/8 in. OD Tygon tube was immersed in the solution and fed through a peristaltic pump. The pump (Buchler Instruments, model 426-2000) was set at the highest speed. The other end of the tube was connected to a 1/8 in. to 1/16 in. in. reducing union. The 1/16 in. OD tube was then fed through a 1/16 in. ID stainless steel sleeve situated over the casting solution by 4 in. to provide rigidity. The 1/16 in. flexible tubing protruded from the end of the stainless steel tubing by 1/8 in..

Before starting a bead casting run, the tubing was flushed with sterile deionized water for 10 minutes. Then alginate solution was introduced to the clean line at a speed setting of 6 on the peristaltic pump. Using a hand counter the number of drops per minute were counted three times to establish a bead formation rate (~96 beads/min). The time to form 100 beads was calculated from this. The beads measured ~3 mm in diameter.

BEAD CASTING APPARATUS

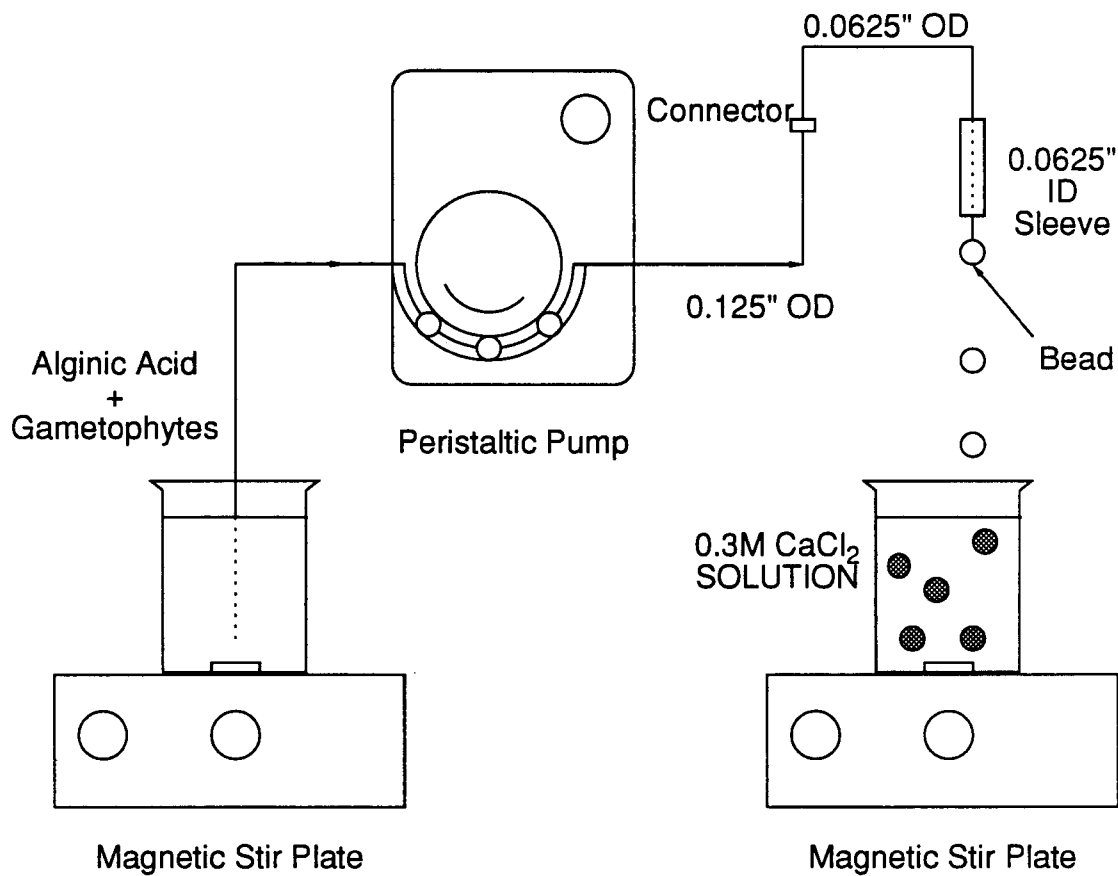


Figure 8: Alginate Bead Casting Apparatus

Experimental Conditions

After establishing a bead formation rate for a set speed, 200 beads were formed and the pump was shut off. The beads were allowed to cure in the casting solution for 10 minutes. Then the casting solution with the beads was filtered through a sterile 105 μm nylon mesh filter and the beads were transferred to a sterile 125 ml Erlenmeyer flask containing 20 ml of GP2 medium.

The flasks were sealed with aluminium foil and placed on an orbital shaker at 100 rpm in a low temperature incubator. The temperature was set at 13 °C and the beads experienced a light intensity of 2500 lux with a photoperiod of 16L:8D.

Sampling was done by first decanting the GP2 culture medium and then adding 20 ml of 0.5M sodium citrate dissolved in GP2 medium. The sodium citrate served to chelate the calcium ions embedded in the alginate matrix, and dissolved the bead. The sample was incubated in 20 ml of 0.5M sodium citrate at room temperature for 30 minutes on an orbital shaker set at 100 rpm.

After 30 minutes the beads were completely dissolved. Three 5.0 ml samples were taken from the flask using a graduated pipet. The samples were filtered through a 20 μm nylon mesh and then washed with 20 ml of deionized water to remove any citrate residues. Then the samples were worked up using the above mentioned chlorophyll assay by placing the filters in clean centrifuge tubes and adding methanol. The experimental conditions are summarized in Table 7.

Table 7: Alginate Bead Experimental Conditions

Condition	Setting
Temperature	12-15 °C
Light Intensity	2500 Lux
Photoperiod	16L:8D
Medium	GP2 (340 mg/l bicarb)
Length of Cultivation	~30 Days
Culture Volume	20 ml
Agitation	100 rpm
# Beads	200
Vol. Beads	~3 ml

Results and Discussion

Porphyra perforata Culture

Protoplast Isolation and Culture

Table 8 shows the results of protoplast isolation from *Porphyra perforata* via enzymatic digestion. Figure 9 shows newly digested protoplasts in 0.33 wt% phenosafrin stain. The best results were obtained under 23 °C, 70 rpm agitation, 16 hour digestion period, *Aplysia* enzyme medium, and pre-frozen fronds. An overall protoplast yield of $1.96 \pm 0.81 \times 10^6$ viable cells per gram of fresh tissue (1s, n=5) was obtained.

This result is comparable to literature results. Waaland et. al. (1990) obtained a yield of 1.25×10^7 cells/g using *Porphyra nereocystis*. Fujita and Saito (1990) obtained yields ranging from 10^6 - 10^8 cells/gram fresh weight for seven different species of *Porphyra*. *Porphyra* is considered a model marine algal species for this culture technique because of its lack of complexity.

Many digestion parameters were tested. These included the type of enzyme, digestion period, post and pre-digestion tissue homogenization, as well as osmoticum concentration and temperature. Of these parameters the most significant were the starting material, freezing of the algae before digestion and the type of enzyme mixture.

All of the best digestions came from one batch of algae. This algae was collected in late August 1992. The algae was at the end of its growth phase and had been severely attacked by local herbivores. This shows the importance that starting material has on the digestion efficiency.

Table 8: Protoplast Isolation Results*

Digest #	Loading (mg)	Yield (Cells/g-ml)
P-5-6	57	2.69×10^6
P-5-12	57	2.06×10^6
P-5-13	57	2.79×10^6
P-5-16	57	1.30×10^6
P-5-31	57	0.98×10^6

* All runs were at the following conditions:

Enzyme: *Aplysia*/2 wt% cellulysin

Time: 19 hours

Temp: 23 °C

Media: 1/2 strength PES

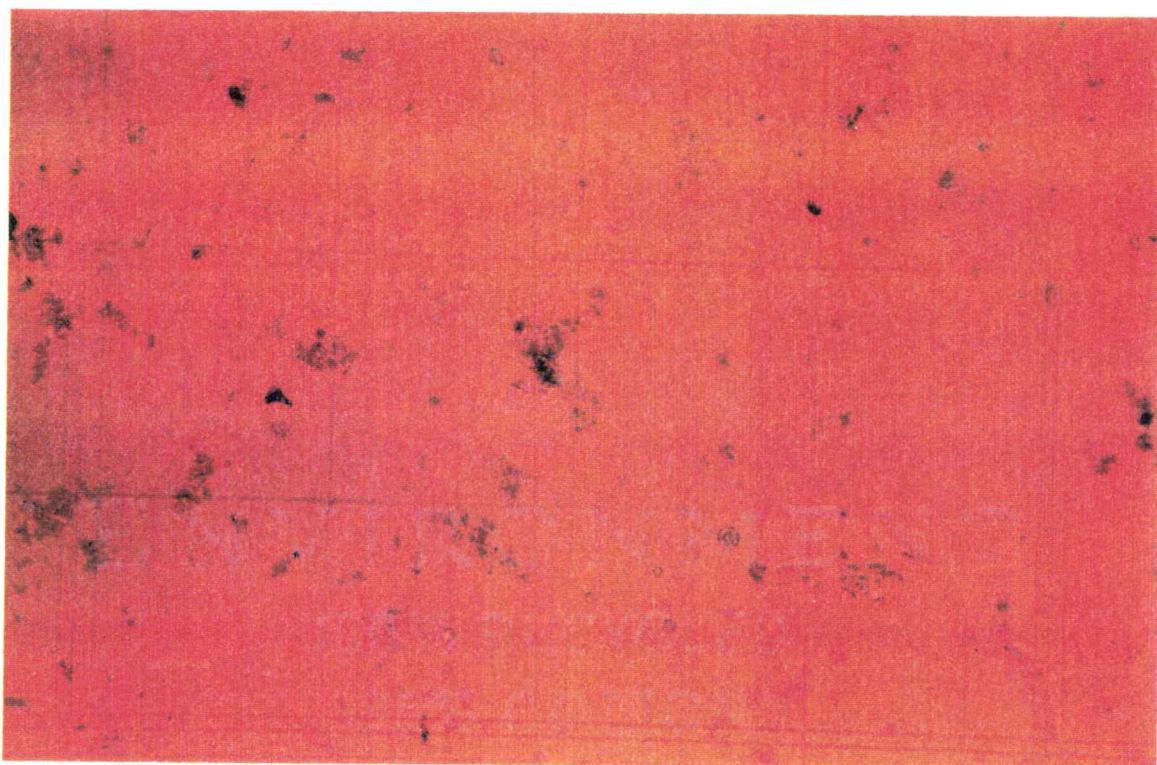


Figure 9: Viable Protoplasts

Figure 10 shows a digestion in progress. It is easy to see that the digestion proceeds along a reaction front radially inward from the cut edges. The protective cuticle on the surface of the algae seems impervious to enzymatic attack. Because better yields were also obtained from the pre-frozen algae, it is believed that by pre-freezing, this cuticle is somehow damaged. It seems logical that by freezing the algae, followed by rapid thawing, prior to digestion serves to crack the cuticle and allow more surface area for enzymatic attack.

The newly isolated protoplasts were cultured in 1/2 strength PES medium with the aforementioned osmoticum concentration (0.4 M sorbitol). Some cultures survived for 1 week, but generally died soon after. Although growth has been reported (Polne-Fuller and Gibor, 1984) under the culture conditions here, I was not able to reproduce these results. Because growing cultures could not be established, an alternative route of algal cultivation had to be adopted. The results of this approach are detailed below.

***Laminaria Saccharina* Gametophyte Culture**

Maintenance Cultures

Female gametophytes of the species *Laminaria saccharina* were successfully cultured in GP2 artificial seawater medium. Figures 11 and 12 show the gametophytes at 100x and 470x magnification respectively. Typical durations of growth in 100 ml of GP2 medium with 0.34 g/L sodium bicarbonate were 30 days and longer. These cultures did not need to be agitated. They grew in filamentous mats roughly 1 cm diameter.

Growth seemed to be primarily affected by subculturing density and temperature. Subculturing at densities less than ~20 mg dry weight/L

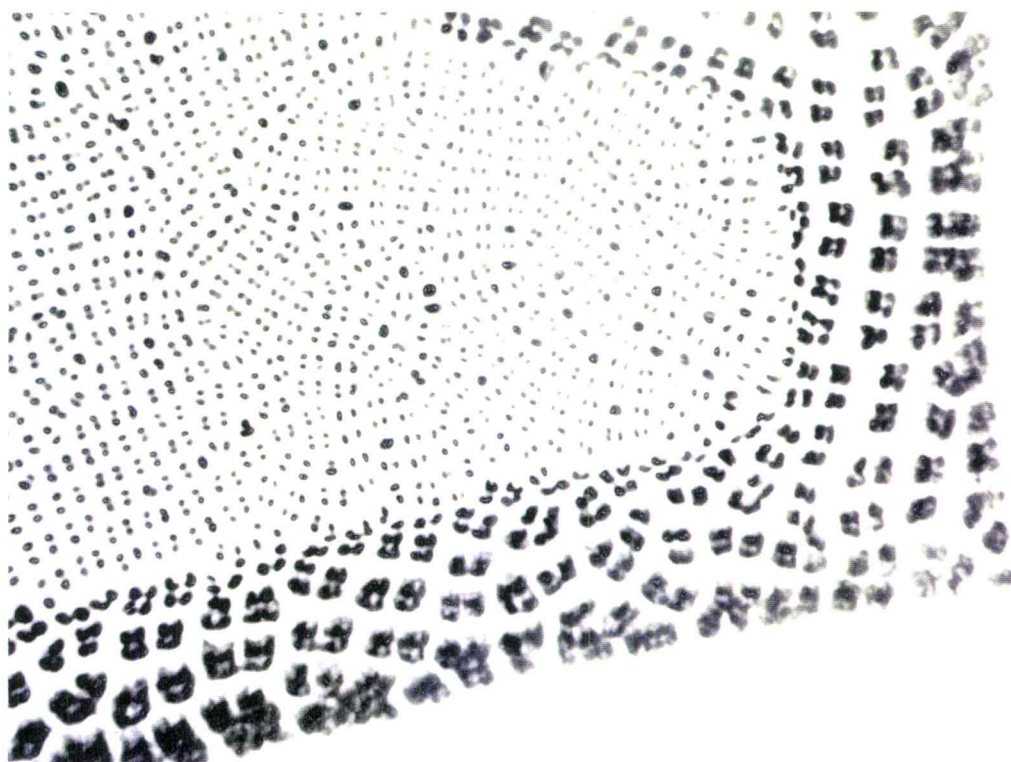


Figure 10: Tissue Digestion in Progress

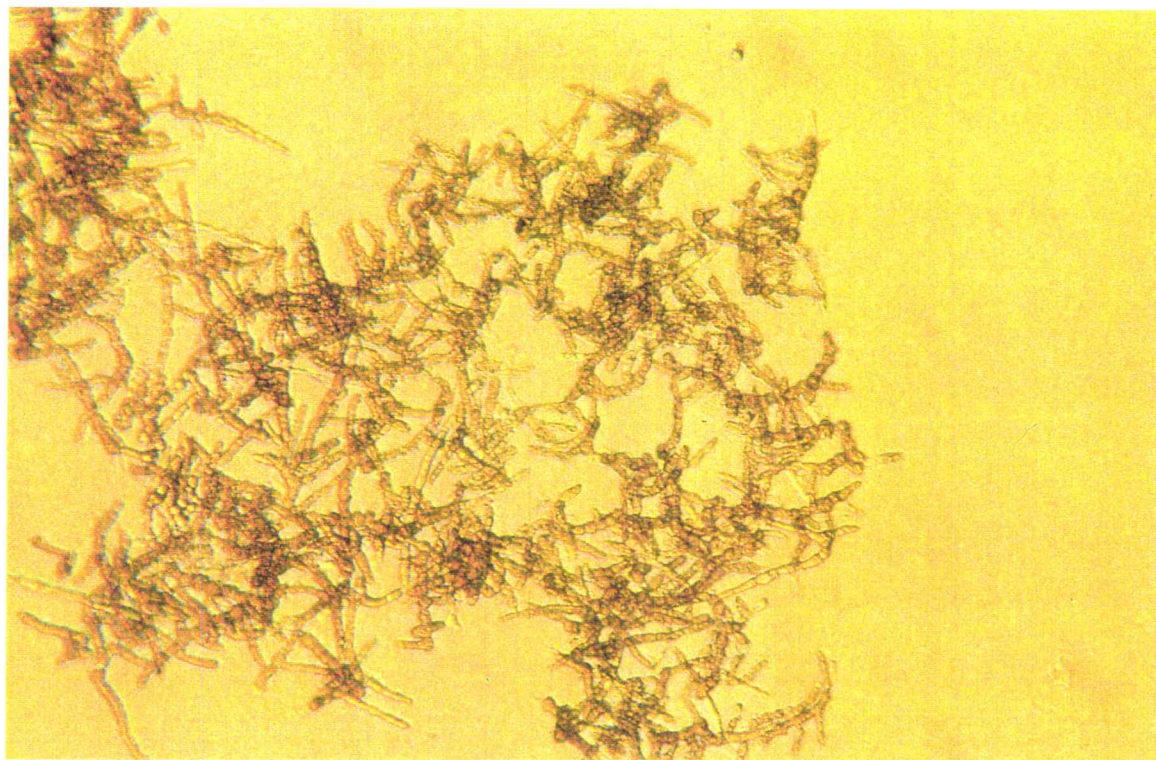


Figure 11: Gametophytes 100x



Figure 12: Gametophytes 470x

produced poor growth characteristics. The gametophytes often coated the bottoms of the culture flasks and refused to stay suspended. Long recovery times and total lack of growth were also characteristics of low inoculum densities. Temperature also may have been a large factor in the quality of gametophytic cultures. Those cultures that were kept at 8 °C became pale in appearance and grew slowly. Those that were kept at the normal 12 °C retained their initial deep brown color and showed noticeable growth within a week.

The optimum conditions for culture maintenance were an inoculum level of >20 mg dry weight/L, 12 °C, 2500 Lux, 16L:8D photoperiod, and no agitation. Those that were agitated on an orbital shaker quickly coagulated into dense clumps. This retarded growth probably as a result of decreased nutrient diffusion.

Static Flask

The results of two static flask culture growth curves are shown in Figure 13. The growth rate of the cultures, determined by linear regression, was found to be 0.075 µg Chl a/ml-day for culture run #1 and 0.082 µg Chl a/ml-day for culture run #3-LG. Both runs grew at nominally the same rate in a zero order manner. The conditions for both runs are shown in Table 5.

Because the growth is linear and no agitation was used on the cultures it seems that the rates are controlled by diffusion of nutrients through the liquid film surrounding the cells.

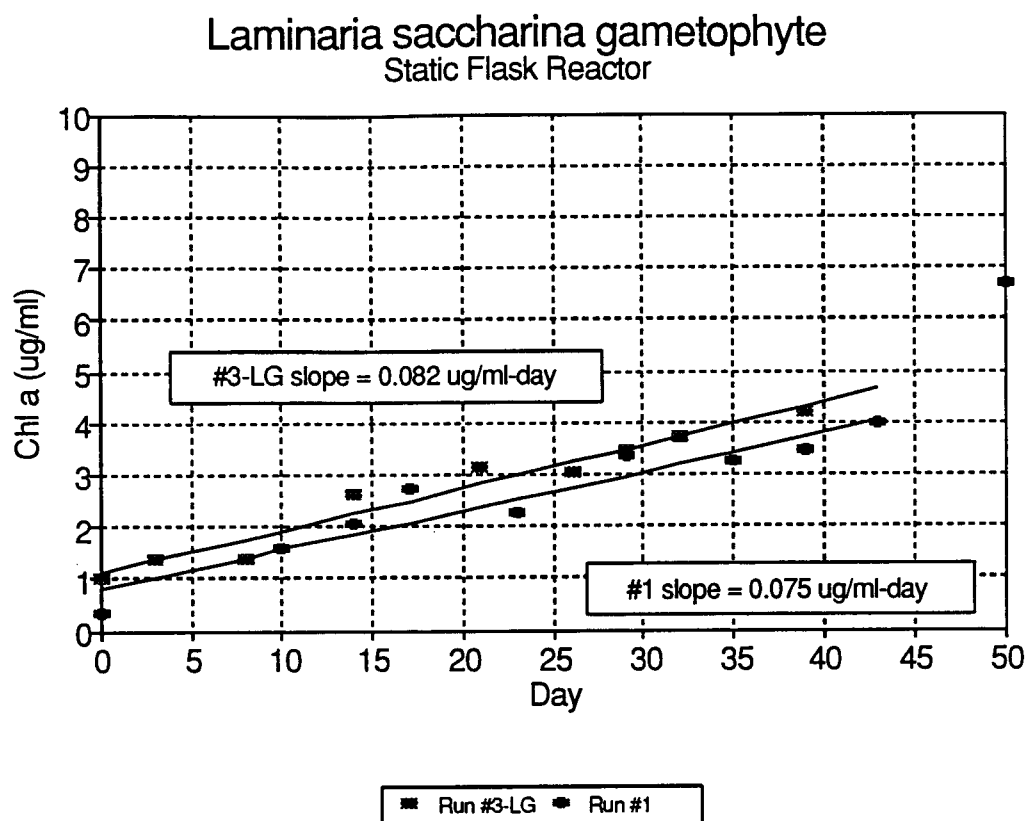


Figure 13: Static Flask Growth Curve

Cell Immobilization

Bead Characteristics

Freshly blended gametophytic cultures of *Laminaria saccharina* were successfully immobilized in an calcium alginate matrix. Each bead was ~3 mm in diameter and had an elastic consistency. The beads appeared suitably transparent to light. This was important because photosynthetic processes are necessary to the health of the gametophytes. In liquid culture the beads did not stick together, solving the problem of agitation with free gametophytes.

Under normal culture conditions the beads lost mechanical strength. This was evidenced by a clouding of the culture solution after 1 week. Because the beads are cast in high concentrations of calcium it is believed that this is a result of the calcium being leached from the binding sites in the alginate matrix. This leaching resulted in a less mechanically strong bead. After 20 days the beads still held their form and size. No gametopyhte release from the beads was noticed.

To improve bead stability several things can be done. It has been reported that a low phosphate concentration significantly reduces this leaching effect (Dainty et. al. , 1986). Phosphate acts as a chelator of calcium in competition with the alginic acid. This was not done to avoid experimental prejudice when comparing with static flask and bubble column cultivation results. Another approach is to lower the pH. The pH of the medium has an influence on the chelating effectiveness of the alginic acid. The stabilizing effect is not noticeable until the pH of 6.5 (Dainty et. al. , 1986) and lower.

The problem here is that the gametophytes naturally enjoy a relatively high pH of 8.0. The survival of the gametophytes in the pH range of ≥ 6.5 has not been tested.

Immobilized Cell Cultivation

The growth curve for gametophyte cultivation in alginate beads is shown in Figure 14. No growth was observed. This is probably due to two factors. One is that the light intensity that reached the cells is somewhat diminished by the alginate matrix. The beads have a cloudy translucent appearance similar to a greenhouse. Lastly, the matrix could have restricted the diffusion of nutrients to the cells, inhibiting their growth. It is possible to cast the beads at a lower calcium concentration. This may have the effect of "opening" the bead to better nutrient transport.

Bubble Column Bioreactor

Two bioreactors were run in parallel to test for the repeatability of the growth curve. The results of those tests are shown in Figure 15. The run conditions were 12-13 °C, 16L:8D photoperiod, 2500 Lux light intensity, 97.1 ml/min constant aeration, and 0.34 g bicarbonate/L. Because of constant aeration the flowrate based on volume of gas per volume of culture (vvm) varied. For this reason Figure 16 shows the changing flowrate on a vvm basis with time.

Both reactors showed good similarity up to the 10th day. At this point reactor 1 suffered from gametophyte coagulation problems. This problem arises when the silanized frit becomes stripped of its hydrophobic layer. This

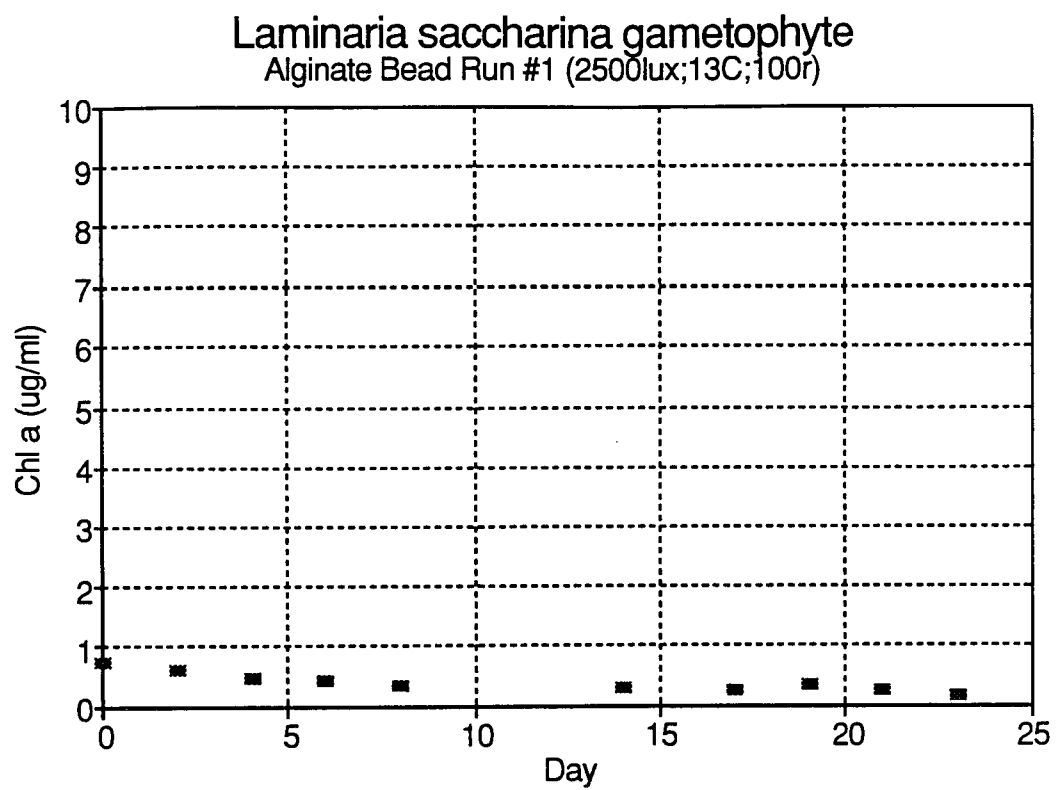


Figure 14: Alginate Bead Growth Curve

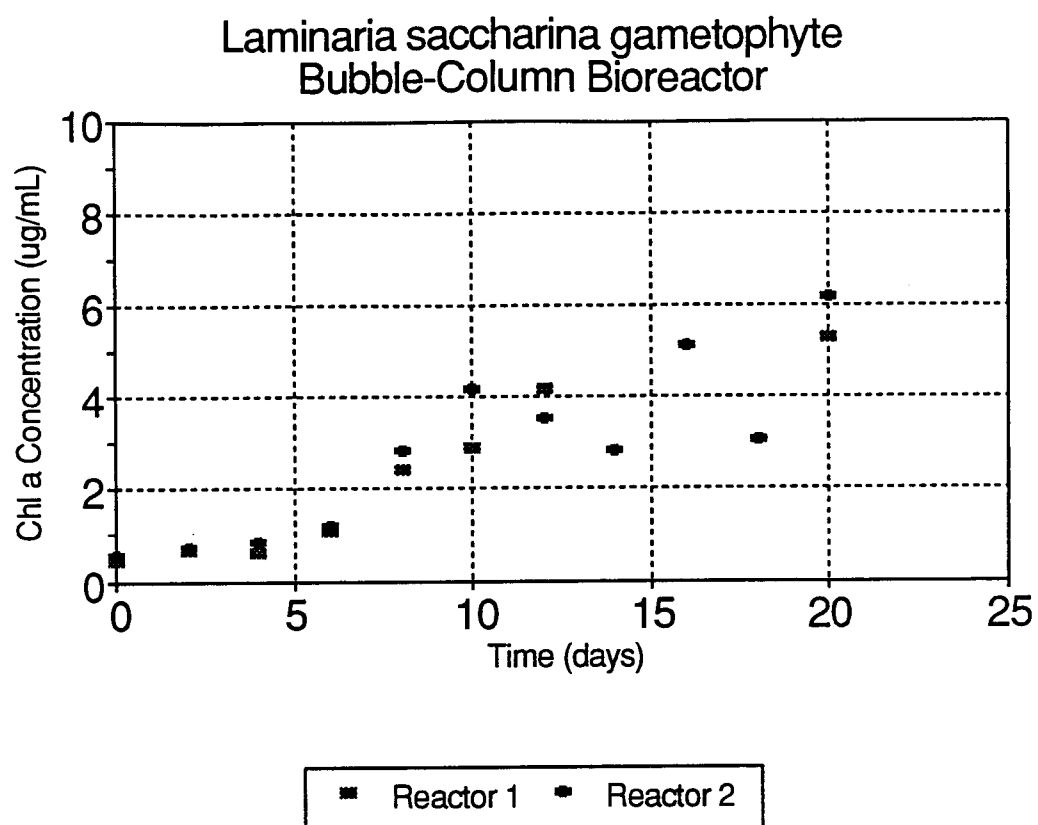


Figure 15: Bubble Column Bioreactor Growth Curve (340 mg/L)

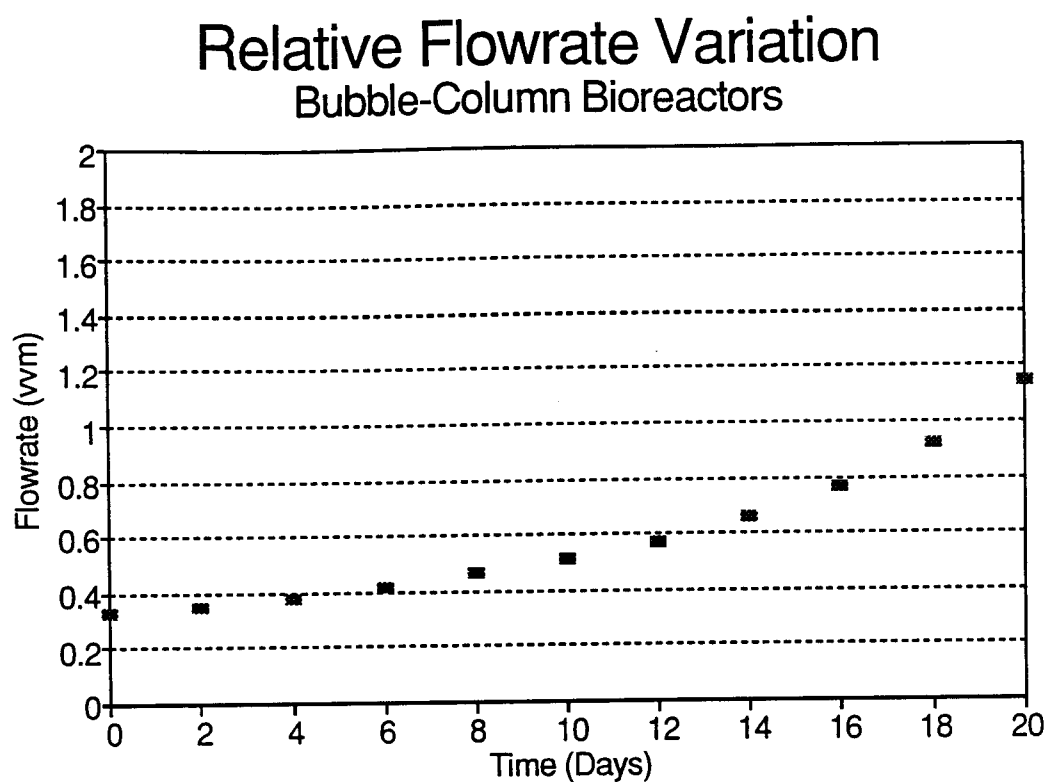


Figure 16: Flowrate Variation

causes the size of bubbles formed at the frit to decrease. As a result the turbulence in the column decreases allowing the gametophytes to settle and coagulate.

In bioreactor 2 this problem can also be seen at 10 days. However, in this case the gametophytes coagulated, but then rose back into the column. This had the effect of creating clumps. Clumping effected the sampling as can be seen. To remedy both these problems samples were taken up through the exponential phase. Then a last sample was taken at the end of the run by blending the remaining contents.

Table 9 shows the overall results of the comparison run. The cultures grew in a first order manner and went from an initial dry weight density of 45 mg/L to a final of 795 mg/L and 850 mg/L for reactor 1 and reactor 2 respectively. Figure 17 shows a semi-log plot for the run. Linear regression was used to determine the slopes of the exponential phase. This slope gave the specific growth rates (μ) directly as 0.272 day^{-1} and 0.291 day^{-1} for reactors 1 and 2 respectively. This analysis resulted in doubling times ($\text{Ln}(2)/\mu$) of 2.55 days and 2.38 days for reactor 1 and reactor 2 respectively.

The doubling times are comprable and indicate that parallel runs may be made at different carbon source levels for comparison. The average of the doubling time for the two runs was 2.46 ± 0.12 days (1s, n=2).

Comparison of Cultivation Systems

From Figures 13, 14, and 15 it can be clearly seen that the bubble column bioreactor gave superior growth rates. Growth rates are compared in Table 10. The improved growth rate is due to improved mixing. In the static flask environment the cells are allowed to grow in mats and are not agitated.

Table 9: Bioreactor Comparison Results*

Measurement	Reactor #1	Reactor #2
Initial Chl a (ug/ml)	0.423	0.538
Final Chl a (ug/ml)	5.310	6.206
Initial Dry Cell Density (mg/L)	45	45
Final Dry Cell Density (mg/L)	795	850
Specific Growth Rate (1/day)	0.272	0.291
Doubling Time (day)	2.55	2.38

* All runs were performed at the following conditions:

Temp: 13 °C
 Light: 2500 Lux / 16L:8D
 Media: GP2 w/ 0.34 g/L bicarbonate
 Volume: 280 ml
 Aeration: 97.1 ml/min constant

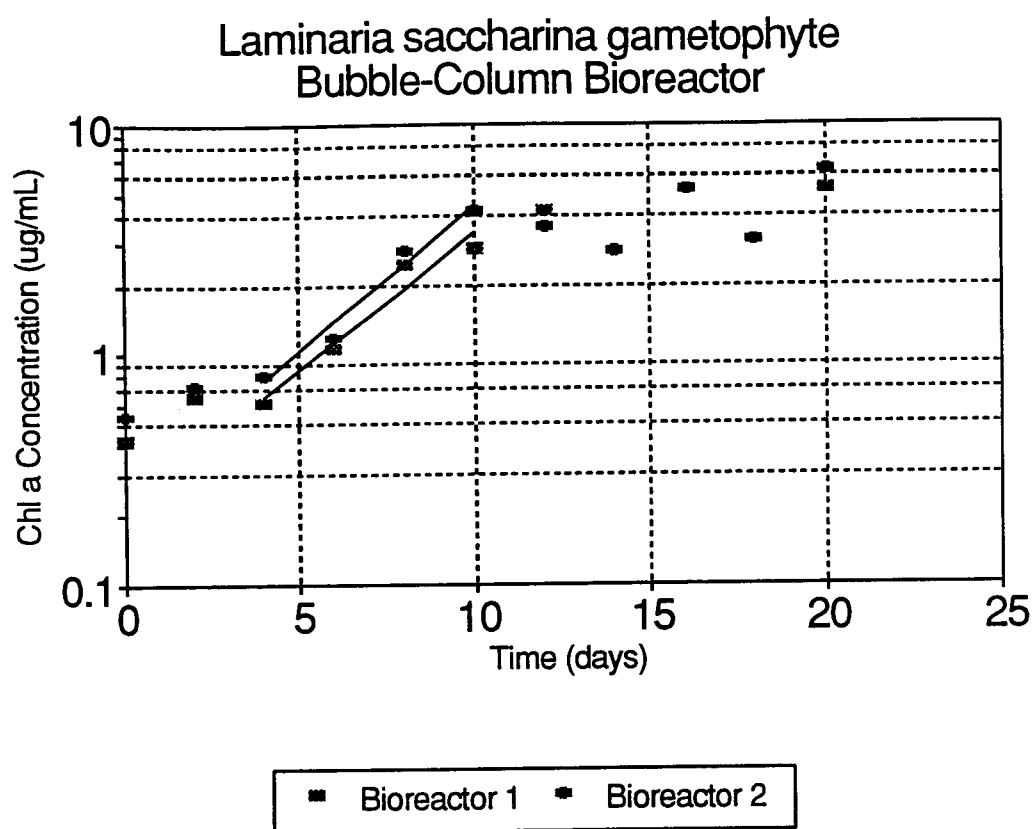


Figure 17: Bubble Column Bioreactor Growth Curve (340 mg/L)

Table 10: Cultivation Comparison

Measurement	Bubble-Column	Immobilized	Static
Initial Chl a (ug/ml)	0.481	0.755	0.684
Final Chl a (ug/ml)	5.759	0.185	5.449
Initial Dry Cell Density (mg/L)	45	(119)	100
Final Dry Cell Density (mg/L)	822.5	(100)	(279)
Max. Growth Rate (ug Chl a/ml-day)	1.00	~0	0.079
Doubling Time (day)	2.47	~0	-

*Numbers in parenthesis calculated from calibration curve

Both situations correspond to an increase in nutrient diffusion resistance. The alginate beads have similar problems, as well as, light reduction outlined above. The bubble column has none of these problems.

Effect of Bicarbonate Concentration on Growth

The effects of bicarbonate concentration on the growth curve is shown in Figure 18. Table 11 shows the effect in tabular form. Three levels were compared; 0 mg/L, 340 mg/L and 680 mg/L. Doubling times were 2.77, 2.47, and 3.40 days for 0, 340, and 680 mg/L bicarbonate respectively. Final dry cell densities were all ~800 mg/L.

Looking at the maximum growth rates, we might conclude that by increasing the carbonate concentration we inversely affect the growth rate. In this case, however, doubling time is a much better indicator of growth rate because of the exponential characteristic of the growth. Comparing, then, the doubling times we do not see a correlation with bicarbonate level. The doubling times seem random with the 340 mg/L run having the lowest doubling time followed by 0 mg/L and then 680 mg/L.

With a reasonable degree of certainty we can then say that bicarbonate concentration is not the limiting nutrient in this system. We are constantly sparging the cultures with air and would expect to reach an equilibrium concentration of bicarbonate ion. As the CO_2 from the air enters solution it will speciate into bicarbonate ion HCO_3^- . Because we are operating in a batch mode, all solutions would reach an equilibrium bicarbonate level with the sparging atmosphere. Therefore, increasing the partial pressure of CO_2 in the sparging gas might increase the growth rate. The investigation of this phenomenon was beyond the scope of this project.

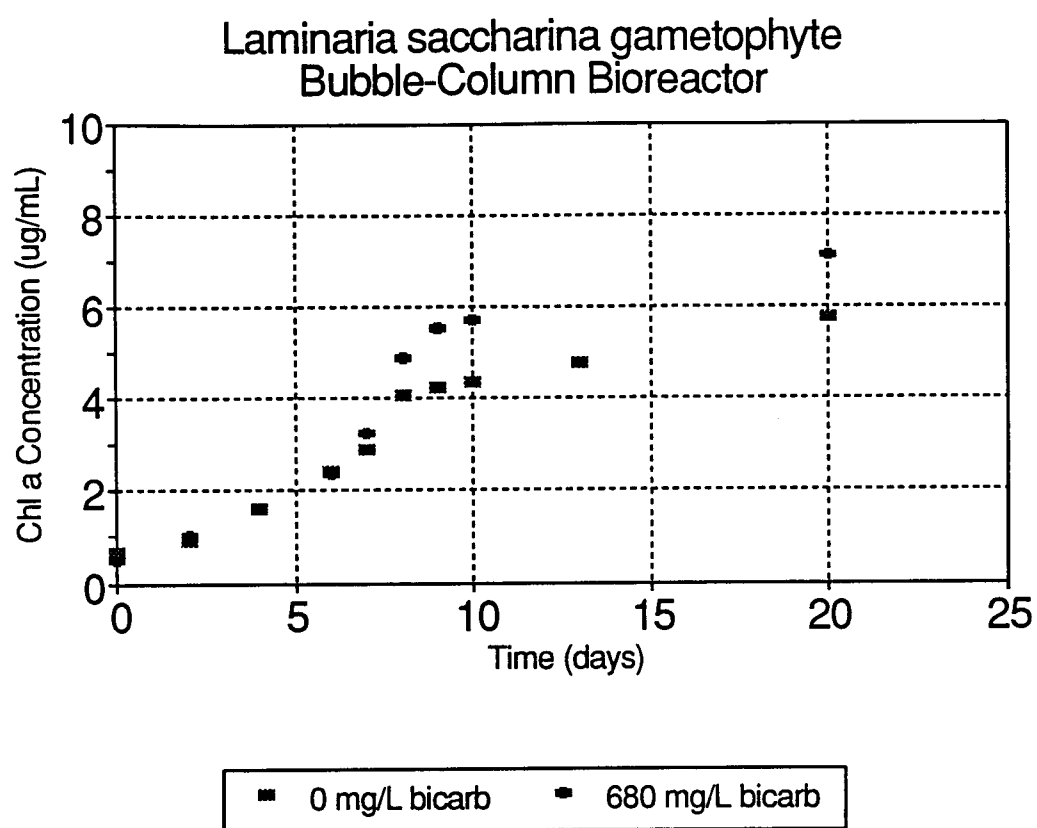


Figure 18: Effect of Bicarbonate Concentration on Growth Rate

Table 11: Effect of Bicarbonate Concentration

Measurement	0 mg/L	340 mg/L	680 mg/L
Initial Chl a (ug/ml)	0.505	0.481	0.668
Final Chl a (ug/ml)	7.119	5.759	5.783
Initial Dry Cell Density (mg/L)	29.4	45	29.4
Final Dry Cell Density (mg/L)	883.3	822.5	865.0
Specific Growth Rate (1/day)	0.250	0.272	0.204
Doubling Time (day)	2.77	2.47	3.40

Calibration Curve

A calibration curve relating dry cell weight to Chl *a* content is shown in Figure 19. The cultures used for the curve were maintained at 12 °C, 16L:8D photoperiod, 2500 Lux, and no agitation for 1 month prior to use. They were then blended and chlorophyll content as well as dry cell weight was assayed.

The curve was taken for one condition and does not correspond well with observations from the cultivation runs. For example, the bioreactor comparison run started at ~0.5 µg Chl *a*/ml. This corresponds to 2.5 µg Chl *a* on the calibration curve (0.5 µg/ml x 5.0 ml sample). Reading off the curve then we find a dry weight of ~0.55 mg dry weight. In a 5 ml sample this is equivalent to 110 mg/L dry cell density. Direct measurement, however, shows an initial dry cell density of 45 mg/L. Furthermore, final dry cell densities of 795 mg/L and 860 mg/L, obtained by direct measurement were much higher than cell densities of 273 mg/L and 304 mg/L obtained from the calibration curve.

The discrepancy arises from two main factors. The first is the period of growth. Freshly subcultured cells will likely show a higher chlorophyll content due to damage caused by the procedure. The cultures are blended then filtered and placed in subculture. This has the effect of removing dead cell matter, increasing the viability on a weight basis. Dead cell fragments will easily break into smaller pieces and pass through the filter. This raises the initial chlorophyll concentration on a dry cell weight basis.

The second reason is that as the cells grow some die off. The pigments in those that die off decay. This has the effect of underestimating the dry cell density because it will not include dead cells in the chlorophyll analysis.

Laminaria Saccharina Gametophyte Chlorophyll A Calibration Curve

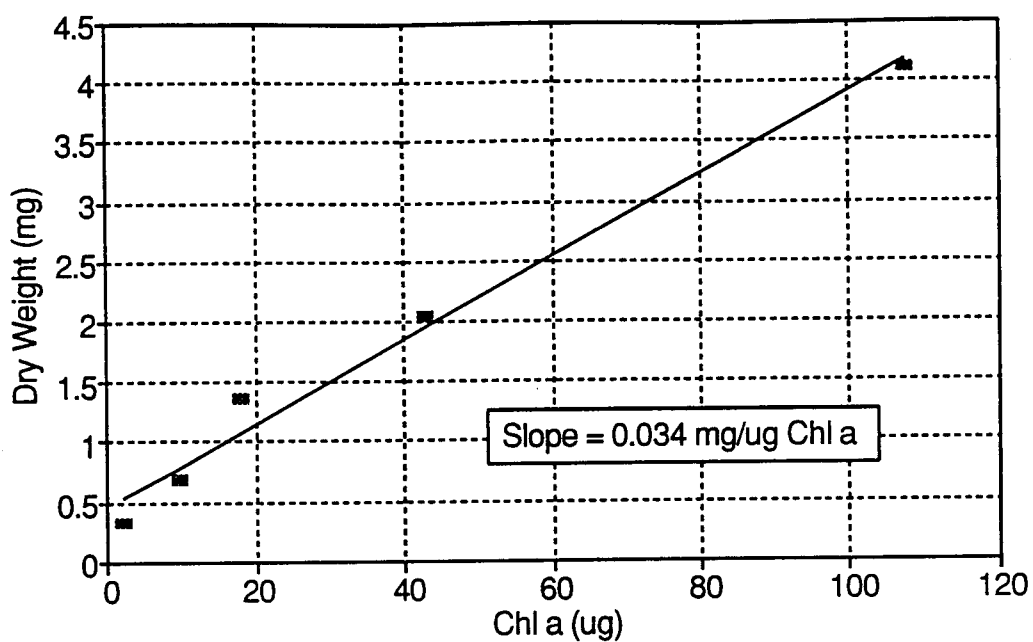


Figure 19: Calibration Curve

Conclusions

Protoplast isolation of the species *Porphyra* was accomplished. A yield of $1.96 \pm 0.81 \times 10^6$ viable cells per gram of fresh tissue was obtained. The yields of viable protoplasts were comparable to literature values. Although the literature review suggests that the cells can then be grown under the culture conditions used here, growth was not seen.

Laminaria saccharina gametophytes were successfully maintained in liquid culture. The gametophytes were used for all cultivation and comparison studies.

A bubble column bioreactor was designed and performed to the specifications required. Those specifications were to be able to support photosynthetic growth, be small in size, and provide a low shear environment. Several sparging configurations were tried and the fixed configuration gave the best fluidizing characteristics.

Three methods of cultivation were compared. The methods were static flask, immobilized bead, and bubble column bioreactor. The static flask appeared to show zero order gametophytic growth with a rate of $0.079 \mu\text{g Chl a/mL-day}$. The immobilized gametophytes showed no growth. The lack of growth was probably due to low light transmission through the bead as well as restricted diffusion of nutrients through the bead.

The bubble column bioreactor showed the best growth characteristics. During the exponential phase of growth, the reactor showed first order behavior with a specific rate of $0.282 \pm 0.013 \text{ day}^{-1}$. However, the variation of an inorganic carbon source (bicarbonate) showed no affect on the growth of

the gametophyte cultures in the bubble column bioreactor. This was attributed to carbon dioxide mass transfer from the sparging gas to the seawater medium.

Macroalgal cultures *Laminaria saccharina* have been established and exhibit growth. A bubble column bioreactor has been developed and operates well. Future studies of macroalgal cultivation systems will benefit from many of the problems solved in this study.

Future Work

Cultivation of *Laminaria saccharina* gametophytes in immobilized alginate beads has many advantages. Ease of recovery and manipulation of reacting configurations are two. This study has shown that, under the conditions studied, alginate immobilization produces poor growth characteristics. To improve these characteristics two effects should be studied. Light intensity should be varied to determine if the lack of growth was due to poor transmittance. Furthermore, different concentrations of calcium casting solution should be investigated. Lower concentrations produce a less mechanically strong bead. This could result in better nutrient transport properties through the bead.

The bubble column bioreactor was found to be the best cultivation system studied. Bicarbonate concentration was varied to determine its effects on growth. Because sparging may have affected these results through equilibrium considerations, future work could look at no bicarbonate with different CO₂ concentrations in the sparging gas.

The effect of other nutrients on growth should also be studied. Phosphate and nitrate concentrations could have limiting effects. It may also be possible to grow the cultures heterotrophically with an organic carbon source. Once these effects have been characterized, the final goal of pharmaceutical production and optimization can be studied.

Bibliography

- Bold, H.C. and Wynne, M.J. (1985). Introduction to the algae, 2nd ed. Prentice-Hall, Englewood Cliffs, NJ.
- Bolton, J.J. and Luning, K. (1982). Optimal growth and maximal survival temperatures of atlantic *Laminaria* species in culture. Mar. Biol. 66: 89-94.
- Butler et al. (1989). Isolation conditions for high yields of protoplasts from *Laminaria saccharina* and *L. digitata* (Phaeophyceae). Journal of Experimental Botany, 40, 1237-1246.
- Butler, D.M., and Evans, L.V. (1990). Cell and tissue culture of macroalgae. In: Introduction to Applied Phycology, I. Akatsuka, Ed., SPB Academic Publishing bv: The Hague, Netherlands, pp. 629-645.
- Butler, D.M., Evans, L.V., and Kloareg, B. (1990). Isolation of protoplasts from marine macroalgae. In: Introduction to Applied Phycology, I. Akatsuka, Ed., SPB Academic Publishing bv: The Hague, Netherlands, pp. 647-668.
- Chen, L.C.M. (1989). Cell suspension culture from *Porphyra linearis* a multicellular marine red alga. Journal of Applied Phycology. 1: 153-159.
- Dainty, A.L., Goulding, K.H., Robinson, P.K., Simpkins, I. and Trevan, M.D. (1986). Stability of alginate-immobilized algal cells. Biotech. and Bioeng. 28: 210-216.
- Fujita, Y. and Saito, M. (1990). Protoplast isolation and fusion in *Porphyra*. Hydrobiologia. 204/205: 161-166.
- Geider, R.J. and Osborne, B.A. (1992). Algal photosynthesis: the measurement of algal gas exchange. Chapman and Hall, NY.
- Gerwick, W.H., Bernart, M.W., Moghaddam, M.F., Jiang, Z.D., Solem, M.L., and Nagle, D.G. (1990). Eicosanoids from the Rhodophyta: new metabolism in the algae. Hydrobiologia, 204/205, 621.
- Kargi, F., and Rosenberg, M.Z. (1987). Plant cell bioreactors: present status and future trends. Biotechnology Progress, 3, 1-8.

- Kerby, N.W., Musgrave, S.C., Codd, G.A., Rowell, P. and Stewart, W.D.P. (1983) in *Biotech '83* p. 1029.
- Luning, K. (1980). Critical levels of light and temperature regulating the gametogenesis of three *Laminaria* species. *J. Phycol.* 16:1-15.
- Luning, K. and Dring, M.J. (1975). Reproduction, growth and photosynthesis of gametophytes of *Laminaria saccharina* grown in blue and red light. *Mar. Biol.* 29: 195-200.
- Luning, K. and Neushul, M. (1978). Light and temperature demands for growth and reproduction of *Laminarian* gametophytes in southern and central california. *Mar. Biol.* 45: 297-309.
- Millner, P.A., Callow, M.E. and Evans, L.V. (1979). Preparation of protoplasts from the green alga *Enteromorpha intestinalis*. *Planta* 147: 174-177.
- Montomura, T. and Sakai, Y. (1984). Regulation of gametogenesis of *Laminaria* and *Desmarestia* (phaeophyta) by iron and boron. *Jap. J. Phycol.* 32: 209-215.
- Muallem, A., Bruce, D., Hall, D.O. (1983). *Biotechnol. Lett.* 5: 365.
- Nelson, N.A., Kelley, R.C., and Johnson, R.A. (1982). Prostaglandins and the arachidonic acid cascade *C&EN*, 60(30), 30-44.
- Polne-Fuller, M., and Gibor, A. (1990). Developmental studies in *Porphyra* (Rhodophyceae). III. Effect of culture conditions on wall regeneration and differentiation of protoplasts. *J. Phycology*, 26, 674-682.
- Polne-Fuller, M., and Gibor, A. (1984). Developmental studies in *Porphyra*. I. Blade differentiation in *Porphyra perforata* as expressed by morphology, enzymatic digestion, and protoplast regeneration. *J. Phycology*. 20: 609-616.
- Robinson, P.K., Dainty, A.L., Goulding, K.H., Simpkins, I., Trevan, M.D. (1985). *Enzyme Microb. Technol.* 7:212.
- Robinson, P.K., Mak, A.L., Trevan, M.D. (1986). Immobilized algae: a review. *Process Biochemistry*, August 1986. pp. 122-127.
- Saga, N. (1984). Isolation of protoplasts from edible seaweeds. *Bot. Mag. Tokyo* 97:423-427.

- Saga, N., and Sakai, Y. (1984). Isolation of protoplasts from *Laminaria* and *Porphyra*. Bull. Jpn. Soc. Sci. Fish., 50, 1085.
- Smith, G.D. and Lambert, G.R. (1981). Botechnol. Bioeng. 23:213.
- Steele, R.L., and Thursby, G.L. (1992). Protocol and original data sheets for kelp sexual reproduction test. U.S. EPA Laboratory, Hatfield Marine Science Center, Newport, OR.
- Steele, R.L., and Thursby, G.L. (1988). Laboratory culture of gametophytic stages of the marine macroalgae *Champia parvula* (Rhodophyta) and *Laminaria saccharina* (Phaeophyta). Environmental Toxicology and Chemistry. 7: 997-1002.
- Tait, M.I., Milne, A.M., Grant, D., Somer, J.A., Staples, J. Long, W.F., Williamson, F.B., and Wilson, S.B. (1990). *Porphyra* cell cultures: isolation, growth and polysaccharide production. Journal of Applied Phycology, 2, 63-70.
- Tang, Y. (1982). Isolation and cultivation of the vegetative cell of *Porphyra suborbiculata*. J. Shandong Coll. Oceanol. 12: 37-50.
- Waaland, J.R., Dickson, L.G., and Watson, B.A. (1990). Protoplast isolation and regeneration in the marine red alga *Porphyra nereocystis*. Planta 181: 522-528.

APPENDICES

Appendix A

Experimental Protocols

Porphyra perforata Disk Preparation

1. Obtain three recently frozen *Porphyra* fronds from -20 °C freezer.
2. Immediately place frozen fronds in 200 ml of sterile PES enriched seawater chilled to 0 °C on ice. This should thaw fronds.
3. Place thawed frond on a clean, ethanol wiped, plexiglass surface.
4. Flatten frond so only one thickness of the plant is resting on the plexiglass.
5. Using a clean, flame sterilized, cork borer 5 mm in diameter, cut 10 disks from the plant. This is most easily accomplished by tilting the borer at an angle and rolling it through its circumference.
6. Pat the disks dry with a Kimiwipe and weigh. This gives an average fresh weight for each disk. Record weight.
7. Cut 30 disks for each digestion to be performed.
8. Cut the disks in 10 count batches and remove them from the main plant.
9. Place freshly cut disks in sterile PES enriched seawater on ice.

***Porphyra perforata* Disk Sterilization**

1. Decant PES medium from freshly cut disks.
2. Add 50 ml of sterile deionized water to disks.
3. Sonicate disks in beaker for 30 seconds.
4. Decant deionized water being careful to retain disks in beaker.
5. In laminar flow hood, add 50 ml of sterile PES medium to disks.
6. Sonicate disks in beaker for 30 seconds.
7. In laminar flow hood, decant PES medium and replace with 50 ml of fresh, sterile, PES medium.
8. Sonicate disks in beaker for 30 seconds.
9. In laminar flow hood, decant PES medium.
10. Using a sterile 50 ml graduated cylinder, measure out 20 ml of fresh, sterile, PES medium.
11. To this add 2 ml of Betadine solution.
12. Add the 10% v/v dilution to the disks. Let the disks soak for 5 minutes, occasionally swirling the liquid during that time.
13. After 5 minutes, decant Betadine solution and wash disks with 30 ml of sterile PES medium.
14. Continue washing disks until there is no apparent brown color from the Betadine left. Then wash the disks twice more with sterile PES medium.
15. Place disks in 20 ml of antibiotic solution (Table[]) in a 100 x 15 mm plastic, sterile petri dish.
16. Seal dish with Parafilm.
17. Place dish in incubator at 12 °C, 2500 lux, 12L:12D photoperiod, for two days.

***Porphyra perforata* Enzyme Digestion**

1. In laminar flow hood, take thirty 5 mm disks from antibiotic solution, after two day incubation period, and place in 100 x 15 mm plastic, sterile petri dish.
2. Obtain clean utility knife and one clean safety razor blade for each digestion.
3. Using a pair of needle nose pliers, fit razor blade into utility knife.
4. Dip blade in 70% ethanol and flame sterilize.
5. Use blade to cut disks into strips by rocking the blade back and forth.
6. Cut again at a 90° angle. Continue in this manner until the tissue is cut into 1 mm² pieces.
7. Into a 60 x 15 mm sterile plastic petri dish, pour 5 ml of enzyme medium.
8. Using blade of utility knife, transfer cut pieces to the enzyme medium.
9. Seal petri dish with Parafilm and place on orbital shaker (70 rpm) under desired digestion conditions (diffuse light, 23 °C).
10. After incubation period, check digestion, using sterile technique, for protoplast release and viability.
11. If digestion is good, filter medium through a sterile 60 µm nylon mesh filter.
12. Wash retained pieces with 30 ml of sterile medium containing 0.4M sorbitol.
13. Transfer filtrate to a sterile, 50 ml, polypropylene centrifuge tube.
14. Centrifuge filtrate for 15 minutes at 1350 rpm.

15. Using a Pasteur pipet and sterile technique, withdraw liquid from the centrifuge tube, leaving only the pelleted protoplasts.
16. Resuspend pelleted protoplasts in growth medium containing 0.4M sorbitol.
17. Repeat washing procedure three times.
18. After washing, resuspend protoplasts in 4 ml of sterile medium containing 0.4M sorbitol. Transfer culture to a 60 x 15 mm sterile petri dish.
19. Take small sample for viability count on hemocytometer.
20. Seal petri dish with Parafilm and place under appropriate culture conditions. (12L:12D, 13 °C, 2500 lux, 70 rpm).
21. Step down sorbitol concentration in 0.2M increments daily.

***Aplysia* Enzyme Medium Preparation**

1. Feed *Aplysia* the targeted algae for at least one week prior to gut juice extraction.
2. Anesthetize animal by injecting 50 ml of 7 wt% MgCl_2 into the foot.
3. Place animal on back in dissection tray filled with seawater and surrounded by ice.
4. Pin head and tail to tray such that the animal is stretched out.
5. Cut animal open with scissors from tail to head.
6. Pin foot back displaying internal organs.
7. Locate gut and hepatopancreas.
8. Tie off gut with surgical thread and cut from body.
9. Cut open gut and drain stomach juice into 50 ml centrifuge tube.
10. Freeze juice at -70°C until needed.
11. To make enzyme medium first make 1 L of buffer solution. Solution is 25 mM Trizma Base and 25 mM MES in PES enriched seawater.
12. pH buffer solution to 6.5
13. Measure out 45 ml of buffer solution to a sterile 100 ml beaker with magnetic stir bar.
14. Chill buffer solution on ice and add 3.64 grams of sorbitol (0.4M).
15. Add 1.0 gram of Cellulysin cellulase (2 wt%) to the mixture.
16. Stir, covered, until components are dissolved.
17. Add 5.0 ml of *Aplysia* stomach juice to the mixture and transfer to centrifuge tube.
18. Centrifuge enzyme medium for 15 minutes at 5000 rpm.
19. Using Pasteur pipet, transfer enzyme medium from centrifuge tube to 115 ml sterilizing filter, containing glass wool as a pre-filter.

20. Filter sterilize the enzyme medium and decant into a sterile 100 ml beaker. Do this in Laminar flow hood.
21. Divide enzyme medium into 5.0 ml aliquots using a pipet. Transfer to clean, sterile, centrifuge tubes.
22. Seal tubes with aluminum foil and freeze at -20 °C until needed.

Acetone Powder Enzyme Medium Preparation

1. To a clean, sterile, 50 ml beaker add 5.0 grams of acetone powder.
2. Add 50 ml of culture medium (PES, GP2, etc..).
3. Mix on stir plate for 1 hour covered.
4. Decant liquid into sterile centrifuge tubes and centrifuge for 20 minutes at 2000 rpm.
5. Using a Pasture pipet transfer liquid to 8" of softened dialysis tubing tied at one end. Tubing is softened by boiling in 2.5 mM EDTA for two 30 minute intervals followed by boiling in deionized water for 30 minutes. Tie off other end of tubing.
6. Immerse filled tubing in a 1 liter Erlenmeyer flask filled with 4 °C Instant Ocean seawater (33 g /l).
7. Place flask with tubing on stir plate in 4 °C refrigerator overnight mixing constantly at low speed.
8. After ~16 hours, remove tubing from seawater and transfer liquid in tubing to a clean, sterile 100 ml beaker using a graduated cylinder. Take note of volume transferred.
9. Using volume transferred as a basis, calculate the quantities of sorbitol and Cellulysin needed to achieve 0.4M and 2 wt% respectively. Add these amounts and stir until dissolved.
10. At this point follow same procedure as *Aplysia* Enzyme Preparation #'s 18-22.

Cell Count and Viability

1. Using sterile Pasture pipet place two drops of culture to be examined on 1/2" x 1" piece of Parafilm.
2. Fill eye dropper with 0.33 wt% phenosafarin. Touch tip of dropper to Parafilm leaving a small drop of stain.
3. Use Pasture pipet to lift stain from the Parafilm by capillary action.
4. Inject stain into drop of culture. Add air bubbles to culture drop with pipet and stir droplet with tip of pipet until stain is evenly distributed throughout the drop.
5. Lift stained culture off of Parafilm with pipet and transfer to hemocytometer. Let stained culture move into hemocytometer through capillary action until chamber is full. Do not over fill.
6. Place hemocytometer on microscope stage and view culture under 100x magnification.
7. Count total number of protoplasts, dead (red) and viable (green), in five 1 mm² squares. Record each square separately.
8. Count total number of viable cells, they will be green (unstained), in the same 1 mm² squares. Record each square separately.
9. Repeat for a total of three samples per culture.
10. Calculate total number of protoplasts per ml by multiplying total count from all samples by $(1 \text{ square}/0.2 \text{ mm}^3) \times (1000 \text{ mm}^3/\text{ml}) \times (1/15 \text{ squares}) = 333.3 \text{ per ml}$.
11. Do the same for total viable count.
12. Calculate protoplast yield by dividing total viable/ml by grams of fresh tissue digested.

***Laminaria saccharina* Subculturing Procedure**

1. Select four maintenance cultures from a line of ten that have deep brown color, no contamination (microscopic analysis), and have no adherence to flask walls.
2. Filter cultures through 60 μ m nylon mesh filter.
3. Back wash gametophytes into blending vessel with 100 ml of sterile, complete GP2 medium.
4. Seal blending vessel and blend for 30 seconds on "liquefy" speed.
5. Keeping the freshly blended culture evenly suspended by swirling the blending vessel, pipet 10 ml of the culture into a 250 ml sterile flask containing 90 ml of sterile, fresh GP2 medium. Use a sterile, wide mouth pipet for this procedure.
6. Seal flask with sterile foam plug and place in incubator at 16L:8D photoperiod, 13 °C, 2500 lux, no agitation.
7. Repeat for each subculture, taking 3 subcultures from two flasks and 2 subcultures from the remaining two flasks.
8. Record subculture data.
9. Subculture should be performed after 3 weeks of growth.

Alginate Bead Cell Immobilization Procedure

1. Weigh out 4 grams of alginic acid (Sigma, A-2033) into a clean sterile 250 ml beaker. Add stir bar.
2. Cover beaker with aluminum foil and autoclave for 15 minutes.
3. In the Laminar flow hood, add 200 ml of GP2 base medium (no nutrients or carbonate or trace metals) containing no multivalent ions (no Ca^{+2} , Mg^{+2} , Sr^{+2}).
4. Mix on hot plate (setting 6), covered, for 30 minutes at $\sim 70^\circ\text{C}$ until alginic acid is dissolved.
5. Allow solution to cool to room temperature.
6. While solution is cooling mix casting solution.
7. Weigh out 44.07 grams CaCl_2 into a clean 100 ml beaker. Cover and autoclave for 15 minutes.
8. To 1 liter of complete GP2 medium (nutrients, metals, carbonate included) add the CaCl_2 . CaCl_2 will be liquid after autoclave.
9. Pour ~ 400 ml of the casting solution into a 500 ml beaker and mix on stirring plate at low speed.
10. After alginate solution has cooled to room temperature, blend two 3 week old maintenance cultures for 20 seconds on "liquefy" speed.
11. Filter blended cultures through a $20\ \mu\text{m}$ nylon mesh filter.
12. Transfer filtered gametophytes to mixing alginate solution with flame sterilized spatula.
13. After gametophytes are evenly distributed throughout the alginic acid solution, insert one end of 1/8" OD Tygon tubing into sterile deionized water.
14. Run other end of tubing through the peristaltic pump.

15. Use reducing union on end of tubing coming from pump to reduce the tubing size to 1/16". Run the 1/16" tubing through the stainless steel tubing support and suspend above slowly mixing casting solution ~4 in.
16. Dial pump to its highest setting and pump sterile water through the tubing for 5 minutes, catching the sterile water in a waste beaker.
17. Remove input end from water and allow line to clear.
18. Insert input end of tubing into alginate solution and cover.
19. Set pump to setting #6 and allow line to fill.
20. Make sure output is ~4 in. above casting solution.
21. Cast 10 beads then shut off pump.
22. Wipe output clean with Kimiwipe and start pump.
23. Count number of beads formed in 1 minute three times.
24. Record casting rate.
25. Calculate time to cast 200 beads.
26. Filter casting solution through 105 μ m nylon mesh filter to remove all beads and debris.
27. Wipe output with Kimiwipe and cast 200 beads into filtered casting solution using calculated time from above.
28. Shut off pump after casting 200 beads. Leave beads in slowly mixing casting solution for 10 minutes to cure.
29. Remove beads from casting solution by filtration through a 105 μ m nylon mesh filter.
30. Transfer beads to clean, sterile, 125 ml flask containing 20 ml of complete GP2 medium. Seal flask.
31. Place flask on orbital shaker (100rpm) in incubator at desired conditions.

Immobilized Cell Culture Sampling Procedure

1. Remove culture flask containing beads from incubator.
2. Decant culture medium into 30 ml vial for possible future use and place in freezer.
3. To the beads add 20 ml of 0.5M sodium citrate in GP2 medium via pipet.
4. Cover flask and place on orbital shaker (100rpm) at room temperature for 30 minutes or until beads have completely dissolved.
5. Remove, via pipet, 5.0 ml of the dissolved bead solution and filter on a 20 μ m nylon mesh filter.
6. Wash filtered cells with 20 ml of deionized water to remove citrate solution.
7. Fold filter and place in centrifuge tube. Repeat three times. Use chlorophyll absorbance protocol to work-up from here.

Dry Cell Weight Measurement

1. Dry three 47 mm, 0.45 μ m pore size, Millipore filters in oven at 70 °C overnight. Dry in covered 100 x 15 mm, marked, glass petri dishes.
2. Remove a filter from the oven in a hot dry glass covered petri dish and transport quickly to analytical balance.
3. Immediately remove filter from petri dish with forceps and place on balance.
4. Wait for balance reading to stabilize on one reading for a five second count. Record initial weight.
5. Do this for all three filters.
6. Place filter on filter stage and wet with deionized water.
7. Pipet 20 ml of culture sample onto filter.
8. Wash filter thoroughly with deionized water to remove any traces of salt. Be careful that cells do not run off of filter.
9. Remove filter from stage with forceps and transfer to marked glass petri dish.
10. Place in 70 °C oven. Watch carefully for first 30 minutes of drying, lifting filter from petri dish occasionally to prevent sticking. If filter does stick, rewet with small amount of deionized water and lift filter.
11. Repeat for each sample (3).
12. After drying overnight cover petri dish and remove from oven to analytical balance.
13. Quickly remove filter from petri dish and place on balance. Record final weight after balance has stabilized for 5 seconds.

14. Calculate weight of cells by subtraction.
15. Divide final weight by ml filtered to obtain dry cell weight per liter.

Chlorophyll *a* Assay: Sample Preparation

1. Obtain culture samples.
2. Place clean dry 20 μ m nylon mesh filter on filter stage.
3. Wash filter with ~20 ml of deionized water.
4. Filter 5.0 ml of culture through mesh.
5. Fold filter into 1/8 ths with forceps, forming a wedge shape.
6. Place filter in clean, dry, 50 ml, polypropylene, centrifuge tube with the point of the wedge touching the bottom of the tube.
7. Add 5.0 ml of HPLC grade methanol to the tube via a volumetric pipet.
8. Seal tube with polypropylene cap.
9. Vortex solution in tube for 30 seconds on speed 4.
10. Place vortexed solution in refrigerator at ~4 °C overnight in the dark.
11. Repeat for each sample taken. This may be done in parallel for each sample.
12. After the filters have soaked overnight in the methanol remove the tubes from the refrigerator.
13. Vortex solutions for 30 seconds on speed 4.
14. Solutions are now ready to be read on spectrophotometer.

Chlorophyll *a* Assay: Spectrophotometer Use

1. Turn spectrophotometer and tungsten lamp on. Allow the machine to warm-up for at least 20 minutes.
2. Turn on voltmeter.
3. Locate 10 mm path length cuvette.
4. Wash cuvette 4 times with fresh HPLC grade methanol.
5. Wipe optical path clean with a Kimiwipe.
6. Visibly inspect optical path of cuvette for any dirt or haze.
7. Load cuvette with ~4.0 ml of blank solution.
8. Make sure spectrophotometer is set at 665 nm wavelength.
9. Place cuvette in spectrophotometer.
10. Note the direction in which the mark on the optical face points. The cuvette must always be placed in this same orientation.
11. Close sample bay door and read absorbance from voltmeter.
12. For the blank adjust the absorbance to read +0.000 using the fine adjust knob.
13. Remove cuvette and wash 4 times with HPLC grade methanol.
14. Load next sample (~4.0 ml)
15. Close bay door and read absorbance.
16. Repeat for each sample. Blank adjustment need only be done at the beginning of a series of samples.

Dry Cell Density Vs. Absorbance Calibration Curve

1. Select 5 three week old maintenance cultures.
2. Blend cultures for 30 seconds at "liquefy" speed.
3. Filter the cultures through a 60 μm nylon mesh filter.
4. Add the filtered gametophytes to 1 L of fresh GP2 medium.
5. Add stir bar to the 1 L flask.
6. Keep gametophytes under continuous agitation while sampling.
7. Take two samples of 5 ml, 10 ml, 20 ml, 30ml.
8. Filter samples through a 20 μm nylon mesh filter and follow chlorophyll assay.
9. Take two samples of 20 ml, 30 ml, 60 ml, 100 ml.
10. Filter through 0.45 μm Millipore filter that has been pre-dried and weighed (dry cell weight procedure).
11. From dry cell weight measurements find average mg/L.
12. Plot absorbance versus dry cell weight.

Preparation of GP2 Medium

1. Refer to Table 4 in making this solution.
2. Obtain 8 liters of 18 megohm deionized water.
3. Place in autoclavable carboy.
4. Add stir bar and set carboy on stir plate.
5. Stir continuously while making base solution.
6. Weigh out on a top loading balance the following:

NaCl	168.2 grams
Na ₂ SO ₄	28.16 grams
KCl	4.88 grams
KBr	0.70 grams
Na ₂ B ₄ O ₇ •10H ₂ O	0.27 grams
MgCl ₂ •6H ₂ O	76.00 grams
CaCl ₂ •6H ₂ O	10.56 grams
SrCl ₂ •6H ₂ O	0.16 grams
7. Add components to mixing 8 liters of deionized water.
8. Allow to mix over night before use.
9. After 24 hours, autoclave 121 °C, 15 psig for 30 minutes.
10. Mix up stock trace metal solutions individually.
11. For each metal mix up 500 ml of stock solution. The weights are shown below (weigh on analytical balance).

Na ₂ MoO ₄ •2H ₂ O	120.0 mg/500ml
KI	420.0 mg/500ml
ZnSO ₄ •7H ₂ O	112.0 mg/500ml
Na ₃ VO ₄	48.0 mg/500ml
MnCl ₂ •4H ₂ O	30.4 mg/500ml

12. Mix vitamin stock solution in 500ml batch.
13. To 500 ml of deionized water add the following:

Thiamine-HCl	2500.00 mg/500ml
B ₁₂	1.25 mg/500ml
Biotin	1.25 mg/500ml
14. Mix citrate stock solution by adding 1.03 g of sodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$) to 100 ml of deionized water.
15. Mix stock nutrient solution.
16. Add the following to make a final volume of 1 liter nutrient stock solution:

NaNO_3	12.70 grams
$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	1.28 grams
Citrate Stock Solution	10.0 ml
Metal Stock Solution (5 metals)	10.0 ml each
Vitamin Stock Solution	10.0 ml
17. Make up to 1 liter with deionized water.
18. Filter sterilize nutrient stock solution.
19. Make up bicarbonate stock solution by adding 34 grams of sodium bicarbonate to 500 ml of deionized water. Autoclave for 20 minutes (212 °C, 15psig).
20. Make final GP2 medium by adding (in flow hood):

Nutrient Stock Solution	5.0 ml
Bicarbonate Stock Solution	5.0 ml
• Base Solution	Add to 1 L
21. Solution is now ready for use and sterile.

Provasoli's Enriched Seawater (PES) Medium Preparation

1. Prepare the following stock solutions in deionized water:

<u>Component</u>	<u>Amount/100ml</u>
NaNO ₃	35.0 grams
Na ₂ glycerophosphate	5.0 grams
B ₁₂	1.0 milligrams
Thiamine	50.0 milligrams
Biotin	0.5 milligrams

2. Vitamin solutions may be made as one solution. Do not autoclave the vitamin solution.
3. Make up the PII trace metal stock solution. It consists of:

<u>Component</u>	<u>Amount/500ml</u>
H ₃ BO ₃	0.57 grams
FeCl ₃ •6H ₂ O	24.50 milligrams
MnSO ₄ •H ₂ O	62.10 milligrams
ZnSO ₄ •7H ₂ O	11.00 milligrams
CoSO ₄ •7H ₂ O	2.40 milligrams
Na ₂ EDTA	0.50 grams

4. Make up chelated iron solution. It consists of:

<u>Component</u>	<u>Amount/500ml</u>
Fe(NH ₂) ₂ (SO ₄) ₂ •6H ₂ O	351.0 milligrams
Na ₂ EDTA	300.0 milligrams

5. Now lastly make up pH correcting solution. It consists of 0.1 grams of Trizma Base in 3 ml of deionized water.
6. You should now have 8 stock solutions.
7. To a 2 liter Erlenmeyer flask add the following:

<u>Stock Solution</u>	<u>Amount</u>
NaNO ₃	10.0 ml
Na ₂ glycerophosphate	10.0 ml
B ₁₂	10.0 ml
Thiamine	10.0 ml
Biotin	10.0 ml
PII Trace Metals	250.0 ml
Chelated Iron	250.0 ml

8. Bring volume to 1250 ml total with deionized water. This is the base media concentrate.
9. Adjust pH with dropwise addition of pH correcting solution. Final pH should be 8.0.
10. To prepare PES medium add 20 ml of the base media concentrate to 1 liter of filtered seawater.
11. Final concentrations of species can be found in Table [].

Bubble Column Bioreactor Set-up

1. Thoroughly wash glass reactor body and headplate with Liquinox detergent.
2. Wash all clamps, hoses, and fittings.
3. Rinse all pieces with tap water ten times. Make sure there is no soap residue left.
4. Rinse all pieces with deionized water ten times.
5. Place all rinsed items in an oven (70 °C) for 3 hours to dry.
6. Remove glass body from dryer and place in ring stand. Allow to cool to room temperature in a hood.
7. Wearing rubber surgical gloves, remove cap from Sigmacote silanizing agent. **Do this in the hood. Agent is very toxic.**
8. Add ~10 ml of the agent to the reactor. This is should be enough agent to silanize two reactors. Use sparingly.
9. Wet all interior glass surfaces with the agent.
10. Remove any excess and allow to air dry in the hood overnight.
11. Rinse reactor ten times with deionized water to remove any trace of the silanizing agent.
12. Assemble the reactor. Headplate, hoses, filters, clamps (sample port and air inlet).
13. Autoclave reactor and humidifying assembly for 20 min (15 psig, 212 °C).
14. Remove from autoclave and allow to cool to room temperature in laminar flow hood.

Bubble Column Bioreactor Inoculation

1. After sterilization, bioreactor is ready for inoculation.
2. Carefully remove headplate from reactor. Be sure to keep all surfaces in contact with the culture sterile.
3. Using a sterile graduated cylinder, add 250 ml of GP2 medium to the reactor.
4. Chose an inoculum source from existing 1 month old maintenance cultures. Criteria outlined under subculturing procedure.
5. Blend inoculum for 20 seconds on "liquefy" speed.
6. Pipet 30 ml of the inoculum into the reactor using a sterile 10 ml graduated pipet.
7. Seal headplate to reactor body and place in stand in incubator.
8. Connect air hoses from humidifier.
9. Connect exhaust hoses to reactor.
10. Turn on air pump and unclamp air inlet.
11. Set flowrate. If air bubbles are not seen check hoses for kinks. Check that humidifier is sealed.
12. Turn on lights and check intensity/photoperiod.

Bubble Column Bioreactor Sampling

1. Unclamp hose leading from sampling port.
2. Withdraw 10 ml from the reactor using a syringe fitted to the
 Leur-lok connector.
3. Clamp the sampling hose.
4. Remove syringe from connector.
5. Place sample in plastic bottle.
6. Remove 5.0 ml of the sample with a graduated pipet. Be sure to
 keep the sample agitated.
7. Filter the sample and work-up as outlined in the chlorophyll *a*
 procedure.

Bubble Column Bioreactor Shut-Down

1. On the last sampling day, clamp air inlet hose.
2. Remove bioreactor from incubator and place in ring stand.
3. Remove headplate.
4. Empty contents of bioreactor into a 250 ml flask.
5. Blend left over culture for 20 seconds on "grind" speed.
6. Take last 5.0 ml samples and work-up as per chlorophyll assay.
7. Take three 20 ml samples and work-up as per dry cell density procedure.
8. Disassemble bioreactor and wash all pieces in Liquinox detergent.
9. Rinse all pieces in deionized water.
10. Store pieces in deionized water until next run.

Sample Calculations

1. Viable Cell Concentration (C_{VI})

$$\begin{aligned} \#_{VI} &= \# \text{ viable cells in 15 squares} \\ &\quad \text{(three samples 5 squares each)} \\ \text{square} &= 1 \text{ mm}^2 \text{ outline on hemocytometer} \\ C_{VI} &= \text{Viable cell concentration} \end{aligned}$$

$$C_{VI} = (\#_{VI}) \times (1/15 \text{ squares}) \times (1 \text{ square} / 0.2 \text{ mm}^3) \times (1000 \text{ mm}^3/\text{ml})$$

2. Protoplast Yield (P_Y)

$$(C_{VI}) \times (1/\text{grams fresh weight digested})$$

3. Dry Cell Density (DCD)

$$DCD = [(\text{final filter weight}) - (\text{initial filter weight})]/(\text{liters filtered})$$

4. Growth Rate

Zero Order:

$$\text{Rate} = \text{Slope of Chl } a \text{ vs. Time} = \mu\text{g Chl } a/\text{day}$$

First Order:

$$\begin{aligned} \text{Rate} &= \text{Slope of linear section on semi-log plot} \\ &= 1/\text{day} \end{aligned}$$

Appendix B

Raw Data

Static Flask Raw Data

Static Flask Run #3-LG

DAY	ABS(665n	Devlatio	linear	Chl a ug/ml
0	0.063	0.005	1.108393	1.02627
3	0.085	0.005	1.354626	1.38465
8	0.086	0.002	1.765015	1.40094
14	0.163	0.023	2.257481	2.65527
21	0.194	0.007	2.832026	3.16026
26	0.188	0.005	3.242415	3.06252
29	0.213	0.004	3.488648	3.46977
32	0.23	0.005	3.734881	3.7467
39	0.257	0.007	4.309425	4.18653

Static Flask Run #1

DAY	ABS(665n	Devlatio	Linear	Chl a ug/ml
0	0.021	0	0.81382	0.34209
10	0.096	0.014	1.559573	1.56384
14	0.127	0.032	1.857874	2.06883
17	0.167	0.019	2.0816	2.72043
23	0.139	0.011	2.529052	2.26431
29	0.206	0.041	2.976503	3.35574
35	0.199	0.006	3.423955	3.24171
39	0.211	0.003	3.722256	3.43719
43	0.245	0.005	4.020557	3.99105
50	0.412	0.008	4.542584	6.71148

Regression Output: #3-LG

Constant	1.108393
Std Err of Y Est	0.254707
R Squared	0.956574
No. of Observations	9
Degrees of Freedom	7
X Coefficient(s)	0.082078
Std Err of Coef.	0.00661

Regression Output: #1

Constant	0.81382
Std Err of Y Est	0.378797
R Squared	0.901343
No. of Observations	9
Degrees of Freedom	7
X Coefficient(s)	0.074575
Std Err of Coef.	0.009325

Alginate Bead Data

DAY	samp1	samp 2	samp 3	average	Chl a ug/ml
0	0.044	0.047	0.048	0.046333	0.75477
2	0.036	0.046	0.034	0.038667	0.62988
4	0.028	0.028	0.03	0.028667	0.46698
6	0.027	0.026	0.026	0.026333	0.42897
8	0.019	0.02	0.022	0.020333	0.33123
14	0.02	0.018	0.018	0.018667	0.30408
17	0.016	0.015	0.015	0.015333	0.24978
19	0.024	0.019	0.021	0.021333	0.34752
21	0.016	0.016	0.016	0.016	0.26064
23	0.012	0.012	0.01	0.011333	0.18462

Regression Output:

Constant			0.03744
Std Err of Y Est			0.005514
R Squared			0.776559
No. of Observations			10
Degrees of Freedom			8
X Coefficient(s)		-0.00115263	
Std Err of Coef.		0.000218594	

Comparison of Bioreactors Raw Data

DAY	Abs (665nm)B #1	Abs (665nm)B #2	B#1 Chl a ug/ml	B#2 Chl a ug/ml	Ln (b1)	Ln(b2)
0	0.026	0.033	0.42354	0.53757	-0.85911	-0.6207
2	0.04	0.045	0.6516	0.73305	-0.42832	-0.3105
4	0.038	0.05	0.61902	0.8145	-0.47962	-0.2052
6	0.066	0.072	1.07514	1.17288	0.072451	0.15946
8	0.151	0.175	2.45979	2.85075	0.900076	1.04758
10	0.177	0.259	2.88333	4.21911	1.058946	1.43962
12	0.2555	0.2165	4.162095	3.526785	1.426019	1.26039
14		0.1745		2.842605	ERR	1.04472
16		0.315		5.13135	ERR	1.63537
18		0.19		3.0951	ERR	1.12982
20	0.326	0.381	5.31054	6.20649	1.669694	1.8256

Regression Output: Bioreactor #1

Constant	-1.5171967732
Std Err of Y Est	0.20400677954
R Squared	0.94680362496
No. of Observations	4
Degrees of Freedom	2
X Coefficient(s)	0.272165791
Std Err of Coef.	0.045617303

Regression Output: Bioreactor #2

Constant	-1.4275153247
Std Err of Y Est	0.16149651038
R Squared	0.970146225
No. of Observations	4
Degrees of Freedom	2
X Coefficient(s)	0.291126751
Std Err of Coef.	0.036111718

Bicarbonate Effect Data

DAY	bio #1 (0 mg/l bi)	bio#2 (680 mg/l bi)	bio #1 chl a ug/ml	Ln(Chl a)	bio #2 chl a ug/ml	Ln(Chl a)
0	0.031	0.041	0.50499	-0.68322	0.66789	-0.4036
2	0.061	0.057	0.99369	-0.00633	0.92853	-0.0742
4	0.099	0.098	1.61271	0.477916	1.59642	0.46776
6	0.146	0.147	2.37834	0.866403	2.39463	0.87323
7	0.201	0.177	3.27429	1.186101	2.88333	1.05895
8	0.299	0.251	4.87071	1.58324	4.08879	1.40825
9	0.34	0.26	5.5386	1.711742	4.2354	1.44348
10	0.352	0.269	5.73408	1.746427	4.38201	1.47751
11						
12						
13		0.292			4.75668	
14						
15						
16						
17						
18						
19						
20	0.437	0.355	7.11873		5.78295	

Regression Output Reactor 1 (0.0 mg/L bicarb)	
Constant	-0.57639922491
Std Err of Y Est	0.116204124802
R Squared	0.985048026325
No. of Observations	8
Degrees of Freedom	6
X Coefficient(s)	0.2498581687717
Std Err of Coef.	0.0125671988444

Regression Output Reactor 2 (680 mg/L bicarb)	
Constant	-0.39289650725
Std Err of Y Est	0.107837944706
R Squared	0.98080995676
No. of Observations	8
Degrees of Freedom	6
X Coefficient(s)	0.2042295715083
Std Err of Coef.	0.011662416428

Calibration Curve Data

ML sample	ABS (665nm)	Corrected ABS	Average ABS	dry wt. (mg)
5	0.073	0.033	0.026	0.341
5	0.059	0.019		0.341
10	0.16	0.12	0.117	0.682
10	0.154	0.114		0.682
20	0.343	0.303	0.218	1.364
20	0.173	0.133		1.364
30	0.553	0.513	0.5275	2.046
30	0.582	0.542		2.046
60	1.34	1.3	1.32	4.092
60	1.38	1.34		4.092

ML sample	Chl a (ug)	ugchl A/mg DCW	DCW (mg)	Chl a (ug)
5	2.118	6.210	0.341	2.118
5			0.682	9.53
10	9.530	13.973	1.364	17.756
10			2.046	42.965
20	17.756	13.018	4.092	107.514
20				
30	42.965	20.999		
30				
60	107.514	26.274		
60				
Average---->		16.095		

Regression Output:		linear plot
Constant	0.4676417021	0.54048696474591
Std Err of Y Est	0.2234450222	0.79541099046769
R Squared	0.9830511486	1.078331259705
No. of Observations	5	1.9453549731994
Degrees of Freedom	3	4.1654158118819
X Coefficient(s)		0.034393419552
Std Err of Coef.		0.002607334848