AN ABSTRACT FOR THE THESIS OF

Hanshi Qi for the degree of Master of Science in Chemical Engineering presented on April 22, 1994.

Title: Cultivation of *Laminaria saccharina* Gametophyte Cell Cultures in a Stirred-Tank Photobioreactor

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Callus tissue from *Laminaria setchellii* and female gametophytes from *Laminaria saccharina* were considered for development of macroalgal liquid suspension cell cultures.

Filamentous *Laminaria setchellii* callus tissue was induced on seawater solid agar medium (15 g/l) at 8 °C in the dark. About 5% of the explants contained dense callus filaments six weeks after plating. The callus tissue liquid culture was then attempted, but no quantitative growth was observed and most cells were not viable after two months.

Stirred-tank photobioreactors were used to cultivate photolithotrophic liquid suspension cell cultures of *Laminaria saccharina* female gametophytes. Cultivation was repeatable in terms of growth rate and final cell density.

The effect of incident illumination intensity on culture growth was studied. Incident light intensities ranging from

1500 to 19,000 lux resulted in specific growth rates of 0.068 to 0.147 day^{-1} and final cell densities of 626 to 890 mg DCW/l respectively in the stirred-tank bioreactor cultivations. Therefore, incident light intensity was a limiting factor in the stirred-tank bioreactor cultivation of *Laminaria saccharina* gametophytes. A maximum specific growth rate (μ_{max}) of 0.154 day^{-1} and a half-saturation constant (K_0) of 1920 lux were determined to correlate the growth rates (μ) to incident light intensity (I_0) in a light limited growth model.

Photosynthetic oxygen evolution rates (P_0), respiration rates (Q_0) and mass transfer coefficients (k_La) of the culture during cultivation period were determined through dissolved oxygen (D.O.) concentration vs. time measurements. The photosynthetic oxygen evolution rates increased during exponential phase, reached a maximum value of 0.08 mg O2/l-min at the 8th day near the end of exponential phase, then decreased during stationary phase. The CO_2 consumption rates and the maximum CO_2 transfer rates were then estimated using oxygen evolution rates and the volumetric oxygen mass transfer coefficients respectively. An analysis of CO_2 consumption and CO_2 transfer rates indicated that the cultures in the stirred-tank bioreactor were not CO_2-limited.

Cultivation of *Laminaria saccharina* Gametophyte Cell Cultures in a Stirred-Tank Photobioreactor

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NOMENCLATURE

C_A dissolved oxygen concentration, mmol O_2/L

C^*_A dissolved oxygen concentration at saturation, mmol O_2/L

C_{CO2} dissolved CO_2 concentration, mmol CO_2/L

C^*_{CO2} dissolved CO_2 concentration at saturation, mmol CO_2/L

I_0 incident cultivation light intensity, lux

K_0 half saturation constant, lux

k_La mass transfer coefficient in culture, 1/hr

P_0 photosynthetic oxygen evolution rate, mmol O_2/L-hr

$P_0\hat{}$ photosynthesis rate, mmol O_2/L-hr

Q_0 respiration rate, mmol O_2/L-hr

r_{CO2} CO_2 consumption rate, mg CO_2/L-hr

t cultivation time, hr

X_i inoculate cell density, g DCW/L

X_f final cell density, g DCW/L

Greek letters

μ specific growth rate, 1/hr

μ_{max} maximum specific growth rate, 1/hr

Cultivation of *Laminaria saccharina* Gametophyte Cell Cultures in a Stirred-Tank Photobioreactor

Introduction

Plants have for a very long time been of great importance not only as food sources but also as a supply of a wide range of chemicals including pharmaceuticals, insecticides, flavors, fragrances and colors. Even with the advances in microbial and chemical production methods plants are still a source of compounds which are too complex or expensive to produce in any other way (Morris et al., 1985). One of the huge plant families is marine algae which contains some 1800 genera and 21,000 species (Alexopoulos et al., 1967) and lives in the oceans covering approximately 71% of our earth's surface (McConnaughey et al., 1989). In the marine environment, inter-species competition for space, nutrients, and light is fierce, and marine plants have evolved unique chemical defense mechanism to enhance survival or ward off predators. These chemical defense mechanisms are often the source of structurally unique natural products (Gerwick, 1987).

One of the very important groups of natural metabolic products to find wide distribution in the marine algae are eicosanoids, which are 20-carbon fatty acids with at least one site of oxidation in addition to the carboxyl group. Recently, a metabolic pathway known as the arachidonic acid cascade has been discovered in some red and brown macroalga on the Oregon coast (Gerwick et al., 1990). In the arachidonic acid cascade, arachidonic acid is enzymatically oxidized to form eicosanoids. These important bioactive compounds contain novel structural features which represent unique analogs of physiologically important mammalian eicosanoids and have the ability to inhibit the mammalian Na+/K+ and H+/K+ ATPase enzymes, modulate the release of superoxide anion by neutrophils, and inhibit the metabolism of arachidonic acid in human cell lines (Gerwick et al., 1993). In fact, many commercial drugs are manufactured from the eicosanoids which include prostaglandins, thromboxanes, prostacylin, and leukotriene (Nelson et al., 1982).

Traditionally, there are two ways of approaching the manufacture of natural products from plants. The first is to attempt the chemical synthesis of the desired biocompounds. This approach is very difficult. The extremely complicated structure and stereochemistry of the target metabolite biocompounds have to be fully understood and controlled in order to use simpler precursor chemicals to organically synthesize those biocompounds. In addition,

chemical synthesis of natural biocompounds usually requires expensive precursors and has low yield. This often makes the approach economically prohibitive. Therefore, despite substantial advances in modern synthetic organic chemistry, many secondary metabolic compounds are either too difficult or too costly to synthesize.

The second traditional approach is to farm the macroalgae that produce the natural products in costal ocean waters and then extract the desired products from the harvested algae by both physical and chemical means. Although this approach is industrially feasible for some natural products such as polysaccharides, most secondary metabolites are in trace concentrations within the intact macroalgal plant. For example, eicosanoids typically constitute less than 5% of extractable lipids (Gerwick et al., 1990), so that extraction of these secondary metabolites is very difficult and economically prohibitive.

A new approach for producing marine plant secondary metabolites is to cultivate macroalgae as cell suspension cultures in medium within a bioreactor under controlled conditions. This approach has several significant advantages compared to the traditional ones. First, marine plant cells can be cultivated anywhere without restriction of weather or geographical conditions. Second, the product quality and yields can be controlled and optimized by manipulating the bioreactor cultivation parameters, such as light intensity,

photoperiod, temperature, medium composition, inoculum source, etc. Third, some secondary metabolites can be produced in much higher quantities in suspension culture than that in the whole plant. Secondary metabolites seem to be produced only in certain environments which are sometimes very different from their natural environment. The culture environment can be easily controlled in a bioreactor.

However, the cultivation of plant cells on a large scale and to maximize the production and accumulation of secondary metabolites is still a challenge today. Plant cells are more difficult to cultivate than microbial cells (Lee, 1992), and techniques for marine plant cell culture lag far behind those of terrestrial plant cell culture (Kawashima et al., 1990). There are no published reports on the cultivation of macroalgal cell suspension cultures in bioreactors under controlled process conditions.

Traditionally, plant cell suspension cultures are established by callus induction techniques. Thus, an attempt will be made to establish a macroalgal cell suspension culture from callus tissue of the brown macroalga *Laminaria setchellii*. If this attempt is not successful, then an alternative technique will be used for brown macroalga of order Laminariales.

Although unstudied, gametophytes of certain brown macroalgae may serve as a suitable macroalgal cell suspension for bioreactor cultivation. Particularly, some brown

macroalgae of order Laminariales possess a reduced gametophyte life phase which approximate the properties of liquid cell suspension. The female gametophytes from the brown alga *Laminaria saccharina* are of particular interest because the parent plant is known to produce 15-lipoxygenase metabolites (Gerwick et al., 1993). These cultures are photolithotrophic and possess a clumped and filamentous morphology. It is possible that gametophytes can be cultivated in a well-mixed stirred-tank bioreactor. However, illumination must be supplied to the bioreactor to promote photolithotrophic growth, and an external CO_2 source (such as CO_2 in air) must be sparged into the culture. Therefore, in order to characterize the bioreactor *Laminaria saccharina* gametophyte cultivation, the specific objectives of this study are to

(1) Develop an illuminated stirred-tank bioreactor for macroalgal gametophyte cell suspension culture;

(2) Study effect of illumination condition on culture growth and biomass productivity in the stirred-tank bioreactor;

(3) Measure photosynthetic oxygen release as a tool to study the relationship between photolithotrophic culture growth and CO_2 transfer rate.

LITERATURE REVIEW

This review focuses on three areas: 1) callus induction and culture of marine macroalgae, particularly brown macroalgae; 2) development of gametophyte cell cultures from brown macroalgae, particularly of genus Laminaria; 3) photolithotrophic cultivation of microalgae in illuminated bioreactors.

Callus Induction and Cultures of Marine Macroalgae

Unorganized cells formed around plant tissues in response to injury are called callus tissues. Some callus masses are soft, whereas others are of harder consistency. All callus tissues form lumps of undifferential cells which represent an abnormal growth form of the plant (Polne-Fuller et al. 1987). Studies on callus cultures derived from marine plants (seaweed) have a much shorter history than those of terrestrial plants (Kawashima et al. 1990). In fact, studies of seaweed callus have only been reported in the last decade (Polne-Fuller et al. 1987).

Callus tissue culture is a convenient tool for studying the breeding, genetics, physiology and biochemistry of seaweeds (Kawashima et al. 1990). In recent years, several

investigators have reported induction of callus tissue from red, brown, and green algae.

Fries (1980) reported that a colorless callus-like tissue grew out from explant pieces of L. digitata and L. hyperborea which had been sterilized for 30 to 60 seconds in hypochlorite solution. All the explant pieces were made from meristematic basal zone of blade tissue. The explants were placed on agar plates made from the artificial seawater ASP6-F2 medium, solidified with 6 g/L agar. After 6 weeks, colorless callus tissue grew out from about 20% of the explant pieces.

Studies on macroalgal callus induction have been conducted only with isolated cells or callus-like tissues obtained spontaneously (Saga et al. 1982) until Saga et al (1982) presented a method for callus induction from the brown alga Dictyosiphon. Callus tissue was induced on ASP 12-NTA solid medium supplemented with 3% mannitol, 0.1% yeast extract and 1.5% agar. The addition of auxins and kinetin to the medium did not show any effect on the formation and growth of the callus.

However, some plant growth hormones are known to stimulate callus induction (e.g., Fries, 1980; Yan, 1984; Liu and Kloareg, 1991). Yan et al. (1984) considered the synthetic plant hormone C-754 (sodium naphthenate). Depending on the concentration, the hormone could induce either callus formation or totipotency of the somatic cells

of the brown algae *Laminaria japonica* and *Undaria pinnatifida*. The best medium was an improved MS medium containing 0.5 mg/L vitamin B2, and 5 mg/L of the hormone. The experiment was conducted at 10-15 ^{0}C and irradiance of 2.9 W/m^2 and photoperiod of 8:16 h (L:D).

From Gusev et al. (1987) obtained callus formation from seven species of agarophyte marine algae, including *Phullophora nervosa*, *Gracilaria verrucosa*, *Furcellaria fastifiata*, *Ceramium kondoi*, *Gelidium vagum*, *Laurencia paniculata*, and *Rhodymenia pertusa*. A new sterilizing agent, chlorhexidin bigluconate, was used to for preparation of axenic explants from thalli tissue. A medium containing macro- and microsalts according to Murashige and Skoog (1962), with the addition of sucrose or mannitol (20 g/L), yeast extract (1 g/L), Na_2 glycerophosphate (10 mg), NaCl (24 g/L), and agar (9 g/L) was used to obtain the callus. The pH of the medium was between 7.6 and 8.0.

Using the brown alga *Ecklonia radiata*, Lawlor et al. (1989) investigated the effects of light and irradiance, organic carbon supply, and vitamin supply on the growth of unpigmented and pigmented callus cells. The two different growth forms varied in their response to light. The growth of unpigmented callus cells was best in the dark but stopped after 10 weeks. The pigmented callus cells formed under illuminated culture conditions, with growth being enhanced by increasing the photon flux density up to 30 μEs/m_2-sec,

with the active spectral component being red light (>600 nm). The addition to the medium of a range of organic carbon sources or vitamins did not stimulate growth of either culture type.

Kamashima et al. (1990) studied callus induction from the brown alga *Ecklonia cava* using six media under several culture conditions. Stipe and meristem explants both developed callus tissue three to six weeks after inoculation on five of the six media. Callus developed on the stipe explants but did not develop on meristem explant at a temperature of 23 ^{0}C. Temperatures from 8 to 13 ^{0}C were favorable for callus development. Callus development on the meristem explants required light, but callus development on the stipe explants did not.

Although many investigators have studied the effects of medium composition, plant growth hormones, and environmental conditions on callus induction and culture from marine macroalgae, quantitative studies on macroalgal callus induction and culture are seriously lacking.

Brown Macroalgal Gametophyte Cultures

The lifecycle of macroalga *Laminaria saccharina* is diagrammed in Figure 1. A highly differentiated diploid sporophyte plant stage and a relatively undifferentiated haploid gametophyte stage form a cycle life history for

Laminaria saccharina. The diploid sporophyte stage releases spores from the sporangia. These spores then develop into either male or female gametophytes. The male gametophytes generate sperm from the antheridiom and the female gametophyte generates one egg on the oogonium. Once the sperm fertilize the eggs, the sporophyte stage starts once again.

Steele et al. (1988) isolated *Laminaria saccharina* gametophytes from sporophytes. The gametophytes were cultured successfully in GP2 artificial seawater medium at a temperature of 12-15 ^{0}C. The GP2 medium contains no iron. This lack of iron inhibits gametogenesis and egg formation, and thus provides faster vegetative growth (Montomura and Sakai, 1984).

Luning and Neushul (1978) studied the light and temperature demands for growth and reproduction of Laminarian gametophytes. They reported that vegetative growth leading to the formation of filamentous gametophytes was light-saturated at an illuminance of 1000 lux. Different temperature optima were found for Laminarian gametophytes depending on the location of their habitat. For example, optimal temperature was 12 ^{0}C for gametophytes from central California versus 17 ^{0}C for the gametophytes from southern California.

Luning (1980) cultivated gametophytes of three Laminaria species, including *Laminaria saccharina*, in artificial 12:12

Life Cycle of Laminaria

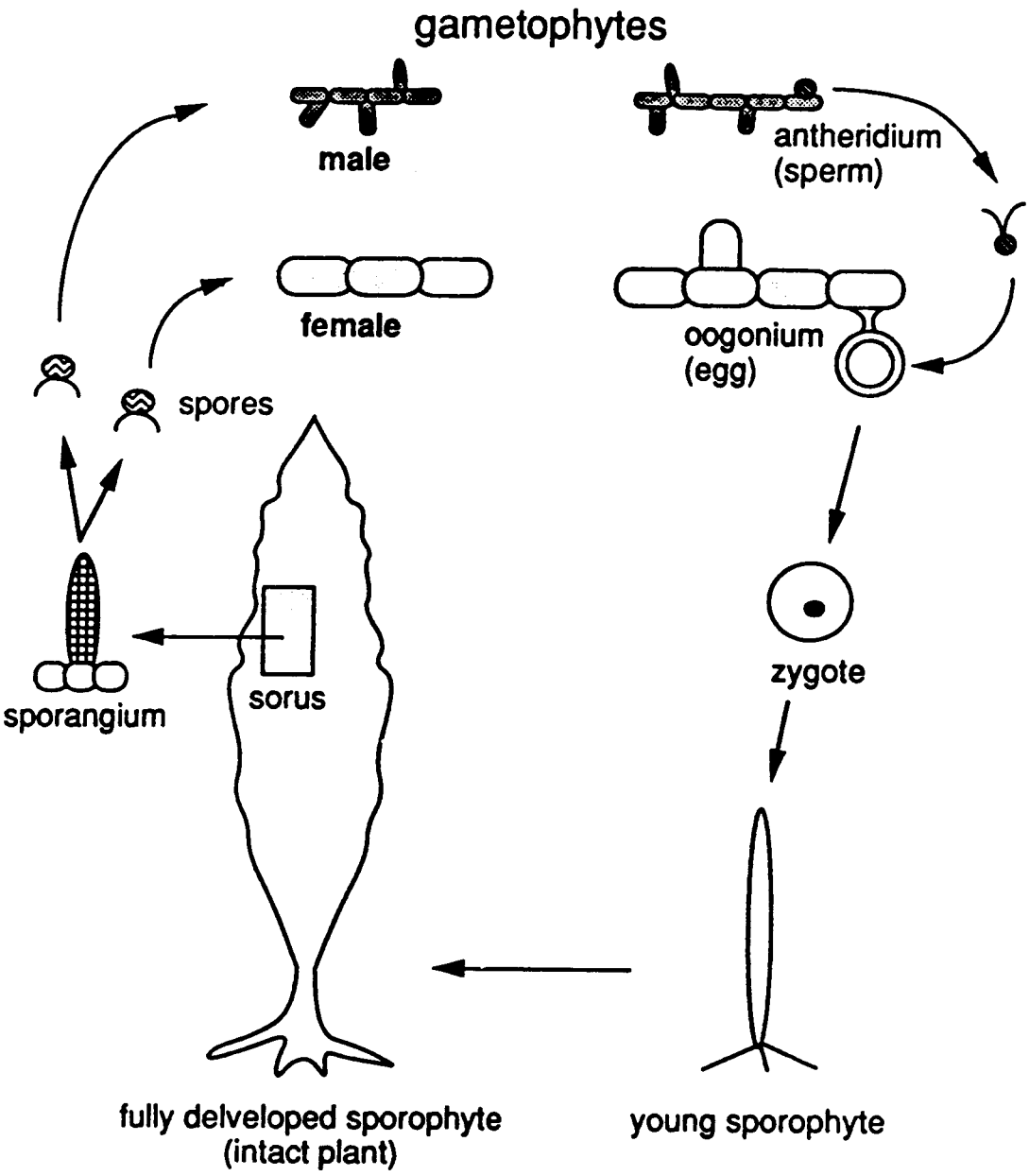


Figure 1. Life cycle of macroalga *Laminaria saccharina*.

LD light fields at different temperatures (from 2 ^{0}C to 21 ^{0}C). The artificial light fields simulated different spectral distributions. The results showed that the rate of vegetative growth did not depend on spectral distribution, and was light-saturated at 4-6 W/m^2. However, the growth rate increased with increasing temperature up to 15 ^{0}C, and then decreased from 15 ^{0}C to 21 ^{0}C (at 22 ^{0}C, the gametophytes died). The cultivation *Laminaria saccharina* exhibited the highest tolerance toward temperature, light intensity and UV spectral radiation. Gametophytes survived in the dark winter months with almost no vegetative growth due to lack of light.

Temperature acclimation of respiration and photosynthesis in the sporophytes of the brown alga *Laminaria saccharina* was investigated by Davison et al. (1991). Sporophytes grown at 15 ^{0}C contained significantly more chlorophyll a (chl a) than did similar sporophytes grown at 5 ^{0}C. The increase in chl a within sporophytes cultivated at 15 ^{0}C were associated with increased photosynthetic efficiencies and reduced respiration rates. The light intensity necessary to reach the compensation point and the light-saturated photosynthesis (I_c and I_k, respectively) were both significantly higher in sporophytes grown at 5 ^{0}C than at 15 ^{0}C.

Photobioreactor Microalgae Cultivation

Microalgae are the micro-organisms which dominate the open waters of the oceans and large lakes (Geider, 1992). There is significant interest in developing high-density microalgal cultures to produce high-value products, such as pharmaceuticals and genetically-engineered products. These include antibacterial, antiviral, antitumor/anticancer, antihistamine, and many other biologically valuable products (Javanmardian et al., 1991). The increased interest in the commercial exploitation of microalgae has led to a requirement for more efficient, economical and controllable algal biomass production systems (Benemann, 1989), such as photobioreactor systems. One of the main objectives of all types of photobioreactor systems is to improve the illumination condition of microalgal cultivations.

Previous photobioreactor studies for microalgae provide the technical basis for new bioreactor studies with macroalgal cell cultures. This review focuses on photobioreactor microalgae cultivation, particularly effect of light intensity on microalgae culture growth.

Almost all microalgae are photosynthetic marine microorganisms. Light energy is an important factor in photosynthetic microalgae cultures, especially in high-density microalgal cell suspensions. Several studies have showed that light intensity affected growth of microalgal

cultures. The cultivation of marine microalga *Isochrysis galbana* Parke under different illumination conditions was studied by Grima et al. (1992). The study showed that culture growth rate by *Isochrysis galbana* increased linearly with increased light intensity up to 124 W/m^2, followed by saturation effect up to 376 W/m^2, while higher intensities inhibited photosynthesis. A specific growth rate of 0.021 h^{-1} was obtained at light intensity of 56 W/m^2 while 0.032 h^{-1} was obtained at the saturation light intensity. Dermoun et al. (1991) cultivated unicellular red alga *Porphyridium cruentum* under non-nutrient-limited conditions and studied the effects of several growth parameters including light intensity were reported. Dermoun et al. showed that the growth rate depended on light intensity at temperatures ranging from 5 ^0C to 35 ^0C. For each temperature, the growth rate increased with increasing of light intensity up to a maximum value (at light intensity of about 400 μmol photon/m^2s), then decreased. The report also showed that the effects of light intensity and temperature on growth of *Porphyridium cruentum* were closely connected.

Like Dermoun's study, several studies involving the effect of light intensity on microalgal culture growth have developed models showing the dependence of microalgal culture growth rate on light intensity. Lee et al. (1986) cultivated the blue-green algae *Spirulina platensis* in a rectangular algal fermentor with uniform lighting on the front and back

sides of the vessel. Turbidostat operation was employed to maintain a constant cell concentration and a constant fraction of absorbed light. Two cell concentrations of 0.04 g DCW/L and 0.05 g DCW/L were investigated. The results showed that when the light intensity was increased, the average specific growth rate increased up to a maximum value of 0.12 h^{-1} at a saturation light intensity of about 400 μmol photon/m^2s^{-1}, then decreased (light inhibition). Several kinetic models were then considered to express the relationship between growth rate and light intensity. They included the Monod kinetic model, the Bannister model, the Aiba model and the modified Aiba model. All of the models were consistent with the experiment results for light-limited growth. Only the Aiba and modified Aiba models could account for the photoinhibition observed at high incident light intensity. The modified form of the Aiba model provided a good fit at all light intensities that were investigated.

Sancho et al. (1991) also studied kinetics of growth in *Chlorella pyrenoidosa* cylindrical tank culture under light-limited conditions. They reported that when the algae grow under low light intensities, a linear relationship is observed between the specific growth rate, μ, and the light intensity, I. However they also reported that at high values of light intensity the specific rate becomes constant. Sancho et al. used equation (1) to fit the experimental data.

$$\mu = \mu_m [1-\exp(-I/I_s)] \quad (1)$$

Furthermore, equation (2) was developed to calculate the average specific growth rate when the cells adapt to an average value of light intensity

$$\mu_{ave}=\mu_m[1-\exp(-I_0[1-\exp(-k_aLC)])/(k_aLCI_s)] \quad (2)$$

where k_a is the extinction coefficient with a value of 2.42 L/g-cm, and L is the equivalent length of the culture chamber in centimeters.

Although many studies have showed that the specific growth rate is affected by light intensity in microalgal cultures, most commercial large-scale microalgal cultures are still cultivated under nutrient sufficiency and light limitation. For this reason, a number of photobioreactor designs and cultivations have been investigated to encourage microalgae growth at light saturation intensities, by culturing algal cells in well mixed systems with short optical pathlengths. Mori (1985) constructed a microalgal photoautotrophic bioreactor incorporating a multitude of fiber-optic light radiators within the tank to provide 5800 cm^2 radiative surface/L algal suspension, allowing biomass growth rates of 0.08 h^{-1} at high densities of 4.0-5.0 g DCW/L. The sun was used as the main light source and xenon lamps were used as a supplement.

Another photobioreactor system was also designed and constructed by Javanmardian et al. (1991) to achieve high photosynthetic rates in high-density microalgae *Chlorella vulgaris* suspension. A fiber-optic based optical transmission system was also used in this photobioreactor system and a high cell densities of up to 10^9 cells/ml were achieved.

Thus, light intensity effects microalgal culture growth and is often the limiting factor in large-scale photobioreactor microaglal cultivations. Typically, microalgal culture growth rate increases with increasing light intensity up to a maximum value at light saturation, then decreases.

MATERIALS AND METHODS

CELL CULTURE DEVELOPMENT

Laminaria setchellii Callus Induction and Culture

Collection

Fresh tissue of the brown alga *Laminaria setchellii* was collected at low tide of -1 ft or lower at Seal Rock, Oregon. All samples were collected between the months of January and August of 1992. The submerged algae were cut off from the rocks at their holdfast sections. The upper blade sections were cut off immediately after collection. These samples were washed in seawater, and placed in a container containing seawater, and then transported to the lab in an ice chest.

Solid Medium Preparation

Callus of *Laminaria setchellii* explants was induced on solid medium. The solid medium used in this study consisted of Instant Ocean sea water and 1.5 wt% agar. The medium preparation procedure is described here. First, 33 g of Instant Ocean powder was completely dissolved in 1 L distilled water within a 1 L erlenmeyer flask. The pH was

adjusted to about 8.0 with 1 N NaOH solution. Second, a 7.5 g agar was added to a 500 ml aliquot of Instant Ocean solution in a 1 L flask to provide 1.5 wt% agar in solution. This medium was autoclaved at 250 ^{0}F, 15 psig for 20 min. The flasks were removed from the autoclave and cooled in the laminar flow hood. The cooled agar solution was still liquid at this time. Then the flask month was flamed, and about 25 ml of liquid agar medium was poured into each 90 mm petri dish to a depth of 5 mm. Each poured dish was covered and then allowed to cool for 1 h in the laminar flow hood. If the dish cover was left slightly ajar, then condensation of water vapor on the cover was avoided. The cooled plates were put in the petri dish sleeve bag. The bag was taped shut, labeled, and stored in a refrigerator.

Pre-Cleaning and Storage of *Laminaria setchellii* Tissue

Collected *Laminaria setchellii* tissue were placed in chilled, aerated holding tanks containing 18 L of Instant Ocean seawater solution (33 g/L). About 200 g of fresh tissue was loaded into each tank. Each tank was maintained at 11 ^{0}C by stainless-steel cooling coils. Cooling water from a chilling circulator flowed through the coils. Aeration at 2 L/min provided both oxygenation and agitation to each tank. All tissue samples were used for callus induction studies within three days. Each piece of tissue

was carefully cleaned and washed first with D.D. water and then with Instant Ocean seawater. All cleaned tissue samples were placed back in the holding tanks.

Explant Preparation, Sterilization, and Plating

Figure 2 shows the explant preparation, sterilization and plating. Only stipe sections of collected *Laminaria setchellii* plants were used for callus induction studies. Stipe sections were cut into 5 cm segments with razor blade in a way so that each 5 cm segment was as straight as possible. All segments were soaked in Instant Ocean seawater in a 250 ml beaker under ice.

All explant preparation and plating procedures were carried out using sterile technique in operating laminar flow hood. The explant plating procedure is described below.

Two sterile petri dishes provided a sterile work surface within the laminar flow hood. Prior to explant sterilization, large forceps and three autoclaved cork borers of size #4, #5, and #6 were emerged in 70% v/v ethanol, flame-sterilized and then allowed to cool for at least 30 seconds on the sterile work surface. The stipe segment was completely wiped with a cheese cloth pre-wetted with 70% ethanol. The whole stipe segment was then dipped in 70% ethanol using sterile forceps and flamed for less than 3 seconds in order to sterilize the explant surface. The

Explant Preparation & Sterilization

(Laminaria setchellii)

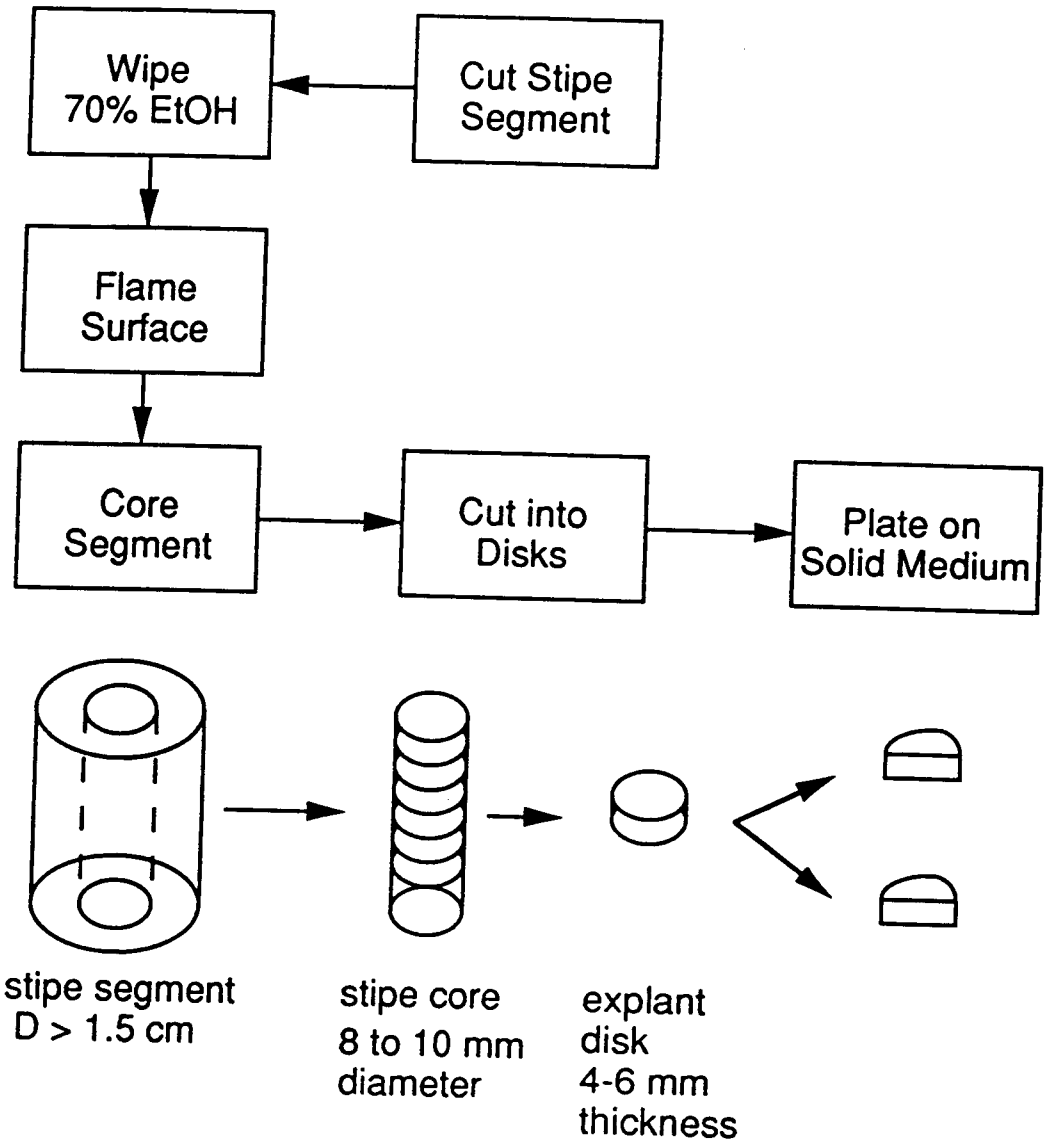


Figure 2. Explant preparation, sterilization and plating.

interior tissue of the sterilized stipe segment was bored out using a # 5 flame-sterilized core borer on the petri-dish work surface. The cored stipe segment was removed with a flame-sterilized reamer and deposited onto the sterile work surface. The cored stipe segment was cut into disks 3 to 5 mm thick with a flame-sterilized razor blade. The ends of the core segment was discarded. The disks were cut in half to clearly expose the cortical/medullary tissue boundary.

Using sterile technique, each explant was placed onto the solid medium in the 90 mm sterile petri dish, and pushed in to a depth of about 1 mm in order to provide ample exposed surfaces for callus induction. About 20 half-disk explants were plated onto each dish. The dish was covered and double wrapped with parafilm around the edge. The bottom of each plate was labeled. Stack of ten plates were covered with aluminum foil and placed in dark refrigerator at 8 ^{0}C. Typically, about 1000 explants were made and plated in 50 petri dishes at one time.

Callus Tissue Induction and Measurement

Callus was induced during a period of about 60 days. The size, morphology and color of the callus on each explant was measured weekly. The explant/callus tissue was classified into four types (Figure 3). In the first classification (type #0) the explant surface is changing its

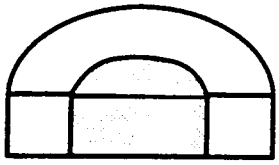
Callus Induction & Growth (Solid Media)

Conditions:

8 °C
Dark
15 g/L agar in seawater

Type 1

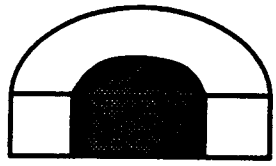
Light Filaments on Medulary Tissue



Explant

Type 2

Filaments Cover Medulary Tissue



Type 3

Dense Filaments Cover Medulary Tissue & Spread over Expant

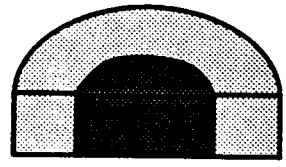


Figure 3. Callus induction classifications.

color from yellow white to yellow but no callus tissue observed on the explant. In the second classification (type #1) a ridge of clear tissue formed along the cortical tissue boundary of the explant and the color of the explant was dark yellow. In the third classification (type #2) filamentous, clear callus tissue completely covered the central core ofthe partially brown explant. In the fourth classification (type #3) large callus tissue clumps almost covered the whole top surface of the explant and the color of the explant changed from partially brown to brown or dark brown.

The classification of each explant was recorded weekly. The callus induction frequency was calculated by:

$$\%induction = \frac{number\ of\ explants\ of\ a\ given\ type}{total\ number\ of\ explants} \quad (3)$$

$$\%total\ induction = \frac{number\ of\ all\ callus\text{-}bearing\ explants}{total\ number\ of\ explants} \quad (4)$$

In addition, the fresh weight callus yield was estimated. Three callus-bearing explants of the same classification were weighed. The callus tissue was then carefully scraped off the explant, and the explant was reweighed. The fresh weight of callus on each explant was determined by weight difference. The three samples were averaged to estimate the fresh weight of callus yield of a

given classification. Then the total callus yield (Y) was calculated by

$$Y = \sum [(\textit{fresh wt of callus per explant}) \; X \; (\quad \textit{of explants})] \qquad (5)$$

Initiation of Liquid Culture

The development of a liquid suspension culture from callus was attempted. All techniques described below were carried out in operating laminar flow hood using sterile technique.

Several of the best callus-bearing explants of type #3 were selected. The tissue around the callus-bearing part of the explant was cut off with a flame-sterilized razor blade. The sectioned callus-bearing tissue was immediately transferred onto a sterile petri dish in laminar flow hood. This callus-bearing tissue was diced up into 1 mm pieces. About 10 mg fresh callus weight was transferred to 2 ml sterile liquid cultivation medium in a 10 ml sterile test tube. The test tube was covered with sterile aluminum foil. The mixture was vortexed gently to disperse callus into liquid medium. The liquid culture was then transferred to a 50 ml sterile erlenmeyer flask and 8 ml sterile liquid cultivation medium was added to bring the total liquid volume in each flask to 10 ml.

Several different liquid cultivation media were used including PES enriched seawater medium and ASP6 artificial sea water medium. The composition of PES enriched seawater medium is shown in Table 1.

The cultivation flasks placed on orbital shaker within a low-temperature incubator and cultivated at 100 rpm, 15 °C and 12L:12D illumination cycle at about 1500 lux.

The viability of the callus cells was periodically checked using Evan blue non-vital stain (0.25% w/v).

Table 1. PES Enriched Seawater medium composition.

Compound	Chemical Formula	mg/L
Sodium nitrate	$NaNO_3$	56.0
Boric acid	H_3BO_3	4.56
Cobalt (II) sulfate hepahydrate	$CoSO_4.7H_2O$	0.019
Iron (III) chloride hexahydrate	$FeCl_3.6H_2O$	0.196
EDTA chelated iron	$Fe(NH_4)_2(SO_2).6H_2O$	2.808
Manganese sulfate tetrahydrate	$MnSO_4.4H_2O$	0.656
Sodium EDTA	Na_2EDTA	6.4
Sodium glycerophosphate hexahydrate	Na_2(glycerophosphate) $.6H_2O$	8.0
Zinc sulfate hepahydrate	$ZnSO_4.7H_2O$	0.088
Biotin		0.0008
B12		0.0016
Thiamine-HCl		0.08

Laminaria saccharina Gametophyte Culture

Maintenance and Subculture

Laminaria saccharina female gametophyte cultures were maintained in 250 ml Erlenmeyer flasks containing 100 ml GP2 artificial seawater medium. The culture flasks were kept in an incubator at 13 ^{0}C under light intensity of 2500 lux and photoperiod of 16 hours light / 8 hours dark. The composition of the GP2 artificial seawater medium is shown in Table 2. The culture flasks were swirled for 2 to 5 seconds once per day to agitate and aerate the cultures. The cultures were sub-cultured every six weeks at 25% v/v by the procedures described below.

Five of the best looking cultures were selected for subculture. Selection was based on a deep brown color and no sign of contamination under 100x magnification. Each of the cultures selected for subculture was then kept sealed and vortexed for 1 min to break the loose filamentous chains that are characteristic of the female gametophytes. After vortexing, the flasks were returned to the laminar flow hood for subculture. To twenty sterile 250 ml flasks, 75 ml of sterile GP2 medium was added in the laminar flow hood. Then, ml of freshly vortexed culture was pipetted to each of the 250 ml sterile flasks containing 75 ml of sterile, fresh GP2 medium. The culture was evenly suspended by swirling the

Table 2. GP2 Artificial Seawater medium composition.

Compound	Chemical Formula	mg/L
Artificial Seawater:		
Sodium chloride	$NaCl$	21,030
Sodium sulfate	Na_2SO_4	3,520
Potassium chloride	KCl	610
Potassium bromide	KBr	88
Sodium tetraborate decahydrate	$Na_2B_4O_7.10H_2O$	34
Magnesium chloride hexahydrate	$MgCl_2.6H_2O$	9,500
Calcium chloride dihydrate	$CaCl_2.2H_2O$	1,320
Strontium chloride hexahydrate	$SrCl_2.6H_2O$	20
Nutrients:		
Sodium nitrate	$NaNO_3$	63.5
Sodium phosphate	$NaH_2PO_4.H_2O$	6.4
Sodium citrate dihydrate	$Na_3C_6H_5O_7.2H_2O$	0.52
Trace Metals:		
Sodium molybdate (VI) dihydrate	$Na_2MoO_4.2H_2O$	0.012
Potassium iodide	KI	0.042
Zinc sulfate hepahydrate	$ZnSO_4.7H_2O$	0.0112
Sodium orthovanadate	Na_3VO_4	0.0048
Manganese chloride tetrahydrate	$MnCl_2.4H_2O$	0.0034
Vitamins:		
Thiamine-HCl		0.25
B_{12}		0.000125
Biotin		0.000125

flask when the culture was pipetted. From the five flasks originally chosen for subculture, twenty flasks of new culture were inoculated. The newly inoculated flasks were then placed in a low-temperature incubator and cultivated at 13 ^{0}C under 2500 lux, 12L:12D illumination cycle.

Decontamination

The cultures were periodically checked for contamination using ATM (Axenic Test Medium) solid agar plates and then given a decontamination treatment if necessary. The decontamination technique described below.

First, 500 ml D.D. water, 1000 ml GP2 medium, and two 60 µm nylon mesh filters were autoclaved at 121 ^{0}C under 15 psig for 20 minutes. A 250 ml polystyrene mini blender cup was carefully cleaned and sonicated with D.D. water for 15 minutes, and then autoclaved at 121 ^{0}C under 15 psig for only 1 minute to avoid melting the plastic. All autoclaved items were allowed to cool down to room temperature in laminar flow hood. About 100 ml of the contaminated culture was poured into the sterilized blender cup and blended at "liquefy" setting for 30 seconds on an Osterizer blender. The blended culture was filtered through the sterile 60 µm nylon mesh filter. The filtered cells were retained on the nylon mesh. The filtered cells were then washed with 20 ml sterile GP2 medium, followed by 10 successive 10 ml washes with sterile

D.D. water. The bottom of the nylon filter containing the cells was then dipped into sterile D.D. water in a 50 ml sterile beaker for 30 seconds to let the cells contained on the nylon filter continuously merged in the D.D. water. The cells were then back washed with 100 ml sterile fresh GP2 medium directly into a sterile 250 ml flask to form a new 100 ml decontaminated culture.

Usually, decontamination was performed concurrently with 33% v/v subculture. About 33 ml of the decontaminated culture was poured into a sterile 250 ml flask. Sterile fresh GP2 medium was then added into the flask to bring up the culture volume to 100 ml (33% v/v decontamination/subculture).

PHOTOBIOREACTOR CULTIVATION STUDIES

Bioreactor Design

Stirred-tank illuminated bioreactor systems (photobioreators) were used in this study. Specifically, two stirred-tank illuminated bioreactor systems with different sized reactor vessels were fabricated. The first bioreactor has an effective cultivation volume of 900 ml (Figure 4). It was designed for macroalgal cell cultivation and photosynthesis studies. The second bioreactor has an

effective cultivation volume of 1300 ml. It was used for cell cultivation studies (Figure 5).

The entire illuminated bioreactor systems, including the bioreactor assembly, the illuminated stage, the aeration system, and the control unit were installed within a VWR Scientific model 2020 low-temperature incubator.

Each stirred-tank bioreactor vessels consists of a glass cultivation vessel, a headplate assembly, and an impeller assembly purchased from Bellco Glass, Inc. The glass vessel for the 1300 ml bioreactor (Bellco 1965 1000 ml spinner flask) has a 100 mm central opening and two 45 mm open sidearms. The diameter is 13 cm and the overall height is 24.5 cm. The glass vessel for the 900 ml bioreactor (Bellco 1965 500 ml spinner flask) has a 70 mm central opening and two 38 mm open sidearms. The diameter is 10.5 cm and the overall height is 20.5 cm. For each vessel, a screw cap over the central opening serves as the headplate, and two screw caps seal the side-arm ports.

The impeller design of a stirred-tank bioreactor system plays a very important role in the suspension of cells, the break-up of air bubbles for enhanced oxygenation, and the break-up of cell aggregates. Specifically, the 1300 ml bioreactor is equipped with two twin-blade paddle impellers (45 mm height x 18 mm width for each blade), each pitched to an angle of 45 degrees. The 900 ml bioreactor is equipped

with a single twin-blade impeller (80 mm height x 26 mm width for each blade), pitched to an angle of 90 degrees. For each bioreactor, the impeller was driven magnetically, and a constant rotor speed was maintained by a magnetic stirrer motor control unit. A stir bar mounted to the bottom of the impeller assembly provided the necessary magnetic coupling.

Both bioreactors are equipped with similar air inlet and outlet assemblies. Ambient air is pumped through a 0.2 μm sterile air filter, and then sparged to the culture by an air inlet pipe tube. The sparger consists of five 1.4 mm holes lined up on the bottom side of the tubing sparger. The air sparger is positioned directly beneath the impeller to break up the air bubbles and mix them into the cell suspension culture. The air flow is metered by a flowmeter.

The 900 ml bioreactor has an oxygen electrode port fitted for a YSI 5750 dissolved oxygen (D.O.) polarographic electrode. The D.O. electrode is connected to a YSI model 54 D.O. meter. Analog readings from the D.O. meter are sent to a computer data acquisition system.

The 1300 ml bioreactor is equipped with a heater assembly to maintain a constant temperature. Specifically a heating cartridge is inserted into a thermowell which

900 mL Stirred-Tank Photobioreactor

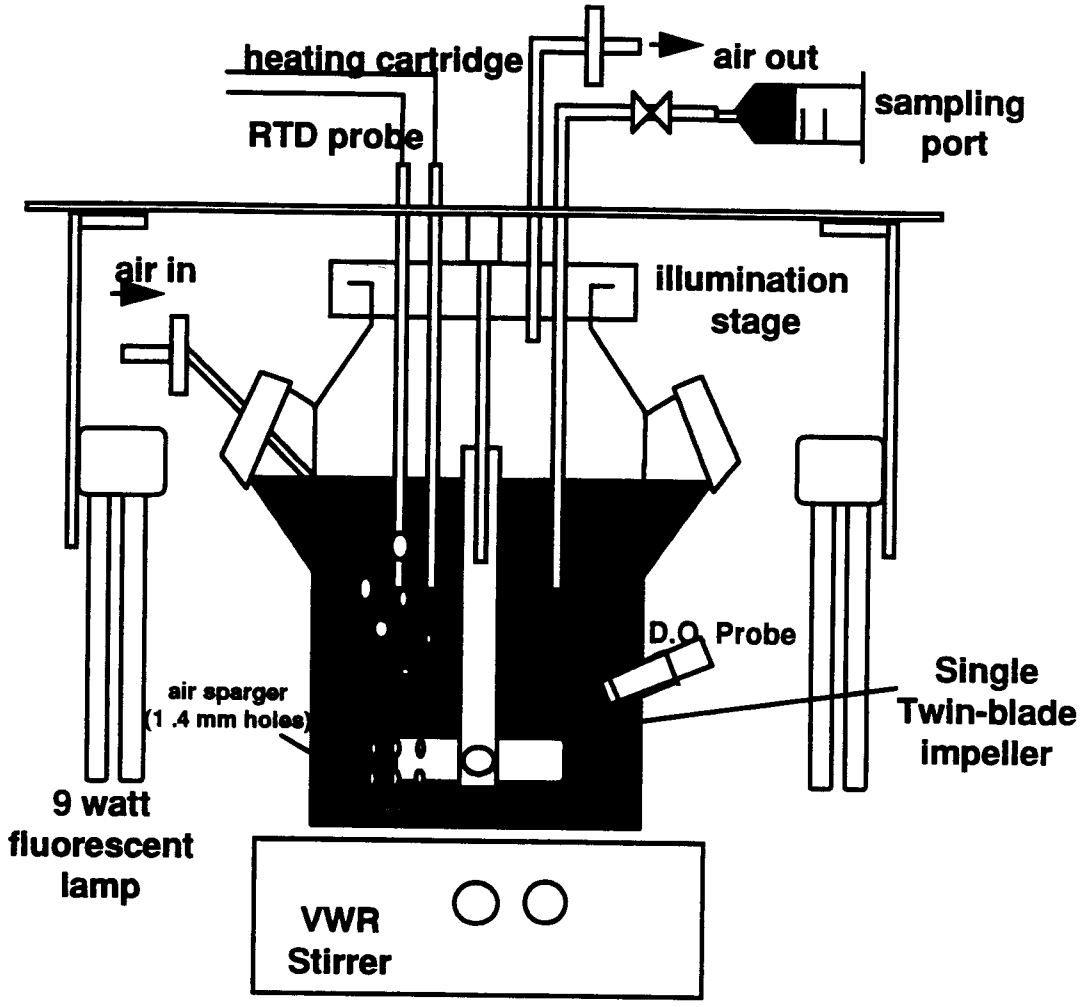


Figure 4. 900 ml stirred-tank photobioreactor.

1300 mLStirred-Tank Photobioreactor

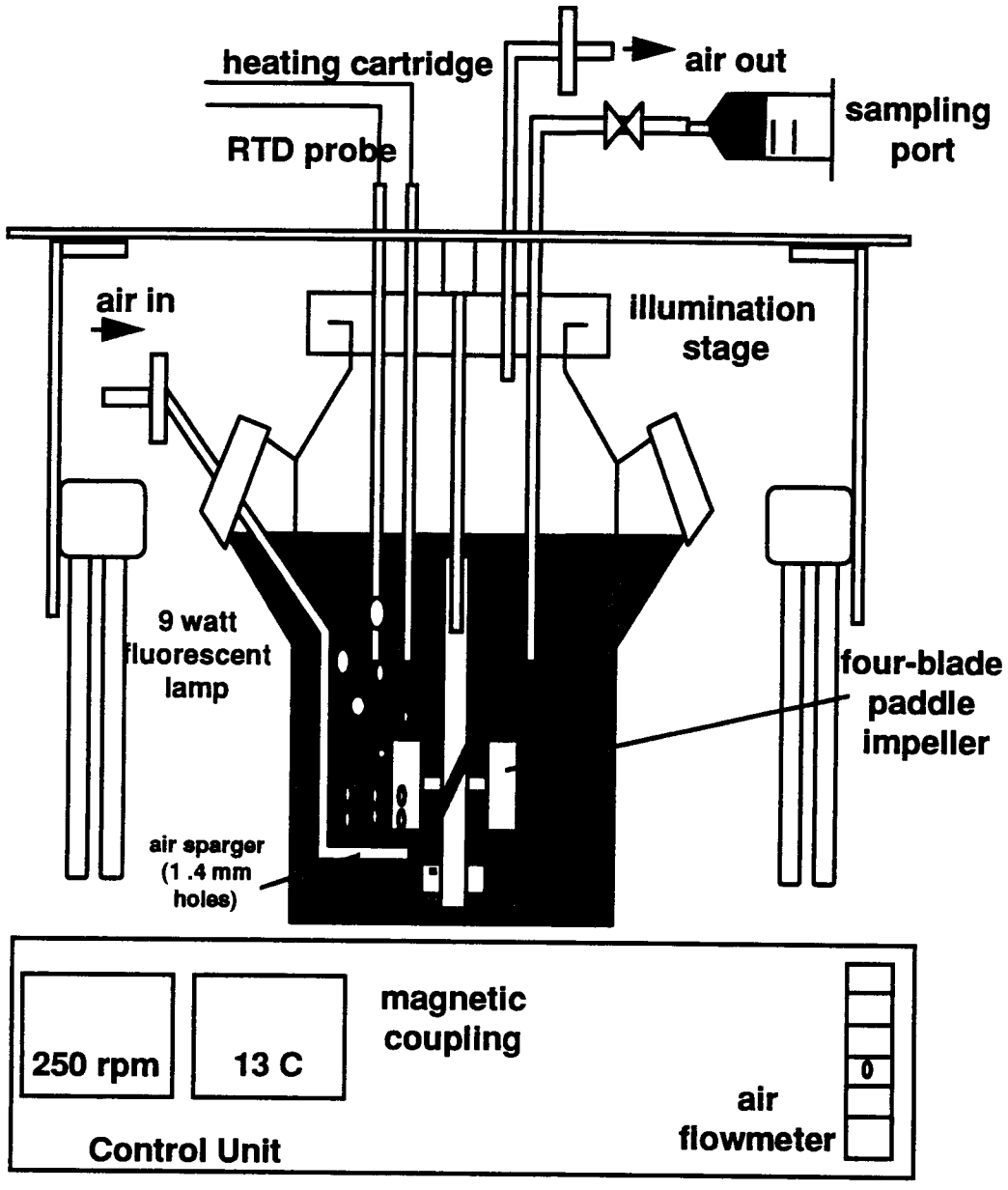


Figure 5. 1300 ml stirred-tank photobioreactor.

penetrates into the cell suspension culture. An RTD temperature probe is inserted into a separate thermowell. A temperature controller connected to both the RTD probe and heating cartridge maintains the culture temperature at the set point value.

Light Stage Design

The *Laminaria saccharina* female gametophyte cell cultures used in this study are photolithotrophic. Therefore, uniform illumination must be supplied to the bioreactor vessel.

The light stage consists of two 9 watt fluorescent lamps, mounted on plexiglass blocks. The plexiglass blocks are fitted with two pairs of vertical referencing plates. The reference plates are supported on the incubator shelf above the bioreactor. Holes drilled 18 mm apart in each referencing plate adjust the vertical position of the lamps. The distance between the lamp and the vessel surface is adjusted by changing the horizontal position of the reference plates.

Experimental Conditions

This experimental design for the cultivation of *Laminaria saccharina* cell culture in an illuminated stirred tank bioreactor focused on two areas: 1) the effect of incident light intensity on specific growth rate and final cell density; 2) measurement of photosynthetic oxygen evaluation and dark-phase oxygen respiration during the batch cultivation period. For all studies the nominal cultivation period was 26 days. Four incident light intensities were considered: 1500 lux, 3000 lux, 8200 lux and 19,000 lux. The base cultivation conditions are summarized in Table 3.

In addition, as shown in Figure 6, light transmission in the bioreactor was investigated by measuring the incident light intensity at the out wall surface of the bioreactor vessel. A digital light meter (EXTECH Instrument) was used for the measurement. When the measurement was performed, the probe of the light meter was placed on the out wall surface of the reactor vessel with its sensor side facing to the vessel.

Table 3. Base conditions for stirred-tank photobioreactor cultivations.

Temperature	13°C
Illumination	3000 lux / 16L:8D
Media	GP2 (no bicarbonate)
Volume	900 ml & 1300 ml
Mixing	250 rpm
Aeration	1000 ml/min constant (for the 900 ml bioreactor)
	1500 ml/min constant (for the 1300 ml bioreactor)

Illumination of Stirred-Tank Bioreactor

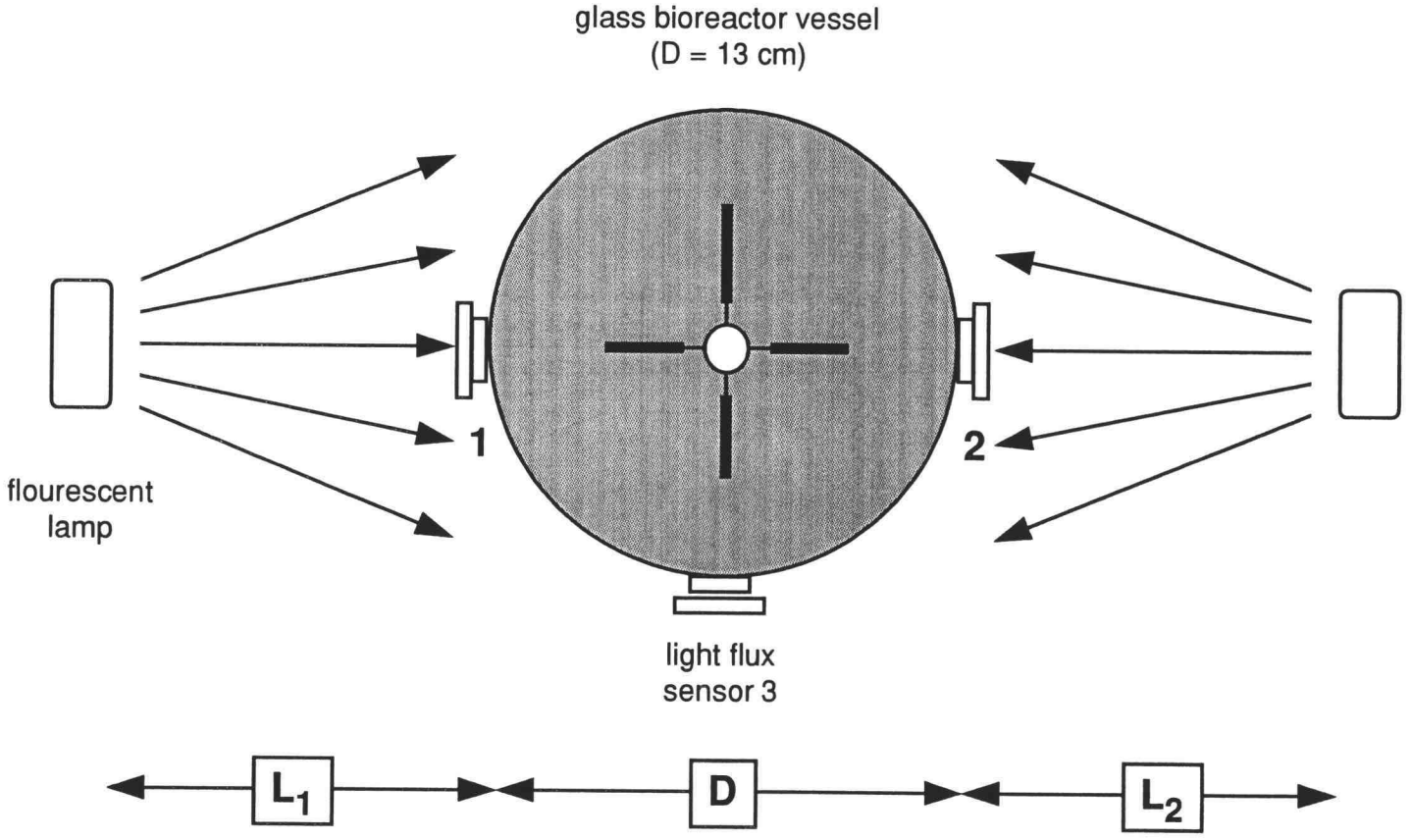


Figure 6. Light transmission measurements of stirred-tank bioreactor

Start-up and Inoculation

The bioreactor operational procedures are divided into three sections: reactor cleaning and sterilization, inoculum preparation and reactor inoculation.

Prior to use, the bioreactor was disassembled and cleaned. All the assemblies and parts (except for the air filters) were soaked in approximately 2-3% phosphate-free soap (Liquinox) solution for three hours. They were washed again with the soap solution, and then rinsed with tap water followed by 6 successive rinses with D.D. water. The entire bioreactor was reassembled. The assembled bioreactor was autoclaved at 121 ^{0}C and 15 psig for 20 minutes. After sterilization, the bioreactor was allowed to cool down in the laminar flow hood.

An inoculum mixture was typically obtained from three representative 250 ml flasks of *Laminaria saccharina* maintenance cultures. The inoculum mixture was blended for 5 seconds on "blend" speed to disperse the culture. The dry cell density ($X_{c,o}$, mg DCW/L) of the inoculum mixture was determined. The volume of inoculum mixture (V_o, ml) needed to inoculate the bioreactor was calculated by:

$$X_{c,i} = X_{c,0}\frac{V_0}{V_0 + V_m} = X_{c,0}\frac{V_0}{V_T} \qquad (6)$$

Sterile GP2 medium (cooled to 13 ^{0}C) of volume V_m was added into the bioreactor vessel. The total cultivation volume V_T ($V_o + V_m = V_T$) was typically 1300 ml or 900 ml, depending on the bioreactor used. The bioreactor was then sealed in the laminar flow hood and placed on the magnetic control unit. The incubator was set at 13 ^{0}C. The air pump was connected to the bioreactor and the aeration rate was set at 1.2 vvm (1500 ml air/min for 1300 ml culture or 1000 ml air/min for 900 ml culture at 13^{0}C). The fluorescent lamp positions were adjusted to set incident cultivation light intensity to the desired value. The photoperiod was set to 16L:8D. The impeller speed was set to 250 rpm.

About 1 hr after inoculation and start-up, three 5 ml culture samples were taken for analysis of initial chlorophyll a concentration and pH.

Sampling

During the 26 day cultivation period, culture samples were taken from the bioreactor for culture concentration and pH measurements at two day intervals.

Just prior to sampling, the silicone tube leading from the sampling port was unclamped. About 10 ml of culture was withdrawn from the bioreactor through the sampling tube, using a 10 ml syringe fitted to a Leur-lock connector. When

the syringe was removed from the connector, the silicone tube was closed in order to prevent the culture liquid from flowing out of the slightly pressurized bioreactor.

Measurement of Chl a Concentration in Culture

Growth of photosynthetic *Laminaria saccharina* cell cultures was followed by measurements of chlorophyll a concentration in culture. The chlorophyll concentration was measured using a spectrophotometer (Hitachi, model 100-10) set at a wavelength at 665 nm. At each sampling time, two trial measurements were performed. For each trial measurement, a 5.0 ml sample was obtained from the culture and vacuum filtered through a 20 μm pore size nylon mesh filter (Spectra Mesh) using an aspirator. The filter and cells were then placed in a 50 ml polypropylene centrifuge tube. For each measurement a blank filter was also prepared to provide a control sample. To each centrifuge tube containing the filter and cells, 5.0 ml HPLC grade methanol was added. Each sample was then mixed on a VWR vortex machine at speed setting 4 for 30 seconds. All the samples were then stored in a dark refrigerator at 4 ^{0}C overnight.

About half hour prior to the spectrophotometer measurement, the samples were removed from the refrigerator and vortexed once again for 30 seconds at speed setting 4.

The spectrophotometer absorbance was read at 665 nm. The chlorophyll a concentration was then calculated by multiplying the absorbance value with 16.29 μg Chl a/abs unit (Geider and Osbornes 1992). To quantify the growth of the culture for different batches of cultivation, the measured chlorophyll concentrations were correlated with dry cell density measurements.

pH Measurements

Prior to Chl a measurement, the pH of the culture sample was also measured using a General Purpose Combination pH electrode (CORNING) and General Purpose pH meter (CORNING).

Cell Density Measurements

At the beginning and the end of cultivation, the dry cell weight density of the culture was measured. The procedure is described here. First, a 47 mm, 0.45 μm pore size Millipore filter was dried in a covered 100 x 15 mm glass petri dish within an oven at 70 ^{0}C for 12 hrs. The dry filter was weighed. The biomass from a 40 ml culture sample was filtered onto the dry filter, and then dried at 70 ^{0}C for 12 hrs. The dried cells/filter were reweighed. The weight of dry cell mass determined by weight difference. Three

duplicate measurements were obtained and a average value for the dry cell weight density was taken.

Total Biomass at Run Completion

All the cell mass produced from the photobioreactor cell cultivations in this study was collected and weighed.

After the bioreactor was shut down, the fresh cell culture in the bioreactor was collected and then was filtered using a 60 μm nylon filter. The collected cell mass was transferred into a pre-weighed small glass bottle with a screw cap. The bottle containing the cell mass was then reweighed. The weight difference was recorded as the net fresh weight of the cell mass. The bottle was then sealed and stored in dark in a deep freezer.

PHOTOSYNTHETIC OXYGEN EVOLUTION AND RESPIRATION RATES IN CELL CULTURE

D.O. vs. Time Measurements for Estimation of P_0 and Q_0

The photosynthetic oxygen evolution rate (P_0) and the oxygen respiration rate (Q_0) per unit volume of culture were measured every two days over a 22 day cultivation period in

the 900 ml illuminated bioreactor. The P_0 and Q_0 were estimated from dissolved oxygen (D.O.) concentration versus time data.

A YSI model 5750 polarographic D.O. electrode fitted into the wall of the vessel measured the D.O. concentration in the culture. The electrode connected to a YSI model 54 D.O. meter. The analog readings from the D.O. meter were sent to a computer data acquisition system comprised of a Data Translation DT2801 I/O Board and IBM PC/XT computer.

Prior to experiments, the YSI D.O. meter was calibrated. When the meter was calibrated, the entire bioreactor containing 900 ml autoclaved GP2 medium was set at the same conditions as those for cell culture, including temperature of 13 ^{0}C, mixing speed of 250 rpm, and air flow rate of 1000 ml/min. The calibration started with setting up air-saturated D.O. concentration. At 13 ^{0}C and under 1 atm the saturation D.O. concentration for water is 10.6 ppm. With an assumption of 1 atm atmosphere pressure, the saturation D.O. value was then set at 10.6 ppm when the medium was fully aerated and agitated with air. After the saturation D.O. value was set, nitrogen was sparged into the medium through the air sparger to strip the medium of dissolved oxygen. When the medium was completely stripped of dissolved O_2, the meter was adjusted with zero reading.

The D.O. concentration versus time measurement technique used for estimation of P_0 was performed in the bioreactor containing 900 ml of sterilized GP2 medium without cells as a control experiment. During the control experiment, the temperature was 13 ^{0}C, the air flow rate was 1000 ml/min, the incident light intensity was 8200 lux and the impeller speed was 250 rpm. The procedures for the D.O. vs. time control measurement are briefly described here. When the liquid medium was fully saturated with dissolved O_2, the measurement was started. After the saturation D.O. concentration was measured for 1.5 min, the air flow was turned off and nitrogen at a flowrate of 1000 ml/min was sparged into the medium through the air sparger to strip medium of dissolved oxygen. When the D.O. level decreased to 0.5 ppm, the nitrogen was turned off and the reactor was immediately sealed. The D.O. concentration was then continuously measured for 15 min after the reactor was sealed. For both the de-aeration and re-aeration phases of the experiment, the D.O. concentration was monitored as a function of time by D.O. meter.

Experiments were then conducted with cell cultures. The inoculum cell density was 100 mg DCW/L and the initial culture volume was 900 ml. The cultivation conditions were set at the same as those for the control experiment described above. The D.O. concentration versus time measurements used

for estimation of P_0 and Q_0 were obtained at a two or three day intervals during the cultivation period. The procedures for each P_0 and Q_0 measurement are described below.

D.O. concentration vs. time measurements used for P_0 estimation were performed 3 hours into the light portion of the photo period. First, the computer data acquisition system was turned on, and baseline D.O. level data for the aerated culture were obtained for 1.5 minutes. Then, the air flow to the reactor was turned off, and nitrogen flow was introduced into the culture at a flowrate identical to the air flowrate. The nitrogen gas stripped the dissolved oxygen out of the culture until the D.O. concentration in the culture decreased to 0.5 ppm. The nitrogen was then shut off and the reactor was sealed. The D.O. concentration increased as oxygen was photosynthetically evolved from the culture. The D.O. concentration rise was linear for about 10 to 15 minutes following shut off of the nitrogen gas flow. The

D.O. concentration versus time data were monitored by the D.O. meter and collected by the PC data acquisition system.

The measurements for the Q_0 estimation were performed 6 hours into the dark photoperiod. First, baseline D.O. level data for the aerated culture were obtained for 3 minutes. Then, the air supply was shut off and the reactor was sealed. After the reactor was sealed, the D.O. concentration in the culture decreased due to cell respiration. The decreasing D.O. concentration was measured for about 15 min.

Models for Estimating P_0 and Q_0 from D.O. Concentration versus Time Data

The unsteady-state mass balance on dissolved oxygen for a photosynthetic cell culture within a well-mixed and isothermal batch bioreactor is described by

$$\frac{dC_A}{dt} = k_L a [C_A^* - C_A] - Q_0 + \hat{P}_0 \qquad (7)$$

where:

C_A is the dissolved oxygen concentration (mmol O_2/L);

C_A^* is the dissolved oxygen concentration at saturation with respect to the oxygen partial pressure of the aerating gas (mmol O_2/L);

$k_L a$ is the volumetric oxygen transfer coefficient.

When the culture in the bioreactor is aerated with air, air bubbles are mixed with the medium, and oxygen is transferred from the gas phase to the liquid phase. The equilibrium between the gas phase oxygen partial pressure (P_A) and the D.O. concentration at saturation (C_A^*) is described by Henry's law.

$$P_A = HC_A^* \tag{8}$$

where H is the Henry's law constant.

When the culture in the bioreactor is de-aerated by nitrogen, the liquid medium is stripped of dissolved oxygen. If the head space gas is completely purged with nitrogen, the oxygen concentration in the head space gas is very low, and thus P_A is close to zero. As a result, C_A^* is close to zero. Therefore, the D.O. balance reduces to

$$\frac{dC_A}{dt} = -k_L a C_A - Q_0 + \hat{P}_0 = -k_L a C_A + P_0 \tag{9}$$

If the nitrogen flow is turned off and the reactor was sealed when the oxygen in the culture is completely stripped off, then the equation (9) reduces to

$$\frac{dC_A}{dt} = \hat{P}_0 - Q_0 = P_0 \qquad (10)$$

and P_0 is estimated from the slope of C_A vs time data during this oxygen evolution phase of the experiment.

During the dark photoperiod,

$$\hat{P}_0 = 0 \qquad (11)$$

and the oxygen balance for no aeration flow into the culture is

$$\frac{dC_A}{dt} = -Q_0 \qquad (12)$$

Thus, Q_0 is estimated from C_A vs time data.

RESULTS AND DISCUSSION

Laminaria setchellii CALLUS INDUCTION

Laminaria setchellii callus was induced by plating cortical tissue explants on seawater solid agar medium (15 g agar/L Instant Ocean seawater) at 8 ^{0}C in the dark. The kinetics of callus induction for *Laminaria setchellii* at three different plating times are provided in Figure 6. The fraction of type 3 callus was below 5% versus 30 % for type 1 callus after two months. Photographs of explants bearing type 3 callus is shown in Figure 7. The typical filamentous callus yield from type 2 and type 3 callus after two months was about 250 mg fresh weight per 1000 explants.

The development of a photosynthetic liquid cell culture from callus tissue was attempted. Specifically, clear, loosely-packed, filamentous callus tissue (type 3) was removed from the explants and cultivated in both PES and 1/2 PES liquid medium at 15 ^{0}C, 100 rpm shaking speed, and 30 $\mu E/m^2$-sec illumination. Figure 8 shows that in the liquid medium, the cells existed as clumps containing both filamentous and spherical shaped cells. In the liquid suspension culture, no quantitative growth measurement was obtained due to the low culture cell density and the highly

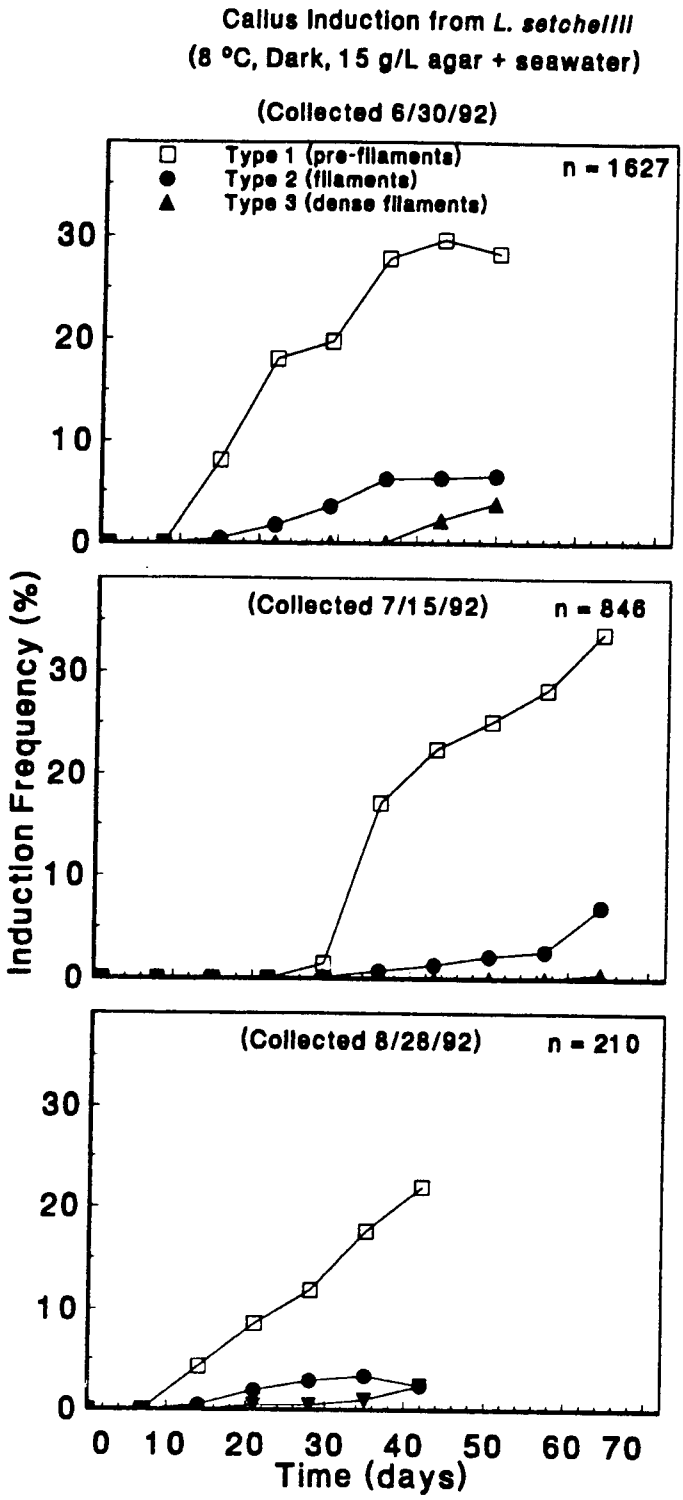


Figure 7. Callus induction frequency vs. time.

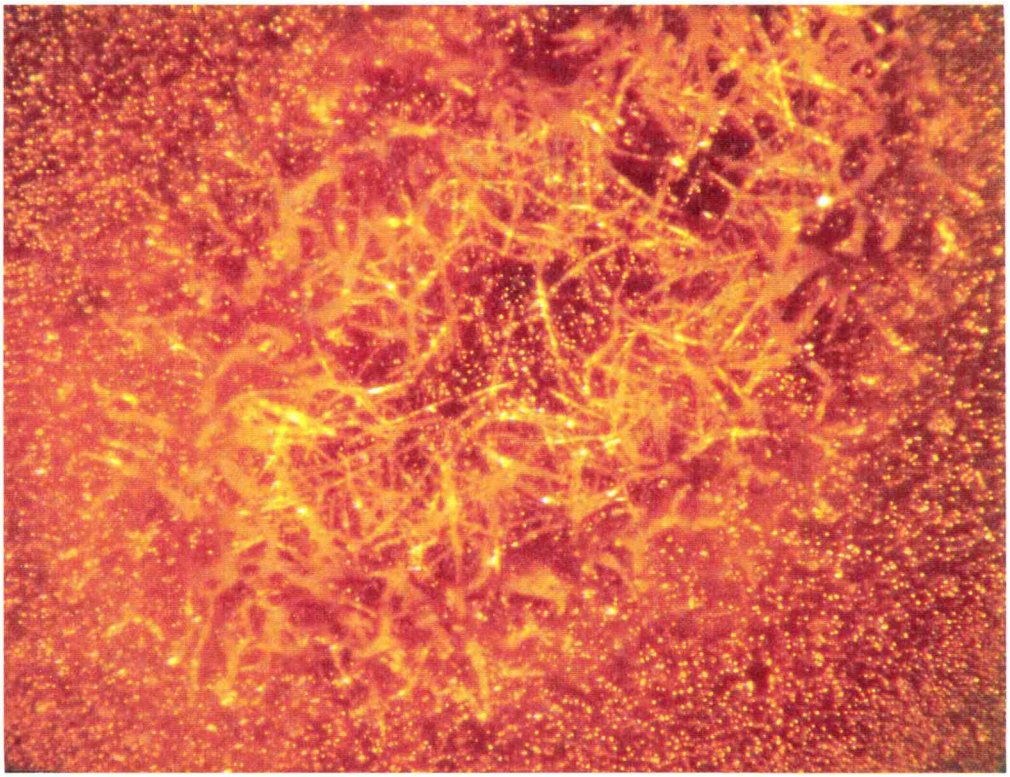


Figure 8. The explant bearing type 3 callus.

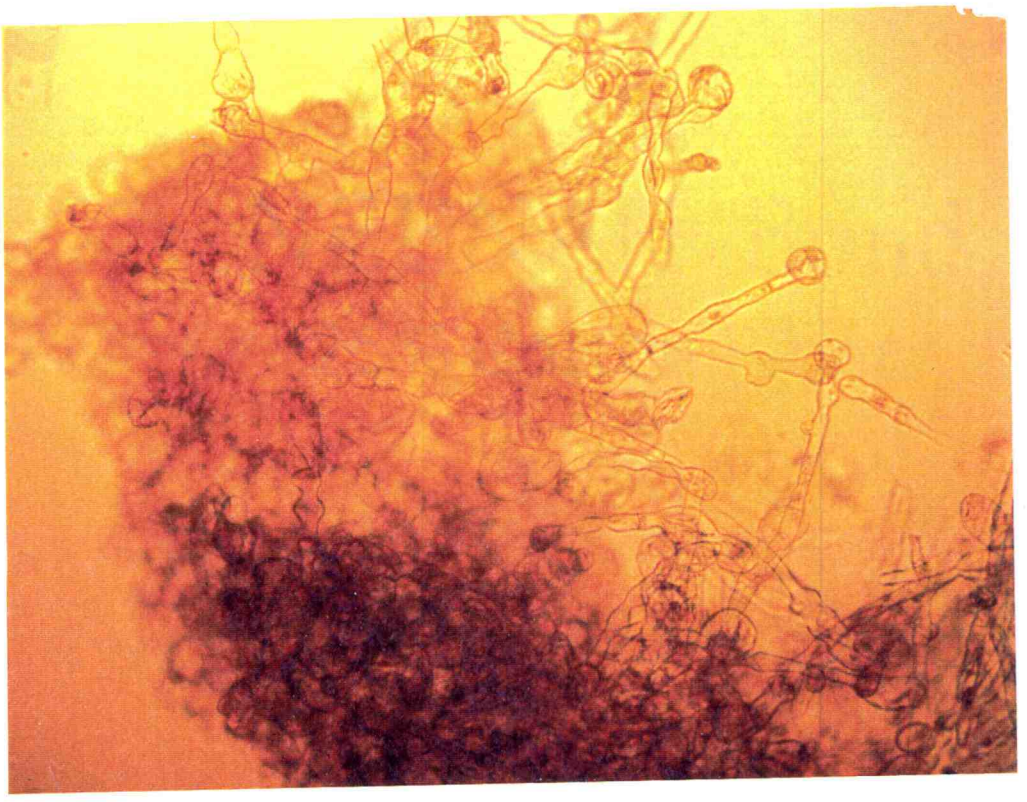


Figure 9. *Laminaria setchellii* callus cell clumps in liquid suspension culture.

clumped morphology at the cell suspension. However, cell staining assays of culture aliquots indicated that the some of the cells were still viable after two months in the liquid cultures.

In summary, (1) the kinetics of callus induction from *Laminaria satchellii* was quantitatively followed at 8 0C without illumination, and (2) callus tissue cultivated in both PES and 1/2 PES liquid medium under illumination remained viable up to two months, but the growth of the culture was not quantitatively followed.

BIOREACTOR CULTIVATION

Cultivation in 900 ml Stirred-tank Photobioreactor

Two cell cultivation runs were performed at the same conditions in the 900 ml stirred-tank bioreactor to assess the repeatability of the growth kinetics of the *Laminaria saccharina* female gametophyte cells. The growth curves are compared in Figure 10. The both cultivations were inoculated with 57 day old flask culture at a initial cell density of 100 mg DCW/l. The conditions for these two cultivations were 13 ^{0}C, 16L:8D photoperiod, 8200 lux incident light intensity, 1000 ml/min aeration rate, and 250 rpm impeller mixing speed.

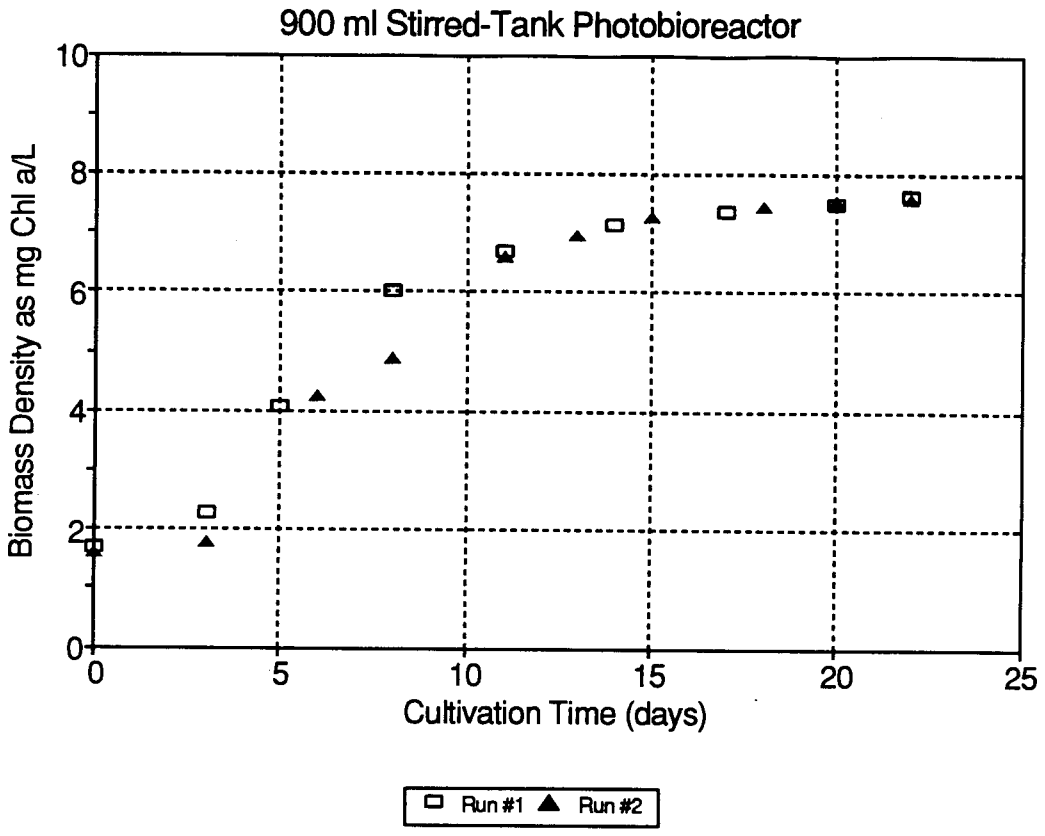


Figure 10. Growth kinetics of *Laminaria saccharina* gametophyte in the 900 ml stirred-tank bioreator.

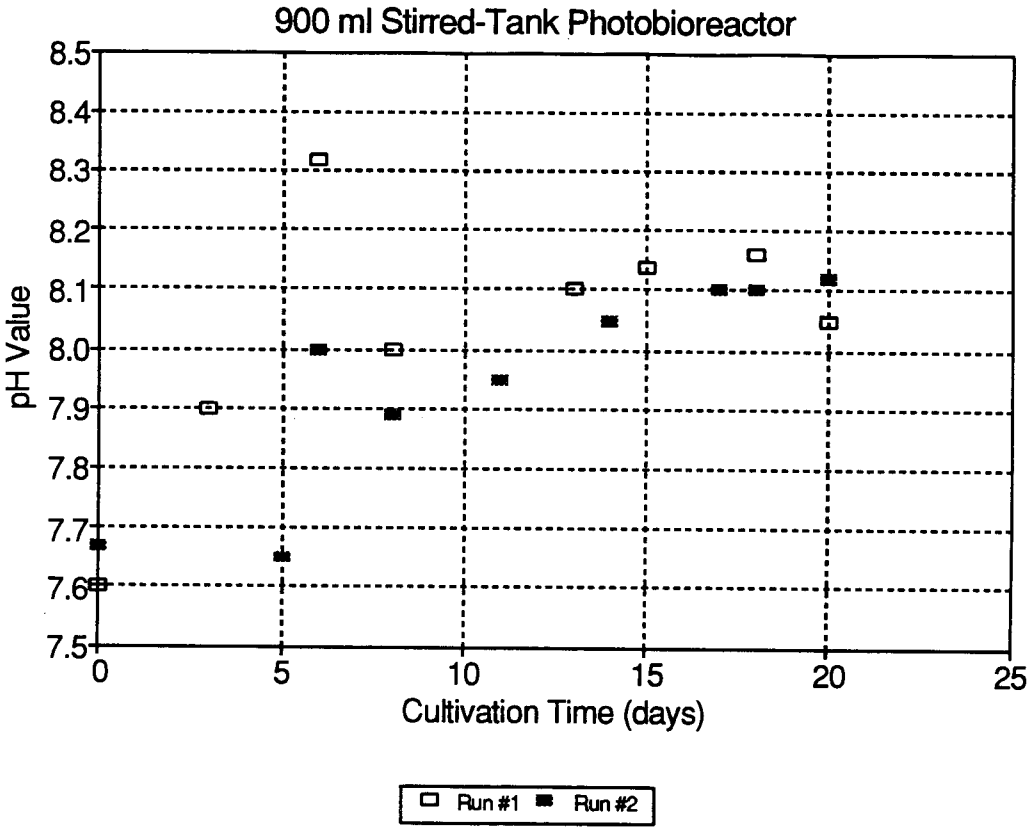


Figure 11. Variation of pH during *Laminaria saccharina* gametophyte cultivation in the 900 ml bioreactor.

Table 4. 900 ml photobioreactor cultivation parameters.

Measurement	Run #1	Run #2
Initial Cell Density, X_0 (mg DCW/l)	100 (H_2O washing)	100 (H_2O washing)
	266* (GP2 washing)	266* (GP2 washing)
Final Cell Density, X_f (mg DCW/l)	608 (H_2O washing)	603 (H_2O washing)
	875 (GP2 washing)	873 (GP2 washing)
Specific Growth Rate, μ (day^{-1})	0.135 ± 0.021	0.143 ± 0.024
Doubling Time, t_d (days)	5.13	4.85

* These are calculated values. The calculation was based on $X(GP2\ washing) = 1.2\ X(H_2O\ washing) + 145.6$.

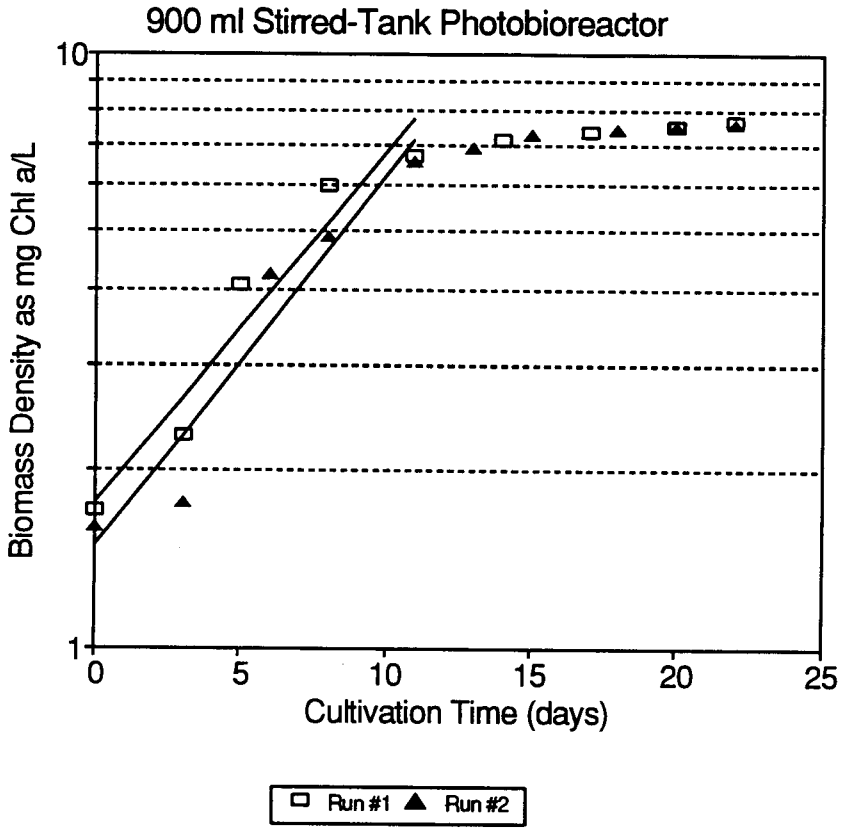


Figure 12. Semi-log plots of the cultivation growth curves in the 900 ml bioreactor.

Liquid GP2 medium without bicarbonate was also used for both cultivations.

The cultivation was repeatable. The both cultivations gave similar growth phases of about 10 days followed by a similar stationary phase. No lag phase was observed due to the high inoculation cell density. During the 25 day cultivation period, the pH of the each culture rose from around 7.6 at the 3rd day of cultivation to about 8.1 after the 22nd day (Figure 11).

Table 4 shows the growth parameters for the two cultivation runs. The final cell density was 608 mg DCW/l and 603 mg/l for each run after 25 days, indicating that the biomass production was repeatable.

Figure 12 shows a semi-log plot for the cultivation growth curves. The specific growth rate (μ) was determined from the slope of the exponential growth phase data. The specific growth rates were 0.135 $\pm$ 0.021 (1s) day^{-1} and 0.143 $\pm$ 0.024 (1s) day^{-1} for run #1 and run #2 respectively. Thus the specific growth rates were also repeatable.

Effect of Incident Light Intensity on Culture Growth

The effect of incident light intensity (1500 - 19,000 lux) on the growth curve in the 1300 ml stirred-tank photobioreactor is shown in Figure 13. The cultivation

conditions are given in Table 3. The cultivation parameters are summarized in Table 5. The semi-log plots of these growth curves are provided in Figure 14 and Figure 15.

The specific growth rate was computed from the slopes of the linear portion of the growth curve. The specific growth rate is plotted vs. incident illumination intensity in Figure 16. From Figure 16, increasing the incident light intensity increased the culture growth rate until a saturation light intensity was achieved, where the culture growth was no longer dependent on light intensity. Therefore, incident light intensity is a limiting factor in stirred-tank bioreactor cultivation of *Laminaria saccharina* gametophytes.

A further analysis of these results suggests a relationship between the specific growth rate and the incident cultivation light intensity. This relationship can be expressed by the following simple model equation suggested by Martinez et al. (1991)

$$\mu = \frac{u_{max} I_0}{K_0 + I_0} \qquad (13)$$

where μ is specific growth rate;

μ_{max} is maximum specific growth rate (day^{-1});

I_0 is incident cultivation light intensity (lux);

K_0 is the light intensity at 0.5 μ_{max} (half-saturation constant, lux).

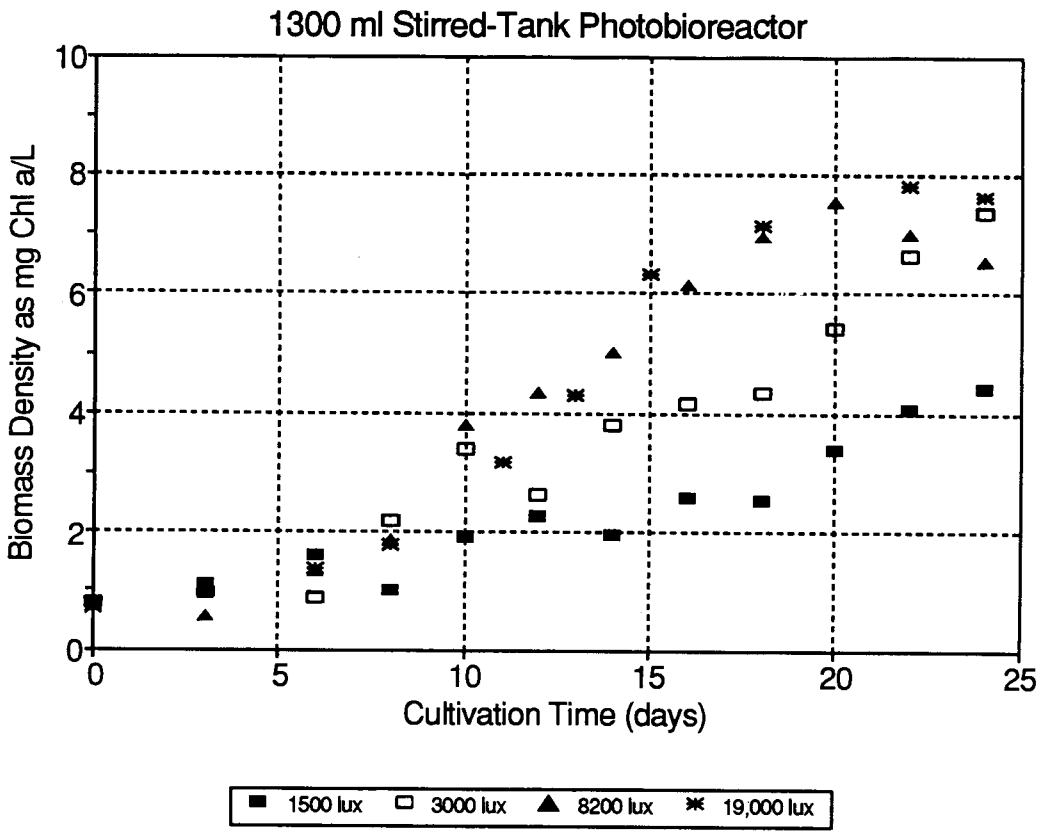


Figure 13. Effect of incident light intensity on growth.

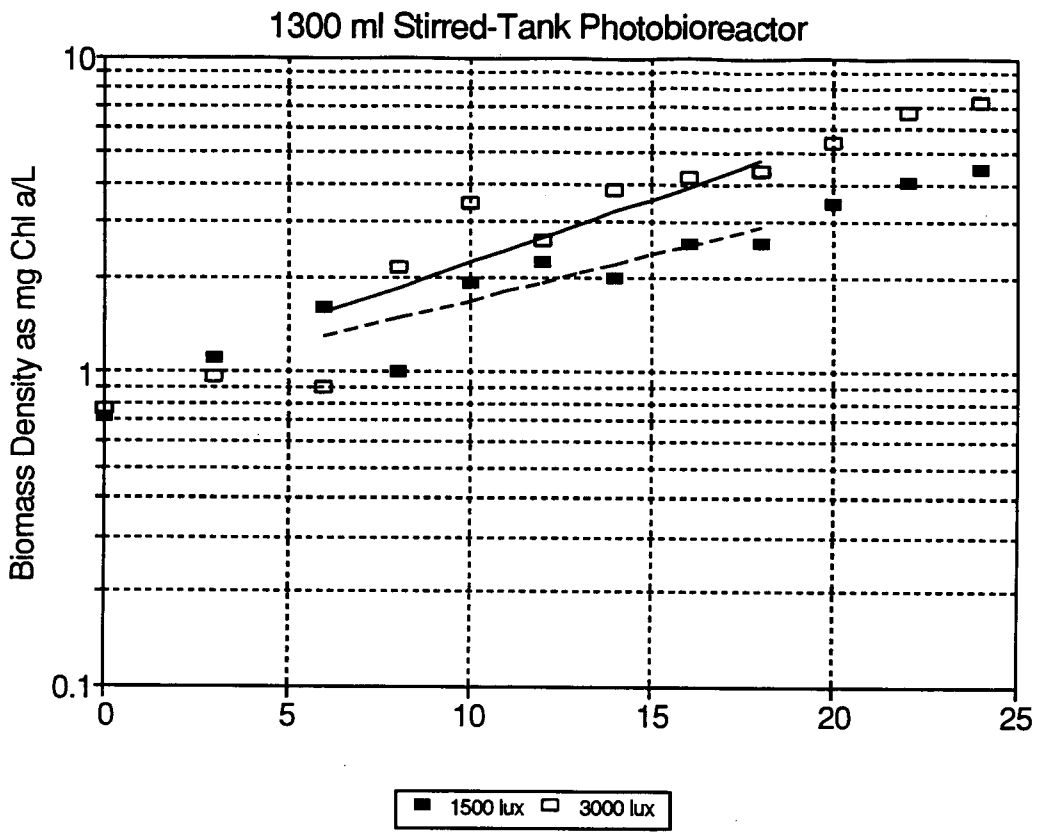


Figure 14. Semi-log plots of culture growth curves at 1500 lux and 3000 lux.

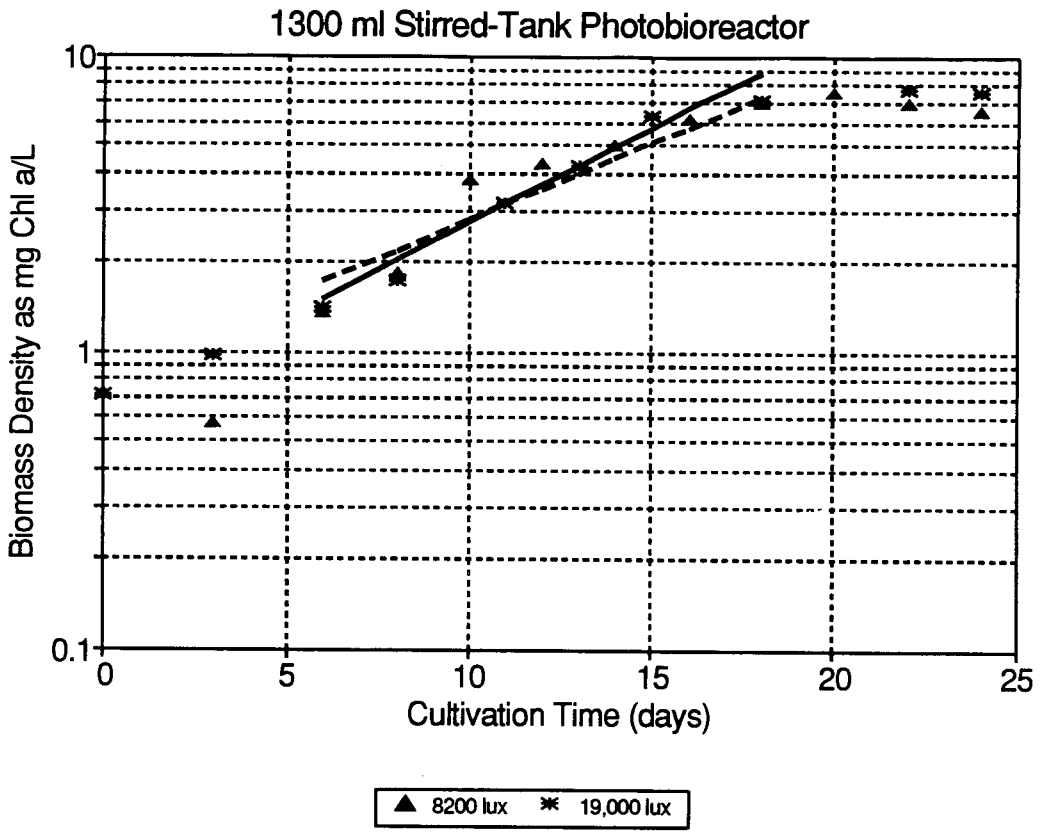


Figure 15. Semi-log plots of culture growth curves at 8200 lux and 19,000 lux.

Table 5. 1300 ml photobioreactor cultivation parameters.

Incident Light Intensity, I_0 [lux]	Specific Growth Rate, μ [day^{-1}]	Final Cell Density (H_2O washed) [mg DCW/L]	Final Cell Density (GP2 washed) [mg DCW/L]
1,500	0.068	400	626*
3,000	0.093	500	765
8,200	0.120	525	776*
19,000	0.147	613	890

* These are calculated values.

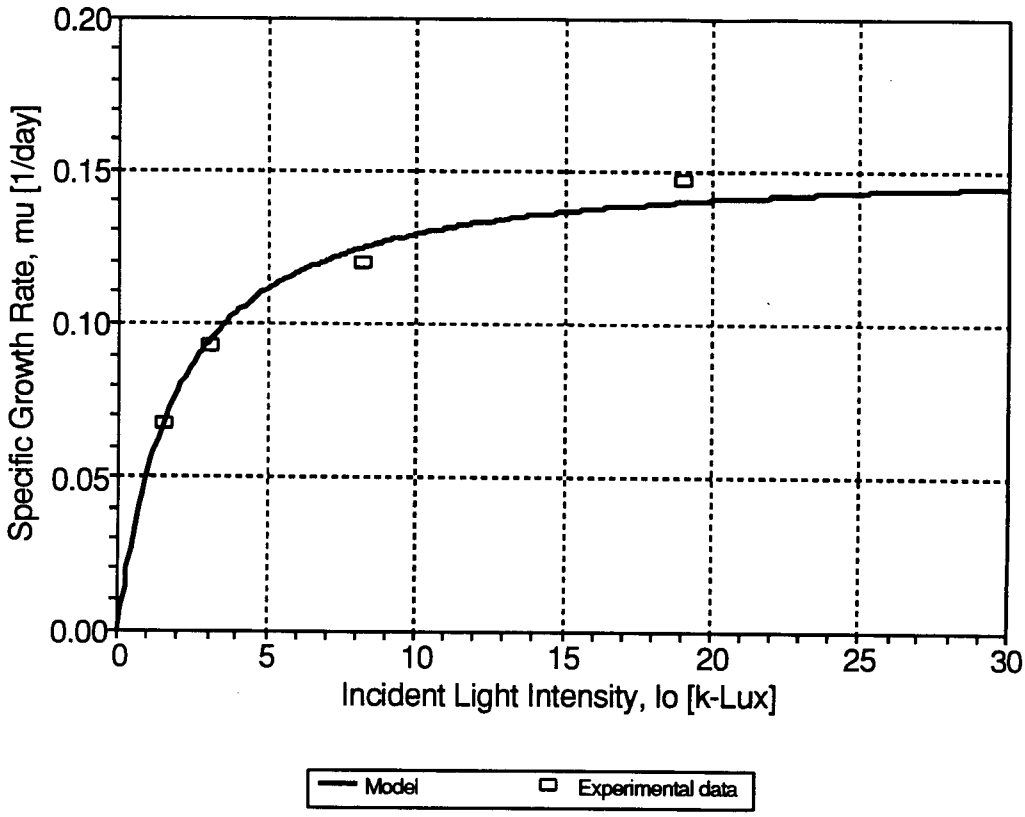


Figure 16. Specific growth rate, μ, versus incident light intensity, I_0.

A rearrangement of equation (13) gives the following linear form

$$\frac{1}{\mu} = \frac{1}{\mu_{max}} + \frac{1}{I_0}\frac{K_0}{\mu_{max}} \qquad (14)$$

As shown in Figure 17, a plot of $1/\mu$ vs. $1/I_0$ data is reasonably linear. Linear regression analysis gives a maximum specific growth rate (μ_{max}) of 0.154 day^{-1} and a half-saturation constant (K_0) of 1920 lux for cultivations of *Laminaria saccharina* female gametophytes in the 1300 ml stirred-tank bioreactor. The solid line in Figure 16 represents model prediction for μ vs I_0 based on equation (13) and the fitted values for μ_{max} and K_0.

Although the incident light intensity was always constant, the light transmission flux through the culture decreased as the cell density increased (Figure 18).

pH Variation in Culture

The pH variation in the culture is showed in Figure 19. During the cultivation, the pH increased during the exponential phase of growth, but was constant during stationary phase. The speciation of dissolved carbon dioxide (CO_2) in aqueous solution is described by

$$CO_2 + OH^{-1} \rightleftharpoons HCO_3^{-1} \qquad (15)$$

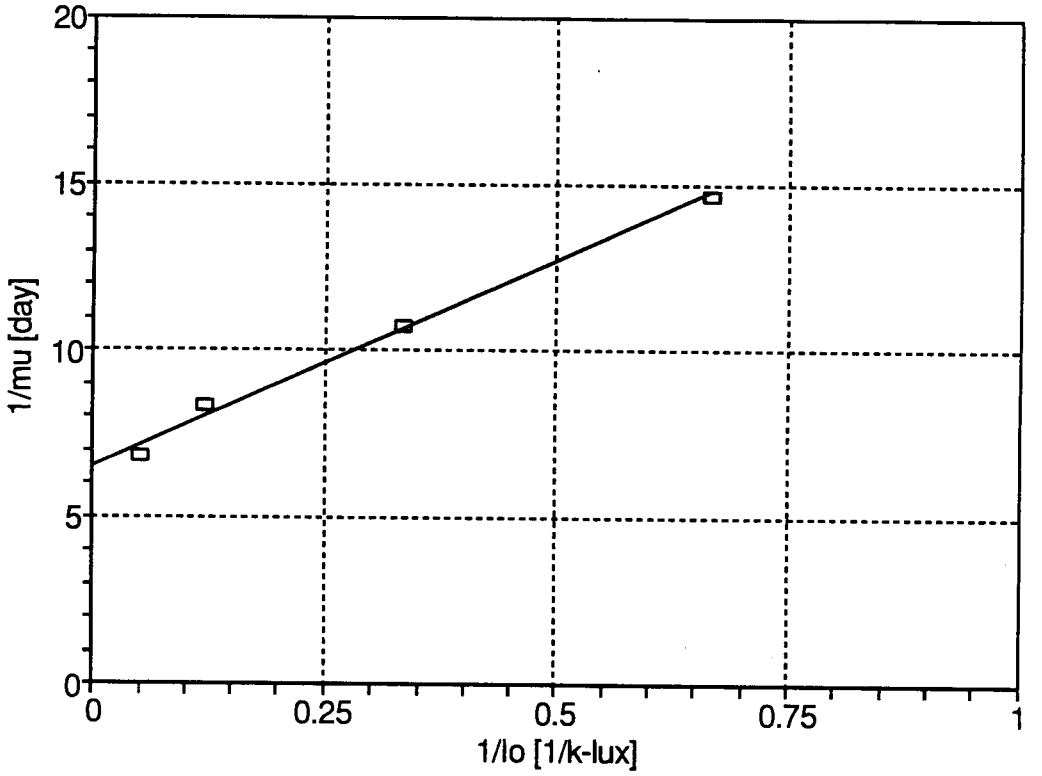


Figure 17. Plot of $1/\mu$ versus $1/I_0$ data.

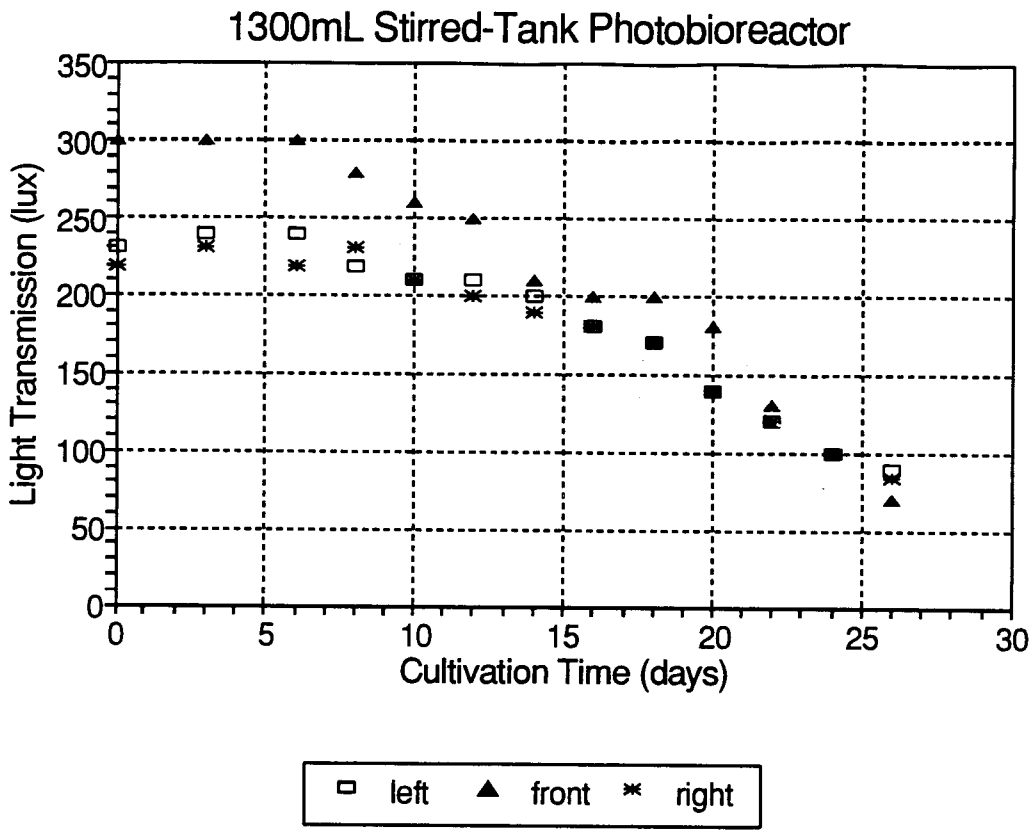


Figure 18. Light transmission versus cultivation time.

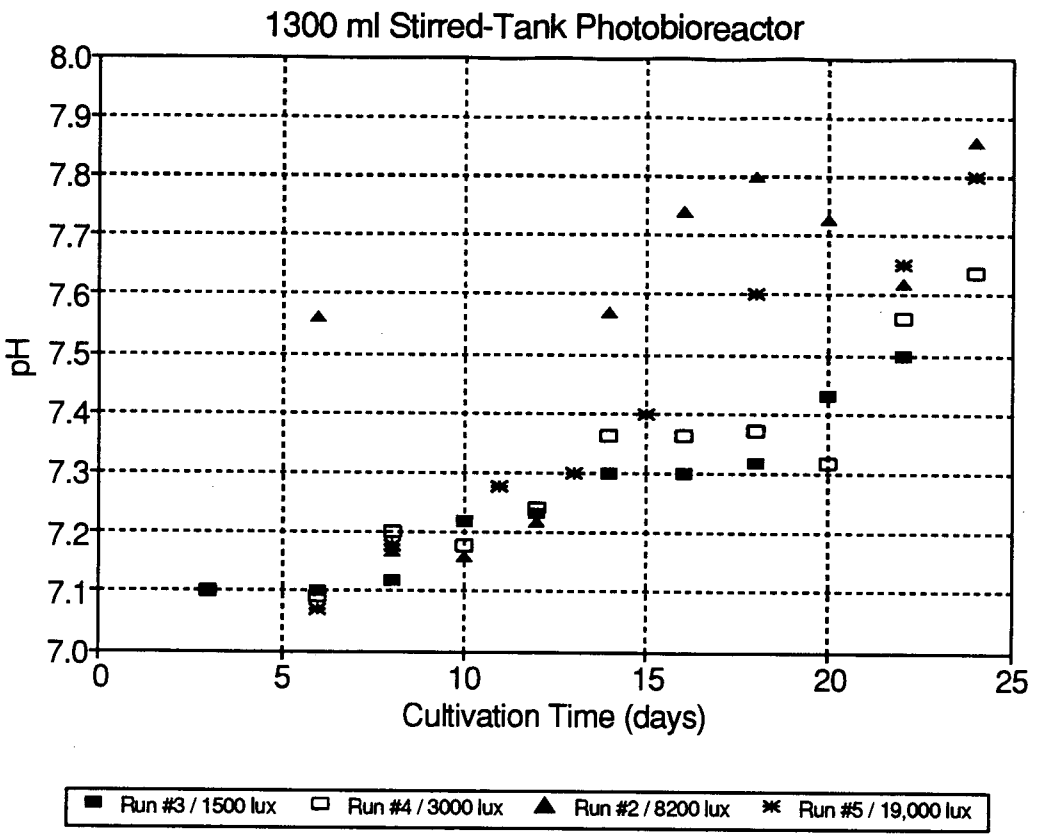


Figure 19. Variation of pH in the 1300 ml photobioreactor cultivations.

As the culture grew and consumed dissolved CO_2, the chemical equilibrium shifted to the left hand side of equation (15) and released OH^{-1} ions. The rise in OH^{-1} ion concentration subsequently increased the pH.

PHOTOSYNTHETIC OXYGEN EVOLUTION AND RESPIRATION RATES IN CELL CULTURE

Control Experiments

Prior to estimation of P_0, control experiments were performed in the bioreactor containing 900 ml of sterile GP2 medium without cells. Figure 20 shows one of the control experiments. The data in Figure 20 had three phases (I, II & III). Phase I started at time t = 0. During this phase, the steady state D.O. concentration in the bioreactor was 10.3 mg O_2/L. Phase II started at 1.5 minutes, where the air flow to the reactor was turned off, and nitrogen flow was introduced into the culture at a flowrate of 1000 ml/min, which was identical to the air flowrate. After 19 minutes, the D.O. concentration decreased to 0.5 mg O_2/L. Phase III started when the nitrogen flowrate was turned off and the reactor was sealed. Since no cells were present to photosynthetically release oxygen, the D.O. concentration in the sealed reactor was constant at about 0.5 mg O_2/L.

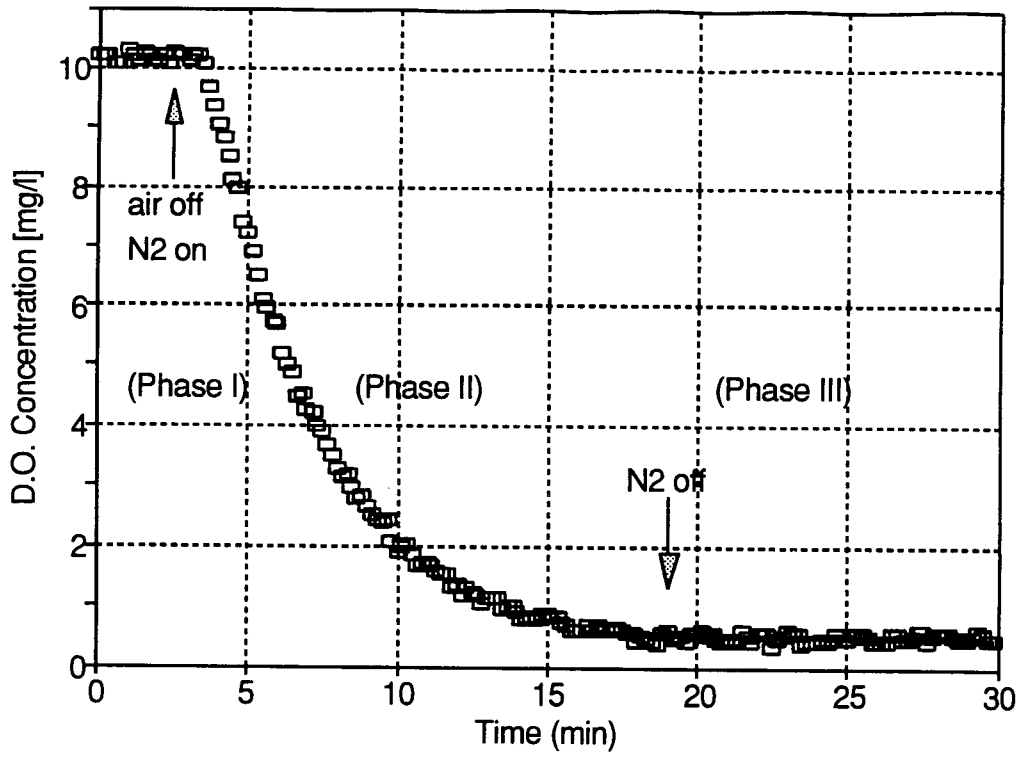


Figure 20. D.O. vs. time data (control experiment).

Estimation of Photosynthetic Oxygen Evolution Rate, P_0

Following the control experiment, a 26-day cell cultivation experiment was performed. At two to three day intervals during the cultivation period, the D.O. concentration was measured as a function of time using the technique described above for control experiments. All experiments were performed 3 hours into the light phase of the photoperiod. Figures 21 to 23 show D.O. concentration vs. time curves measured on the second, 8th and 20th days of cultivation respectively. The photosynthetic oxygen evolution rate (P_0) at each cultivation time was determined from the slope of the linear portion in phase III, in accordance with equation (10). All measurements were repeated in duplicate, and averaged values were reported.

Figure 28 shows the P_0 vs. time data over the 26 day cultivation period. The photosynthetic oxygen evolution rate increased during exponential phase, reached a maximum value at the 8th day near the end of exponential phase, then decreased during stationary phase. The experiment was repeatable, as shown in Figure 29.

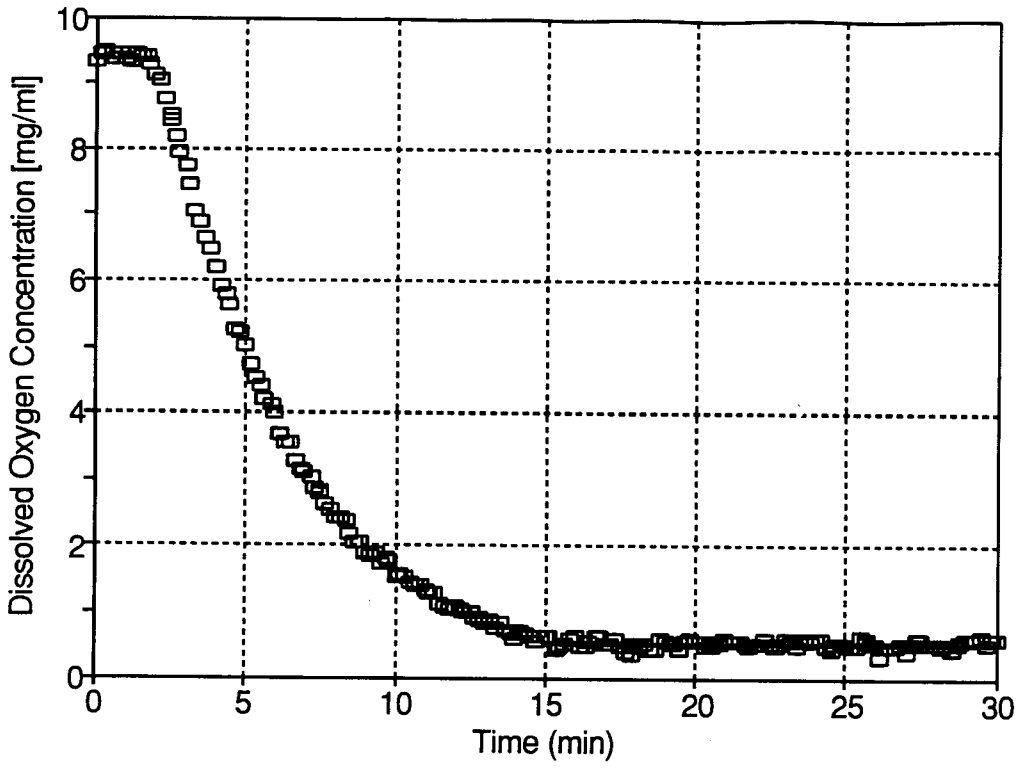


Figure 21. D.O. vs. time data curve for estimation of P_0 on the 2nd cultivation day.

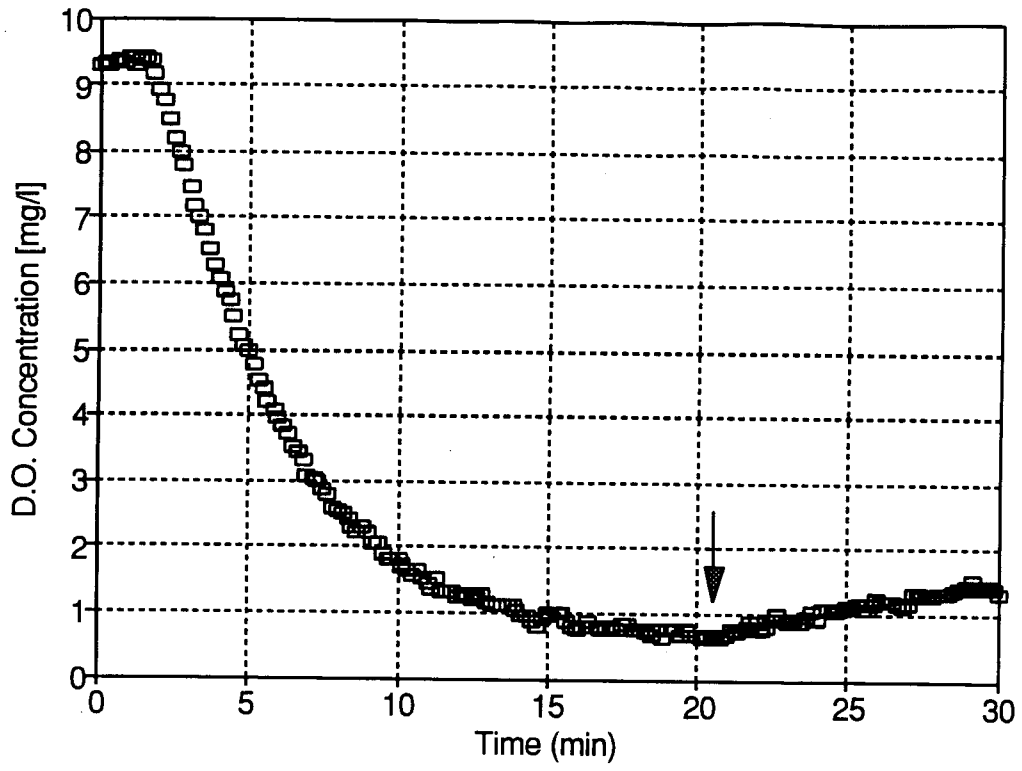


Figure 22. D.O. vs. time data curve for estimation of P_0 on the 8th cultivation day.

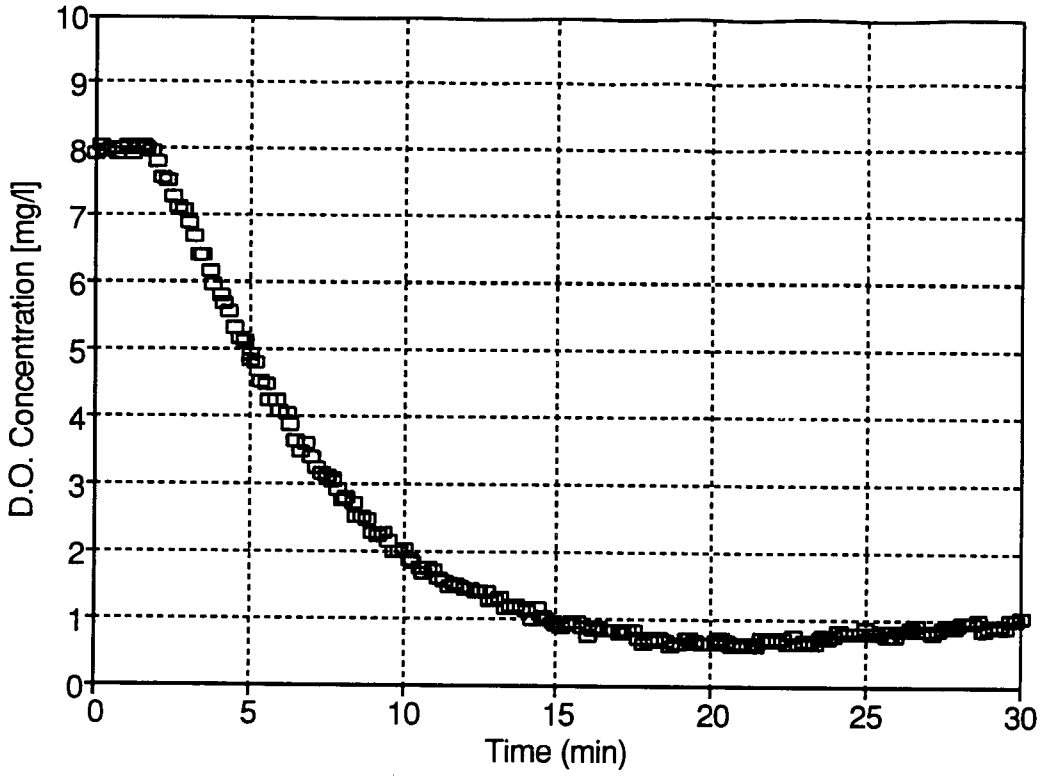


Figure 23. D.O. vs. time data curve for estimation of P_0 on the 20th cultivation day.

Estimation of Respiration Rate, Q_0

At two to three day intervals during the cultivation period, the respiration rate (Q_0) was also estimated from the D.O. vs. time data. (see Figures 24 to 27). All experiments were performed 6 hours into the dark phase of the photoperiod. Each of these curves consists of two phases (I & II). Phase I started at time t = 0 and ended after 3 minutes to provide the saturated D.O. concentration baseline. Phase II started at 3 minutes, once the air supply was turned off and reactor was sealed. As described by equation (12), the D.O. concentration linearly decreased during phase II. The respiration rates at each time was estimated from the slope of the phase II curve. A plot of Q_0 vs cultivation time is provided in Figure 29. Values for Q_0 are much lower than values for P_0. For this reason, the $\dot{Q}_0$ vs. cultivation time data is shown again in Figure 30 on a different scale.

In bioreactor culture under the incident light intensity of 8200 lux, cell respiration rate also increased with increasing culture growth until stationary phase was achieved.

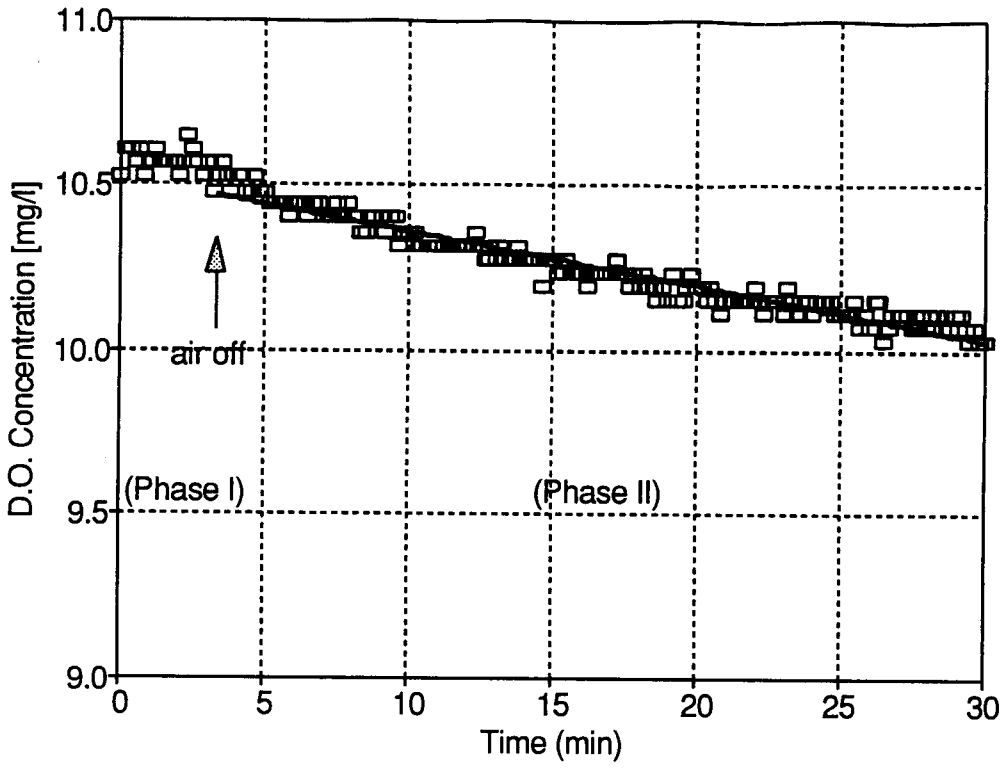


Figure 24. D.O. vs. time data curve for estimation of Q_0 on the 2nd cultivation day.

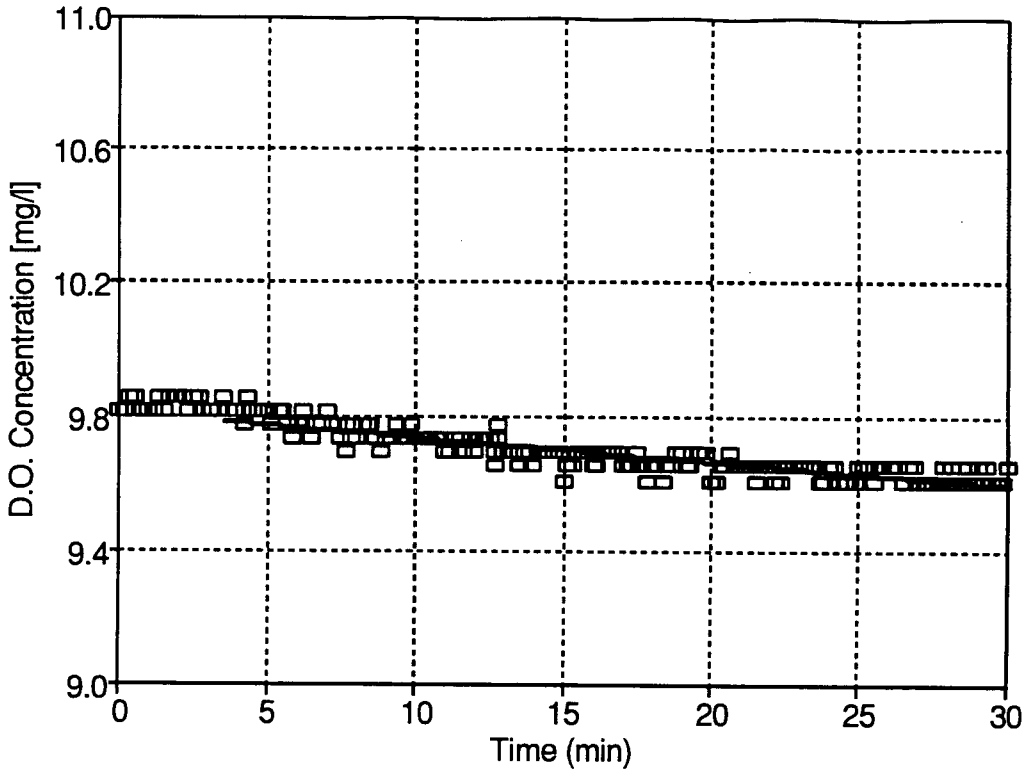


Figure 25. D.O. vs. time data curve for estimation of Q_0 on the 3rd cultivation day.

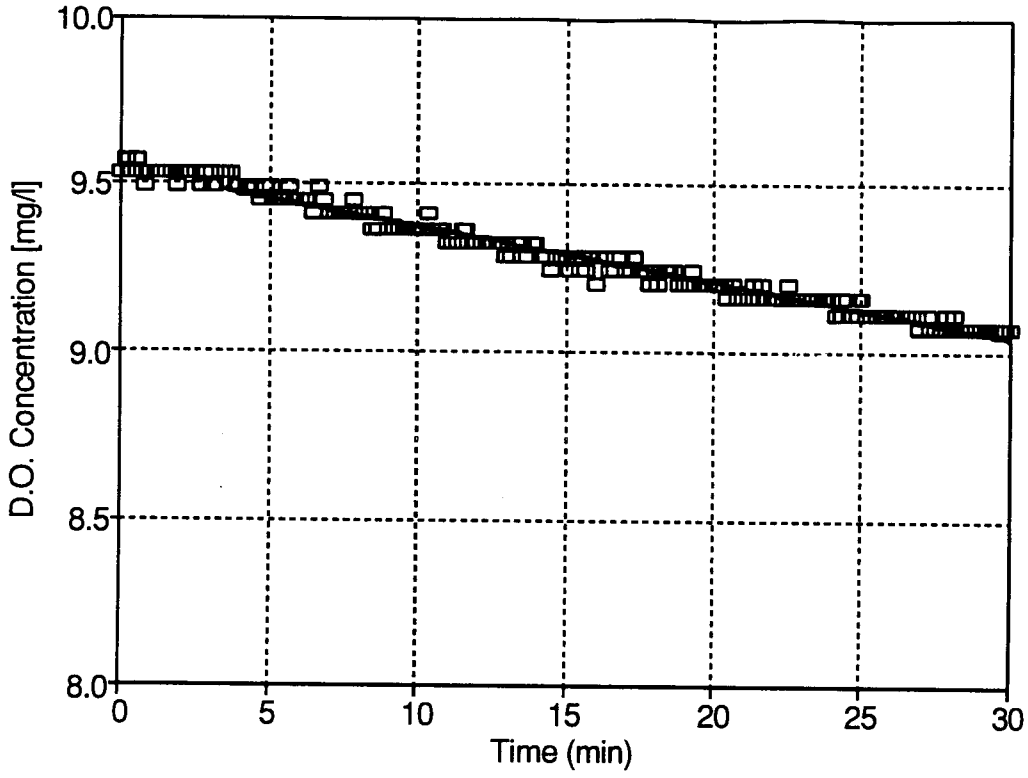


Figure 26. D.O. vs. time data curve for estimation of Q_0 on the 8th cultivation day.

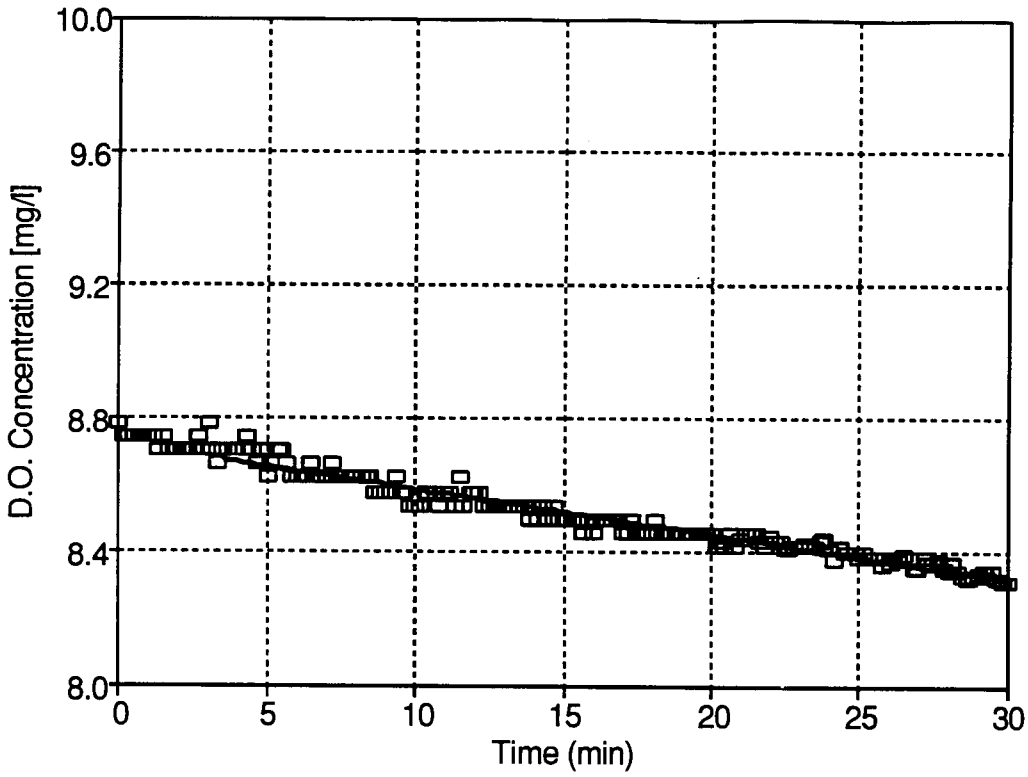


Figure 27. D.O. vs. time data curve for estimation of Q_0 on the 18th cultivation day.

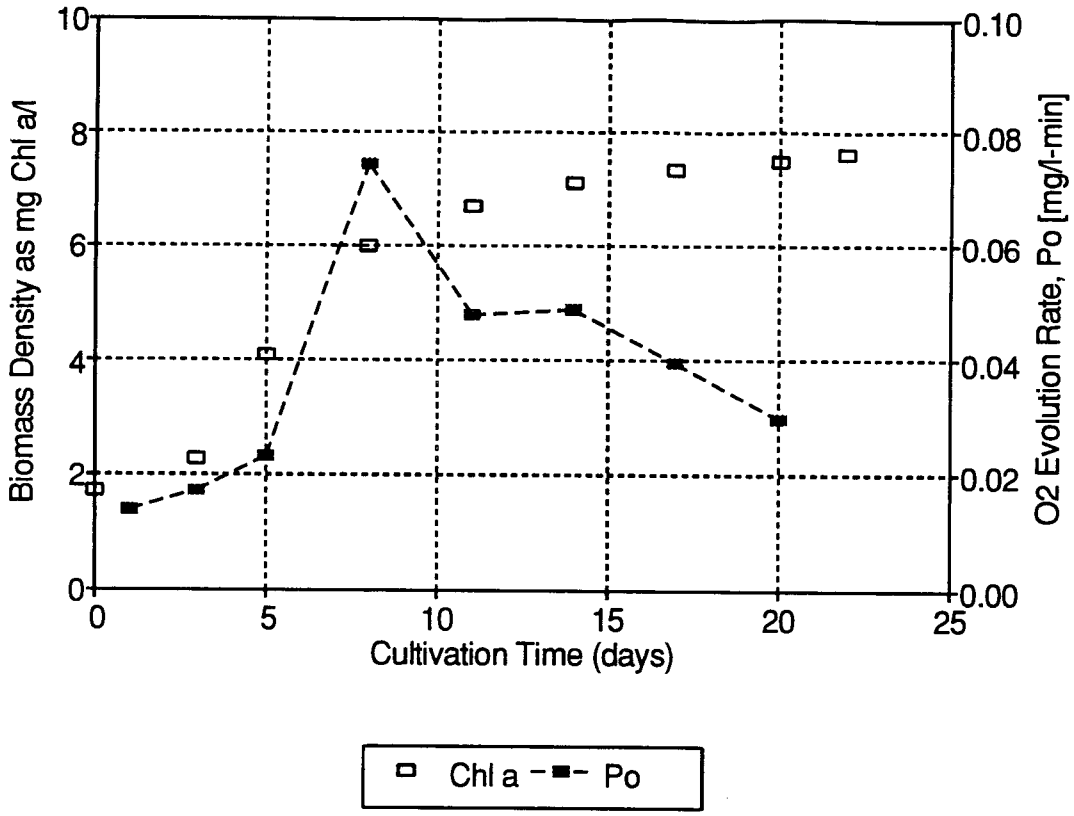


Figure 28. P_0 versus cultivation time curve, run #1.

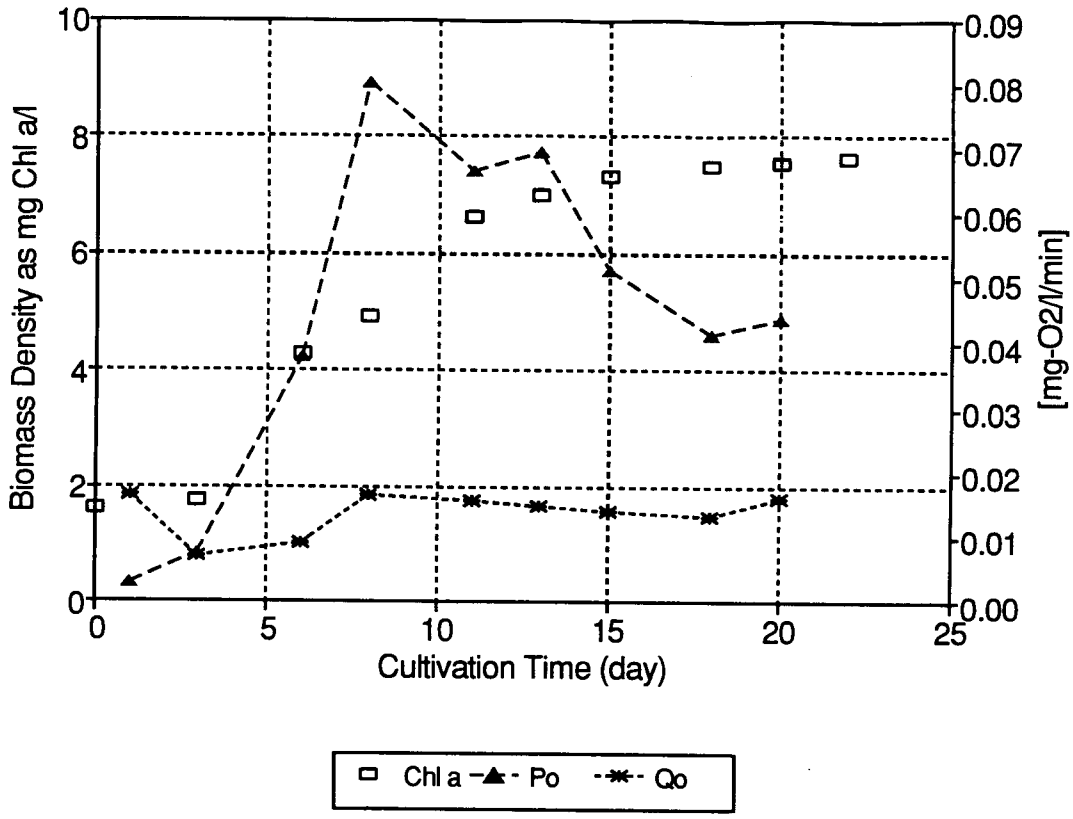


Figure 29. P_0 and Q_0 versus cultivation time curves, run #2.

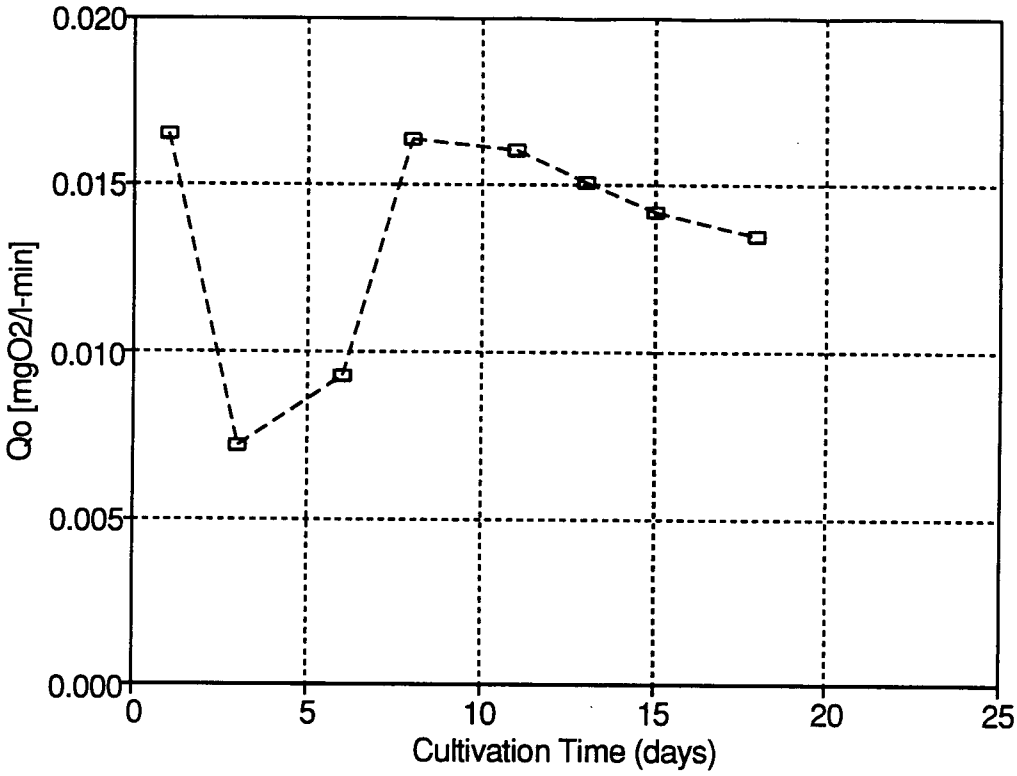


Figure 30. Q_0 versus cultivation time curve.

Estimation of Carbon Dioxide Consumption Rate

The oxygen evolution rate data can be used to estimate the rate of carbon dioxide consumption using simple photosynthesis stoichiometry

$$CO_2 + H_2O \rightarrow CH_2O\ (cell\ biomass) + O_2 \qquad (16)$$

From equation (16) one mole of CO_2 is consumed for every mole of O_2 evolued. Therefore

$$-r_{CO_2} = P_0 \qquad (17)$$

At the peak O_2 evolution rate (P_0) of 0.08 mg O2/l-min (8th day of cultivation), the maximum CO_2 consumption rate was computed to be 6.6 mg CO_2/l-hr.

The volumetric oxygen mass transfer coefficient, k_La, can be determined from the phase II data of the oxygen evolution experiments. The flowrate of 1000 ml/min of nitrogen employed for de-aeration during the phase II of the experiments was identical to that used for aeration of the culture. Integration of the equation (9) gives

$$C_A = \frac{P_0}{k_La} - \frac{P_0 - k_La C_A^*}{k_La} \exp\left[-k_La(t-t_0)\right] \qquad (18)$$

A value of 13.6 hr^{-1} for k_La was obtained by fitting phase II data at the peak O_2 evolution rate to equation (18) using a least squares minimization technique, and the value for P_0 obtained from phase III data.

In order to convert k_La for O_2 transfer to k_La for CO_2 transfer, Grima et al. (1993) recommend the correlation

$$(k_La)_{CO_2} = \left(\frac{D_{CO_2}}{D_{O_2}}\right)^{0.5} (k_La)_{O_2} = 0.91(k_La)_{O_2} \qquad (19)$$

By equation (19), a value of 12.4 hr^{-1} was obtained for the volumetric CO_2 mass transfer coefficient, $(k_La)_{CO2}$. Therefore maximum CO_2 mass transfer rate is

$$CO_2\text{-}TR = (k_La)_{CO_2} C^*_{CO_2} \qquad (20)$$

The value of C_{CO2}^* for 35 Pa CO_2 in air (1 atm) in equilibrium with seawater at 13 ^{0}C is 0.0145 mM (Skirrow 1975). Thus the maximum CO_2-TR is 7.9 mg CO_2/l-hr.

It can be concluded that in this cell cultivation, there was just enough CO_2 transferred from air supply into culture to avoid CO_2-limited growth at peak CO_2 demand. But CO_2 concentration in the culture was close to zero at the maximum

P_0 point in the exponential phase. From this point of view, a larger k_La should be attempted to guarantee non-CO_2-limited growth.

CONCLUSIONS AND RECOMMENDATIONS

Filamentous *Laminaria setchellii* callus tissue was induced on seawater solid agar medium (15 g/l) at 8 ^{0}C in the dark. About 5% of the explants contained dense callus filaments six weeks after plating. Photosynthetic liquid cell cultures from this callus tissue were initialized in PES liquid medium at 8 ^{0}C and 2500 lux, but culture growth was not quantitatively observed, and most cells were not viable after two months.

Two stirred-tank photobioreactors were feasible for cultivating *Laminaria saccharina* female gametophyte cell suspensions in liquid culture. The first bioreactor had a working volume of 1300 ml and was equipped with two 45^{0} pitched blade impellers. The second bioreactor had a working volume of 900 ml and was equipped with a flat blade impeller and a D.O. electrode. All cultivations were carried out in GP2 liquid medium at 13 ^{0}C, 1.2 vvm aeration rate, and 250 rpm impeller speed. The cultivation period was 26 days, and the inoculation density was 100 mg DCW/l for the 900 ml bioreactor and 50 mg DCW/l for the 1300 ml bioreactor. Cultivation was repeatable in terms of specific growth rate and final biomass density.

The effect of incident illumination intensity (1500 - 19,000 lux) on gametophyte culture growth in the 1300 stirred-tank bioreactor was studied. The results showed that increasing the incident light intensity increased the culture growth rate until a saturation light intensity was achieved. Therefore, incident light intensity was a limiting factor in stirred-tank bioreactor cultivation of *Laminaria saccharina* gametophytes. The growth rates (μ) were correlated to incident light intensity (I_0) by the following model

$$\mu = \frac{\mu_{max} I_0}{K_0 + I_0} \qquad (21)$$

where a maximum specific growth rate (μ_{max}) of 0.154 day^{-1} and a half-saturation constant (K_0) of 1920 lux were determined. The final biomass density after a 26 day cultivation time increased from 400 mg DCW/l at 1500 lux to 613 mg DCW/l at 19,000 lux.

During the cultivation, the pH increased during the exponential phase of growth, but was constant during stationary phase. This was due to increase in OH^{-1} ion concentration in the culture as the biomass grew and consumed dissolved CO_2, shifting the CO_2-HCO_3^- equilibrium back towards CO_2 and OH^-.

Photosynthetic oxygen evolution rate during the light phase of the photoperiod and oxygen respiration rate during

the dark phase of the photoperiod in the 900 ml stirred-tank bioreactor were determined at an incident light intensity of 8200 lux during the cultivation period. The photosynthetic oxygen evolution rate (P_0) increased during exponential phase, reached a maximum value of 0.08 mg O_2/l-min at the 8th day near the end of exponential phase, then decreased during stationary phase. The cell respiration rate also increased with increasing culture growth until stationary phase was achieved.

The oxygen evolution rate and the volumetric oxygen mass transfer coefficient (k_La) were used to estimate the CO_2 consumption rate and the maximum CO_2 transfer rate respectively. At peak CO_2 demand, there was just enough CO_2 transferred from the air supply to culture to avoid CO_2-limited growth.

This study showed the effect of incident illumination intensity on culture growth as well as the variation of photosynthetic oxygen evolution and respiration rates during the cultivation period. However, the photosynthetic oxygen evolution and respiration rates were determined at only one light intensity near light saturated growth. Therefore, oxygen evolution and respiration rates should be determined at a higher incident illumination intensity to make sure the growth is light limited.

In this study, reproducible cell biomass production under controlled cultivation conditions in a stirred-tank bioreactor has been shown. A long-term goal of this study is to determine if eicosanoid biopharmaceutical compounds can be produced from *Laminaria saccharina* gametophyte cell cultures. In future work, arachidonic acid can be added into *Laminaria saccharina* gametophyte cultures to stimulate production of eicosanoids. The consumption of arachidonic acid and the production of target eicosanoids can be then followed by GC-MS analysis.

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APPENDICES

Appendix A
Experimental Protocols

D.O. Electrode Cleaning and Sterilization

1. Wash D.O. electrode in sterile D.D. water. Be gentle to the membrane.
2. Wipe the whole probe body except the membrane in the laminar flow hood with 70% ethanol solution.
3. Rinse the probe in sterile D.D. water until no ethanol residue is left.
4. Keep the probe in the laminar flow hood. The D.O. electrode is ready for installation into the sterile bioreactor.

Stirred-Tank Bioreactor Set-up

1. Carefully disassemble the bioreactor. Gently remove the D.O. electrode.
2. Thoroughly wash the glass reactor vessel and associated headplate assembly (except D.O. electrode and air filters) with Liquinox detergent solution.
3. Wash all clamps, hoses, and fittings.
4. Rinse all pieces with tap water ten times. Make sure there is no soap residue left.
5. Rinse all pieces with D.D. water six times.
6. Reassemble the reactor, including headplate, arm caps, hoses, filters, clamps (sample port and air inlet). Keep the headplate and arm caps and all the fittings loose at this time.
7. Cover all open tubing ends with aluminum foil.
8. Autoclave reactor and humidifying assembly for 20 min at 15 psig, 250 ^{0}F without D.O. electrode. (See the protocol for D.O. electrode cleaning and sterilization).
9. Remove bioreactor from autoclave and allow it to cool down to room temperature in the laminar flow hood.
10. After the autoclaved bioreactor has cooled in the laminar flow hood, check on the impeller blades inside the reactor. They should be pitched at 45 degrees.

11. Tighten the headplate, arm caps and all fittings. Keep open tubing ends covered by aluminum foil.
12. Remove the bioreactor from the laminar flow hood and place on the magnetic stirrer unit inside the incubator. Run the bioreactor at the desired impeller speed.
13. Adjust the positions of all the tubes in the reactor vessel. Make sure that the air sparger is positioned directly beneath the impeller and the thermowells are positioned as low as possible without touching the impeller. Make sure the impeller is spinning at the proper orientation.
14. Remove the bioreactor from the stirrer and place it back in the laminar flow hood.
15. The bioreactor now is ready for inoculation.

Stirred-Tank Bioreactor Inoculation

1. Have the sterilized bioreactor ready for inoculation in the laminar flow hood.
2. Prepare 2 L complete sterile fresh GP2 medium.
3. Have sufficient inoculum culture of pre-determined cell density ready.
4. Prepare new culture of the desired volume and cell density in a 2000 ml sterile flask in the laminar flow hood using the sterile GP2 medium and the inoculum culture of known cell density.
5. Carefully remove one of the arm caps from reactor. Be sure to keep all surfaces in contact with the culture sterile.
6. Transfer the newly inoculated culture into reactor from the 2000 ml flask through the open arm of the reactor.
7. Recap the open arm of the reactor and seal it.
8. Carefully remove the loaded reactor from the laminar flow hood. Place the reactor onto the stirrer unit in the incubator.
9. Gently fit the reactor into a suitable position.
10. Connect air inlet and outlet hoses to reactor.
11. Insert heating cartridge and temperature probe all the way down into the thermomells.

12. Connect the oxygen electrode to the pre-calibrated oxygen meter.
13. Turn on air pump and unclamp air inlet.
14. Set flowrate.
15. Turn on lights (the light stages are pre-adjusted to have the desired incident light intensity).
16. Check incident light intensity and photoperiod.

Stirred-Tank Photobioreactor Sampling

1. Unclamp hose leading from sampling port.
2. Withdraw 10 ml from reactor using a syringe fitted to the Leur-lock connector.
3. Clamp the sampling hose.
4. Remove syringe from connector.
5. Place sample in plastic centrifuge tube.
6. Remove 5.0 ml of the sample with a graduated pipet. Be sure to keep the sample agitated.
7. Filter out the cells from the 5.0 ml sample with a clean 20 μm nylon mesh filter on filter stage.
8. Place the filter containing cells in a clean, dry, 50 ml, polypropylene, centrifuge tube.
9. Add 5.0 ml of HPLC grade methanol to the tube via a volumetric pipet.
10. Repeat for each sample taken.

Stirred-Tank Photobioreactor Shut-Down

1. After take the last regular "unblended" samples, turn off agitation stirrer, clamp air inlet hose.
2. Disconnect air inlet hose.
3. Disconnect D.O. electrode from D.O. meter.
4. Remove heating cartridge and temperature probe from thermowells.
5. Remove bioreactor from incubator and place in laminar flow hood.
6. Empty contents of bioreactor into a flask.
7. Blend the culture for 5 seconds on "grind" speed.
8. Take three 5 ml "blended" samples for chlorophyll a measurements.
9. Take two 40 ml samples for dry cell density measurements
10. Measure the volume of the left over culture.
11. Filter out the cell biomass from the above culture using a 20 μm nylon filter.
12. Transfer all the cell biomass into a pre-weighed small glass bottle from the filter.
13. Weigh the bottle containing the cell biomass.
14. Cap and seal the bottle.
15. Label the bottle with run number, date, volume of the culture used to filter out the cell biomass, and the fresh weight of the cell biomass.

16. Store the bottle containing the cell biomass in deep freezer for future analysis.

Dissolved Oxygen Meter Calibration

1. Load sterile bioreactor with sterile 900 ml GP2 medium.
2. Set up the bioreactor and run it at the same conditions as those used in the bioreactor cell cultivation (13 ^{0}C).
3. Initially calibrate the YSI D.O. meter by the calibration procedure given by the manufacturer, then perform steps 4 to 6. Make sure the D.O. probe membrane is emersed in GP2 medium in the bioreactor when calibrating it.
4. De-aerate the medium with 1000 ml/min N_2 for at least 1 hour to strip off the dissolved oxygen in the medium. After the dissolved oxygen in the medium is completely stripped off, set the zero reading on the D.O. meter.
5. Re-aerate the medium with 1000 ml/min air for at least 1 hour. After the medium is completely saturated with air and meter reading is constant, set the D.O. meter reading to the value for C_{O2}^{*} (10.6 ppm) at 13 ^{0}C.
6. Repeat steps 4 and 5. The zero and C_{O2}^{*} settings may need to be reasonably adjusted again.

Photosynthetic Oxygen Evaluation Experiment

1. Calibrate the D.O. meter.
2. Inoculate the bioreactor with an initial culture volume of 900 ml and an initial cell density of 100 mg DCW/L.
3. Take samples at a two or three-day interval for chlorophyll a measurements.
4. On the sampling day, at 3 hours into the light portion of the photoperiod, perform measurements, as described below.
5. Carefully plan and write down all the file names by which the D.O. data will be recorded by computer.
6. Load program in computer, chose reading parameters, type in the file name and prepare for D.O. readings.
7. Start computer reading at time 0 min (use a stop watch to set time) to establish baseline D.O. concentration at air saturation.
8. At 1.5 min, turn off air supply and turn on nitrogen supply. Set nitrogen flowrate to the same value for the air flowrate.
9. When D.O. level reaches to 0.5 ppm (watch it from the meter reading), turn off nitrogen gas flow and seal the bioreactor by clamping the inlet and outlet tubes.
10. At 30 min, stop reading D.O. concentration vs. time data.

11. Restore air supply immediately.
12. When the culture D.O. level is totally restored (D.O. concentration at saturated value for 5 min), repeat from step 9.
13. Backup the D.O. data onto a labeled backup disk.

Respiration Experiment

1. Calibrate the D.O. meter.
2. Inoculate the bioreactor with an initial culture volume of 900 ml and an initial cell density of 100 mg/L DCW.
3. Take samples at a two or three-day interval for chlorophyll a measurements.
4. On the sampling day, at 6 hours into the dark portion of the photoperiod, perform measurements, as described below.
5. Carefully plan and write down all the file names by which the D.O. data will be recorded by computer.
6. Load program in computer, chose reading parameters, type in the file name and prepare for D.O. readings.
7. Start computer reading at time 0 min (use a stop watch to set time) to establish baseline saturate D.O. concentration at saturation.
8. At 3 min, turn off air supply and seal bioreactor.
9. At 15 min, stop reading D.O. concentration vs. time data.
10. Restore air supply immediately.
11. Backup the D.O. data onto a labeled backup disk.

Appendix B

Tabulated Data

Table B-1.

Laminaria saccharina Gametophyte Cell Cultivation
900 mL Stirred-tank Photobioreactor#1,Run#4 QPRO Data Spreadsheet
Cultivation Period: 11:00am, December 20,1993 to 11:00am, January 11,1994. Total Cultivation Time: 528 Hours.

RUN IDENTIFICATION		CULTURE LOADING		PROCESS PARAMETERS	
Run#:	#4	Inoc. Volume:	319 ml	Impeller type:	Twin flat blade
Description:	900 ml Stirred-tank Photobioreactor #1	Inoc. Source:	L.saccharina gametophyte LS-4-1,LS-4-2,LS-4-7	Aeration Rate:	1000 ml/min
Time Started:	11:00 am			Mixing Speed	250 rpm
Date Started:	12/20/1993	Age of Inoculum:	57 days	setpoint temp.:	13 C
		Total Culture Vol.:	900 ml	Illum. Position:	Two sides
		Bicarbonate:	0	Incident Illumination:	8200 Lux
		Inoculum (g/20 mL):	0.0055	Photoperiod:	16 hr ON 8 hr OFF
		Inoculum (g/20 mL):	0.0058		
		Inoculum (g/40 mL):	0.00565 *2		
		avg =	0.0057 g/20 mL		

DATE	DAY	Abs(665nm) Trial#1	Abs(665nm) Trial#2	Abs(665nm) Trial#3	Abs(665nm) Average	Chl a /average [ug/ml]	ln(Chl)	ln(Chl) predicted	Chl a [ug/ml] predicted	DCW [mg/L]	PH	Po (trial#1) [mgO2/L-min	Po (trial#2) [mgO2/L-min	Po (average) [mgO2/L-min]
12/20/93	0.0	0.118	0.097	0.099	0.105	1.705	0.534	0.565	1.759	100.1	N/A			
21	1.0											0.014		0.014
23	3.0	0.129	0.152	N/A	0.141	2.289	0.828	0.970	2.638	N/A	7.60	0.018	0.017	0.017
25	5.0	0.244	0.259	N/A	0.252	4.097	1.410	1.240	3.456	N/A	7.90	0.023	0.023	0.023
28	8.0	0.383	0.355	N/A	0.369	6.011	1.794	1.645	5.181	N/A	8.32	0.075		0.075
31	11.0	0.441	0.380	N/A	0.411	6.687	1.900	2.050	7.768	N/A	8.36	0.054	0.043	0.048
01/03/94	14.0	0.445	0.430	N/A	0.438	7.127	1.964			N/A	8.10	0.049		0.049
6	17.0	0.453	0.450	N/A	0.452	7.355	1.995			N/A	8.14	0.041	0.039	0.040
9	20.0	0.465	0.458	N/A	0.462	7.518	2.017			N/A	8.16	0.031	0.028	0.030
11	22.0	0.468	0.484	0.459	0.470	7.662	2.036			607.5	8.05			

Regression Output:

Constant		0.565
Std Err of Y Est		0.178
R Squared		0.934
No. of Obeervations		5.000
Degrees of Freedom		3.000
X Coefficient(s)	u = 0.135	1/day
Std Err of Coef.	0.021	

Final DCW:

DD water washing:				GP2 medium washing:		
Trial#1	0.013		g/20 ml	Trial#1	0.018	g/20 ml
Trial#2	0.012	*1.5	g/30 ml	Trial#2	0.017	g/20 ml
Xmax =	0.012	g/20 ml		Xmax =	0.018	g/20 ml
	607.5	mg DCW/L			875.0	mg DCW/L

Table B-2.

Laminaria saccharina Gametophyte Cell Cultivation
900 mL Stirred-tank Photobioreactor #1, Run#5 QPRO Data Spreadsheet
Cultivation Period: 11:00 am, January 12,1994 to 11:00 am, February 3,1994. Total Cultivation Time: 528 hours.

RUN IDENTIFICATION		CULTURE LOADING		PROCESS PARAMETERS	
Run #:	#5	Inoc. Volume:	250 mL	Impeller Type:	Twin flat blade
Description:	900 mL Stirred-Tank Photobioreactor #1	Inoc. Source:	L. saccharina gametophyte LS-5-7,L-5-2,LS-5-7	Aeration Rate:	1000 mL/min
				Mixing Speed	250 rpm
Time Started:	11:00 am	Age of Inoculum:	58 days	Setpoint Temp.:	13 C
Date Started:	01/12/1993	Total Culture Vol.:	900 mL	Illuminator Position:	Two sides
		Bicarbonate:	0 mg/L	Incident Illumination:	8200 Lux
		Inoc. DCW (g/20 mL):	0.0075	Photoperiod:	16 hr ON 8 hr OFF
		Inoc. DCW (g/20 mL):	0.0073		
		Inoc. DCW (g/20 mL):	0.0070		
		avg =	0.0073 g/20 mL		

DATE	DAY	Abs (665 nm) Trial #1	Abs (665 nm) Trial #2	Abs (665 nm) Trial #3	Abs (665 nm) Average	Chl a [mg/L]	ln(Chl)	ln(Chl) predicted	Chl a [mg/L] predicted	DCW [mg/L]	pH	Po [mg O2/L-min]	Qo [mg O2/L-min]
01/12/94	0.0	0.093	0.084	0.120	0.099	1.613	0.478	0.395	1.484	100.9			
13	1.0											0.003	0.017
15	3.0	0.109	0.107		0.108	1.759	0.565	0.824	2.280		7.67	0.008	0.007
18	6.0	0.255	0.268		0.262	4.260	1.449	1.253	3.501		7.65	0.038	0.009
20	8.0	0.318	0.285		0.302	4.911	1.592	1.539	4.660		8.00	0.080	0.016
23	11.0	0.395	0.417		0.406	6.614	1.889	1.968	7.156		7.89	0.067	0.016
25	13.0	0.422	0.434		0.428	6.972	1.942				7.95	0.069	0.015
27	15.0	0.440	0.457		0.449	7.306	1.989				8.05	0.051	0.014
30	18.0	0.449	0.470		0.460	7.485	2.013				8.10	0.041	0.014
02/01/94	20.0	0.462	0.466		0.464	7.559	2.023				8.10	0.044	0.016
03	22.0	0.470	0.466	0.467	0.468	7.618	2.031			602.5	8.12		

Regression Output:

Constant		0.395
Std Err of Y Est		0.201
R Squared		0.925
No. of Observations		5.000
Degrees of Freedom		3.000
X Coefficient(s)	u =	0.143 1/day
Std Err of Coef.		0.024

Final DCW

DD water washing:			GP2 medium washing:		
Trial #1	0.012	g/20 mL	Trial #1	0.018	g/20 mL
Trial #2	0.012	g/20 mL	Trial #2	0.017	g/20 mL
Xmax =	0.012	g/20 mL	Xmax =	0.017	g/20 mL
	602.5	mg DCW/L		872.5	mg DCW/L

Table B-3.

Laminaria saccharina Gametophyte Cell Cultivation

1300 mL Stirred-Tank Photobioreactor#2,Run#2 QPRO Data Spreadsheet
Cultivation period: 1:00pm, September 22,1993 to 2:30pm, October 18,1993. Total Cultivation Time: 625.5 Hours.

RUN IDENTIFICATION		CULTURE LOADING			PROCESS PARAMETERS	
Run#:	#2	Inoc. Volume:	120	mL	Impeller type:	Four 45-Degree Pitch Blade
Description:	1300 mL Stirred-tank Photobioreactor #2	Inoc. Source:	L.saccharina Gametophyte L-3-8;L-3-1;L-3-24;L-3-7		Aeration Rate:	1500 ml/min
Time Started:	1:00pm				Mixing Speed	250 rpm
Date Started:	09/22/1993	Age of Inoculum:	57	days	Setpoint Temp.:	13 C
		Total Culture Vol.:	1300	mL	Illum. Position:	Two sides
		Bicarbonate:	0	mg/L	Incident Illumination:	8200 lux
		Inoculum (g/20 mL):	0.0105		Photoperiod:	16 hr ON 8 hr OFF
			0.0106			
			0.0106			
		avg =	0.0106	g/20 mL		

DATE/1993	DAY	Abs (665 nm) Trial #1	Abs (665 nm) Trial #2	Abs (665 nm) Trial #3	Abs (665 nm) Average	Chl a [ug/ml]	mg DCW/L	pH	ln(Chl a)	ln(Chl a) Predicted	Chl a (ug/mL) Predicted
09/22	0.0	0.042	0.045	0.047	0.045	0.728	48.8				
25	3.0	0.039	0.032		0.036	0.578		7.10			
28	6.0	0.081	0.086		0.084	1.360		7.56	0.308	0.547	1.728
30	8.0	0.107	0.120		0.114	1.849		7.17	0.615	0.787	2.197
10/02	10.0	0.228	0.241		0.235	3.820		7.16	1.340	1.027	2.793
04	12.0	0.261	0.273		0.267	4.349		7.22	1.470	1.267	3.550
06	14.0	0.319	0.296		0.308	5.009		7.57	1.611	1.507	4.513
08	16.0	0.373	0.380		0.377	6.133		7.74	1.814	1.747	5.737
10	18.0	0.398	0.459		0.429	6.980		7.80	1.943	1.967	7.294
12	20.0	0.457	0.470		0.464	7.550		7.73	2.022	2.227	9.272
14	22.0	0.424	0.437		0.431	7.013		7.62			
16	24.0	0.398	0.407		0.403	6.557		7.86			
18	26.0	0.477	0.460	0.487	0.475	7.732	525.0	7.88			

Regression Output:			Final DCW		
Constant		-0.173	DD water washing:		
Std Err of Y Est		0.218		0.011	g/20ml
R Squared		0.895		0.010	g/20ml
No. of Observations		8.000		0.010	g/20ml
Degrees of Freedom		6.000	Xmax =	0.011	g/20ml
				525.0	mg DCW/l
X Coefficient(s)	u =	0.120 1/day			
Std Err of Coef.		0.017			

Table B-4.

1300 ml Stirred-Tank Bioreactor#2, Run#3 QPRO Data Spreadsheet
Cultivation Period: 11:00am, October 24, 1993 to 11:00am, November 19, 1993. Total Cultivation Time: 625.5 Hours.

RUN IDENTIFICATION		CULTURE LOADING			PROCESS PARAMETERS	
Run#:	#3	Inoculum Volume:	62.8	ml	Impeller type:	Four 45-degree pitch blades
Description:	1300ML Stirred-Tank Photobioreactor #2	Inoc. Source:	L.saccharina Gametophyte L-2-1,L-4-8,L-4-4		Aeration Rate:	1500 ml/min
Time Started:	11:00am				Mixing Speed	250 rpm
Date Started:	10/24/1993	Age of Inoculum:	57	days	Setpoint Temp.:	13 C
		Total Culture Vol.:	1300	ml	Illum. Position:	Two sides
		Bicarbonate:	0	mg/L	Insident Illumination:	1500 lux
		Inoculum (g/20 mL	0.0205		Photoperiod:	16 hr ON 8 hr OFF
			0.0210			
			0.0206			
		avg =	0.0207	g/20 mL		

DATE/1993	DAY	Abs (665 nm) Trial#1	Abs (665 nm) Trial#2	Abs (665 nm) Trial#3	AU/average	Chl a [ug/ml]	DCW [mg/L]	pH	ln(Chl a)	ln(Chl a) predicted	Chl a[ug/ml] predicted
10/24	0.0	0.046	0.040	0.047	0.044	0.722	50.0				
27	3.0	0.068	0.069	N/A	0.069	1.116	N/A	7.10			
30	6.0	0.100	0.096	N/A	0.098	1.596	N/A	7.10	0.468	0.253	1.288
11/1	8.0	0.065	0.059	N/A	0.062	1.010	N/A	7.12	0.010	0.389	1.475
3	10.0	0.114	0.123	N/A	0.119	1.930	N/A	7.22	0.658	0.525	1.690
5	12.0	0.143	0.135	N/A	0.139	2.264	N/A	7.23	0.817	0.661	1.936
7	14.0	0.123	0.120	N/A	0.122	1.979	N/A	7.30	0.683	0.797	2.219
9	16.0	0.155	0.163	N/A	0.159	2.590	N/A	7.30	0.952	0.933	2.542
11	18.0	0.153	0.161	N/A	0.157	2.558	N/A	7.32	0.939	1.069	2.912
13	20.0	0.214	0.206	N/A	0.210	3.421	N/A	7.43	1.230	1.205	3.337
15	22.0	0.247	0.253	N/A	0.250	4.073	N/A	7.50	1.404	1.341	3.823
17	24.0	0.271	0.273	N/A	0.272	4.431	N/A	7.60	1.489	1.477	4.380
19	26.0	0.407	0.410	0.409	0.409	6.654	400.0	7.62			

Regression Output:

Constant			-0.155	
Std Err of Y Est			0.183	
R Squared			0.851	
No. of Observations			10.000	
Degrees of Freedom			8.000	
X Coefficient(s)	u =	0.068	1/day	
Std Err of Coef.		0.010		

Final DCW
DD water washing:

	0.017	g/40ml
	0.018	g/40ml
	0.013	g/40ml
Xmax =	0.016	g/40ml
	400.0	mg DCW/l

Table B-5.

1300 ml Stirred-Tank Bioreactor#2, Run#4 QPRO Data Spreadsheet

Cultivation Period: 11:00am, December 3,1993 to 11:00am, December 29,1993. Total Cultivation Time: 625.5 Hours.

RUN IDENTIFICATION		CULTURE LOADING		PROCESS PARAMETERS	
Run#:	#4	Inoculum Volume:	150 ml	Impeller type:	Four 45-degree pitch blades
Description:	1300ML Stirred-Tank Photobioreactor #2	Inoc. Source:	L.saccharina gametophyte L-2-1,L-4-8,L-4-4	Aeration Rate:	1500 ml/min
Time Started:	11:00 am			Mixing Speed	250 rpm
Date Started:	12/03/1993	Age of Inoculum:	55 days	Setpoint Temp.:	13 C
		Total Culture Vol.:	1300 ml	Illum. Position:	Two sides
		Bicarbonate:	0 mg/L	Incident Illumination:	3000 lux
		Inoculum (g/20 mL):	0.0090	Photoperiod:	16 hr ON 8 hr OFF
		Inoculum (g/20 mL):	0.0086		
		Inoculum (g/40 mL):	0.0087 *2		
		avg =	0.0088 g/20 mL		

DATE/1993	DAY	Abs(625nm) Trial#1	Abs(625nm) Trial#2	Abs(625nm) Trial#3	AU/average	Chl a [ug/mL]	DCW [mg/L]	pH	ln(Chl a)	ln(Chl a) predicted	Chl a[ug/ml] predicted	Light transmission [Lux] left	front	right
12/03	0.0	0.050	0.044	0.046	0.047	0.760	50.6		-0.274			230	300	220
6	3.0	0.065	0.053	N/A	0.059	0.961	N/A	7.10	-0.040			240	300	230
9	6.0	0.057	0.052	N/A	0.055	0.888	N/A	7.09	-0.119	0.429	1.536	240	300	220
11	8.0	0.128	0.139	N/A	0.134	2.175	N/A	7.20	0.777	0.616	1.851	220	280	230
13	10.0	0.214	0.206	N/A	0.210	3.421	N/A	7.18	1.230	0.802	2.231	210	260	210
15	12.0	0.154	0.171	N/A	0.163	2.647	N/A	7.24	0.973	0.989	2.689	210	250	200
17	14.0	0.232	0.236	N/A	0.234	3.812	N/A	7.36	1.338	1.176	3.240	200	210	190
19	16.0	0.263	0.250	N/A	0.257	4.178	N/A	7.36	1.430	1.362	3.905	180	200	180
21	18.0	0.266	0.249	N/A	0.268	4.358	N/A	7.37	1.472	1.549	4.707	170	200	170
23	20.0	0.340	0.329	N/A	0.335	5.449	N/A	7.32	1.695	1.736	5.673	140	180	140
25	22.0	0.392	0.427	N/A	0.410	6.671	N/A	7.56	1.898	1.922	6.837	120	130	120
27	24.0	0.449	0.455	N/A	0.452	7.363	N/A	7.64	1.996	2.109	8.240	100	100	100
29	26.0	0.501	0.468	0.497	0.489	7.960	500.0	7.70	2.074			90	70	85

Regression Output:

Constant		-0.131
Std Err of Y Est		0.265
R Squared		0.837
No. of Observations		10.000
Degrees of Freedom		8.000
X Coefficient(s)	u =	0.093 1/day
Std Err of Coef.		0.015

Final DCW [g/20ml]:
DD water washing:

Trial #1	0.010		[g/20ml]
Trial #2	0.011		[g/20ml]
Trial #3	0.010	*2	[g/40ml]
Xmax =	0.010		[g/20ml]
	500.0		[mg DCW/L]

Final DCW [g/20ml]:
DD water washing:

Trial #1	0.015		[g/20ml]
Trial #2	0.016		[g/20ml]
Trial #3	0.015	*2	[g/40ml]
Xmax =	0.015		[g/20ml]
	766.7		[mg DCW/L]

Table B-6.

1300 ml Stirred-Tank Bioreactor#2, Run#5 QPRO Data Spreadsheet
Cultivation Period: 11:00am, January 12,1994 to 11:00am, February 7,1994. Total Cultivation Time: 625.5 Hours.

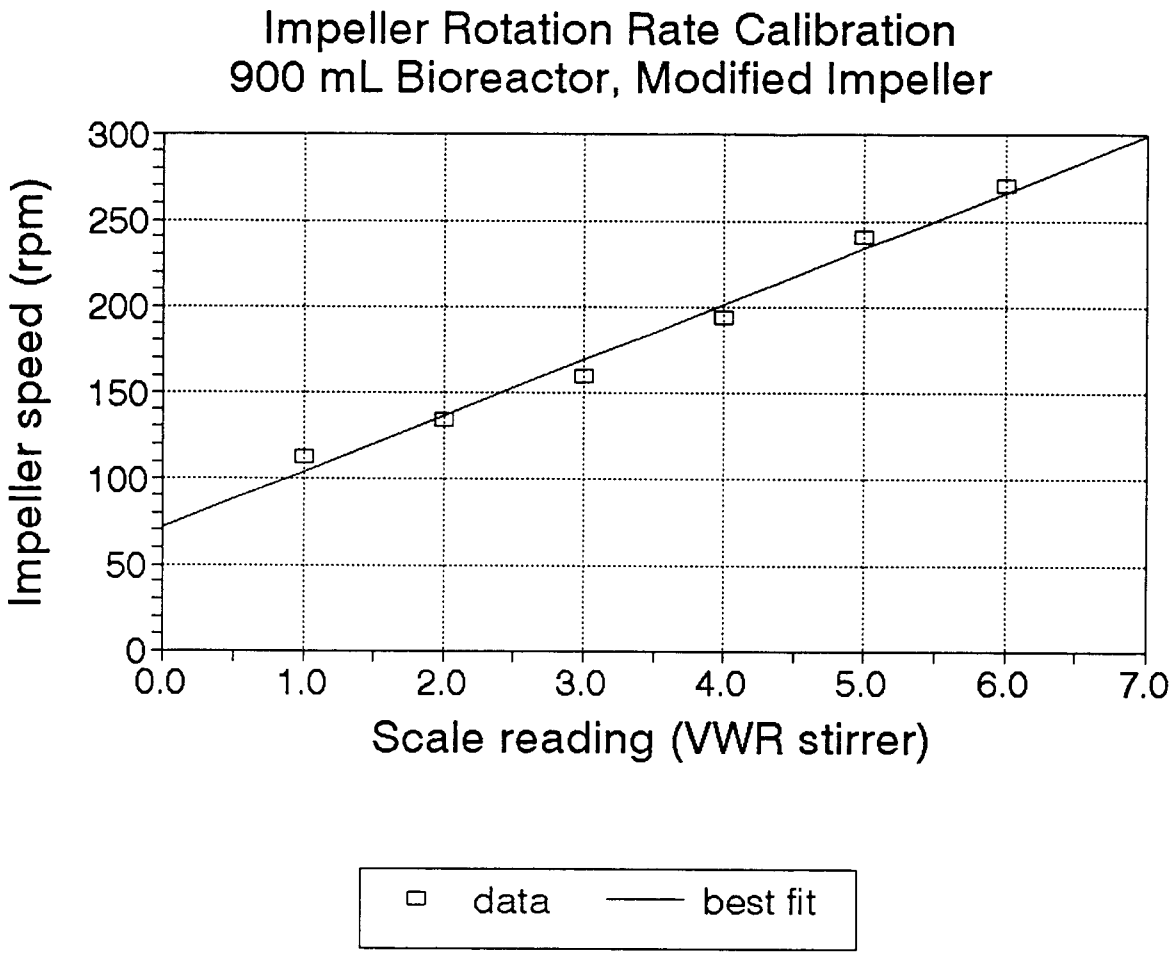
RUN IDENTIFICATION		CULTURE LOADING		PROCESS PARAMETERS	
Run#:	#5	Inoc. Volume:	180.5 ml	Impeller type:	Four 45-degree pitch blades
Description:	1300ML Stirred-Tank Photobioreactor #2	Inoc. Source:	L.s. Gametophyte	Aeration Rate:	1500 ml/min
Time Started:	11:00am		L-5-2,LS-3-7,LS-3-6	Mixing Speed	250 rpm
Date Started:	01/12/1994	Age of Inoculum:	58 days	Setpoint Temp.:	13 C
		Total Culture Vol.:	1300 ml	Illum. Position:	Two sides
		Bicarbonate:	0 mg/L	Incident Illumination:	19000 lux
		Inoculum (g/20 mL):	0.0075	Photoperiod:	16 hr ON 8 hr OFF
		Inoculum (g/20 mL):	0.0073		
		Inoculum (g/30 mL):	0.0070 *1.5		
		avg =	0.0073 g/20 mL		

DATE/1994	DAY	Abs(625nm) Trial#1	Abs(625nm) Trial#2	Abs(625nm Trial#3	AU/average	Chl a [ug/mL]	DCW [mg/L]	pH	ln(Chl a) [ug/ml]	ln(Chl a) predicted	Chl a[ug/ml] predicted
01/12	0.0	0.041	0.044	0.047	0.044	0.717	50.4		-0.333	-0.461	0.631
15	3.0	0.062	0.057	N/A	0.060	0.969	N/A	7.10	-0.031	-0.020	0.980
18	6.0	0.088	0.084	N/A	0.086	1.401	N/A	7.07	0.337	0.421	1.523
20	8.0	0.115	0.100	N/A	0.108	1.751	N/A	7.18	0.560	0.715	2.044
23	11.0	0.188	0.205	N/A	0.197	3.201	N/A	7.28	1.163	1.156	3.177
25	13.0	0.273	0.254	N/A	0.264	4.292	N/A	7.30	1.457	1.450	4.263
27	15.0	0.388	0.390	N/A	0.389	6.337	N/A	7.40	1.846	1.744	5.720
30	18.0	0.413	0.465	N/A	0.439	7.151	N/A	7.36	1.967		
02/01	20.0	0.492	0.470	N/A	0.481	7.835	N/A	7.65	2.059		
04	23.0	0.470	0.467	N/A	0.469	7.632	N/A	7.80	2.032		
07	26.0	0.516	0.507	0.515	0.512	8.340	612.5	7.85	2.121		

Regression Output:			Final DCW:			Final DCW:		
Constant		-0.461	DD water washing:			GP2 water washing:		
Std Err of Y Est		0.108	trial #1	0.012	g/20ml	trial #1	0.018	g/20ml
R Squared		0.985	trial #2	0.012	g/20ml			
No. of Observations		7	Xmax =	0.012	g/20ml	Xmax =	0.018	g/20ml
Degrees of Freedom		5		612.5	mg DCW/L		900.0	mg DCW/L
X Coefficient(s)	u =	0.147						
Std Err of Coef.		0.008						

Appendix C

Calibration Curves


Impeller Rotation Rate Calibration
900 mL Bioreactor, Modified Impeller
Impeller speed (rpm)
300
250
200
150
100
50
0
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
Scale reading (VWR stirrer)
data
best fit

Approximate Conversion Factors for White Fluorescent Light

To convert	Multiply by
W/m^2 to $\mu mol/s\text{-}m^2$	4.6
klux to $\mu mol/s\text{-}m^2$	12
klux to W/m^2	2.7