AN ABSTRACT OF THE DISSERTATION OF

Yao-ming Huang for the degree of Doctor of Philosophy in Chemical Engineering, presented on March 21, 2001. Title: Photobioreactor Cultivation of the Cell and Tissue Cultures Derived from Marine Red Macroalga *Agardhiella subulata*

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Abstract approved: _____
Gregory L. Rorrer

Macrophytic marine algae are a rich source of unique natural products. Controlled biological production of these compounds first requires the development of an engineered biomass production system to illustrate the application of bioprocess engineering principles to this new area of marine biotechnology. Toward this end, two axenic liquid suspension cultures were established for *Agardhiella subulata,* including an undifferentiated filament clump culture established by induction of callus-like tissue from thallus explants, and a microplantlet culture established by regeneration of callus filaments. The microplantlet culture was selected for bioreactor cultivation studies because it was morphologically stable.

Controlled cultivation of *Agardhiella subulata* microplantlet suspension was successful in both externally-illuminated bubble-column and stirred-tank bioreactors. Limiting process parameters on biomass production, including temperature, pH, CO_2 delivery, light transfer, macronutrient consumption, agitation intensity, and microplantlet morphology were assessed. The optimal growth temperature was 24 °C. The optimal pH environment for cultivation was centered around pH 8. The growth was not CO_2-limited in either bioreactor system when aerated at 0.3 vvm containing 3500 ppm CO_2 in the aeration gas. Light transfer limitations were addressed by comparing the mean light intensity (I_m) to the light intensity at 63% of photosynthetic saturation (I_k). The optimal photoperiod was 16:8 LD. The biomass yield coefficients based on N and P during the bioreactor cultivation were 1.0 gDCW/mmol N and 26.0 gDCW/mmol P respectively. Medium perfusion rate of 20% per day during bioreactor cultivation prevented nitrate and phosphate depletion, maintained the specific oxygen evolution rate at 0.12 mmol O_2 g^{-1} DCW h^{-1}, and prolonged active growth phase. Two exponential phases of growth were observed during prolonged biomass production under resource-saturated conditions. Bubble aeration did not fragment the plantlets. Impeller rotation rates from 60 and

250 rpm in the 500 mL stirred tank bioreactor did not affect the specific growth rate during the exponential phase of growth.

Photobioreactor Cultivation of the Cell and Tissue Cultures Derived from Marine Red Macroalga *Agardhiella subulata*

by
Yao-ming Huang

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Yao-ming Huang, Author

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CONTRIBUTION OF AUTHORS

Sanjiv Maliakal and Dr. Donald P. Cheney from Northeastern University were involved in the cell and tissue culture development of *Agardhiella subulata*.

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NOMENCLATURE

$C^*_{CO_2}$ dissolved CO_2 concentration equilibrated with aeration gas CO_2 partial pressure, mmol CO_2 L^{-1}

C_N nitrate concentration in culture liquid, mmol L^{-1}

$C_{N,O}$ initial concentration of nitrate in the reactor, mmol L^{-1}

$C_{N,f}$ final concentration of nitrate in the reactor, mmol L^{-1}

$C_{N,i}$ measured nitrate concentration in the i^{th} sample, mmol L^{-1}

$C^*_{O_2}$ dissolved oxygen concentration equilibrated with aeration gas O_2 partial pressure, mmol O_2 L^{-1}

$C_{O_2,0}$ dissolved oxygen concentration at the onset of re-aeration during the k_La measurement, mmol O_2 L^{-1}

C_P phosphate concentration in culture liquid, mmol L^{-1}

d inner diameter of photobioreactor vessel, cm

d_p plantlet diameter, mm

$\bar{d}_p$ average plantlet diameter, mm

D_i overall diameter of the impeller assembly, cm

D_T inner diameter of 500 mL Bellco jacketed spinner flask, cm

E specific concentration of active photosynthetic apparatus within cell biomass, mol g^{-1} biomass

E_o total specific concentration of active and inactive photosynthetic apparatus within cell biomass, mol g^{-1} biomass

E' specific concentration of inactivated photosynthetic apparatus within cell biomass, mol g^{-1} biomass

f fractional illumination photoperiod (h light on / 24 h photoperiod)

f' light to dark (LD) ratio, h light h^{-1} dark within a 24 h photoperiod

F_A molar flowrate of CO_2 in the aeration gas, mol CO_2 min^{-1}

H Henry's law constant for CO_2 in 36 ppt seawater at 24 °C

NOMENCLATURE, CONTINUED

k'	apparent light attenuation constant, cm^{-1}; apparent rate constant for photodamage, day^{-1}
k_c	specific light attenuation constant, L g^{-1} DCW cm^{-1}
k_d	specific rate constant for photodamage during illumination, h^{-1}
$k_L a$	volumetric mass transfer coefficient, h^{-1}
k_o	light attenuation constant of liquid medium, cm^{-1}
k_r	specific rate constant for photodamage repair, h^{-1}
K_f	proportionality constant, equivalent to f' at 63.2% of photoperiod saturation in the absence of photodamage, h^{-1} light h^{-1} dark
I	light intensity, μE m^{-2} s^{-1}
I_c	light intensity at compensation point, μE m^{-2} s^{-1}
I_k	light intensity at 63% of photosynthetic saturation, μE m^{-2} s^{-1}
I_m	mean light intensity within microplantlet culture suspension, μE m^{-2} s^{-1}
I_o	incident light intensity to vessel surface, μE m^{-2} s^{-1}
m'	apparent specific growth rate in the absence of photodamage, day^{-1}
M_N	total molar consumption of nitrate, μmol Nitrate
N	impeller rotation rate, rev sec^{-1}
n_A	CO_2 delivery rate, mmol CO_2 L^{-1} min^{-1}
p_{CO_2}	partial pressure of CO_2, atm
P_o	specific O_2 evolution rate of microplantlet biomass, mmol O_2 g^{-1} DCW h^{-1}
$P_{o,max}$	specific O_2 evolution rate at photosynthetic saturation, mmol O_2 g^{-1} DCW h^{-1}
$P_{o,\infty}$	maintenance O_2 evolution rate, mmol O_2 g^{-1} DCW h^{-1}
P^*	specific oxygen evolution rate normalized to the control photoperiod (10:14 LD)
q_{CO_2}	specific CO_2 consumption rate of microplantlet biomass, mmol O_2 g^{-1} DCW h^{-1}
Q_o	specific dark-phase O_2 respiration rate of microplantlet biomass, mmol O_2 g^{-1} DCW h^{-1}

NOMENCLATURE, CONTINUED

Q^*	specific dark-phase respiration rate normalized to the control photoperiod (10:14 LD)
r_c	radius of the forced vortex zone within a stirred tank, m
r_i	radius of the impeller diameter within a stirred tank, m
r_t	radius of the tank diameter within in a stirred tank, m
R	gas constant, atm L mol^{-1} K^{-1}
Re	impeller Raynolds number
s	distribution spread of microplantlet diameter
t	cultivation time, days
t_c	cultivation time when microplantlet culture reaches CO_2-limited growth
t_{mix}	mixing time constant, sec
t_{mt}	mass transfer time constant, sec
t_{rxn}	CO_2 consumption time constant, sec
T	temperature, K
v	superficial gas velocity, m s^{-1}
v_f	volumetric flowrate associated with culture removal, mL day^{-1}
v_g	volumetric gas flowrate, mL min^{-1}
v_o	volumetric flowrate of fresh medium, mL day^{-1}
V	culture volume, L
V_0	initial volume in the reactor after inoculation, L
V_f	final volume in the reactor in the end of experiment, L
V_i	medium volume withdrawn for nitrate and phosphate analysis, L
X	biomass cell density of microplantlet culture suspension, mg DCW L^{-1}
$X_{c,N}$	critical cell density associated with nitrate limitation, g FW L^{-1}
$X_{c,P}$	critical cell density associated with phosphate limitation, g FW L^{-1}

NOMENCLATURE, CONTINUED

X_F biomass cell density of microplantlet culture suspension, g FW L^{-1}

$X_{F,o}$ initial fresh cell density of the bioreactor cultivation, g FW L^{-1}

$X_{F,1}$ fresh cell density at cultivation time t_1, g FW L^{-1}

$X_{F,c}$ critical fresh cell density associated with CO_2 limitation, g FW L^{-1}

$X_{F,f}$ final fresh cell density of the bioreactor cultivation, g FW L^{-1}

$X_{F,o}$ initial fresh cell density of the bioreactor cultivation, g FW L^{-1}

X_N number density of plantlets in culture suspension, # plantlets L^{-1}

X'_N apparent number density of plantlet in culture suspension, # plantlets L^{-1}

Y_a average shear rate of the stirred tank bioreactor

Y_{X/CO_2} biomass yield coefficient based on CO_2 consumption, mmol CO_2 g^{-1}DCW

$Y_{X/N}$ biomass yield coefficient based on nitrate consumption, mmol N g^{-1}DCW

$Y'_{X/N}$ growth-associated biomass yield coefficient based on nitrate consumption in the exponential phase of growth. mmol N g^{-1}DCW

$Y_{X/P}$ biomass yield coefficient based on phosphate consumption, mmol P g^{-1}DCW

Y_{X/O_2} biomass yield coefficient based on total oxygen evolution rate, mmol O_2 g^{-1}DCW

W_f final fresh weight of microplantlet culture, g

W_0 initial fresh weight of microplantlet culture, g

W_s solids content ratio of dry cell weight to fresh weight, mg DCW g^{-1} FW

z position within light path of microplantlet suspension, cm

α number of planes of illumination to photobioreactor vessel surface

β growth rate during the CO_2-limited growth

ϕ frequency of the microplantlet diameter d_p

$\bar{\phi}$ frequency at average plantlet diameter $\bar{d}_p$

μ specific growth rate at time t, day^{-1}

NOMENCLATURE, CONTINUED

μ_1 specific growth rate of the first exponential phase of growth, day^{-1}

μ_2 specific growth rate of the second exponential phase of growth, day^{-1}

μ' apparent specific growth rate μ scaled to the illumination phase of the photoperiod, day^{-1}

μ_{app} apparent specific growth rate, day^{-1}

μ_{max} maximum specific growth rate at cultivation time t, day^{-1}

μ^{o}_{max} maximum specific growth rate in the absence of photodamage, day^{-1}

μ^* apparent specific growth rate normalized to the control photoperiod (10:14 LD)

ν_{CO_2} stoichiometric coefficient for CO_2 based on overall photosynthetic biomass production stoichiometry

ν_{O_2} stoichiometric coefficient for CO_2 based on overall photosynthetic biomass production stoichiometry

ν_L kinematic viscosity of the liquid medium, $m^2\ s^{-1}$

θ medium perfusion rate, L fresh medium L^{-1} reactor volume day^{-1}

Photobioreactor Cultivation of the Cell and Tissue Cultures Derived from Marine Red Macroalga *Agardhiella subulata*

Chapter 1

INTRODUCTION

Background

The search for bioactive compounds of natural origin is important for securing future pharmaceutical agents. The use of natural marine resources for production of bioactive metabolites is a new and promising area of marine biotechnology.

Anatomically complex marine macroalgae, commonly known as seaweeds, are a rich source of novel products with diverse pharmacological activities. Although seaweeds fall into three primary classes - Rhodophyta (red macroalgae), Phaeophyta (brown macroalgae), and Chlorophyta (green macroalgae)- red seaweeds are a particularly prolific source of bioactive compounds. The literature review below will overview the bioactive compounds possessed by marine marophytic red algae, explain the bioprocess development from the marine biotechnology prospective, and summarize "the state of the art" with respect to current macroalgal cell and tissue culture development and photobioreactor cultivation.

Literature Review

Bioactive compounds from marine macrophytic red algae. There are two novel systems for producing bioactive compounds in red macroalgae. In the first system, arachidonic acid metabolism biosynthesizes eicosanoids by enzymes that promote oxidation, including lipoxygenases (Gerwick, 1994). Several pharmaceuticals derived from eicosanoids are commercially important (Nelson et al., 1992). Red macroalgae possess many eicosanoids and oxylipins, including 12S-HETE from *Gracilariopsis lemaneiformis* (Jiang and Gerwick, 1991), hepoxilin B_3 from *Platysiphonia miniata* (Moghaddam et al., 1990), and 6E-leukotriene B_4 from *Murrayella periclados* (Bernart and Gerwick, 1994).

In the second system, haloperoxidases halogenate organic compounds from chloride and bromide ions found in seawater (Fenical, 1975). Sponges and red macroalgae from the marine environment are the two largest groups of organisms in nature to contain halogenated

compounds (Gribble, 1992). Red macroalgae within genera *Ochtodes, Plocamium* and *Portieria* have an unique ability to synthesize a diverse array of halogenated monoterpenes (Dev et al., 1982; Gribble, 1992). Monoterpenes are a class of chemopreventive agents for anti-tumor activities (Kelloff et al, 1994; Crowell and Gould, 1994).

Bioprocess Development Cell and tissue suspension culture systems established from macroalgae have the potential to biosynthesize these compounds in a controlled environment at a scale required for continued drug development or commercial production. The bioprocess development scheme is summarized in Figure 1.1. This proposed research will focus on the first two important steps of the bioprocess technology: cell culture development and bioreactor cultivation.

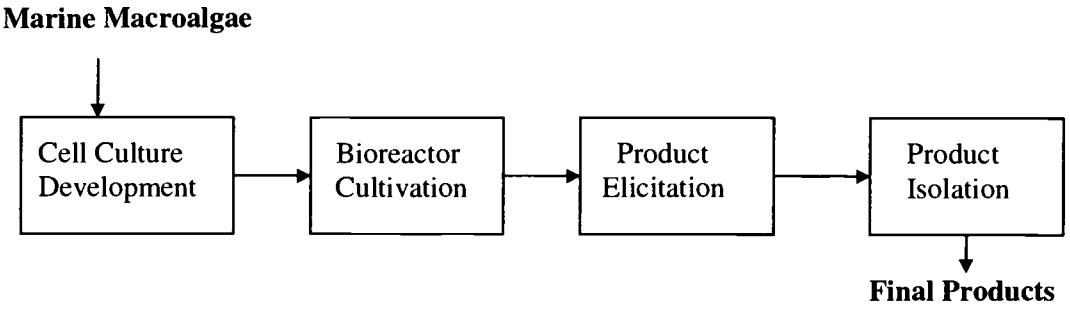

Figure 1.1 Bioprocess Development Flowsheet

There are four advantages for cultivating macroalgal suspensions in bioreactors. First, bioreactors promote rapid and controlled production of cell mass bearing bioactive compounds Second, direct biosynthesis of secondary metabolites within these living cells can preserve the stereochemistry and bioactivity of a novel complex product. Third, the target metabolites can be elicited to enhance the productivities in the closed controlled system. Finally, bioreactor cultivation avoids ecological impact on the marine environment as the biomass can be supplied consistently throughout the year without the need for field collection.

Macroalgal cell and tissue culture. Macroalgal cell biotechnology is still in its infancy compared with terrestrial plant cell biotechnology. However, a few cell and tissue cultures of marine macroalgae can be established by techniques adapted from terrestrial plant cell biotechnology. The specific protocols for the development of cell and tissue culture of marine macroalgae significantly lag behind those for terrestrial plant cell and tissue culture (Butler and Evans, 1990). In contrast to higher plants, callus development in macroalgal culture depends on

photosynthesis. In general, seaweed calli are slow growing and small in size, about 1 to 3 mm in diameter (Aguirre-Lipperheide, 1995). Most calli cease growth upon excision from the explant (Robaina et al., 1990; Kaczyna and Megnet 1993). There are only a few previous studies documenting the successful development of sustainable liquid cell suspension culture systems from red macroalgae. These include a liquid cell suspension culture of *Porphyra* species by protoplast isolation techniques (Chen, 1989; Tait et al. 1990), and clumped cell suspension culture of *Pterocladia capillacea* (Liu et al., 1990) by callus induction techniques.

Reliable routes for the development of axenic liquid suspension culture systems for macrophytic red algae will be needed for future biotechnology and bioprocess engineering applications. The temperate macrophytic red alga *Agardhiella subulata* is an excellent model system for culture development, as it possesses a highly branched, thallus morphology where cell growth occurs at apical meristems. Generic techniques developed for *A. subulata* have the potential to be extended to other red macroalgae of similar morphology and growth patterns that possess bioactive compounds, including species within *Bonnemaisonia* (McConnel and Fenical, 1980), *Ochtodes* (Paul et al., 1987), *Plocamium* (Crews, 1977), and *Portieria* (Fuller et al., 1994). *Agardhiella subulata* itself provides eicosanoids 8-HETE and a novel tricyclioxylipin, Agardhilactone, from 8-lipoxygenase metabolism of eicosapentaenoate (Graber et al., 1996).

The development of novel cell and tissue culture systems representing brown, green, and red macroalgae suitable for bioreactor cultivation are ongoing efforts in our laboratory since 1991. Specific phototrophic culture systems include a female gametophyte cell suspension culture from the macrophytic brown alga *Laminaria saccharina* (Qi and Rorrer, 1995), a semi-differentiated tissue suspension culture of the macrophytic green alga *Acrosiphonia coalita* (Rorrer et al., 1996), a callus-derived filament clump culture and a regenerated microplantlet suspension culture from the macrophytic red alga *Agardhiella subulata* (Huang et al., 1998) and a regenerated microplantlet culture from the macrophytic red alga *Ochtodes secundiramea* (Maliakal, 1996).

Photobioreactor Cultivation of Macroalgal Suspensions. Macroalgal cell and tissue suspension cultures are phototrophic, requiring light, externally supplied inorganic carbon, and a well-mixed suspension environment to support photosynthetic growth. Therefore, all photobioreactors need to meet these requirements. Continuous bubbling aeration of the culture with CO_2 in air is used to supply inorganic carbon. Illumination of photobioreactors can be either external or internal. The culture vessel wall is constructed of a transparent material to allow the transfer of light to phototrophic culture. A well-mixed environment is accomplished through mechanical (e.g. stirred tank) or pneumatic (e.g. bubble column) agitation.

The controlled cultivation of *L. saccharina* gametophyte cell suspension culture was accomplished in externally illuminated stirred-tank (Qi and Rorrer, 1995), bubble-column (Zhi and Rorrer, 1996), and tubular recycle (Mullikin and Rorrer, 1998) photobioreactors. Growth of this organism on GP2 artificial sea water medium at initial nitrate concentrations of 63.5 mg/L and higher had no significant effect on the specific growth rate of the culture (Zhi and Rorrer, 1996). However, fed-batch addition of nutrients enhanced the final cell density during stirred tank photobioreactor cultivation (Ramanan, 1997).

Attempts to grow the *Acrosiphonia coalita* culture were successful in a stirred tank bioreactor, but not a bubble column photobioreactor (Rorrer et al. 1996). The addition of 3500 ppm CO_2 to the aeration gas provided a maximum CO_2 transfer rate of six times the maximum CO_2 consumption rate, stabilized the pH to 8.0, and modestly enhanced the specific growth rate from 0.185 to 0.245 day^{-1}, but did not improve the final biomass productivity.

Limiting Metabolic Factors for Phototrophic Cultures. Light intensity is known to affect the pattern of macromolecular synthesis from photosynthetically fixed CO_2 (Darley, 1982). Total CO_2 fixation declines at light intensities below light saturation, and so it is essential to maintain light saturated growth. As the light intensity decreases, the percentage of the fixed carbon incorporated into protein increases, and the percentage incorporated into carbohydrate decreases. At high light intensities, the rate of carbon fixation exceeds the rate of protein synthesis, which is limited by nitrogen assimilation. Consequently, excess carbon is stored as carbohydrate (Konopka & Schnur 1980). When cells are grown under light-dark cycles, the synthesis of protein and other macromolecules continues into the dark period at the expense of carbon and energy stored in carbohydrate. Therefore, proper light and dark cycles as well as sufficient irradiance are the keys to optimizing the photosynthetic growth of macroalgal cultures.

Objectives

This proposed research plan will focus on the development of new marine biotechnology to provide a generic process for biomass production from red macroalgae. Cell and tissue suspension cultures can provide reliable biomass production and compound biosynthesis within the living cells under controlled conditions. Controlled cultivation is a way to establish a "biological platform" for these compounds using bioengineering techniques.

The macrophytic marine red macroalga *Agardhiella subulata* represents an excellent model system for macrophytic red algae. In nature, *A. subulata* possesses a highly branched,

terete thallus morphology, and cell growth occurs at apical meristems. These characteristics are common to many anatomically complex red algae, which produce bioactive compounds.

The project has three major objectives, each of which emphasizes the first two important steps for bioprocess engineering of marine organisms - cell culture development and bioreactor cultivation. The specifics of each objective are summarized below.

1. Develop cell and tissue suspension culture systems for the macrophytic red alga *Agardhiella subulata*.
2. Compare the biomass productivity of *Agardhiella subulata* cultures in bioreactor systems, including bubble column, and stirred tank bioreactors in batch, fed-batch, and perfusion cultivation modes.
3. Identify the major process engineering factors that limit microplantlet suspension biomass productivity in photobioreactors. Determine light delivery conditions for light-saturated growth, including the saturation light intensity and optimal photoperiod. Other process variables include temperature, pH, agitation intensity, and limiting macronutrients.

Chapter 2 addresses the cell and tissue culture development and compares the stability and the growth characteristics of two axenic, *in vitro* suspension cultures: undifferentiated filament clumps, and microplantlets regenerated from filament clumps. In Chapter 3, microplantlet suspension cultures were cultivated within a 2 L bubble-column photobioreactor. Limiting factors on biomass production, including light transfer, CO_2 transfer, macronutrient consumption, temperature, pH, agitation intensity, and microplantlet morphology were assessed. In Chapter 4, the optimal photoperiod and temperature were investigated in a bubble-column bioreactor with constant medium replacement. In Chapter 5, microplantlet suspension cultures were cultivated within a stirred-tank photobioreactor. Limiting process factors, including CO_2 delivery, macronutrient consumption, pH, agitation intensity, and microplantlet morphology, were assessed. Chapter 6 summarizes and concludes all the bioreactor cultivation studies.

Chapter 2

COMPARISON OF DEVELOPMENT AND PHOTOSYNTHETIC GROWTH FOR FILAMENT CLUMP AND REGENERATED MICROPLANTLET CULTURES OF *AGARDHIELLA SUBULATA* (RHODOPHYTA, GIGARTINALES)

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Introduction

The first goal of this study is to develop a stable liquid suspension culture system for *A. subulata* which could be subcultured indefinitely. Specifically, two *in vitro* liquid suspension culture systems will be developed for *A. subulata*, including a clumped filament culture established by an induction of undifferentiated filaments from explant tissue and a microplantlet culture established by controlled regeneration of filamentous clumps. The second goal of this study is to compare the morphology, growth rates, and photosynthetic activity of the undifferentiated filament clumps and the regenerated microplantlets in order to access which culture system may be more suitable for further bioprocess development. The analysis of natural products found in these culture systems is beyond the scope of this present study, but may be considered in future work.

Materials and Methods

Explant preparation, sterilization, and induction of callus-like tissue. Mature female gametophytes of *Agardhiella subulata* (C. Agardh) Kraft et Wynne (Gabrielson and Hommersand, 1982) were collected near the Millstone Nuclear Power Station (Waterford, Connecticut) on July 25, 1995 at 1 m depth and 26 °C ambient water temperature. Non-epiphytic portions of plants containing cystocarps were isolated and shaken with 0.5 mm glass beads for one min. Following this treatment, plants were incubated in sterile-filtered natural seawater for one week at 24 °C and 10 μmol photons•m^{-2}•s^{-1} within 100 mm by 80 mm glass culture dishes. Spores released after one week were collected and cultured in 500 mL bubbler flasks containing sterile-filtered seawater supplemented with ESS nutrients (Saga, 1986). When tetrasporic plants reached 10 cm in length, primary thalli were isolated and surface sterilized with 1% betadine in filter-sterilized seawater, then incubated in sterile-filtered seawater supplemented with ESS nutrients for three days. Primary thalli obtained near the tip or branch points were cut into segments of 1-2 mm length and 0.5-1.0 mm diameter. The segments were placed in 24-well culture plates. Each culture well contained 2 mL of filter-sterilized ASP12 medium supplemented with 12 mM nitrate, 0.01 mg L^{-1} zeatin, and 0.01 mg L^{-1} phenylacetic acid, prepared according to Bradley and Cheney (1990). Only 2 explants were loaded in each well to minimize contamination. A total of 144 explants were prepared. The explants were incubated in the culture wells without agitation at 24 °C under 10 μmol photons•m^{-2}•s^{-1} cool white fluorescent light (10:14 LD photoperiod) within an illuminated incubator. After one

week, elongated, clear, uniseriate filaments appeared on the cut faces of the explant. After formation of filaments, ASP12 medium was completely replaced once per week. After 4 weeks, about three quarters of the explants possessed a thick bushy mass of proliferating filaments at the cut face of the explant. Explant sections bearing the most extensive filamentous growth were selected for culture development. The filamentous mass was cut from the explant near the origin, carrying over a small portion of the explant. After three weeks each section was divided in half and placed in new culture wells with 2 mL new medium, 2 sections per well. After 3 weeks, the disk was divided half again and placed in new medium. After three more subcultures, the parent explant was completely removed. The final cell mass consisted of a compact core of pigmented cells with uniseriate, lightly pigmented filaments emanating from the core cell mass.

Maintenance of undifferentiated filament clump culture. Filamentous cell mass was subcultured by cutting each clump into four to six pieces with a razor blade. Each contained a portion of the compact core cells and lightly-packed, uniseriate filaments. New growth continued from the uncut filaments after the first week and then from the cut face after the second week. If the subcultured filament mass did not contain a portion of the compact core cells, it usually died within two weeks. Filament clumps were maintained in 6-well culture plates (16.8 mL per well) with 8-10 clumps per 8 mL medium in each well. Filter-sterilized ASP12 medium supplemented with 12 mM sodium nitrate and no plant-growth regulators served as the culture growth medium. The medium was buffered with 10 mM sodium bicarbonate and sodium HEPE to pH 8. The culture well plates were not agitated. Gas exchange, including absorption of carbon dioxide and off-gassing of photosynthetic oxygen, was accomplished by surface aeration (1.2 cm^2 surface area per 1 cm^3 culture volume). The filament clump culture was maintained at 24 °C under 8 μmol photons•m^{-2}•s^{-1} cool white fluorescent light (10:14 LD photoperiod) within an illuminated incubator. The medium volume was completely replaced every 4 days (replacement rate of 25% per day) to prevent nutrient starvation and wash away waste products.

Regeneration and maintenance of microplantlets from filament clumps. To induce regeneration, well-plate cultures of 18 and 30 day old filament clumps described above were placed directly on an orbital shaker and continuously mixed at 100 rev•min^{-1} under 20 μmol photons•m^{-2}•s^{-1} cool white fluorescent light (10:14 LD photoperiod) with medium replacement every 10 days. Within four weeks, one or more shoots were produced from the filament clumps. The shoots continued to develop, and grew to a nominal length of 8-10 mm after an

additional three weeks. During this time, most of the core cells in the regenerating clump bearing the shoots turned white, lysed, and disintegrated into the medium.

About 50 pieces of tissue mass bearing newly regenerated shoot tissue and some residual filament mass were inoculated into 75 mL of filter-sterilized ASP12 medium within a 250 mL foam-stoppered erlenmeyer flask. The ASP12 medium was supplemented with 12 mM sodium nitrate, 10 mM sodium bicarbonate, and no plant growth regulators. The regenerated microplantlet culture was maintained at 24 °C under 12 or 36 μmol photons•m^{-2}•s^{-1} cool white fluorescent light (10:14 LD photoperiod) within an illuminated incubator. Although flasks were not continuously mixed or bubble aerated they were swirled once per day for 5 s to promote gas exchange. For routine maintenance, the medium was completely replaced every 14 days, and the tissues were subcultured every 28 days. The microplantlet shoots formed branches from a common center. During subculture a given microplantlet was cut through the center into two to four pieces, and the shoots were trimmed to 3-5 mm length from the tip. Microplantlets of 3-10 mm length could be readily suspended in liquid culture. The cut microplantlets were then inoculated into fresh medium.

For growth curve experiments, microplantlets were grown in both flask and six-well plate culture, and the medium was completely replaced every 4 or 5 days (replacement rate of 20% or 25% per day).

Photosynthetic oxygen evolution rate measurements. Photosynthetic oxygen evolution rate measurements for the filament clump culture and the microplantlet culture were performed in the apparatus shown in Figure 2.1. The dissolved oxygen (D.O.) measurement cell consisted of a jacketed glass vessel (65 mL total volume, 4.5 cm inner diameter) with a tapered port fitted for a YSI model 5750 dissolved oxygen (D.O.) electrode. The liquid within the vessel was mixed with a 2.5 cm magnetic stir bar. Temperature was maintained at 24 °C by a circulating water bath connected to the vessel jacket. The YSI D.O. electrode was connected to a YSI model 58 D.O. meter interfaced to a computer data acquisition system. The response time of the D.O. electrode was 30 s. Two horizontally-mounted 9 W fluorescent lamps were positioned on opposite sides of the vessel to uniformly illuminate the culture. The distance from the lamp to the vessel surface was adjusted to set the desired incident light intensity to the vessel. The D.O. concentration vs. time baseline in ASP12 medium without cells had a consistent negative drift of 0.1 % of O_2 saturation in air per minute. The liquid medium had to be mixed at a rate of at least 6.5 rev•s^{-1} (stir bar tip speed of 52 cm•s^{-1}) to provide sufficient fluid convection over the surface of the D.O. electrode.

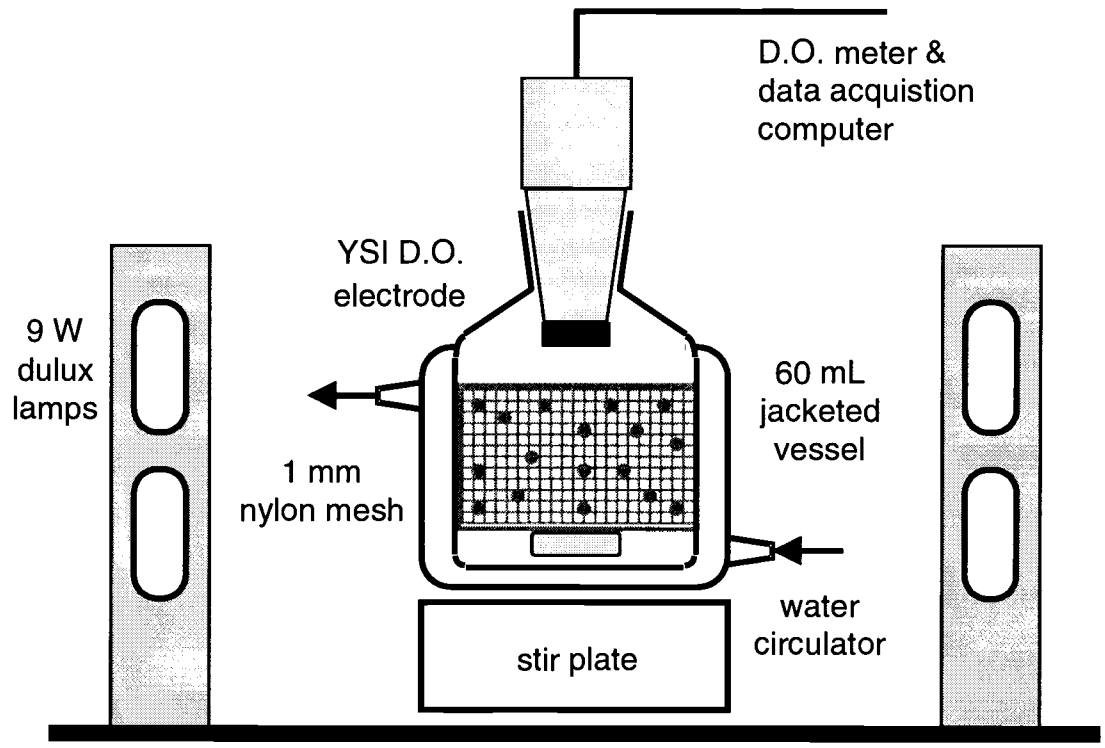

Figure 2.1 Apparatus for oxygen evolution rate measurements.

Initial culture development efforts demonstrated that the undifferentiated filament clumps in liquid suspension were very sensitive to agitation and died off within 4 hours under continuous mixing at 6.5 $rev \cdot s^{-1}$ within the vessel. However, filament clumps immobilized on nylon mesh were protected from the damaging effects of agitation. In the immobilization procedure, 0.5 g of fresh cell mass was weighed to precision of ± 0.001 g and then evenly placed between two 12 cm (width) by 3 cm (height) sheets of clear, 1 mm nylon mesh. The mesh assembly was rolled up and then inserted into the vessel through the D.O. electrode port. The mesh assembly expanded to fit against the entire inner circumference of the vessel. 60 mL of aerated ASP12 medium supplemented with 12 mM nitrate and 10 mM bicarbonate were added to the vessel. The culture was mixed at 6.5 $rev \cdot s^{-1}$ for at least 30 min to equilibrate the culture to the new environment. Then, the calibrated D.O. electrode was inserted into the vessel. Since there was no gas headspace within the vessel, all the evolved oxygen was transferred to the liquid medium. The same procedures were performed for the microplantlet cultures.

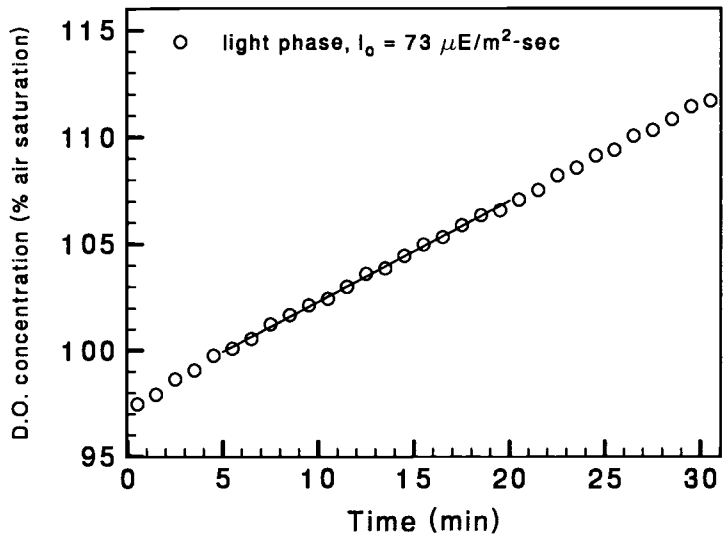

Figure 2.2 Representative D.O. concentration versus time profile for *A. subulata* regenerated microplantlet culture at 24 °C and 73 µmol photons•m^{-2}•s^{-1} incident light intensity.

A representative D.O. concentration vs. time profile is shown in Figure 2.2. The oxygen evolution rate at a given incident light intensity was determined from the least-squares slope of the linear portion of the D.O. concentration versus time curve, typically from 5 to 20 min. The experiment started with the lowest light intensity under consideration and ended with the highest. Incident light intensity was measured in units of µmol photons m^{-2} s^{-1} with a LI-COR 190SA PAR quantum sensor and LI-COR 189 quantum radiometer. Between measurements, the D.O. electrode was removed for 10 min to allow the dissolved gas concentrations in the medium to re-equilibrate. Typically, for photosynthetic oxygen evolution rate measurements, two- or three-week old filament clumps 3 h into the light phase of the photoperiod were used. Duplicate measurements were obtained at each light intensity. For oxygen respiration rate measurements, the same two-week old filament clumps 3 h into the dark phase of the photoperiod were used, and the vessel was wrapped in aluminum foil to eliminate light. At the end of a series of measurements, the dry cell mass was determined, and all oxygen evolution rates were reported as the specific oxygen evolution rate P_o (mmol O_2•g^{-1} DCW•h^{-1}) for light phase measurements or specific respiration rate Q_o (mmol O_2•g^{-1} DCW•h^{-1}) for dark phase

measurements. Estimates for P_o were correlated to I_o using an exponential saturation model of the form

$$P_o = P_{o,\max}\left(1 - e^{-I_o / I_k}\right) + Q_o \qquad (2.1)$$

where Q_o is the specific respiration rate, $P_{o,max}$ is the maximum photosynthetic oxygen evolution rate at light saturation, and I_K is the light intensity at which 63.2% of the $P_{o,max}$ value is achieved. The value for Q_o was obtained from D.O. concentration vs. time measurements during the dark phase of the photoperiod. With Q_o known, P_o vs. I_o data were fitted to equation (2.1) by least-squares nonlinear regression to obtain estimates for $P_{o,max}$ and I_k. Once these constants were determined, the light intensity at the photosynthetic compensation point (I_c) was estimated by setting P_o equal to zero. The concentration of dissolved oxygen in the medium at 100% of air saturation and 24 °C was 0.2 mM.

Other analytical techniques. Biomass density as fresh cell weight was determined by filtering the entire contents of the culture vessel (well plate or flask) on 20 μm nylon mesh under sterile vacuum to remove entrained water not associated with the cell mass. The filtered cell mass was weighed under sterile conditions to precision of ± 0.001 g. Immediately after each fresh cell weight measurement, the biomass was re-suspended in a measured volume of new medium and returned to the culture vessel. Fresh cell weight measurements were based on the cell mass averaged from two well plates. The specific growth rate was determined from the least-squares fit of fresh cell density vs. time growth curve data on a semi-log plot. Dry cell density was determined at the end of cultivation. Filtered cells were dried at 80 °C and 1 atm for 24 h then weighed using the procedures described by Zhi and Rorrer (1996). Chlorophyll *a* content was measured spectrophotometrically at 665 nm following maceration of biomass samples with HPLC-grade methanol in a Potter-Elvehjem tissue homogenizer, 18-24 h of extraction with methanol in the dark at 4 °C, and centrifugation at 4000 rev•min^{-1} for 10 min. Other details are provided by Qi and Rorrer (1995).

Results

Development and morphology of undifferentiated filament clump and microplantlet liquid suspension culture systems. The culture development scheme for the macrophytic red alga *A. subulata* is summarized in Figure 2.3. The induction of undifferentiated filaments from

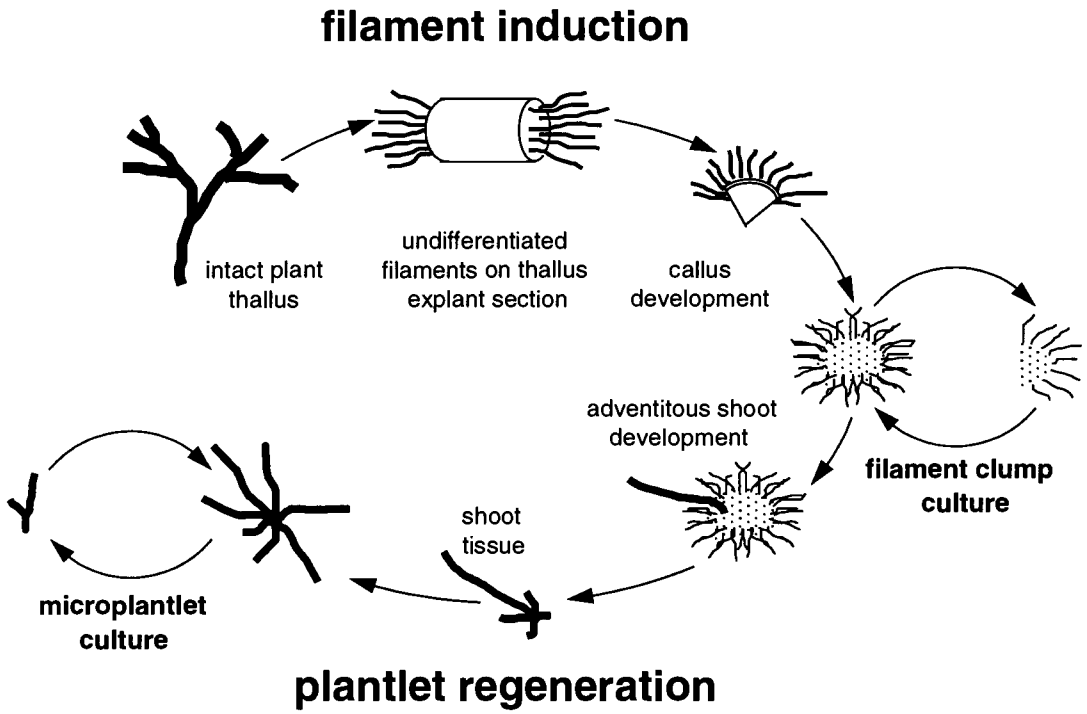

Figure 2.3 Development of undifferentiated filament clump culture and regenerated microplantlet culture systems for *A. subulata*

explant tissue and the development of adventitious shoots from filament clumps are shown in Figure 2.4. Filamentous cells formed at the cut face of the explant tissue, presumably as a wound response (Figure 2.4a). The filaments and a portion of the explant bearing the filaments were excised and cultured. The resulting cell mass was undifferentiated, consisting of tightly meshed filaments and some round cells at the core of the clump with uniseriate filaments emanating from the core cell mass as a thick bushy mass (Figure 2.4b). The overall diameter of the cell clump ranged from 2 to 8 mm. The uniseriate filaments were about 10 μm diameter and 10-30 μm length (Figure 2.4c). The filaments were clear or lightly pigmented, whereas the cells near the core of the clump were darkly pigmented. The filamentous mass could not be dispersed by agitating the liquid, i.e. the cell mass was not friable.

The undifferentiated filament clumps were susceptible to regeneration. The filament clumps could only be maintained under a narrow set of culture conditions which promoted proliferation of undifferentiated cell mass but not regeneration. Adventitious shoots developed from the filament clumps by imposing stress conditions on the culture, such as continuous

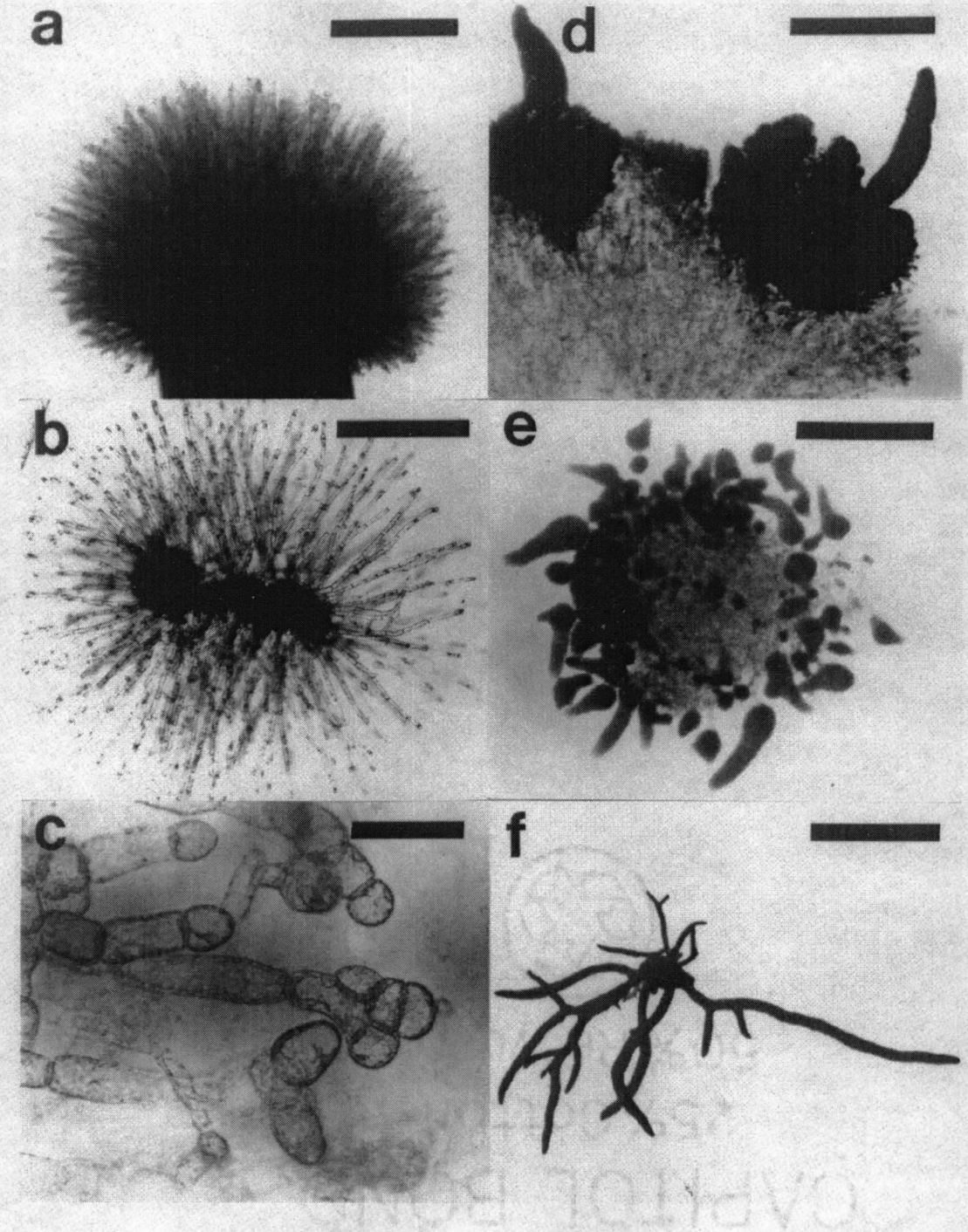

Figure 2.4 Photomicrographs of the development of undifferentiated filament clumps regenerated microplantlets for *A. subulata.* (a) filaments form at the cut face of explant tissue (scale bar 500 μm); (b) undifferentiated filament clump (scale bar 500 μm); (c) adventitous shoot formation, as observed by a change in the plane of cell division at the filament tip (scale bar 20 μm) ; (d) continued differentiation into shoots (scale bar 200 μm); (f) visible formation of several shoots from a 1 mm filament clump fragment (scale bar 500 μm); (f) freely suspended microplantlet (scale bar 2 mm).

agitation or reducing the nutrient medium replacement rate. Agitation was a particularly sensitive factor. When flasks containing a liquid suspension of the filament clumps were mixed on an orbital shaker or bubble-aerated, adventitious shoots formed from the filament clumps. Higher levels of agitation intensity, such as mixing a liquid suspension of filament clumps with a magnetic stir bar, killed the cells. The medium also had to be completely replaced at least every 7 days. The filament clumps generally died within 14 days without medium replacement.

Regeneration was gently and reliably initiated at 24 °C by continuously mixing a liquid suspension of three-week old filament clumps on an orbital shaker at 100 rev•min^{-1} under 20 µmol photons•m^{-2}•s^{-1} incident light intensity (10:14 LD photoperiod), with medium replacement every 10 days. The combination of these conditions put a sublethal stress on the culture. Adventitious protoshoot formation marking the initiation of the regeneration process was observed microscopically by a change in the plane of division of the uniseriate filaments (Figure 2.4c). One or more shoots ultimately formed from each filament clump undergoing regeneration (Figures 2.4d, 2.4e). Unlike the filament clumps, the shoots were not uniseriate and the cells were highly pigmented. Many, but not all of the shoots formed branches. As the microplantlet developed, the inner core of compact cells turned white, died off, and disintegrated away from the microplantlet. The shoots elongated and formed new branches but did not fully regenerate to intact plants with highly branched thallus and holdfast structures (Figure 2.4f). The kinetics of the regeneration process for 18 and 30 day old filament clumps are shown in Figure 2.5. Although there appears to be some variability in regeneration frequency, approximately 40-70% of the filament clumps consistently regenerated. The remainder of the filament clumps either died or remained undifferentiated.

Growth curves for filament clump and microplantlet suspension cultures. Representative growth curves for filament clumps in well plate culture are shown in Figure 2.6 at three different initial biomass densities. The incident light intensity was maintained at 8 µmol photons m^{-2} s^{-1} to avoid the possibility of light-induced regeneration. Cell biomass increased for 50 to 60 days until stationary growth phase was finally attained. Typically, the filament clumps were initially 2-3 mm in diameter but increased to over 5 mm by stationary phase. Since the filament clumps did not disperse during culture, the final biomass density was dependent on the initial biomass density. At each initial biomass density, the biomass increased by a factor of three from inoculation to stationary phase, and specific growth rate was nominally 2-3% per day (Table 2.1). The pH was measured just before medium replacement.

Representative growth curves for the regenerated microplantlets grown in six-well plate culture at conditions consistent with the filament clump culture are presented in Figure 2.7 at 24

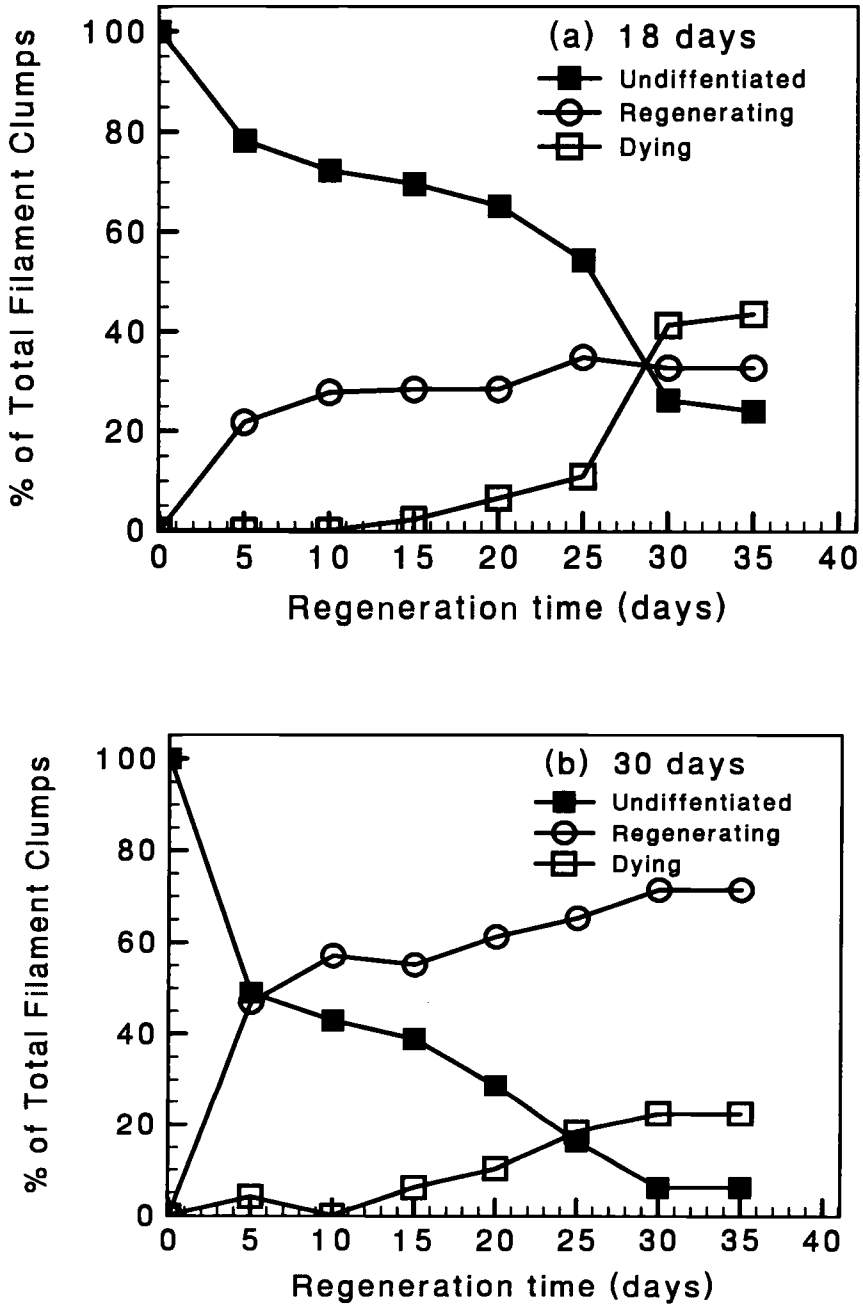

Figure 2.5 Kinetics of the regeneration process for filament clumps on an orbital shaker at 100 rev min^{-1} (rpm). (a) 18 day old filament clumps, 46 total clumps; (b) 30 day old filament clumps, 49 total clumps. The percentage of filament clumps regenerating is the sum of both microscopic evidence of regeneration (change in plane of cell division of filament tips), and macroscopic evidence of regeneration (formation of visible shoots).

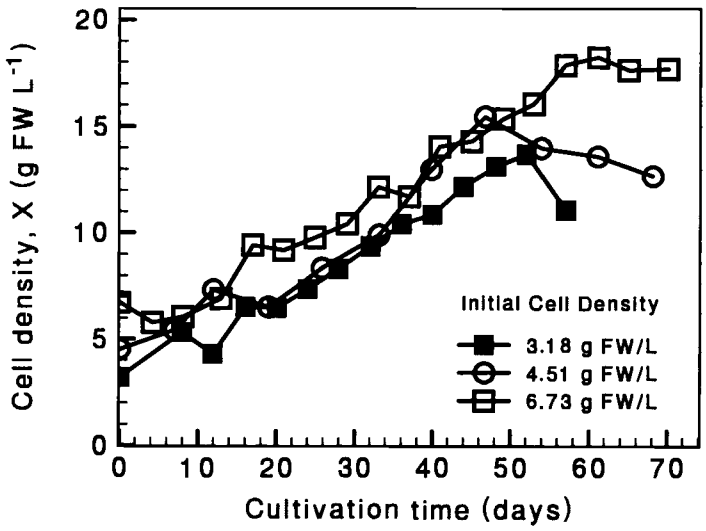

Figure 2.6 Growth curves for *A. subulata* filament clumps in nonagitated, 8 mL well-plate culture at 24 ° C and 8 µmol photons•m^{-2}•s^{-1} incident light intensity for initial cell densities ranging from 3.2 to 6.7 g FW•L^{-1}. The medium was completely replaced every 4 days following fresh weight measurement. Therefore, the medium replacement rate was 25% per day.

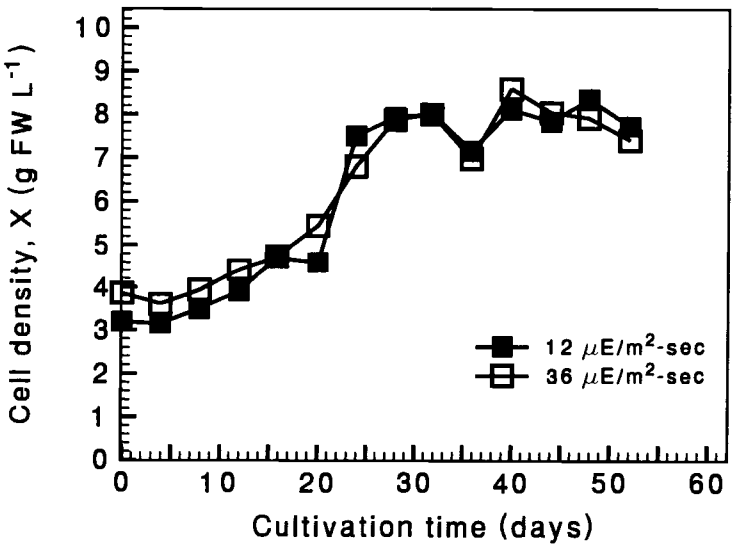

Figure 2.7 Growth curves of *A. subulata* regenerated microplantlets in nonagitated, 8 mL well-plate culture at 24 ° C for incident light intensities of 12 and 36 µmol photons•m^{-2}•s^{-1}. The medium was completely replaced every 4 days following fresh weight measurement. Therefore, the medium replacement rate was 25% per day.

Table 2.1 Comparison of biomass productivities for cell and tissue cultures of *Agardhiella subulata* at 24 °C.

Culture System	Incident Light Intensity (μmol photons$\cdot m^{-2} \cdot s^{-1}$)	Medium Replacement (% per day)	Initial Cell Density (g FW$\cdot L^{-1}$)	Maximum Cell Density (g FW$\cdot L^{-1}$)	Log-Phase Growth (days)	Specific Growth Rate (% per day ± 1s)
Cell clumps,	8	25	3.2	13.7	0-28	3.2 ± 0.5
six-well plate	8	25	4.5	15.4	0-26	2.2 ± 0.6
	8	25	6.7	18.2	4-29	2.6 ± 0.1
Microplantlets,	12	25	3.2	8.4	8-28	4.3 ± 0.7
six-well plate	36	25	3.9	8.6	8-28	3.6 ± 0.4
Microplantlets,	12	20	1.0	3.7	0-25	4.1 ± 0.8
Flask	24	20	0.9	5.2	0-25	6.0 ± 1.2
	36	20	1.5	4.2	0-25	3.2 ± 0.2

°C and incident light intensities of 12 and 36 μmol photons•m^{-2}•s^{-1}. Cultivation experiments were carried out in parallel with a common inoculum source. At inoculation, the shoot tissue length was nominally 3-5 mm, but grew to a final length of about 10 mm. The biomass also increased by a factor of two to three and stationary phase achieved after 30 days. The specific growth rates of the microplantlets were about 50% higher than the filament clumps in six-well plate culture. Increasing the light intensity from 12 to 36 μmol photons•m^{-2}•s^{-1} had no effect on growth. However, the well-plate cultures were not agitated or bubble-aerated, and so it is possible the CO_2 and O_2 mass transfer rates were limiting the culture growth rate. However, it was necessary to cultivate the microplantlets under surface aeration without agitation so that their growth conditions were comparable to those for the filament clump culture. Growth curves of the microplantlets in flask culture were also determined at 12, 24, and 36 μmol photons•m^{-2}•s^{-1} (Figure 2.8).

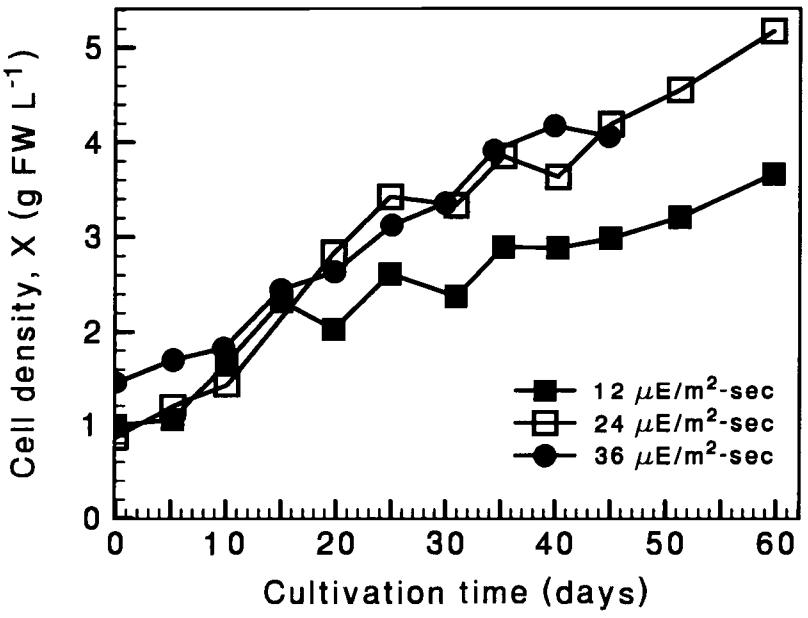

Figure 2.8 Growth curves for *A. subulata* regenerated microplantlets in flask culture at 24 °C for incident light intensities of 12, 24, and 36 μmol photons•m^{-2}•s^{-1}. The medium was completely replaced every 5 days following fresh weight measurement. Therefore, the medium replacement rate was 20% per day.

The flask cultures were swirled for 5 seconds once per day to promote gas exchange. The specific growth rate of the microplantlets in flask culture during exponential phase ranged from 3-6 % per day. At 12 and 24 μmol photons•m^{-2}• s^{-1}, no stationary phase of growth was observed over the 60 day cultivation period, and microplantlets continued to grow slowly after this initial exponential phase. Doubling the light intensity from 12 to 24 μmol photons•m^{-2}•s^{-1} increased the final biomass density and specific growth rate over the 60 day cultivation period.

In all growth experiments for filament clumps and microplantlets, the pH was measured just before medium replacement. The culture pH was always between 8.6 and 9.0. The chlorophyll *a* content of the microplantlet and filament clump biomass was comparable at 0.3 wt% on a dry biomass basis for 30 day old cultures grown at the maintenance conditions.

Photosynthetic light saturation curves for filament clump and microplantlet cultures. Representative photosynthetic light saturation curves, expressed as specific oxygen evolution rate (P_o , mmol O_2•g^{-1} DCW•h^{-1}) versus incident light intensity I_o (μmol photons•m^{-2}•s^{-1}) for filament clump and microplantlet cultures at 24 °C are compared in Figure 2.9.

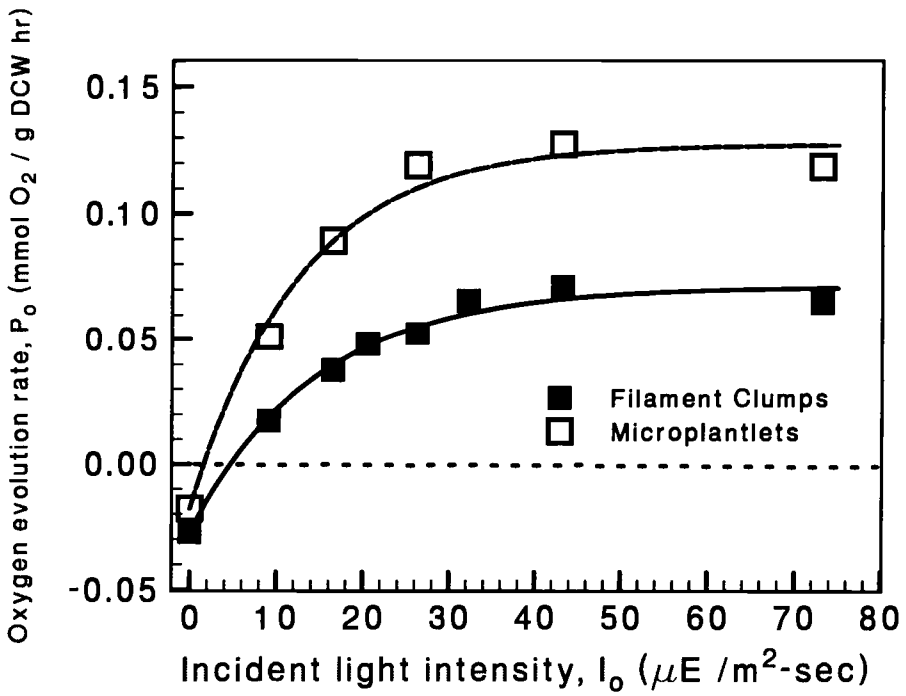

Figure 2.9 Representative oxygen evolution rate versus irradiance curves for filament clumps and regenerated microplantlets immobilized on nylon mesh.

All measurements were performed on cultures in the exponential phase of growth maintained at the conditions described in the Materials and Methods. The pH was within the 8.6 to 9.0 range in all experiments. Within a given P_o vs. I_o curve, repeat measurements for P_o at a given I_o generally had standard errors of less than 10%, and so error bars are not shown. Photosynthetic growth parameters defined by equation (2.1) were estimated for a given P_o vs. I_o curve. Then, photosynthetic growth parameters obtained for both filament clump and microplantlet cultures at three separate subculture cycles were averaged. The final results are summarized in Table 2.2. Both the filament clumps and microplantlet cultures had similar I_k values. The incident light intensity for light-saturated growth was $3\ I_k$ or nominally 50 μmol photons•m^{-2}•s^{-1}. The maximum photosynthesis rate $P_{o,max}$ at light saturation was higher for the microplantlet culture relative to the filament clump culture. The specific respiration rate Q_o for the microplantlets was generally lower than for the filament clumps, but varied significantly between subculture cycles.

Table 2.2 Comparison of photosynthetic growth parameters[a] for filament clump and regenerated microplantlet cultures of *Agardhiella subulata* at 24 °C.

Parameter	Undifferentiated Filament Clumps	Regenerated Microplantlets
Dark-phase respiration rate, Q_o (mmol O_2•g^{-1} DCW•h^{-1})	-0.040 ± 0.013	-0.033 ± 0.017
Maximum oxygen evolution rate, $P_{o,max}$ (mmol O_2•g^{-1} DCW•h^{-1})	0.130 ± 0.023	0.181 ± 0.035
$Q_o / P_{o,max}$	31%	18%
Irradiance at 63.2% of Saturation, I_k (μmol photons•m^{-2}•s^{-1})	14.9 ± 2.6	17.5 ± 3.9
Photosynthetic Compensation Point, I_c (μmol photons•m^{-2}•s^{-1})	5.5 ± 1.7	3.8 ± 2.3
Specific Growth Rate (equation 2), μ_{max} (% per day)	3.9 ± 0.7	5.4 ± 1.0

[a] Reported photosynthetic growth parameters were estimated from P_o vs. I_o curves averaged over three subculture cycles.

The specific growth rate of the cultures at light saturation can also be approximately calculated from photosynthetic growth measurements by

$$\mu_{max} = \frac{P_{o,max}}{Y_{X/O_2}} f \qquad (2.2)$$

where f is the fraction of the photoperiod that is illuminated (f = 0.42 for 10:14 LD photoperiod), and $Y_{X/O2}$ is the biomass yield coefficient based on total oxygen evolution. Assuming Calvin photosynthesis stoichiometry where one mole of CO_2 consumed generates one mole of O_2, $Y_{X/O2}$ is equal to 33.3 mmol $O_2 \cdot g^{-1}$ DCW. Estimates for μ_{max} based on equation 2.2 and the average $P_{o,max}$ values are also compared in Table 2.2 for filament clump and microplantlet culture. These values agree reasonably the specific growth rates given in Table 2.1.

Discussion

The first goal of this study was to develop a liquid suspension culture system for the macrophytic red alga *A. subulata* which could be subcultured indefinitely. We report the successful development of two *in vitro* culture systems (Figure 2.3). The filament clump culture was established by the induction of undifferentiated filaments from the cut faces of thallus explants. The filament clumps were undifferentiated, and individual filaments were uniseriate. The clump diameter ranged from 2 to 8 mm. The microplantlet culture was established by regeneration of undifferentiated filament clumps. The microplantlets consisted of lightly branched shoot tissues of 3-10 mm in length. Both cultures grew as a free suspension in liquid medium and could be subcultured indefinitely if the cell mass cut down by hand to 1-2 mm in size and placed in fresh liquid medium.

The undifferentiated filament clump culture was susceptible to regeneration. Proliferation of undifferentiated cell mass was only possible with no agitation and periodic medium replacement. In previous work with macrophytic red algae, Liu et al. (1990) reported that callus cultures of *Pterocladia capillacea* were friable owing to the release of bud cells from the callus clump. Chen (1989) and Tait et al. (1990) also reported that cell suspension cultures of *Porphrya* spp. established by protoplast isolation techniques were friable. In this present study, the undifferentiated filament clumps of *A. subulata* could not be dispersed by mixing and were not morphologically stable. The formation of adventitious shoots from uniseriate filaments leading to the development of the microplantlet was reliably and gently initiated when three-week old filament clumps were cultivated under continuous mixing at a sublethal agitation intensity,

e.g. 100 rev•min^{-1} on an orbital shaker. Formation of adventitious shoot tissues from an intervening callus-like tissue stage has been demonstrated for several genera of macrophytic red algae, including *Chondrus* (Chen and Taylor, 1978), *Porphyra* and *Sargassum* (Polne-Fuller et al., 1984, Polne-Fuller and Gibor, 1986, 1987, Polne-Fuller 1990), *Eucheuma* and *Kappaphycus* (Dawes and Koch, 1991), *Agardhiella* (Bradley and Cheney, 1990), and *Grateloupia* (Yokoya et al., 1993). However, in all of these studies, regeneration was viewed as a fortuitous or stochastic event, with light, explant/callus morphology, and plant growth regulators (auxins or cytokinins) implicated in the regeneration process (Huang and Fujita, 1997).

A second major goal of this study was to compare the growth characteristics of the cell and tissue culture systems developed for *A. subulata*. Specific growth rates and photosynthetic activity of *in vitro* cell and tissue cultures derived from macrophytic red algae have not been reported prior to this study. Microplantlets were faster growing and exhibited higher photosynthetic activity than the undifferentiated filament clumps at consistent cultivation conditions (Tables 2.1 and 2.2). The filament clump cultures were very fragile and were susceptible to regeneration or cell lysis under even gentle liquid mixing. Therefore, in order to meaningfully compare growth curves for the filament clump and microplantlet cultures, cultivations for both systems were carried out under surface aeration without agitation. It was possible that under these conditions that CO_2 and O_2 mass transfer rates were limiting the culture growth rate. However, the oxygen evolution rate measurements were used to estimate the photosynthetic growth parameters were carried out in an agitated vessel with the cell mass (filament clumps or microplantlets) immobilized on a nylon mesh. Between oxygen evolution rate measurements, the liquid phase was bubble aerated. In the environment for the short-term oxygen evolution rate measurements, the liquid phase was well mixed, CO_2 and O_2 gas exchange was vigorously promoted, and the immobilized filament clumps survived for at least 8 hours without loss of viability. Hence, the photosynthetic oxygen evolution rate measurements provide a rapid and complimentary means to compare the intrinsic growth rates for each culture system in response to light because growth rate data could be obtained under well-mixed, well-aerated conditions within a time frame that would not compromise the viability of the filament clump culture.

The specific growth rate of *A. subulata* regenerated microplantlet suspension cultures was typically 6% per day in nonagitated, surface aerated flask culture at 24 μmol photons•m^{-2}•s^{-1}. In comparison, aquaculture of *A. subulata* field-collected plants grown in nitrate-enriched seawater had maximum specific growth rates of 10% per day under 140 μmol photons•m^{-2}•s^{-1}, 16:8 LD photoperiod, in surface-aerated, continuous flow culture at 22 °C (DeBoer et al., 1978). If the

photoperiod of our *A. subulata* microplantlet culture is scaled linearly from a 10:14 to a 16:8 LD photoperiod, then the specific growth rate is also 10% per day. The low I_k values of our *in vitro* cultures of *A. subulata* reflect that they were maintained at relatively low light intensities below 20 μmol photons•m^{-2}•s^{-1}. However, optimization of growth conditions for maximum biomass productivity of the microplantlet culture system was beyond the scope of this present paper and will be reserved for future work, as described below.

The microplantlet culture system is best suited for further bioprocess development of *A. subulata* because it is morphologically stable and exhibits higher maximum oxygen evolution rates at light saturation relative to the filament clump culture. Our future work will focus on the development of suitable bioreactor systems to optimally cultivate the microplantlets in liquid suspension culture. Initial studies will focus on the cultivation of the microplantlet suspension culture in a continuously-aerated, bubble-column bioreactor with continuous medium perfusion in order to eliminate the possibility of CO_2 / O_2 mass transfer limitations and the buildup of waste products in the medium. The undifferentiated filament clump culture is still important for strain improvement, since microplantlet somaclones exhibiting enhanced growth rates, intrinsically high concentrations of desired natural products, or other desirable properties can be potentially obtained.

Chapter 3

CULTIVATION OF MICROPLANTLETS DERIVED FROM THE MACROPHYTIC MARINE RED ALGA *AGARDHIELLA SUBULATA* IN A BUBBLE-COLUMN PHOTOBIOREACTOR WITH MEDIUM PERFUSION

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Introduction

For macrophytic marine red algae, the phototrophic microplantlet suspension culture system is best suited for controlled production of cell biomass in a bioprocess environment. Toward this end, the overall goals of this work were to develop a suitable photobioreactor system for the *A. subulata* microplantlet suspension culture, and assess the limiting factors for cell biomass production. Specific limiting processes included light transfer, CO_2 transfer and consumption, macronutrient (nitrate, phosphate) utilization, and microplantlet morphology. Cultivation experiments were conducted with a bubble-column photobioreactor in batch, fed-batch, and liquid medium perfusion modes of operation at illumination and aeration conditions designed to avoid light and CO_2 transfer limitations. Culture growth and maintenance requirements were determined from measurements of cell density, photosynthetic oxygen evolution rate, and macronutrient concentration vs. time profiles. The efficiency of light and CO_2 transfer was assessed by estimation of mean light intensity, photosynthetic P-I curve parameters, and interphase CO_2 mass transfer rate during photobioreactor cultivation.

Materials and Methods

Culture maintenance. Agardhiella subulata microplantlet suspension cultures were maintained on modified ASP12 artificial seawater medium (Table 3.1) supplemented with 10X nitrate (11.8 mM), and 1X phosphate (44.9 μM). After addition of macronutrient and micronutrient stocks to the artificial seawater base medium, the final medium was sterile-filtered under vacuum through a 0.2 μm filter (Nalgene #155-0020) within a laminar flow hood. The sterilized medium was stored in autoclaved one-liter screw-cap bottles at 4 °C.
Development of the *A. subulata* microplantlet suspension culture from controlled regeneration of callus filaments is detailed in our previous work (Huang et al., 1998). Microplantlet suspension cultures were maintained in 250 mL foam-stoppered Erlenmeyer flasks, each with 100 mL of culture, without agitation at 24 °C under 20-40 $\mu E\ m^{-2}\ s^{-1}$ cool white fluorescent light (10:14 LD photoperiod) within an illuminated incubator. Flask cultures were swirled for five seconds once per day, and the medium was completely replaced every two weeks. Microplantlet shoot tissues were subcultured every four weeks. Four randomly-selected flask cultures were pooled into a single 500 mL Erlenmeyer flask. Individual microplantlets consisted of branched shoot tissues emanating from a central core. Therefore, each microplantlet was cut through its central branch

Table 3.1 APS 12 artificial seawater medium composition.

Compound	Chemical formula	mg L^{-1}
Artificial seawater base		
Sodium chloride	NaCl	28,020
Potassium chloride	KCl	700
Magnesium chloride heptahydrate	$MgSO_4 \cdot 7H_2O$	7,000
Magnesium chloride hexahydrate	$MgCl_2 \cdot 6H_2O$	4,000
Calcium chloride dihydrate	$CaCl_2 \cdot 2H_2O$	400
Nitriotriacetic acid	$C_6H_9NO_6$	100
Sodium HEPE (buffer)	$C_8H_{17}N_2O_4S$ Na	1,300
Sodium bicarbonate (buffer)	$NaHCO_3$	840
Macronutrients		
Sodium nitrate	$NaNO_3$	1,000
Sodium glycerophosphate	$C_3H_7O_6PNa_2$	9.70
Micronutrients – Vitamins		
B_{12}	$C_{63}H_{88}CoN_{14}O_{14}P$	0.0002
Biotin	$C_{10}H_{16}N_2O_3S$	0.001
Thiamine-HCl	$C_{12}H_{18}N_4OSCl \cdot HCl$	0.100
Micronutrients - P2 Metals		
Zinc chloride	$ZnCl_2$	0.109
Manganese chloride tetrahydrate	$MnCl_2 \cdot 4H_2O$	1.44
Cobalt chloride hexahydrate	$CoCl_2 \cdot 6H_2O$	0.04
Ferric chloride hexahydrate	$FeCl_3 \cdot 6H_2O$	0.49
Sodium ethylenedinitrilo-tetraacetate	$Na_2EDTA \cdot 2H_2O$	9.00
Boric acid	H_3BO_3	11.4
Micronutrients - S2 Metals		
Potassium bromide	KBr	14.9
Strontium chloride hexahydrate	$SrCl_2 \cdot 6H_2O$	5.20
Rubidium chloride	RbCl	2.78
Lithium chloride	LiCl	1.22
Potassium iodide	KI	0.013
Sodium molybdate (VI) dihydrate	$Na_2MoO_4 \cdot 2H_2O$	1.26

point into three pieces with a sterile scalpel within a sterile petri dish containing an overlay of fresh medium. The cut individual shoots formed new branches to continue growth. The excised tissues contained less than 10 shoots per microplantlet. Elongated shoots greater than 10 mm were trimmed to 3-5 mm in length. The remaining medium in the petri dish was pipetted out completely, and the trimmed microplantlets were re-suspended in fresh medium. Each flask contained about 100 microplantlets in 100 mL ASP12 medium. All subculturing procedures were carried out in a laminar flow hood using sterile technique.

2-L Bubble-column photobioreactor. The 2-L bubble-column perfusion photobioreactor shown in Figure 3.1 was used for all cultivation experiments. Process cultivation conditions are summarized in Table 3.2. The glass jacketed vessel is 41 cm in height, 8.5 cm inner diameter, and has a working volume of 1900 mL at the liquid medium exit port. The vessel jacket is connected to a temperature-controlled water circulation bath maintained at 24 °C. The vessel head plate has five ports, including a sampling port with a 12 mm inner diameter glass sampling tube, thermocouple well, pH electrode port, optional D.O. electrode port, and two air outlet ports connected to 0.2 μm Gelman sterilizing air filters. The illumination stage is equipped with two 20 cm 15 W cool-white fluorescent lamps vertically mounted on opposite sides of the glass vessel. The lamps are positioned 4.5 cm from the vessel surface to provide an incident light intensity of 43 $\mu E\ m^{-2}\ s^{-1}$. A timer sets the lamps to 10 h on and 14 h off per day (10:14 LD photoperiod).

The liquid culture within the bubble-column bioreactor was agitated and aerated by rising stream of fine air bubbles introduced into the base of the bioreactor vessel through a 4.0 cm diameter glass frit of 40-60 μm pore size. House air was metered, passed through a 0.2 μm Gelman autoclaved filter, and then bubbled through a sterilized humidifier before being introduced into the vessel. Carbon dioxide (CO_2) in the aeration gas served as the sole carbon source for biomass growth. The ambient CO_2 concentration in air was 350 ppm. In some experiments, supplemental CO_2 gas from a CO_2 tank was metered (1.9 mL min^{-1}) and then mixed with the inlet air stream to supply 3791 ppm of CO_2 in the aeration gas. The total aeration rate was 0.552 L min^{-1}, or 0.290 L air L^{-1} culture min (vvm) for all cultivation experiments (superficial gas velocity of $1.62 \cdot 10^{-3}$ m s^{-1}). The pH of the liquid medium in the bioreactor was set by the CO_2 partial pressure in the aeration gas using methods described previously (Rorrer et al., 1996). The measured equilibrium pH of the aerated ASP12 medium containing the 5.0 mM Na-HEPE / 10 mM $NaHCO_3$ buffer system (see Table 3.1.) was 8.7 at 350 ppm in the CO_2 aeration gas versus 8.0 at 3791 ppm CO_2 in the aeration gas. The bubble diameter of the aerated liquid medium in the vessel was estimated photographically at midpoint of the vessel height. All measured bubble diameters were below 2.0 mm, with at least 90% at or below 1.0 mm.

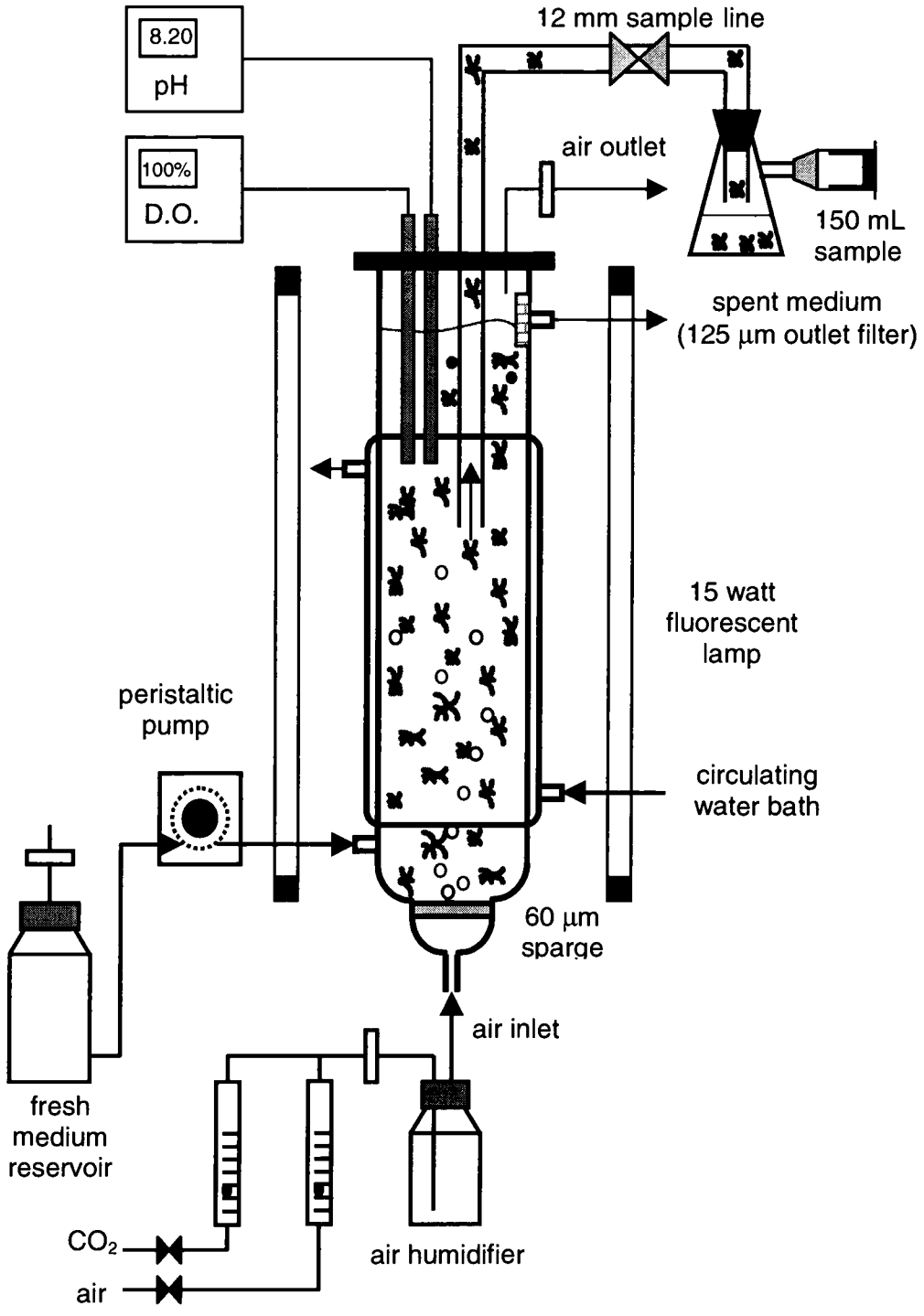

Figure 3.1 2-L bubble-column photobioreactor with medium perfusion.

Table 3.2 Bubble-column photobioreactor process conditions.

Variable	250 mL photobioreactor	2 L photobioreactor
Working volume, V	250 mL	1900 mL
Vessel inner diameter, d	4.5 cm	8.5 cm
Aeration rate, v_g / V	0.400 L air L^{-1} min^{-1}	0.290 L air L^{-1} min^{-1}
Mass transfer coefficient, $k_L a$	90 ± 2.3 h^{-1}	141 ± 3.5 h^{-1}
Nominal bubble size	1 mm	1 mm
Partial pressure CO_2, p_{CO_2}	35.5 Pa	35.5, 384 Pa
	(350 ppm CO_2)	(350, 3971 ppm at 1.0 atm)
pH	8.7 (350 ppm CO_2)	8.0 (3791 ppm CO_2),
		8.7 (350 ppm CO_2)
Medium	ASP12 (Table I)	ASP12 (Table I)
Medium perfusion rate	20 % per day	0, 2.6, 20.3 % per day
Culture removal rate	0.0 % per day	2.6% per day
Incident light intensity, I_o	38 µE m^{-2} s^{-1}	43 µE m^{-2} s^{-1}
α	2.0	2.0
Photoperiod (h ON / h OFF)	10:14 LD	10:14 LD
Temperature	24 °C	24 °C

In the perfusion cultivation experiments, fresh ASP12 medium (Table 3.1) was pumped into the bottom of the vessel by a peristaltic pump (Buchler 426-2000) at 38.6 mL h^{-1} to provide 386 mL day^{-1} total fresh medium during the 10h light phase of the 10:14 LD photoperiod. At 1900 mL total volume, the medium perfusion rate was 20.3% per day. The medium outlet port was set 5 cm from the top of the vessel to keep the culture volume constant at 1900 mL. A 125 µm nylon mesh glued onto the medium outlet port retained the biomass within the vessel. To provide a net liquid medium perfusion rate of 2.6% per day, immediately after removal of culture for sampling (150 mL suspension every three days), the medium flowrate was set at 150 ml h^{-1} for 1 h to replace the 150 mL liquid volume removed by sampling. In the batch cultivation experiments, liquid medium was not replaced after sampling and the total culture volume decreased proportionally to the cumulative amount of sample removal.

The autoclaved (121 °C, 205 kPa, 30 min) bioreactor assembly was inoculated in a laminar flow hood using sterile technique. To prepare the bioreactor inoculum, microplantlets

from 6 four-week old flasks were pooled. Microplantlets were sectioned into three pieces and trimmed as described above, and then rinsed with sterilized ASP12 medium under sterile filtration. The fresh tissue was weighed and re-suspended in 100 mL of filter-sterilized ASP12 medium. The tissue suspension and 1800 mL of filter-sterilized ASP12 medium (Table 3.1) were loaded into the bioreactor vessel to provide a total culture volume of 1900 mL. The suspension typically contained 1500-2000 microplantlets at a fresh cell density of 1.0-1.3 g FW L^{-1}. Culture samples, typically 150 mL of the microplantlet tissue suspension, were removed from the bioreactor through the 12 mm inner-diameter sampling tube under sterile suction at three day-intervals three hours into the light phase of photoperiod.

Cell biomass, nitrate, and phosphate. Immediately following removal of the 150 mL culture samples, the sample volume was measured to precision of ±1 mL. The liquid was decanted down to 50 mL. Aliquots of this supernatant liquid were saved and stored at -20 °C for later nitrate (2 mL sample) and phosphate (20 mL sample) concentration measurement. The total number of microplantlets in the sample was counted. The fresh cell weight (FW) and dry cell weight (DCW) of the microplantlet sample were also measured. To determine FW, the culture suspension was vacuum-filtered on a 50 mm by 20 μm nylon mesh with a 42.5 mm Whatman No. 1 filter paper resting directly underneath the mesh to prevent the nylon mesh from retaining medium. The retained biomass and nylon mesh were gently blotted with a paper towel, placed into a petri dish, and weighed to precision of ± 0.001 g. Immediately after each FW measurement, the microplantlet tissues were transferred with forceps onto an oven-dried, preweighed 47 mm x 0.45 μm filter (Millipore HWAP04700) and then dried at 80 °C for 24 h. The dried cell mass and filter were weighed to precision of ± 0.1 mg. FW and DCW values were determined by mass difference. Number cell density, fresh cell density, and dry cell density were determined from tissue number, FW, DCW and the original sample volume.
The nitrate (NO_3^-) concentration in seawater was assayed with a LaMotte nitrate test kit (model NCR 3110). Prior to the assay, a liquid sample aliquot of 2 mL was thawed to room temperature and diluted 1:100 in distilled water. The nitrate concentration in the sample was measured spectrophotometrically at 530 nm. For determination of glycerophosphate concentration in seawater, the glycerophosphate was first hydrolyzed to free phosphate (PO_4^{-3}) using a LaMotte auxiliary phosphorous test kit (model APT, code 7884). Specifically, 20 mL of the liquid sample was acidified with 1.0 mL of 36 wt% sulfuric acid in a 50 mL flask, and then 0.5 g ammonium persulfate was added to the acidified sample. The liquid mixture was boiled gently for 30 minutes, cooled to room temperature, neutralized with 1 M NaOH, then brought back up to 20 mL in distilled water. Free phosphate was then assayed with a LaMotte standard phosphorous

test kit (model VM-12 4408), where the phosphate concentration in the sample was measured spectrophotometrically at 605 nm after a color development time of 30 minutes. All nitrate and phosphate assays were performed in duplicate.

Photosynthetic oxygen evolution rate. Photosynthetic oxygen evolution rate (OER) measurements for the sampled microplantlets were performed in the dissolved oxygen (D.O.) cell described by Huang et al. (1998). Typically, 95 mL of liquid medium in the 150 mL culture sample was pipetted out to concentrate the microplantlet suspension to 65 mL. The suspension was then loaded into the 65 mL D.O. measurement cell. The stir bar tip speed within the D.O. cell was set at 52 cm s^{-1}. The volumetric oxygen evolution rate (P'_o, mmol O_2 L^{-1} h^{-1}) was determined from the least-squares slope of the linear portion of the D.O. concentration versus time curve, typically from 3 to 10 min. All data were collected at D.O. concentration below 120% saturation to avoid any possibility of dissolved oxygen toxicity effects on the culture. The D.O. concentration vs. time baseline in the sampled ASP12 medium with the tissue removed was also measured for the blank control. The base line had a consistent negative drift ranging from 0.05 to 0.12% of O_2 saturation in air per minute.

The OER measurements were designed to represent the conditions within the 2-L bubble-column photobioreactor. Therefore, OER measurements within the D.O. cell were carried out at an incident light intensity of 43 μE m^{-2} s^{-1} and 24 °C. Specific oxygen evolution rate (P_o, mmol O_2 g^{-1} DCW h^{-1}) was determined by the volumetric oxygen evolution rate (P_o) divided by the cell mass loaded into the D.O. test cell. All reported values were averaged from triplicate measurements and corrected for the medium blank. Dark-phase oxygen respiration rate measurements were also conducted in triplicate. Photosynthesis-light response (P-I) curves of the microplantlet suspension culture were measured at selected times during photobioreactor cultivation. For a given sample, the OER was measured at incident light intensities ranging from 4 to 67 μE m^{-2} s^{-1}.

Volumetric oxygen mass transfer coefficient. The volumetric oxygen mass transfer coefficient (k_La) for interphase mass transfer of oxygen into 1900 mL of ASP12 liquid medium within the 2-L bubble column photobioreactor was determined by the dynamic gassing-in method at 24 °C. The tip of the YSI model 5750 D.O. electrode was placed 5 cm below the liquid surface. Dissolved oxygen in the liquid medium was removed by sparging with N_2 through the sparger at a constant rate of 550 mL min^{-1}. When the D.O. concentration reached 10% of O_2 saturation in air, air was re-introduced through the sparger at a given flowrate ranging from 16 to 1360 mL min^{-1} (superficial gas velocities of $4.6 \cdot 10^{-5}$ - $4.0 \cdot 10^{-3}$ m s^{-1}). At each flowrate, the D.O. concentration vs. time data was recorded every 0.5 sec until the medium was saturated with

dissolved oxygen, and the value for k_La was estimated from the least-squares slope of the normalized D.O. concentration time data on a semi-log scale. The average response time for the D.O. electrode was 5.2 sec. The k_La values were corrected for the D.O. electrode response using the step method described by Blanch and Clark (1997). The uncorrected and corrected k_La values vs. superficial gas velocity are presented in Figure 3.2.

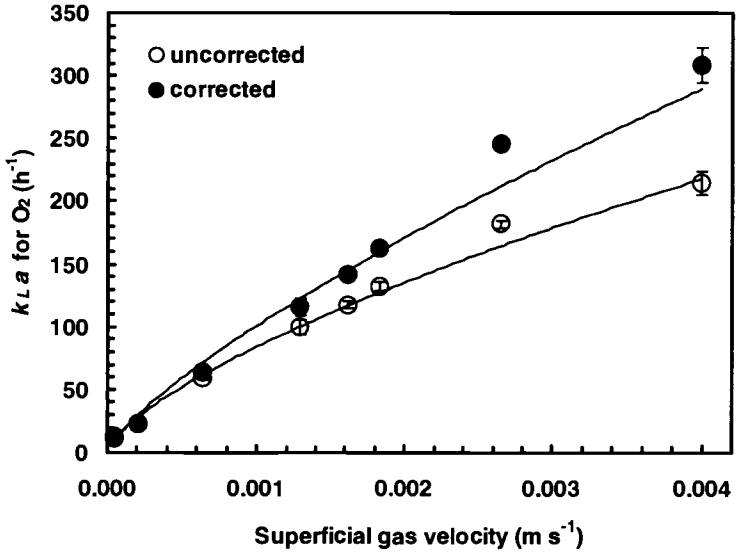

Figure 3.2 Volumetric mass transfer coefficient for O_2 (k_La) versus superficial gas velocity in 2-L bubble-column photobioreactor. Cultivation experiments were carried out at a superficial gas velocity of 0.0016 m s^{-1} (0.29 L air L^{-1} culture· min^{-1}).

250 mL Bubble-column photobioreactor. A 250 mL bubble-column photobioreactor described in our previous work (Zhi and Rorrer, 1996) was used to cultivate *A. subulata* microplantlets without biomass dilution by sample removal. Process cultivation conditions are summarized in Table II. Every five days, the entire 250 mL suspension was removed from the photobioreactor and filtered. The fresh weight of the microplantlets was measured. The microplantlets were immediately resuspended in 250 mL of fresh ASP12 medium (Table 3.1) and loaded back into the photobioreactor vessel, providing a medium replacement rate of 20% per day. Since all of the biomass was returned to the vessel, no other measurements on the biomass were performed. Fresh weight measurements and culture transfer procedures were carried out using sterile technique within a laminar flow hood.

Light attenuation constant. Values for the apparent light attenuation constant k' were determined for the *A. subulata* microplantlets at various sizes and cell densities. Specifically, microplantlets maintained in flask culture were trimmed to a mean equivalent diameter of 8.4 mm, where the equivalent diameter of a given microplantlet was the arithmetic average of the shortest and longest axes. The trimmed microplantlets (0.163 g FW, 0.0486 g DCW) and 180 mL ASP12 medium were added to 96 mm glass culture dish with opaque sides. A fluorescent lamp bank was placed directly above the culture dish, and a Li-Cor SA 190 PAR quantum sensor was positioned underneath the dish facing the lamp. The incident irradiance to the liquid suspension surface (I_o) was increased from 15 to 152 μE m^{-2} s^{-1} by moving the light source closer to the dish. At each incident light intensity, the average light intensity exiting the well-mixed culture suspension (I_z) was measured at a culture depth (z) of 2.5 cm, and k' was estimated from the least squares slope of I_z vs. I_o data with the intercept forced to zero (see equation 3.6). The procedure was repeated with increasing fresh cell mass ranging from 0.376 to 1.52 g to obtain the dependence of k' on the biomass density in the suspension.

Data Analysis

Specific growth rate for perfusion cultivation. The *Agardhiella subulata* microplantlet suspension was cultivated in a well-mixed, aerated bubble column photobioreactor with medium perfusion. Removal of the culture by sampling and addition of fresh medium by perfusion were modeled as continuous processes. At constant liquid volume, the unsteady-state material balance on cell biomass in the bioreactor during the exponential phase of the growth is

$$\frac{dX}{dt} = \left(-\frac{v_f}{V} + \mu \right) X = \mu_{app} X \tag{3.1}$$

where X is the cell density, v_f is the volumetric flow rate of culture suspension removal by sampling, V is the total culture volume, and μ is the specific growth rate averaged over the photoperiod. The apparent specific growth rate (μ_{app}) is estimated from the least-squares slope of linear portion of the ln(X) vs. t data. The medium perfusion rate is defined as v_o / V, where v_o is the volumetric flowrate of the entering fresh liquid medium less v_f.

Microplantlet friability. The normalized fragmentation rate (F) of individual microplantlets in the liquid suspension culture is defined as

$$F = \frac{1}{X_N} \cdot \left(\frac{dX_N}{dt} \right) \tag{3.2}$$

where X_N is the number density of individual microplantlets in the culture. If F is positive, the microplantlet shoot tissues break apart into smaller pieces. If F is negative, the microplantlet shoot tissues aggregate. If F is assumed equal to zero, the number of microplantlets per liter of culture suspension corrected for sample removal is estimated by

$$X_N = X'_N + \frac{\sum_{i=1}^{n} N_{s,i}}{V} \tag{3.3}$$

where $N_{s,i}$ is the number of microplantlets in the culture volume of a given sample, n is the current sample, and X'_N is the measured apparent number of microplantlets in the n^{th} sample. The slope of X_N vs. time data over the entire cultivation experiment must be statistically zero to justify the assumption that F is equal to zero.

Photosynthetic oxygen evolution rate (OER). The photosynthesis-light response (P-I) saturation model defines the effect of light intensity (I) on the net specific oxygen evolution rate (P_o). Estimates for P_o are correlated to light intensity by an exponential saturation model of the form

$$P_o = P_{o,\max}\left(1 - e^{-I/\alpha I_k}\right) + Q_o \tag{3.4}$$

where Q_o is the specific respiration rate, $P_{o,max}$ is the gross photosynthetic oxygen evolution rate at light saturation, and I_k is the light intensity at which 63.2% of the $P_{o,max}$ is achieved. Estimates for $P_{o,max}$ and I_k are determined by nonlinear regression of P_o vs. I data, whereas Q_o is independently estimated from dark-phase respiration measurements.

The specific oxygen evolution rate versus cultivation time data were fit to an exponential model of the form

$$P_o(t) = P_{o,\infty} + k_1 t e^{-k_2 t} \tag{3.5}$$

where $P_{o,\infty}$ is apparent specific oxygen evolution rate associated with metabolic activities of the culture after the exponential phase of growth, and $1/k_2$ is the time at which the peak specific oxygen evolution rate $P_{o,peak}$ is achieved. Least-squares estimates of $P_{o,\infty}$, k_1, and k_2 are determined by nonlinear regression of P_o vs. t data.

Mean light intensity. Light transfer into the microplantlet suspension is attenuated by the absorption of photons into the cells and the scattering of photons from the surface of individual microplantlets. The light intensity distribution is approximated by the Beer-Lambert Law, given by

$$I_z = I_o\, e^{-k' z} \tag{3.6}$$

where k' is the apparent light attenuation constant, I_z is the measured irradiance at culture depth z, I_o is the incident irradiance at the culture surface, and X is the biomass density of the suspension culture. The mean light intensity (I_m) within the photobioreactor control volume is the volume integral of light intensity distribution. Specifically, for one-dimensional light transfer across the vessel width (d) with symmetrical two-sided illumination, the conservative estimate for I_m *is*

$$I_m = \frac{\alpha}{d}\int_0^d I_z dz = \frac{\alpha\, I_o}{k'd}\left(1 - e^{-k'd}\right) \tag{3.7}$$

where α equals to 2.0 for symmetric two-sided illumination. The apparent light attenuation constant (k') is a linear function of cell density X

$$k' = k_o + k_c \cdot X \tag{3.8}$$

where k_c is the specific light attenuation constant of the biomass, and k_o is the light attenuation constant of the cell-free medium.

CO_2 consumption and mass transfer. The CO_2 consumed by the microplantlets for photosynthetic growth is supplied to the liquid medium by the aeration gas. Interphase mass transfer of CO_2 from the aeration gas bubbles to the culture suspension is liquid-phase controlling, due to the low solubility of CO_2 in liquid medium. The mass balance for CO_2 in the constant volume perfusion photobioreactor with sample removal is

$$v_o C_{CO_2,o} + V \cdot (k_L a)_{CO_2} (C^*_{CO_2} - C_{CO_2}) - q_{CO_2}(t) \cdot X(t) - v_o C_{CO_2} = 0 \tag{3.9}$$

where $C_{CO_2,o}$ is the dissolved CO_2 concentration in the fresh medium feed stream, C_{CO_2} is the dissolved CO_2 concentration within the well-mixed liquid volume of the photobioreactor, $C^*_{CO_2}$ is the dissolved CO_2 concentration in equilibrium with the partial pressure of CO_2 in the aeration gas (mmol CO_2 L^{-1}), $(k_L a)_{CO_2}$ is the volumetric mass transfer coefficient for CO_2 (h^{-1}), and q_{CO_2} is the specific CO_2 consumption rate by the microplantlets (mmol CO_2 g^{-1} DCW h^{-1}). Usually, the v_o terms are neglected. If C_{CO_2} goes to zero, then photosynthetic biomass production is limited by interphase mass transfer of CO_2. Consequently, equation (3.9) reduces to

$$(k_L a)_{CO_2} C^*_{CO_2} \geq q_{CO_2}(t) \cdot X(t) \tag{3.10}$$

with the required CO_2 transfer rate defined as

$$CO_2 - TR = (k_L a)_{CO_2} \cdot C^*_{CO_2} = (k_L a)_{CO_2} \frac{p_{CO_2}}{H} \tag{3.11a}$$

or $$CO_2 - TR = n_A = \frac{v_g \; p_{CO_2}}{RT\,V} \tag{3.11b}$$

where the Henry's law constant for CO_2 in 36 ppt seawater (H) at 24 °C is 0.0346 atm L mmol^{-1} (Raven, 1984). At pH 8 to 9, dissolved inorganic carbon in seawater is speciated as bicarbonate (HCO_3^-)

$$CO_2(aq) + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \tag{3.12}$$

$$HCO_3^- \leftrightarrow CO_3^{-2} + H^+ \tag{3.13}$$

where the pK_a values for HCO_3^- and carbonate (CO_3^{-2}) dissociation in seawater at 24 °C are 6.05 and 9.10 respectively (Raven, 1984). The Na-HEPE (5.0 mM, pK_a = 7.5) in the ASP12 medium buffers the pH increase in response to dissolved CO_2 demand by the suspension culture at pH 8-9. At pH 8-9, CO_2 transfer is affected by chemical reaction in alkaline medium, and the $(k_L a)_{CO_2}$ for CO_2 mass transfer is related to $k_L a$ for O_2 mass transfer by

$$(k_L a)_{CO_2} = k_L a \left(\frac{D_{CO_2}}{D_{O_2}} \right)^{1/2} \tag{3.14}$$

where the ratio of molecular diffusion coefficients for CO_2 to O_2 in seawater medium at 24 °C is 0.93, using data provided by Raven (1984).

The phototrophic *A. subulata* microplantlet cultures utilize dissolved CO_2 as the sole carbon source and rely on the Calvin photosynthesis cycle to incorporate carbon from CO_2 into cellular material. The overall stoichiometry for photosynthetic biomass production is approximated as

$$CO_2 + H_2O \rightarrow CH_2O + O_2 \tag{3.15}$$

The contribution of nitrate and phosphate assimilation into cellular material is assumed to have only a small effect on the CO_2 consumption to O_2 evolution ratio. The C:N:P composition of phototrophic organisms cultivated in a large excess of all nutrients generally follows the Redfield ratio of 120:16:1, although experimentally measured C:N:P values for marine macroalgae are typically 550:30:1 (Atkinson and Smith, 1983). At C:N:P of 550:30:1, the overall photosynthetic biomass stoichiometry is

$$\begin{aligned} &550CO_2 + 580H_2O + 30NO_3^- + PO_4^{-3} + 30H^+ \\ &\rightarrow (CH_2O)_{550}(NH_3)_{30}(H_3PO_4) + 610O_2 \end{aligned} \tag{3.16}$$

The specific CO_2 consumption rate is estimated from the specific oxygen evolution rate by

$$q_{CO_2} = P_o \cdot \frac{\nu_{CO_2}}{\nu_{O_2}} \cong P_o \tag{3.17}$$

where ν_{CO_2} and ν_{O_2} are stoichiometric coefficients for CO_2 and O_2 based on overall photosynthetic biomass production stoichiometry. To avoid CO_2 mass transfer limited growth, CO_2-TR must be larger than $q_{CO_2} \cdot X$ at all times during the cultivation.

Results and Discussion

Photosynthetic biomass production. Process conditions for cultivation of *A. subulata* microplantlet suspensions in the bubble-column photobioreactor are summarized in Table 3.2. Representative photographs of the microplantlets are presented in Figure 3.3. Batch cultivation experiments are presented in Figure 3.4, whereas cultivation experiments at fresh medium perfusion rates of 2.6% per day and 20% per day are presented in Figures 3.5 and 3.6 respectively. In all cultivation experiments, dry cell density (X) and specific oxygen evolution rate (P_o) vs. time profiles were measured over a 50 day cultivation period. Specific growth rates estimated from dry cell density vs. time data are presented in Table 3.3, whereas Table 3.4 presents the parameters described by equation (3.5) that were fitted to P_o vs. cultivation time data. Table 3.4 also provides specific respiration rate (Q_o), peak volumetric CO_2 consumption rate, and CO_2-TR values for each cultivation experiment.

In the batch cultivation experiments, the total culture volume decreased as the suspension was sampled, whereas in the perfusion photobioreactor cultivation experiments, the total culture volume was kept constant. The cultivation experiment at the perfusion rate of 2.6% per day was designed to approximate a constant-volume batch experiment, as this perfusion rate equaled the volumetric sample removal rate. Increasing the perfusion rate from 2.6 to 20.3% per day did not have a significant effect on the rate of biomass production, which was nominally 10% per day (Table 3.3). However, medium perfusion did affect specific oxygen evolution rate (P_o) vs. time curve, which is a measure of the photosynthetic activity of the cell biomass. The specific oxygen evolution rate increased to a maximum value during the early exponential phase, then settled down to a constant value in perfusion culture but moved toward zero in batch culture. Specifically, at a medium perfusion rate of 20% per day, the maintenance oxygen evolution rate ($P_{o,\infty}$) was 0.12 mmol O_2 g^{-1} DCW h^{-1}, two times higher than the value for $P_{o,\infty}$ from 2.6 % per day medium perfusion and batch cultivation experiments (Table 3.4).

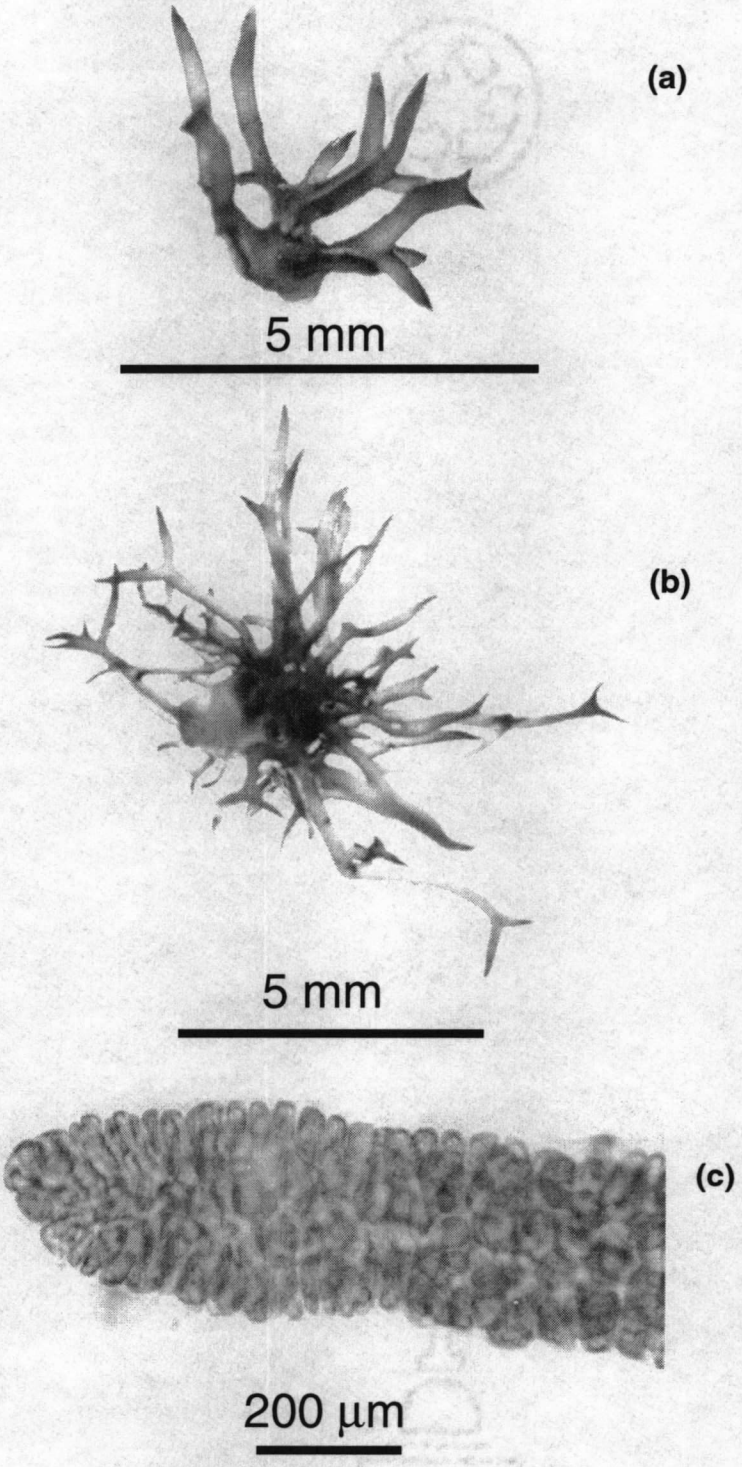

Figure 3.3 Photographs of *A. subulata* microplantlets (a) inoculation, scale bar = 5.0 mm; (b) 50 days old, scale bar = 5.0 mm; (c) shoot tip, scale bar = 200 µm.

Table 3.3 Biomass productivity of *A. subulata* microplantlets in batch and perfusion modes of cultivation within the 2-L bubble-column photobioreactor.

Run #	Medium Perfusion Rate (% per day)	CO_2 Partial Pressure (Pa)	Average pH	Growth Phase (days)	Apparent Growth Rate, μ_{app} (day^{-1})	Culture Removal Rate (% per day)	Specific Growth Rate, μ (day^{-1})	Solids Content (wt %) Initial	Final
23	0.0	35.5	8.65 ± 0.06	0-26	0.062 ± 0.014	0.0	0.062 ± 0.014	20.4	27.1
25	0.0	35.5	8.71 ± 0.05	0-27	0.083 ± 0.006	0.0	0.083 ± 0.006	19.7	30.3
27a	Fed-batch P addition	35.5	8.72 ± 0.05	0-26	0.058 ± 0.009	0.0	0.058 ± 0.009	15.1	28.2
8	2.6	384	8.10 ± 0.12	0-30	0.076 ± 0.005	2.6	0.102 ± 0.005	16.8	30.5
10	2.6	384							
11	20.3	384	8.06 ± 0.07	0-14	0.093 ± 0.017	2.6	0.119 ± 0.017	15.3	31.6
				14-51	0.020 ± 0.003	2.6	0.046 ± 0.003		
13	20.3	384	7.98 ± 0.04	0-17	0.072 ± 0.0140	2.8	0.100 ± 0.014	15.8	32.2
				17-51	0.015 ± 0.003	2.8	0.043 ± 0.003		

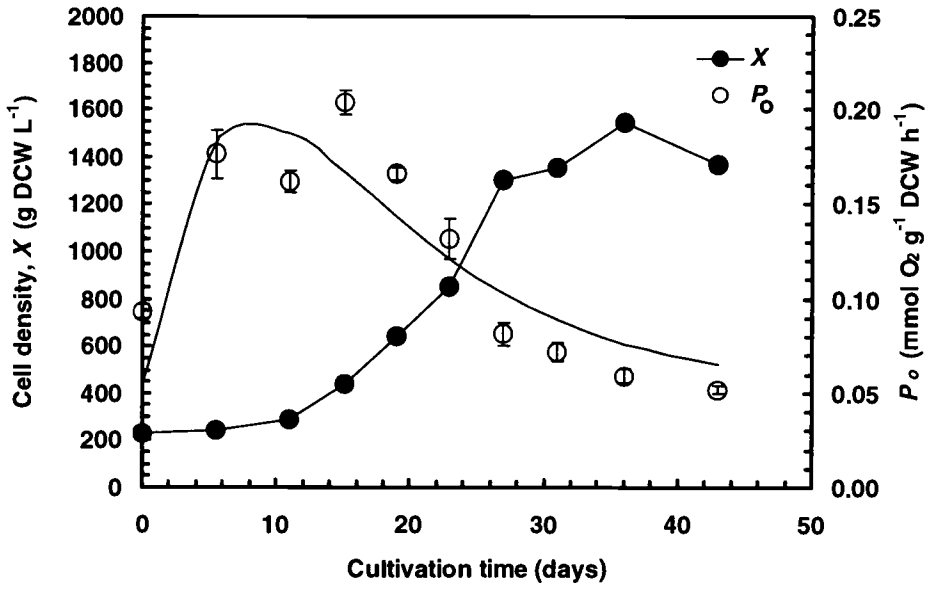

Figure 3.4 Batch cultivation of *A. subulata* microplantlet suspension in 2-L bubble-column photobioreactor: cell density (X) and specific oxygen evolution rate (P_o) versus time.

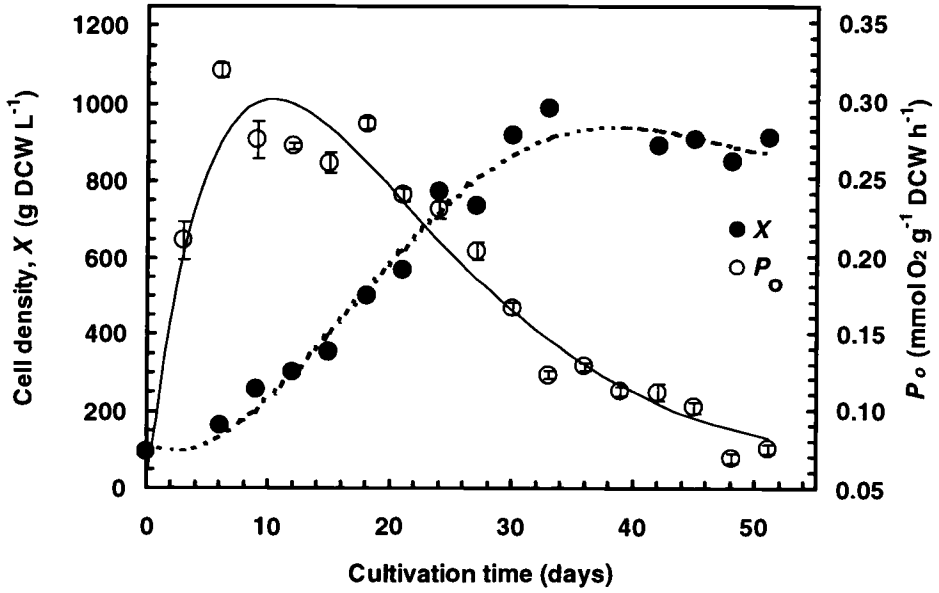

Figure 3.5 Cultivation of *Agardhiella subulata* microplantlet suspension in 2-L bubble-column photobioreactor at a medium perfusion rate of 2.6% per day: cell density (X) and specific oxygen evolution rate (P_o) versus time.

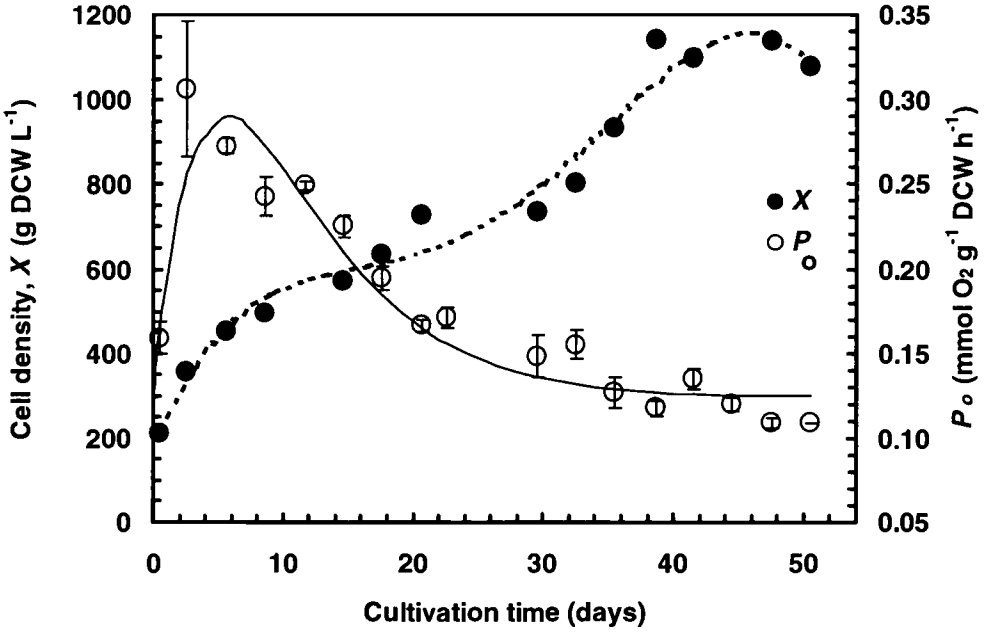

Figure 3.6 Cultivation of *Agardhiella subulata* microplantlet suspension in 2-L bubble-column photobioreactor at medium perfusion rate of 20% per day: cell density (X) and specific oxygen evolution rate (P_o) versus time.

The cell density versus time data on a semi-log scale (graph not shown) reveals that cultivation at a medium perfusion rate of 20% per day has two log phases of growth: an initial phase of relatively high growth within the first 14 days of cultivation, followed by a slower but sustained growth phase for the remainder of the cultivation period. This observation is also reflected in the P_o vs. time curve, which has a sharp maximum within the first two weeks of growth. In contrast, no second log phase of growth was observed for 2.6% per day medium perfusion or batch cultivation experiments. Fresh medium perfusion also continuously removed used medium to avoid potential build-up of metabolite wastes. At the end of the batch cultivation experiments, the medium was cloudy and yellow. However, the liquid medium was always clear during the 20% per day medium perfusion cultivation experiments. All of these observations point to the conclusion that medium perfusion maintains the *A. subulata* microplantlets in a viable state.

Table 3.4 Photosynthetic growth parameters of *A. subulata* microplantlets in batch and perfusion modes of cultivation within the 2-L bubble-column photobioreactor.

Medium Perfusion Rate (% per day)	CO_2 Partial Pressure (Pa)	Average PH	$P_{o,\infty}$ (mmol O_2 g^{-1} DCW h^{-1})	k_1 (mmol O_2 g^{-1} DCW h^{-1} day^{-1}) k_2 (day^{-1})	Specific Q_o (mmol O_2 g^{-1} DCW h^{-1})	Peak $q_{CO_2} \cdot X$ (mmol CO_2 L^{-1} h^{-1})	CO_2-TR & n_A (mmol CO_2 L^{-1} h^{-1})
0.0	35.5	8.65	0.0403 ± 0.0104	0.045 (k_1)	0.0155 ± 0.0055	0.0891	1.36 (CO_2-TR)
				0.119 (k_2)			0.25 (n_A)
0.0	35.5	8.71	0.0537 ± 0.0192	0.059 (k_1)	0.0192 ± 0.0056	0.112	1.36 (CO_2-TR)
				0.190 (k_2)			0.25 (n_A)
Fed-batch P addition	35.5	8.72	0.0627 ± 0.0104	0.0498 (k_1)	0.0162 ± 0.0099	0.118	1.36 (CO_2-TR)
				0.1241 (k_2)			0.25 (n_A)
2.6	384	8.10	0.0585 ± 0.0143	0.064 (k_1)	0.0212 ± 0.0045	0.177	14.8 (CO_2-TR)
				0.096 (k_2)			2.74 (n_A)
20.3	384	8.06	0.124 ± 0.0079	0.081 (k_1)	0.0232 ± 0.0047	0.170	14.8 (CO_2-TR)
				0.180 (k_2)			2.74 (n_A)
20.3	384	7.98	0.126 ± 0.0086	0.059 (k_1)	0.0234 ± 0.0077	0.121	14.8 (CO_2-TR)
				0.192 (k_2)			2.74 (n_A)

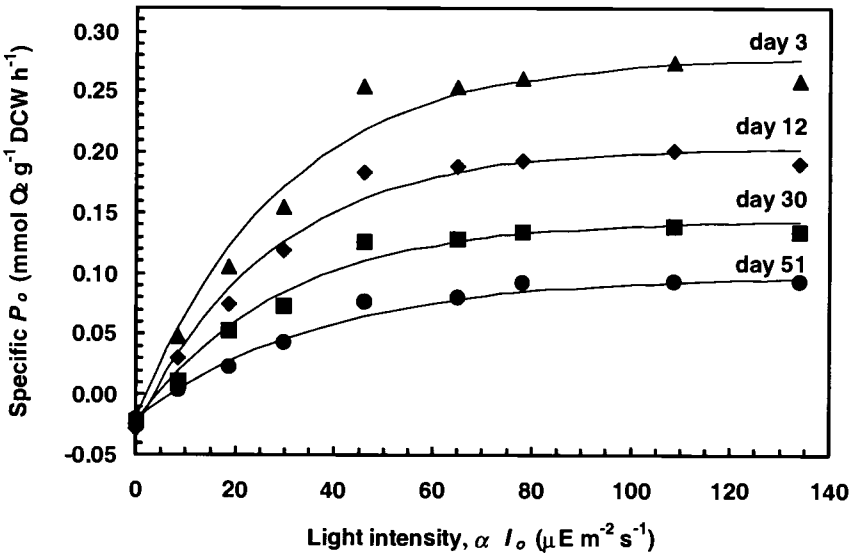

Figure 3.7 P_o-αI curves during cultivation of the *A. subulata* microplantlet suspension in the 2-L bubble-column photobioreactor at a medium perfusion rate of 20% per day.

Representative P_o -αI curves at various times during cultivation of the microplantlets at a medium perfusion rate of 20% per day are presented in Figure 3.7. The P_o –αI curves level off but do not increase with increasing light intensity, demonstrating that the microplantlets were not subject to photoinibition at αI values below 134 μE m^{-2}s^{-1}. The specific O_2 respiration rates (Q_o) did not change significantly over the cultivation period. Specific Q_o values averaged over the 50 day cultivation period ranged from 0.015 to 0.025 mmol O_2 g^{-1} DCW h^{-1}, about 10% of the peak P_o values.

Growth rates for the *A. subulata* microplantlets in the exponential phase of growth, ranged from 0.06-0.12 day^{-1} (Table 3.3), similar to nitrate-saturated growth of aqua-cultured *Agardhiella* plants collected from the field (De'Boer et al., 1978). Macroalgal cell and tissue cultures previously developed in our laboratory had higher growth rates. For example, cultivation of gametophyte cells derived from the macrophytic brown alga *Laminaria saccharina* in stirred-tank and bubble-column photobioreactors achieved specific growth rates of 0.15 day^{-1} (Qi and Rorrer, 1995; Zhi and Rorrer, 1996), whereas cultivation of filamentous tissue suspensions derived from the macrophytic green alga *Acrosiphonia coalita* in a stirred-tank photobioreactor achieved specific growth rates of 0.19 day^{-1} (Rorrer et al., 1996).

Nitrate and phosphate utilization. For batch cultivation and 20% per day medium perfusion cultivation experiments, nitrate and phosphate concentration vs. time profiles in the

photobioreactor liquid medium were also measured, as shown in Figures 3.8 and 3.9 respectively. Nitrate and phosphate are the required macronutrients for phototrophic growth that are supplied by the liquid medium. The N:P ratio in the ASP12 medium (Table 3.1) was 262:1 on a molar basis, about 10 times higher than the typical N:P ratio of 30:1 in marine macroalgae (Atkinson and Smith, 1983), to circumvent the effects of N-limitation on photosynthesis in carbon-nitrogen metabolism of algae (Turpin, 1991). This medium formulation also moved microplantlet growth toward phosphate limitation. During batch cultivation, the phosphate concentration in the liquid culture went from 0.045 mM to zero within 14 days of cultivation, during the mid-exponential phase of growth (Figure 3.8). However, biomass production reached stationary phase after about 30 days of cultivation, implying that phosphate was assimilated and stored before being incorporated into new cells. The nitrate concentration decreased modestly during the cultivation period because the medium was ballasted with a large stoichiometric excess of nitrate. Consequently, batch cultivation was phosphate limited. Based on the initial cell density of 229 mg DCW L^{-1} , and the average final cell density of 1390 ± 107 mg DCW L^{-1} (± 1 s.d., n=4, 27-43days), the biomass mass yield coefficient based on phosphate limitation ($Y_{X/P}$) was 25.8 g DCW $mmol^{-1}$ P, versus 17.2 g DCW $mmol^{-1}$ P estimated from equation 3.16. As shown in Figure 3.9, microplantlet growth was not limited by nitrate or phosphate during medium perfusion cultivation at a rate of 20% per day.

The microplantlet cultivation data presented in Figures 3-6 were obtained in the 2 L bubble column photobioreactor, which required periodic removal of 150 mL suspension culture samples. In constant-volume medium perfusion operation (Figures 3.5 and 3.6), this sample removal process diluted microplantlets from the suspension culture and therefore affected the volumetric biomass productivity, as described by equations (3.1) and (3.3). In order to simulate medium perfusion without sample removal, cultivation experiments were conducted in a 250 mL bubble column photobioreactor by procedures which measured the fresh cell weight of the microplantlets in the entire vessel (see Materials and Methods). Figure 3.10 presents a representative fresh cell density vs. time profile in the 250 mL bubble-column photobioreactor at a medium replacement rate of 20% per day (complete medium changeover every five days). The process cultivation conditions were similar to the 2 L bubble column photobioreactor (Table 3.2). The fresh cell density continuously increased with time over the 50 day cultivation period. In comparison, the fresh cell density for batch cultivation in the 2 L bubble-column photobioreactor achieved stationary phase after 30 days. After 50 days, the fresh cell density for perfusion culture at a medium replacement rate of 20% per day was approximately two times the fresh cell density for batch culture.

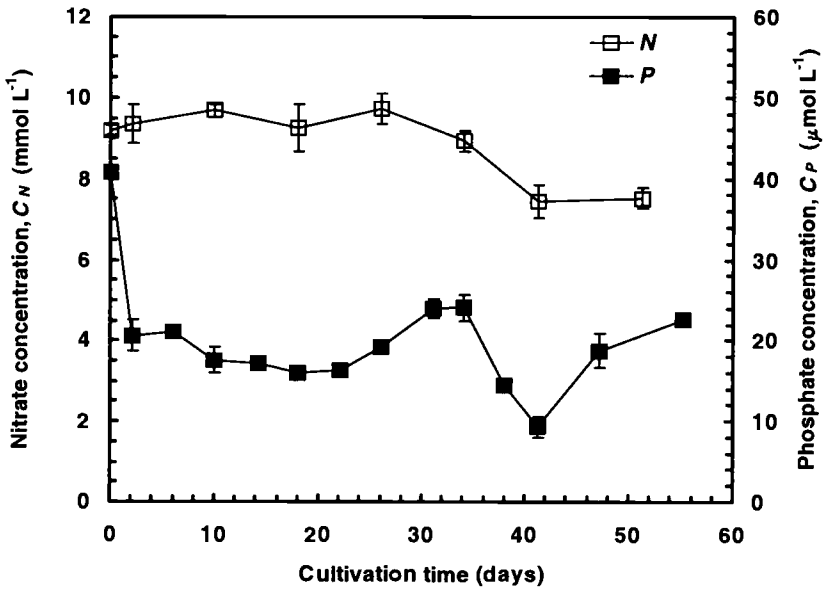

Figure 3.8 Batch cultivation of *A. subulata* microplantlet suspension in 2-L bubble-column photobioreactor: nitrate and phosphate concentration versus time.

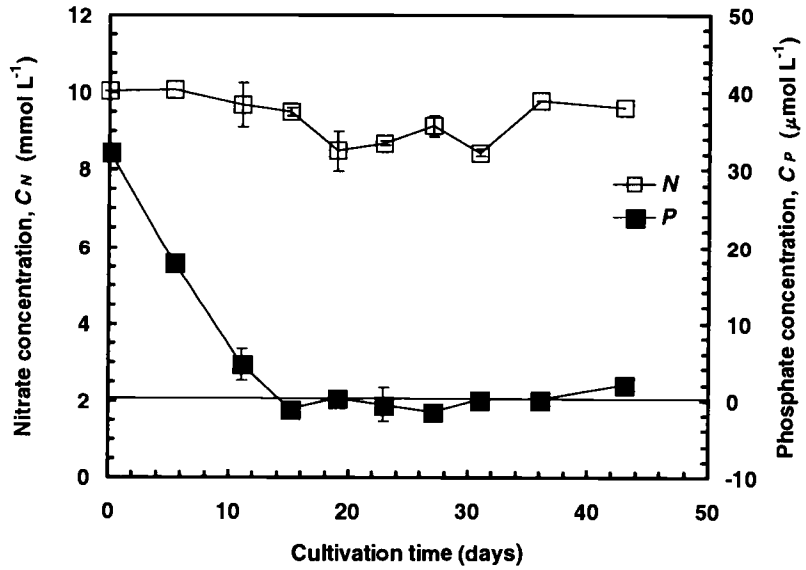

Figure 3.9 Cultivation of *A. subulata* microplantlet suspension in 2-L bubble-column photobioreactor at medium perfusion rate of 20% per day: nitrate and phosphate concentration versus time.

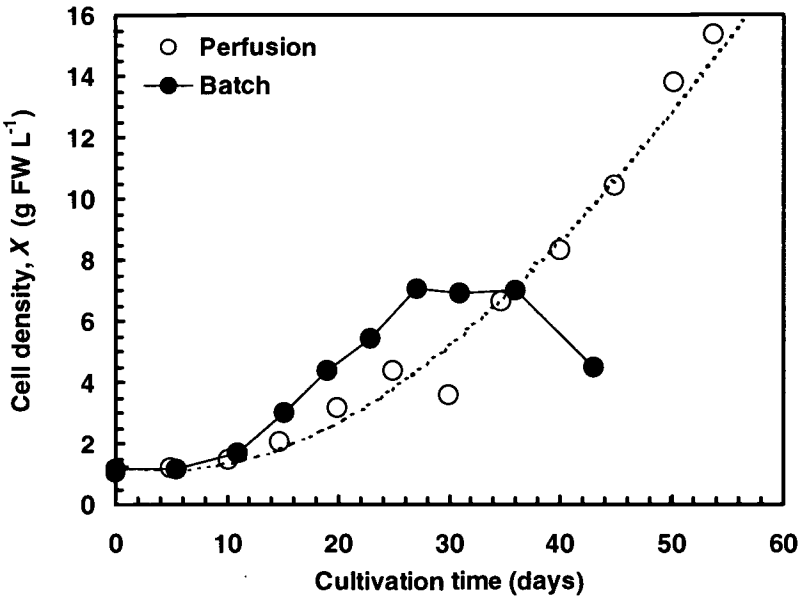

Figure 3.10 Comparison of *A. subulata* microplantlet suspension growth curve: batch cultivation in the 2-L bubble-column photobioreactor versus 20% per day perfusion cultivation with no sample removal in the 250-mL bubble-column photobioreactor.

As mentioned above, batch cultivation of microplantlets in the 2 L bubble-column photobioreactor was phosphate limited. In order to avoid phosphate limitation during batch cultivation, phosphate was fed to the microplantlet culture suspension at a linearly increasing rate, described by

$$r_P = 0.189t + 1.16 \tag{3.18}$$

where r_P is the total rate of phosphate addition (μmol day^{-1}), and t is cultivation time (days). Phosphate addition was carried out by periodically adding aliquots of 100X sodium glycerophosphate solution (4.5 mM) to the culture. The cell density and specific oxygen evolution rate vs time profiles for fed-batch phosphate cultivation are presented in Figure 3.11a, and additional growth parameters are presented in Tables 3.3 and 3.4. The phosphate concentration in the liquid medium and the phosphate conversion estimated by material balance is presented in Figure 3.11b. Fed-batch addition of phosphate avoided phosphate limitation and maintained biomass production over the 50 day cultivation period, but did not increase the specific rate of biomass production or the specific oxygen evolution rate.

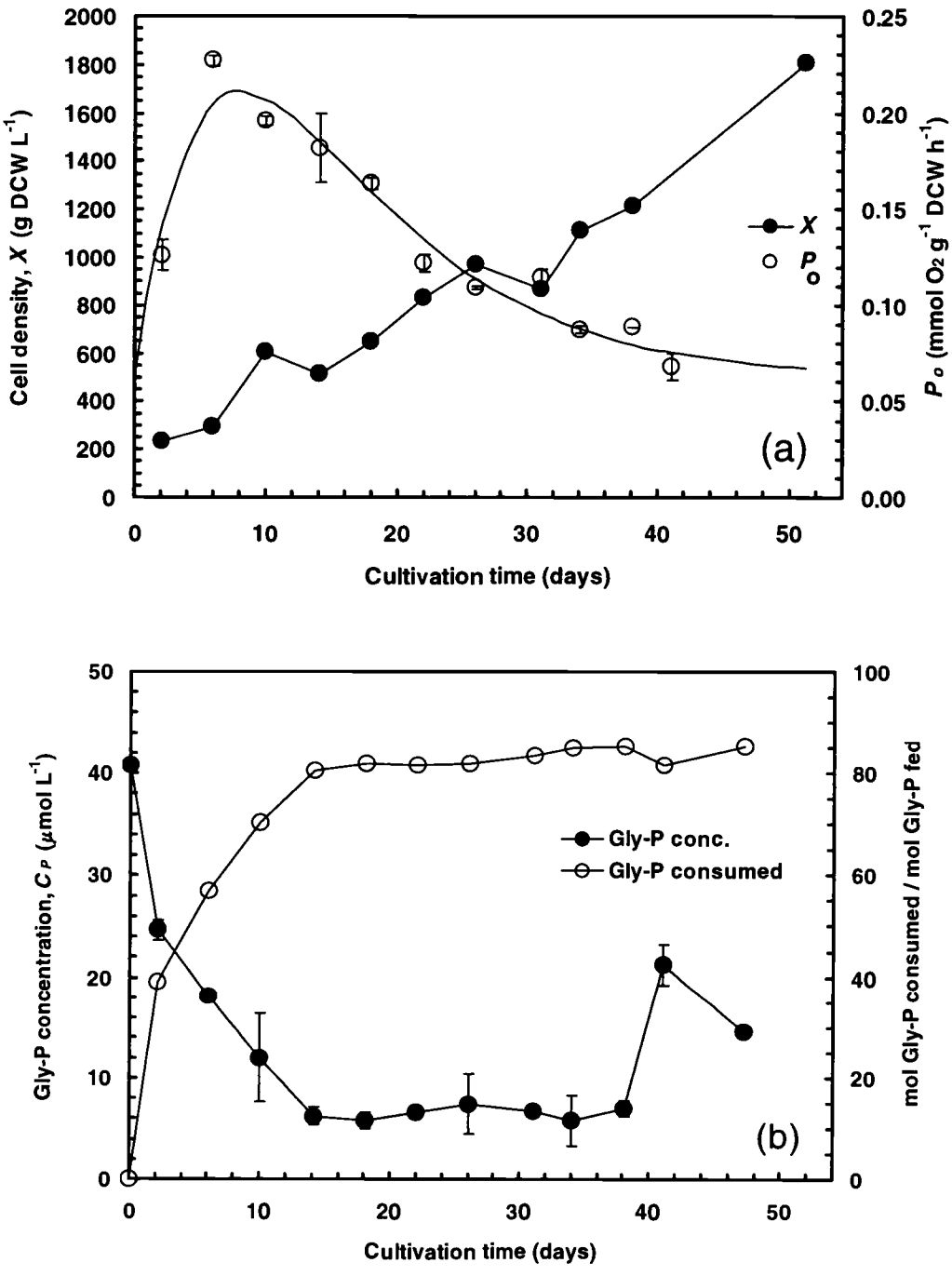

Figure 3.11 Cultivation of *A. subulata* microplantlet suspension in the 2-L bubble-column photobioreactor under fed-batch phosphate addition. (a) Cell density (X) and specific oxygen evolution rate (P_o); (b) Phosphate concentration in liquid, and phosphate consumption per phosphate fed.

Previous studies on phosphate-limited growth of aqua-cultured *Agardhiella* plants collected from the field showed that the specific growth rate was saturated at seawater phosphate concentrations of 10 μM whereas the specific P content of the biomass was saturated at 20 μM (Chopin et al., 1990). Based on the phosphate concentration profiles provided in Figures 3.8 and 3.11b, both fed-batch phosphate cultivation and perfusion cultivation at a rate of 20% per day were at saturation conditions with respect to phosphate.

Light and CO_2 transfer. Based on the above results, cultivation of *A. subulata* microplantlets at a medium perfusion rate of 20% per day was not limited by delivery of macronutrients nitrate and phosphate to the liquid suspension. In addition to medium macronutrient limitations, growth of the phototrophic *A. subulata* microplantlets may be subject to light and CO_2 transfer rate limitations, as external illumination is the sole energy source for photosynthesis and dissolved CO_2 supplied by the aeration gas is the sole carbon source for growth. The effect of biomass density on the apparent light attenuation constant (k') for the *A. subulata* microplantlets is presented in Figure 3.12. The dependence of k' on dry cell density X was linear, with specific light attenuation constant k_c of $1.07 \cdot 10^{-4} \pm 5.2 \cdot 10^{-6}$ L cm^{-1} mg^{-1} DCW and k_o of 0.241 ± 0.010 cm^{-1} (±1 s.d., n = 8). Light transfer limitations were addressed by comparing the mean light intensity (I_m) to the light intensity at 63% of light saturation (I_k) of the microplantlet suspension as a function of cultivation time (Figure 3.13). The I_k values were estimated by fitting P_o - αI curve data in Figure 3.8 to equation (3.4). As the cell density X increased with time, I_m decreased due to light attenuation, whereas values for αI_k increased modestly with time to adapt to the decreased light availability at higher cell density. By day 51, the mean light intensity was 27 μE m^{-2} s^{-1} at 1100 mg DCW L^{-1} cell density, whereas αI_k was 37 μE m^{-2} s^{-1}. In equation (3.4), if I_m and αI_k are inserted into equation (3.4), then growth was reduced to 52% of photosynthetic saturation, but clearly not shut down by light attenuation through the suspension.

Interphase CO_2 mass transfer rate limitations were addressed by comparing the volumetric CO_2 volumetric rate to the CO_2-TR defined by equation (3.10). The CO_2-TR and peak volumetric CO_2 consumption rate ($q_{CO_2} \cdot X$) for each cultivation experiment are presented in Table 3.4. In all cultivation experiments, the CO_2-TR was always at least two times higher than the peak $q_{CO_2} \cdot X$. Clearly, growth of the *A. subulata* microplantlet suspension in the bubble-column photobioreactor was not limited by the CO_2 mass transfer rate. The high CO_2 transfer rate was accomplished by 1) the high k_La provided by fine-bubble aeration of the liquid suspension, and 2) the elevated CO_2 concentration in the aeration gas (3800 ppm, 11 times ambient air

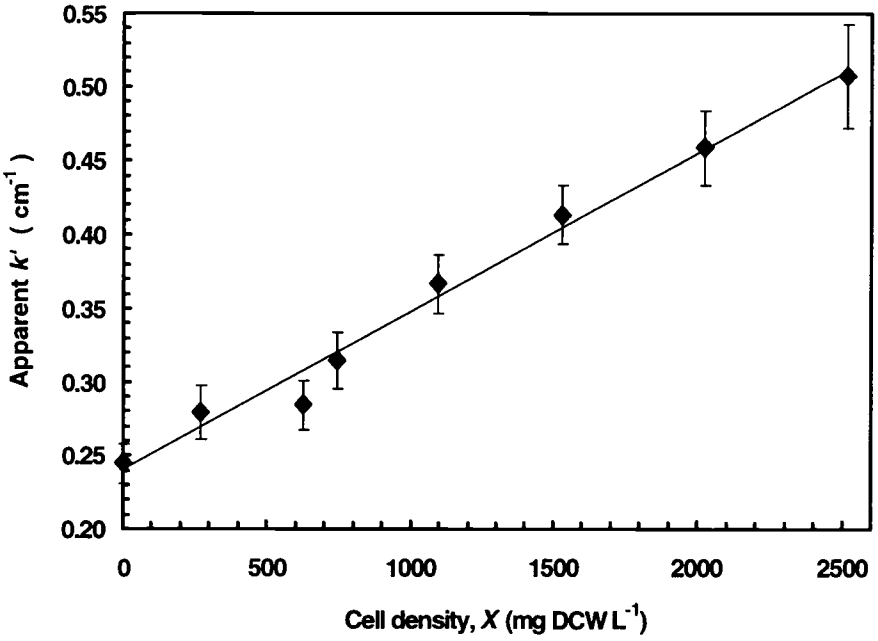

Figure 3.12 Effect of cell density (X) on the apparent attenuation constant k' for *Agardhiella subulata* microplantlet suspension for microplantlets of 8.4 mm average diameter.

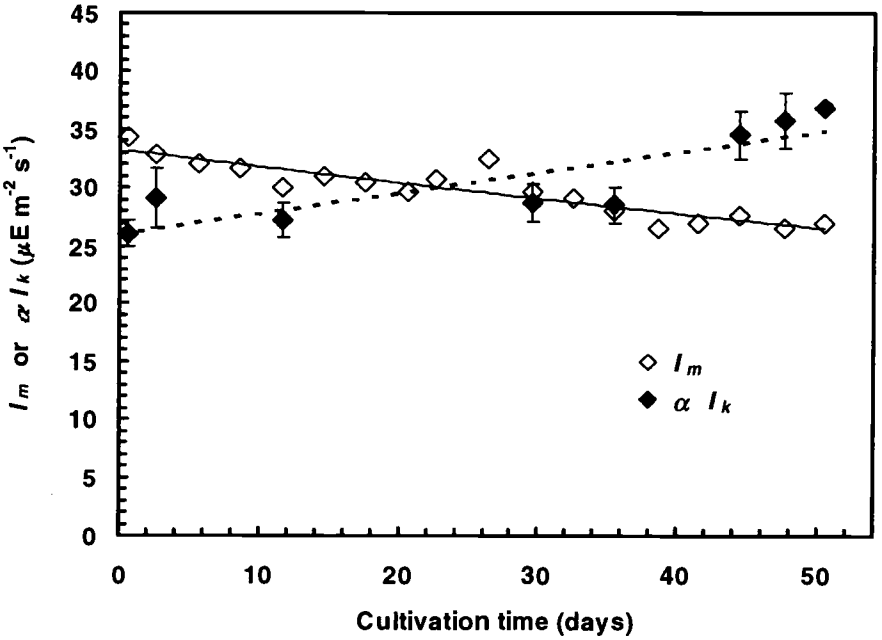

Figure 3.13 Cultivation of *A. subulata* microplantlet suspension in 2-L bubble-column photobioreactor at medium perfusion rate of 20% per day: mean light intensity (I_m) and light intensity at 63.2% of saturation (I_k) versus time.

concentration). The high CO_2-TR also maintained the culture pH at the setpoint value by buffering H^+ consumption during speciation of dissolved CO_2 to bicarbonate and carbonate, as described by equations (3.12-13).

Microplantlet growth morphology. In bubble-aerated liquid suspension, the *A. subulata* microplantlets appeared rigid. An aeration rate of 0.29 L air L^{-1} culture min^{-1} (Table 3.2) was sufficient to uniformly and individually suspend the microplantlets in the liquid culture. Figure 3.14 presents the apparent and corrected number density of microplantlets in suspension with time during medium perfusion cultivation at a rate of 20% per day. The microplantlets in pneumatically agitated suspension culture did not break apart during the 50 day cultivation period. The average number density of microplantlets in the liquid suspension corrected for dilution of microplantlets during sampling (X_N) was 1131 ± 58 plantlets per liter (±1 s.d., n = 18). Application of the Null Hypothesis test to these data showed no dependence of X_N on time at the 95% confidence level. Furthermore, a semi-log plot of the apparent number density (X_N') vs. cultivation time had a least-squares slope of 2.5 ± 0.2 % per day (±1 s.d., n = 18), close to the volumetric sample removal rate of 2.6% per day. Therefore, the decrease in X_N' was due to

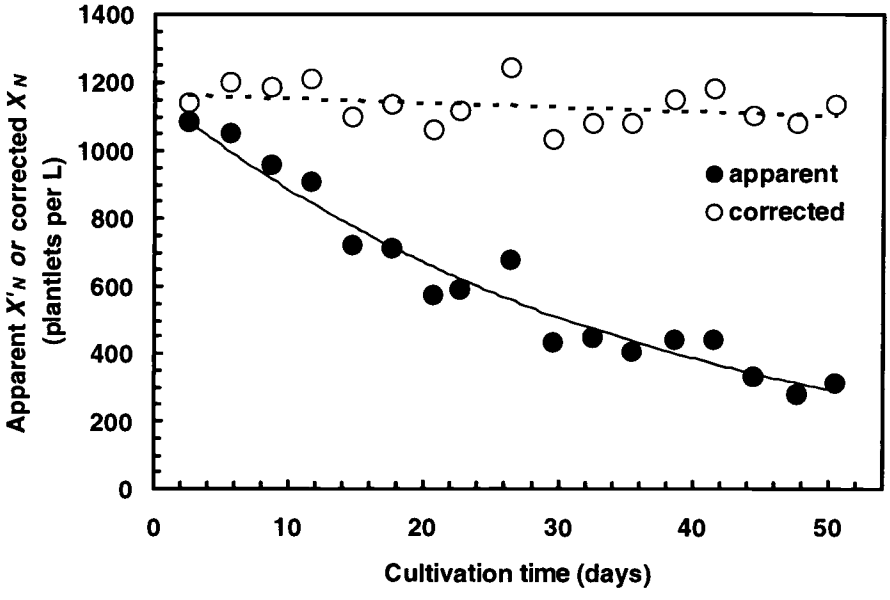

Figure 3.14 Cultivation of *A. subulata* microplantlet suspension in 2-L bubble-column photobioreactor at medium perfusion rate of 20% per day: apparent plantlet number density (X'_N) and corrected plantlet number density (X_N).

biomass dilution by sample removal and not the fragmentation of microplantlets by pneumatic agitation. Consequently, chemostat cultivation with sterile feed will not be possible for this organism.

The morphology of individual *A. subulata* microplantlets consisted of highly branched shoot tissues emanating from a central core (Figure 3.3). At inoculation, the overall diameter of microplantlets containing trimmed shoot tissues was nominally 3-5 mm. After 50 days of cultivation, the overall microplantlet diameter increased to 8-12 mm. During medium perfusion cultivation at a rate of 20% per day, the microplantlet shoot tissues maintained a dark red pigmentation over the 50-day cultivation period. However, microplantlets in batch culture turned yellow at the shoot tips after reaching the stationary phase of growth. In all modes of cultivation, the girth of the shoot tissues also increased with time, and active shoot tips formed new branches. Cell division was observed microscopically only at the shoot tips, i.e. the apical meristems, where the growth rate decreased from 0.12 day^{-1} (0-14 days) to 0.046 day^{-1} (14-51 days). The solids content of microplantlet tissue ranged from 15 to 20% in exponential phase, but steadily increased to approximately 30% after 50 days of cultivation (Table 3.3), further suggesting that biomass production was moving from cell division to photosynthate accumulation. Consequently, it appears that as the nonvascular thallus tissue enlarges, growth slows down. All of these observations show that these structured growth characteristics of the microplantlet will ultimately limit biomass production if all other process limiting factors are minimized, and so shoot tissues must be periodically cut down to maintain high growth rates.

Conclusions

New bioprocess cultivation technologies are needed for macrophytic marine organisms. This work describes the first comprehensive bioreactor study of a novel microplantlet suspension culture derived from an anatomically complex, macrophytic marine red alga (seaweed). A bubble-column photobioreactor with medium perfusion is a suitable biomass production platform for microplantlet suspension cultures derived from the macrophytic marine red alga *Agardhiella subulata*. At the cultivation conditions provided in Table 3.2, microplantlet growth at a fresh medium perfusion rate of 20% per day was not limited by insufficient CO_2 transfer or macronutrient delivery, although light attenuation through the suspension reduced specific biomass production to 50% of light saturation. Microplantlets did not break apart within the pneumatically agitated bioreactor, and the structured growth characteristics of the microplantlets

factor into specific biomass production. With this new biomass production platform in place, process optimization and secondary metabolite production studies will be conducted in the future.

Chapter 4

OPTIMAL TEMPERATURE AND PHOTOPERIOD FOR CULTIVATION OF *AGARDHIELLA SUBULATA* IN A BUBBLE-COLUMN PHOTOBIOREACTOR

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Introduction

Macrophytic marine algae, commonly known as seaweeds, are an emerging source of bioactive natural products (Carte, 1996). For example, macrophytic red algae within the genus *Agardhiella* are a rich and diverse source of phycocolloids (Cheney et al., 1987), novel eicosanoids (Graber et al., 1996), and sulfonated galactans with activity against HIV (Witrvouw et al., 1994). Development of natural products from macrophytic marine organisms is often limited by a reliable supply of biomass bearing the target compound (Rhouhi, 1995), and macrophytic marine red algae are no exception (Fuller et al., 1994). Unlike phototrophic marine microorganisms, macrophytic marine red algae are anatomically complex and grow anchored to the benthic marine environment. A major bioprocess technology barrier for production of new compounds from macrophytic marine algae is the development of *in vitro* culture systems suitable for bioreactor cultivation (Rorrer et al., 1998).

Techniques for establishing cell and tissue cultures from macrophytic marine algae are significantly underdeveloped relative to land plants (Aguirre-Lipperheide et al., 1995). Recently, we developed reliable routes for establishing liquid suspension cultures from macrophytic marine red algae using callus induction and somoclonal regeneration techniques, focusing on *Agardhiella subulata* as the model plant (Huang et al., 1998; Rorrer, 2000). Ultimately, we developed a phototrophic "microplantlet" suspension culture, and determined that this liquid suspension culture system was best suited for bioprocess development.

Temperature and light delivery are two important process variables in the design of algal photobioreactors (Richmond, 1996). In general, the effects of temperature on algal growth are significant (for review, see Raven and Geider, 1988). In the cultivation of macrophytic marine algae, light delivery has two components: the light flux intensity to the culture, and the diurnal photoperiod, defined as the light-dark illumination cycle (LD) within a 24 hour day. Although photoperiod is important to the development of macrophytic marine algae in the natural environment (Dring, 1984), the effects of photoperiod on biomass production remain unclear.

Given the potential significance of temperature and photoperiod effects on the growth of *A. subulata* microplantlets in photobioreactor systems, this work had two objectives. The first objective was to study the short- and long-term effects of temperature on the photosynthetic oxygen evolution rate of the microplantlet suspension culture, in order to determine the optimum temperature for cultivation. The second objective was to study the effect of diurnal photoperiod on biomass production in a bubble-column photobioreactor under conditions where light flux intensity, inorganic nutrient delivery, and CO_2 delivery did not limit phototrophic culture growth.

Toward this end, we developed a mathematical model to predict the effect of photoperiod on biomass production under these resource-saturated conditions. Both data and model assert that cumulative photodamage at photoperiods approaching continuous light ultimately limits biomass production, and that *A. subulata* microplantlets possess an optimal photoperiod for growth.

Materials and Methods

Microplantlet flask culture. Development of the phototrophic *Agardhiella subulata* microplantlet suspension culture is detailed in our previous work (Huang et al., 1998). Individual microplantlets consisted of branched, terete shoots emanating from a central core thallus, with growth occurring at apical meristems. Shoot tissues were pigmented red and ranged from 3 mm to 10 mm in length. Microplantlet suspension cultures were maintained in 250 mL foam stoppered Erlenmeyer flasks. Each flask contained approximately 50 microplantlets in 100 mL of filter-sterilized ASP12 artificial seawater medium as modified by Bradley and Cheney (1990), supplemented with 10X nitrate (11.8 mM), 1X phosphate (44.9 μM), 10 mM bicarbonate buffer, and 5 mM Na-HEPE buffer, but containing no plant growth hormones. Flasks were cultured without agitation at 24 °C under 24 μmol photons $m^{-2} s^{-1}$ cool white fluorescent light within an illuminated incubator. The illumination photoperiod was 10 h light /14 h dark on a 24 h cycle (10:14 LD). The medium was completely replaced every two weeks, and microplantlets were subcultured every four weeks. Prior to subculture, each microplantlet was cut through its central branch point into three pieces. The excised tissues contained less than 10 shoots per microplantlet. Elongated shoots greater than 10 mm were trimmed to 3-5 mm in length. Detailed maintenance and subculture procedures are described by Huang et al. (1998).

For the acclimation temperature studies, six culture flasks maintained at 24 °C and 24 μmol photons $m^{-2} s^{-1}$ were randomly selected 9 days after subculture. The microplantlets were removed from the flasks, pooled, and then evenly re-loaded into 24 flasks, each containing about 50 microplantlets in 100 mL fresh ASP12 medium. The 24 flasks were divided into six groups of four flasks. Each group of flasks was maintained at a new temperature (12, 18, 21, 24, 28 and 33 °C) within a temperature-controlled illuminated incubator or environmental chamber. The light flux intensity was maintained at 24 μmol photons $m^{-2} s^{-1}$ under a 10:14 LD photoperiod. The ASP12 liquid medium in each was completely exchanged every 7 days (14.3 % per day) to avoid nutrient depletion, and flasks were swirled for 5 sec once per day to promote gas exchange. After a given acclimation time of 1 h, 24 h, 7 days, and 24 days, a flask was removed for oxygen evolution rate (OER) measurements.

Cultivation of microplantlets in bubble-column photobioreactor. An externally-illuminated bubble-column bioreactor (Zhi and Rorrer, 1996) was used for all *A. subulata* microplantlet cultivation experiments. The glass vessel of the bioreactor had an effective cultivation volume of 250 mL, and consisted of a 12.7 cm straight section of 4.5 cm diameter and a 15.2 cm conical riser section of 1.3 cm inner diameter at the base. The small vessel diameter minimized the attenuation of light through the microplantlet suspension. House air was metered through a flow meter, humidified in a bubbler, sterilized through a 0.2 μm Gelman autoclaved filter, and then introduced into the base of the riser section through a 1.3 cm diameter glass frit of 40-60 μm pore size. The liquid suspension culture in the vessel was uniformly agitated and aerated by rising air bubbles of 1.0 mm nominal diameter. Ambient CO_2 in the aeration gas (nominally 350 ppm CO_2) served as the carbon source for photosynthetic biomass growth. The illumination stage consisted of two 6.0 W cool-white fluorescent lamps (F6T5) vertically mounted on opposing sides of the glass vessel. A referencing plate set the distance between the each lamp and the vessel wall to deliver the desired incident light flux intensity to the culture, and a programmable timer set the illumination period for each lamp. The photobioreactor was maintained at 24 °C within a temperature-controlled room.

Two identical 250 mL bubble-column photobioreactors described above were operated in parallel, each inoculated with microplantlets from a common inoculum source. To prepare the inoculum source, microplantlets from two randomly selected flask cultures (28 days after subculture) were pooled. Microplantlets cut and trimmed as described earlier and then rinsed with filter-sterilized ASP12 medium under sterile filtration. The shoot tissues were then re-suspended with 250 mL fresh ASP12 medium and then loaded into a given autoclaved bioreactor vessel. The target initial cell density in each vessel was 1.0 g fresh cell weight per liter of suspension (1.0 g FW L^{-1}). The process cultivation conditions are given in Table 4.1. Every five days, the liquid was completely replaced with fresh medium, and the total fresh cell weight (FW) within each vessel was determined. Specifically, the entire suspension within each 250 mL vessel vacuum-filtered on a 20 μm nylon mesh filter (50 mm diameter) with Whatman No. 1 filter paper (42.5 mm diameter) resting beneath the nylon mesh to prevent the mesh voids from retaining liquid medium. The filtered microplantlets and nylon mesh were placed into a sterile Petri dish and immediately weighed to precision of ± 0.001 g. The bioreactor vessel reloaded with 250 mL of fresh ASP12 medium, and microplantlets were immediately transferred back to the bioreactor. The mesh and Petri dish were re-weighed, and total fresh cell mass was determined by mass difference. The pH of the filtered medium was also measured. All fresh cell mass measurements

and culture transfer procedures were carried out using sterile technique within a laminar flow hood.

Table 4.1 Cultivation conditions for *Agardhiella subulata* microplantlets.

Process Variable	250 mL Bubble Column	Flask
Cultivation volume, V	250 mL	100 mL
Vessel inner diameter	4.5 cm	
ASP12 medium replacement rate	250 mL every 5 days (20% per day)	100 mL every 7 days (14.3 % per day)
Temperature (reference condition)	24 °C	24 °C
Incident light intensity, αI_o	76 μmol photons $m^{-2}s^{-1}$	24 μmol photons $m^{-2}s^{-1}$
Planes of illumination, α	2.0	1.0
Photoperiod (reference condition)	10:14 LD	10:14 LD
Aeration rate	100 mL min^{-1}	Surface aeration
O_2 mass transfer coefficient, k_La	89.5 ± 2.3 h^{-1}	n.d.
CO_2 partial pressure, p_A	0.00035 atm	0.00035 atm
CO_2-TR (mass transfer)	0.873 mmol CO_2 L^{-1} h^{-1}	n.d.
CO_2-TR (CO_2 in aeration gas)	0.345 mmol CO_2 L^{-1} h^{-1}	n.d.
Cultivation pH	8.7	8.7

Photosynthetic oxygen evolution rate. Photosynthetic oxygen evolution rate (OER) measurements for the microplantlets were performed in a well-mixed, temperature-controlled photosynthetic test cell described by Huang et al. (1998), equipped with The YSI model 58 dissolved oxygen (D.O.) meter and YSI model 5750 D.O. electrode. The volumetric OER was determined from the least-squares slope of the linear portion of the D.O. concentration versus time curve, typically from 3 to 10 min, where all D.O. concentrations below 120% of air saturation. The D.O. concentration vs. time baseline in the ASP12 medium without tissue was also measured, and all OER measurements were corrected for the sample blank. The D.O. electrode and meter compensated for OER measurements at temperatures other than 24 °C. The

dissolved oxygen concentration for air-saturated seawater medium of 3.75 ppt salinity at 24 °C and 1.0 atm total pressure is 0.2122 mM (Benson and Krause, 1984).

For the temperature acclimation studies, the liquid medium in a given 100 mL flask culture was decanted down to 65 mL, and the concentrated biomass suspension was loaded into the 65 mL D.O. cell. The volumetric OER was measured at a total incident light flux intensity (αI_o) of 134 μmol photons $m^{-2} s^{-1}$. The temperature of the OER assay was set to the acclimation temperature of the particular sample (12-33 °C). Duplicate measurements were performed for each sample.

The photosynthetic oxygen evolution rate vs. light intensity curve (*PI* curve) of a representative microplantlet sample was measured at 24 °C in fresh ASP12 liquid medium. The OER of the same sample was measured at increasing light flux (αI_o) ranging from 8.4 to 134 μmol photons $m^{-2} s^{-1}$. OER measurements for the dark-phase oxygen respiration rate were conducted after the last illumination OER measurement. The D.O. cell was covered with aluminum foil to exclude light. Duplicate measurements were performed at each light intensity.

Volumetric mass transfer coefficient. The volumetric oxygen mass transfer coefficient (k_La) for O_2 into the bubble column photobioreactor vessel loaded with 250 mL ASP12 liquid medium was determined by the dynamic gassing-in method at 24 °C. The tip of the YSI model 5750 D.O. electrode was immersed 5.0 cm below the liquid surface. Dissolved oxygen in the liquid medium was removed by sparging with N_2 gas through the sparger. When the D.O. concentration reached 1.0 % of O_2 saturation, air was re-introduced through the sparger at 100 mL min^{-1}, and k_La was estimated from the least-squares slope of D.O. concentration versus time data on a semi-log plot. The response time for the D.O. electrode was 5.2 sec, and all k_La values were corrected for the D.O. electrode response.

Other techniques. Dry cell weight (DCW) was measured following the final OER measurement on a given sample and at the termination of all cultivation experiments. Microplantlets were transferred onto an oven-dried, pre-weighed 47 mm x 0.45 μm filter (Millipore HWAP04700) and then dried at 80 °C for 24 h. The dry cell mass and filter were weighed to precision of ± 0.1 mg, and the DCW was determined by mass difference. The solids content in the biomass was determined from the DCW and FW measurements. Light flux intensity was measured in units of μmol photons $m^{-2} s^{-1}$ with a LI-COR 189 quantum radiometer and a LI-COR 190SA PAR quantum sensor. The total incident light flux intensity was defined as αI_o, where α is the number of planes of illumination (e.g. $\alpha = 2$ for two-sided illumination), and I_o is the incident light flux intensity to the vessel surface.

Cumulative Photodamage Growth Model

The photosynthetic apparatus within the chloroplast of an algal cell contains a complex array of proteins and enzymes that are sensitive to excess light. In particular, the D1 reaction center of Photosystem II (PSII) is damaged at light saturation conditions (Powles, 1984), which initiates a repair cycle commensurate with a reversible photoinhibition response to thermally dissipate excess absorbed photons. Net photodamage occurs if the rate of D1 protein damage exceeds that rate of its repair processes. Photodamage and repair in phototrophic microorganisms can be kinetically modeled as a reversible process (Lesser et al., 1994). *Agardhiella subulata* may be susceptible to photodamage due to an unusually high concentration of PSII in its phycobilisome (Kursar and Alberte, 1984). Under illumination conditions, consider that the rate processes underlying photodamage are described by

$$E \underset{k_r}{\overset{k_d}{\rightleftharpoons}} E' \tag{4.1}$$

Consequently, within the cell, the net rate of photodamage during illumination is

$$\frac{dE}{dt} = -k_d \cdot E + k_r (E_o - E) \tag{4.2}$$

which upon integration yields

$$\frac{E}{E_o} = \frac{k_r}{k_d + k_r} + \frac{k_d}{k_d + k_r} e^{-(k_d + k_r)t} \tag{4.3}$$

where E is the specific concentration of *active* photosynthetic apparatus within the cell biomass, E' is the specific concentration of the inactivated photosynthetic apparatus after photodamage, E_o is the fixed, total specific concentration of active and inactive photosynthetic apparatus ($E + E'$), k_d is the rate constant for photodamage, and k_r is the rate constant for photodamage repair.

Below, we describe a new model to predict the cumulative effect of photodamage on the cell density vs. time curve under light flux intensity and nutrient saturation conditions. Biomass production in a well-mixed batch reactor at constant volume is described by

$$\frac{dX}{dt} = \mu X \tag{4.4}$$

where X is the cell density, and μ is the specific growth rate determined by cultivation conditions at time t. The specific growth rate of phototrophic algae can exhibit saturation behavior with respect to dissolved CO_2 concentration, limiting macronutrient concentration C_N, incident light flux intensity I, and fractional photoperiod f. If growth is not limited by CO_2 availability, then

the superimposing effects of each variable on the specific growth rate can be described by a multiplicative saturation model

$$\mu = \mu_{\max}\left(\frac{C_N}{K_N + C_N}\right)\cdot\left(1 - e^{-I/I_k}\right)\cdot\left(1 - e^{-f'/K_f}\right) \tag{4.5}$$

where μ_{max} is the maximum specific growth rate, K_N is the half-saturation constant for the limiting macronutrient, I_k is the light flux intensity at 63.2% of photosynthetic saturation, K_f is a proportionality constant, and f' is

$$f' = \frac{f}{1-f} \tag{4.6}$$

where f is the fractional illumination photoperiod (h illumination / 24 h photoperiod, $0 \leq f \leq 1$). In batch cultivation, C_N will decrease toward zero at finite I and f. However, under periodic medium replacement, macronutrients are provided so $C_N >> K_N$ during cultivation. Furthermore, if $I > 3 \cdot I_k$, then culture growth is at 95% of saturation with respect to light flux intensity. Under these conditions, equation (4.5) reduces to

$$\mu \approx \mu_{\max}\left(1 - e^{-f'/K_f}\right) \tag{4.7}$$

Note that $\mu = 0$ at $f = 0$, $\mu = \mu_{max}$ at $f = 1$, and μ is linearly proportional to f at low values for f. We now assume that μ_{max} is proportional the specific concentration of photosynthetic apparatus within the cell

$$\mu_{\max} \propto k_p \cdot E \tag{4.8}$$

where k_p is a proportionality constant. Furthermore, under light-saturated growth, we assume that the rate of photodamage significantly exceeds the rate of repair so that $k_d >> k_r$. Under these assumptions, equation (4.3) reduces significantly to

$$E = E_o\, e^{-k_d t} \tag{4.9}$$

Combining equations (4.8) and (4.9) yields

$$\mu_{\max} = \mu^o_{\max} e^{-k'(f)t} \tag{4.10}$$

where μ^o_{max} is the maximum specific growth rate under conditions were irreversible photodamage is negligible, and k' is the apparent rate constant for photodamage. Since the growth rate also depends on photoperiod (equation 4.7), k' is now also dependent on photoperiod.

When photodamage occurs, cell biomass still exists but further photosynthetic growth and division is disabled. Although E is not measured, its effect on the specific growth rate can be assessed through the X *vs.* t growth curve. Combining equations (4.4), (4.7) and (4.10) yields

$$\frac{dX}{dt} = \mu_{\max}^{o} e^{-k' \cdot t}\left(1 - e^{-f'/Kf}\right)X \tag{4.11}$$

which upon integration with respect to time gives

$$\ln\left(\frac{X}{X_o}\right) = \frac{m'}{k'}\left(1 - e^{-k't}\right) \tag{4.12}$$

where m' is the saturation component of the photoperiod effect on biomass production in the absence of photodamage, given by

$$m' = \mu_{\max}^{o}\left(1 - e^{-f'/Kf}\right) \tag{4.13}$$

Results and Discussion

Requirements for light-saturated photosynthesis. A representative *PI* curve for *Agardhiella subulata* microplantlets 30 days after subculture at 24 °C is presented in Figure 4.1.

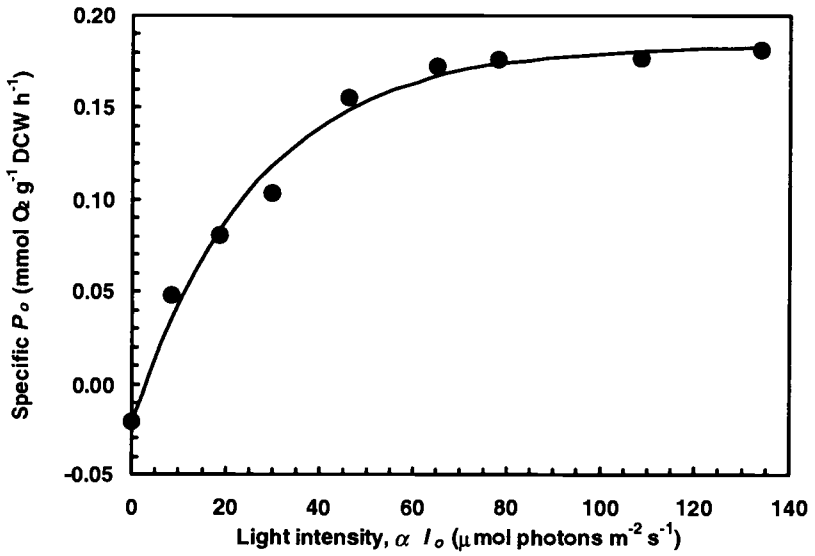


Figure 4.1 Specific oxygen evolution rate versus incident light intensity (*PI* curve) for *Agardhiella subulata* microplantlets at 24 °C, measured 30 days after subculture.

The *PI* curve data was fit to the exponential saturation model

$$P_o = P_{o,\max}\left(1 - e^{\alpha I_o / \alpha I_k}\right) + Q_o \tag{4.14}$$

where Q_o is the specific dark-phase respiration rate, P_o is the net specific oxygen evolution rate, $P_{o,max}$ is the total oxygen evolution rate at light saturation, and I_k is the light flux intensity at 3.2% of saturation (Table 4.2). Microplantlets were not subject to photoinhibition in the measured range of 8.4-134 μmol photons m^{-2} s^{-1}. The *PI* curve response to photoinhibition, marked by a decrease in OER with increasing light flux intensity, is observed in many macroalgae at light intensities exceeding 500 μmol photons m^{-2} s^{-1} (Coutinho and Zingmark, 1987). Light-saturated growth for many macroalgae occurs near 70 μmol photons m^{-2} s^{-1} (Fortes and Lüning, 1980), similar to this study where αI_o at 95% of $P_{o,max}$ was 86 μmol photons m^{-2} s^{-1} ($3 \cdot \alpha I_k$). Red macroalgae tolerate a wider range of light intensities than any other group of photosynthetic plants, and their saturating irradiance values generally increase if the maintenance cultivation light flux intensity is increased (Gantt, 1990).

Table 4.2 Typical photosynthetic growth parameters for *Agardhiella subulata* microplantlets in the exponential phase of growth at 24 °C.

Parameter	Value ± 1.0 S.D.	
$P_{o,max}$	0.167 ± 0.006	mmol O_2 g^{-1} DCW h^{-1} (n = 9)
αI_k	28.7 ± 3.3	μmol photons $m^{-2}s^{-1}$ (n = 9)
Q_o	0.023 ± 0.002	mmol O_2 g^{-1} DCW h^{-1} (n = 3)

Effect of temperature on light-saturated photosynthesis. The transient effects of temperature on algal growth characteristics are conveniently assessed by photosynthetic oxygen evolution rate measurements (Davidson, 1987; Fan et al., 1994). Therefore, the effect of temperature on the specific OER of *A. subulata* microplantlets was determined. Flask cultures were subjected to a step change from the maintenance temperature of 24 °C to the temperature of interest (12 to 33 °C), and then allowed to acclimate to the new temperature at times ranging from 1.0 h to 28 days. The results are presented in Figure 4.2. After short acclimation times of 1.0 days or less, P_o increased significantly from 12-24 °C, and then was constant at 24-33 °C (Figure 4.2a). However, after acclimation times of 7 and 28 days, the optimum temperature was 24 °C,

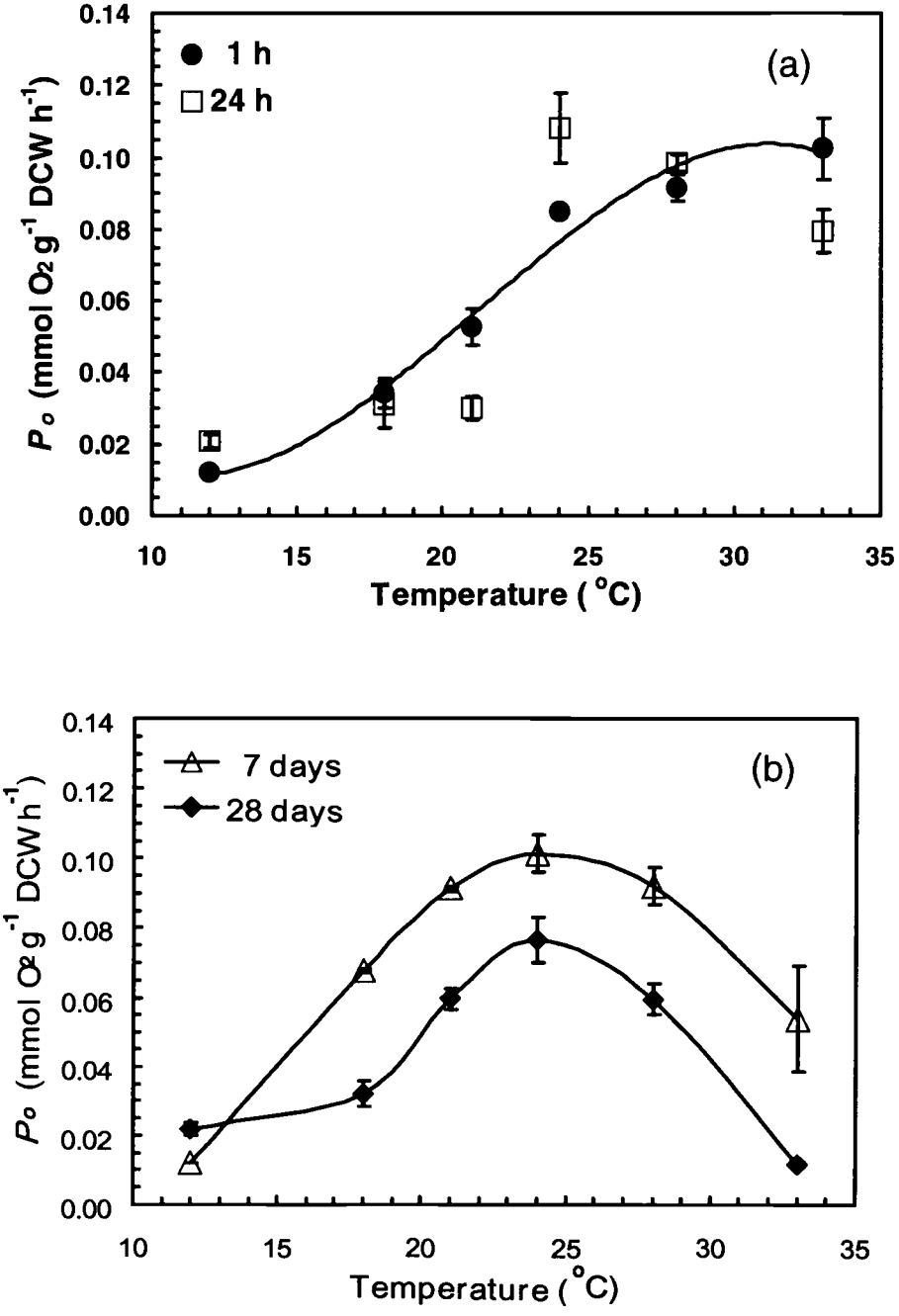


Figure 4.2 Specific oxygen evolution rate at light saturation for *Agardhiella subulata* microplantlets exposed to a step change in temperature ranging from 12 to 33 °C. (a) Short exposure times (1 h, 24 h); (b) longer exposure times (7 days, 28 days).

and high OER values at 33 °C could not be sustained (Figure 4.2b). After 28 days at 33 °C, microplantlet pigmentation bleached out, with shoot tips turning pale red to white.

The optimum temperature for the *A. subulata* microplantlets was 24 °C, which was identical to the maintenance cultivation temperature. It is well established that a given algal genotype and its associated phenotypes in nutrient-saturated environments possess unique optima temperature for growth determined by the ensemble rates of metabolic processes within the cell (Raven and Geider, 1988). Native *Agardhiella subulata* plants belong to the northeast American tropical-to-temperate group (Yarish et al., 1984), where summer temperatures of up to 28 °C bound the upper limit for growth, and temperatures of 35 °C are lethal. Since the maximum growth rate of red algae is often near the top of its tolerated temperature range (Kain and Norton, 1990), optimal cultivation will be bounded within this temperature window. Furthermore, since the short-term acclimation of macroalgae to a new temperature may induce transient effects on photosynthetic rates in ways not predicted at longer acclimation times (Hanelt, 1996), the *P-T* curve at 28 days (Figure 4.2b) best reflects the temperature response of *A. subulata* microplantlets.

Effect of photoperiod on apparent growth rate and OER. The effect of illumination photoperiod on growth of *A. subulata* microplantlets was assessed in a 250-mL bubble-column photobioreactor operated at the conditions provided in Table 4.1. In all experiments, flask cultures were inoculated into the bioreactor and then exposed to a step change in photoperiod.

During the illumination portion of the photoperiod, photosynthesis and biomass production occurs. As a first approximation, consider that the apparent growth rate during the illumination phase of the photoperiod (μ') is scaled to the apparent growth rate (μ) during the overall photoperiod (light and dark phases of photoperiod) by

$$\mu = \mu' \cdot f \tag{4.15}$$

where f is the fractional photoperiod. The apparent growth rate (μ) was estimated by from the least-squares slope of cell density (X) vs. time data (semi-log scale) from the first 30 days of cultivation. At the control photoperiod of 10:14 LD, $\mu = 0.066 \pm 0.021$ day^{-1} based on three independent inoculates. It was convenient to normalize (μ') obtained under a given value for f with respect its control:

$$\mu^* = \frac{\mu'}{\mu'_{10:14}} = \frac{\mu}{\mu_{10:14}} \frac{f_{10:14}}{f} \tag{4.16}$$

The effect of fractional photoperiod (f) on μ^* is presented in Figure 4.3. Values for μ^* were fairly constant between fractional photoperiods of 0.2 and 0.67. However, fractional photoperiods

above 0.67 had a profoundly negative effect on μ*. At continuous illumination ($f = 1$), μ* was close to zero.

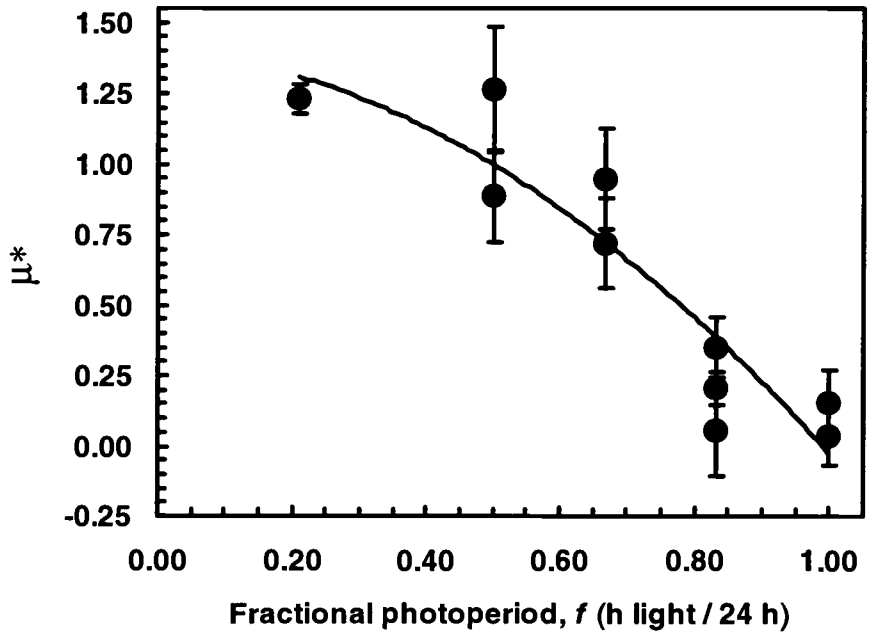


Figure 4.3 Effect of photoperiod on normalized growth rate μ* for *Agardhiella subulata* microplantlets. Cultivation conditions are provided in Table I. Error bars reflect propagated errors (± 1.0 S.D.); r^2 values were generally above 0.95.

The specific oxygen evolution rate (P_o) and specific dark-phase respiration rate (Q_o) of the *A. subulata* microplantlets were also measured after at least 30 days of cultivation in the 250 mL bubble-column photobioreactor. Again, it was convenient to define

$$P^* = \frac{P_o}{P_{o,10:14}} \text{ and } Q^* = \frac{Q_o}{Q_{o,10:14}} \tag{4.17}$$

The effect of fractional photoperiod (f) on P^* and Q^* is presented in Figure 4.4. Values for P^* were maximized at a fractional photoperiod of 0.5 (12:12 LD), then decreased markedly towards zero as f increased to 1.0 (continuous light). Hobson et al. (1979) also showed that the optimum photoperiod for the microalga *Isochrysis galbana* Parke was 12:12 LD, based also on photosynthesis measurements. In Figure 4.4, Q^* was independent of f (Null Hypothesis, 95% confidence), which agreed with previous studies on photosynthetic microalgae (Laws and Chalup, 1990) and oceanic picoplankters (Iriate and Purdie, 1993).

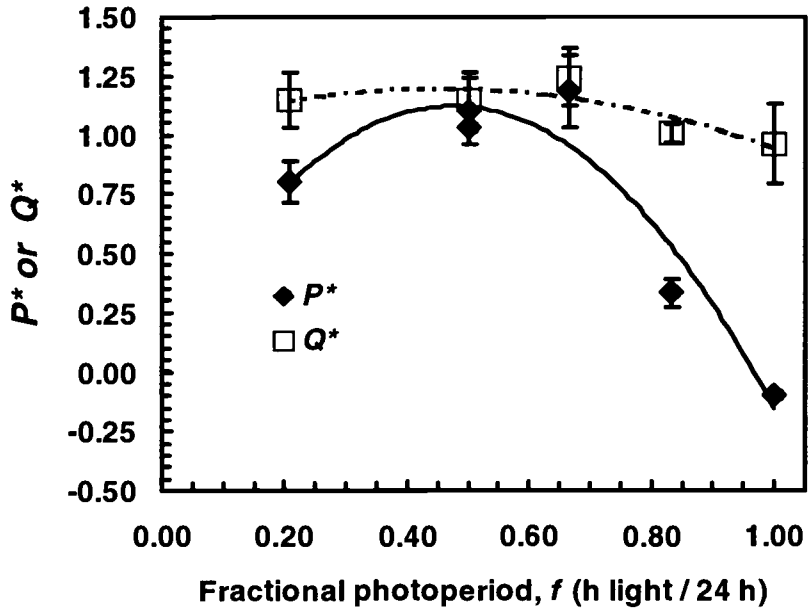


Figure 4.4 Effect of photoperiod on specific oxygen evolution rate and specific respiration rate for *Agardhiella subulata* microplantlets, normalized with respect to a control cultivation at photoperiod of 10:14 LD. Cultivation conditions are provided in Table 4.1. All measurements were taken after at least 30 days into the cultivation period. Error bars reflect propagated errors (± 1.0 S.D.) for P_o or Q_o estimation and its control.

Effect of photoperiod on growth curve in bubble-column photobioreactor. Figure 4.5 presents growth curves for *A subulata* microplantlets over an extended 60 day cultivation time at photoperiods of 5:19 LD ($f = 0.21$), 16:8 LD ($f = 0.67$), and 20:4 LD ($f = 0.83$). Each growth curve was compared to a control cultivation at photoperiod of 10:14 LD ($f = 0.42$). All cultivations were carried out at the conditions provided in Table 4.1. The average pH of each cultivation experiment was between 8.7 and 8.8, and pH varied by no more than ±0.05 pH units during the 60 day cultivation. In this resource-saturated environment, active growth was extended so that the *cumulative* effect of photoperiod on biomass production could be readily observed. Relative to the control cultivation at 10:14 LD photoperiod ($f = 0.42$), cumulative biomass production was maximized at a photoperiod of 16:8 LD. After 60 days of cultivation, cell densities exceeding 14 g FW L^{-1} were achieved, which corresponded to a dry cell density near 3.6 g DCW L^{-1}, based on a 25.5 ± 2.8 wt% solids content. Biomass production was proportional to photoperiod at low fractional photoperiods ($f \leq 0.42$), and the biomass production rate was sustained. However, high fractional photoperiods ($f \geq 0.83$) shut down cumulative biomass production (Figure 4.6).

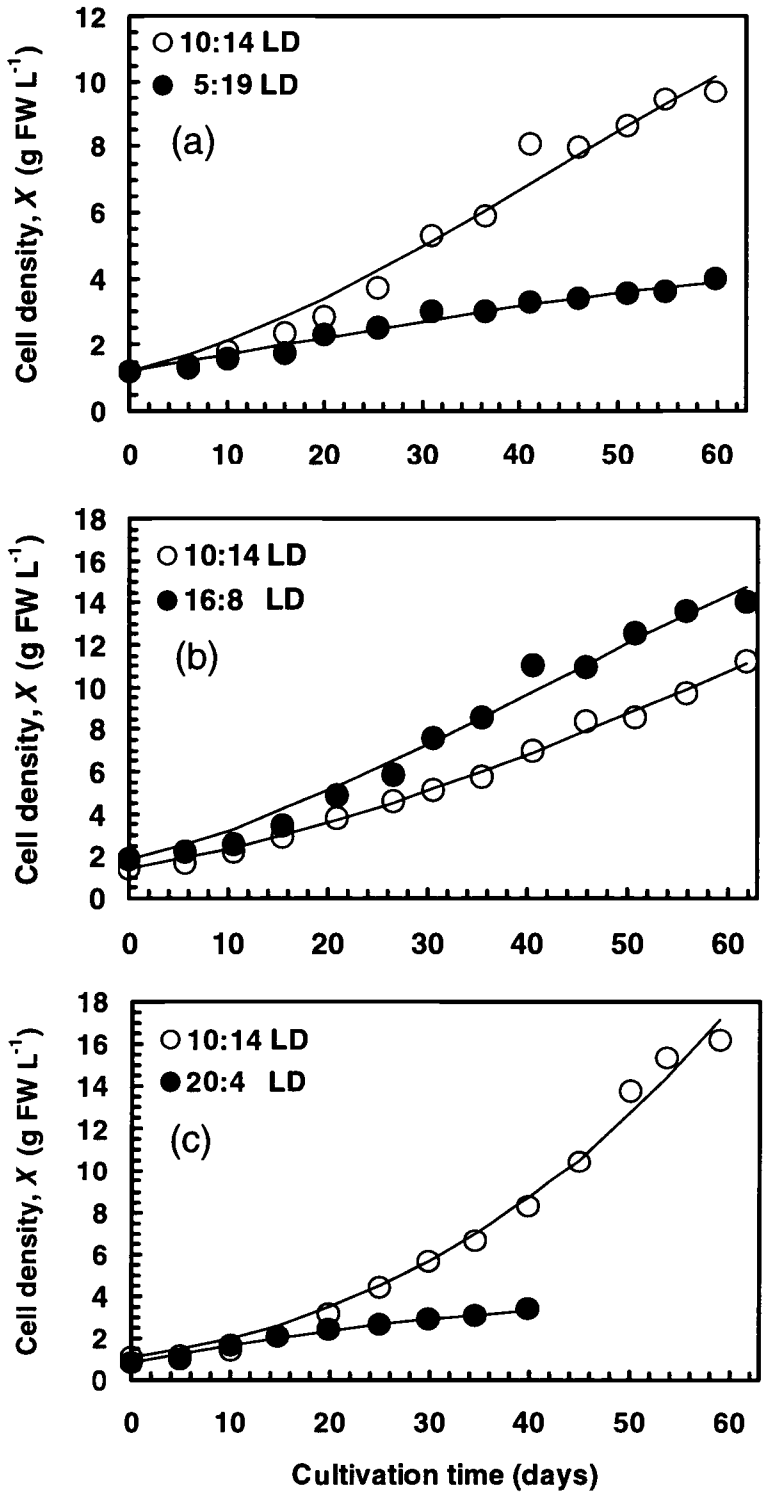


Figure 4.5 Cell density vs. time for cultivation of *Agardhiella subulata* microplantlets at various photoperiods. (a) 10:14 LD (control) vs. 5:19 LD; (b) 10:14 LD (control) vs. 16:8 LD; (c) 10:14 LD (control) vs. 20:4 LD. Cultivation conditions are provided in Table I. Solid lines represent the nonlinear, least-squares fit of data to equation (4-12); r^2 values ranged from 0.89 to 0.98.

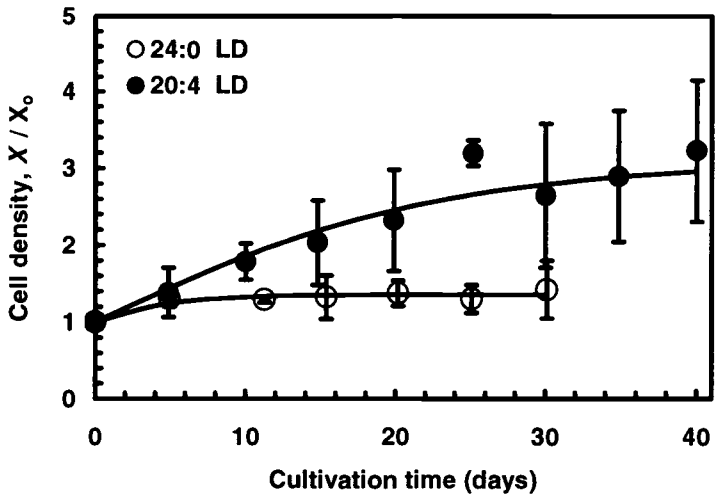


Figure 4.6 Cell density vs. time for cultivation of *Agardhiella subulata* microplantlets at photoperiods of 20:4 LD and 24:0 LD, using averaged data from two separate inoculates (± 1.0 S.D. error bars). Cultivation conditions are provided in Table 1. Solid lines represent fit of averaged data to equation (4-12).

In limited previous work, Yarish et al. (1984) conducted qualitative temperature-photoperiod cultivation studies with tetrasporic plants of *Agardhiella subulata*, who observed maximum growth at 40 μmol photons m^{-2} s^{-1} light flux intensity and 16:8 LD photoperiod. However, this study considered photoperiods of only 8:16 LD and 16:8 LD, and did not determine the saturation light intensity.

The CO_2 consumed by the microplantlets in liquid suspension culture during photosynthetic growth was continuously supplied to the culture by the aerating gas stream. The CO_2 delivery was assessed in two ways. First, the maximum interphase mass transfer rate of CO_2 is

$$CO_2 - TR = k_L a \left(\frac{D_{CO_2}}{D_{O_2}} \right)^{1/2} \frac{p_{CO_2}}{H} \tag{4.18}$$

where $k_L a$ is the O_2 volumetric mass transfer coefficient (h^{-1}), H is Henry's law constant for CO_2 (0.0346 atm mM^{-1}) in 36 ppt seawater at 24 °C (Raven, 1984), p_{CO_2} is the partial pressure of CO_2 in the aeration gas (350 ppm), and the ratio of diffusion coefficients is 0.93. Second, the CO_2 delivery by the aeration gas itself is

$$CO_2 - TR = \frac{v_g \; p_{CO_2}}{RT\,V} \tag{4.19}$$

where R is the gas constant, T is temperature of the aeration gas, and v_g is the volumetric flowrate of the aeration gas, and V is the culture volume.

The P_o data was also used to estimate CO_2-TR requirements for photobioreactor cultivation. To avoid CO_2-limited growth, a conservative criterion is

$$P_o(t) \cdot X(t) > CO_2 - TR \tag{4.20}$$

where it is assumed that one mole of CO_2 is consumed for every mole of O_2 evolved. For example, after 60 days in culture P_o was 0.083 ± 0.009 mmol O_2 g^{-1} DCW h^{-1} for the control cultivations in Figure 5. From equation (4.20), the minimum required CO_2-TR at 4.0 g DCW L^{-1} cell density is 0.33 mmol CO_2 L^{-1} h^{-1}. As shown in Table I, CO_2-TR defined by either equation (4.18) or (4.19) met or exceeded this requirement, and so cultivations were not limited by insufficient CO_2 delivery until near the end of the 60 day cultivation period.

Cumulative photodamage growth model. Figures 4.4 and 4.5 show that photoperiod affects photosynthetic biomass production by a cumulative process. The cumulative photodamage growth model summarized by equation (4.12) predicts the effect of fractional photoperiod (f) on cell density (X) versus time within a batch reactor under cultivation conditions not limited by light flux intensity, nutrient delivery, and CO_2 delivery. The model predicts that cumulative photodamage will eventually limit biomass productivity even in a resource saturated environment, i.e.

$$X_\infty = X_o \cdot e^{m'/k'} \quad at \quad t \to \infty \tag{4.21}$$

As shown in Figure 4.6, at photoperiods of 20:4 LD and 24:0 LD, the cell density leveled off within 30 days of cultivation. Cultivation under continuous light prevented biomass growth, and the tissue was pigmented pale red to white at the shoot tips.

Model sensitivity to f is modulated through parameters m' and k'. Each set of X vs. t data given in Figures 4.5 and 4.6 were fitted to equation (4.12) by nonlinear least-squares regression to obtain m' and k' at each f, as shown in Figure 4.7. Estimates for m' vs. f were then fitted to equation (4.13) by nonlinear least-squares regression to obtain μ°_{max} = 0.0782 ± 0.009 day^{-1} and K_f = 0.453 ± 0.185 h^{-1} light h^{-1} dark (n = 4, r^2= 0.782). Parameters m' and k' at 24:0 LD could not be estimated simultaneously due to the low sensitivity of X to t (Figure 4.6). Consequently, k' was estimated from X vs. t data using m' = 0.0782 day^{-1} (equation 4.13) to obtain k' = 0.257 ± 0.015 day^{-1} at 24:00 LD. At $f \geq 0.83$, $k' > m'$, suggesting that photodamage was the true limiting factor to biomass production at photoperiods approaching continuous light.

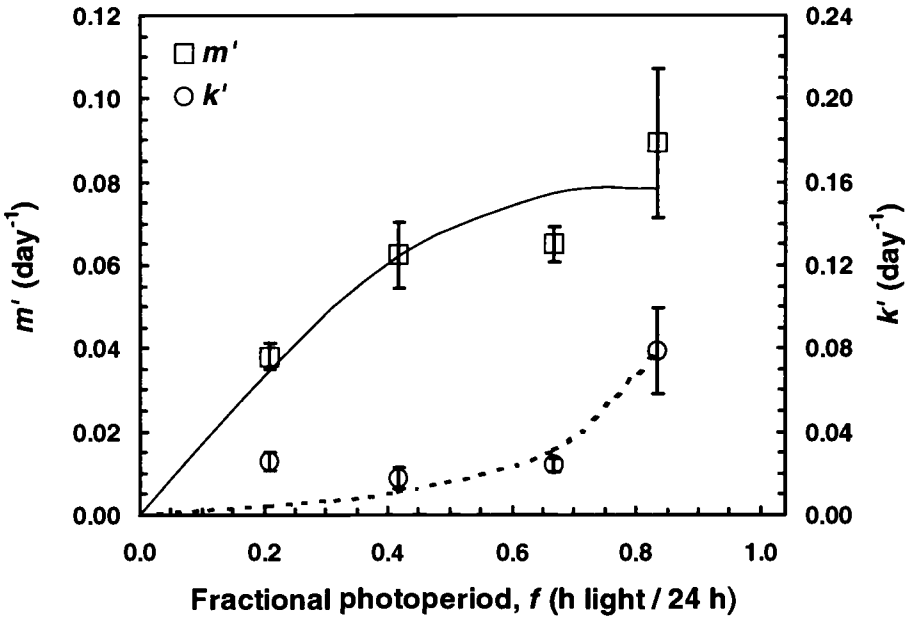


Figure 4.7 Effect of photoperiod on parameters m' and k'. Error bars on m' and k' values (± 1.0 S.D.) obtained from nonlinear least-squares regression of data in Figure 5 to equation (12). Values for m' ranged from 0.0381 ± 0.003 to 0.0893 ± 0.018 day^{-1}; values for k' ranged from 0.0177 ± 0.005 to 0.0788 ± 0.020 day^{-1}. Solid line represents fit of m' vs. f to equation (13), with μ°_{max} = 0.0782 ± 0.009 day^{-1} and K_f = 0.453 ± 0.185 h^{-1} light h^{-1} dark (n = 4, r^2= 0.782). Dotted line represents least-squares fit of k' vs. f to equation (4-22).

If photodamage is selectively acting on the light harvesting components of photosynthesis, then k_d should only be finite during the illumination phase of the photoperiod. Consider that k' scales to the light: dark ratio, i.e.

$$k'(f) = k_d f' \tag{4.22}$$

for $f < 1$. This predicted trend is observed in Figure 4.7, where the least-squares estimate of k_d is 0.0156 ± 0.0025 day^{-1} (n = 4, r^2 = 0.765).

Conclusions

In summary, Figures 4.3 to 4.7 clearly show that photoperiod has a profound affect on the cultivation of *A. subulata* microplantlets. The optimum photoperiod for cumulative biomass

production is near 16:8 LD, and continuous light halts culture growth. Although the developmental aspects of photoperiodism on macroalgal plants in the natural marine environment are well established (Dring, 1984), the effects of photoperiod on biomass production are not well studied, due in part to the difficulty of cultivating field-collected macroalgae in long-term laboratory culture. For example, Fortes and Lüning (1980) showed that the growth rate of four species of macroalgae exhibited a saturation dependence on photoperiod, where the growth rate was maximized at continuous illumination. However, the growth rates of Fortes and Lüning (1980) were based on only a one-week cultivation time following field collection. In this study, we were able to observe the cumulative effects of photoperiod in long-term laboratory culture, using an *in vitro* microplantlet suspension culture recently developed in our previous work (Huang et al., 1998). Furthermore, we attributed the long-term effects of photoperiod on cumulative biomass production to irreversible photodamage, which has some precedence in the cultivation of phototrophic marine microorganisms (Brand and Guillard, 1981; Brunet et al., 1996).

Chapter 5

STIRRED-TANK CULTIVATION OF MICROPLANTLETS DERIVED FROM THE MARINE RED MACROALGA *AGARDHIELLA SUBULATA*

Yao-ming Huang and Gregory L. Rorrer

In preparation for *Biotechnology Progress*

Introduction

Macrophytic marine algae are an emerging source of pharmacologically active compounds (Carte, 1996). However, development of new compounds from delicate marine organisms is often limited by a reliable supply of biomass bearing the target compound (Rhouhi, 1995). Macrophytic red marine algae, unlike most phototrophic marine microorganisms (e.g. cyanobacteria or microalgae), must be introduced into cell or tissue suspension culture before they can be cultivated in bioreactor systems to produce compounds within a controlled environment at a scale required for continued product development. At present, the major bioprocess technology barrier for production of new compounds from macrophytic marine algae is the development of a suitable biomass production platform (Rorrer et al., 1998).

In this study *A. subulata* was selected as a model plant to demonstrate the cultivation of an algal culture derived from a macrophytic marine red alga. First, *A. subulata* possesses a terete thallus morphology with cell growth at the apical meristem (Gabrielson and Hommersan, 1982), a feature common to many genera of red algae, and a feature that is also amenable to *in vitro* culture development (Cheney et al., 1987). Second, *Agardhiella* species are a rich and diverse source of potentially valuable natural products, including phycocolloids (Cheney et al., 1987), novel eicosanoids deriving from 8-lipoxygenase metabolism of arachidonic acid (Graber et al., 1996), and sulfonated galactans with strong activity against the human immunodeficiency virus (Witrvouw et al., 1994).

The stirred tank bioreactor is an important bioreactor configuration. The goal of this study is to assess biomass growth of *Agardhiella* microplantlets within a stirred-tank photobioreactor, and investigate limiting factors for cell biomass production within the stirred tank, including CO_2 transfer and consumption, macronutrient utilization, pH, agitation intensity, and microplantlet morphology. The microplantlet suspension culture consists of multicellular tissue with a high solid content (20-30%) that requires an agitation intensity sufficient to prevent sedimentation and maintain a well-mixed environment. A well-mixed environment is also desirable for nutrient, CO_2 and light delivery. Furthermore, for investigating CO_2-limited growth, the stirred-tank configuration also offers sufficient mechanical agitation to maintain the microplantlet in suspension under low aeration rates.

Materials and Methods

Culture maintenance. Development of the *Agardhiella subulata* microplantlet suspension culture is detailed in our previous work (Huang et al., 1998). Microplantlet suspension cultures were contained in 250ml foam-stoppered Erlenmeyer flasks, each with up to 50 microplantlets in 100 mL nitrate-enriched ASP12 artificial seawater medium with 10.2 mM nitrate. The culture flasks were maintained without agitation at 24 °C under 20-40 μmol photons $m^{-2} s^{-1}$ cool white fluorescent light (10:14 LD photoperiod) within an illuminated incubator. Flask cultures were swirled for five seconds once per day, and the medium was completely replaced every two weeks. Microplantlet shoot tissues were subcultured every four weeks within a laminar flow hood.

500 mL stirred-tank photobioreactor design and operation. An illuminated stirred-tank bioreactor with a working volume of 500 mL was used to study phototrophic growth of *A. subulata* microplantlet cultures. A schematic of the bioreactor system is shown in Figure 5.1. A Bellco jacketed spinner flask of diameter 10.5 cm and height 20.5 cm (model 1965-00100) served as the bioreactor vessel. The bioreactor was equipped with a magnetically driven, twin-blade paddle impeller (2.6 cm in height) pitched vertically. The overall diameter of the impeller assembly was 5.5 cm ($D_i / D_T = 0.52$), and was positioned 1.0 cm above the bottom of the vessel. Commonly, two vessels were operated in parallel. Each bioreactor vessel was either mounted on a VirTis Omni-Culture control unit or a VWR stirrer plate (Model 110), and the impeller assembly was driven magnetically at constant rotation speed. Air was metered through a flowmeter, sterilized with a 0.2 μm Gelman autoclavable filter, and introduced to the culture by a stainless steel sparger positioned next to the impeller assembly. The sparger produced fine bubbles with 1 mm nominal size. The outlet air line was connected to 0.2 μm filter. The vessel jackets were connected to a water circulator set at 24°C. Constant illumination to the glass vessel was provided by two cool-white 11 watt compact fluorescent lamps positioned on opposite sides of the bioreactor to set incident light intensity at 80 μE m^{-2} sec^{-1}. Each lamp was set on a timer at 10:14 light/dark photoperiod. The bioreactor was inoculated using sterile technique within a laminar flow hood. The fresh cell weight was measured every five days. The medium was also completely replaced every five day after each fresh cell weight measurement. For the batch cultivation experiments, the medium was not replaced.

250-mL bubble-column photobioreactor design and operation. The details of the bubble-column photobioreactor design are detailed by Zhi and Rorrer (1996). The inner diameter was 4.45 cm for the straight section and 1.27 cm at the base of the riser section. The

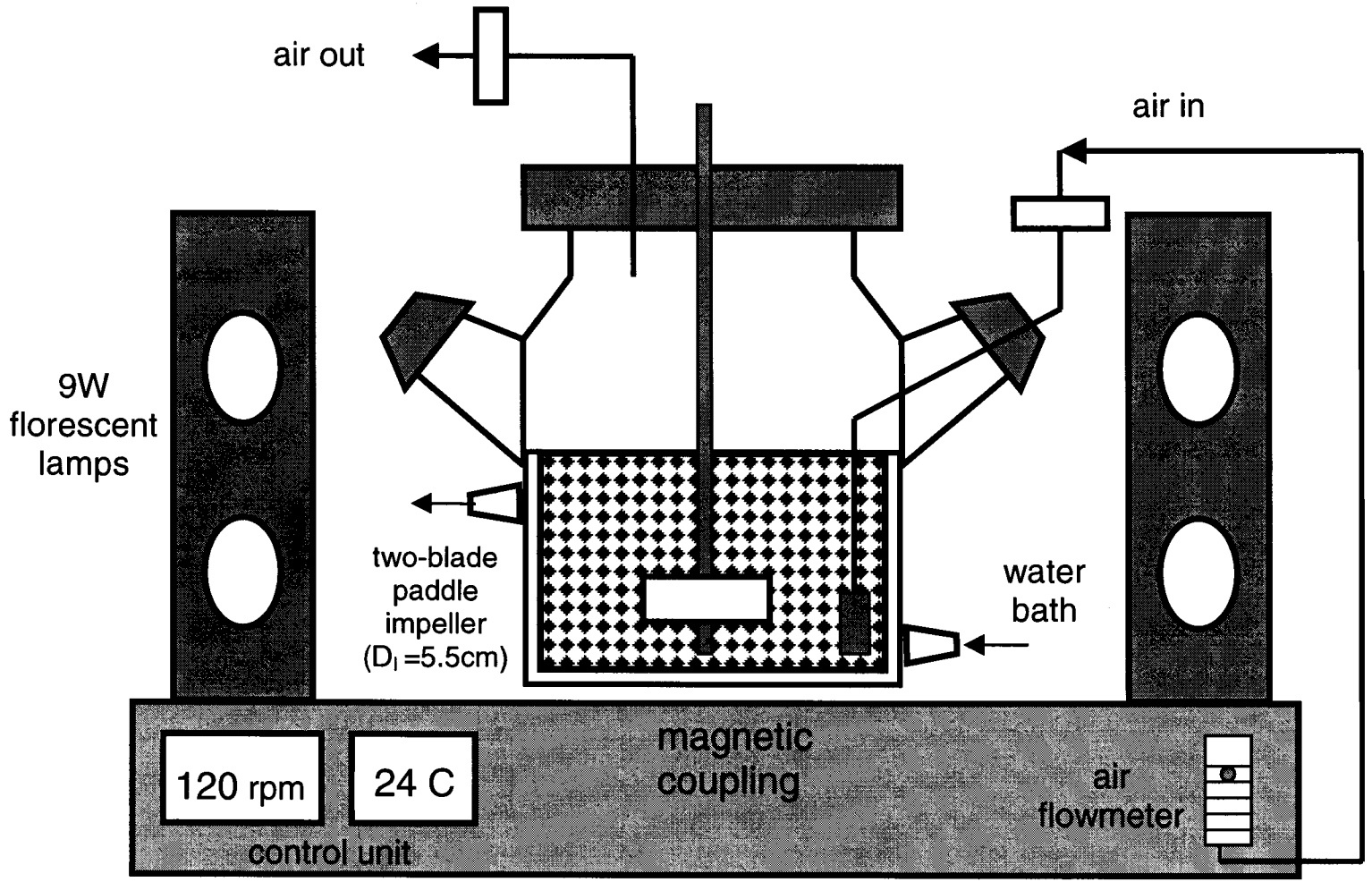


Figure 5.1 Schematic of 500 mL stirred-tank photobioreactor

illumination intensity incident to the vessel outer wall (I_o) was 38 μE m^{-2} sec^{-1}. The operation conditions were summarized in Table 4.1. Medium was completely replaced every five days (20% per day medium replacement rate).

Cell biomass and plantlet size measurements. To determine fresh weight (*FW*) of microplantlet biomass, the whole culture suspension within the culture vessel was vacuum-filtered on a 50 mm by 20 μm nylon mesh with a 42.5 mm Whatman No. 1 filter resting directly underneath the mesh to prevent the nylon mesh from retaining medium. The liquid sample (40 mL) was frozen at 20 °C and reserved for later nitrate and phosphate analysis. The retained biomass and nylon mesh were placed into a petri dish, and weighed to precision of ± 0.001 g. The biomass was then re-suspended using fresh ASP12 medium back to the bioreactor vessel. Dry cell weight (*DCW*) was usually measured for the last biomass sample, immediately after FW measurement. The microplantlet tissues were transferred with forceps onto an oven-dried, pre-weighed 47 mm x 0.45 μm filter (Millipore HWAP04700) and then dried at 80 °C for 24 h. The dried cell mass and filter were weighed to precision of ± 0.1 mg. *FW* and *DCW* values were determined by mass difference. Fresh cell density and dry cell density were determined from tissue *FW*, *DCW* and the original sample volume.

For the size analysis, microplantlets from the stirred-tank were randomly selected after biomass production reached the stationary phase. The microplantlets were placed on a petri dish with a ruler lying beneath. The longest and shortest axis of each plantlet was measured to precision of 1 mm. The plantlet diameter d_p was estimated by the average of the longest and shortest axis on a given plantlet. The data of microplantlet size were fit to a symmetric distribution function of the form

$$\phi = \bar{\phi} \exp\left(-\frac{(d_p - \bar{d}_p)^2}{2s^2} \right) \tag{5.1}$$

where $\bar{d}_p$ is the average plantlet diameter, $\bar{\phi}$ is the frequency at $\bar{d}_p$, and s is the distribution spread.

Nitrate, and phosphate measurements. The nitrate (NO_3^-) concentration in seawater was assayed with a LaMotte nitrate test kit (model NCR 3110). Prior to the assay, a liquid sample aliquot of 2 mL was thawed to room temperature and diluted 1:100 in distilled water if necessary. The nitrate concentration in the sample was measured spectrophotometrically at 530 nm. For determination of glycerophosphate concentration in seawater, the glycerophosphate was first hydrolyzed to free phosphate (PO_4^{-2}) using a LaMotte auxiliary phosphorous test kit (model APT, code 7884). Specifically, 20 mL of the liquid sample was acidified with 1 mL of

36 wt% sulfuric acid in a 50 mL flask, and then 0.5 g ammonium persulfate was added to the acidified sample. The liquid mixture was boiled gently for 30 minutes, cooled to room temperature, neutralized with 1 M NaOH, then brought back up to 20 mL in distilled water. Free phosphate was then assayed with a LaMotte standard phosphorous test kit (model VM-12 4408), where the phosphate concentration in the sample was measured spectrophotometrically at 605 nm after a reaction time of 30 minutes. All nitrate and phosphate assays were performed in duplicate.

During the batch cultivation experiment, the total molar consumption of nitrate M_N in the bioreactor was determined by

$$M_N = C_{N,O} \cdot V_0 - C_{N,f} \cdot V_f - \sum_{i=1}^{n} V_i C_{N,i} \tag{5.2}$$

where $C_{N,0}$ and $C_{N,f}$ are the initial and final concentration of nitrate in the reactor, V_0 and V_f are the initial and final volume in the reactor, and V_i is the volume withdrawn for nitrate and phosphate analysis, and $C_{N,i}$ is the measured nitrate concentration in the i^{th} sample.

The biomass yield coefficient based on nitrate limitation $Y_{X/N}$, was calculated by the increase of biomass over the total consumption of nitrate

$$Y_{X/N} = \frac{(W_f - W_o) \cdot W_s}{M_N} \tag{5.3}$$

where W_f and W_0 are the final and initial fresh weight of *A. subulata* in the stirred-tank cultivation, and W_s is the solid content ratio of dry cell weight to fresh weight. The growth-associated biomass yield coefficient based on nitrate consumption, $Y'_{X/N}$, was estimated by linear regression of the net biomass increase vs. nitrate consumption during the exponential phase of growth.

Photosynthetic oxygen evolution rate. Photosynthetic oxygen evolution rate (OER) measurements for the microplantlets were performed in the dissolved oxygen (D.O.) cell described by Huang et al. (1998). The suspension was loaded into the 65 mL D.O. measurement cell. The stir bar tip speed within the D.O. cell was set at 52 cm s^{-1}. The volumetric oxygen evolution rate (mmol O_2 L^{-1} h^{-1}) was determined from the least-squares slope of the linear portion of the D.O. concentration versus time curve, typically from 3 to 10 min. All data were collected at D.O. concentrations below 120% of O_2 in air saturation to avoid any possibility of dissolved oxygen toxicity effects on the culture. The D.O. concentration vs. time baseline in the sampled ASP12 medium with the tissue removed was also measured for the blank control.

The OER measurements were designed to represent the photosynthetic oxygen evolution rate at light saturation. Therefore, OER measurements within the D.O. cell were carried out at an incident light intensity of 134 μmol photons $m^{-2} s^{-1}$ and 24 °C. Specific oxygen evolution rate (P_o, mmol O_2 g^{-1} DCW h^{-1}) was determined by the volumetric oxygen evolution rate and the cell mass in the D.O. test cell. All reported values were averaged from duplicate measurements and corrected for the medium blank. Dark-phase oxygen respiration rate measurements were also conducted right after oxygen evolution rate in duplicate.

Volumetric oxygen mass transfer coefficient. The volumetric oxygen mass transfer coefficient (k_La) for interphase mass transfer of oxygen into cell-free liquid medium within the 500 mL stirred-tank photobioreactor was determined by the dynamic gassing-in method at 24 °C. The vessel was loaded with 500ml of filter-sterilized ASP12 liquid medium. The tip of the YSI model 5750 D.O. electrode was placed 5 cm below the liquid surface. Dissolved oxygen in the liquid medium was removed by sparging with N_2 through the sparger. When the D.O. concentration reached 1.0 % of O_2 saturation in air, air was re-introduced through the sparger at a given flow rate and impeller speed. The D.O. concentration vs. time data was recorded every 0.5 sec until the medium was saturated with dissolved oxygen. The unsteady-state material balance on oxygen in the liquid phase is given by

$$\ln\left[\frac{C^*_{O_2} - C_{O_2,o}}{C^*_{O_2} - C_{O_2}}\right] = k_L a \cdot (t - t_o) \tag{5.4}$$

where $C^*_{O_2}$ is the D.O. concentration of the medium at air saturation, and $C_{O_2,o}$ is the D.O. concentration at the onset of re-aeration at time t_o. The value for k_La was estimated from the least-squares slope of ln $C_{O_2,o}$ versus time data. The response time for the D.O. electrode is 5.2 sec and k_La values are corrected for the D.O. electrode response. To obtain the dependence of k_La on superficial gas velocity, inlet gas flowrates ranging from 12.7 to 250 mL min^{-1} at a given impeller stirred rate were considered. To test the dependence of k_La on the impeller Reynolds number, the impeller rotation rates were set from 60 to 250 rpm under a given aeration rate.

CO_2 consumption and mass transfer. The CO_2 consumed by the microplantlets for photosynthetic growth is supplied to the liquid medium by the aeration gas. Interphase mass transfer of CO_2 from the aeration gas bubbles to the culture suspension is liquid-phase controlling, due to the low solubility of CO_2 in liquid medium. To ensure microplantlets growth is not limited by CO_2 demand, CO_2 mass transfer rate must be larger than CO_2 consumption rate.

$$CO_2 - TR \geq q_{CO_2}(t) \cdot X(t) \tag{5.5}$$

where q_{CO_2} is the specific CO_2 consumption rate by the microplantlets (mmol CO_2 g^{-1} DCW h^{-1}). The maximum possible CO_2 transfer rate is defined as

$$CO_2 - TR = (k_L a)_{CO_2} \cdot C^*_{CO_2} = (k_L a)_{CO_2} \frac{p_{CO_2}}{H} \tag{5.6}$$

where $C^*_{CO_2}$ is the dissolved CO_2 concentration in equilibrium with the partial pressure of CO_2 in the aeration gas (mmol CO_2 L^{-1}), $(k_L a)_{CO_2}$ is the volumetric mass transfer coefficient for CO_2 (h^{-1}), and the Henry's law constant for CO_2 in 36 ppt seawater (H) at 24 °C is 0.0346 atm L $mmol^{-1}$. The $(k_L a)_{CO_2}$ for CO_2 mass transfer is related to $k_L a$ for O_2 mass transfer by

$$(k_L a)_{CO_2} = k_L a \left(\frac{D_{CO_2}}{D_{O_2}} \right)^{1/2} \tag{5.7}$$

where the ratio of molecular diffusion coefficients for CO_2 to O_2 in seawater medium at 24 °C is 0.93, using data provided by Raven (1984).

At pH 8 to 9, dissolved inorganic carbon in seawater is speciated as bicarbonate (HCO_3^-)

$$CO_2(aq) + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \tag{5.8}$$

$$HCO_3^- \leftrightarrow CO_3^{-2} + H^+ \tag{5.9}$$

where the pK_a values for HCO_3^- and carbonate (CO_3^{-2}) dissociation in seawater at 24 °C are 6.05 and 9.10 respectively (Raven, 1984). The Na-HEPE (5.0 mM, pK_a = 7.5) in the ASP12 medium also buffers the pH increase in response to dissolved CO_2 demand by the suspension culture at pH 8-9. However, bicarbonate in ASP12 medium remains at 10 mM (Table 3.1) and does not contribute to the CO_2 consumption by the suspension culture.

The CO_2 mass transfer analysis (equation 5.6) assumes that the aeration gas flowrate is high, and CO_2 is slightly soluble in the liquid so that P_{CO_2} and $C^*_{CO_2}$ are essentially constant. However, when the liquid medium is alkaline, CO_2 in the aeration gas can be absorbed more efficiently since dissolved CO_2 speciates to bicarbonate and increases the total dissolved inorganic carbon concentration. If the liquid medium captures all of the gas phase CO_2 delivered to the culture, then the CO_2 delivery rate is defined by

$$n_A = \frac{F_A}{V} = \frac{v_g P_{CO_2}}{RTV} \tag{5.10}$$

where F_A is the molar flowrate of CO_2 in the aeration gas (mol CO_2 /min), R is the gas constant, T is temperature (K) of the aeration gas, and v_g is the volumetric flowrate of the aeration gas (L/min), and V is the culture volume.

The overall stoichiometry for photosynthetic biomass production is approximated as

$$CO_2 + H_2O \rightarrow CH_2O + O_2 \tag{5.11}$$

From equation 5.11, the biomass yield coefficient based on CO_2 (Y_{X/CO_2}) is 30 mg DCW $mmol^{-1}$ CO_2. Since the photosynthetic growth occurs during the illumination phase when CO_2 is consumed to evolve oxygen, the CO_2 consumption rate (mmol CO_2 L^{-1} h^{-1}) during the light phase is estimated by

$$q_{CO_2} \cdot X(t) = \frac{\mu}{f} \cdot \frac{X(t)}{Y_{X/CO_2}} \tag{5.12}$$

where X is the dry cell density (mg DCW L^{-1}) at cultivation time t, and f is the fractional illumination photoperiod (e.g. $f = 24$ at continuous light). Equation (5.11) assumes that the molar ratio of CO_2 consumption to O_2 evolution is equal to one. Therefore, when the specific oxygen evolution rate is available, the specific CO_2 consumption rate is also estimated by

$$q_{CO_2} = P_o \cdot \frac{v_{CO_2}}{v_{O_2}} \cong P_o \tag{5.13}$$

where v_{CO_2} and v_{O_2} are stoichiometric coefficients for CO_2 and O_2 based on overall photosynthetic biomass production stoichiometry. To avoid CO_2 mass transfer limited growth, CO_2-TR and n_A must be larger than $q_{CO_2} \cdot X$ at all times during the cultivation.

Average shear rate analysis. To examine how microplantlet growth responded to agitation intensity in the stirred tank, the impeller revolution rates were set at 60, 120, 140 and 250 rpm. For an unbaffled vessel operated in the turbulent regime, an equation described by Croughan et al. (1987) was used to estimate the average shear rate Y_a of the stirred tank bioreactor

$$Y_a = \frac{112.8 N r_i^{1.8} (r_t^{0.2} - r_i^{0.2})(r_c / r_i)^{1.8}}{r_t^2 - r_i^2} \tag{5.14}$$

where N is impeller rotation rate (rev s^{-1}), r_i and r_t are the radius of impeller and tank respectively, r_c is the radius of the forced vortex zone can be estimated from

$$\frac{r_c}{r_i} = \frac{\mathrm{Re}}{1000 + 1.6\mathrm{Re}} \tag{5.15}$$

In equation (5.15), Re is the impeller Reynolds number, defined as

$$\mathrm{Re} = \frac{N_i D_i^2}{\nu_L} \tag{5.16}$$

where ν_L is the kinematic viscosity of the liquid medium ($m^2\ s^{-1}$), D_i is the impeller diameter (m) and N_i is the impeller rotation rate (rev sec^{-1}). If impeller Reynolds number exceeds approximately 1000, the flow field becomes completely turbulent (Nagata, 1975). All stirred-tank experiments were agitated in the turbulent regime with Re ranging from 3380 (60 rpm) to 14083 (250 rpm).

Results and Discussion

Agardhiella subulata microplantlets were successfully cultivated in a 500 mL stirred-tank bioreactor. The biomass production with or without medium replacement was first compared. The long-term effects of CO_2 delivery on biomass growth were then assessed under medium replacement. Finally, the experiments were conducted at various impeller rotation speeds to address mixing and effect of agitation on growth.

Biomass production without medium replacement (Nitrate-limited growth). To study the effect of a nitrate-limiting condition on the microplantlet culture, two aerated 500 mL stirred-tank photobioreactor cultivation experiments were conducted in parallel with a common inoculum at initial cell densities of 1.26 and 2.93 g FW/L. Common process conditions for cultivation of *A. subulata* microplantlet suspensions in the 500 mL stirred-tank photobioreactors are summarized in Table 5.1. The initial nitrate and phosphate concentration of the ASP12 medium in each reactor was 1.28 mM (1X) and 0.45 mM (10X) respectively, N:P of 2.7:1 to move the cultivation toward nitrate limitation. The system was in a 10-fold excess of phosphate based on N:P values for marine algae (Atkinson and Smith, 1983). Biomass density, nitrate concentration, and phosphate concentration vs. time profiles are presented in Figures 5.2a,b. Without medium replacement, the biomass production reached stationary phase shortly before nitrate depletion, showing the effects of N-limitation on photosynthesis in carbon-nitrogen metabolism of algae (Turpin, 1991). Figure 5.2b shows that phosphate concentration continued to decrease even though culture reached stationary phase, implying that phosphate was assimilated and stored without being incorporated into new cell biomass. In the end of batch cultivation experiment, phosphate concentration was at least 66 μM, which was more than the 1X phosphate concentration in ASP12 medium (45 μM).

Table 5.1 Process parameters for the effects of N *A. subulata* microplantlet suspension culture within a 500 mL stirred-tank photobioreactor

Process Variables	
Temperature	24 °C
Incident light intensity	80 μmol photon m^{-2}-s^{-1}
α	2
Photoperiod	10:14 LD
Aeration gas flowrate (mL min^{-1})	150 (0.00035 atm CO_2) 292 (0.00720 atm CO_2)
Medium replacement	None
Initial nitrate concentration, $C_{N,0}$	1.28 mM
Initial phosphate concentration, $C_{p,0}$	0.45 mM
Impeller rotation rate	120 rpm

Previous studies on phosphate-limited growth of aqua-cultured *Agardhiella* plants collected from the field showed that the specific growth rate was saturated at seawater phosphate concentrations of 10 μM whereas the specific P content of the biomass was saturated at 20 μM (Chopin et al., 1990).

The stationary phase was triggered by nitrate-limited growth, and the growth was completely shut down after complete nitrate consumption. In previous work, DeBoer et al. (1978) reported that the optimum medium nitrate concentrations for macroscopic marine algae were in the range of 120-2140 μM. In this present study, biomass production ceased when nitrate concentration went below 200 μM (Figure 5.2a). The biomass yield coefficient based on nitrate limitation $Y_{X/N}$ was 0.91 ± 0.05 g DCW $mmol^{-1}N$, whereas the growth-associated biomass yield coefficient in the exponential phase of growth $Y'_{X/N}$ was 1.31 ± 0.07 g DCW $mmol^{-1}N$. This result indicates that nitrate consumption in the late exponential phase of growth was not used for biomass production.

Biomass production under medium replacement. The representative biomass growth curves of the microplantlets within 250 mL bubble-column and 500 mL stirred-tank

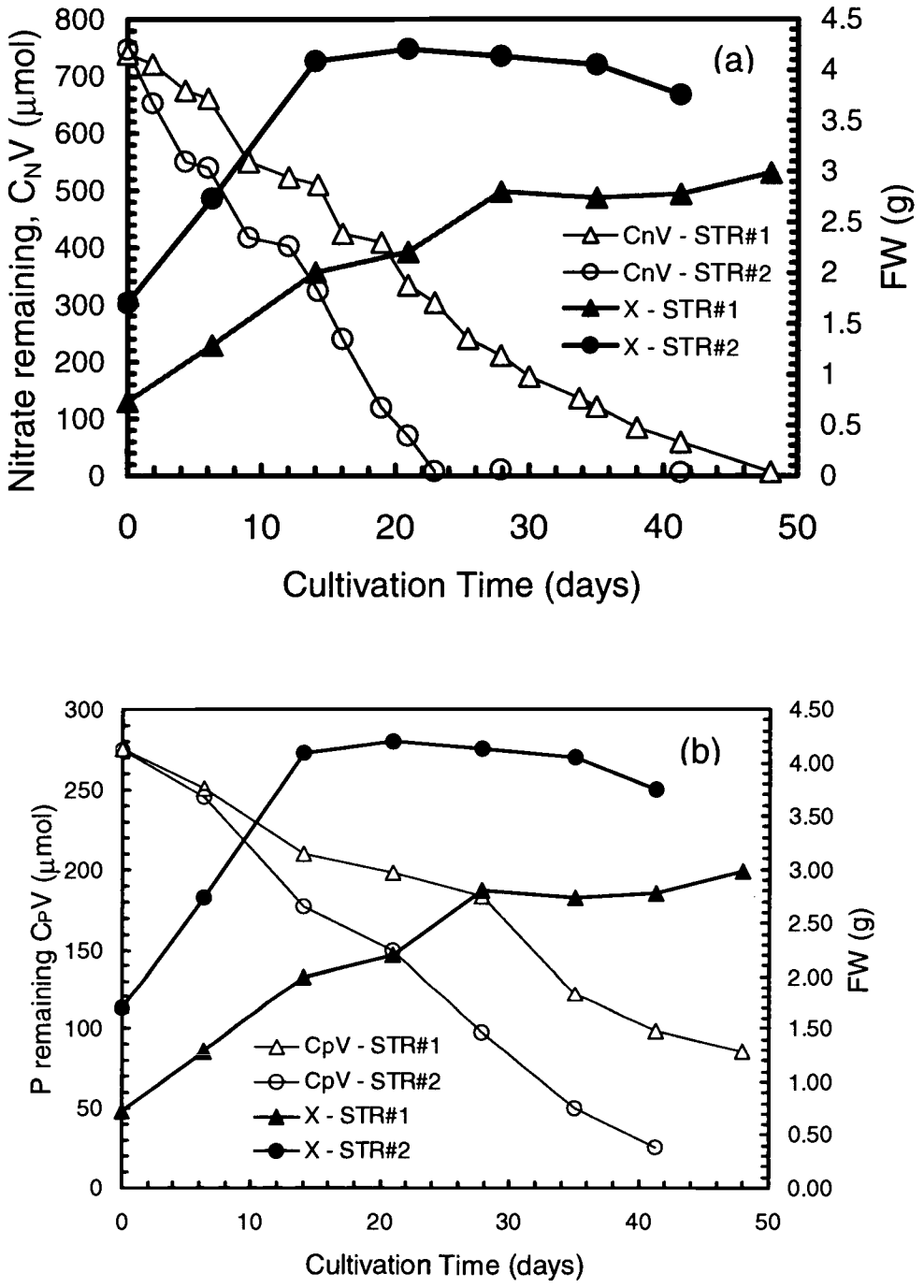


Figure 5.2 Nitrate-limited batch cultivation experiments. (a) FW and remaining nitrate vs. time curves; (b) FW and remaining phosphate vs. time curves

photobioreactors are presented in Figure 5.3. Bubble-column bioreactor process conditions were presented earlier in Table 4.1. At CO_2 partial pressure of 0.00035 atm in the aeration gas stream, microplantlets can sustain active growth under 20% per day of medium replacement rate for 50 days without reaching the stationary phase of growth in either stirred tank or bubble column. The extension of the growth phase is desirable for the study of the long-term effects of process variables on microplantlet growth, including agitation intensity, CO_2 delivery, and pH. Therefore, all future experiments were conducted under medium replacement of 20% per day to avoid nutrient depletion. Without sample removal, the critical cell densities for N or P limited growth are

$$X_{c,N} = \frac{Y_{X/N} \cdot C_N \cdot \theta}{\mu} \tag{5.17}$$

$$X_{c,P} = \frac{Y_{X/P} \cdot C_P \cdot \theta}{\mu} \tag{5.18}$$

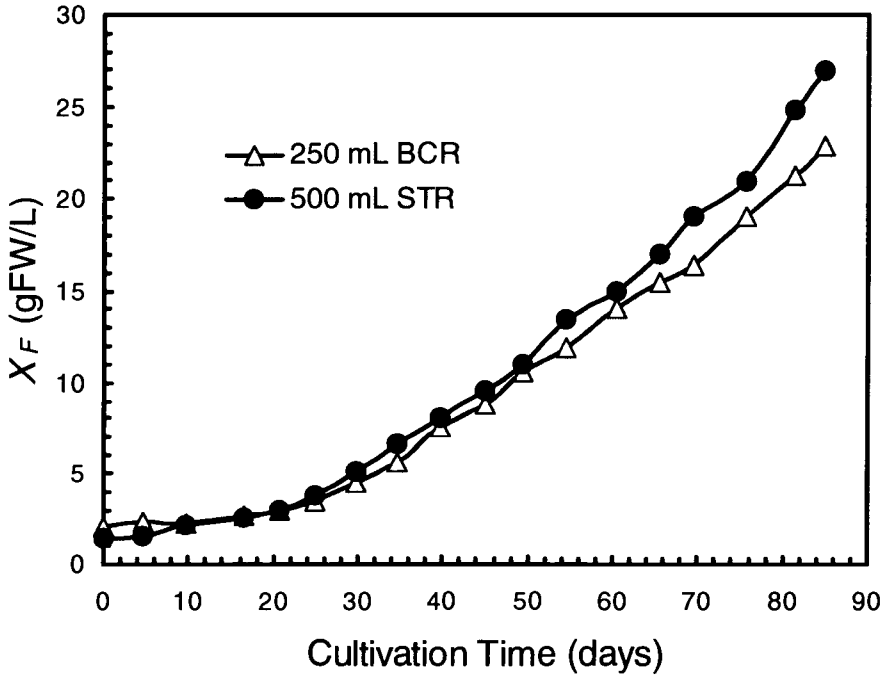


Figure 5.3 Comparison of representative growth curves of *Agardhiella subulata* in 250 mL bubble-column (BCR) and 500 mL stirred-tank (STR)

where C_N and C_P are the nitrate and phosphate concentration in the bioreactor respectively, μ is the specific growth rate in the extended exponential phase of growth (day^{-1}) and θ is the medium replacement rate (e.g. $\theta = 0.2$ means 20 % per day). The biomass yield coefficient based on phosphate limitation ($Y_{X/P}$) was 25.8 g DCW $mmol^{-1}$ P (Chapter 3). The critical cell density for either N or P limitation under medium replacement of 20% per day was above 30 g FW/L.

Growth morphology in bioreactors. The morphology of microplantlets cultivated in the bubble-column bioreactors was different from the microplantlets cultivated in the stirred tank (Figure 5.4). For microplantlets growing in a bubble-column bioreactor, shoots and their secondary branches tended to elongate freely, resulting in asymmetric shape. For microplantlets growing in the stirred tank bioreactor, more linear shoots emanated from the central core, and the shape of the plantlets was spherical and compact. Since the microplantlet shoots appeared rigid and seldom broke apart within the stirred vessel, the physical contact of impeller with the plantlets within the stirred tank may have forced microplantlets to grow into a compact and spherical shape.

Microplantlet size distribution. Figure 5.5 compares final size distribution of microplantlets from the nitrate-limited batch cultivation experiments from two initial cell densities of 1.26 and 2.93 g FW/L. The adjustable parameters $\bar{d}_p$ and s from equation (5.1) are summarized in Table 5.2. As stated in chapter 3, microplantlets appeared rigid and not easily broken apart (see Figure 3.14). The final size of microplantlets d_p was related to the initial cell density X_0. Low X_0 led to larger d_p. The larger size average at lower initial cell density also led to wider spread of the plantlet size distribution.

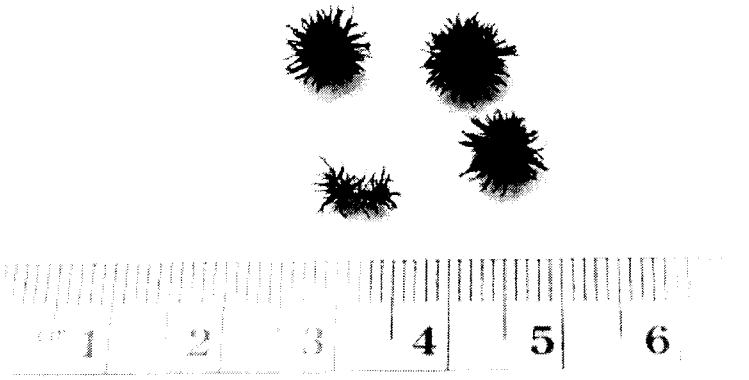


Figure 5.4 *A.subulata* microplantlets cultivated in 500 mL stirred-tank bioreactor

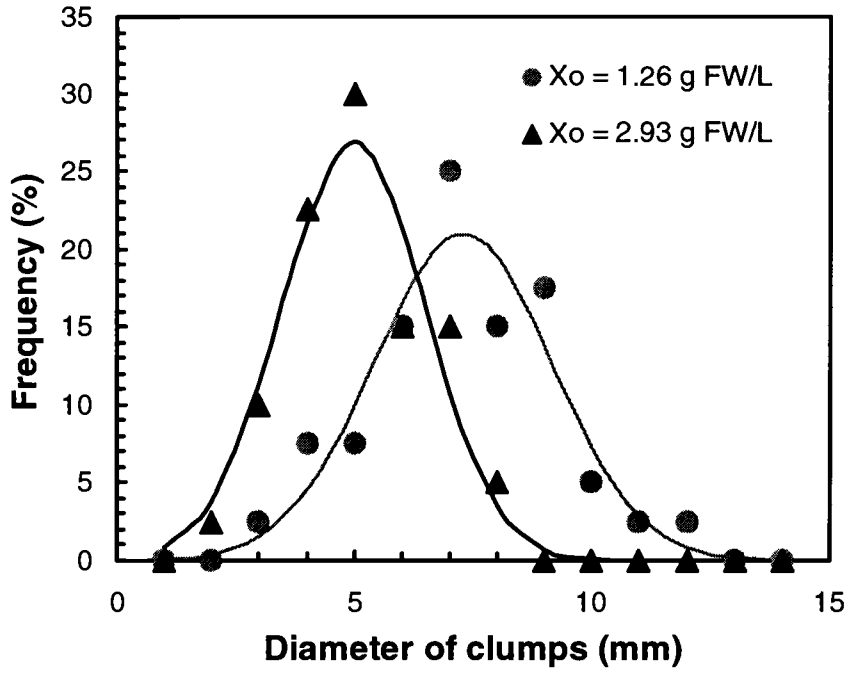


Figure 5.5 Size distribution of *A. subulata* microplantlets in nitrate-limited batch cultivation experiments

Table 5.2 Nitrate-limited stirred tank cultivation experiment. Comparison of the size distribution parameters, biomass growth and nitrate yield coefficients.

Growth Parameters	As-STR-11#1	As-STR-11#2
$X_{F,0}$, g FW	0.73	1.70
$\overline{d}_P$ (distribution function), mm	7.3 ± 0.2 (n= 40)	5.0 ± 0.1(n=40)
$\overline{d}_P$ (measured), mm	8 ± 2 (n=40)	5 ± 1 (n=40)
s (distribution spread), mm	1.87 ± 0.19 (n=40)	1.50 ± 0.13 (n=40)
$X_{F,f} - X_{F,0}$, g FW	2.10	2.32
$Y_{X/N}$, g DCW mmol^{-1} N	0.98 ± 0.05 (n=4)	1.01 ± 0.10 (n=5)
$Y'_{X/N}$, g DCW mmol^{-1} N	1.36 ± 0.20 (n=5)	1.26 ± 0.31 (n=4)
	r^2=0.94, day 0-28	r^2=0.89, day 0-21
M_N, mmol N	605.8	653.7
μ (day 0-14), % day^{-1}	7.13 ± 0.93 (n=3)	6.21 ± 0.65 (n=3)

Mass transfer coefficient and correlation. The effects of agitation and aeration on the oxygen mass transfer coefficient of k_La in the stirred-tank bioreactor were determined. The measured k_La values were fitted into a power law correlation of the form

$$k_La = Av^a + Bv^a \,\mathrm{Re}^b \tag{5.19}$$

where v is superficial gas velocity (m s^{-1}), and Re is the impeller Reynolds number. The first term Av^a represents the part of mass transfer when the vessel was only pneumatically agitated without mechanical agitation. The fitted parameters A, B, a and b, obtained from nonlinear regression are summarized in Table 5.3. Figure 5.6 shows that the correlation of the measured k_La to equation (5.19) in the range from 6 to 246 hr^{-1}.

Table 5.3 Fitted parameters for k_La correlation according to equation (5.19)

Fitted Parameters	Reactor 1	Reactor 2
A	4483.30	699.55
B	8.48E-11	2.70E-3
a	0.50 ± 0.13	0.51 ± 0.10
b	3.39 ± 0.72	1.52 ± 0.13
n	7	9
R^2	0.98	0.92

CO_2 delivery. CO_2 delivery limitations were addressed by comparing the volumetric CO_2 consumption rate to the CO_2-TR defined by equation (5.6), and the CO_2 delivery rate n_A, defined by equation (5.10). It was first necessary to determine whether CO_2-TR or n_A was the limiting mode of CO_2 delivery to the culture. Figure 5.7 showed the operation line where CO_2-TR equals to n_A. This line was calculated by setting CO_2-TR equals to n_A, i.e.

$$k_La \cdot \frac{P_{CO_2}}{H} = \frac{v_g P_{CO_2}}{RT \cdot V} \tag{5.20}$$

or $$Av^a + Bv^a \,\mathrm{Re}^b = \frac{\pi v D_T^2 H}{4RT \cdot V} \tag{5.21}$$

The operation line in Figure 5.7 is independent of CO_2 partial pressure. The area below the line is the operation region where CO_2 delivery rate is determined by CO_2-TR (equation 5.6),

whereas the area above the line is where the CO_2 delivery rate is determined by n_A (equation 5.10).

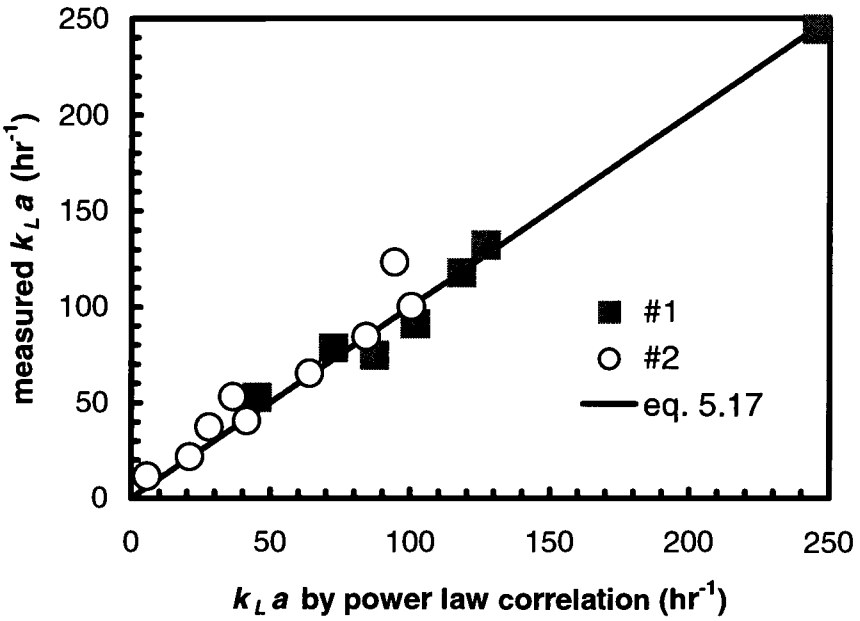


Figure 5.6 Correlations of k_La from equation (5.17) vs. measured data

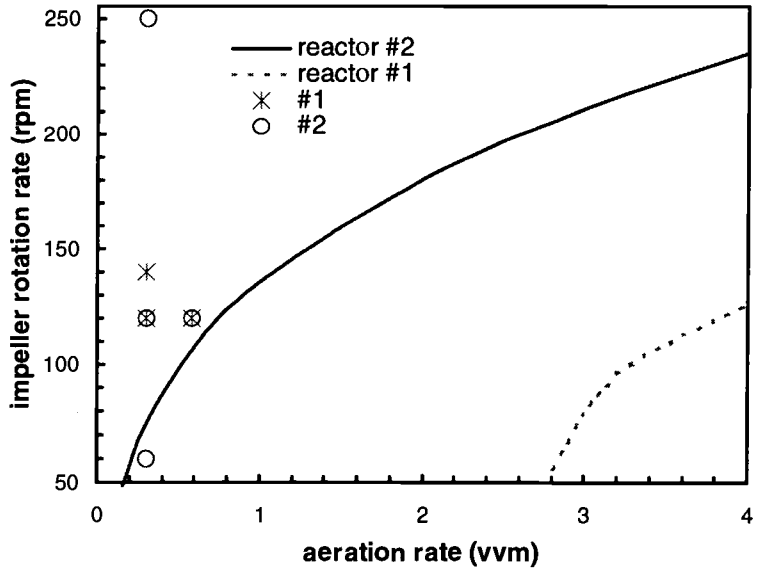


Figure 5.7 CO_2-TR regime map. The operation line is calculated according to equation (5.21)

Figure 5.7 shows that at 0.3 vvm (L air L^{-1} culture min^{-1}) aeration rate, CO_2 delivery is determined by n_A in reactor #1. The CO_2 delivery in reactor #2 is also determined by n_A when the impeller rotation rate is above 75 rpm. Therefore, among all the stirred tank cultivation experiments, As-STR-5 was the only one defined by CO_2-TR as opposed to n_A.

The microplantlet cultivation went through three growth phases, including two exponential phases of growth, followed by a linear CO_2-limited growth phase. In the rate-limited exponential phases of growth, the equations used to estimate the growth curve in terms of fresh cell density (X_F) are

$$X_F = X_{F,0} e^{\mu_1 t} \qquad \text{for } t = 0 \text{ to } t_1 \tag{5.22}$$

$$X_F = X_{F,1} e^{\mu_2 (t - t_1)} \qquad \text{for } t = t_1 \text{ to } t_c \tag{5.23}$$

When the CO_2 consumption rate matched the CO_2 delivery rate, the growth curve is

$$X_F = X_{F,c} + \beta (t - t_c), \ \beta = \frac{n_A Y_{X/CO_2} f}{W_s} \qquad \text{for } t > t_c \tag{5.24}$$

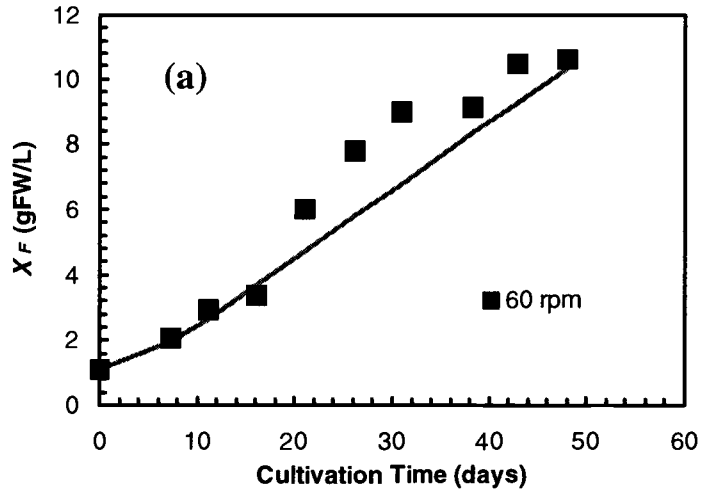
The transition to CO_2-limited growth phase occurs at the critical cell density of

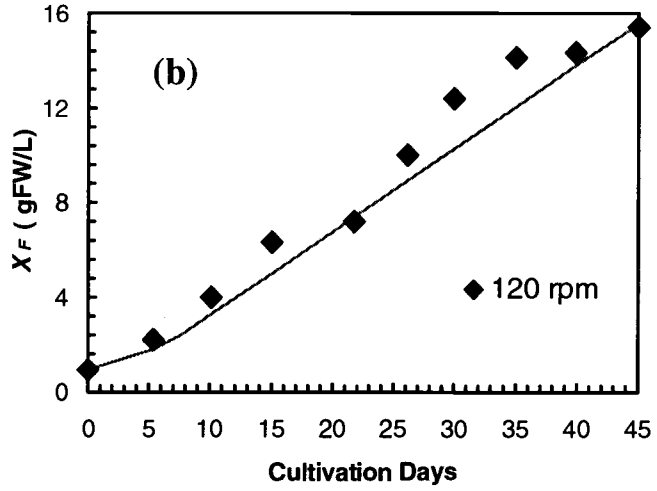
$$X_{F,c} = \frac{n_A \cdot f \cdot Y_{X/CO_2} \cdot W_s}{\mu_2} \tag{5.25}$$

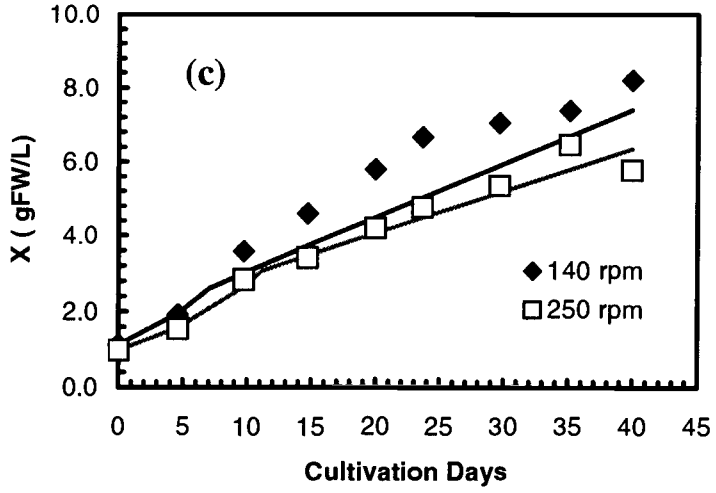
at critical cultivation time of

$$t_c = t_1 + \frac{1}{\mu_2} \ln\left(\frac{X_{F,c}}{X_{F,1}}\right) \tag{5.26}$$

Representative growth curves for *A subulata* microplantlet over an extended 45 to 90 day cultivation time are presented in Figure 5.8a-d. All cultivation experiments were carried out with 20% per day medium replacement to sustain active growth so that the effects of CO_2 delivery on biomass production could be readily observed. Table 5.4 compares the growth parameters derived from equations 5.22 to 5.26. The predicted growth curves were generated from the model equations 5.22-26 using the measured μ_1, μ_2, and β (Table 5.4). In some stirred-tank experiments (Figure 5.8a-c), CO_2 limitation occurred before the second exponential phase of growth, and so the specific growth rate for the second exponential phase of growth was not reported in Table 5.4. In Figure 5.8d, CO_2-limited growth occurred in the middle of second exponential phase of growth (day 56 and day 61), and the culture switched to a linear phase of continued growth at CO_2 delivery rate (n_A) of 0.26 mmol CO_2 L^{-1} h^{-1}. After 90 days of cultivation, cell densities exceeding 14 g FW L^{-1} were achieved. In Figure 5.8e, CO_2 was added


(a)
X_F (gFW/L)
60 rpm
Cultivation Time (days)


(b)
X_F (gFW/L)
120 rpm
Cultivation Days


(c)
X (gFW/L)
140 rpm
250 rpm
Cultivation Days

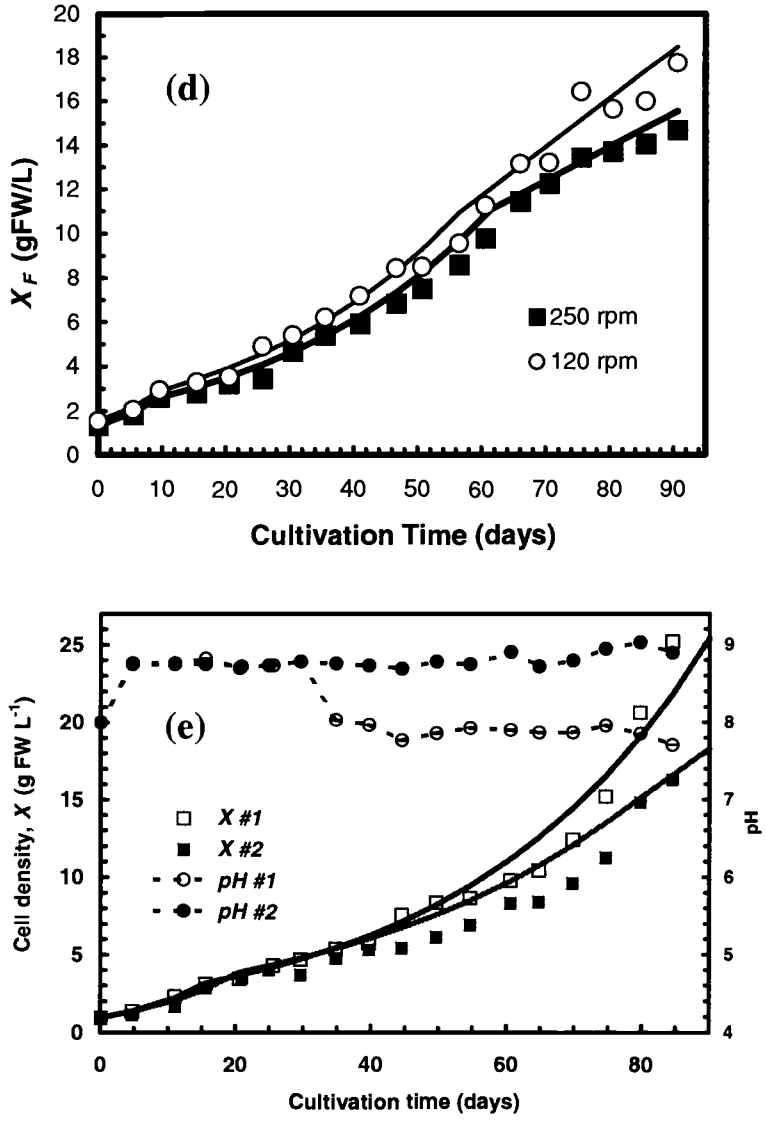


Figure 5.8 Cultivation of microplantlets in the 500ml Stirred tank bioreactors under 20% per day of medium replacement rate at 60-250 rpm (a) As-STR-5; (b) As-STR-6; (c) As-STR-7; (d) As-STR-8; (e) As-STR-9.

to the aeration gas stream (720 pa) in the middle of exponential phase (day 30) to prevent CO_2 limitation. Therefore, microplantlets sustained the second exponential phase of growth and achieved 25 g FW L^{-1} in 85 days. The biomass growth curve of *A. subulata* microplantlets with two exponential phase of growth was also observed in the 2-L bubble-column bioreactor with constant medium perfusion and elevated CO_2 (350 pa) in the aeration gas stream (Table 3.3).

Table 5.4 Comparison of process parameters, growth characteristics and time constants of *A.subulata* microplatlet culture in 500 mL stirred-tank bioreactor. The cultivation was under 20% per day of medium replacement rate.

Run I.D. (As-STR-#)	5	6,9#2	9#1	7#1, 8#1	7#2, 8#2
Impeller rotation rate	60 rpm	120 rpm	120 rpm	140 rpm	250 rpm
CO_2 concentration (ppm, day range)	35	35	35 (0-35) 720 (35-85)	35	35
Impeller Re	3380	6759	6795	7886	14082
Y_a, sec^{-1}	1.86	4.31	4.31	5.14	9.74
μ_1, % day^{-1} (day range, ID#)	7.6 ± 0.6 (0-21)	12.9 ± 1.2 (0-15, 6) 6.7 ± 1.2 (0-15, 9#2)	7.8 ± 3.4 (0-15)	11.9 ± 0.2 (0-10, 7#1) 6.6 ± 1.0 (0-10, 8#1)	10.8 ± 0.6 (0-10,7#2) 7.0 ± 0.9 (0-10, 8#2)
μ_2, % day^{-1} (day range)	-	2.2 ± 0.1 (15-75,9#2)	2.8 ± 0.1 (15-85)	2.8 ± 0.2 (10-50, 8#1)	2.8 ± 0.2 (10-45,8#2)
X_c, g FW L^{-1} (ID#)	3.33	2.4 (6) 14.15 (9#2)	-	2.63 (7#1) 10.92 (8#1)	3.01 (7#2) 11.03 (8#2)
t_c (days)	14.36	7.54 (6) 83.94 (9#2)	-	7.06 (7#1) 56.43 (8#1)	11.14 (7#2) 61.38 (8#2)
β, g FW day^{-1} (eq. 5.17)	0.25	0.31	-	0.31	0.31
β, g FW day^{-1}, measured (day range, ID#)	0.21± 0.03 (16-48)	0.35 ± 0.02 (6-45, 6) 0.32 (80-85, 9#2)	-	0.15 ± 0.02 (10-40, 7#1) 0.22 ± 0.01 (56-91, 8#1)	0.11 ± 0.02 (10-40, 7#2) 0.15 ± 0.02 (56-91, 8#2)
n_A, mmol CO_2 L^{-1} h^{-1} (day range)	0.258	0.258	0.258 (0-35) 10.3 (35-85)	0.258	0.258
$k_L a\, C^*_{CO_2}$, mmol CO_2 L^{-1} h^{-1}	0.209	0.394	0.890 (0-35) 25.5 (35-85)	0.983	0.975
t_{mt}, sec, (Run ID#)	173.8	92.4 (6)	40.9 (0-35) 29.4 (35-85)	37.1	37.3
$t_{rxn,c}$, sec, at $X_{F,\,c}$	173.8	140.9	-	140.9	140.9
Minimum $t_{rxn,f}$ (s) at the end of experiments	54.5	22 (6) 122.4 (9#2)	62	44.8 (7#1) 105.7 (8#1)	74.6 (7#2) 106.2 (8#2)
t_{mix} sec	4.6	2.3	2.3	2.0	1.1

Effect of impeller rotation rate. The specific growth rates of *A. subulata* microplantlet suspensions in the 500 mL stirred-tank at four different impeller rotation rates (60, 120, 140 and 250 rpm) are summarized in Table 5.4. The minimum impeller rotation rate to uniformly suspend the microplantlet suspension was 60 rpm, whereas the 250 rpm was fastest stable impeller rotation rate experimentally achievable in 500 mL Bellco stirred tank. Under these agitation conditions, average shear rates ranging from 2 to 10 s^{-1} at impeller tip speed of 20 to 72 cm s^{-1} were obtained by equation 5.14. Comparing the same inoculum agitated at 140 and 250 rpm (As-STR-7 and As-STR-8), the average specific growth rates for the first and second exponential growth phase were not significantly different. However, once the culture reached the linear growth phase under CO_2-limited condition, microplantlets cultivated at 250 rpm achieved only about 74% of those cultivated at 140 rpm (Table 5.4). No substantial physical damage on shoot tissue was observed at the end of the stirred-tank cultivation experiments.

The hydrodynamic shear generated in the present study was considered harmful for many other cell cultures. The critical shear rate was 3 to 7 s^{-1} for animal cell culture grown in geometrically similar stirred-tank system (Croughan et al., 1987). According to the flow regime map for Rushton turbines with Di/D_T of 0.5 (Doran, 1999), the 500 mL STR in this study was operated in the damage region for terrestrial plant cell suspension (i.e. 0.3 vvm aeration rate and N of 4.2 sec^{-1}).

Time constant analysis. Comparison of time constants can further verify if the system is well mixed and to determine which processes are rate-limiting. The time constants for CO_2 consumption (t_{rxn}) and gas-liquid mass transfer (t_{mt}) are

$$t_{rxn} = \frac{C^*_{CO_2}}{q_{CO_2} \cdot X} = \frac{C^*_{CO_2} Y_{X/CO_2} f}{\mu X} \tag{5.23}$$

$$t_{mt} = \frac{1}{(k_L a)_{CO_2}} \tag{5.24}$$

The mixing time, proposed by Kossen and Oosterhuis (1985), for Rushton turbine in a baffled tank, is

$$t_{mix} = \frac{1.54V}{D_i^3 N} \tag{5.25}$$

The time constant analysis is summarized in Table 5.4. A small time constant represents a fast process. In the present analysis, t_{mt} and t_{mix} are considered constant over the cultivation period, while t_{rxn} is a function of cell density X. Therefore, t_{rxn} at critical cell density X_c and the final cell density are reported in Table 5.4. The t_{mix} values were low (e.g. 1.1-4.6 sec) due to the

small size of the vessel and relatively large impeller diameter to tank diameter. Even though t_{mix} may be underestimated for the unbaffled Bellco spinner flask, the t_{mt} values were 14 to 30 times higher than t_{mix}, more than an order of magnitude. Therefore, the systems were well mixed, at least relative to gas-liquid mass transfer.

Table 5.4 also indicates that CO_2 mass transfer (t_{mt}) and consumption rate ($t_{rxn,}$) time constants were within the same order of magnitude at the critical cell density and near the end of cultivation experiments. At 60 rpm, the $t_{rxn,c}$ value matched t_{mt}, suggesting that the process was limited by gas-to-liquid mass transfer. At 120-250 rpm, the $t_{rxn,c}$ remained at 141 sec due to the limiting n_A delivery rate. Increasing n_A is necessary to sustain its exponential growth and increase critical cell density.

Effects of pH environment on microplantlet growth. The pH cultivation experiments for *A. subulata* microplantlets were conducted in an aerated 500 mL stirred-tank photobioreactor. The experiment had two stages: a constant pH profile during the first 41 days, followed by a transient pH profile for the remainder of the cultivation period. Microplantlets were not sampled during the first 17 days in order to generate enough biomass for later OER measurements. The pH change in the second stage was bounded within the nominal physiological range of 6.5 to 9.0. During the entire cultivation period, the medium was replaced at a rate of 20% per day to prolong active growth. The specific OER measurements captured photosynthetic growth characteristics of the microplantlets during the constant and transient pH cultivation stages.

Specific growth rate, OER, and CO_2-TR for the constant pH stage of cultivation are presented in Table 5.5. During the first 41 days of cultivation, the aeration gas CO_2 partial pressure was set at 0.00035 atm CO_2 (1X ambient) to provide a cultivation pH of 8.8 in the 5.0 mM HEPE / 10.0 mM bicarbonate buffer system. Figure 5.10 shows that specific oxygen evolution rate was decreasing at pH near 9 during CO_2-limited growth (from day 19 to 41). Then from day 42 to day 60, the CO_2 partial pressure was incrementally increased then decreased according to the profile shown in Figure 5.9 to provide the pH profile shown in Figure 5.10. The culture pH equilibrated to the new value within 2 hours of the step change in CO_2 partial pressure. Just before the next step change in CO_2 level, the specific OER was also measured (Figure 5.10) 3 h into the 10:14 LD photoperiod. As the pH was lowered from 8.9 to 6.7, the specific OER went through a sharp optimum at pH 7.8. Past day 50, the culture was moving toward stationary phase and so the specific OER was insensitive to increasing the pH back up from 6.7 to 8.0.

Based on these studies, the optimal pH environment for cultivation of *A. subulata* microplantlets is centered around pH 8. Sustained cultivation at average pH of 7.9 significantly improved cumulative biomass production relative to average pH of 8.8 (Figure 5.8e). When the

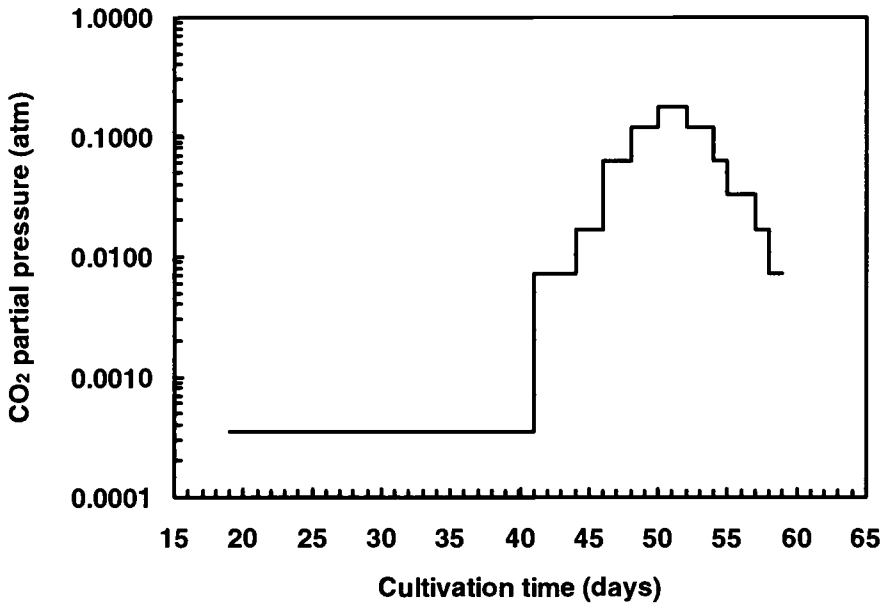


Figure 5.9 Aeration gas CO_2 partial pressure vs. time profile for 500 mL stirred tank bioreactor during the pH experiment.

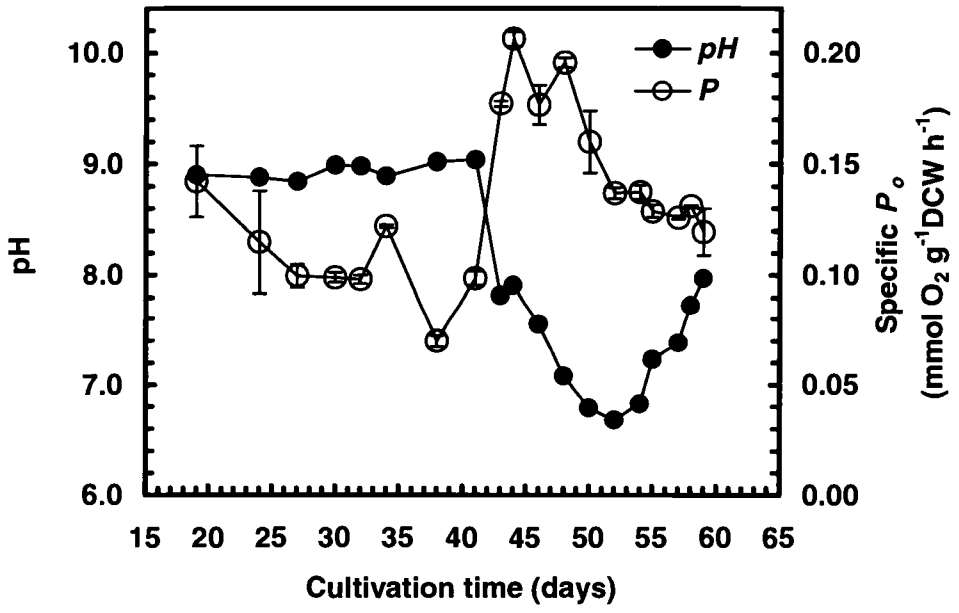


Figure 5.10 Culture pH and specific oxygen evolution rate (P_o) of *A. subulata* microplantlets in response to aeration gas CO_2 partial pressure profile in 500 mL stirred tank bioreactor during the pH experiment

pH was re-set to 7.9, biomass production increased relative to the pH 8.8 control, and a cell density of 25 g FW L^{-1} was achieved after 85 days in culture.

Table 5.5 Process conditions of 500-mL stirred-tank bioreactor under 20% per day of medium replacement rate for pH experiment.

Process Variable	As-STR-10 #1
CO_2 partial pressure (atm)	0.00035 (day 0-41)
Cultivation pH	8.84 ± 0.07 (n = 6) (day 0-23)
Specific growth rate (day^{-1})	0.063 ± 0.008 (n=5, day 0-19)
Aeration gas flowrate (mL min^{-1})	150 (day 0-41) 71-290 (day 41-60)
CO_2-TR or $k_L a\, C^*_{CO_2}$ (mmol CO_2 L^{-1} h^{-1})	0.4 (day 0-41) 10-135 (day 41-60)
n_A (mmol CO_2 L^{-1} h^{-1})	0.26 (day 0-41)
Specific OER, P_o (mmol O_2 g^{-1} DCW h^{-1})	0.106 ± 0.021 (n=8, day 19-41)
Respiration rate, Q_o (mmol O_2 g^{-1} DCW h^{-1})	0.028 ± 0.013 (n=8, day 19-41)
Average pH for P_o and Q_o	8.94 ± 0.07 (n = 8) (day 19-41)
Peak CO_2 consumption rate (mmol CO_2 L^{-1} h^{-1})	0.34 (day 44)

Conclusions

The *Agardhiella* microplantlet culture can sustain active growth under 20% per day medium replacement for at least 50 days within a 500 mL stirred-tank bioreactor. For batch cultivation, the final plantlet size was related to the initial cell density. Medium replacement was required to look at long-term effects of agitation and CO_2 delivery on growth. When the microplantlet were cultivated at 0.3 vvm using ambient air as the aeration gas (n_A, of 0.26 mmol CO_2 L^{-1} h^{-1}), microplantlets reached CO_2 limited growth phase at the critical cell density of 3 g FW/L, and then continued a linear phase of CO_2-limited growth. The optimal pH environment for cultivation was centered around pH 8. Time constant analysis showed that all stirred-tank

cultivation experiments were well mixed. The impeller rotation rate did not affect the specific growth rate during the exponential phase of growth. However, the growth of microplantlets during linear CO_2-limited growth phase was modestly reduced at impeller rotation speeds higher than 140 rpm.

Chapter 6

SUMMARY AND CONCLUSIONS

This work describes the first successful development and bioreactor cultivation of a novel microplantlet suspension culture derived from an anatomically complex, macrophytic marine red alga. Bubble-column and stirred-tank photobioreactors with medium perfusion are suitable biomass production platforms for microplantlet suspension cultures derived from the macrophytic marine red alga *Agardhiella subulata*. Important findings from culture development and photobioreactor studies are presented below.

Cell and Tissue Culture Development

Callus induction and microplantlet regeneration. The reliable routes for the development of suspension cultures of macrophytic red algae of terete thallus morphology were achieved for biotechnology applications. Specifically, two axenic liquid suspension cultures were established for *Agardhiella subulata*, including an undifferentiated filament clump culture and a regenerated microplantlet culture. The filamentous clump culture was undifferentiated, consisting of tightly meshed, uniseriate filaments and some round cells at the core of each clump. The undifferentiated filament clumps were susceptible to regeneration, and could be maintained only under a narrow set of culture conditions that promoted proliferation of undifferentiated cell mass but not regeneration. Adventitious shoots could be initiated from the filament clumps by imposing a sub-lethal stress condition on filament clump. Agitation was a particularly sensitive factor. Regeneration was gently and reliably initiated at 24°C by continuously mixing a liquid suspension of 3-week-old filament clumps on an orbital shaker at 100 rev-min^{-1}.

Well plate and flask culture studies. The specific growth rate of the microplantlets was higher than the filament clumps in nonagitated well plate culture (4-6 % per day for microplantlets vs. 2-3 % per day for filament clumps) at 24 °C and 8-36 μmol photons•m^{2}•s^{-1} irradiance (10:14 LD photoperiod) when grown on ASP12 artificial seawater medium at pH 8.6-8.9 with 20 to 25% per day medium replacement. Oxygen evolution rate vs. irradiance measurements showed that relative to the filament clumps, microplantlets had a higher maximum specific oxygen evolution, but comparable respiration, compensation point, and light intensity at 63.2% of saturation (I_k). The microplantlet culture was more suitable for suspension culture development than the filament

clump culture because it was morphologically stable. Therefore, continued photobioreactor cultivation studies focused on the microplantlet suspension culture.

The optimal growth temperature for microplantlets in flask culture was 24 °C.

Photobioreactor Cultivation

Agardhiella subulata microplantlet suspension was successfully cultivated in both 2 L bubble-column and 500 mL stirred-tank photobioreactors. Limiting process parameters on biomass production, including macronutrient delivery, agitation intensity, light transfer, CO_2 transfer, pH, and growth morphology were assessed.

Macronutrient delivery under batch or medium perfusion cultivation. The cell density versus time data on a semi-log scale revealed that cultivation at a medium perfusion rate of 20% per day had two log phases of growth in either the 2 L bubble-column or the 500 mL stirred-tank photobioreactor. The initial phase of relatively high growth (10-14 % per day) occurred within the first 14 days of cultivation, followed by a slower but sustained growth phase (3-5% per day) for the remainder of the cultivation period. This observation was also reflected in the O_2 evolution vs. time curve, which had a sharp maximum within the first two weeks of growth. In contrast, no second log phase of growth was observed for 2.6% per day medium perfusion or batch cultivation experiments. Fresh medium perfusion also continuously removed the used medium to avoid potential build-up of metabolite wastes. In batch cultivation, the biomass production generally ceased within 30 days due to phosphate depletion. The specific growth rate was about 6% per day for batch culture. Medium perfusion prolonged active growth phase of the microplantlet culture and facilitated the study of long-term growth. With 1.0 g FW/L of inoculum, the biomass density under medium perfusion reached up to 25 g FW/L after 85 days of cultivation (6.3 g DCW/L, based on 25% solids content) without sample removal.

The two major macronutrients in ASP12 medium are nitrate and phosphate. Nitrogen and phosphorus are required elements for biomass production. Batch cultivation of microplantlets in ASP12 medium with 1X N and 10X P was nitrate limited. However, batch cultivation at 1X P and 10X N was phosphate limited. When nitrate concentration went below 0.2 mM, the stationary phase of growth was triggered, and biomass production ceased after nitrate depletion. Fed-batch addition of phosphate during the phosphate-limited cultivation experiment prolonged the growth phase, but not the specific growth rate. Medium perfusion or replacement rate of 20% per day prevented both nutrients from being depleted. The critical cell density for either N or P limitation under medium perfusion rate of 20% per day was above 30 g FW/L. The

biomass yield coefficients based on N and P limitation were 1.0 g DCW $mmol^{-1}$ N and 26 g DCW $mmol^{-1}$ P respectively. Therefore, the N:P composition in *Agardhiella* microplantlets was 26:1. This value was a bit lower than the typical N:P ratio of 30:1 in marine macroalgae (Atkinson and Smith, 1983), but almost identical to the N:P ratio in the ASP12 medium with 1X nitrate (i.e. 26.2 :1 in Table 3.1).

Mixing and agitation. The microplantlet suspension culture consists of multicellular tissue with a high solid content (20-30%) that requires an agitation intensity sufficient to prevent sedimentation and maintain a well-mixed environment. An aeration rate of 0.29 L air L^{-1} culture min^{-1} (Table 3.2) was sufficient to uniformly and individually suspend the microplantlets in the liquid culture in the 2-L bubble-column. In the 500 mL stirred-tank, impeller rotation rates of at least 60 rpm were needed. From time constant analysis, the stirred-tank bioreactor was well mixed. The impeller rotation speeds from 60 rpm to 250 rpm within the 500 mL stirred-tank bioreactor did not significantly affect the specific growth rate during the exponential phase of growth. However, the growth of microplantlets during the linear CO_2-limited growth phase was modestly reduced at impeller rotation speeds higher than 140 rpm. Impeller rotation rates higher than 250 rpm (tip speeds of 72 cm s^{-1}) are needed to determined the critical shear rate for the microplantlets under resource-saturated cultivation conditions.

Light transfer. The light intensity required to prevent light-limited growth was determined by the photosynthesis-light response curve (P-αI curve). The $P_o - \alpha I$ curves indicated that the microplantlets were not subject to photoinhibition at αI values below 134 μE m^{-2} s^{-1}. In the 2 L bubble-column perfusion bioreactor (I_0 = 43 μE m^{-2} s^{-1}, α=2), αI_k obtained from P_o - αI curves gradually increased from 25 to 37 μE m^{-2} s^{-1} over the cultivation period.

Light transfer limitations were addressed by comparing the mean light intensity (I_m) to the I_k of the microplantlet suspension. As the cell density increased with time, I_m decreased due to light attenuation. After 50 days cultivation, I_m decreased to 27 μE m^{-2} s^{-1} at 1100 mg DCW L^{-1} cell density. At this cell density, total incident light intensity (αI_0) of 86 μE m^{-2} s^{-1} provided 50% of light saturated growth. The specific O_2 respiration rates (Q_o) did not change significantly over the cultivation period. Specific Q_o values averaged over the 50 day cultivation period ranged from 0.015 to 0.025 mmol O_2 g^{-1} DCW h^{-1}, about 10% of the peak P_o values.

Photoperiod. The effects of illumination LD photoperiod (h light: h dark within a 24 h cycle) on biomass production was studied within a 250 mL bubble-column photobioreactor. The photobioreactor was maintained at saturation conditions with respect to light flux intensity (76 μmol photons m^{-2} s^{-1}), nutrient medium delivery (20% nutrient replacement per day), and CO_2 delivery (0.35 mmol CO_2 L^{-1} h^{-1}) to observe the cumulative effects of photodamage on the cell

density vs. time curve at photoperiods approaching continuous light. Biomass production was proportional to photoperiod at low fractional photoperiods ($\leq$ 10:14 LD), but high fractional photoperiods approaching continuous light ($\geq$ 20:4 LD) shut down biomass production. Biomass production was maximized at 16:8 LD. Cell density vs. time profiles under resource-saturated cultivation conditions were adequately described by a cumulative photodamage growth model, which coupled irreversible photodamage and photoperiod-based light saturation processes to the specific growth rate. The maximum specific growth rate in the absence of photodamage was 0.078 day^{-1}, whereas the apparent rate constant for photodamage ranged from 0.018 day^{-1} (10:14 LD) to 0.26 day^{-1} (24:0 LD), suggesting that photodamage was the true limiting factor to biomass production at photoperiods approaching continuous light.

CO_2 delivery. The bioreactor systems in the present study have relatively large k_La because of fine bubbles generated by spargers. Therefore, the CO_2 liquid-gas mass transfer rate (CO_2-TR) was often higher than the CO_2 delivery rate (n_A). For the 2 L bubble-column reactor, CO_2 delivery was determined by n_A. The growth of microplantlets cultivated in the 2 L bubble-column bioreactor was never CO_2-limited. However, the growth of microplantlets cultivated in the 500 mL stirred-tank bioreactor at 0.3 vvm using ambient air as the aeration gas reached CO_2 limited growth at 3 g FW/L. The culture continued a linear phase of CO_2-limited growth beyond the critical cell density. However, increasing CO_2 concentration in the aeration gas to 3500 ppm prolonged the exponential phase of growth by increasing the critical cell density for CO_2-limited growth.

The optimal culture pH was centered around pH 8, which required at least 0.0035 atm CO_2 partial pressure in the aeration gas. Therefore, addition of CO_2 in the aeration gas stream not only prevented CO_2-limited growth but also improved the specific growth rate by lowering the culture pH to the optimal range.

Growth morphology. A. subulata microplantlets appeared rigid during the bioreactor cultivation. The microplantlets in pneumatically agitated suspension culture did not break apart, and so the number cell density remained constant during the cultivation period. Consequently, chemostat cultivation with sterile feed will not be possible for this organism.

The morphology of individual *A. subulata* microplantlets consisted of branched shoot tissues emanating from a central core (Figure 3.3). At inoculation, the overall diameter of microplantlets containing trimmed shoot tissues was nominally 3-4 mm. After 50 days of resource-saturated cultivation, the overall microplantlet diameter increased to about 8-12 mm in both the bubble column and the stirred tank. In all cultivations, it was observed that the girth of the shoot tissues also increased with time, and active shoot tips formed new branches. For

microplantlets growing in a bubble-column bioreactor, shoots and their secondary branches tended to elongate freely, resulting in asymmetric shape. For microplantlets growing in the stirred tank bioreactor, linear shoots emanated from the central core, and the shape of the plantlets was spherical and compact. The physical contact of impeller with the plantlets within the stirred tank may have forced microplantlets to grow into this compact and spherical shape.

All of these observations show that the structured growth characteristics of the microplantlet will ultimately limit biomass production if all other process limiting factors are minimized. Therefore, shoot tissues must be periodically sectioned and trimmed to maintain high growth rates.

Future Work

The scope of this thesis was limited to develop a photosynthetic biomass production platform for marine red seaweed by using *A. subulata* as a model plant. Based on this study, there are three recommendations for future work.

1. Develop an intrinsic structured growth model for microplantlet suspension. This may first require an extensive observation of microplantlet growth in a well-defined flow field similar to the bioreactor cultivation conditions.
2. Determine the critical shear rate detrimental to the microplantlet growth. The importance of shearing actions increases with scale-up because agitation also provides the mass transfer needs to the cultured tissues. Growth inhibition rather than cell death should be the deciding factor in the selection of impeller and agitation speed.
3. Conduct secondary metabolite production study. This will include identification of the key compounds of interests in the metabolic pathway and development of the elicitation strategy to optimize the product production.

Successful completion of the future work can provide a generic approach for bioprocess development extendible to other macrophytic red algae with the morphology similar to *Agardhiella subulata*.

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APPENDICES

Appendix A

Protocols

Appendix A-1 Preparation of ASP12 artificial seawater medium

1. Prepare 100X P2 Metal stock solution in deionized distilled water. Stir the solution overnight before storing at 4°C. It consists of:

Component	Chemical formula	mg L^{-1}
Zinc chloride	$ZnCl_2$	10.9
Manganese chloride tetrahydrate	$MnCl_2 \cdot 4H_2O$	144
Cobalt chloride hexahydrate	$CoCl_2 \cdot 6H_2O$	4
Ferric chloride hexahydrate	$FeCl_3 \cdot 6H_2O$	49
Disodium Ethylenedinitrilo-tetraacetate	$Na_2EDTA \cdot 2H_2O$	900
Boric acid	H_3BO_3	1140

2. Prepare 100X S2 Metal Stock solution in deionized distilled water. Stir the solution overnight before storing at -20°C. It consists of

Component	Chemical formula	mg L^{-1}
Potassium bromide	KBr	1490
Strontium chloride hexahydrate	$SrCl_2 \cdot 6H_2O$	520
Rubidium chloride	RbCl	278
Lithium chloride	LiCl	122
Potassium Iodide	KI	1.3
Sodium molybdate (VI) dihydrate	$Na_2MoO_4 \cdot 2H_2O$	126

3. Prepare Vitamin solution. Store the solution in 20ml aliquot in a plastic vial at -20°C. It consists of

Component	Chemical formula	mg L^{-1}
B_{12}	$C_{63}H_{88}CoN_{14}O_{14}P$	0.2
Biotin	$C_{10}H_{16}N_2O_3S$	1
Thiamine-HCl	$C_{12}H_{18}N_4OSCl\text{-}HCl$	100

Appendix A-1, CONTINUED

4. Prepare artificial seawater base in deionized distilled water. It consists of

Component	Chemical formula	mg L^{-1}
Sodium chloride	NaCl	28,020
Potassium chloride	KCl	700
Magnesium chloride heptahydrate	$MgSO_4 \bullet 7H_2O$	7,000
Magnesium chloride hexahydrate	$MgCl_2 \bullet 6\ H_2O$	4,000
Calcium chloride dihydrate	$CaCl_2 \bullet 2H_2O$	400
Sodium Bicarbonate	$NaHCO_3$	840
Sodium Nitrate	$NaNO_3$	1,000
Sodium Glycerophosphate	$C_3H_7O_6PNa_2$	9.7
Nitriotriacetic acid	$C_6H_9NO_6$	100

Note: The phosphate source in the base medium has been changed to 54 mg/L of Sodium monophosphate ($NaH_2PO_4 \bullet H_2O$) since September 2000.

5. Mix the artificial seawater base for 24 h to equilibrate the liquid with dissolved CO_2 and O_2.
6. One hour before making ASP12 medium, thaw S2 Metal stock solution and Vitamin solution to room temperature.
7. Add 10 ml each of P2 and S2 stock solutions and 1.0 ml of the vitamin stock solution into one liter of the base medium.
8. Adjust the pH of the medium to 8.0 with either 0.1N NaOH or 0.1N HCl.
9. Sterile filter through 0.2μm filter (Nalgene #155-0020) connected with autoclaved 1000 mL Wheaton orange-capped bottle under vacuum within a laminar flow hood. It is advisable to filter sterilize ASP12 medium containing glycerophosphate within 48 hours; otherwise medium will easily clog the filter.
10. Store the sterilized medium at 4°C.

Appendix A-2 Maintenance and subculture of *Agardhiella* filament clumps in well-plate culture

Maintenance

1. Completely replace the medium every 4 days (replacement rate of 25% per day) to prevent nutrient starvation and wash away waste products.
2. To replace medium, take a 6-well culture plate containing cultures to the laminar flow hood.
3. Unwrap the seal and open the cap.
4. Use autoclaved 10-mL glass pipette to completely remove the spent medium from each well. Pipette the medium slowly to avoid disturbing the filament clumps resting in each well.
5. Use a clean sterilized pipette to add 8 mL of fresh ASP12 medium into each well.
6. Recap the well plate. Wrap and seal the well plate with Paraffin before returning it to the incubator.

Subculture

1. Filamentous cell mass was subcultured every two weeks. Clumps generally ranged from 6 mm to 12 mm in diameter before subculture.
2. Take a 6-well culture plate to the laminar flow hood.
3. Unwrap the seal and open the cap.
4. Transfer all the cell clumps using autoclaved forceps into a sterile petri dish with 50 mL of fresh ASP12 medium in it.
5. Cut each clump through center into four to six pieces with an autoclaved scalpel. Each subcultured clump contains a portion of the compact core cells and lightly-packed, uniseriate filaments.
6. Prepare a new sterile 6-well plate and write down the subculture date on it.
7. Use forceps to transfer and distribute each subcultured piece back to 6-well plate. Generally there are 8-10 piece for each well, a total of 50-60 pieces in one plate.
8. Wrap and seal the well plate with Paraffin before placing it back to the incubator.

Appendix A-3 Initiation of *Agardhiella* microplantlets from filament clumps

1. Place well-plate cultures of 18 and 30-day-old filament clumps on an orbital shaker.
2. Maintain the cultures at 24 °C in an air-conditioned room.
3. Set the orbital shaker to continuously mix at 100 rev•min^{-1} under 20 µmol photons•m^{-2}•s^{-1} cool white fluorescent light (10:14 LD photoperiod).
4. Replace ASP12 medium every 10 days. While pipetting the medium, remove any filament mass that has turned white or dead.
5. Within four weeks, one or more shoots were regenerated from the filament clumps. The shoots continued to develop, and grew to a nominal length of 8-10 mm after an additional three weeks.
6. Transfer 50 pieces of tissue mass bearing newly regenerated shoot tissue and some residual filament mass into a 250 mL Erlenmeyer flask containing 100 mL of filter-sterilized ASP12 medium. Cap the flask with a foam-stopper.
7. Place the regenerated microplantlet culture flasks at 24 °C under 36 µmol photons•m^{-2}•s^{-1} cool white fluorescent light (10:14 LD photoperiod) within an illuminated incubator.
8. Swirl the flasks once per day for 5 s to promote gas exchange.

Appendix A-4 Maintenance and subculture of *Agardhiella subulata* microplantlets in flask culture

Maintenance

1. The frequency of medium replacement for maintenance depends on the composition of ASP12 medium. When ASP12 medium containing 9.7 mg/L of glycerophosphate disodium salt are used, replace the medium every two weeks. When ASP12 medium containing 54.1 mg/L $NaHPO_4 \cdot H_2O$ are used, no medium replacement is needed between subculture.
2. Transfer selected 2-week-old flask culture into laminar flow hood.
3. Decant the medium carefully so that biomass is retained within 250 mL Erlenmeyer flask.
4. Prepare 100 ml of ASP12 medium in a graduate cylinder and pour it into flasks to re-suspend microplantlets.

Subculture

1. Pool selected flask cultures, generally 3 or 4 flasks, into a 500 mL Erlenmeyer flask.
2. Decant the culture medium till about 40 mL remaining.
3. Carefully pour the contents containing microplantlet suspension into a 90mm sterile petri dish.
4. Cover the microplantlet tissues with ASP12 medium during subculture.
5. Cut a given single microplantlet through its central branch point into two to four pieces with a sterile scalpel so that the excised tissues contain less than 10 shoots.
6. Trim the elongated shoots greater than 10 mm to 3-5 mm in length.
7. Pick out and discard those dying shoot tissues with light yellow or white color.
8. After subculture, pipette out the remaining medium in the petri dish.
9. Prepare eight autoclaved flasks, each filled with 100 mL fresh ASP12 medium.
10. Use sterilized forceps to transfer microplantlets from the petri dish to the flasks. Each flask contains about 50 microplantlets in 100 mL ASP12 medium.

Appendix A-5 P_0, Q_0 and *P-I* curve measurements with D.O. cell

P_0 and Q_0 measurements for filament clumps and microplantlets culture

1. Calibrate YSI model 58 dissolved oxygen (D.O.) meter and model 5750 D.O. electrode. The D.O. bottle contains at least 1 inch of D.I. water. Place YSI D.O. oxygen electrode onto the tapered port fitted for the electrode and wait for at least 20 minutes. Set D.O. concentration at 100%.
2. Place D.O. measurement cell on VWR model 310 stir plate.
3. Turn on the water circulation bath connected with the jacket of the D.O. cell and set the temperature at 24 °C.
4. Connect YSI model 58 D.O. meter to a computer data acquisition system.
5. Turn on the two adjustable horizontally-mounted 9 W fluorescent lamps positioned on the opposite sides of the D.O. cell. Adjust the distance from the lamp to the vessel surface to set the desired incident light intensity to the vessel.
6. Add 60 mL of aerated ASP12 medium and a 2.5 cm magnetic stir bar into the cell.
7. Set the stir plate at scale 6 or at a rate of at least 6.5 $rev \cdot s^{-1}$ (stir bar tip speed of 52 $cm \cdot s^{-1}$) to provide sufficient fluid convection over the surface of the D.O. electrode.
8. Insert the calibrated D.O. electrode in the electrode port. The medium should overflow during the electrode insertion to ensure no headspace or bubble within D.O. cell. Measure the D.O. concentration vs. time baseline in ASP12 medium without biomass tissues.
9. Decant the medium after each measurement. Re-aerate the used ASP12 medium and repeat the baseline measurement.
10. Weigh about 0.5 g of fresh cell mass to precision of ± 0.001 g.
11. Resuspend the fresh cell mass in a petri dish containing ASP12 medium. Evenly place the filament clumps between two 12 cm (width) by 3 cm (height) sheets of clear, 1 mm nylon mesh using forceps.
12. Roll up the mesh assembly and then insert into the vessel through the D.O. electrode port.
13. Repeat step 6 to 9 for the photosynthetic oxygen evolution rate P_0 measurements three times, preferably at 3 h into the light phase of the photoperiod.
14. Right after the P_0 measurements, turn off the lights of the two-sided illumination stage and the room.
15. Perform the oxygen respiration rate Q_0 measurements with the vessel wrapped in aluminum foil to eliminate light.

Appendix A-5, CONTINUED

16. The same procedure is applied to microplantlets except no immobilization is needed. Microplantlets is freely suspended in the medium during the D.O. measurements.

P_0 vs I_0 curve measurement

1. Measure the incident light intensity in units of μmol photons m^{-2} s^{-1} with a LI-COR 190SA PAR quantum sensor and LI-COR 189 quantum radiometer at selected illuminated stage positions.
2. Start P_0 measurement with biomass tissue at least 3 h into the light phase of photoperiod from the lowest given incident light intensity I_0.
3. Discard the spent medium after each measurement. Always use the same batch of fresh well-aerated ASP12 medium for all measurements.
4. Move the adjustable lamp stage closer to increase I_0 for next P_0 measurement. Duplicate measurements at each given light intensity.
5. Perform Q_0 measurements at the last after all the P_0 measurements.
6. Determine the dry cell mass at the end of the P_0 vs I_0 curve measurement.

Appendix A-6 Cultivation of *Agardhiella* microplantlets in a 2-L perfusion bubble-column photobioreactor: bioreactor startup & sampling, shutdown

2 L perfusion bubble-column bioreactor startup

1. Prepare 100mL of 1 M NaOH solution. Pour the solution into the bubble column. Aerate the NaOH solution with air to prevent it from penetrating through the frit for at least 30 minutes in order to form a hydrophilic layer on the grass frit sparger. Then rinse thoroughly in cold tap water, followed by a 3X final rinse with distilled water.
2. Completely reassemble headplate assembly. Attach tubings, air filters and humidifier assembly. Add 200 mL of DD H_2O to humidifier vessel prior to assembly. Stuff cotton into all tubing ends exposed to the open atmosphere before wrapping them with aluminum foil.
3. Autoclave bioreactor assembly at 121 °C, 205 kPa for 30 min. Also autoclave one 1 L graduate cylinder, forceps, scalpel, filtration cup unit, and humidifier. After autoclave, allow the bioreactor assembly to cool down to room temperature.
4. Pool microplantlets from 6 representative four-week-old flask cultures.
5. Perform the subculture procedure on the inoculum tissues.
6. Measure the fresh weight of inoculum and rinse the inoculum with sterilized ASP12 medium under sterile filtration.
7. Resuspend the microplantlets using 100 mL filter-sterilized ASP12 medium in a 250 mL autoclaved flask.
8. Place the bioreactor in a laminar flow hood. Clamp the aeration gas stream tubing, inlet medium port and outlet medium port tightly before the inoculation.
9. Load the tissue suspension and 1800 mL filter-sterilized ASP12 medium from the top of the bioreactor vessel to provide a total culture volume of 1900 mL. The suspension typically contained 1500-2000 microplantlets at a fresh cell density of 1.0-1.3 g FW L^{-1}.
10. Carefully transfer and secure the inoculated bioreactor assembly to the assigned experiment station.
11. Hook up a sterilized pre-calibrated pH electrode onto the headplate of the bioreactor.
12. Insert the thermocouple into bioreactor headplate thermowell, and turn on digital temperature meter.
13. Connect the inlet gas tubing with an autoclaved humidifier. Connect the humidifier with the inlet gas flow meter.
14. Turn on optional CO_2 gas tank and house air. Set both flow meters at desired flow rates.

Appendix A-6, CONTINUED

15. Turn on the water bath circulator and the illumination lamps.
16. Connect the medium inlet tubing with medium supply and outlet tubing to the spent medium flask. Turn on the peristaltic pump (Buchler 426-2000) set at 38.6 mL h^{-1} to provide 386 mL day^{-1} total fresh medium during the 10h light phase of the 10:14 LD photoperiod and medium perfusion rate of 20.3% per day.
17. To approximate a constant-volume batch cultivation (i.e. a net liquid medium perfusion rate of 2.6% per day), immediately after biomass sample removal (150 mL suspension every three days), the medium flowrate was set at 150 ml h^{-1} for 1 h to replace the 150 mL liquid volume removed by sampling.
18. For the batch cultivation experiments, liquid medium was not replaced after sampling and the total culture volume decreased proportionally to the cumulative amount of sample removal.

Sampling

1. Unclamp hose leading from sampling port.
2. Withdraw 150 ml from reactor using a syringe fitted on the sample receiver flask. Generally, each pool can withdraw 50 ml sample. The first pull will fill the sample in the sampling tube without receiving much samples in the receiver flask. Therefore, perform step 3 four times.
3. Pull the syringe quickly, clamp the tubing, detach the syringe, and push the piston of the syringe to the end, and reattach the syringe.
4. Remove the sample receiver flask, pour the sample into a graduate cylinder to measure the sample volume, and reattach the receiver flask back to the sampling assembly.

Shutdown

1. After taking the last sample, turn off inlet gas stream and clamp inlet gas tubing.
2. Disconnect pH electrode from the pH meter, and remove it from headplate.
3. Remove thermocouple from the thermowell. Detach the sampling assembly.
4. Remove headplate from bioreactor vessel.
5. Disconnect the water circulation tubings. Drain the water from the jacket into a flask.
6. Empty contents of bioreactor into a 2-L flask.
7. Measure the final volume and total fresh weight of the content if desired.
8. Clean the headplate and the glass vessel with Liquinox soap using clean bottlebrush.
9. Return and secure the glass vessel back on the experiment station.

Appendix A-6, CONTINUED

10. Pour 100 mL of bleach solution into the bubble column to clean and disintegrate the remaining tissue residue resting on the glass frit. Let the bleach work on the frit overnight.
11. Decant the bleach, rinse with DD water and replace with 100 mL of 1 N HCl. Let the acid works on the frit overnight.
12. Decant the HCl solution, rinse with DD water, and replace with 100 mL of 1 N NaOH. Let the base solution works on the frit for at least 1 hour.
13. Decant the base solution, rinse with DD water, and store the bioreactor for next use.

Appendix A-7 250 mL Bubble-column photobioreactor: startup, inoculation, operation & sampling, and shutdown

Start-up and inoculation

1. Prepare 50 mL of 1 M NaOH solution to clean and create a hydrophilic layer on the grass frit for fine bubbles. Aerate the NaOH solution with air to prevent it from penetrating through the frit for at least 30 minutes. Then rinse thoroughly in cold tap water, followed by a 3X final rinse with distilled water.
2. Wash glass reactor body and headplate with Liquinox detergent. Rinse thoroughly with DD water afterward.
3. Completely assemble headplate assembly. Attach tubings, air filters and assembly. Stuff cotton into all tubing ends exposed to the open atmosphere before wrapping them with aluminum foil.
4. Autoclave bioreactor assembly at 121 °C, 205 kPa for 30 min. Also autoclave one 250 mL graduate cylinder, forceps, scalpel, and filtration cup unit. After autoclave, allow the bioreactor assembly to cool down to room temperature.
5. Pool microplantlets from 2 representative four-week-old flasks.
6. Perform the subculture procedure on the inoculum tissues.
7. Measure the fresh weight of inoculum and rinse the inoculum with sterilized ASP12 medium under sterile filtration.
8. Re-suspend the microplantlet in 100 mL of filter-sterilized ASP12 medium in a 250 mL autoclaved flask.
9. Place bioreactor on the illumination stage in the laminar flow hood. Carefully remove headplate from reactor. Be sure to keep all surfaces in contact with the culture sterile.
10. Load the tissue suspension and 150 mL of filter-sterilized ASP12 medium from the top of the bioreactor vessel to provide a total culture volume of 250 mL.
11. Seal headplate to reactor body and carefully transfer to a experiment bench.
12. Connect the inlet gas tubing with the flow meter. Set flow meters at desired flow rates.
13. Turn on the illumination lamps. Set the timer at desired photoperiod.
14. Add an auxiliary fan (fix and secure in front of light stage using wire) to promote air circulation.

Appendix A-7, CONTINUED

Sampling

1. Turn off the water bath circulation, stirred plate and the aeration gas flow meter.
2. Calibrate pH meter using pH 7 buffers.
3. Unplug the lamp. Transfer the bioreactor along with the illumination stage to the laminar flow hood.
4. Wait till the biomass settle in the bottom of bioreactor. Carefully remove the headplate and pour about 150 ml of medium into a 250 ml flask for pH measurement.
5. Assemble the filter unit and hook up with the house air suction for fresh cell weight measurement.
6. Pour the remaining content (100 ml of culture + medium) into the filter cup to measure the total fresh cell weight.
7. Measure the pH value of the spent medium. Record the pH value.
8. Transfer the tissues using forceps back to the bubble column.
9. Using the graduate cylinder to measure 250mL of ASP12 fresh medium and load it into the reactor.
10. Close and seal the headplate and put the bubble column and light stage back on the experiment bench.
11. Turn the light and auxiliary fan back on.

Shutdown

1. Follow the sampling procedure to measure the last sample.
2. Clean the glass vessel with Liquinox soap using clean bottle brush.
3. Return and secure the glass vessel back on the experiment station in a flow hood.
4. Pour 30 mL of bleach solution into the glass vessel to clean and disintegrate the remaining tissue residue on the glass frit. Let the bleach work on the frit overnight.
5. Decant the bleach, rinse with DD water and replace with 100 mL of 1 N HCl. Let the acid work on the frit overnight (preferred) or at least 2 hours.
6. Decant the HCl solution, rinse with DD water, and replace with 100 mL of 1 N NaOH. Let the base work on the frit for at least 1 hour to create a hydrophilic layer.
7. Decant the base solution, rinse with DD water, and store the bioreactor for next use.

Appendix A-8 Light attenuation constant k_c measurements

1. Select a few representative microplantlet flask cultures with a total fresh weight of about 1.5 g. Pool the microplantlets into a petri dish. Trim them if necessary.
2. Place a ruler underneath the petri dish containing ASP12 medium and microplantlets. Use forceps to secure and place each plantlet right above the ruler. Measure and record the longest and shortest axes of the plantlet to the precision of 1 mm. Report the arithmetic average as the size (diameter) of the given plantlet.
3. Measure at least 40 plantlets to get a statistical average of plantlet size.
4. Add the trimmed microplantlets and 180 mL ASP12 medium to 96 mm glass culture dish with opaque sides. By calculation the culture depth (z) in culture dish should be 2.5 cm.
5. Place A fluorescent lamp bank directly above the culture dish, and a Li-Cor SA 190 PAR quantum sensor underneath the dish facing the lamp.
6. Measure the incident irradiance to the liquid suspension surface (I_o) from 15 to 152 $\mu E\ m^{-2}\ s^{-1}$ by moving the light source closer to the dish. Record the distance required for each incident irradiance.
7. At each incident light intensity, measure the average light intensity exiting the well-mixed culture suspension (I_z). Stir the medium to keep the culture suspended during the measurements. The measured I_z values will fluctuate a lot during measurements, so it is desirable to record at least twenty I_z values in the notebook and report the average value for a given I_o.
8. After a set of I_z vs. I_o is obtained, measure the total fresh weight in the culture dish.
9. Remove about 0.2 g of fresh weight from the previous experiment. Record the exact value and repeat step 4-7 to get another set of I_z vs. I_o data.
10. Repeated step 9 (i.e. decrease fresh cell mass ranging from 1.5 to 0.3 g) to obtain the dependence of k' on the biomass density.
11. The last measurement will be 180 mL of blank ASP12 medium in the culture dish.
12. Measure the dry cell weight of the microplantlets to obtain the solids content percentage of microplantlets.
13. At each fresh weight density, the light attenuation constant k' was estimated from the least squares slope of I_z vs. I_o data with the intercept forced to zero.

$$I_z = I_o\ e^{-k'z}$$

Appendix A-8, CONTINUED

11. Plot k' vs. X (dry cell density), the k_c will be the slope of the plot. The apparent light attenuation constant (k') is a linear function of cell density X

$$k' = k_o + k_c \cdot X$$

where k_c is the specific light attenuation constant of the biomass, and k_o is the light attenuation constant of the cell-free medium.

Appendix A-9 Mass transfer coefficient k_La measurements

1. The volumetric oxygen mass transfer coefficient (k_La) for interphase mass transfer of oxygen into cell-free liquid medium within the bubble-column and stirred-tank photobioreactor was determined by the dynamic gassing-in method at 24 °C.
2. Load the desired amount of the filter-sterilized ASP12 liquid medium in the vessel. Place the tip of the YSI model 5750 D.O. electrode at least 5 cm below the liquid surface.
3. Saturate the liquid medium with oxygen by sparging with ambient air for at least 15 minutes before the experiment. Allow the D.O. reading to establish a saturation baseline and record the value.
4. Turn on the nitrogen cylinder. Remove dissolved oxygen in the liquid medium by sparging with N_2 through the sparger.
5. When the D.O. concentration reached 1.0 % of O_2 saturation in air, turn off nitrogen. Air was re-introduced through the sparger at desired flow rates. For 500 mL Bellco stirred- tank, the flow rates range from 50 to 250 mL min^{-1} at a given impeller stirred rate to test the dependence of k_La on aeration rate. To test the dependence of k_La on impeller stirred rate, the stirred rate of impeller were set from 60 to 250 rpm during the re-aeration process under a given aeration rate.
6. To make D.O. concentration accurate, you may want to manually shake the electrode every 15 second during the re-aeration process to generate enough turbulence and avoid an oxygen depletion layer around the membrane. This is particularly necessary when aeration rate and/or impeller spinning rates are low.
7. The D.O. concentration vs. time data was recorded every 0.5 sec until the medium was saturated again with dissolved oxygen. The unsteady-state material balance on oxygen in the liquid phase is given by

$$\ln\left[\frac{C^*_{O_2} - C_{O_2,o}}{C^*_{O_2} - C_{O_2}}\right] = k_L a \cdot (t - t_o)$$

where $C^*_{O_2}$ is the D.O. concentration of the medium at air saturation, and $C_{O_2,o}$ is the D.O. concentration at the onset of re-aeration at time t_o.

8. The value for k_La was estimated from the least-squares slope of ln $C_{O_2,o}$ versus time data. The response time for the D.O. electrode is 5.2 sec and k_La values are corrected for the D.O. electrode response.

Appendix A-10 Cultivation of *Agardhiella subulata* microplantlets in Bellco 500 mL stirred-tank bioreactor: startup & inoculation, operation & sampling, shutdown

Start-up & inoculation

1. Carefully rinse and sonicate the spargers using 1 N NaOH solution for 30 minutes before assembling them onto the inlet gas tube.
2. One day before the startup, apply a layer of silicon to seal the gap between impeller paddle and the shaft. Allow the glue to harden and seal completely overnight.
3. Completely reassemble headplate assembly. Attach tubings, air filters and assembly. Stuff cotton into all tubing ends exposed to the open atmosphere before wrapping them with aluminum foil.
4. Autoclave bioreactor assembly at 121 °C, 205 kPa for 30 min. Also autoclave one 500 mL graduate cylinder, forceps, scalpel, and filtration cup unit. After autoclave, allow the bioreactor assembly to cool down to room temperature.
5. Pool microplantlets from 6 representative four-week-old flasks.
6. Perform the subculture procedure on the inoculum tissues.
7. Measure the fresh weight of inoculum and rinse the inoculum with sterilized ASP12 medium under sterile filtration.
8. Resuspend the microplantlet in 100 mL filter-sterilized ASP12 medium in a 250 mL autoclaved flask.
9. Place bioreactor in the laminar flow hood. Carefully remove one of the arm caps. Be sure to keep all surfaces in contact with the culture sterile.
10. Load the tissue suspension and 400 mL filter-sterilized ASP12 medium through the open arm of the bioreactor vessel to provide a total culture volume of 500 mL.
11. Carefully transfer the inoculated bioreactor assembly to the assigned stirrer unit.
12. Insert the thermocouple into bioreactor headplate thermowell, and turn on digital temperature meter.
13. Connect the inlet gas tubing with the flow meter. Set flow meters at desired flow rates.
14. Turn on the water bath circulator and the illumination lamps.

Operation & sampling

1. Turn off the water bath circulation, stirred plate and the aeration gas flow meter.
2. Calibrate pH meter using pH 7 buffers.

Appendix A-10, CONTINUED

3. Clamp the water circulation tubings (a total of 4) for both stirred tanks before removing the bioreactor from the experiment station.
4. Transfer the stir tank to a laminar flow hood. Wait till biomass settle in the bottom of bioreactor. Carefully remove one of the arm caps and pour about 350 ml medium into a 500 mL flask.
5. If necessary, save 2 and 20 mL aliquot of liquid medium for later nitrate and phosphate assays respectively. Label the bottle with run number, date, and saved liquid volume. Store the bottle in deep freezer.
6. Assemble the filter unit and hook it up with the house air suction for fresh cell weight measurement.
7. Pour the remaining content (150 ml of culture + medium) into the filter cup to measure the total fresh cell weight.
8. Measure the pH value of the spent medium. Record the pH value.
9. Transfer the tissues using forceps back to the stirred tank.
10. Using the graduate cylinder to measure 500mL ASP12 fresh medium and pour it into the stirred tank.
11. Close the cap and put the stirred tank back on the stirred plate.
12. Reconnect the stirred tank with inlet gas stream and water circulation.
13. Add distilled water to the water circulation bath to maintain the water level.

Shutdown

1. Follow the sampling procedure to measure the last sample.
2. Clean the glass vessel with Liquinox soap using clean bottlebrush.
3. To clean the stainless steel spargers, dissemble and put them in a 50 mL beaker filled with 20 mL of bleach. Sonicate the sparger for at least 1 hour. Rinse the spargers thoroughly with DD water and replace with 1 N HCl solution in the beaker. Sonicate the sparger again for at least one hour. Rinse the spargers thoroughly with DD water, replace with 1 N NaOH solution in the beaker, sonicate the sparger for at least another hour.
4. After three steps of sonication, rinse the sparger thoroughly with DD water before assembling them back on inlet gas tube.

Appendix A-11 Nitrate assay: LaMotte nitrate test kit (model NCR 3110)

1. Thaw a liquid sample of 2 mL to room temperature.
2. Turn on the spectrophotometer at least 30 minutes before the first measurement. Zero the instrument with DD water at 530 nm.
3. Pipette 1 mL thawed liquid sample and dilute it 100X in a measuring flask.
4. Fill one glass test tube to the 2.5 mL line with the diluted liquid sample.
5. Dilute to 5 mL line with Mixed Acid Reagent (V-6278). Cap and mix. Wait 2 minute.
6. Use the 0.1g spoon to add one level measure of Nitrate Reducing Reagent (V-6279). Use spatula to remove excess.
7. Replace the cap of Nitrate Reducing Reagent bottle right away to avoid moisture.
8. Cap and invert the test tube gently 50-60 times in one minute. Wait another 10 minute.
9. Pipette the first sample into the spectrophotometer cuvette. Measure the absorbance in the spectrophotometer and record in lab notebook.
10. Clean the cuvette and glass test tube with DD water between the measurements.
11. Repeat step 4-9 with second sample.

Appendix A-12 Phosphate assay: LaMotte phosphate (model 4408) and auxiliary phosphorus (model APT 7884) test kit

1. Thaw 20 mL frozen liquid sample to room temperature.
2. Turn on the spectrophotometer at least 30 minutes before the first measurement. Zero the instrument with DD water at 605 nm.
3. Measure 20 mL of each thawed liquid sample to precision of 1 mL in a graduate cylinder and pour in a 50 mL flask. Add 1.0 mL 36 wt% sulfuric acid using a 1 mL pipet. Swirl to mix.
4. Use the 0.5 g spoon to add one measure of Ammonium Persulfate. Swirl until the salt is completely dissolved.
5. Add 5-7 pieces of boiling stones into each flask. Place flasks on a hot plate (VWR model 310) in a flow hood. Turn on the heater and set at 10.
6. Boil the acidified solution gently for 30 minutes. Add DD water to the sample during the boiling step to maintain a volume at least 10 mL.
7. Permit the volume to decrease to approximately 10 mL (about 0.5 cm of sample) at the end of the boiling step but do not allow the sample to go to dryness or to dense white sulfur trioxide fumes.
8. Remove from hot plate and let them cool down to room temperature.
9. Pipette 1 mL of the thawed liquid sample and dilute it 100X in a measuring flask.
10. Add one drop of Phenolphthalein Indicator, 1% to the cooled sample. The sample should remain colorless.
11. While swirling flask, use a 1 mL pipet to add 15% Sodium Hydroxide dropwise until the solution turns faint pink. Generally, a volume of slightly over 3 mL is required.
12. While swirling flask, add 36% Sulfuric Acid, one drop at a time, until pink color disappears. In general, one drop is enough.
13. Add DD water to dilute the sample and bring the volume back to precisely 20 mL.
14. Apply LaMotte standard phosphorous test kit (model VM-12 4408) to measure the free phosphorus concentration.
15. First, fill the test tube (0230) to 5 mL mark.
16. Use the 1.0 mL pipet to add 1.0 mL of VM Phosphate Reagent. Cap the stopper and mix by inverting several times. Wait 5 minutes.
17. Add 3 drops of Reducing Reagent. Cap and mix. Record the time of addition. Blue color will develop within 10 seconds.
18. Wait 30 minutes from the time when Reducing Reagent is added.

Appendix A-12, CONTINUED

19. Pipette the first sample into the spectrophotometer cuvette. Measure the absorbance in the spectrophotometer and record in lab notebook.
20. Clean the cuvette and glass test tube with DD water between the measurements.
21. Repeat step 15-20 with second sample.

Appendix A-13 Cell density: dry cell weight, fresh cell weight, plantlet number density

Aseptic non-invasive fresh weight (FW) measurement for 500-mL stirred tank or 250-mL bubble column

1. Turn off the water bath circulation, stirred plate and the aeration gas flow meter.
2. Calibrate pH meter using pH 7 buffer.
3. Clamp the water circulation tubing before disconnection.
4. Transfer bioreactor assembly to laminar flow hood. Wait until the biomass settle in the bottom of bioreactor. For stirred tank, remove one of the arm caps and pour about 350 mL medium into a 500 mL flask through the side arm. For bubble column, disassemble the headplate and pour about 100 mL of medium into a 250 mL flask.
5. To determine FW, vacuum-filter the culture suspension on a 50 mm by 20 μm nylon mesh with a 42.5 mm Whatman No. 1 filter paper resting directly underneath the mesh to prevent the nylon mesh from retaining medium. Assemble the autoclaved filter unit and hook it up with the house air suction.
6. Pour the remaining content (150 mL of culture + medium) into the filter cup. After filtration, separate the unit cup from the filter flask.
7. Use forceps to transfer the nylon mesh (NM) along with tissue resting on it to a sterile petri dish (PD). Carefully collect any tissues left around the filter cup.
8. Weigh on the analytical balance. Record the FW+NM+PD value.
9. Measure the pH value of the spent medium. Record the pH value.
10. Transfer the petri dish back to the laminar flow hood. Transfer the tissues using forceps back to the bioreactor. Weight and record NM+PD value.
11. Use the graduate cylinder to measure 500 mL or 250 mL of fresh ASP12 medium and pour it into the bioreactor.
12. Close the cap or the headplate and place the bioreactor back to where it was.
13. Repeat step 4 to 13 for bioreactor #2.
14. Reconnect the stirred tank with inlet gas stream and water circulation. Turn on water circulation.

Appendix A-13, CONTINUED

Sampling of FW, DCW, ND (destructive FW measurement)

1. Immediately after the removal of the 150 mL culture samples, measure the sample volume using a graduate cylinder to precision of ±1 mL.
2. Decant 50 mL of liquid medium. Save aliquots of this supernatant liquid in a 25 mL plastic vial and store at -20 °C for later nitrate (2 mL sample) and phosphate (20 mL sample) concentration measurement.
3. After oxygen evolution rate measurements, pour the liquid suspension into a petri dish and count the total number of microplantlets in the sample.
4. Vacuum filter the remaining culture samples using a filter cup unit described at step 5 above.
5. Use forceps to transfer the nylon mesh (NM) along with tissue resting on it to a sterile petri dish (PD). Carefully collect any tissues left around the filter cup.
6. Gently blot the retained biomass and nylon mesh with a paper towel. Place them into a petri dish, and weigh to precision of ± 0.0001 g on an analytical balance.
7. Immediately after each FW measurement, transfer the microplantlet tissues with forceps onto an oven-dried, preweighed 47 mm x 0.45 μm filter (Millipore HWAP04700) and then dried at 80 °C for 24 h.
8. Weight the dried cell mass and filter on an analytical balance to precision of ± 0.1 mg. The FW and DCW values are determined by mass difference. Number cell density, fresh cell density, and dry cell density are determined from tissue number, FW, DCW and the original sample volume.

Appendix B

Spreadsheets

k_La measurements in the 500 mL Bellco spinner flask
Method: dynamic gassing-in (re-aeration data)
Impeller D_I 5.5 cm
Vessel diameter D_T 10.5 cm
Medium Volume 0.5 L
D.O. electrode: YSI 5750
D.O. electrode time constant: 5.16 sec or 698.0 +/- 5.4 hr^{-1}

Correlation STR#1
source files: 823c.xls; 823d.xls; 823e.xls; 823f.xls; 823g.xls

A	4483.30
B	8.48E-11
a	0.50
b	3.39

Sparger #1, cup-shape HPLC filter

Date	Aeration rate vvm	Aeration rate ml / min	Superfical gas velocity m / s	Impeller rate rpm	Re	Trial#1 k_La hr^{-1}	Trial#2 k_La hr^{-1}	Average k_La hr^{-1}	Std dev k_La hr^{-1}	Calibrated k_La hr^{-1}	Std dev k_La hr^{-1}	Correlation $Av^a+Bv^aRe^b$ hr^{-1}
8-25-00	0.1	50	9.62E-05	120	6759	38.3	46.5	42.4	5.80	45.1	6.2	52.8
	0.2	100	1.92E-04	120	6759	73.8	81.2	77.5	5.23	87.2	5.9	74.5
	0.5	250	4.81E-04	120	6759	99.0	103.2	101.075	2.99	118.2	3.6	117.6
	0.3	150	2.89E-04	120	6759	88.0	89.6	88.8	1.13	101.7	1.5	91.2
	0.3	150	2.89E-04	60	3380	65.0	66.3	65.65	0.92	72.5	1.2	78.6
	0.3	150	2.89E-04	180	10139	111.4	103.5	107.45	5.59	127.0	6.7	132.2
	0.3	150	2.89E-04	250	14082	183.8	179.4	181.6	3.11	245.5	4.6	244.4

Correlation STR#2
source files: 906a(-d).xls;911a(-d).xls

A	699.55
B	2.70E-03
a	0.51
b	1.52

Sparger #2, Stainless steel sparger of Aplikon STR

Date	Aeration rate vvm	Aeration rate ml / min	Superfical gas velocity m / s	Impeller rate rpm	Re	Trial#1 k_La hr^{-1}	Trial#2 k_La hr^{-1}	Average k_La hr^{-1}	Std dev k_La hr^{-1}	Calibrated k_La hr^{-1}	Std dev k_La hr^{-1}	Correlation $Av^a+Bv^aRe^b$ hr^{-1}
9-06-00	0.3	150	2.89E-04	120	6759	38.9	42.7	40.82	2.72	43.4	2.90	40.4
	0.3	150	2.89E-04	60	3380	19.2	19.1	19.16	0.08	19.7	0.18	21.5
	0.3	150	2.89E-04	180	10139	59.1	60.8	59.96	1.19	65.6	1.40	65.1
	0.3	150	2.89E-04	250	14082	98.1	95.3	96.68	1.99	112.2	2.47	100.0
9-11-00	0.3	150	2.89E-04	120	6759	41.9	36.4	39.16	3.90	41.5	4.15	40.4
	0.3	150	2.89E-04	60	3380	20.5	20.4	20.45	0.07	21.1	0.18	21.5
	0.3	150	2.89E-04	180	10139	63.4	53.9	58.67	6.75	64.1	7.38	65.1
	0.3	150	2.89E-04	250	14082	86.5	88.9	87.715	1.66	100.3	2.05	100.0
9-11-00	0.1	50	9.62E-05	180	10139	29.6	24.4	26.965	3.68	28.0	3.84	37.4
	0.2	100	1.92E-04	180	10139	37.4	32.1	34.735	3.70	36.6	3.90	53.1
	0.5	250	4.81E-04	180	10139	75.0	75.2	75.1	0.10	84.2	0.66	84.3
	1.1	529	1.02E-03	180	10139	83.2	83.1	83.165	0.04	94.4	0.73	123.2
	0.3	150	2.89E-04	180	10139	63.4	53.9	58.665	6.75	64.0	7.39	65.1
10-11-00	0.0254	13	2.44E-05	120	6759	5.0	6.20	5.6	0.85	5.6	0.86	11.6

Run Identification		**Culture Loading**		**Process Parameters**		**CO_2 growth model**	
Bioreactor Run#:	AS-STR-5	Culture:	A. subulata microplantlets	Air supply design:	Sparger #2 (Applikon)	R =	0.08206 L-atm/mol-K
Bioreactor Description:		Cell line I.D.	As-15-11,12,13,14 3/3/98	Air flow setting: 23 =	150 ml/ min	P_{CO2} =	3.50E-04 atm (air)
500 ml Bellco spinner flask		Age of Inoculum:	34 days	Air flow S/N	059360	$Y_{X/CO2}$ =	30.0 mg DCW $mmol^{-1}$ CO_2
Time started:	2:00 PM	Inoculum cell weight:	0.5617 g FW	Impeller design: flat-paddle Di =	5.5 cm	μ =	0.0756 day^{-1} (0-21 days)
		ASP12 medium volume:	500 mL	Impeller speed:	60 rpm	H =	0.0346 atm-L/mmol
Date started:	4-6-98	Initial cell density:	1.12 g FW/L	Setpoint temperature:	24 °C	k_La for O_2 =	21.5 h^{-1}
		Solid Content W_s	250 mg DCW/ g FW	Illuminater position:	10.5 cm from the center	n_A =	0.258 mmol CO_2 /L-hr
				Illumination intensity:	80 $\mu E/m^2.s$	$k_La C_A^*$ =	0.210 mmol CO_2 /L-hr
				Photoperiod :	10 hr ON / 14 hr OFF	X_c =	3.33 g FW/L
				mixing time t_{mix}	4.6 sec	t_c =	14.36 day
				mass transfer time t_{mt}	173.6 sec	f =	10 hr / day
				CO_2 consumption time t_{rxn}	173.6 sec, at X_c	measured β =	0.208 g FW / L-day (14-48 days)
				CO_2 consumption time t_{rxn}	54.5 sec, at X_f	theory β =	0.252 g FW / L-day

Sample Identification				Culture	Biomass Measurements						t_{rxn}	n_A	qco_2	Biomass Prediction	
Date	Run # Sample # Trial #	Cultivation Time (day:hr:min)	Cultivation Time (days)	pH	Sample Volume (mL)	pd + nm + cells (g)	pd + nm (g)	FW (g)	X (g FW/L)	LN(X)	(sec)	(mmol CO_2/L-hr)	(mmol CO_2/L-hr)	Cultivation Time (days)	X (g FW/L)
4-6-98	6-1-1	0- 2:00 pm	0.00	8.00	500	9.0210	8.4595	0.5615	1.12	0.12	514.5	0.210	0.071	0.00	1.12
4-13-98	6-2-1	7- 9:00 pm	7.29	8.93	500	9.4204	8.3797	1.0407	2.08	0.73	277.6	0.210	0.131	7.29	1.95
4-17-98	6-3-1	11- 6:30 pm	11.19	8.72	500	9.9370	8.4827	1.4543	2.91	1.07	198.6	0.210	0.183	11.19	2.62
4-22-98	6-4-1	16- 4:30 pm	16.10	8.81	500	10.0654	8.3753	1.6901	3.38	1.22	173.6	0.210	0.213	14.36	3.33
4-27-98	6-5-1	21- 4:00 am	21.08	8.85	500	11.4430	8.4384	3.0046	6.01	1.79	173.6	0.210	0.379	16.10	3.69
5-2-98	6-6-1	26- 6:00 pm	26.17	8.78	500	12.3376	8.4446	3.8930	7.79	2.05	173.6	0.210	0.491	21.08	4.73
5-7-98	*6-7-1	31-12:30 pm	30.94	8.88	500	12.7253	8.2360	4.4893	8.98	2.19	173.6	0.210	0.566	26.17	5.79
5-14-98	6-8-1	38- 11:00pm	38.38	8.85	500	13.0105	8.4576	4.5529	9.11	2.21	173.6	0.210	0.574	30.94	6.78
5-19-98	6-9-1	43- 1:30pm	42.94	8.81	500	13.5808	8.3419	5.2389	10.48	2.35	173.6	0.210	0.660	38.38	8.33
5-24-98	7-10-1	48- 2:00pm	47.96	8.84	500	13.7754	8.4711	5.3043	10.61	2.36	173.6	0.210	0.669	42.94	9.28
														47.96	10.32

Run Identification

Bioreactor Run#: AS-STR-6
Bioreactor Description:
500 ml Bellco spinner flask
Time started: 3:30 PM
Date started: 6-11-98

Culture Loading

Culture: A. subulata microplantlets
Cell line I.D. As-17-1,2,3,4 5 /1 / 98
Age of Inoculum: 41 days
Inoculum cell weight: 0.45 g FW
ASP12 medium volume: 500 mL
Initial cell density: 0.91 gFW/L
Solid Content W_s 0.25 g DCW / g FW

Process Parameters

Air supply design: Applikon sparger (#2)
Air flow setting: 23 = 150 ml/ min
Air flow S/N 059360
Impeller design: flat-paddle Di = 5.5 cm
Impeller speed: 120 rpm
Setpoint temperature: 24 °C
Illuminater position: 10.5 cm from the center
Illumination intensity: 80 µE/m^2.s
Photoperiod : 10 hr ON / 14 hr OFF
mixing time, t_{mix} 2.3 sec
mass transfer time t_{mt} 92.4 sec
CO_2 consumption time t_{rxn} 140.9 sec, at X_c
CO_2 consumption time t_{rxn} 22.0 sec, at X_f

CO_2 growth model

R = 0.08206 L-atm/mol-K
P_{CO2} = 3.50E-04 atm (air)
$Y_{X/CO2}$ = 30.0 mg DCW mmol^{-1} CO_2
μ = 0.129 day^{-1} 0-15 days
H = 0.0346 atm-L/mmol
k_La for O_2 = 40.4 h^{-1}
n_A = 0.2584 mmol CO_2 /L-hr
$k_La C_A^*$ = 0.394 mmol CO_2 /L-hr
X_c = 2.40 g FW/L
t_c = 7.54 day
f = 10 hr / day
measured β = 0.3514 g FW / L-day 5-45 days
theory β = 0.3100 g FW / L-day

Sample Identification				Culture	Biomass Measurements						t_{rxn}	n_A	qco_2	Biomass Prediction	
Date	Run # Sample # Trial #	Cultivation Time (day:hr:min)	Cultivation Time (days)	pH	Sample Volume (mL)	pd + nm + cells (g)	pd + nm (g)	FW (g)	X (g FW/L)	LN(X)	(sec)	(mmol CO_2/L-hr)	(mmol CO_2/L-hr)	Cultivation Time (days)	X (g FW/L)
6-11-98	7-1-1	0- 3:30 pm	0.00	8.00	500	8.9706	8.5162	0.4544	0.91	-0.10	372.8	0.258	0.098	0.00	0.91
6-17-98	7-2-1	6- 1:00 am	5.40	9.02	500	9.3658	8.2522	1.1136	2.23	0.80	152.1	0.258	0.239	5.40	1.82
6-21-98	7-3-1	10- 3:00 pm	10.02	8.92	500	10.8789	8.8920	1.9869	3.97	1.38	140.9	0.258	0.427	7.54	2.40
6-26-98	7-4-1	15- 3:00 am	15.02	8.86	500	11.5390	8.3692	3.1698	6.34	1.85	140.9	0.258	0.682	10.20	3.34
7-3-98	7-5-1	22- 9:00 am	21.75	8.85	500	12.0567	8.4470	3.6097	7.22	1.98	140.9	0.258	0.776	15.02	5.03
7-7-98	7-6-1	26- 6:00 pm	26.13	8.85	500	13.0852	8.0916	4.9936	9.99	2.30	140.9	0.258	1.074	21.75	7.40
7-11-98	7-7-1	30-1:00 pm	29.92	8.92	500	14.4646	8.2714	6.1932	12.39	2.52	140.9	0.258	1.332	26.13	8.93
7-16-98	7-8-1	35- 4:00pm	35.04	9.10	500	16.1104	9.0297	7.0807	14.16	2.65	140.9	0.258	1.522	29.92	10.27
7-21-98	7-9-1	40- 1:30pm	39.94	9.10	500	12.3693	8.3419	4.0274	14.30	2.66	140.9	0.258	1.538	35.04	12.07
7-26-98	7-10-1	45- 2:00pm	44.96	8.83	500	24.673	16.98	7.6930	15.39	2.73	140.9	0.258	1.654	39.94	13.79
	7-9-2					11.3194	8.1952	3.1242						44.96	15.55

Run Identification

Bioreactor Run#: AS-STR-7a
Bioreactor Description:
500 ml Bellco spinner flask
Time started: 10:00 PM
Date started: 7-28-98

Culture Loading

Culture: A. subulata microplantlets
Cell line I.D. As3-19-5,6,7 7/ 12 / 98
Age of Inoculum: 16 days
Inoculum cell weight: 0.45 g FW
ASP12 medium volume: 500 mL
Initial cell density: 1.13 g FW/L
Solids content W_s = 0.25 g FW/gDCW

Process Parameters

Air supply design: sparger #1 (HPLC filter cup)
Air flow setting: 65 = 150 ml/ min
Air flow S/N 052530
Impeller design: flat-paddle Di = 5.5 cm
Impeller speed: 140 rpm
Setpoint temperature: 24 °C
Illuminater position: 10.5 cm from the center
Illumination density: 80 µE/m^2.s
Photoperiod : 10 hr ON / 14 hr OFF
mixing time t_{mix} 2.0 sec
mass transfer time t_{mt} 37.1 sec
CO_2 consumption time t_{rxn} 140.9 sec, at X_c
CO_2 consumption time t_{rxn} 44.8 sec, at X_f

CO_2 growth model

R = 0.08206 L-atm/mol-K
P_{CO2} = 3.50E-04 atm (air)
$Y_{X/CO2}$ = 30.0 mg DCW mmol^{-1} CO_2
μ = 0.119 day^{-1} 0-10 days
H = 0.0346 atm-L/mmol
$k_L a$ for O_2 = 100.7 h^{-1}
n_A = 0.258 mmol CO_2 /L-hr
$k_L a C_A^*$ = 0.982 mmol CO_2 /L-hr
X_c = 2.61 g FW/L
t_c = 7.06 day
f = 10 hr / day
measured β = 0.1457 g FW / L-day 5-45 days
theory β = 0.3100 g FW / L-day

Sample Identification				Culture	Biomass Measurements						t_{rxn}	n_A	qco_2	Biomass Prediction	
Date	Run # Sample # Trial #	Cultivation Time (day:hr:min)	Cultivation Time (days)	pH	Sample Volume (mL)	pd + nm + cells (g)	pd + nm (g)	FW (g)	X (g FW/L)	LN(X)	sec	(mmol CO_2/L-hr)	(mmol CO_2/L-hr)	Cultivation Time (days)	X (g FW/L)
7-28-98	7-1-1	0- 10:00 pm	0.00	8.00	500	8.8562	8.2920	0.5642	1.13	0.12	326.0	0.258	0.112	0.00	1.13
8-2-98	7-2-1	5- 1:00 pm	4.63	8.74	500	9.1980	8.2408	0.9572	1.91	0.65	192.1	0.258	0.190	4.63	1.95
8-7-98	7-3-1	10- 3:30 pm	9.73	8.86	500	10.1027	8.3111	1.7916	3.58	1.28	140.9	0.258	0.355	7.06	2.61
8-12-98	7-4-1	15- 3:00 am	14.71	8.86	500	10.6352	8.3416	2.2936	4.59	1.52	140.9	0.258	0.454	14.71	3.72
8-17-98	7-5-1	20- 9:00 pm	19.96	8.85	500	11.8567	8.9567	2.9000	5.80	1.76	140.9	0.258	0.574	19.96	4.49
8-21-98	7-6-1	24- 1:00 pm	23.63	8.80	500	20.3406	17.0123	3.3283	6.66	1.90	140.9	0.258	0.659	23.63	5.02
8-27-98	7-7-1	30-1:00 pm	29.63	8.78	500	20.5386	17.0109	3.5277	7.06	1.95	140.9	0.258	0.698	29.63	5.90
9-2-98	7-8-1	36- 0:30am	35.10	8.81	500	20.7553	17.0606	3.6947	7.39	2.00	140.9	0.258	0.732	35.10	6.70
9-7-98	7-9-1	40- 1:30pm	39.94	9.10	500	11.5830	8.0175	3.5655	8.20	2.10	140.9	0.258	0.812	39.94	7.40
	7-9-2					8.5537	8.0175	0.5362							

Run Identification

Bioreactor Run#: AS-STR-7b
Bioreactor Description:
500 ml Bellco spinner flask
Time started: 10:00 PM
Date started: 7-28-98

Culture Loading

Culture: A subulata microplantlets
Cell line I.D. As3-19-5,6,7 7/ 12 / 98
Age of Inoculum: 16 days
Inoculum cell weight: 0.45 g FW
ASP12 medium volume: 500 mL
Initial cell density: 0.99 g/L
W_s = 250 mg DCW/gFW

Process Parameters

Air supply design: Applikon sparger (#2)
Air flow setting: 23 = 150 ml/ min
Air flow S/N 059360
Impeller design: flat-paddle Di = 5.5 cm
Impeller speed: 250 rpm
Setpoint temperature: 24 °C
Illuminater position: 10.5 cm from the center
Illumination density: 80 µE/m^2-s
Photoperiod : 10 hr ON / 14 hr OFF
mixing time, t_{mix} 1.1 sec
mass transfer time t_{mt} 37.3 sec
CO_2 consumption time t_{rxn} 140.9 sec, at X_c
CO_2 consumption time t_{rxn} 74.6 sec, at X_f

CO_2 growth model

R = 0.08206 L-atm/mol-K
P_{CO2} = 3.50E-04 atm (air)
$Y_{X/CO2}$ = 30.0 mg DCW mmol^{-1} CO_2
μ = 0.101 day^{-1} 0-10 days
H = 0.0346 atm-L/mmol
k_La for O_2 = 100 h^{-1}
n_A = 0.258 mmol CO_2 /L-hr
$k_LaC_A^*$ = 0.976 mmol CO_2 /L-hr
X_c = 3.06 g FW/L
t_c = 11.14 day
f = 10 hr / day
measured β = 0.1139 g FW / L-day 5-45 days
theory β = 0.3100 g FW / L-day

Sample Identification				Culture	Biomass Measurements						t_{rxn}	n_A	qco_2	Biomass Prediction	
Date	Run # Sample # Trial #	Cultivation Time (day:hr:min)	Cultivation Time (days)	pH	Sample Volume (mL)	pd + nm + cells (g)	pd + nm (g)	FW (g)	X (g FW/L)	LN(X)	(sec)	(mmol CO_2/L-hr)	(mmol CO_2/L-hr)	Cultivation Time (days)	X (g FW/L)
7-28-98	7-1-1	0- 10:00 pm	0.00	8.00	500	8.7798	8.2848	0.4950	0.99	-0.01	435.7	0.258	0.084	0.00	0.99
8-2-98	7-2-1	5- 1:00 pm	4.63	8.75	500	9.0375	8.2600	0.7775	1.56	0.44	277.4	0.258	0.131	4.63	1.58
8-7-98	7-3-1	10- 3:30 pm	9.73	8.81	500	9.7262	8.3082	1.4180	2.84	1.04	152.1	0.258	0.239	9.73	2.65
8-12-98	7-4-1	15- 3:00 am	14.71	8.86	500	10.0481	8.3413	1.7068	3.41	1.23	140.9	0.258	0.288	11.14	3.06
8-17-98	7-5-1	22- 9:00 am	19.96	8.85	500	11.0567	8.9567	2.1000	4.20	1.44	140.9	0.258	0.355	15.70	3.58
8-21-98	7-6-1	24- 1:00 pm	23.63	8.85	500	11.0572	8.6770	2.3802	4.76	1.56	140.9	0.258	0.402	19.96	4.06
8-27-98	7-7-1	30-1:00 pm	29.63	8.81	500	11.0107	8.3320	2.6787	5.36	1.68	140.9	0.258	0.452	23.63	4.48
9-2-98	7-8-1	36- 0:30am	35.10	8.85	500	11.9252	8.6920	3.2332	6.47	1.87	140.9	0.258	0.546	29.63	5.17
9-7-98	7-9-1	40- 1:30pm	39.94	9.10	500	11.2299	8.3399	2.8900	5.78	1.75	140.9	0.258	0.488	35.10	5.79
														39.94	6.34

Run Identification

Bioreactor Run#: AS-STR-8a
Bioreactor Description:
500 ml Bellco spinner flask
Time started: 11:00 PM
Date started: 9-9-98

Culture Loading

Culture: A. subulata microplantlets
Cell line I.D. As3-20-2,8,9 8 /6/ 98
Age of Inoculum: 33 days
Inoculum cell weight: 1.53 g FW / L
ASP12 medium volume: 500 mL
Initial cell density: 1.53 g FW/L
Solid content Ws 250 mgDCW/gFW

Process Parameters

Air supply design: sparger#1(HPLC filter cup)
Air flow setting: 23 = 150 ml/ min
Air flow S/N 059360
Impeller design: flat-paddle D_i = 5.5 cm
Impeller speed: 140 rpm
Setpoint temperature: 24 °C
Illuminater position: 10.5 cm from the center
Illumination intensity: 80 µE/m^2.s
Photoperiod : 10 hr ON / 14 hr OFF
mixing time t_{mix} 2.0 sec
mass transfer time t_{mt} 37.1 sec
CO_2 consumption time t_{rxn} 140.9 sec
CO_2 consumption time t_{rxn} 105.7 sec

CO_2 growth model

R = 0.08206 L-atm/mol-K
P_{CO2} = 3.50E-04 atm (air)
$Y_{X/CO2}$ = 30.0 mg DCW mmol^{-1} CO_2
μ_1 = 0.0657 day^{-1} (0-10 days)
μ_2 = 0.0284 day^{-1} (10-50 days)
H = 0.0346 atm-L/mmol
k_La for O_2 = 100.7 h^{-1}
n_A = 0.2584 mmol CO_2 /L-hr
$k_LaC_A^*$ = 0.982 mmol CO_2 /L-hr
X_c = 10.91 g FW/L
t_c = 56.43 day
measured β = 0.2209 gFW/L-day 56-91 days
theory β = 0.3100 gFW/L-day theory

Sample Identification				Culture	Biomass Measurements						t_{rxn}	n_A	qco_2	Biomass Prediction	
Date	Run # Sample # Trial #	Cultivation Time (day:hr:min)	Cultivation Time (days)	pH	Sample Volume (mL)	pd + nm + cells (g)	pd + nm (g)	FW (g)	X (g FW/L)	LN(X)	sec	(mmol CO_2/L-hr)	(mmol CO_2/L-hr)	Cultivation Time (days)	X (g FW/L)
9-9-98	8-1-1	0- 11:00 pm	0.00	8.00	500	9.1089	8.3422	0.7667	1.53	0.43	434	0.258	0.084	0.00	1.53
9-15-99	8-2-1	6- 12:30 pm	5.56	8.69	500	9.0250	8.0062	1.0188	2.04	0.71	326	0.258	0.112	5.56	2.20
9-20-99	8-3-1	11- 3:00 pm	9.67	8.86	500	9.6990	8.2390	1.4600	2.92	1.07	228	0.258	0.160	9.67	2.89
9-25-98	8-4-1	16- 12:00 pm	15.54	8.86	500	9.9352	8.3016	1.6336	3.27	1.18	471	0.258	0.077	15.54	3.41
9-30-98	8-5-1	21- 1:30 pm	20.60	8.86	500	9.9530	8.1917	1.7613	3.52	1.26	436	0.258	0.083	20.60	3.94
10-5-98	8-6-1	26- 7:00 pm	25.83	8.78	500	10.6220	8.1768	2.4452	4.89	1.59	314	0.258	0.116	25.83	4.57
10-10-98	8-7-1	31-12:30 pm	30.56	8.73	500	10.8113	8.1162	2.6951	5.39	1.68	285	0.258	0.128	30.56	5.23
10-15-98	8-8-1	36- 2:00pm	35.63	8.79	500	11.4130	8.3180	3.0950	6.19	1.82	248	0.258	0.147	35.63	6.04
10-20-98	8-9-1	41- 11:30 pm	41.02	8.77	500	11.5768	7.9954	3.5814	7.16	1.97	215	0.258	0.170	41.02	7.04
10-26-98	8-10-1	47- 4:00 pm	46.71	8.79	500	21.5435	17.3340	4.2095	8.42	2.13	183	0.258	0.199	46.71	8.27
10-30-98	8-11-1	51- 5:00 pm	50.75	8.88	500	21.7440	17.5014	4.2426	8.49	2.14	181	0.258	0.201	50.75	9.28
11-4-98	8-12-1	56- 12:00 pm	56.54	8.82	500	21.7786	17.0100	4.7686	9.54	2.26	161	0.258	0.226	56.43	10.91
11-9-98	8-13-1	60- 12:30 pm	60.60	8.91	500	22.8135	17.1987	5.6148	11.23	2.42	141	0.258	0.266	60.60	11.83
11-15-98	8-14-1	67- 12:00 am	66.04	8.76	500	23.3330	16.7691	6.5639	13.13	2.57	141	0.258	0.311	66.04	13.03
11-19-98	8-15-1	70- 12:00 pm	70.58	8.69	500	24.0405	17.4468	6.5937	13.19	2.58	141	0.258	0.312	70.58	14.03
11-24-98	8-16-1	76- 1:00 pm	75.58	8.97	500	25.0707	16.8770	8.1937	16.39	2.80	141	0.258	0.388	75.58	15.14
11-29-98	8-17-1	81- 12:30 pm	80.56	8.85	500	25.1900	17.3900	7.8000	15.60	2.75	141	0.258	0.369	80.56	16.24
12-4-98	8-18-1	85- 3:30 pm	85.69	8.98	500	25.5181	17.5440	7.9741	15.95	2.77	141	0.258	0.378	85.69	17.37
12-9-98	8-19-1	91- 3:00 pm	90.67	8.95	500	26.2801	17.4230	8.8571	17.71	2.87	141	0.258	0.420	90.67	18.47

Run Identification

Bioreactor Run#: AS-STR-8b

Bioreactor Description:

100 ml Bellco spinner flask

Time started: 10:00 PM

Date started: 7-28-98

Culture Loading

Culture: A subulata microplantlets

Cell line I.D. As-19-1,2,3,4 7 /1 / 98

Age of Inoculum: 28 days

Inoculum cell weight: 0.4012 g FW

ASP12 medium volume: 500 mL

Initial cell density: 1.32 g FW/L

Solid content Ws 250 mgDCW/gFW

Process Parameters

Air supply design: sparger#2

Air flow setting: 23 = 150 ml/ min at 21°C

Air flow S/N 026947

Impeller design: flat-paddle D_i = 5.5 cm

Impeller speed: 250 rpm

Setpoint temperature: 24 °C

Illuminater position: 10.5 cm from the center

Illumination intensity: 80 µE/m^2.s

Photoperiod : 10 hr/24 hrs

mixing time t_{mix} 1.1 sec

mass transfer time t_{mt} 37.3 sec

CO_2 consumption time t_{rxn} 140.9 sec, at X_c

CO_2 consumption time t_{rxn} 106.2 sec, at X_f

CO_2 growth model

R = 0.08206 L-atm/mol-K

P_{CO2} = 3.50E-04 atm (air)

$Y_{X/CO2}$ = 30.0 mg DCW mmol^{-1} CO_2

μ_1 = 0.0695 day^{-1} 0-10 days

μ_2 = 0.0281 day^{-1} 10-45 days

H = 0.0346 atm-L/mmol

k_La for O_2 = 100 h^{-1}

n_A = 0.2584 mmol CO_2 /L-hr

$k_La C_A^*$ = 0.976 mmol CO_2 /L-hr

X_c = 11.05 g FW/L

t_c = 61.38 day

measured β = 0.1531 gFW/L-day

theory β = 0.3100 gFW/L-day

Sample Identification				Culture	Biomass Measurements						t_{rxn}	n_A	qco_2	Biomass Prediction	
Date	Run # Sample # Trial #	Cultivation Time (day:hr:min)	Cultivation Time (days)	pH	Sample Volume (mL)	pd + nm + cells (g)	pd + nm (g)	FW (g)	X (g FW/L)	LN(X)	(sec)	(mmol CO_2/L-hr)	(mmol CO_2/L-hr)	Cultivation Time (days)	X (g FW/L)
9-9-98	8-1-1	0- 11:00 pm	0.00	8.00	500	8.9896	8.3288	0.6608	1.32	0.28	475.8	0.258	0.077	0.00	1.32
9-15-99	8-2-1	6- 12:30 pm	5.56	8.84	500	8.9144	8.0070	0.9074	1.81	0.60	346.5	0.258	0.105	5.56	1.95
9-20-99	8-3-1	11- 3:00 pm	9.67	8.81	500	9.5345	8.2312	1.3033	2.61	0.96	241.2	0.258	0.151	9.67	2.59
9-25-98	8-4-1	16- 12:00 pm	15.54	8.85	500	9.7481	8.3413	1.4068	2.81	1.03	553.4	0.258	0.066	15.54	3.05
9-30-98	8-5-1	21- 1:30 pm	20.60	8.82	500	9.8222	8.2127	1.6095	3.22	1.17	483.7	0.258	0.075	20.60	3.52
10-5-98	8-6-1	26- 7:00 pm	25.83	8.78	500	9.8403	8.1212	1.7191	3.44	1.23	452.9	0.258	0.080	25.83	4.07
10-10-98	8-7-1	31-12:30 pm	30.56	8.81	500	10.5141	8.1806	2.3335	4.67	1.54	333.6	0.258	0.109	30.56	4.65
10-15-98	8-8-1	36- 2:00pm	35.63	8.66	500	10.9798	8.2882	2.6916	5.38	1.68	289.2	0.258	0.126	35.63	5.36
10-20-98	8-9-1	41- 11:30 pm	41.02	8.00	500	10.9679	8.0152	2.9527	5.91	1.78	263.7	0.258	0.138	41.02	6.24
10-26-98	8-10-1	47- 4:00 pm	46.71	8.02	500	11.6142	8.2030	3.4112	6.82	1.92	228.2	0.258	0.160	46.71	7.32
10-30-98	8-11-1	51- 5:00 pm	50.75	8.05	500	11.8592	8.1122	3.7470	7.49	2.01	207.8	0.258	0.175	50.75	8.20
11-4-98	8-12-1	56- 12:00 pm	56.54	8.00	500	12.8435	8.5600	4.2835	8.57	2.15	181.7	0.258	0.200	56.54	9.64
11-9-98	8-13-1	60- 12:30 pm	60.60	7.98	500	12.8155	7.9327	4.8828	9.77	2.28	159.4	0.258	0.228	61.38	11.05
11-15-98	8-14-1	67- 12:00 am	66.04	8.06	500	14.0967	8.3837	5.7130	11.43	2.44	136.3	0.258	0.267	66.04	11.76
11-19-98	8-15-1	70- 12:00 pm	70.58	7.96	500	23.5392	17.4228	6.1164	12.23	2.50	127.3	0.258	0.286	70.58	12.46
11-24-98	8-16-1	76- 1:00 pm	75.58	8.78	500	23.6403	16.9291	6.7112	13.42	2.60	116.0	0.258	0.314	75.58	13.22
11-29-98	8-17-1	81- 12:30 pm	80.56	8.80	500	24.1669	17.3264	6.8405	13.68	2.62	113.8	0.258	0.320	80.56	13.98
12-4-98	8-18-1	85- 3:30 pm	85.69	8.90	500	24.4694	17.4550	7.0144	14.03	2.64	111.0	0.258	0.328	85.69	14.77
12-9-98	8-19-1	91- 3:00 pm	90.67	8.90	500	24.7557	17.4230	7.3327	14.67	2.69	106.2	0.258	0.343	90.67	15.53

Run Identification

Run#:	AS-STR-9
STR #	#1
Bioreactor Description:	
500 mL Bellco spinner flask	
Time started:	11:00 PM
Date started:	5-12-99

Culture Loading

Culture:	*Agardhiella subulata* microplantlets		
Cell line I.D.	As3-29-1,2,3,4	4/19/99	
Age of inoculum:		23	days
Inoculum cell weight:		0.4682	g FW
Initial ASP12 medium volume:		500.0	mL
Initial cell density:		0.94	g FW/L
$NaNO_3$ conc. :		1.00	g/L
$NaHCO_3$ conc. :		0.84	g/L
Average pH (day 5-30):		8.75 +/-	0.04
Average pH (day 35-85):		7.88 +/-	0.09
Medium replacement:		100	% per sample
Solid content Ws		250	mgDCW/gFW

Process Parameters

Sparger:	Stainless steel filter #2		
Air flowmeter S/N:	26947 Setting: 22		
Air flowrate:	150 mL/ min (day 0-30)	290 mL/min (day 30-85)	
CO_2 flowmeter S/N:	082623 Setting: 0	Setting: 10	
CO_2 flowrate:	0 mL/ min (day 0-30)	2 mL/min (day 30-85)	
Total flowrate:	150 mL/ min (day 0-30)	292 mL/min (day 30-85)	
CO_2 partial pressure:	3.50E-04 atm (day 0-30)	0.0072 atm (day 30-85)	
Impeller design:	Two blade flat-paddle (d_i = 5.5 cm, H = 2.5 cm)		
Stir plate # / setting	VWR 310 Setting: 2.00		
Impeller speed:	120 rpm		
Setpoint temperature:	24 °C		
Lamp position:	10.2 cm from the center		
Incident illumination inter	80 µE/m^2-sec		
photoperiod	10 hr ON / 14 hr OFF		
mixing time t_{mix}	2.3 sec		
mass transfer time t_m	40.9 sec	0-30 days	
CO_2 consumption time t_{rm}	n.a. sec	at X_c	
CO_2 consumption time t_{rm}	62.0 sec	at X_f	

CO_2 growth model

R =	0.08206	L-atm/mol-K	
P_T =	1	atm	
P_{CO2} =	3.50E-04	atm (air)	
$Y_{X/CO2}$ =	30.0	mg DCW mmol^{-1} CO_2	
μ_1 =	0.0781	day^{-1}	(0-10 days)
μ_2 =	0.028	day^{-1}	
H =	0.0346	atm-L/mmol	
k_La =	91.2	h^{-1}	0-30 days
k_La =	127.1	h^{-2}	30-85 days
n_A =	0.258	mmol CO_2 /L-hr	0-30 days
n_A =	10.3420	mmol CO_2 /L-hr	30-85 days
$k_LaC_A^*$ =	0.890	mmol CO_2 /L-hr	0-30 days
$k_LaC_A^*$ =	25.495	mmol CO_2 /L-hr	30-85 days
X_c =	n.a.	g FW/L	
t_c =	n.a.	day	

Sample Identification				Culture	Biomass Measurements						OER	t_{rm}	n_A	qco_2	Biomass Prediction	
Date	Run # Sample # Trial #	Cultivation Time (day:hr:min)	Cultivation Time (days)	pH	Sample Volume (mL)	pd + nm + cells (g)	pd + nm (g)	FW (g)	X (g FW/L)	ln(X)	Sample (mL)	(sec)	(mmol CO_2/L-hr)	(mmol CO_2/L-hr)	Cultivation Time (days)	X (g FW/L)
															0.00	0.94
5-12-99	9-1-1	0- 11:00 pm	0.00	8.00	500	8.6849	8.2167	0.4682	0.94	-0.066		597.5	0.258	0.061	4.67	1.35
5-17-99	9-2-1	5- 3:00 pm	4.67	8.74	500	8.9510	8.2770	0.6740	1.35	0.299		415.1	0.258	0.088	11.00	2.21
5-23-99	9-3-1	11- 11:00 pm	11.00	8.74	500	9.5180	8.3692	1.1488	2.30	0.832		243.5	0.258	0.150	15.58	3.16
5-28-99	9-4-1	16- 1:00 pm	15.58	8.82	500	9.7280	8.1714	1.5566	3.11	1.136		179.7	0.258	0.203	20.58	3.64
6-2-99	9-5-1	21- 1:00 pm	20.58	8.70	500	10.2550	8.5370	1.7180	3.44	1.234		162.8	0.258	0.224	25.60	4.19
6-7-99	9-6-1	26- 1:30 pm	25.60	8.73	500	10.5434	8.4043	2.1391	4.28	1.454		364.8	0.258	0.100	29.63	4.69
6-11-99	9-7-1	30- 2:00 pm	29.63	8.78	500	10.709	8.3705	2.3385	4.68	1.543		333.7	0.258	0.109	34.83	5.42
6-16-99	9-8-1	35- 7:00 pm	34.83	8.03	500	11.2479	8.5700	2.6779	5.36	1.678		291.4	10.342	0.125	39.67	6.21
6-21-99	9-9-1	40- 3:00 pm	39.67	7.97	500	11.1718	8.3072	2.8646	5.73	1.746		272.4	10.342	0.134	44.54	7.11
6-26-99	9-10-1	45- 12:00 pm	44.54	7.77	500	11.9722	8.1995	3.7727	7.55	2.021		206.8	10.342	0.176	49.63	8.20
7-1-99	9-11-1	50- 2:00 pm	49.63	7.86	500	21.0580	16.8827	4.1753	8.35	2.122	30	186.9	10.342	0.195	54.63	9.44
7-6-99	9-12-1	55- 2:00 pm	54.63	7.93	470	21.1099	17.0482	4.0617	8.64	2.157		180.6	10.342	0.202	60.54	11.14
7-12-99	9-13-1	61- 12:00 pm	60.54	7.90	470	21.9478	17.3450	4.6028	9.79	2.282		159.4	10.342	0.229	64.75	12.53
7-16-99	9-14-1	65- 5:00 pm	64.75	7.87	470	22.1003	17.2000	4.9003	10.43	2.344		149.7	10.342	0.243	69.73	14.40
7-21-99	9-15-1	70- 4:30 pm	69.73	7.87	470	23.2034	17.3800	5.8234	12.39	2.517		126.0	10.342	0.289	74.63	16.52
7-26-99	9-16-1	75- 2:00 pm	74.63	7.96	470	24.3600	17.2200	7.1400	15.19	2.721	20	102.7	10.342	0.354	79.75	19.07
7-31-99	9-17-1	80- 5:00 pm	79.75	7.85	450	26.6062	17.3205	9.2857	20.63	3.027		75.6	10.342	0.481	84.50	21.78
8-5-99	9-18-1	85- 11:00 am	84.50	7.71	450	28.5949	17.2623	11.3326	25.18	3.226		62.0	10.342	0.588	90.00	25.41

Run Identification

Run#:	AS-STR-9
STR #	#2
Bioreactor Description:	
500 mL Bellco spinner flask	
Time started:	11:00 PM
Date started:	5-12-99

Culture Loading

Culture:	*Agardhiella subulata* microplantlets
Cell line I.D.	As3-29-1,2,3,4 4/19/99
Age of inoculum:	23 days
Inoculum cell weight:	0.4894 g FW
Initial ASP12 medium volume:	500.0 mL
Initial cell density:	0.98 g FW/L
$NaNO_3$ conc. :	1.00 g/L
$NaHCO_3$ conc. :	0.84 g/L
Solids content Ws	250.00 mgDCW/ gFW
Average pH (day 21-85):	8.80 +/- 0.10
Medium replacement:	100 % per sample

Process Parameters

Sparger:	Stainless steel filter #1
Air flowmeter S/N:	059360 Setting: 22
Air flowrate:	150 mL/ min at 21°C
CO_2 flowmeter S/N:	082623 Setting: 0
CO_2 flowrate:	0 mL/ min at 21°C
Total flowrate:	150 mL/ min at 21°C
CO_2 partial pressure:	0.00035 atm
Impeller design:	Two blade flat-paddle (d_i = 5.5 cm, H = 2.5 cm)
Stir plate # / setting	VirTis Omni Culture
Impeller speed:	120 rpm
Setpoint temperature:	24 °C
Lamp position:	10.2 cm from the center
Incident illumination intens	80 µE/m^2-sec
Photoperiod :	10 hr ON / 14 hr OFF
mixing time t_{mix}	2.3 sec
mass transfer time t_{mt}	40.9 sec
CO_2 consumption time t_{rxn}	140.9 sec at X_c
CO_2 consumption time t_{rxn}	116.1 sec at X_f

CO_2 growth model

R =	0.08206 L-atm/mol-K
P_{CO2} =	3.50E-04 atm (air)
$Y_{X/CO2}$ =	30.0 mg DCW mmol^{-1} CO_2
μ_1 =	0.067 day^{-1} (0-15 days)
μ_2 =	0.023 day^{-1} (15-75 days)
H =	0.0346 atm-L/mmol
k_La for O_2 =	91.2 h^{-1}
n_A =	0.258 mmol CO_2 /L-hr
$k_La C_A^*$ =	0.890 mmol CO_2 /L-hr
X_c =	13.42 g FW/L
t_c =	74.42 day
f =	10 hr day^{-1}
measured β =	0.3139 gFW/L-day 80-85 days
theory β =	0.3100 gFW/L-day

Sample Identification				Culture	Biomass Measurements						OER	t_{rxn}	n_A	qco_2	Biomass Prediction	
Date	Run # Sample # Trial #	Cultivation Time (day:hr:min)	Cultivation Time (days)	pH	Sample Volume (mL)	pd + nm + cells (g)	pd + nm (g)	FW (g)	X (g FW/L)	ln(X)	Sample (mL)	(sec)	(mmol CO_2/L-hr)	(mmol CO_2/L-hr)	Cultivation Time (days)	X (g FW/L)
															0.00	0.98
5-12-99	9-1-1	0- 11:00 pm	0.00	8.00	500	8.7061	8.2167	0.4894	0.98	-0.02		668.5	0.258	0.054	4.67	1.34
5-17-99	9-2-1	5- 3:00 pm	4.67	8.76	500	8.8365	8.2595	0.5770	1.15	0.14		567.0	0.258	0.064	11.00	2.04
5-23-99	9-3-1	11- 11:00 pm	11.00	8.76	500	9.1766	8.3520	0.8246	1.65	0.50		396.7	0.258	0.092	15.58	2.77
5-28-99	9-4-1	16- 1:00 pm	15.58	8.74	500	9.5962	8.1714	1.4248	2.85	1.05		229.6	0.258	0.159	20.58	3.87
6-2-99	9-5-1	21- 1:00 pm	20.92	8.72	500	10.2550	8.5370	1.7180	3.44	1.23		190.4	0.258	0.191	25.60	4.35
6-6-99	9-6-1	25- 1:30 pm	24.94	8.73	500	10.4086	8.4195	1.9891	3.98	1.38		475.5	0.258	0.077	29.63	4.77
6-11-99	9-7-1	30- 2:00 pm	29.63	8.78	500	10.1277	8.2982	1.8295	3.66	1.30		517.0	0.258	0.070	34.83	5.38
6-16-99	9-8-1	35- 7:00 pm	34.83	8.75	500	11.1478	8.7619	2.3859	4.77	1.56		396.4	0.258	0.092	39.67	6.01
6-21-99	9-9-1	40- 3:00 pm	39.67	8.73	500	10.9618	8.3080	2.6538	5.31	1.67		356.4	0.258	0.102	44.54	6.73
6-26-99	9-10-1	45- 12:00 pm	44.54	8.69	500	10.7707	8.0689	2.7018	5.40	1.69		350.1	0.258	0.104	49.63	7.57
7-1-99	9-11-1	50- 2:00 pm	49.63	8.78	500	11.1967	8.1441	3.0526	6.11	1.81	30	309.9	0.258	0.118	54.63	8.50
7-6-99	9-12-1	55- 2:00 pm	54.63	8.74	470	20.5300	17.2734	3.2566	6.93	1.94		273.0	0.258	0.133	60.54	9.74
7-12-99	9-13-1	61- 12:00 pm	60.54	8.90	470	21.4349	17.5330	3.9019	8.30	2.12		227.9	0.258	0.160	64.75	10.74
7-16-99	9-14-1	65- 5:00 pm	64.75	8.72	470	21.3719	17.4210	3.9509	8.41	2.13		225.0	0.258	0.162	69.73	12.04
7-21-99	9-15-1	70- 4:30 pm	69.73	8.79	470	21.8752	17.3667	4.5085	9.59	2.26		197.2	0.258	0.185	74.63	13.49
7-26-99	9-16-1	75- 2:00 pm	74.63	8.94	470	22.513	17.224	5.2890	11.25	2.42	20	168.1	0.258	0.217	74.42	13.42
7-31-99	9-17-1	80- 5:00 pm	79.75	9.03	450	23.8934	17.2333	6.6601	14.80	2.69		140.9	0.258	0.285	84.50	16.59
8-5-99	9-18-1	85- 11:00 am	84.50	8.89	450	24.8974	17.5664	7.3310	16.29	2.79		140.9	0.258	0.314	90.00	18.31

Run Identification

Run#:	AS-STR-10
STR #	#1
Bioreactor Deacription:	
500 mL Bellco spinner flask	
Time started:	3:00 PM
Date started:	9-25-99

Culture Loading

Culture:	*Agardhiella subulata* microplantlets	
Cell line I.D.	As3-33-6,7,8,9	9/15/99
Age of inoculum:	11 days	
Inoculum cell weight:	0.9288 g FW	
Initial ASP12 medium volume:	500.0 mL	
Initial cell density:	1.86 g FW/L	
$NaNO_3$ conc. :	1.00 g/L	
Solid content Ws	250 mg DCW/ gFW	
Average pH:	7.84 +/- 0.23	
P_{CO2} =	7.20E-03 atm (air)	
Solid content Ws	244 mg DCW/ g FW	

Process Parameters

Sparger:	Stainless steel, #1 HPLC cup filter	
Air flowmeter S/N:	026947 Setting:	30
Air flowrate:	290 mL/ min at 21°C	
CO_2 flowmeter S/N:	082623 Setting: 0	
CO_2 flowrate:	2 mL/ min at 21°C	
Total flow rate	292 mL/ min at 21°C	
Impeller design:	Two blade flat-paddle (d_I = 5.5 cm, H = 2.5 cm)	
Stir plate # / setting	VWR 310 Setting: 2.00	
Impeller speed:	120 rpm	
Setpoint temperature:	24 °C	
Lamp position:	10.2 cm from the center	
Incident illumination intens	80 $\mu E/m^2$-sec	
Photoperiod *f*:	10 hr ON / 14 hr OFF	
mixing time t_{mix}	2.3 sec	
mass transfer time t_{ml}	29.4 sec	
CO_2 consumption time t_{rxn}	291.5 sec	at X_c
CO_2 consumption time t_{rxn}	1802.1 sec	at X_f

CO_2 growth model

R =	0.08206 L-atm/mol-k	
P_{CO2} =	7.20E-03 atm (air)	
$Y_{X/CO2}$ =	30.0 mg DCW $mmol^{-1}$ CO_2	
μ_1 =	0.0843 day^{-1}	(0-15 days)
μ_2 =	0.0437 day^{-1}	(15-24 days)
H =	0.0346 atm-L/mmol	
$k_L a$ for O_2 =	127.1 h^{-1}	
n_A =	10.3464 mmol O2/L-hr	
$k_L a C_A^*$ =	25.506 mmol O2/L-hr	
X_c =	291.54 g FW/L	
t_c =	97.80 day	
f =	10 hr day^{-1}	

Sample Identification				Culture	Biomass Measurements						t_{rxn}	n_A	qco_2
Date	Run # Sample # Trial #	Cultivation Time (day:hr:min)	Cultivation Time (days)	pH	Sample Volume (mL)	pd + nm + cells (g)	pd + nm (g)	FW (g)	X (g FW/L)	ln(X)	(sec)	(mmol CO_2/L-hr)	(mmol CO_2/L-hr)
9-25-1999	10-1-1	0- 3:00 pm	0.00	8.00	500.0	9.0968	8.1680	0.9288	1.86	0.619	5884.7	10.3464	0.127
9-30-99	10-2-1	5- 3:00 pm	5.00	7.76	500.0	10.4123	8.2591	2.1532	4.31	1.460	2538.4	10.3464	0.295
10-5-99	10-3-1	10- 1:00 pm	9.92	7.81	500.0	19.7030	17.2480	2.4550	4.91	1.591	2226.3	10.3464	0.336
10-10-99	10-4-1	15- 9:00 pm	15.25	7.68	500.0	11.9903	8.0230	3.9673	7.93	2.071	1377.7	10.3464	0.544
10-14-99	10-5-1	19- 1:00 pm	18.92	7.73	500.0	13.3825	8.2173	5.1652	10.33	2.335	2043.4	10.3464	0.367
10-19-99	10-6-1	24- 1:30 pm	23.94	8.24	467.0	13.7755	8.3053	5.4702	11.71	2.461	1802.1	11.0775	0.416

Run Identification

Run#:	AS-STR-10
STR #	#2
Bioreactor Description:	
500 mL Bellco spinner flask	
Time started:	3:00 PM
Date started:	9-25-99

Culture Loading

Culture:	*Agardhiella subulata* microplantlets	
Cell line I.D.	As3-33-6,7,8,9	9/15/99
Age of inoculum:	11 days	
Inoculum cell weight:	0.9350 g FW	
Initial ASP12 medium volume:	500.0 mL	
Initial cell density:	1.87 g FW/L	
$NaNO_3$ conc. :	1.00 g/L	
$NaHCO_3$ conc. :	0.84 g/L	
Average pH:	8.84 +/- 0.07	
Solid content Ws	226 mg DCW/ g FW	

Process Parameters

Sparger:	#2, Stainless steel Applikon sparger
Air flowmeter S/N:	059360 Setting: 22
Air flowrate:	150 mL/ min at 21°C
CO_2 flowmeter S/N:	082623 Setting: 0
CO_2 flowrate:	0 mL/ min at 21°C
Impeller design:	Two blade flat-paddle (d_I = 5.5 cm, H = 2.5 cm)
Stir plate # / setting	VirTis Omni Culture
Impeller speed:	120 rpm
Setpoint temperature:	24 °C
Lamp position:	10.2 cm from the center
Incident illumination intens	80 µE/m²-sec
Photoperiod *f*:	10 hr ON / 14 hr OFF
mixing time t_{mix}	2.3 sec
mass transfer time t_{mt}	92.4 sec
CO_2 consumption time t_{rxn}	140.9 sec at X_c
CO_2 consumption time t_{rxn}	104.8 sec at X_f

CO_2 growth model

R =	0.08206 L-atm/mol-k
P_{CO2} =	3.50E-04 atm (air)
$Y_{X/CO2}$ =	30.0 mg DCW mmol^{-1} CO_2
μ_l =	0.0629 day^{-1} (0-15 days)
H =	0.0346 atm-L/mmol
k_La for O_2 =	40.4 h^{-1}
n_A =	0.2584 mmol O2/L-hr
$k_La C_A^*$ =	0.394 mmol O2/L-hr
X_c =	5.46 g FW/L
t_c =	17.03 day
f =	10 hr day^{-1}
measured β =	0.2187 gFW/L-day 9-23 days
theory β =	0.3430 gFW/L-day

Sample Identification				Culture	Biomass Measurements						t_{rxn}	n_A	qco_2	Biomass Prediction	
Date	Run # Sample # Trial #	Cultivation Time (day:hr:min)	Cultivation Time (days)	pH	Sample Volume (mL)	pd + nm + cells (g)	pd + nm (g)	FW (g)	X (g FW/L)	ln(X)	(sec)	(mmol CO_2/L-hr)	(mmol CO_2/L-hr)	Cultivation Time (days)	X (g FW/L)
														0.00	1.87
9-25-99	10-1-1	0- 3:00 pm	0.00	8.00	500	9.1056	8.1706	0.9350	1.87	0.63	411.3	0.258	0.089	5.00	2.56
9-30-99	10-2-1	5- 3:00 pm	5.00	8.74	500	9.8508	8.2282	1.6226	3.25	1.18	237.0	0.258	0.154	9.92	3.49
10-5-99	10-3-1	10- 1:00 pm	9.92	8.88	500	19.2630	17.0740	2.1890	4.38	1.48	175.7	0.258	0.207	15.25	4.88
10-10-99	10-4-1	15- 9:00 pm	15.25	8.82	500	10.7519	8.0740	2.6779	5.36	1.68	143.6	0.258	0.254	17.03	5.46
10-14-99	10-5-1	19- 1:00 pm	18.92	8.90	500	11.4752	8.1911	3.2841	6.57	1.88	117.1	0.258	0.311	18.92	5.87
10-19-99	10-6-1	24- 1:30 pm	23.94	8.88	467	11.7310	8.3053	3.4257	7.34	1.99	104.8	0.258	0.347	23.94	6.97

Run Identification

Run#: AS-STR-10
STR # #1
Bioreactor Description:
500 mL Bellco spinner flask
Time started: 3:00 PM
Date started: 9-25-99

Culture Loading

Culture: *Agardhiella subulata* microplantlets
Cell line I.D. As3-33-6,7,8,9 9/15/99
Age of inoculum: 11 days
Inoculum cell weight: 0.9288 g FW
Initial ASP12 medium volume: 500.0 mL
Initial cell density: 1.86 g FW/L
$NaNO_3$ conc. : 1.00 g/L
$NaHCO_3$ conc. : 0.84 g/L
P_{CO2} = 3.50E-04 atm (air)
P_T = 1.0 atm

Process Parameters

Sparger: #1, Stainless steel Applikon filter
Air flowmeter S/N: 026947
CO_2 flowmeter S/N: 082623
Impeller design: Two blade flat-paddle (d_i = 5.5 cm, H = 2.5 cm)
Stir plate # / setting VWR 310 setting 2.00
Impeller speed: 137 rpm
Setpoint temperature: 24 °C
Lamp position: 10.2 cm from the center
Incident illumination inter 80 $\mu E/m^2$-sec
Photoperiod : 10 hr ON / 14 hr OFF (10am - 8pm)
OER were usually measured between 1-5pm, at least 3 hours into light phase

D.O. Cell and OER

D.O. cell volume: 65.0 mL
Sat. D.O. conc.: 0.2055 mmol O_2 / L (air in seawater, 24 °C)

Summary

Range (days)	P_o (mmol O_2 /g DCW-hr)		Q_o (mmol O_2 /g DCW-hr)		pH	
	Average	+/- 1 s.d.	Average	+/- 1 s.d.	Average	+/- 1 s.d.
19-32	0.193	0.014	0.048	0.007	7.75	0.08

Sample Identification		Aeration Gas Composition					Culture	Biomass				OER									
Date	Cultivation Time (days)	Target CO_2 (X ambient)	Air (mL/min)	CO_2 (mL/min)	Total (mL/min)	P_{CO2} (atm)	pH	Sample Vol. (mL)	FW (g)	DCW (g)	Solids (wt.%)	Blank Trials % O2/min		OER Trials (P_o) % O2/min		OER Trials (-Q_o) % O2/min		P_o mmol O_2/g DCW hr		Q_o mmol O_2/g DCW hr	
												1	2	1	2	1	2	Ave	+/- 1 s.d.	Ave	+/- 1 s.d.
10/14/99*	19	20X	290.0	2.0	292.0	0.00720	7.73	100.00	0.3332	0.0691	20.7	-0.1183		1.5900	1.5260	0.3884	0.3435	0.194	0.0052	0.056	0.0037
10/25/99	30	20X	290.0	2.0	292.0	0.00720	7.83	100.00	0.4940	0.1119	22.7	-0.2098		2.2542	2.3213	0.4630	0.4142	0.179	0.0034	0.046	0.0025
10/27/99	32	50X	120.0	2.0	122.0	0.01674	7.68	100.00	0.2340	0.0576	24.6	-0.0708		1.3761	1.4618	0.2260	0.2403	0.207	0.0084	0.042	0.0014
10/29/99	34	100X	120.0	4.0	124.0	0.03260	7.31	100.00	0.4370	0.1071	24.5	-0.1234		2.2788	2.3293	0.3393	0.4071	0.182	0.0027	0.037	0.0036
10/31/99	36	200X	120.0	8.0	128.0	0.06283	7.08	100.00	0.3752	0.1035	27.6	-0.0743		2.5559	2.6034	0.2583	0.2905	0.205	0.0026	0.027	0.0018
11/2/99	38	400X	113.4	15.4	128.8	0.11987	6.88	100.00	0.3280	0.0812	24.8	-0.0464		1.5302	1.4906	0.2147	0.3178	0.154	0.0028	0.031	0.0072
11/5/99	41	1000X	48.0	15.4	63.4	0.24317	6.43	100.00	0.2973	0.0790	26.6	-0.0167		1.2226	0.9850	0.1495	0.1265	0.114	0.0170	0.016	0.0016
11/6/99	42	20X	300.0	2.0	302.0	0.00697	7.82	100.00	0.2206	0.0551	25.0	-0.0655		1.1001	1.1878	0.1075	0.1178	0.176	0.0090	0.026	0.0011
11/8/99	44	1X	902.0	0.0	902.0	0.00035	8.75	100.00	0.7070	0.1682	23.8	-0.0528		1.6847	1.6686	0.5085	0.5280	0.082	0.0005	0.027	0.0007

Run Identification

Run#: AS-STR-10
STR # #2
Bioreactor Description:
500 mL Bellco spinner flask
Time started: 3:00 PM
Date started: 9-25-99

Culture Loading

Culture: *Agardhiella subulata* microplantlets
Cell line I.D. As3-33-6,7,8,9 9/15/99
Age of inoculum: 11 days
Inoculum cell weight: 0.9350 g FW
Initial ASP12 medium volume: 500.0 mL
Initial cell density: 1.87 g FW/L
$NaNO_3$ conc. : 1.00 g/L
$NaHCO_3$ conc. : 0.84 g/L
P_{CO2} = 3.50E-04 atm (air)
P_T = 1.0 atm

Process Parameters

Sparger: Stainless steel, #2 applikon sparger
Air flowmeter S/N: 059360
CO_2 flowmeter S/N: 082623
Impeller design: Two blade flat-paddle (d_i = 5.5 cm, H = 2.5 cm)
Stir plate # / setting VirTis Omni Culture
Impeller speed: 120 rpm
Setpoint temperature: 24 °C
Lamp position: 10.2 cm from the center
Incident illumination inter 80 µE/m²-sec
Photoperiod : 10 hr ON / 14 hr OFF (10am-8pm)
OER were usually measured between 1-5pm, at least 3 hours into light phase

D.O. Cell and OER

D.O. cell volume: 65.0 mL
Sat. D.O. conc.: 0.2055 mmol O_2 / L (air in seawater, 24 °C)

Summary

Range (days)	P_o (mmol O_2 /g DCW-hr)		Q_o (mmol O_2 /g DCW-hr)		pH	
	Average	+/- 1 s.d.	Average	+/- 1 s.d.	Average	+/- 1 s.d.
19-41	0.106	0.021	0.028	0.013	8.94	0.07

Sample Identification		Aeration Gas Composition					Biomass	Biomass				OER									
Date	Cultivation Time (days)	Target CO_2 (X ambient)	Air (mL/min)	CO_2 (mL/min)	Total (mL/min)	P_{CO2} (atm)	Culture pH	Sample Vol. (mL)	FW (g)	DCW (g)	Solids (wt.%)	Blank Trials % O2/min		OER Trials (P_o) % O2/min		OER Trials ($-Q_o$) % O2/min		P_o mmol O_2/g DCW hr		Q_o mmol O_2/g DCW hr	
												1	2	1	2	1	2	Ave	+/- 1 s.d.	Ave	+/- 1 s.d.
10/14/99	19	1X	150.0	0.0	150.0	0.00035	8.90	100.00	0.2199	0.0440	20.0	-0.0797	-0.0764	0.7646	0.6403	0.1932	0.2241	0.142	0.0160	0.052	0.0040
10/19/99	24	1X					8.88	100.00	0.2946	0.0632	21.5	-0.0297	-0.0288	1.0047	0.7477	0.2061	0.3295	0.115	0.0230	0.038	0.0111
10/22/99	27	1X					8.84	100.00	0.5866	0.1224	20.9	-0.1260		1.3408	1.4539	0.4512	0.4289	0.100	0.0052	0.037	0.0010
10/25/99	30	1X					8.99	100.00	0.7201	0.1810	25.1	-0.0599	-0.1032	2.1911	2.1212	0.4957	0.4827	0.099	0.0022	0.025	0.0004
10/27/99	32	1X					8.98	100.00	0.6611	0.1585	24.0	-0.0264	-0.0233	1.9407	1.8888	0.3768	0.3734	0.098	0.0019	0.020	0.0001
10/29/99	34	1X					8.89	100.00	0.6123	0.1479	24.2	-0.0240		2.2202	2.2362	0.3117	0.3294	0.122	0.0006	0.019	0.0007
11/2/99	38	1X					9.02	100.00	0.7710	0.1736	22.5	-0.0642		1.4888	1.4070	0.3968	0.3430	0.070	0.0027	0.020	0.0018
11/5/99	41	1X					9.04	100.00	0.4899	0.1022	20.9	-0.0167		1.2703	1.2124	0.1638	0.2048	0.099	0.0032	0.016	0.0023
11/7/99	43	20X	290.0	2.0	292.0	0.00720	7.82	100.00	0.8064	0.1528	18.9	-0.0843		3.3116	3.2771		0.2639	0.177	0.0013	0.018	
11/8/99	44	20X					7.91	100.00	0.4924	0.1245	25.3	-0.0318		3.1368	3.2101	0.3146		0.206	0.0033	0.022	
11/10/99	46	50X	120.0	2.0	122.0	0.01674	7.55	100.00	0.5897	0.1186	20.1	-0.0828		2.4396	2.6249	0.3254	0.3071	0.177	0.0089	0.027	0.0009
11/12/99	48	200X	120.0	8.0	128.0	0.06283	7.08	100.00	0.5408	0.1081	20.0	-0.0692		2.5904	2.5509	0.2354		0.196	0.0021	0.023	
11/14/99	50	400X	113.4	15.4	128.8	0.11987	6.79	100.00	0.5564	0.1122	20.2	-0.0487		2.0517	2.3281	0.2628	0.2823	0.160	0.0140	0.023	0.0010
11/16/99	52	650X	71.0	15.4	86.4	0.17853	6.68	100.00	0.4500	0.1004	22.3	-0.1017		1.5924	1.6355	0.1968	0.2192	0.137	0.0024	0.025	0.0013
11/18/99	54	400X	113.4	15.4	128.8	0.11987	6.83	100.00	0.3465	0.0804	23.2	-0.0662		1.2885	1.3339	0.1386	0.1990	0.137	0.0032	0.023	0.0043
11/19/99	55	200X	120.0	8.0	128.0	0.06283	7.23	100.00	0.4012	0.0930	23.2	-0.0668		1.4048	1.4507	0.2060	0.1806	0.129	0.0028	0.022	0.0015
11/21/99	57	100X	120.0	4.0	124.0	0.03260	7.38	100.00	0.4476	0.1059	23.7	-0.0319		1.6249	1.6403	0.2056	0.2295	0.126	0.0008	0.019	0.0013
11/22/99	58	50X	120.0	2.0	122.0	0.01674	7.73	100.00	0.3414	0.0718	21.0	-0.0531		1.1223	1.1168	0.1729	0.1613	0.131	0.0004	0.025	0.0009
11/23/99	59	20X	290.0	2.0	292.0	0.00720	7.97	100.00	0.3252	0.0862	26.5	-0.0549		1.3098	1.1503	0.2279	0.2161	0.119	0.0105	0.026	0.0008
11/24/99	60	20X	290.0	2.0	292.0	0.00720	7.83	100.00	0.3127	0.0755	24.1	-0.0518		1.13591	1.1699	0.1601	0.1899	0.128	0.0026	0.024	0.0022
11/27/99	63	1X	150.0	0.0	150.0	0.00035	8.73	100.00	0.3875	0.0917	23.7	-0.0560		1.1645	1.1484	0.2481	0.2072	0.106	0.0010	0.025	0.0025
11/30/99	66	10X	405.0	1.4	406.4	0.00379	8.06	100.00	0.2825	0.0732	25.9	-0.1083		1.1680	1.1884	0.2072		0.141	0.0016	0.035	

*After Oct. 19, medium were completely replaced every 2 or 3 day after sampling
* 100 ml is needed liquid sample volume for OER measurements. The actual biomass amount may vary

Run Identification

Bioreactor Run#:	AS-STR-11a
Bioreactor Description:	
500 ml Bellco spinner flask	
Time started:	3:00 PM
Date started:	1-26-00

Culture Loading

Culture:	*Agardhiella subulata* microplantlets	
Cell line I.D.	As3-37-1,2,3	1 /5 / 00
Age of Inoculum:	21 days	
Inoculum cell weight:	0.7303 g FW	
ASP12 medium volume:	580 mL	
Initial cell density:	1.26 g./L	
$NaNO_3$ conc. :	0.100 g/L	1.18 mM
$NaHCO_3$ conc. :	0.840 g/L	10.00 mM
Na Glycerophosphate:	0.097 g/L	0.45 mM
Solids content measurement	1/26/00	
Fresch weight:	0.0432	
Dry cell weight:	0.0123	
Solids content W_s	285 mg DCW/g FW	

Process Parameters

Air supply design:	sparger #1 (HPLC filter cup)
Air flow setting:	23 = 150 ml/ min at 21°C
Air flow S/N	059360
Impeller design: flat-paddle *Di* =	5.5 cm
Impeller speed:	120 rpm
Setpoint temperature:	24 °C
Illuminater position:	10.5 cm from the center
Illumination intensity:	80 $\mu E/m^2$-s
Photoperiod :	10 hr ON / 14 hr OFF
P_{CO2} =	3.50E-04 atm (air)
P_T =	1.0 atm
$Y_{X/CO2}$ =	30.0 mg DCW $mmol^{-1}$ CO_2
μ =	0.0713 day^{-1} (0-14 days)
H =	0.03455 atm-L/mmol
R =	0.08206 L-atm/mol-K
k_La =	91.2 h^{-1}
CO2-TR	0.8910 mmol CO_2/L-hr

Sample Identification				Culture	Biomass Measurements						Phosphate measurement					n_A	qco_2
Date	Run # Sample # Trial #	Cultivation Time (day:hr:min)	Cultivation Time (days)	pH	Sample Volume (mL)	pd + nm + cells (g)	pd + nm (g)	X (g FW/L)	FW (g)	ln(X)	Gly-P Trial 1 A605	Gly-P Trial 2 A605	Gly-P A605	Gly-P conc. (µmol/L)	Average +/-1s.d. (µmol/L)	(mmol CO_2/L-hr)	(mmol CO_2/L-hr)
1-26-00	11-1-1	0- 3:00 pm	0.00	8.27	580	9.1056	8.3753	1.26	0.7303	-0.31	506	513	509.5	474.7	24.4	0.223	0.085
2-1-99	11-2-1	6- 11:00 pm	6.33	8.72	580	9.5896	8.3012	2.22	1.2884	0.25	471		471.0	432.3	30.1	0.223	0.150
2-9-99	11-3-1	14- 4:00 pm	14.04	8.72	540	10.3706	8.3688	3.71	2.0018	0.69	426	437	431.5	388.8	29.0	0.239	0.251
2-16-9	11-4-1	21- 1:00 pm	20.92	8.72	510	10.6235	8.4182	4.32	2.2053	0.79	431		431.0	388.2	33.3	0.253	0.293
2-23-99	11-5-1	28- 12:00 pm	27.88	8.86	480	11.1787	8.3762	5.84	2.8025	1.03	435	417	426.0	382.7	40.7	0.269	0.395
3-1-99	11-6-1	35- 4:00 pm	35.04	8.69	440	11.1297	8.5350	5.90	2.5947	0.95	572	594	583.0	277.8	229.0	0.294	0.399
3-7-99	11-7-1	41-10:00 pm	40.79	8.70	400	10.9710	8.3372	6.58	2.6338	0.97	502	548	525.0	245.9	199.4	0.323	0.446
3-14-99	11-8-1	48- 12:30Am	47.90	8.81	370	11.5829	8.7408	7.68	2.8421	1.04	493	505	499.0	231.6	203.5	0.349	0.520
3-22-00	11-9-1	56- 3:00 pm	56.00	8.81	340	11.2898	8.3137	8.75	2.9761	1.09	-	-	-	-	-	0.380	0.592
4-4-00	11-18-1	69- 12:00 pm	68.88	8.89	320	10.2361	8.2148	6.32	2.0213	0.70	-	-	-	-	-	0.404	0.427

Run Identification

Bioreactor Run#:	AS-STR-11b
Bioreactor Description:	
100 ml Bellco spinner flask	
Time started:	3:00 PM
Date started:	1-26-00

Culture Loading

Culture:	Agardhiella subalata microplantlets	
Cell line I.D.	As3-37-1,2,3 1 /5 / 00	
Age of Inoculum:	21 days	
Inoculum cell weight:	1.7011 g FW	
ASP12 medium volume:	580 mL	
Initial cell density:	2.93 g/L	
$NaNO_3$ conc. :	0.1 g/L	1.18 mM
$NaHCO_3$ conc. :	0.84 g/L	10.00 mM
Na Glycerophosphate:	0.10 g/L	0.45 mM

Process Parameters

Air supply design:	sparger #2 (Applikon sparger)
Air flow setting:	23 = 150 ml/ min
Air flow S/N	026947
Impeller design: flat-paddle *Di* =	5.5 cm
Impeller speed:	120 rpm
Setpoint temperature:	24 °C
Illuminater position:	10.5 cm from the center
Illumination intensity:	80 $\mu E/m^2$-s
Photoperiod :	10 hr ON / 14 hr OFF
P_{CO2} =	3.50E-04 atm (air)
P_T =	1.0 atm
$Y_{X/CO2}$ =	30.0 mg DCW $mmol^{-1}$ CO_2
μ =	0.0713 day^{-1} (0-14 days)
H =	0.03455 atm-L/mmol
R =	0.08206 L-atm/mol-K
k_La =	40.4 h^{-1}
CO2-TR	0.3947 mmol CO_2/L-hr

Sample Identification				Culture	Biomass Measurements						Phosphate measurement						n_A	qco_2
Date	Run # Sample # Trial #	Cultivation Time (day:hr:min)	Cultivation Time (days)	pH	Sample Volume (mL)	pd + nm + cells (g)	pd + nm (g)	X (g FW/L)	FW (g)	ln(X)	Gly-P Trial 1 A605	Gly-P Trial 2 A605	Gly-P Trial 3 A605	Gly-P A605	Gly-P conc. (µmol/L)	Average +/-1s.d. (µmol/L)	(mmol CO_2/L-hr)	(mmol CO_2/L-hr)
1-26-00	11-1-1	0- 3:00 pm	0.00	8.27	580	10.1087	8.4076	2.93	1.7011	0.53	503	513		508	473.02	7.79	0.223	0.198
2-1-99	11-2-1	6- 11:00 pm	6.33	8.73	580	11.0100	8.2756	4.71	2.7344	1.01	463			463	423.48	-	0.223	0.319
2-9-99	11-3-1	14- 4:00 pm	14.04	8.73	520	12.5916	8.5044	7.86	4.0872	1.41	413	377	374	388	340.90	23.89	0.248	0.532
2-16-9	8-4-1	21- 1:00 pm	20.92	8.85	480	12.6222	8.4178	8.76	4.2044	1.44	361			361	311.18	-	0.269	0.593
2-23-99	8-5-1	28- 12:00 pm	27.88	8.82	440	12.4921	8.3580	9.40	4.1341	1.42	287	274		281	222.55	10.12	0.294	0.636
3-1-99	8-6-1	35- 4:00 pm	35.04	8.84	410	12.5525	8.5027	9.88	4.0498	1.40	284	316		300	122.01	24.91	0.315	0.668
3-7-99	8-7-1	41-10:00 pm	40.79	8.81	390	11.9975	8.3888	9.25	3.6087	1.28	198	200		199	66.41	1.56	0.331	0.626

As-STR-11 microplantlet size measurement

Date: 3/9/00 #1 X_o=1.26 g FW/L

Time: 5:20 PM #2 X_o=2.93 g FW/L

#1			#2		
long axis mm	short axis mm	average mm	long axis mm	short axis mm	average mm
12	8	10.0	6	5	5.5
6	5	5.5	5	4	4.5
5	4	4.5	9	6	7.5
7	7	7.0	4	3	3.5
9	6	7.5	5	3	4.0
8	5	6.5	4	4	4.0
9	5	7.0	8	6	7.0
10	9	9.5	5	3	4.0
11	12	11.5	12	5	8.5
11	6	8.5	5	4	4.5
8	4	6.0	4	4	4.0
4	3	3.5	8	5	6.5
7	5	6.0	7	7	7.0
9	7	8.0	3	3	3.0
10	7	8.5	6	6	6.0
6	5	5.5	7	3	5.0
7	7	7.0	9	6	7.5
9	7	8.0	6	5	5.5
5	5	5.0	4	3	3.5
6	6	6.0	7	7	7.0
5	4	4.5	6	6	6.0
11	7	9.5	6	5	5.5
12	6	9.0	7	4	5.5
13	7	9.5	10	3	6.5
12	8	10.0	3	2	2.5
8	6	7.0	6	6	6.0
8	5	6.5	5	5	5.0
9	6	7.5	5	3	4.0
8	6	7.0	6	5	5.5
7	6	6.5	8	6	7.0
10	7	8.5	4	4	4.0
9	9	9.0	5	5	5.0
8	7	7.5	6	4	5.0
10	8	9.0	9	7	8.0
9	6	7.5	5	3	4.0
10	6	8.0	7	5	6.0
10	8	9.0	4	3	3.5
5	4	4.5	6	5	5.5
14	10	12.0	6	4	5.0
8	6	7.0	6	5	5.5
Average		7.5	Average		5.3
Std. Dev.		1.9	Std. Dev.		1.4

Run # As-STR-11

Nitrate Calibration			Phosphate Calibration		#1 total N consumption, M_N	599.7 µmol			
slope	0.3295 µM / mAU		slope	0.1101 µM / mAU	#2 total N consumption, M_N	653.7 µmol			
intercept	-7.3962 µM		intercept	-8.6284 µM	#1 total biomass increase	0.60	+/-	0.03	g DCW
date:	1-26-2000		date:	10-11-1999	#2 total biomass increase	0.66	+/-	0.07	g DCW
#1 X_f	2.83 +/-	0.11 g FW	NaNO3	85 g/mol	#1 $Y_{X/N}$	1.00	+/-	0.05	g DCW / mmol N
#2 X_f	4.02 +/-	0.24 g FW	NaGlyP	216 g/mol	#2 $Y_{X/N}$	1.01	+/-	0.10	g DCW / mmol N
					average (#1&#2)	1.00	+/-	0.01	g DCW / mmol N

Date	Time	Days	FW (g)		A_{530} (mAU)						Ave. A_{530} (mAU)		V (L)		C_N (µM)		C_NV (µmol)		C_P (µM)		C_PV (µmol)	
			STR #1	STR#2	#1a	#1b	#1c	#2a	#2c	#2b	#1	#2	#1	#2	#1	#2	#1	#2	#1	#2	#1	#2
26-Jan	3:00 PM	0.00	0.7303	1.7011	401	339	353	354	371	393	364	369	0.58	0.58	1274.4	1288.2	739.2	747.1	474.7	473.0	275.3	274.4
28-Jan	12:30PM	1.90			377	345	344	327	311		355	319	0.58	0.58	1244.8	1125.1	722.0	652.5				
30-Jan	11:00 PM	4.33			317	345		275	256		331	266	0.58	0.58	1164.6	948.8	675.5	550.3				
1-Feb	4:00 PM	6.04	1.2884	2.7344	316	331		254	266		324	260	0.58	0.58	1139.9	930.7	661.1	539.8	432.3	423.5	250.7	245.6
4-Feb	4:00 PM	9.04			283	290		226	216		287	221	0.54	0.52	1018.0	802.2	549.7	417.1				
7-Feb	4:00 PM	12.04			264	279		199	228	209	272	212	0.54	0.52	968.6	772.5	523.0	401.7				
9-Feb	8:00 PM	14.21	2.0018	4.0872	275	256	262	169	165	164	264	166	0.54	0.52	944.9	620.9	510.3	322.9	388.8	340.9	210.0	177.3
11-Feb	4:30 PM	16.06			229	231		129	129		230	129	0.51	0.48	831.8	499.0	424.2	239.5				
14-Feb	2:00 PM	18.96			232	223	207	290	268		221	279	0.51	0.48	801.1	248.3	408.5	119.2				
16-Feb	12:00 PM	20.92	2.2053	4.2044	208	176	656	152	156		192	154	0.51	0.48	653.7	145.3	333.4	69.8	388.2	311.2	198.0	149.4
18-Feb	1:00 PM	22.92			180	158		31			169	33	0.48	0.44	630.8	18.3	302.8	8.0				
21-Feb	1:00 AM	25.42			575	597					586	-	0.48	0.44	501.2	-	240.6	-				
23-Feb	12:00 PM	27.88	2.8025	4.1341	500	523		50			512	50	0.48	0.44	439.8	23.9	211.1	-	382.7	222.5	183.7	97.9
25-Feb	3:00 PM	30.00			497	409	465				457	-	0.44	0.41	394.9	-	173.8	-				
29-Feb	9:00 AM	33.75			364	340					352	-	0.44	0.41	308.5	-	135.7	-				
1-Mar	4:00 PM	35.04	2.5947	4.0498	331	292		50			312	-	0.44	0.41	275.1	-	121.0	-	277.8	122.0	122.2	50.0
4-Mar	4:00PM	38.04			229	237					233	-	0.4	0.39	210.4	-	84.2	-				
7-Mar	10:00PM	41.29	2.6338	3.6087	159	152					156	24	0.4	0.39	146.6	15.3	58.6	6.0	245.9	66.4	98.3	25.9
14-Mar	3:00 PM	48.00	2.8421		20	20					20		0.37		35.0		12.9		231.6	-	85.7	

Run Identification

Bioreactor Run#: AS-STR-12a

Bioreactor Description:

500 ml Bellco spinner flask

Time started: 3:00 PM

Date started: 10-11-00

Culture Loading

Culture:	A subulata microplantlets	
Cell line I.D.	As3-37-1,2,3	1 /5 / 00
Age of Inoculum:	21 days	
Inoculum cell weight:	0.6040 g FW	
ASP12 medium volume:	500 mL	
Initial cell density:	1.21 g./L	
Solid content Ws	250 mg DCW/g FW	

Process Parameters

Air supply design:	sparger#1	
Air flowmeter S/N:	059360	Setting: 22
Air flowrate:	150 mL/ min at 21°C	
Impeller design: flat-paddle D_i =	5.5 cm	
Impeller speed:	120 rpm	
Setpoint temperature:	24 °C	
Stir plate # / setting	VirTis Omni Culture	
Illuminater position:	10.5 cm from the center	
Illumination intensity:	80 µE/m^2.s	
Photoperiod :	10 hr ON / 14 hr OFF	
mixing time t_{mix}	2.3 sec	
mass transfer time t_{mt}	29.4 sec	
CO_2 consumption time t_{rxn}	324.5 sec	at X_f

CO_2 growth model

R =	0.08206	L-atm/mol-K
P_{CO2} =	3.50E-04	atm (air)
$Y_{X/CO2}$ =	30.0	mg DCW mmol^{-1} CO_2
µ =	0.0362 day^{-1}	0-20 days
H =	0.0346	atm-L/mmol
$k_L a$ =	127.1	h^{-1}
n_A =	0.258	mmol CO_2 /L-hr
$k_L a C_A^*$ =	1.240	mmol CO_2 /L-hr
f =	10	hr / day

Sample Identification				Culture	Biomass Measurements						t_{rxn}	n_A	qco_2
Date	Run # Sample # Trial #	Cultivation Time (day:hr:min)	Cultivation Time (days)	pH	Sample Volume (mL)	pd + nm + cells (g)	pd + nm (g)	X (g FW/L)	FW g	LN(X)	(sec)	(mmol CO_2/L-hr)	(mmol CO_2/L-hr)
10-11-00	12-1-1	0- 3:00 pm	0.00	8.00	500	8.9165	8.4965						
			0.00			8.6501	8.4661	1.21	0.6040	0.19	999.3	0.258	0.036
10-16-00	12-2-1	5- 9:00 pm	5.25	8.73	500	8.8987	8.2204	1.36	0.6783	0.30	889.8	0.258	0.041
10-22-00	12-3-1	11- 4:00 pm	11.04	8.86	500	9.2669	8.4192	1.70	0.8477	0.53	712.0	0.258	0.051
10-26-00	12-4-1	15- 11:30 pm	15.35	8.85	500	9.4708	8.4395	2.06	1.0313	0.72	585.3	0.258	0.062
10-31-00	12-5-1	20- 11:00 pm	20.33	8.82	500	9.6071	8.3750	2.46	1.2321	0.90	489.9	0.258	0.074
11-5-00	12-6-1	25- 5:00 pm	25.08	8.84	500	9.5226	8.2564	2.53	1.2662	0.93	476.7	0.258	0.076
11-10-00	12-7-1	30- 6:30 pm	30.15	8.70	500	9.6087	8.1640	2.89	1.4447	1.06	417.8	0.258	0.087
11-16-00	12-8-1	36- 3:30pm	36.02	8.74	500	10.2176	8.4525	3.53	1.7651	1.26	342.0	0.258	0.106
11-21-00	12-9-1	41- 11:00 pm	41.33	8.66	500	9.9765	8.2586	3.44	1.7179	1.23	351.3	0.258	0.104
11-26-00	12-10-1	46- 10:30am	45.81	8.70	500	10.0844	8.424	3.32	1.6604	1.20	363.5	0.258	0.100
12-3-00	12-11-1	53- 2:00 pm	52.96	8.80	500	10.3358	8.4756	3.72	1.8602	1.31	324.5	0.258	0.112

Run Identification

Bioreactor Run#: AS-STR-12b
Bioreactor Description:
500 ml Bellco spinner flask
Time started: 3:00 PM
Date started: 10-11-00

Culture Loading

Culture: *Agardhiella subalata* microplantlets
Cell line I.D. As3-42-1,2,3 9 /5 / 00
Age of Inoculum: 36 days
Inoculum cell weight: 0.6361 g FW
ASP12 medium volume: 500 mL
Initial cell density: 1.27 g./L
Solid content Ws 250 mg DCW/g FW

Process Parameters

Air supply design: sparger #2, Applikon
Air flow setting: 10 = 12.7 ml/ min
Air flow S/N 059360
impeller design: flat-paddle (d_i = 5.5 cm)
Impeller speed: 120 rpm
Setpoint temperature: 24 °C
Illuminater position: 10.5 cm from the center
Illumination intensity: 80 µE/m².s
Photoperiod : 10 hr ON / 14 hr OFF
mixing time t_{mix} 2.3 sec
mass transfer time t_{ml} 321.8 sec
CO_2 consumption time t_{rxn} 1664.7 sec, at X_c
CO_2 consumption time t_{rxn} 311.4 sec, at X_f

CO_2 growth model

R = 0.08206 L-atm/mol-K
P_{CO2} = 3.50E-04 atm (air)
$Y_{X/CO2}$ = 30.0 mg DCW $mmol^{-1}$ CO_2
µ = 0.0455 day^{-1} 0-10 days
H = 0.0346 atm-L/mmol
$k_L a$ = 11.6 hr^{-1}
n_A/V = 0.022 mmol CO_2 /L-hr
$k_L a C_A^*$ = 0.113 mmol CO_2 /L-hr
Xc = 0.58 g FW/L
tc = 0.00 day
f = 10 hr / day
measured β = 0.0218 g FW / L-day 11-53 days
theory β = 0.0262 g FW / L-day

Sample Identification				Culture	Biomass Measurements						t_{rxn}	n_A	qco_2	Biomass Prediction	
Date	Run # Sample # Trial #	Cultivation Time (day:hr:min)	Cultivation Time (days)	pH	Sample Volume (mL)	pd + nm + cells (g)	pd + nm (g)	X (g FW/L)	FW g	LN(X)	(sec)	(mmol CO_2/L-hr)	(mmol CO_2/L-hr)	Cultivation Time (days)	X (g FW/L)
10-11-00	12-1-1	0- 3:00 pm	0.00	8.00	500	9.5526	8.9165	1.27	0.6361	0.24	754.9	0.022	0.048	0.00	1.27
10-16-00	12-2-1	5- 9:00 pm	5.25	8.79	500	9.0170	8.2268	1.58	0.7902	0.46	607.7	0.022	0.060	5.25	1.41
10-22-00	12-3-1	11- 4:00 pm	11.04	8.81	500	9.4770	8.4261	2.10	1.0509	0.74	457.0	0.022	0.080	11.04	1.56
10-26-00	12-4-1	15- 11:30 pm	15.35	8.68	500	9.5678	8.4385	2.26	1.1293	0.81	425.2	0.022	0.086	15.35	1.67
10-31-00	12-5-1	20- 11:00 pm	20.33	8.68	500	9.6713	8.3534	2.64	1.3179	0.97	364.4	0.022	0.100	20.33	1.80
11-5-00	12-6-1	25- 5:00 pm	25.08	8.68	500	9.6369	8.2407	2.79	1.3962	1.03	343.9	0.022	0.106	25.08	1.93
11-10-00	12-7-1	30- 6:30 pm	30.15	8.70	500	9.6367	8.2037	2.87	1.4330	1.05	335.1	0.022	0.109	30.15	2.06
11-16-00	12-8-1	36- 3:30pm	36.02	8.76	500	10.0393	8.4834	3.11	1.5559	1.14	308.6	0.022	0.118	36.02	2.22
11-21-00	12-9-1	41- 11:00 pm	41.33	8.66	500	9.7238	8.2636	2.92	1.4602	1.07	328.9	0.022	0.111	41.33	2.35
11-26-00	12-10-1	46- 10:30am	45.81	8.70	500	9.9025	8.4256	2.95	1.4769	1.08	325.1	0.022	0.112	45.81	2.47
12-3-00	12-11-1	53- 2:00 pm	52.96	8.80	500	9.9175	8.3756	3.08	1.5419	1.13	311.4	0.022	0.117	52.96	2.66

Run Identification

Bioreactor Run#: AS-STR-13a

Bioreactor Description:

500 ml Bellco spinner flask

Time started: 3:00 PM

Date started: 12-13-00

Culture Loading

Culture: A. subulata microplantlets

Cell line I.D. As4-28-7,8,9,10,11,12 10/26/00

Age of Inoculum: 48 days

Inoculum cell weight: 1.7806 g FW

ASP12 medium volume 500 mL

Initial cell density: 3.56 g./L

Solid content Ws 250 mg DCW/g FW

Process Parameters

Air supply design: Through 20 um stainless steel sparger#1

Air flow setting: 5 = 46.7 ml/ min

Air flow S/N 026947

impeller design: flat-paddle (d_i = 5.5 cm)

Impeller speed: 120 rpm

Setpoint temperature: 24 °C

$k_L a$ (measured) 51 hr^{-1}

Illuminater position: 10.5 cm from the center

Illumination intensity: 80 $\mu E/m^2.s$

Photoperiod : 10 hr ON / 14 hr OFF

CO_2 growth model

R = 0.08206 L-atm/mol-K

P_{CO2} = 3.50E-04 atm (air)

$Y_{X/CO2}$ = 30.0 mg DCW $mmol^{-1}$ CO_2

μ = 0.036 day^{-1} 0-10 days

H = 0.0346 atm-L/mmol

$k_L a$ = 51 hr^{-1}

n_A = 0.080 mmol CO_2 /L-hr

$k_L a C_A^*$ = 0.498 mmol CO_2 /L-hr

X_c = 2.71 g FW/L

t_c = 0.00 day

f = 10 hr / day

meaured β = 0.2165 g FW / L-day 0-25 days

0.0949 g FW / L-day 10-45 days

theory β = 0.0965 g FW / L-day

Sample Identification				Culture	Biomass Measurements						t_{rxn}	n_A	qco_2	Biomass Prediction	
Date	Run # Sample # Trial #	Cultivation Time (day:hr:min)	Cultivation Time (days)	pH	Sample Volume (mL)	pd + nm + cells (g)	pd + nm (g)	X (g FW/L)	FW g	LN(X)	(sec)	(mmol CO_2/L-hr)	(mmol CO_2/L-hr)	Cultivation Time (days)	X (g FW/L)
12-13-00	13-1-1	0- 3:00 pm	0.00	8.00	500	10.0210	8.2404	3.56	1.7806	0.58	344.1	0.080	0.106	0.00	3.56
12-18-00	13-2-1	5- 4:00 pm	5.04	8.98	500	10.8674	8.4290	4.88	2.4384	0.89	251.3	0.080	0.145	5.04	4.05
12-23-00	13-3-1	11- 1:00 pm	9.92	9.01	500	11.3394	8.4429	5.79	2.8965	1.06	211.5	0.080	0.172	9.92	4.52
12-28-00	13-4-1	15- 6:00 pm	15.13	8.85	500	19.8555	16.4350	6.84	3.4205	1.23	179.1	0.080	0.203	15.13	5.02
1-3-01	13-5-1	21- 5:00 pm	21.08	8.95	500	12.1287	8.2668	7.72	3.8619	1.35	158.6	0.080	0.230	21.08	5.60
1-7-01	13-6-1	25- 8:00 pm	25.21	9.02	500	13.0178	8.4564	9.12	4.5614	1.52	134.3	0.080	0.271	25.21	5.99
1-12-01	13-7-1	30- 4:30 pm	30.06	8.70	500	12.9847	8.3655	9.24	4.6192	1.53	132.6	0.080	0.275	30.06	6.46
1-16-01	13-8-1	34- 11:00pm	34.33	8.78	500	13.0873	8.3492	9.48	4.7381	1.56	129.3	0.080	0.282	34.33	6.88
1-22-01	13-9-1	40- 2:00 pm	39.96	8.78	500	13.055	8.2443	9.62	4.8107	1.57	127.4	0.080	0.286	39.96	7.42
1-29-01	13-10-1	47- 1:30 pm	46.94	8.78	500	13.0612	8.5515	9.02	4.5097	1.51	135.9	0.080	0.268	46.94	8.09

Run Identification

Bioreactor Run#:	AS-STR-13b
Bioreactor Description:	
500 ml Bellco spinner flask	
Time started:	3:00 PM
Date started:	12-13-00

Culture Loading

Culture:	A. subulata microplantlets	
Cell line I.D.	As4-28-7,8,9,10,11,12	10/26/00
Age of Inoculum:	48 days	
Inoculum cell weight:	3.4994 g FW/L	
ASP12 medium volume:	500 mL	
Initial cell density:	1.75 g./L	
Solid content Ws	250 mg DCW/g FW	

Process Parameters

Air supply design:	sparger#2, Applikon	
Air flow setting:	10 =	12.7 ml/ min
Air flow S/N	059360	
impeller design:	flat-paddle (d_I = 5.1 cm)	
Impeller speed:	120	rpm
Setpoint temperature:	24	°C
k_La (measured)	5.6	hr^{-1}
Illuminater position:	10.5	cm from the center
Illumination intensity:	80	$\mu E/m^2.s$
Photoperiod :	10 hr ON / 14 hr OFF	

CO_2 growth model

R =	0.08206	L-atm/mol-K
P_{CO2} =	3.50E-04	atm (air)
$Y_{X/CO2}$ =	30.0	mg DCW $mmol^{-1}$ CO_2
μ =	0.0651 day^{-1}	0-10 days
H =	0.0346	atm-L/mmol
k_La =	11.6 hr^{-1}	
n_A =	0.022	mmol CO_2/L-hr
$k_LaC_A^*$ =	0.113	mmol CO_2/L-hr
X_c =	0.40	g FW/L
t_c =	0.00	day
f =	10	hr / day
meaured β =	0.1470	g FW / L-day
theory β =	0.0262	g FW / L-day

Sample Identification				Culture	Biomass Measurements						t_{run}	n_A	qco_2	Biomass Prediction	
Date	Run # Sample # Trial #	Cultivation Time (day:hr:min)	Cultivation Time (days)	pH	Sample Volume (mL)	pd + nm + cells (g)	pd + nm (g)	FW (g)	X (g FW/L)	LN(X)	(sec)	(mmol CO_2/L-hr)	(mmol CO_2/L-hr)	Cultivation Time (days)	X (g FW/L)
12-13-00	13-1-1	0- 3:00 pm	0.00	8.00	500.0	10.0217	8.2720	1.7497	3.4994	0.56	191.8	0.022	0.190	0.00	3.50
12-18-00	13-2-1	5- 4:00 pm	5.04	8.69	500.0	10.9599	8.5305	2.4294	4.8588	0.89	138.2	0.022	0.264	5.04	3.63
12-23-00	13-3-1	11- 1:00 pm	9.92	8.81	500.0	11.2048	8.4370	2.7678	5.5356	1.02	121.3	0.022	0.300	9.92	3.76
12-28-00	13-4-1	15- 6:00 pm	15.13		500.0	19.7711	16.6787	3.0924	6.1848	1.13	108.5	0.022	0.336	15.13	3.90
1-3-01	13-5-1	21- 5:00 pm	21.08	9.18	500.0	12.0163	8.3926	3.6237	7.2474	1.29	92.6	0.022	0.393	21.08	4.05
1-7-01	13-6-1	25- 8:00 pm	25.21	9.06	500.0	11.8800	8.2207	3.6593	7.3186	1.30	91.7	0.022	0.397	25.21	4.16
1-12-01	13-7-1	30- 4:30 pm	30.06	9.16	500.0	11.7684	8.4037	3.3647	6.7294	1.21	99.8	0.022	0.365	30.06	4.29
1-16-01	13-8-1	34- 11:00pm	34.33	8.98	500.0	11.8378	8.3507	3.4871	6.9742	1.25	96.2	0.022	0.378	34.33	4.40
1-22-01	13-9-1	40- 2:00 pm	39.96	8.79	500.0	11.5586	8.2443	3.3143	6.6286	1.20	101.3	0.022	0.360	39.96	4.55
1-29-01	13-10-1	47- 1:30 pm	46.94	8.86	500.0	11.7753	8.5547	3.2206	6.4412	1.17	104.2	0.022	0.349	46.94	4.73