



Towards high productivities of microalgae in photobioreactors

Rouke Bosma



Towards high productivities of microalgae in photobioreactors

Rouke Bosma

Thesis committee

Thesis Supervisors

Prof. dr. ir. R.H. Wijffels

Professor of Bioprocess Engineering, Wageningen University

Prof. dr. ir. J. Tramper

Professor of Bioprocess Engineering, Wageningen University

Thesis co-supervisor

Dr. ir. M. H. Vermuë

Assistant professor, Bioprocess Engineering, Wageningen University

Other members

Prof. dr. dr. O. Pulz

IGV Institut für Getreideverarbeitung GmbH
Bergholz-Rehbrücke, Germany

Prof. dr. ir. H.H.M. Rijnaarts

Wageningen University

Dr. ir. A.J.B. van Boxtel

Wageningen University

Dr. ir. A.M. Verschoor

Ingrepro BV, Borculo

This research was conducted under auspices of the Graduate school VLAG.

Towards high productivities of microalgae in photobioreactors

Rouke Bosma

Thesis

submitted in fulfillment of the requirements for the degree of doctor

at Wageningen University

by the authority of the Rector Magnificus

Prof. dr. M.J. Kropff

in the presence of the

Thesis Committee appointed by the Academic Board

to be defended in public

on Friday 5 March 2010

at 1:30 p.m. in the Aula.

Rouke Bosma

Towards high productivities of microalgae in photobioreactors

PhD. Thesis Wageningen University, the Netherlands, 2010 – with summary in Dutch and English

ISBN: 978-90-8585-559-0

Voor Evelien

Contents

Chapter 1	Introduction and thesis outline	9
Chapter 2	Prediction of volumetric productivity of an outdoor photobioreactor	15
Chapter 3	Growth inhibition of <i>Monodus subterraneus</i> by free fatty acids	41
Chapter 4	Ultrasound, a new separation technique to harvest microalgae	53
Chapter 5	Marine biotechnology in education: a competitive approach	71
Chapter 6	General discussion	83
References		105
Summary		115
Samenvatting		119
Publications		123
Dankwoord		125
Training activities		129
Curriculum Vitae		131
Support		133

General introduction

Algae produce approximately half of the atmospheric oxygen of our planet and are therefore indispensable for the respiration by man and animals (Hoek et al. 1995). Two types of algae exist: macroalgae, also known as seaweed, and microalgae. In this thesis we address only microalgae. The term “microalgae” refers to microscopic (± 1 to $50\ \mu\text{m}$) eukaryotic algae like *Chlorella* and *Monodus* (Figure 1.1). They range from small, unicellular particles to

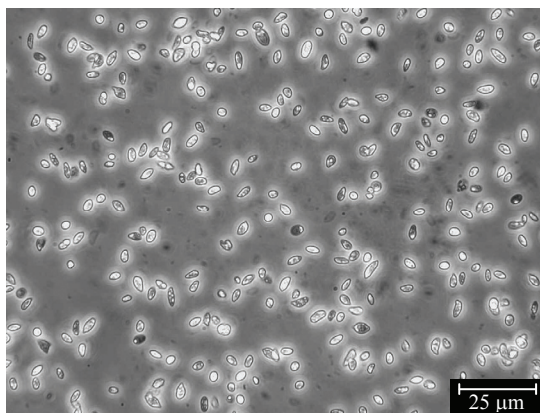


Figure 1.1. *Monodus subterraneus*, a freshwater species, 400x magnified, contrast microscope

more complex aggregated multicellular ones. Unlike higher plants, microalgae do not differentiate and lack specialized organs such as roots, stems, flowers and leaves. However, like higher plants, algae are capable of photosynthesis.

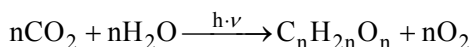
The biodiversity of microalgae is enormous and they represent an almost untapped resource. It has been estimated that about two hundred thousand species exist of which about thirty five thousand species have been described (Hu et al. 2008; Norton et al. 1996). Over fifteen thousand novel compounds originating from algal biomass have been chemically characterized (Cardozo et al. 2007). Most microalgae contain unique products ranging from carotenoids, antioxidants, fatty acids to enzymes, polymers, peptides, toxins, sterols and more (Pulz 2001; Spolaore et al. 2006). These products can be accumulated in the microalgae by changing environmental factors like temperature, illumination, pH, CO_2 supply, salt and nutrients (Richmond 2004a; Wijffels 2008).

The presence of these unique products makes a wide variety of algal applications interesting for agriculture as well as for the pharmaceutical, cosmetic and food industry. They are used as livestock feed and as additives for their high content of vitamins, pigments or essential fatty acids (Becker 1994). Commercial production of microalgae can be considered as an attractive way of transforming light into biomass and into valuable

compounds, therefore algae are presently seen as a potential source for bulk chemicals and as feedstock for biodiesel (Hu et al. 2008, Lehr and Posten 2009).

Photosynthesis

The key process for microalgae to obtain energy is photosynthesis. Photosynthesis is the process of using light energy ($h \cdot \nu$) to fix carbon dioxide into hydrocarbons with discharge of oxygen as waste product:



Photosynthesis consists of light and dark reactions (Figure 1.2). In the light reaction, chlorophyll supported by accessory pigments, also called the photosystem antenna complex, absorbs photons with wavelengths between 400 and 700 nm (Photosynthetic Active Radiation, PAR). This energy is used to generate ATP and NADPH. ATP serves as chemical energy and NADPH serves as reducing power for the dark reactions. In these dark reactions, ATP and NADPH are

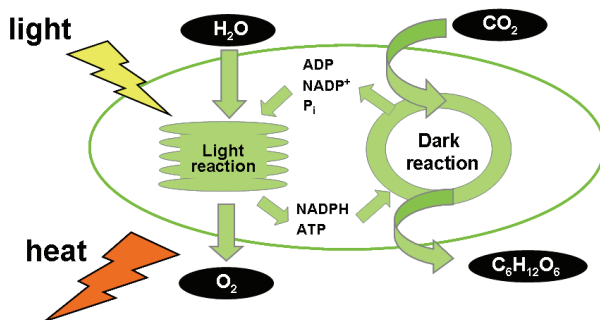


Figure 1.2. Simplified scheme of photosynthesis.

used by enzymes in the Calvin cycle to convert carbon dioxide into organic molecules (Berg et al. 2006). These enzymes are temperature sensitive and therefore predominantly define the optimal cultivating temperature of the species (Fawley 1984). For microalgae, the conversion of light energy into biomass via photosynthesis has a maximum theoretical efficiency of 21% on PAR basis. Since PAR represents 42% of total sunlight, a maximum theoretical photosynthetic efficiency (MTPE) of 9% can be reached with microalgae cultivated on sunlight (Wijffels 2008).

The rate of photosynthesis can be measured by carbon dioxide consumption or oxygen production and is not simply proportional to the rate of photon absorption (Figure 1.3). In darkness, the alga consumes oxygen by converting organic carbon into carbon dioxide and water via dark respiration (R_d). At low light intensities, the photosynthetic rate increases linearly with light intensity and in this linear phase, the alga shows a maximum photosynthetic yield. At higher light intensities the photosynthetic rate levels off. The light intensity at which this occurs depends on the algal species and the adaption of the species to environmental conditions (e.g. temperature and light) (Gordillo et al. 2001; Grobbelaar and

Kurano 2003; Hancke et al. 2008). Eventually, the alga reaches its maximal photosynthetic rate ($P_{\max}$). This phenomenon is called light saturation and at these conditions, the highest productivity of the microalgae is reached. A further increase in light intensity does not result in higher productivity and the surplus of light energy is dissipated in the form of heat. This means that potential energy, which could have been used for cultivating algae, is wasted. At even higher light intensities the overdose of excitation energy can even damage the photosynthetic apparatus in a process called photoinhibition (Richmond 2004a). So, in order to reach highest algal biomass productivities, the algae need to be cultivated at light conditions close to light saturation and photoinhibition has to be prevented.

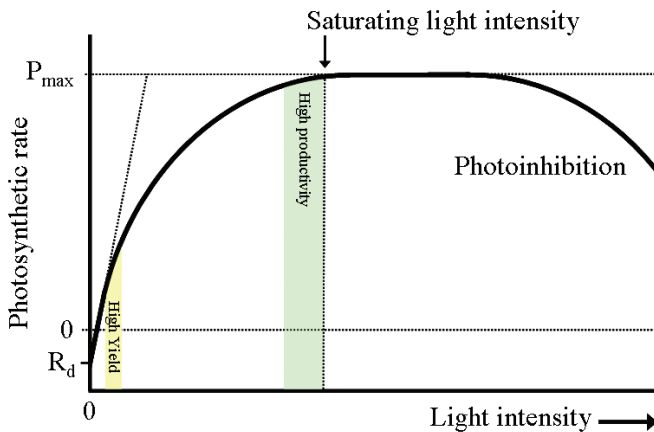


Figure 1.3. Schematic representation of photosynthetic rates vs. light intensity. R_d : dark respiration rate. $P_{\max}$: maximum photosynthetic rate. Adapted from Richmond (2004a).

Commercial production

Currently, there is a niche market for high-value algal products in agriculture, pharmaceutical, cosmetic, and food industry as a source of livestock feed, vitamins, pigments or essential fatty acids (Becker 1994). For these high-value products, algae are produced at relatively small scale. Recently, microalgae have become a hot topic in scientific research as organism to produce bulk products like proteins, polysaccharides and lipids to be used as feedstock for industrial chemical processes and biofuel. However, to make large scale commercial production of these bulk products with microalgae economically feasible, still much research should be done (Figure 1.4).

Nowadays, a few algal species that can be grown under extreme conditions dominate the microalgal market. These species are cultivated in open ponds under extreme conditions (extreme pH, high salinity), thus outcompeting other organisms. However, the conditions in

these open systems are not controlled and low volumetric productivities (productivity per reactor volume per time) and areal productivities (productivity per ground area including empty space per time) are achieved (Borowitzka 1999; Richmond 2004a). Areal productivities reached in these open ponds represent a photosynthetic efficiency of less than 1% (Jimenez et al. 2003a). With microalgae cultivated in closed photobioreactors outdoors, average photosynthetic efficiencies of 2-3% are already obtained (Fernandez et al. 2001; Richmond 2004a; Tredici and Zittelli 1998).

It is of prime importance that much higher areal and volumetric productivities are reached to bridge the gap between real and theoretical maximum efficiency. This can be partly achieved by cultivating microalgae in innovative photobioreactors in which the incident sunlight is diluted to below the saturation light intensity (Lehr and Posten 2009). In addition, more productive strains should be developed or strains should be genetically altered to have better characteristics. Media should be optimized to get highest growth rates of the improved species and maximal product per amount of biomass. To achieve these optimizations, fast screening methods should be developed in which many media or conditions can be tested simultaneously. But, besides getting higher productivities, the complete cultivation process needs to be analyzed in terms of energy and total costs. This chain includes supply of feedstock, cultivation of microalgae but also the harvesting, isolation of products and product derivation into valuable products (biorefinery). Companies that cultivate microalgae on commercial scale should be started and to be commercially successful, personnel should be educated. Finally, products should be identified and the market for these products should be developed (Olaizola 2003).

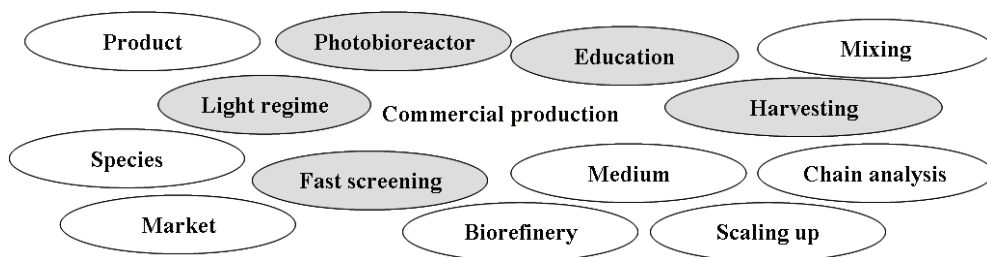


Figure 1.4. Bottlenecks to achieve commercial production of microalgae in photobioreactors. The bottlenecks in gray areas are addressed in this thesis.

Thesis outline

This thesis addresses several of these bottlenecks: light regime, harvesting, development of a fast screening method and education (gray areas Figure 1.4).

It is important to predict how much energy is converted into microalgal biomass. To get better understanding on how light is used in a photobioreactor, we modeled the algal biomass productivity of a bubble column placed outdoors in the Netherlands (Chapter 2). Two extreme modeling approaches were explored and modeled data were compared with real time data. These models can be used to determine minimal and maximal volumetric productivities at any geographical location using the independent data from the lab-scale experiments.

To select strains with better production characteristics, develop optimized media and investigate toxic effects of chemicals, fast screening methods for microalgae are needed. We developed an efficient and fast screening method in which the growth rate of 32 different algae or media can be tested simultaneously in only one week time. This bioassay was used to investigate the growth inhibiting effects of free fatty acids on the microalga *Monodus subterraneus* (Chapter 3).

In commercial processes, the cultivation and downstream processing account both for 40% of the total cost (Shen et al. 2009). Conventional processes to harvest microalgae are centrifugation, membrane filtration and chemical flocculation. These three processes all have their own problems (Ryll et al. 2000). In Chapter 4, we investigated whether ultrasound could be used to harvest microalgae and determined the relevance of this innovative process to harvest microalgae.

To establish a new technology, adequate training of people is needed to get acquainted with this technology. The chief executive officer (CEO) of new companies should focus on personnel issues, identifying educational needs and building relationships with universities to train their personnel (Mogee 1993). Personnel of such companies, but also new researchers in the field of microalgae should get practical and theoretical background. This thesis covers the development of a practical to learn students the basics of designing a complex production process (Chapter 5). In this practical, they learn how to cultivate algae and to understand how different conditions (temperature, light, nutrients) influence the algal growth and the formation of a secondary metabolite such as β -carotene.

As already stated, to produce bulk products with microalgae commercially, productivities should increase drastically. Partly, this can be obtained via the development of low-cost high production systems. Also attention should be paid to media optimization and strain improvement of microalgae. The last chapter in this thesis reviews state-of-the-art photobioreactor developments in algal research and reflects on further steps needed to overcome the bottlenecks that are addressed in this thesis.

Prediction of volumetric productivity of an outdoor photobioreactor

Abstract

Volumetric productivity of *Monodus subterraneus* cultivated in an outdoor pilot-plant bubble column was predicted with a mathematical model. Two extreme approaches to model the photobioreactor were chosen. Firstly, a model with growth integration in which it is assumed that microalgae can adapt immediately to local light conditions. Secondly, full light integration implicating that microalga can convert all absorbed light with a photosynthetic yield based on the average light intensity. Because temperature and light conditions in our photobioreactor changed during the day, photosynthetic yields at any combination of temperature and light intensity were needed. These were determined in repeated-batch lab-scale experiments with an experimental design. The model was evaluated in an outdoor bubble column at different natural light conditions and different temperatures. Volumetric productivities in the bubble column were predicted and compared with experimental volumetric productivities. The light integration model over-estimated productivity, while the model in which we assumed growth integration under-estimated productivity. Light integration occurred partly (47%) during the period investigated. The average observed biomass yield on light was 0.60 g mol^{-1} . The model of partly light integration predicted an average biomass yield on light of 0.57 g mol^{-1} and predicted that productivity could have been increased by 19% if culture temperature would have been maintained at 24°C .

Published as:

Bosma R, van Zessen E, Reith JH, Tramper J, Wijffels RH. 2007. Prediction of volumetric productivity of an outdoor photobioreactor. *Biotechnology and Bioengineering* 97(5):1108-1120.

Introduction

In closed photobioreactors, high volumetric productivities are desired in order to reduce photobioreactor size (Janssen et al. 2003). To study microalgal productivity in the Dutch climate, a pilot-plant bubble column was constructed and operated continuously at different dilution rates outdoors. In a photobioreactor, high light intensities occur at the reactor wall and because of absorption of light by microalgae, light intensities will decrease with increasing radial depth (Figure 2.1A). Individual cells grown in dense cultures experience a fluctuating light environment; they are exposed to light/dark cycles with high light intensities close to the reactor surface and by travelling through a light gradient, they experience darkness in the interior of the photobioreactor.

Our aim was to develop a mathematical model that predicts daily volumetric productivities of this outdoor pilot-plant bubble column in the Dutch climate. Such a model can be used to optimize volumetric productivity, to estimate the effect of temperature control and to predict productivities in other climates. To model the photobioreactor two border cases were chosen, i.e. full light integration and growth integration (Terry 1986). The growth-integrated approach assumed that microalgae that move along the light gradient adapt instantaneously their growth rate to the new conditions (Grima et al. 1996). This means that they experience local light intensities and because of that, photosynthetic yields are calculated with local light intensities. Growth rates are calculated by multiplying absorbed light with photosynthetic yield and these growth rates are integrated. At the reactor wall, where local light intensities are highest (Figure 2.1A), photosaturation or photoinhibition occurs and photosynthetic yields are low (Figure 2.1B). When algae are located more inside the photobioreactor, photosynthetic yields increase until the maximum yield is obtained in the dark interior where local light intensities are lowest.

In the second approach, it is assumed that the reactor is fully light integrated, meaning that algae are adapted to the average light intensity in the photobioreactor and photosynthetic yield is calculated with this average light intensity (Figure 2.1). Light integration takes place when light/dark cycles approach the turnover time of the photosynthetic unit, the time it takes to convert one molecule of carbon dioxide (Richmond et al. 2003). This phenomenon was called the flashing light effect and was observed at light/dark cycles smaller than several ms and a light/dark ratio of about 1:10 (Janssen et al. 2001; Kok 1956; Qiang et al. 1998). In this approach, overall microalgal productivity is high because photosaturation and photoinhibition are prevented (Grima et al. 1997; Richmond et al. 2003; Terry 1986).

We chose as model organism the freshwater algae species *Monodus subterraneus*, which can produce eicosapentaenoic acid at a 4% w/w concentration (Cohen 1994; Cohen 1999).

Eicosapentaenoic acid has therapeutic potential in the treatment of cardiovascular problems, a variety of cancers and inflammatory diseases (Simopoulos 2004). *Monodus subterraneus* is generally grown between temperatures of 23-32 °C (Cohen 1994; Cohen 1999; Lu et al. 2002; Qiang et al. 1997; Richmond et al. 2003; Vonshak et al. 2001). The optimal temperature to grow this species was not known. In addition, it was not known how light and temperature affected photosynthetic yield. To be able to model the outdoor photobioreactor, a central composite design was used to determine photosynthetic yields at different combinations of temperature and light intensities in lab-scale bubble columns. The response area was fitted by a second-order polynomial function and this function was used to predict photosynthetic yields at any combination of temperature and light intensity.

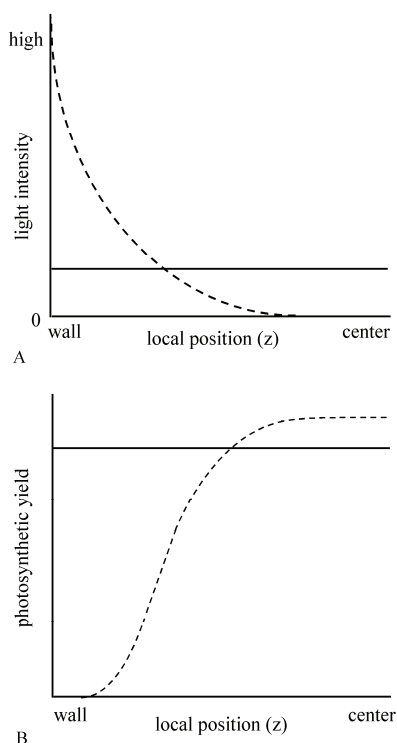


Figure 2.1

- A. Light intensity at different positions in a photobioreactor. Dotted line: local light intensities. Solid line: average light intensity.
- B. Photosynthetic yield at different positions in a photobioreactor for both modeling approaches. Dotted line: growth integration; solid line: light integration.

Finally, predicted volumetric productivities via both approaches were compared with measured volumetric productivities and the amount of light integration in our photobioreactor was determined. We used the model to predict the increase in photosynthetic yield if culture temperature in our photobioreactor would have been maintained at 24 °C.

Materials and methods

First, the outdoor pilot-plant bubble column to be modeled is presented. After that, both modeling approaches are addressed. Then, the methods to determine the effects of temperature and light intensity on photosynthetic yield in lab-scale experiments are given. Finally, the method to calculate measured volumetric productivity in the outdoor bubble column is given.

Outdoor experiments

Pilot-plant bubble column

A Plexiglas bubble column with dimensions (H x D) 2.0 x 0.21 m was constructed on a roof at the Energy research Centre of the Netherlands (ECN) located in Petten, The Netherlands (52° 46' N, 4° 40' E) (Figure 2.2). An overflow tube was placed at a height of 1.85 m giving a culture volume of 63 L. The photobioreactor was diluted from sunrise to sunset with dilution rates between 0.03 to 0.38 d⁻¹. The pump (Iwaki metering pump) was turned off during the night. Carbon dioxide enriched air (gas velocity 10 L min⁻¹) was supplied via mass flow controllers (Brooks Instruments) and sterile filtered with a 0.2 µm filter (Acro®50 Vent filter, Pall). Temperature was continuously measured with a thermocouple and when culture temperature rose above 28°C, a thin water film was sprayed over the reactor wall to cool the algal culture. On top of the reactor, a LI-COR 190-SA 2π quantum sensor measured total horizontal solar radiation within Photosynthetic Active Radiation (PAR) range (400-700 nm). Biomass concentration was measured with a turbidity sensor (Solids Content Sensor CUS41-W, Endress & Hauser) that was calibrated with off-line dry-weight determinations. pH was measured with a Yokogawa electrode (SC21/AGP24) and dissolved oxygen was measured with an AppliSens Dissolved Oxygen sensor (APS101). Measured data was stored using the data acquisition program WizCon for off-line analysis.

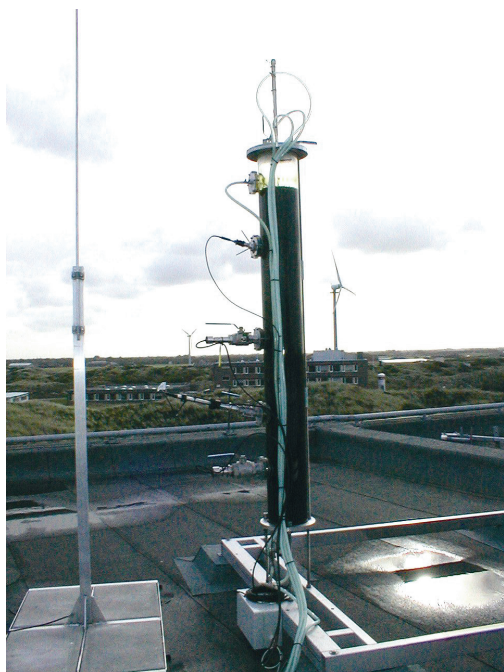


Figure 2.2. Outdoor pilot-plant bubble column.

Organism and cultivation conditions

Monodus subterraneus UTEX 151 was obtained from the University of Texas Culture Collection and cultivated in test tubes containing BG-11 medium (Rippka et al. 1979) containing 1% agar. The cultures were grown in a light climate cabinet at a temperature of 25 °C, a light intensity of $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ and a 16h/8h day/night rhythm. After growing, the algae were transposed to 250 mL Erlenmeyer flasks containing adjusted BG-11 medium and grown under the same conditions. In the modified medium iron-ammonium-citrate was replaced by iron-chloride and citric acid was omitted to prevent bacterial growth. Medium was pumped through a sterile $0.2 \mu\text{m}$ filter (Capsule filter, Pall) before entering the reactor.

Mathematical model

Short overview

Our goal was to predict volumetric daily productivity in the outdoor bubble column from measured total solar radiation on a horizontal surface, temperature and biomass concentration. The general structure of our model is shown in Figure 2.3. The model was programmed in Mathcad 11.0.

Measured total horizontal solar radiation (also called global irradiance) is the sum of incident diffuse radiation and direct normal irradiance projected onto the horizontal surface. Direct light is characterized by having a specific direction while diffuse light is characterized as coming from all directions. In our model, measured total horizontal light intensities were first converted to direct and diffuse horizontal light intensities. These direct and diffuse light intensities were converted to direct and diffuse light intensities falling on the surface inside the vertical photobioreactor. Light gradients inside the photobioreactor were calculated yielding local light intensities in the photobioreactor. The growth integrated approach used these local light intensities to determine local photosynthetic yields. Local absorbed light was multiplied by local photosynthetic yields to obtain local productivities; these were summed to get total productivity. The integrated approach started by calculating the average light intensity in the photobioreactor from local light intensities. Then, this average light intensity was used to calculate average photosynthetic yield. Productivity was calculated by multiplying the total amount of absorbed light inside the photobioreactor by this average photosynthetic yield. In both modeling approaches, for each half hour, productivity was calculated. These productivities were summed and divided by reactor volume to get the daily volumetric productivity of the photobioreactor.

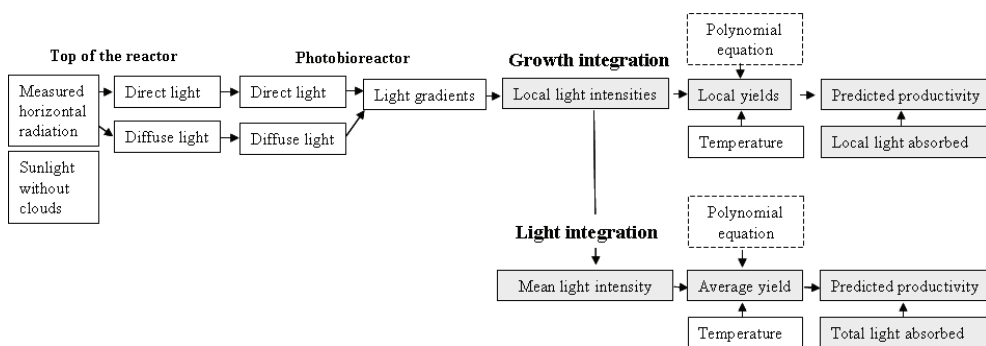


Figure 2.3. Schematic overview of both modeling approaches. Grey boxes indicate were both models differ. Dotted boxes indicate the polynomial function of photosynthetic yield that was determined in lab-scale experiments.

Conversion of measured total horizontal irradiance in a diffuse and direct component

First, the theoretical amount of sunlight, which would have been measured if no clouds were present, was calculated (Velds 1992). Total horizontal radiation, consisting of a diffuse and a direct fraction depending on cloudiness, was measured on top of the reactor each half hour. This measured total horizontal radiation was compared with the theoretical amount of sunlight that would fall on the sensor if no clouds had been present. Via equations derived by de Jong for De Bilt (Netherlands), described in Velds (1992), measured total radiation was split into a direct and diffuse component, which made it possible to calculate light gradients in our reactor.

Calculation of light at the photobioreactor walls

Light could not enter the photobioreactor via the horizontal area at the top of the reactor. For that, light falling on the reactor top was converted to the amount of light falling on the vertical surface of the bubble column (Camacho et al. 1999). With a combination of Fresnel's Law, Snell's Law and refractive indices, the amount of light entering the photobioreactor at the walls was calculated (Heel 1964). These light intensities at the wall were used to calculate light gradients inside the photobioreactor.

Light gradients

With the amount of diffuse light at the wall, biomass concentration and absorption coefficient known, a diffuse light gradient inside the photobioreactor was calculated (Evers 1991). Camacho determined the direct light path (p_{direct}) for radial positions using sun altitude and refractive indexes of air and water (Camacho et al. 1999). This equation was adapted to determine local light intensities (I_{dir}) for each half hour (a), photobioreactor angle (b) and changing locations (z) as shown in Equation 1, which is further explained in Figure 2.4.

$$I_{\text{dir}}(a,b,z) = I_{\text{dir}}(a,b + \Delta b(a,b,z)) \cdot e^{[-\alpha \cdot X \cdot p_{\text{direct}}(a,b,z)]} \quad (1)$$

Where α is the corrected absorption coefficient, X is biomass concentration and I_{dir} is the direct light intensity falling on the wall. Adaptation was needed because local productivities in the no light integrated approach were first summed for each local position in a radial position, while Camacho integrated light intensity over the whole reactor starting with radial position.

A sunlight corrected absorption coefficient of $0.21 \text{ m}^2 \text{ g}^{-1}$ was used that was measured in lab-scale experiments at $23.5 \text{ }^\circ\text{C}$ and $50 \text{ } \mu\text{mol m}^{-2}\text{s}^{-1}$. This absorption coefficient was chosen because a light intensity of $50 \text{ } \mu\text{mol m}^{-2}\text{s}^{-1}$ was about the average light intensity (integrated over the whole reactor) that was experienced by the microalgae during cultivation in our photobioreactor. It was assumed that cells adapted to this average light intensity because acclimation processes are much slower than the light/dark cycles in the photobioreactor (Torzillo et al. 2003; Zonneveld 1998). It was also assumed that the absorption coefficient was not affected by temperature differences.

Productivity

In the growth integrated approach, local absorbed light (I_{locabs}) was calculated by taking the difference between two local light intensities. To calculate productivity, local absorbed light was multiplied by the average photosynthetic yield (Y_{av}) between these two locations and illuminated photobioreactor surface (A_{light}). Productivity was summed for locations (z), radial directions (b) and finally for each half hour (a) (Eq. 3).

$$P_{growth} = \sum_a \sum_b \sum_z Y_{av}(T(a), I_{s_{tot}}(a, b, z)) \cdot I_{locabs}(a, b, z) \cdot A_{light} \cdot 0.5hr \quad (3)$$

In the approach of light integration, the total amount of absorbed light ($\sum_z I_{locabs}(a, b, z)$) was multiplied by average photosynthetic yield ($Y(T, I_{av_{tot}})$) and illuminated photobioreactor surface and summed for each half hour (Eq. 4).

$$P_{int} = \sum_a \left[Y(T(a), I_{av_{tot}}(a)) \cdot \sum_b \sum_z I_{locabs}(a, b, z) \cdot A_{light} \cdot 0.5hr \right] \quad (4)$$

Average weighed temperature

Temperature in our photobioreactor was measured each half hour. It affected photosynthetic yield and thus productivity if light was available. If more light was available, and potentially more algae could be produced, temperature had a larger effect on productivity. To account for that, instead of taking the average temperature during the day, an average weighed temperature was calculated (Eq.5).

$$T_{weighed} = \frac{\sum T \cdot I}{I_{average}} \quad \text{for } I > 0 \mu\text{mol m}^{-2} \text{ s}^{-1} \quad (5)$$

Model criteria

Days from 18 July until 26 October 2001 were modeled if the average weighed temperature during the light period was between 17.5 °C and 29.5 °C and biomass concentration at sunrise did not deviate more than 10% from biomass concentration at sunset. Criteria of temperature were established because outside these temperatures photosynthetic yield could not be predicted accurately by the polynomial equation (Eq. 6). Criterion of 10% deviation of biomass concentration was chosen, because our model assumed steady state during the day. With these criteria, 72 of in total 98 days were modeled.

Determination of photosynthetic yield in lab-scale experiments

Experimental design

A central composite design was chosen to determine photosynthetic yield as a function of temperature and light intensity. Both parameters were varied at five levels. The centre of the experimental domain was measured four times to estimate repeatability of experimental measurements. Table 2.1 shows parameters and their minimal and maximal tested levels. The program Design-Expert version 6 was used to construct, analyze and optimize the design. Photosynthetic yield was fitted with the following polynomial equation:

$$Y = b_0 + b_1 \cdot X_1 + b_2 \cdot X_2 + b_3 \cdot X_1^2 + b_4 \cdot X_2^2 + b_5 \cdot X_1 \cdot X_2 \quad (6)$$

Table 2.1. Parameter levels in central composite design.

Parameter	Unit	Coded value	Level $-\sqrt{2}$	Level -1	Level 0	Level +1	Level $\sqrt{2}$
Light intensity	$\mu\text{mol m}^{-2}\text{s}^{-1}$	X_1	50	270	800	1330	1550
Temperature	$^{\circ}\text{C}$	X_2	17.5	19.3	23.5	27.8	29.5

In this equation, Y is the predicted response and X_i variables are coded values of the parameters. The b values correspond to estimated polynomial coefficients: b_0 is the intercept term, b_1 and b_2 represent the main effect for each variable, b_3 and b_4 describe square effects and b_5 describes the interaction effect between temperature and light. Statistical significant coefficients were estimated by the method of backward stepwise elimination ($P \leq 0.05$). To establish model hierarchy, the linear term of a parameter was introduced when an interaction or a square effect of that parameter was significant.

Organism and cultivation conditions

In lab-scale experiments, the same strain was used as outdoors. Medium was not sterile filtered, but medium compounds except iron and phosphate were mixed from stock solutions and heat sterilized at 121 $^{\circ}\text{C}$. Separate from this solution, phosphate solution was heat sterilized and iron solution was sterilized by filtration (0.2 μm). Phosphate and iron solution were added aseptically before inoculation.

Experimental set-up

Monodus subterraneus was grown in repeated-batch mode in small bubble columns (450 mL) with an internal diameter of four cm. Ten Sylvania CF-LE 55W dimmable fluorescent lamps provided light. A 16/8h day/night rhythm was applied in all experiments. Air was

sparged through each reactor with a flow of 2 L min^{-1} by using flow controllers (Brooks, GT1357). Reactors were kept at the desired temperature $\pm 0.1^\circ\text{C}$ by water baths. In all experiments, pH was maintained at 8.0 ± 0.4 by adding pure carbon dioxide via a pump. Before measuring growth rates, conditions were slowly changed to new ones and then cultures were allowed to adapt for two weeks to these new conditions.

Optical density

The optical densities at 530 nm (OD_{530}) and 680 nm (OD_{680}) were measured as absorbance on a spectrophotometer (Spectronic 20, Genesys) against medium as blank. Samples reaching an absorbance above 0.9 were diluted with medium.

Specific growth rate

Specific growth rates (μ) were calculated by linear regression of the natural logarithm of OD_{530} versus time between OD_{530} values of 0.05 and 1. Between these values, microalgae grew exponentially with a constant growth rate showing that no light limitation occurred. For the lowest light intensity ($50 \mu\text{mol m}^{-2}\text{s}^{-1}$), specific growth rate was determined between OD_{530} values of 0.05-0.5, because at higher biomass concentrations linear growth was observed. At least five OD_{530} measurements were done per specific growth rate calculation. When the first run had a significantly lower growth rate than succeeding runs, the first run was disregarded because the organism was still not adapted.

Photosynthetic yield

At the end of a batch run, biomass was used to determine the specific absorption coefficient (Janssen et al. 2000). Instead of protein dry weight, biomass dry weight was used to determine the specific absorption coefficient on dry weight basis (Dubinsky et al. 1986). In the repeated-batch runs, the relative spectral distribution of the Sylvania lamps was used for yield calculations; this distribution was determined from 400 to 750 nm with 0.5 nm interval with a SR9910 spectroradiometer (Macam, UK). The relative spectral distribution of sunlight (Wozniac et al. 2003) was used to determine photosynthetic yields of our microalgae on sunlight. These yields were used to model volumetric productivity of our outdoor pilot-plant bubble column.

Dry weight determination

A membrane filter (Schleicher & Schuell, NC45) was dried at 80°C for at least 12 hours. It was placed in a desiccator to cool to room temperature. It was weighed and 10 mL of the same solution as used for the specific absorption coefficient (OD_{530} of 1) was filtrated under

vacuum for 5 minutes. Then again, the filter was dried at 80°C for at least 12 hours, allowed to cool to room temperature in a desiccator and weighed.

Photon flux density

Photo flux density (PFD) was measured in the PAR-range (400-700 nm) with a LI-COR 190-SA 2 π sensor at both sides of reactor and averaged.

Biomass yield

With specific growth rate, absorption coefficient and light intensity known, photosynthetic yield was calculated (Eq. 7) representing the ratio of biomass production over energy consumption, including maintenance requirements (Janssen, 2002).

$$Y_{x,E} = \frac{\mu}{I \cdot \alpha \cdot 10^{-6} \cdot 3600 \cdot 16} \quad (\text{g} \cdot \text{mol}^{-1}) \quad (7)$$

This equation should be used for optically thin cultures. Here, specific growth rate was determined from several measurements in a repeated-batch run and it was assumed that the amount of light absorbed was constant. However, a light gradient occurred at the end of the batch phase, which could lead to an under estimation of photosynthetic yield.

Model validation

To validate predicted volumetric productivities, measured productivities were calculated by Eq. 8 taking into account biomass loss via the effluent and biomass accumulation.

$$P_{\text{measured}} = D \cdot t \cdot \left(\frac{X_{\text{sunset}} - X_{\text{sunrise}}}{2} \right) + V \cdot (X_{\text{sunset}} - X_{\text{sunrise}}) \quad (8)$$

Results and Discussion

Determination of photosynthetic yield

To be able to model the outdoor pilot-plant bubble column, photosynthetic yields at any combination of temperature and light intensity had to be known. These were determined in lab-scale experiments with a central composite design and a second-order polynomial function was derived.

Lab-scale experimental results

Table 2.2 shows the number of repeated batches (n), measured growth rates (μ), absorption coefficients (α) and calculated photosynthetic efficiencies of experiments (Y) with 95% confidence intervals. Yield was corrected with the relative spectral distribution of Sylvania lamps that were used to grow the microalga. Mostly, specific growth rate was constant over different runs, implicating that algae adapted to the new conditions. Then, average growth rate over the sequential batches was taken. However, in four cases, growth rates dropped during sequential batches and algae died within four batches. In those cases, algae were not able to adapt to the new conditions and growth rate was set to zero.

Table 2.2. Measured experimental values of the central composite design.

run	Temperature °C	Light intensity $\mu\text{mol m}^{-2}\text{s}^{-1}$	μ day ⁻¹	Conf. interval day ⁻¹	α m ² g ⁻¹	Conf. interval m ² g ⁻¹	Y g mol ⁻¹	Conf. interval g mol ⁻¹	n
1	19.3	270	0.50	0.01	0.11	0.02	0.29	0.07	4
2	27.8	270	0.69	0.07	0.11	0.03	0.40	0.14	8
3	19.3	1330	0		0.08		0		
4	27.8	1330	0.42	0.15	0.06	0.01	0.09	0.05	5
5	23.5	800	0.56	0.08	0.09	0.02	0.14	0.05	7
6	23.5	800	0.61	0.05	0.09	0.02	0.15	0.05	7
7	29.5	800	0		0.04		0		
8	17.5	800	0		0.07		0		
9	23.5	1550	0		0.08		0		
10	23.5	50	0.41	0.08	0.20	0.02	0.73	0.21	4
11	23.5	800	0.62	0.03	0.05	0.03	0.25	0.15	4
12	23.5	800	0.56	0.09	0.07	0.04	0.18	0.14	4

Statistical model

Model coefficients were estimated using Design-Expert version 6 and Equation 6. Figure 2.5 shows that the polynomial fit predicted photosynthetic yield well with an R^2 of 0.92.

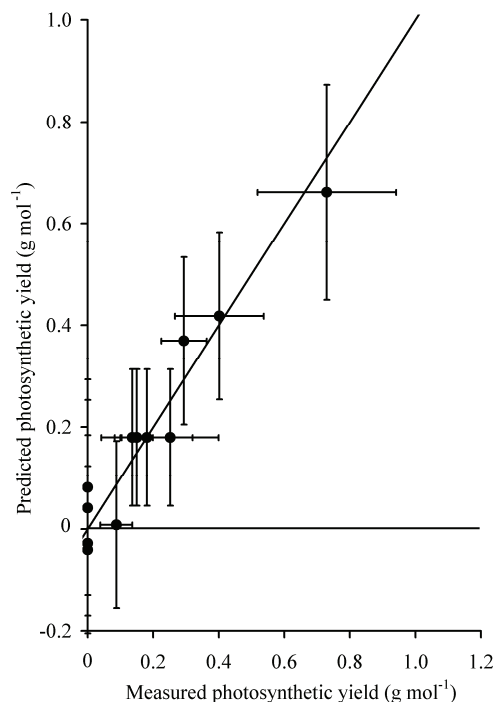


Figure 2.5. Parity plot of measured photosynthetic yield vs. predicted photosynthetic yield. The solid line presents a perfect match. Error bars are 95% confidence intervals.

The ANOVA (analysis of variance) for the model had four degrees of freedom, F-value of 20.3 and probability value of 0.0005. Table 2.3 shows the ANOVA table for model coefficients that were corrected with the relative spectral distribution of sunlight.

Table 2.3. ANOVA table for coefficients of photosynthetic yield.

Coefficient	Parameter	Value	F-value	P-value
b_0	Constant	-1.70		
b_1	X_1	$-9.45 \cdot 10^{-4}$	57.2	0.0001
b_2	X_2	0.20	0.8	0.39
b_3	$X_1 \cdot X_1$	$3.60 \cdot 10^{-7}$	12.2	0.011
b_4	$X_2 \cdot X_2$	$-4.07 \cdot 10^{-3}$	6.4	0.021

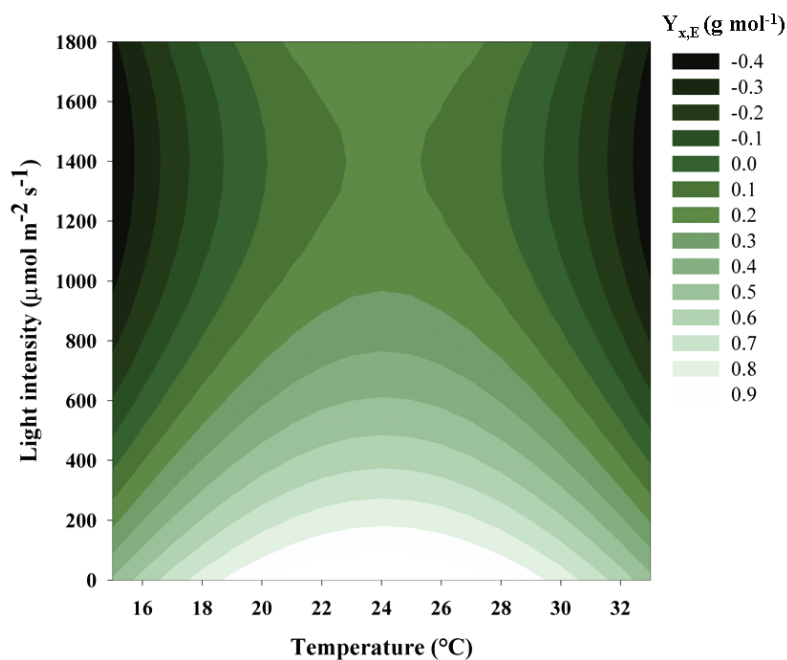


Figure 2.6. Surface plot showing effects of light intensity and temperature on photosynthetic yield.

Influence of light and temperature on photosynthetic yield

Photosynthetic yields were affected by light and temperature (Figure 2.6). Especially light had a large effect on photosynthetic yield, shown by its low probability values (P-value linear effect 0.0001, P-value squared effect 0.010). The optimal photosynthetic yield for *Monodus subterraneus* was predicted at a temperature of 24 °C and a low light intensity. Microalgae can adapt to low light intensities by increasing their antenna size or increase the amount of antennas (Gordillo et al. 2001). At these low light intensities, microalgae try to capture all photons available and are able to convert this absorbed light into carbohydrates. This gives a maximum photosynthetic yield because every photon is captured and converted into biomass and no photons are wasted in the form of heat. At higher light intensities, algae cannot convert all the light because too much light is received. This leads to waste of energy in the form of heat, thus resulting in lower photosynthetic yields. At high light intensities photoinhibition occurred, giving low growth rates. At even higher light intensities, culture death occurs. Photoinhibition occurs when the photosynthetic apparatus is exposed to excessively high irradiances and photooxidation degrades protein D1 in photosystem II (Gordillo et al. 2001; Han et al. 2000). One should realize that diluted cultures were used and so each organism was subjected constantly to high light intensities.

At sub-optimal temperatures, light was absorbed by photosystems, but could not be converted to carbohydrates by enzymes due to lower enzyme activities (Kirk 1994). Photoinhibition occurred and light energy was wasted in the form of heat and photosynthetic yield dropped severely.

Modeling results

As stated before, our goal was to predict volumetric daily productivities in an outdoor bubble column. To validate the model, predicted productivities of both approaches were compared with measured productivities and the amount of light integration was determined. With the final model, the influence of parameters on productivity was determined. Finally, the final model was used to predict the effect of temperature control on productivity.

Model validation

Between 18 July and 26 October 2001, 73% of all days were modeled. Cloudy and cloudless days were included; biomass concentrations ranged between 0.4 and 1.4 g L⁻¹, and weighed temperature varied between 17.5 and 29.5 °C. To validate the model, predicted productivities were compared with measured volumetric productivities (Figure 2.7).

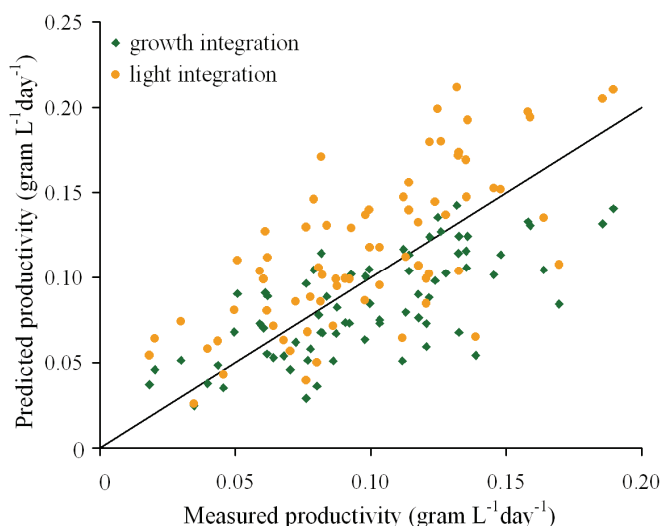


Figure 2.7. Parity plot of measured productivity vs. predicted productivity by both modeling approaches. The solid line presents a perfect match.

Measured productivities ($0.03\text{--}0.20\text{ g L}^{-1}\text{d}^{-1}$) were in the same range as reported previously in which *Monodus subterraneus* was grown in a similar bubble column (Lu et al. 2002). The light integration approach over-predicted productivity because in this model no photoinhibition occurred while the growth integrated approach under-predicted productivity because it over-estimated photoinhibition. Grima et al. described a model, also based on average irradiance, which used an affinity constant and a fitting parameter to determine the amount of photoinhibition and its effect on productivity (Grima et al. 1996). With the models described here, from independent lab-scale experiments, productivities can be estimated if the amount of sunlight, geographical location, day in the year and temperature are known. Because both border cases are modeled, insight is obtained about minimal and maximal productivities reachable without the need to measure productivities. The amount of light integration can be determined if volumetric productivities are measured or can be estimated using literature data (Terry 1986).

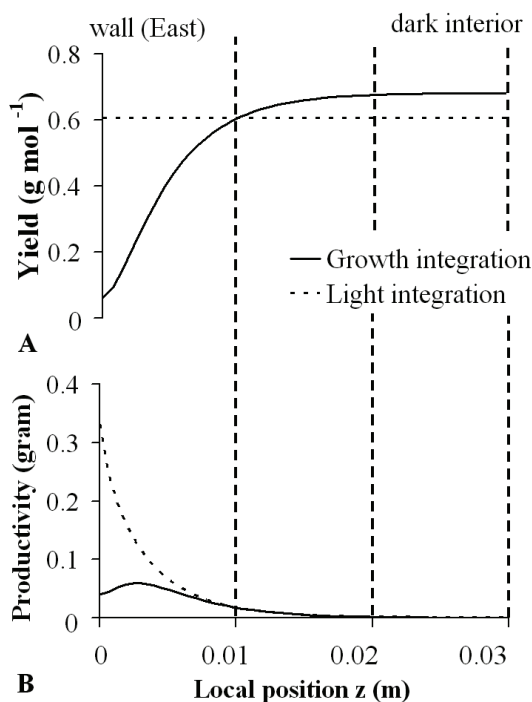


Figure 2.8.

A. Photosynthetic yield vs. position in the photobioreactor for both modeling approaches.

B. Productivity vs. position in the photobioreactor for both modeling approaches.

Model predictions are shown for 22 July 2001 10:00 A.M. for the first 3 cm in our bubble column outdoors in the East position ($b = -90^\circ$).

Figure 2.8 shows local productivities at a certain time in the reactor for both models. The light integrated approach assumes that light/dark cycles are sufficiently fast and the flashing light effect occurs. This means that microalgae can convert absorbed light during the time that they travel in the dark interior of the bubble column with a high photosynthetic yield based on the average light experienced in the light gradient (Figure 2.8A). Productivity in the first five mm is high, because much light is available and this light is converted with a high photosynthetic yield because photoinhibition is absent (Figure 2.8B). The other approach, in which no light integration occurs, assumes that absorbed light is converted into biomass with a photosynthetic yield based on the light intensity experienced at that position. This means that in this approach, photosynthetic yield at the wall ($z = 0.105\text{m}$) is lowest, because at this position light intensity is maximal and photoinhibition occurs (Figure 2.6). Because light is absorbed by the microalgae, less light becomes available when moving to the dark interior and photosynthetic yields increase until it is maximal (Figure 2.8A). Productivity is severely reduced in the growth integrated approach because much energy is wasted in the form of heat, because light intensity in the first five mm is excessively high (Figure 2.8B).

Measured productivities were between both modeling approaches. It was found that at light/dark cycle times below 100 ms, full light integration occurred and high photosynthetic yields could be obtained (Qiang et al. 1998; Terry 1986). However, in our reactor, light/dark cycles of an order of seconds were present, implicating that full light integration could not occur. By illuminating diluted cultures with a cycle time of four seconds, about 30% light integration took place (Terry 1986). We calculated the amount of light integration by minimizing the absolute error in volumetric productivity for the period investigated. In our reactor, 47% light integration occurred, which is a bit higher than the value reported by Terry. This was probably because some mixing occurred between the different light zones and consequently, more light integration was obtained. This model of partly light integration, predicted measured productivity well if it is taken into account that during each day, temperature and light fluctuated continuously and photosynthetic yield was calculated with a polynomial function obtained by independent lab-scale experiments (Figure 2.9).

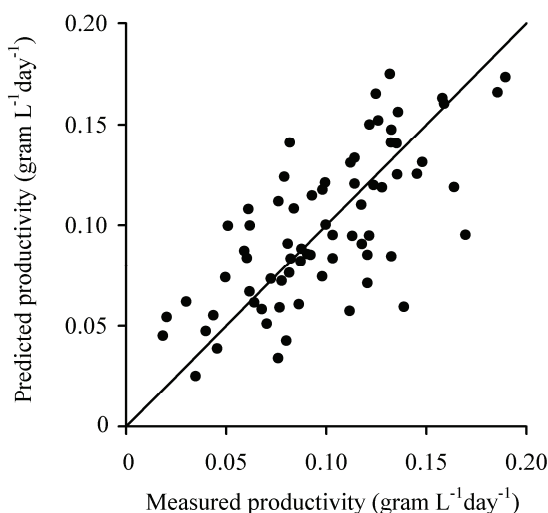


Figure 2.9. Parity plot of measured productivity vs. predicted productivity for the model of partly light integration (47%). The solid line presents a perfect match.

Photosynthetic yield

Measured averaged photosynthetic yield for the period investigated was 0.60 g mol^{-1} . As mentioned earlier, the light integrated approach over-predicted productivity as shown by its photosynthetic yield of 0.67 g mol^{-1} . The growth integrated approach under-predicted productivity and predicted a yield of 0.49 g mol^{-1} . With the model of partly light integration, an average photosynthetic yield of 0.57 g mol^{-1} was obtained.

Sensitivity analysis absorption coefficient

Three external parameters influenced productivity: biomass concentration, temperature and light intensity. As mentioned in the model description, absorption coefficient was set (for all days) at $0.21 \text{ m}^2 \text{ g}^{-1}$. This parameter was used to calculate light gradients in our reactor. To evaluate if it was justified to use this absorption coefficient, eight days were selected that had minimal and maximal values for the parameters affecting productivity. For these days, the absorption coefficient value was halved and doubled and the effect on productivity was determined. It was found that the maximum relative error on predicted productivity was 4.2%. Therefore, it was justified to use a constant absorption coefficient of $0.21 \text{ m}^2 \text{ g}^{-1}$.

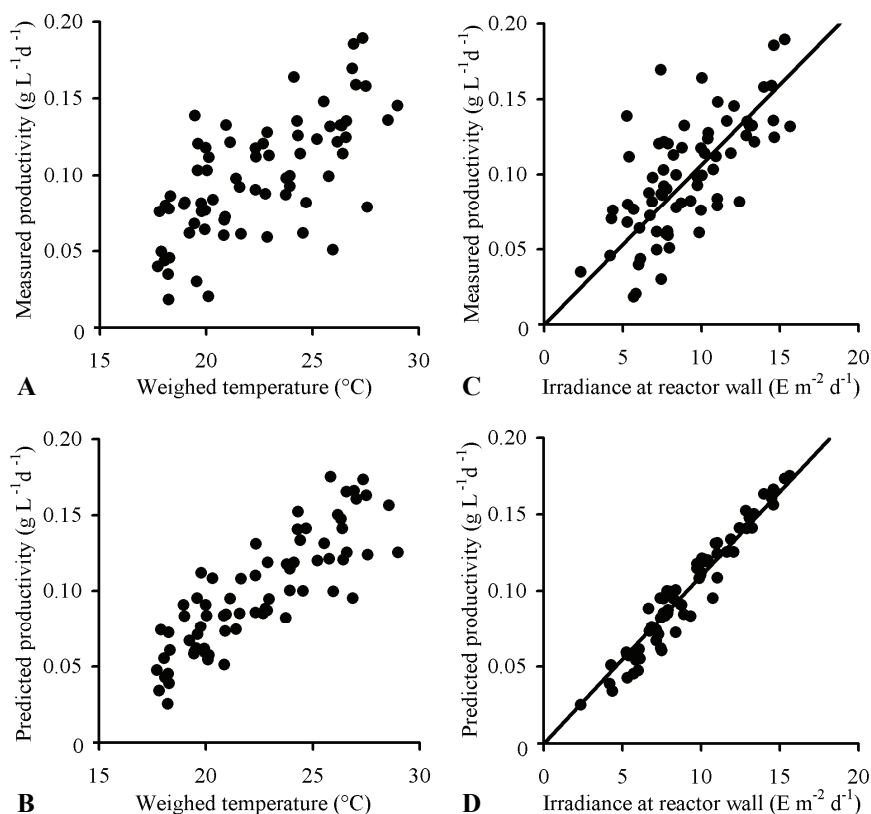


Figure 2.10. Daily volumetric productivity versus biomass concentration and weighed temperature.

- A. Measured productivity versus weighed temperature.
- B. Predicted productivity versus weighed temperature.
- C. Measured productivity versus irradiance falling on the reactor wall.
Regression line with intercept set at zero $y = 0.011x$; $R^2 = 0.43$
- D. Measured productivity versus irradiance falling on the reactor wall.
Regression line with intercept set at zero $y = 0.011x$; $R^2 = 0.94$

Parameters influence on volumetric productivity.

Because biomass concentration, temperature and light intensity changed each day, it was investigated if productivity was correlated to those parameters. As known from other work, at controlled conditions, an optimum of biomass concentration was found where productivity was highest (Qiang et al. 1997; Richmond et al. 2003). Here, no biomass optimum was observed, because light and temperature had more impact on algal

productivity than biomass concentration. For weighed temperature, measured productivities had a positive relation but varied widely (Figure 2.10A). Predicted productivities showed also a positive relationship between weighed temperature and productivity, but we could not distinguish if the relationship was linear or exponential (Figure 2.10B). However, this figure shows that by controlling temperature higher productivities can be attained.

Measured productivity showed a direct correlation with light intensity, implicating that our reactor was limited mainly by light (Figure 2.10C). This same correlation was also reported in outdoor cultures of other microalgae (Qiang et al. 1998; Tredici et al. 1991; Zhang et al. 1999; Zittelli et al. 1996). Zhang also showed that if temperature was not controlled, like here, that this correlation was less pronounced due to the interaction effect between temperature and light intensity. Our model predicted this linear relationship very well (Figure 2.10D) showing that also our model predicted that this photobioreactor was mainly limited by light.

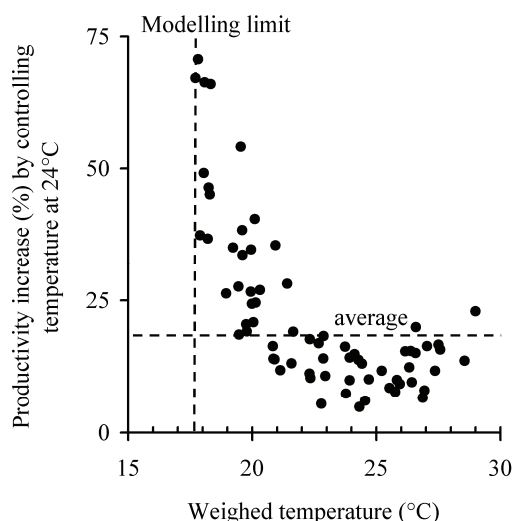


Figure 2.11. Percentage of increase in predicted productivity (with the model of partly light integration) versus average weighed temperature of the culture during the daylight period when culture temperature would have been controlled at 24 °C.

Controlling temperature

Temperature control can be used to enhance volumetric productivity by growing the species at its optimal temperature constantly. Here, it was predicted how much effect temperature control has on volumetric productivity using the partly light integrated model (Figure 2.11). Productivity could be increased by 19% for all days modeled and an average photosynthetic

yield of 0.69 g mol^{-1} could have been reached. This was close to the photosynthetic maximum of our species (0.72 g mol^{-1} , Figure 2.5) and consequently a bubble column is a good photobioreactor to grow *Monodus subterraneus* in the Dutch climate. Firstly, this is caused by the vertical arrangement of the bubble column that prevents high light intensities during noon and by that photoinhibition (Camacho et al. 1999). Secondly, light intensities in the Dutch climate are in comparison to southern countries a factor two lower. In countries where more light is available, photoinhibition reduces productivity much more (Lu et al. 2002). In those countries, it is better to use a photobioreactor with a smaller optical path in which more light integration is obtained and photoinhibition can be prevented (Qiang et al. 1998).

Temperature control had only a small effect when the average temperature of the culture during the daylight period was higher than 22°C (Figure 2.11). However, at temperatures below 22°C , during spring and autumn, controlling temperature can have a large effect because photosaturation and photoinhibition, due to lower activity of the enzymes in the Calvin-Benson cycle, was prevented. In countries with a warmer climate than the Netherlands, for example Israel, also heating in the morning can be used (Vonshak et al. 2001). They reported, by only heating culture for 2 hours in the morning to 28°C , a sixty percent increase of daily productivity. They found a higher increase in volumetric productivity than we did because light intensities in their case were much higher (up to $2000 \mu\text{mol m}^{-2}\text{s}^{-1}$) and by heating their culture, photoinhibition was prevented.

Conclusion

Photobioreactors operated outdoors are mostly limited by sunlight, which is determined by geographic location. The models described in this paper can be used to determine minimal and maximal volumetric productivities at any geographical location from independent lab-scale experiments. Ideally, full light integration is obtained because then productivity is maximal, photoinhibition and photosaturation are then prevented. In our photobioreactor, partly light integration (47%) occurred because light/dark cycles were too long to obtain complete light integration. Higher productivities can be reached by reducing optical path to about 0.5-1.0 cm and optimize gas flow rates (Richmond et al. 2003). Then, light/dark cycles start to approach photosynthetic unit turnover time and if growth inhibition is prevented and mass transfer is sufficient, full light integration and by that maximum productivity can be obtained.

Acknowledgements

This research was financially supported through a grant from the Programme Economy, Ecology and Technology (E.E.T.) by the Netherlands Department of Economic Affairs, the Department of Public Housing, Spatial Planning and Environmental Protection, and the Department of Education, Cultural Affairs and Sciences (K99005/398510-1010). We want to thank Wim A. van Spronsen for measuring growth rates and absorption coefficients in the lab-scale bubble columns and Marcel Janssen for his idea to use photosynthetic yield instead of growth rate as a modeling tool.

Nomenclature

a	= counter for time at half hour interval (-)
A_{light}	= illuminated photobioreactor surface (m^2)
b	= surface azimuth angle (N -180°, E -90°, S 0°, W 90°) (rad)
b'	= surface azimuth angle in the case of direct light at different z (rad)
D	= dilution speed (L day^{-1})
I	= light intensity ($\mu\text{mol m}^{-2}\text{s}^{-1}$)
I_{average}	= average light intensity in the daylight period ($\mu\text{mol m}^{-2}\text{s}^{-1}$)
$I_{\text{av,tot}}$	= average light intensity in the whole reactor ($\mu\text{mol m}^{-2}\text{s}^{-1}$)
I_{dir}	= direct irradiance falling at the reactor wall inside the photobioreactor ($\mu\text{mol m}^{-2}\text{s}^{-1}$)
I_{locabs}	= light absorbed between z and z- Δz inside the reactor ($\mu\text{mol m}^{-2}\text{s}^{-1}$)
I_{Sdir}	= local direct light intensities ($\mu\text{mol m}^{-2}\text{s}^{-1}$)
I_{Stot}	= total irradiance inside reactor ($\mu\text{mol m}^{-2}\text{s}^{-1}$)
P_{int}	= total productivity calculated with the light integrated approach (g)
$p_{\text{dir,2D}}$	= direct light path projected over cross-sectional area (2D) (m)
p_{direct}	= direct light path (3D) (m)
P_{growth}	= total productivity calculated with the growth integrated approach (g)
P_{measured}	= measured productivity (g)
r	= reactor radius (m)
T	= temperature (°C)
T_{weighed}	= temperature weighed average in the daylight period (°C)
t	= total time that pump was on (sunrise to sunset) (hr)
V	= reactor volume (m^3)
X	= average biomass concentration during a day (kg m^{-3})

X_{sunrise}	= biomass concentration at sunrise (kg m^{-3})
X_{sunset}	= biomass concentration at sunset (kg m^{-3})
Y	= photosynthetic yield (g mol^{-1})
Y_{av}	= average photosynthetic yield (g mol^{-1})
z	= distance from reactor wall (m)

Greek symbols

α	= absorption coefficient ($0.21 \text{ m}^2 \text{ g}^{-1}$)
Δb	= angle between b and b' (rad)
μ	= specific growth rate (d^{-1})
ξ, ξ'	= angle between solar angle and surface azimuth angle (rad)
χ_1, χ_2	= angles need to calculate direct light path (rad)
ω	= solar angle (N -180° , E -90° , S 0° , W 90°) (rad)

Growth inhibition of *Monodus subterraneus* by free fatty acids

Abstract

Monodus subterraneus is a microalga, which is known for its high eicosapentaenoic acid (EPA; 20:5 ω 3) content. To produce EPA commercially, high volumetric productivities of microalgae are required. These high productivities can be reached in flat panel photobioreactors with small optical paths that have to be operated at high cell densities ($>10 \text{ g L}^{-1}$). However, at these cell densities a reduction of productivity is observed. This growth inhibition is probably caused by growth inhibitors released by the microalgae, which have been suggested to be fatty acids.

Our aim was to investigate if free fatty acids produced by *Monodus subterraneus* inhibited growth of this species. Therefore a bioassay was developed and saturated, unsaturated and poly-unsaturated fatty acids occurring in *Monodus* were tested on their growth inhibiting properties.

Growth of *Monodus subterraneus* was completely inhibited at a saturated concentration (96 μM) of palmitoleic acid (16:1 ω 9). But, the saturated fatty acid palmitic acid (16:0) and the mono-saturated oleic acid (18:1 ω 9) were much stronger inhibitors. Growth was inhibited for 50% already at concentrations of 0.4 μM 16:0 and 3 μM 18:1 ω 9, respectively. These fatty acids probably cause the growth inhibition in high cell density cultures of *Monodus subterraneus*.

Published as:

Bosma R, Miazek K, Willemsen SM, Vermuë MH, Wijffels RH. 2008. Growth inhibition of *Monodus subterraneus* by free fatty acids. *Biotechnology and Bioengineering* 101(5): 1108-1114.

Introduction

Monodus subterraneus is a microalga that is known for its high eicosapentaenoic acid (EPA, 20:5 ω 3) content (Cohen 1994). Eicosapentaenoic acid is a nutraceutical, which can prevent coronary heart disease, high blood pressure and inflammatory disorders (Simopoulos 1999). Currently, EPA is produced from fish oil; however this source is inadequate to supply the expanding market in terms of productivity and product quality (Gill and Valivety 1997). Therefore production of EPA by microalgae is considered.

To produce EPA commercially in photobioreactors, a high volumetric productivity is needed to decrease photobioreactor size and downstream processing costs. High volumetric productivities can be reached in flat panel photobioreactors with small optical paths. Such photobioreactors have to be operated at high cell densities ($> 10 \text{ g L}^{-1}$) to obtain maximum volumetric productivities (Hu et al. 1996; Richmond et al. 2003). At these biomass concentrations, productivity decreases because microalgal growth is inhibited. This growth inhibition can be completely prevented by daily replenishment of the medium (Qiang et al. 1996; Richmond et al. 2003; Richmond and Zou 1999).

Fatty acids and their oxidation products are often considered as algal growth inhibitors (Ikawa et al. 1984; Ikawa et al. 1997; McCracken et al. 1980; Spruell 1984; Wu et al. 2006; Yamada et al. 1993). Fatty acids are known to be important physiological and ecological markers that can be excreted in the culture medium (Sushchik et al. 2001; Sushchik et al. 2003). They can inhibit the growth of the microalgae by binding to the chloroplast membrane, resulting in an alternation in membrane permeability, which firstly leads to leakage of potassium ions from the cellular interior and finally to disintegration of membrane functional integrity or pigment dissociation from the thylakoid membranes (McCracken et al. 1980; Wu et al. 2006; Yamada et al. 1993). Further, it was reported that unsaturated fatty acids can inhibit eukaryotic cell division by interfering with the microtubule assembly (Namikoshi et al. 2002).

We hypothesized that in high density cultures of *Monodus subterraneus*, free fatty acids occurring in *Monodus subterraneus* cause growth inhibition of this species. A bioassay was developed in which the effect of free fatty acids on the specific growth rate was determined. In this paper, first the development of the bioassay is presented. Secondly, it is determined which fatty acids in *Monodus subterraneus* inhibit growth of this species.

Material and methods

Reagents

The fatty acids used were myristic acid (14:0, $\geq 99\%$), palmitic acid (16:0, $\geq 99\%$), palmitoleic acid (16:1 ω 9, $>99\%$), stearic acid (C18:0, 99%), oleic acid (18:1 ω 9, $>98\%$), linoleic acid (18:2 ω 6, 97%), linolenic acid (18:3 ω 3, $\geq 99\%$) and eicosapentaenoic acid (20:5 ω 3, $\geq 98,5\%$). They were obtained from Sigma-Aldrich, Germany.

Organism and cultivation conditions

Monodus subterraneus (UTEX 151) was precultivated in a shake incubator (100 r.p.m.) with a 2% carbon dioxide enriched airflow (2 L min⁻¹) at a temperature of 25 °C, a light intensity of 41 $\mu\text{mol m}^{-2} \text{s}^{-1}$, pH 7.8 and a 16h/8h day/night rhythm. The airflow was turned off during the night.

Medium

In our initial bioassay experiments, modified BG-11 medium was used (Rippka et al. 1979). Iron-ammonium-citrate was replaced by iron-chloride, citric acid was removed and concentrations of magnesium (60 x decreased) and EDTA (14 x increased) were adjusted. In all other experiments, the medium 3N-BBM (Bold-Basal Medium with 3-fold Nitrogen) was used, because this medium led to higher growth rates. In addition, 25 mM TRIS buffer and 0.5% ethanol with or without fatty acids were added and the pH was set at 7.8.

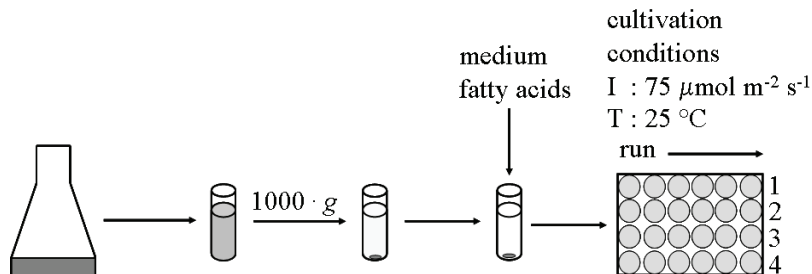


Figure 3.1. Schematic overview of the bioassay procedure.

Bioassay

A bioassay was developed to determine specific growth rates of growth inhibited microalgae (Figure 3.1). Two weeks old precultures were used as inoculum. Cultures were centrifuged at 1000g for 10 minutes to remove old medium. The pellet was resuspended in medium with or without the fatty acids to be tested (final ethanol concentration 0.5%), to give an optical density at 530 nm (OD_{530}) between 0.1-0.2. The concentration of the tested fatty acids ranged from 25% saturation to full saturation. Well plates (Costar 3524) were filled with one mL of this culture and shaken (180 r.p.m.) at a temperature (T) of 25 °C. Light was supplied at a 16h/8h day/night rhythm by ten dimmed fluorescent lamps (Sylvania CF-LE 55W) giving a light intensity (I) of 75 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Moistened air enriched with 2% carbon dioxide was sparged over the well plates at a rate of 2 L min^{-1} . This airflow was turned off when no light was supplied.

For six days, the optical densities at 530 nm (OD_{530}) and 680 nm (OD_{680}) were measured (Spectronic 20 genesys, Spectronic Instruments). Specific growth rates (μ) were calculated by linear regression of the natural logarithm of OD_{530} versus time. Relative growth rates of inhibited microalgae (μ_{rel}) were calculated with the specific growth rate of algae without inhibitor added as a control. The ratio $\text{OD}_{680}/\text{OD}_{530}$ is used as indicator for the amount of chlorophyll per biomass.

Measurement of saturated fatty acid concentrations

Saturated fatty acid solutions in water with 0.5% ethanol were prepared at 25 °C and filtered through 0.20 μm filters (Sartorius Minisart®). To 50 mL of this solution, 0.75 g NaCl and 2 mL H_2SO_4 were added. Then, chloroform (1-5 mL) was added containing as internal standard 0.10 mg mL^{-1} nonadecanoic acid (19:0). After mixing (30 min. at 500

r.p.m.), 100 μL chloroform was collected and 50 μL 0.25 M Trimethylsulfonium hydroxide in methanol was added. Gas chromatographic analysis was done on a Nukol™ column (30m530 μm 1.00 μm). The initial oven temperature was 90°C, followed by a temperature program of 20°C min⁻¹ to 200°C. The injector temperature was 250°C, flame ionization detector temperature 270°C and a split ratio of 0.1:1 was used. Fatty acid concentrations were calculated by comparing each peak area with that of the internal standard and corrected accordingly.

Results and Discussion

Bioassay development

To study growth inhibitory properties of fatty acids, a bioassay had to be developed. Inoculum age, inoculum concentration and light conditions were optimized to obtain exponential growth of the microalgae. Further, the negative effects of centrifugation, TRIS buffer and ethanol, which was needed to dissolve fatty acids, were determined. Finally, the bioassay was tested with different concentrations of an inhibiting compound and the reproducibility of the bioassay was determined.

Centrifugation

To remove old medium, centrifugation of the microalgae was needed. To test any negative effect of centrifugation on cell growth, different centrifugation speeds were tested. Centrifugation did not affect cell growth significantly up to 1000 g. Therefore, this centrifugation speed was used, because a strong pellet was obtained.

Light conditions

Some microalgal species are more viable when a light/dark rhythm for synchronizing cell processes is applied (Suzuki and Johnson 2001). With *Monodus*, growth rates in continuous light compared to microalgae grown in a 16h/8h day/night rhythm were not significantly different. However, with continuous light, the ratio OD₆₈₀/OD₅₃₀ was lower at the end of the exponential phase compared to microalgae cultivated in a day/night rhythm (data not shown), implicating that those cells could not adapt their chlorophyll per biomass when less light per biomass was available. Because we wanted our culture to be as viable as possible, a 16h/8h day/night rhythm and a light intensity of 75 $\mu\text{mol m}^{-2} \text{s}^{-1}$ were used.

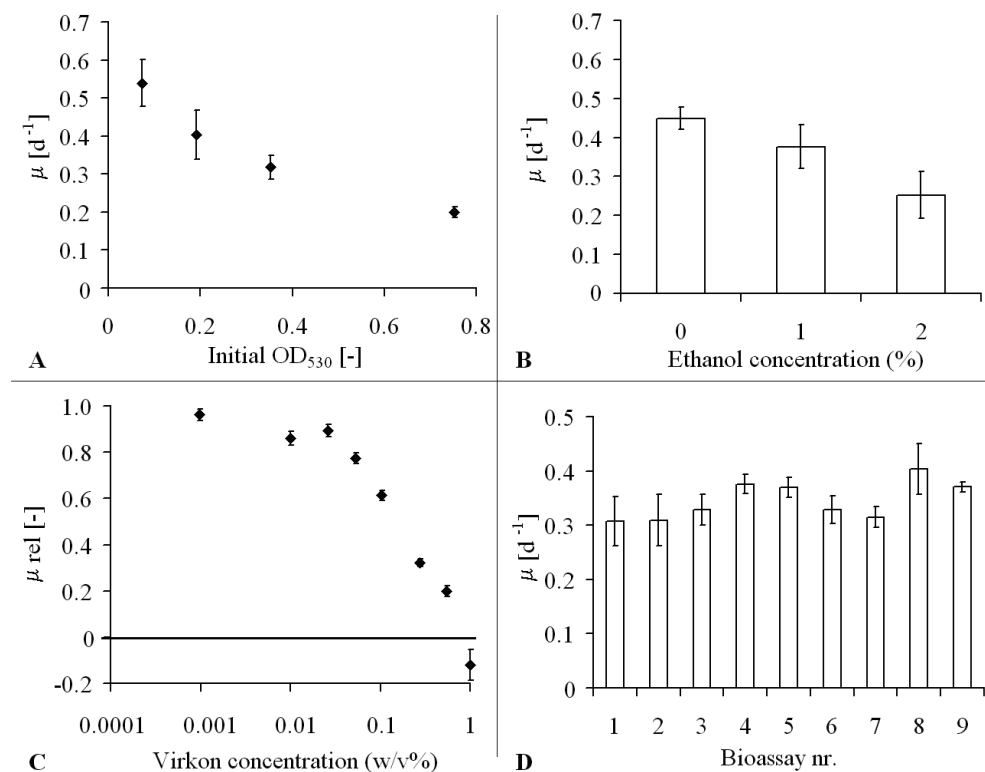


Figure 3.2. Growth rates of *Monodus* at different conditions. Error bars represent 95% standard deviations (n=3).

- A.** Effect of initial biomass concentrations, measured as OD₅₃₀, on specific growth rate. Algae were grown in BG-11 medium and lighted continuously (I: 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$).
- B.** Effect of ethanol concentration on specific growth rate. Algae were grown in BG-11 medium and lighted continuously (I: 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$).
- C.** Relative growth rate versus different disinfectant (Virkon[®]) concentrations.
- D.** Average specific growth rates of controls in the bioassays.

Inoculum

Figure 3.2A shows the effect of initial biomass concentration on specific growth rate. At low initial biomass concentrations, high specific growth rates were obtained because there was hardly a light gradient present and cells grew exponentially during the bioassay. At higher initial biomass concentrations light limitation occurred and the cells no longer experienced maximum growth conditions. In addition, no significant differences in specific growth rates of *Monodus* were found using inoculum of cultures of one, two and four weeks old (results not shown). In the rest of our bioassays, a culture of two weeks old was taken as inoculum and an initial biomass concentration between 0.1-0.2 OD₅₃₀ was used.

Ethanol and buffer

Ethanol was needed to disperse the fatty acids in the aqueous medium. However, ethanol affected *Monodus subterraneus* negatively (Figure 3.2B). Therefore an ethanol concentration of 0.5% was used, which was sufficient to get saturated fatty acid solutions and still obtain a high specific growth rate. A TRIS buffer (25 mM) was used to keep the pH constant although it slightly affected growth rate (20% lower growth, data not shown).

Bioassay test and reproducibility

Different concentrations of a standard inhibiting compound, *i.e.* the disinfectant Virkon® (Fisher), were tested in the bioassay. As expected, high concentrations of this inhibitor led to a strong inhibition of algal growth and at the lowest concentration, growth rate was no more affected (Figure 3.2C). Our results show that relative growth rate depends on disinfectant concentration and that our bioassay could be used to determine these inhibiting effects.

A high reproducibility in the bioassay is important to be able to compare different runs with each other. Figure 3.2D shows the different growth rates of controls. The average growth rate of controls was $0.35 \pm 0.035 \text{ d}^{-1}$. These results show the high reproducibility of our bioassay.

Saturated fatty acid concentrations

Saturated fatty acid concentrations were measured by gas chromatography (Table 3.1).

Table 3.1. Saturated concentration of fatty acids

Fatty acid	Abbreviation	Saturated concentration (μM)	Standard deviation (μM)	n	Saturated concentration* (μM)
Myristic acid	14:0	43	2	2	20-30
Palmitic acid	16:0	0.5	0.2	2	<1
Palmitoleic acid	16:1 ω 7	96	43	3	
Stearic acid	18:0	0.22		1	<1
Oleic acid	18:1 ω 9	3.3	0.9	2	<1
Linoleic acid	18:2 ω 6	46	2.9	3	
Linolenic acid	18:3 ω 3	80	26	2	
Eicosapentaenoic acid	20:5 ω 3	130	0.07	2	

* *Vorum et al. (1992)*

The values for solubilities that we obtained are in agreement with the results of Vorum et al. (1992), who measured saturated fatty acid concentrations at 37°C in phosphate buffer (pH 7.4). Several expected trends in fatty acid solubilities can be observed such as low solubilities of saturated fatty acids compared to unsaturated fatty acids. Further, a decrease in fatty acid solubility is observed for longer and more saturated fatty acids.

Growth inhibition tests

As a typical example, the growth inhibition test of palmitic acid will be presented first. After that, the effects of different concentrations of several fatty acids on growth of *Monodus* will be addressed.

Inhibition test of palmitic acid (16:0)

Monodus subterraneus cultivated in control medium grew with a specific growth rate of $0.33 \pm 0.03 \text{ d}^{-1}$ (Figure 3.3A).

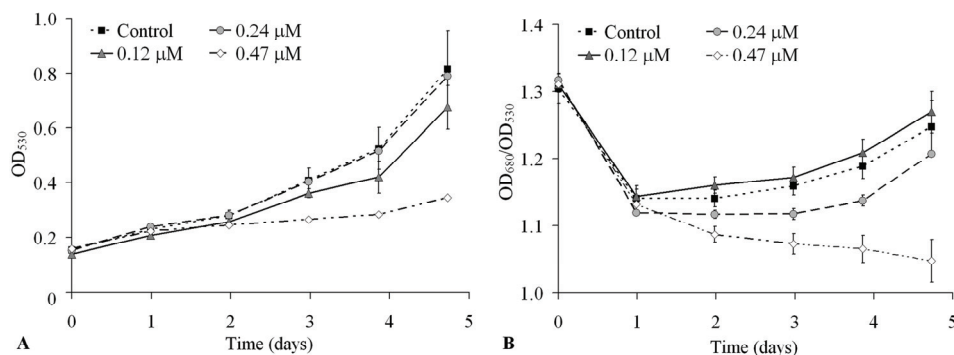


Figure 3.3. Effects of different concentrations of 16:0 on growth and chlorophyll concentrations of *Monodus* (n=3).

A. Biomass concentration, measured as OD₅₃₀, versus time.

B. Ratio of OD₆₈₀ and OD₅₃₀, representing chlorophyll per biomass, versus time. Error bars represent 95% standard deviations.

At a concentration of 0.12 and 0.24 μM 16:0, no significant differences in growth rate were observed. However, at the highest concentration (0.47 μM) *Monodus* grew much slower than in the other cultures ($\mu=0.14 \pm 0.01 \text{ d}^{-1}$).

Figure 3.3B shows the OD₆₈₀/OD₅₃₀ ratio versus time for microalgae cultivated in medium with different concentrations of 16:0 added. This ratio represents the amount of chlorophyll per biomass. In the control, this ratio decreased during the first day because the inoculum was adapted to a low amount of light per cell and when the inoculum was diluted, cells were exposed to more light per cell and therefore decreased their chlorophyll per biomass. When cells started to grow, less light per cell became available and the OD₆₈₀/OD₅₃₀ ratio increased such that microalgae could capture more light. *Monodus* cultivated with the lowest concentration of 16:0 (0.12 μM) showed the same pattern as the control. But surprisingly, the culture with 0.24 μM 16:0 added, which had a similar growth rate, could not adapt its amount of chlorophyll per biomass as fast as the control. At the highest concentration of 16:0 (0.47 μM) microalgae could not adapt the chlorophyll concentration at all. Inhibition of chlorophyll synthesis was also found with the allelochemical nostocyclamide affecting *Anabaena variabilis* by Todorova & Jüttner (1996), but was not reported for fatty acids before.

Our results show that palmitic acid is a growth inhibitor of *Monodus subterraneus* and that the amount of chlorophyll per biomass is affected at lower concentrations of palmitic acid than the specific growth rate.

Inhibition test with fatty acids

Most fatty acids at saturated concentrations inhibited *Monodus subterraneus* to some extent (Figure 3.4).

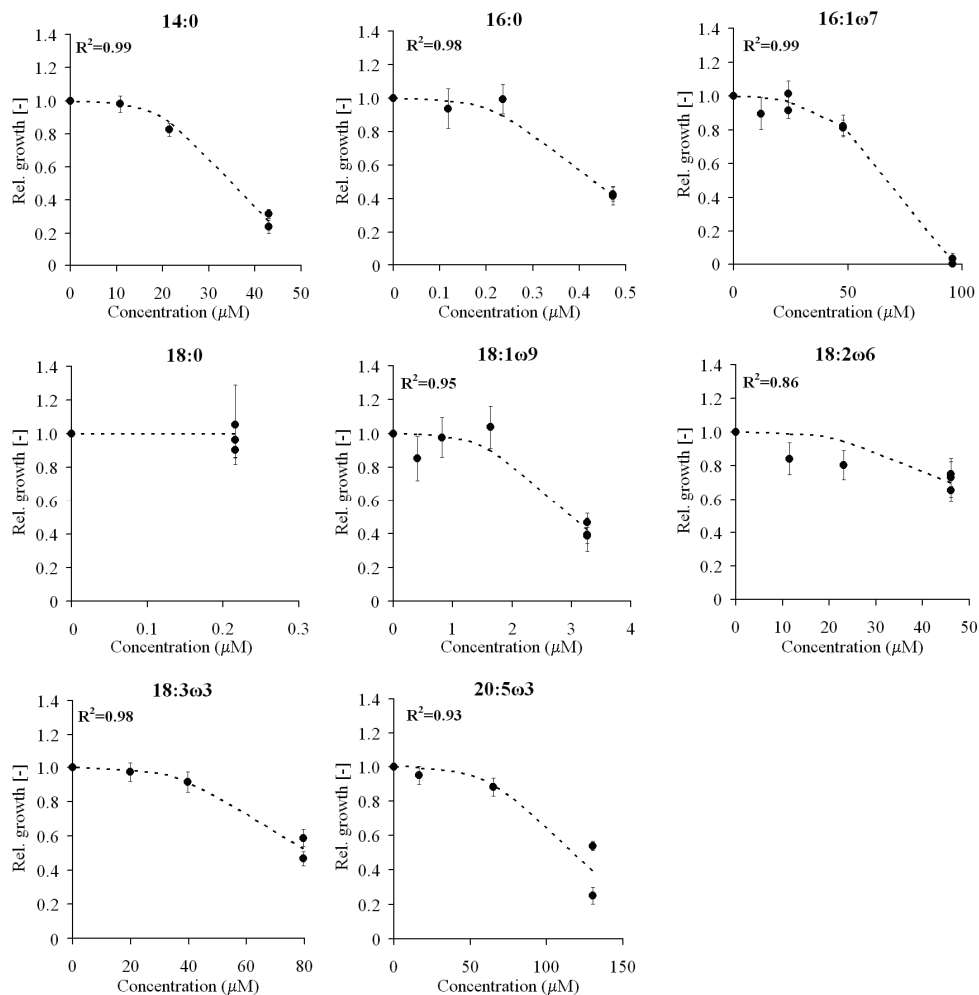


Figure 3.4. Effects of different concentrations of fatty acids on the relative growth rate of *Monodus*. Bars represent 95% standard deviations (n=3). Dashed lines are fits of the data with the function $y = 1 + a \cdot x^{2.5}$ with $y = \mu_{\text{rel}}$, a = fit parameter and x = fatty acid concentration.

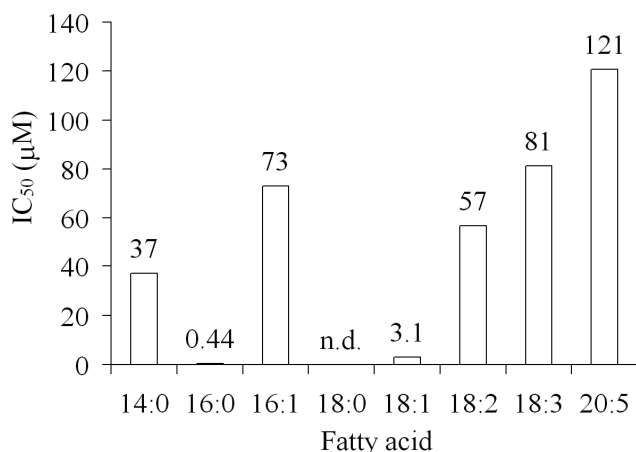


Figure 3.5. Fatty acid concentrations at which 50% growth inhibition (IC₅₀) of *Monodus* occurred. Growth inhibition with 18:0 could not be detected (n.d.) due to poor solubility.

Palmitoleic acid (16:1ω7) was the only fatty acid that inhibited *Monodus* completely at saturated concentration (96 μM). Our data were fitted with the equation $y = 1 + a \cdot x^{2.5}$ with $y = \mu_{\text{rel}}$, a = fit parameter and x = fatty acid concentration, which fitted most data well ($R^2 > 0.95$). These results clearly indicate that growth inhibition of *Monodus* is fatty acid concentration dependent. For 18:2ω6 and 20:5ω3 this dependency was less pronounced with an R-squared of 0.86; 0.93, respectively.

This equation was used to determine the concentration (x) at which 50% growth inhibition ($y = 0.5$) occurred (IC₅₀). Large differences in IC₅₀ between the different fatty acids were found (Figure 3.5). Such differences are also observed for other microalgae (Wu et al. 2006; Yamada et al. 1993). Very strong inhibitors of *Monodus* were palmitic acid (16:0) and oleic acid (18:1ω9) with an IC₅₀ of 0.44 μM and 3.1 μM, respectively. Other fatty acids needed at least a tenfold higher concentration to give the same amount of growth inhibition. Up till now, mainly unsaturated or polyunsaturated fatty acids of C₁₈ or C₂₀ or their oxygenated products are mentioned as the main growth inhibiting compounds for microalgae (Ikawa et al. 1984; Ikawa et al. 1997; McCracken et al. 1980; Spruell 1984; Yamada et al. 1993). However, our results show that the inhibition can also be caused by saturated fatty acids. For *Monodus* we found that palmitic acid was the strongest inhibitor. Also Wu et al. (2006) found this fatty acid to be a strong inhibitor of *Monoraphidium contortum*. Therefore, saturated fatty acids should not by forehand be excluded in growth inhibitory studies.

Concluding remarks

In high density cultures of *Monodus subterraneus* 16:0 and 18:1 ω 9 are likely to cause growth inhibition, since both fatty acids are present in *Monodus* (Cohen 1994) and our study indicates that tiny amounts of these fatty acids in the medium cause growth inhibition. Furthermore, it is known that saturated as well as unsaturated fatty acids can be excreted as free fatty acids while polyunsaturated acids cannot be excreted by most microalgae or only in minor amounts (Sushchik et al. 2001; 2003).

Acknowledgement

We want to thank Marcel Janssen for his useful help in developing the bioassay.

Ultrasound, a new separation technique to harvest microalgae

Abstract

In this article it is proven that ultrasound can be used to harvest microalgae. The separation process is based on gentle acoustically induced aggregation followed by enhanced sedimentation. In this paper, the efficiency of harvesting and the concentration factor of the ingoing biomass concentration are optimized and the relevance of this process compared to other harvesting processes is determined.

For the optimization, five parameters were modeled simultaneously by the use of an experimental design. An experimental design was chosen, because of possible interaction effects between the different parameters. The efficiency of the process was modeled with an R-squared of 0.88. The ingoing flow rate and the biomass concentration had a large influence on the efficiency of the process. Efficiencies higher than 90% were reached at high biomass concentrations and flow rates of 4-6 L day⁻¹. At most, 92% of the organisms could be harvested and a concentration factor of 11 could be achieved at these settings. It was not possible to harvest this microalga with higher efficiencies due to its small size and its small density difference with water.

The concentration factor of the process was modeled with an R-squared of 0.75. The ingoing flow rate, biomass concentration and ratio between harvest flow and ingoing flow rate had a significant effect on the concentration factor. Highest concentration factors, up to 20, could be reached at low biomass concentrations and low harvest flows.

On industrial scale, centrifuges can better be used to harvest microalgae, because of lower power consumption, better efficiencies and higher concentration factors. On lab- or pilot-plant scale, an ultrasonic harvesting process has the advantage that it can be operated continuously, it evokes no shear stress and the occupation space is very small. Also, when the algae excrete a soluble high valued product this system can be used as a biofilter.

Published as:

Bosma R, van Spronsen WA, Tramper J, Wijffels RH. 2003. Ultrasound, a new separation technique to harvest microalgae. *Journal of Applied Phycology* 15(2-3):143-153.

Introduction

At this moment, conventional processes as centrifugation, membrane filtration and chemical flocculation are used to harvest microalgae. All these three processes have their own problems (Ryll et al. 2000):

1. Centrifugation has the disadvantage of high operation costs and mechanical problems due to freely moving parts.
2. Membrane filtration has the drawback of membrane fouling and clogging due to the small size of the microalga.
3. Chemical flocculation has the shortcoming that it is an expensive technique due to the costs of flocculants and operators.

In this study an ultrasonic separation process was used to harvest algae. This process uses ultrasound together with enhanced sedimentation as a separation technique. The organisms are continuously pumped into a resonator chamber, consisting of a transducer and a reflector (Figure 4.1a). The chamber size and the frequency are exactly defined so that a standing wave occurs. When the apparatus is turned on, it creates fields of maximum potential energy (bellies) and fields of minimum potential energy (nodes). Time-averaged forces (primary radiation force) acting on the cells drive them instantly to the node planes, the fields of minimum acoustic potential energy (Coakley et al. 2000; Gröschl 1998; Hawkes et al. 1997). Vertical lines of algae can be seen into the planes of the pressure nodes at half-wavelength intervals (Figure 4.1b).

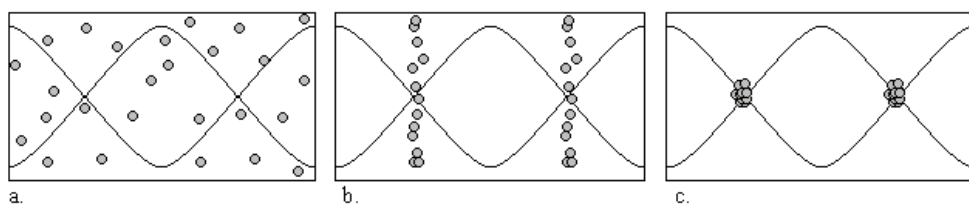


Figure 4.1. Principal of the ultrasound harvesting process. **a.** No ultrasonic field. **b.** The field has just been turned on; the cells have migrated to the pressure node planes. **c.** The cells have aggregated into the knots of the ultrasonic field.

The total ultrasonic field which an individual cell experiences, is the primary field of itself and the scattered fields of the other cells. This interaction effect between the particles results in an attractive force between those cells which slowly drives them together into the knots of the ultrasonic wave. Subsequently, agglomeration of the cells occurs, aided by the acoustic interaction forces and particle-particle interaction forces. Cells can be seen as agglomerates positioned into the knots of the ultrasonic wave (Figure 4.1c).

Then, the ultrasonic field is relieved and these larger agglomerates sediment rapidly from the fluid due to gravity forces. After that the process repeats itself. In summary, the ultrasonic field concentrates the cells due to acoustic forces, but the final separation is based on gravity forces.

Advantages of the new separation device in comparison to conventional processes are: this technique is non-fouling, arouses no shear, has the absence of mechanical failures because this device has no freely moving parts and has the possibility of continuous operation (Berg et al. 2001; Bierau et al. 1998; Gröschl et al. 1998).

When ultrasound is used to capture the cells, the cells experience no shear stress due to the high frequency (in the order of MHz) and the low sound pressure amplitude of the ultrasound. Further, the acoustic field is exactly defined in the form of a standing wave. In this field, the cells move almost instantly from the fields of high energy to the fields of minimum energy (nodes) and stay there until the field is relieved. In these nodes, the amplitude of the sound wave is almost zero, which means that the cell experiences no shear stress. Even, shear sensitive mammalian and hybridoma cells still have an high viability when ultrasound is used to capture the cells (Doblhoff-Dier et al. 1994; Trampler et al. 1994; Kilburn et al. 1989). However, when ultrasound is used to break down cells, lower frequencies (in the order of kHz) and higher pressure amplitudes are used (Gröschl et al. 1998). The principal of disruption of the cells is founded on shear forces due to cavitation instead of precise directed forces.

The alga *Monodus subterraneus* was used as a model organism. Five parameters (biomass concentration, ingoing flow rate, ratio between harvest and ingoing flow, time frequency before the field was switched off and power input) were optimized simultaneously by the use of an experimental design. The efficiency and the concentration factor of the process were measured at different settings of the apparatus. The efficiency of the process determines the number of organisms that are lost during the harvesting process. The concentration factor is important for downstream processing. When the alga is harvested, also a lot of water has to be removed. Higher concentration factors mean more water removal. This implicates that further concentration steps can be performed on smaller scale and this leads to lower costs of the total downstream process.

This paper is intended to determine the relevance of this harvesting process. First, the efficiency of the separation process and the concentration factor of biomass concentration are separately modeled by the use of an experimental design. Secondly, the new process is optimized by determining the optimal settings of the device. Then, the separation process is compared with conventional harvesting systems.

Materials and methods

Experimental design

By use of traditional methods, such as “one factor at a time” or trial and error, the optimization of this process would take a lot of time. This is especially true when the parameters are closely correlated.

To solve this problem a statistical experimental design was chosen. These designs focus on well-designed experiments which leads towards faster progress and problem solution (Haaland 1989). Here, two experimental designs could be used: a factorial face centered central composite design or a Box-Behnken experimental design. The central composite design predicts the whole area well, while the Box-Behnken design predicts especially the centre of the surface area well and lacks accuracy at the extremes (Myers and Montgomery 1995). Because it was not known where the optimum was located, the factorial central composite design was chosen.

The program Design-Expert version 6 was used to construct, analyze and optimize the design. All five parameters were varied on three levels. The experimental domain was defined taking into account both instrumental constraints and experimental limits. The power input could be set only at 3, 4, 5, 6, 8 and 10 W. Levels of 4, 6 and 8 W were chosen for the experimental design because symmetry is needed. Also the time frequency of the ultrasonic field could not be freely chosen because the instrumental set-up limited it.

The center of the experimental domain was measured five times to estimate the repeatability of the experimental measurements. Table 4.1 shows the parameters and their minimal and maximal tested levels. As responses, the efficiency of the process (Y_1) and concentration factor (Y_2) were calculated and modeled independently.

Table 4.1. Levels of parameters in the central composite design.

Parameter	Unit	Coded value	Low (-1)	Medium (0)	High (+1)
Biomass concentration	10^7 cells mL^{-1}	X_1	1.0	17	33
ϕ_{in}	L day^{-1}	X_2	2.0	10	18
Time frequency	s	X_3	60	180	300
Power input	W	X_4	4	6	8
$\phi_{\text{harv}} / \phi_{\text{in}}$	-	X_5	2.0	6.0	10

The separating efficiency was calculated by dividing the cells per day in the return flow by those in the ingoing flow:

$$\text{Efficiency} = 100\% - 100\% \cdot \frac{(C_{\text{return}} \cdot \phi_{\text{return}})}{(C_{\text{in}} \cdot \phi_{\text{in}})} \quad (1)$$

The return flow was taken instead of the harvest flow because the harvest flow was sometimes very small and the concentration of cells was high. This should lead to large experimental errors in the efficiency calculation. The concentration factor was calculated by dividing the amount of the cells in the harvest flow by the amount of cells in the ingoing flow.

The efficiency and concentration factor were fitted with the following polynomial equation:

$$Y = b_0 + \sum_{i=1}^5 b_i X_i + \sum_{i=1}^5 \sum_{j=1}^5 b_{ij} X_i X_j + \sum_{ii=1}^5 b_{ii} X_i^2 \quad (2)$$

In this equation Y is the predicted response and the X_i variables are the coded values of the parameters. The b values correspond to the estimated polynomial coefficients: b_0 is the intercept term, b_i coefficients represent the main effect for each variable, b_{ij} ($i \neq j$) coefficients describe the interaction effect between the parameters and b_{ii} values describe the square effects of the different parameters.

Statistical significant coefficients were estimated by the method of backward stepwise elimination. A probability value (P-value) was used to distinguish signals from noise signals. All 21 coefficients were put in both models and then each coefficient with the highest probability value was removed. Removal of coefficients continued until only coefficients were left with probability values smaller than 0.10. To establish model hierarchy, the linear term of a parameter was introduced when an interaction effect or a square effect of that parameter was significant. Then again, coefficients were removed (not those needed for hierarchy) with probability values larger than 0.10.

Organism and cultivation conditions

Monodus subterraneus UTEX 151 was obtained from the University of Texas Culture Collection and cultivated in test tubes containing BG-11 medium (Rippka et al. 1979) containing 1% agar. The cultures grew in a light climate cabinet at a temperature of 25 °C and a light intensity of about 200 $\mu\text{mol m}^{-2}\text{s}^{-1}$.

After growing, the alga was transposed to 250 mL Erlenmeyer flasks containing BG-11 medium and grew under the same conditions. This culture inoculated an air-lift-loop reactor with a diameter of 6 cm and height of 70 cm. This reactor was used to produce the material needed for the ultrasonic harvesting experiments. The organisms were grown at 25 °C and

pH 7.8, air was sparged through the reactor to establish mixing. Ten Sylvania CF-LE 55W fluorescent lamps provided light. The light intensity was kept low (ca. $200 \mu\text{mol m}^{-2}\text{s}^{-1}$) when the culture was very dilute. At higher biomass concentrations ($> \text{OD}_{530} 0.5$) the light intensity was increased to $\pm 1200 \mu\text{mol m}^{-2}\text{s}^{-1}$. The light intensity was measured with a LiCor LI-190 SA air quantum sensor.

In some experiments, higher biomass concentrations than the concentration of algae in the air-lift-loop reactor were needed. In those cases an amount of suspension was taken from the cultivation reactor and this suspension was concentrated using a centrifuge (9600 g, 5 min.). The pellets were resuspended to attain the desired biomass concentration.

Experimental set-up of the ultrasonic harvesting process

The schematic experimental set-up is illustrated in Figure 4.2. A bubble column with a diameter of 4 cm and a volume of 450 mL was used to maintain the algae during the harvesting process. Air was sparged through the column with a flow of 2 L min^{-1} by using mass flow controllers. The reactor was kept at $25^\circ\text{C} \pm 0.1^\circ\text{C}$.

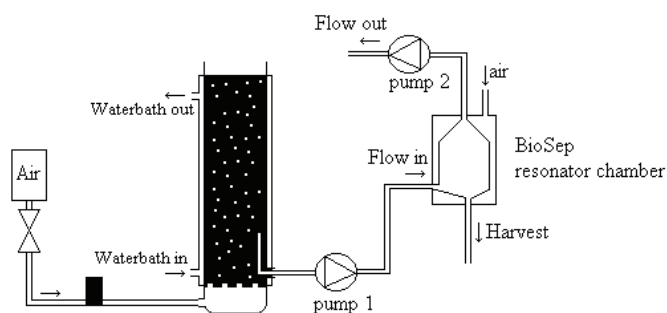


Figure 4.2. Schematic of the harvesting process.

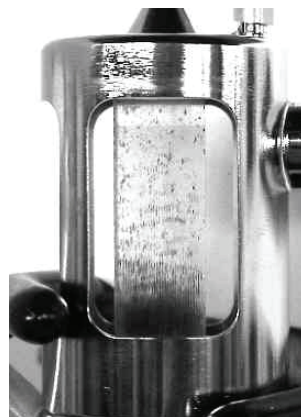


Figure 4.3. Resonator chamber

Our separation system consisted of an ultrasonic resonator chamber (Figure 4.3, Applisens, dimensions $45.0 \times 12.5 \times 12.5 \text{ mm}$, volume 7.0 mL) and a control unit (Applisens, BioSep ADI 1015). The control unit adjusted the power input of the ultrasonic field, controlled the timer that turned the acoustic field on and off and also regulated the operating frequency to the desired resonance frequency of 2.1 MHz automatically. The time frequency between turning off the field changed between 60-300 seconds. The time that the field was off, to allow cell sedimentation, was kept at 3 seconds.

Two pumps controlled the flow into the resonator chamber (ϕ_{in}) and the return flow (ϕ_{return}). The harvest flow (ϕ_{harv}) represents the difference between those two flows. Air bubbles disturb the ultrasonic field in the resonator chamber. To prevent the entrance of air bubbles into the resonator chamber, a small metal tube (diameter 1 mm) was put into the bubble column from which the first pump pumped algae into the chamber.

Procedure

An algae suspension of the desired biomass concentration was put into the bubble column and kept at 25 °C. The power input of the field and the timer settings were adjusted and the pumps were set on the right flow rates by adjusting them according to a calibration graph.

Pump 1 was turned on and algal suspension was collected from the harvest tube. The tube was submerged into the harvest liquid. Pump 2 was switched on and the resonator chamber filled with biomass. The ultrasonic field was turned on and the separation of the organisms started. The harvest flow and return flow were collected and recycled to allow the separation process to reach steady state for 30 minutes.

Then, the harvest flow and the return flow were collected separately. At least 1 gram of the harvest flow and 20 grams of the return flow was assembled and the time was recorded. The ingoing flow was disconnected from the resonation chamber and this flow was measured. At least 7 grams of the ingoing flow was assembled. The diameter, the number of cells and the optical density at 530 nm in the different flows were measured.

Analytical procedures

The number of algal cells and their volumes were counted using a CASY analyzer system (Schärfe system, model TTC, Reutlingen, Germany) according to the method by Winkelmeier (Winkelmeier et al. 1993). The diameter of the cells was calculated from their volume. Particle counting and volume determination is based on the Coulter counter principle. The signals generated by the cells suspended in electrolyte are evaluated by pulse area analysis. The pulse area of the signal is strictly proportional to the volume of the particle generating the signal.

A 60 μ m capillary was installed to count the organisms. A volume of 200 μ L sample was analyzed in triplicate. The sample was diluted when too many cells were counted. The number of algal cells per mL was calculated by multiplying the total number of cells with the dilution factor. Then, a duplicate sample was measured and the total number of algal cells of both samples was averaged.

For comparison with other studies the optical density at 530 nm (OD_{530}) was measured with a spectrophotometer (Spectronic 20, Genesys). A calibration line was made between the OD_{530} and the amount of cells as counted by the CASY analyzer system. 1 OD_{530} corresponded with $4 \cdot 10^7$ cells mL^{-1} . When comparing this to dry weight, a cell concentration of $1 \cdot 10^8$ cells mL^{-1} corresponded with a dry weight of 0.4 - 0.5 g L^{-1} .

Results

Table 4.2 shows the measured values of the experiments with calculated efficiencies and concentration factor. The experiments were performed in chronological order. A maximum efficiency of 85 % was obtained in experiment 18 and 29.

At low ingoing flow rates (2 $L day^{-1}$), due to bad heat removal inside the separation chamber, the ultrasonic field was not stable and the frequency changed continuously. At flow rates of 10 $L day^{-1}$ the field collapsed approximately once per 180-300 seconds, but this did not interfere with the separation process because the field was quickly restored. At flow rates of 18 $L day^{-1}$ the field was stable at all times.

Mass balances of each experiment were made and Figure 4.4 shows that large deviations ($>20\%$) mainly occur at low harvest flow rates. The relative measurement error at these low flow rates ($< 0.2 L day^{-1}$) is high and at these low flow rates it takes probably longer than a half hour to reach steady state. At the other three experiments, maybe air bubbles were introduced into the separation chamber. Air bubbles make the ultrasonic field instable and influence the separation process.

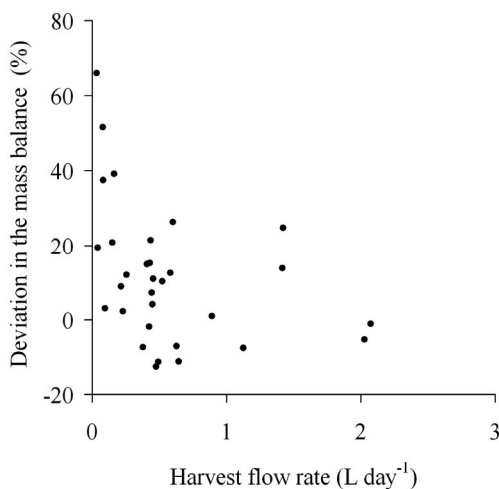


Table 4.2. Measured experimental values of the factorial experimental design.

Exp.	Parameters					Responses	
	Biomass concentration (10^7 cells mL ⁻¹)	ϕ_{in} (L day ⁻¹)	Time field on (s)	Power input (W)	ϕ_{harv}/ϕ_{in} (%)	Efficiency (%)	Conc. factor (-)
1	0.97	18.2	60	8	7.79	58.9	4.4
2	1.3	18.3	60	4	3.18	29.0	5.2
3	1.4	2.04	300	8	10.6	58.8	4.7
4	1.8	2.20	60	8	7.46	73.7	4.6
5	0.97	18.2	300	4	7.78	34.6	2.7
6	1.4	2.13	60	4	12.1	75.8	5.3
7	1.3	18.2	300	8	3.55	46.5	16.3
8	41	9.85	180	6	3.84	50.4	15.1
9	18	10.0	180	6	4.91	79.0	18.4
10	43	18.1	60	8	3.47	9.2	4.7
11	39	18.3	300	8	11.1	16.1	1.9
12	39	18.4	60	4	11.3	28.1	2.6
13	31	18.0	300	4	2.35	11.3	5.6
14	34	2.09	60	8	10.9	78.8	7.0
15	38	2.15	300	8	6.94	67.5	6.7
16	19	10.1	180	6	5.93	70.0	7.4
17	0.49	10.0	180	6	4.75	81.4	19.8
18	6.6	9.90	180	6	4.60	84.6	16.0
19	6.9	9.94	180	6	5.26	82.4	13.7
20	19	9.86	180	4	4.50	60.2	11.8
21	18	9.91	60	6	4.52	76.9	16.1
22	20	9.93	300	6	4.39	84.7	14.4
23	20	1.99	180	6	4.81	66.2	13.2
24	17	9.94	180	8	4.33	69.4	12.5
25	13	9.94	180	6	0.81	54.4	20.8
26	18	18.0	180	6	6.25	19.0	4.2
27	17	10.0	180	6	8.89	50.9	5.6
28	15	9.94	180	6	4.11	60.0	11.0
29	29	1.94	300	4	4.04	84.8	8.2
30	29	1.94	60	4	1.70	83.3	10.2
31	1.8	1.96	300	4	2.13	57.0	17.6

Modeling

As stated before, the coefficients for the models were estimated using the program Design-Expert version 6 with Equation 2. The R^2 value for the model of efficiency was 0.88 and for the model of concentration 0.75. The R^2 value for the prediction of efficiency was 0.76 and for the model of concentration 0.62.

Table 4.3 shows the ANOVA (Analysis of variance) for both models. It includes the degrees of freedom (n), the explained sum of squares by the model towards the total (Exp. SS%), the F-value (F) and the probability value. Tables 4.4 and 4.5 show the ANOVA tables for the coefficients of both models. These include their coded parameter, values, standard error, statistical value for the calculation of confidence limits (t_{stat}) and the probability value of the coefficients.

Table 4.3. ANOVA for the two models.

Model	n	Exp. SS%	F	P-value
Efficiency	10	88	17.13	<0.0001
Concentration	6	75	14.99	<0.0001

Table 4.4. ANOVA for the coefficients of the model of efficiency.

Coefficient	Parameter	Value	F-value	P-value
b_0	Constant	75		
b_1	X_1	$1.2 \cdot 10^{-7}$	3.65	0.069
b_2	X_2	5.2	75.47	<0.0001
b_3	X_3	-0.33	2.36	0.14
b_4	X_4	0.32	0.31	0.58
b_5	$X_2 \cdot X_2$	-0.41	25.67	<0.0001
b_6	$X_3 \cdot X_3$	$8.5 \cdot 10^{-4}$	5.38	0.031
b_7	$X_1 \cdot X_2$	$-6.6 \cdot 10^{-9}$	13.79	0.0013
b_8	$X_1 \cdot X_4$	$-1.4 \cdot 10^{-8}$	3.75	0.066
b_9	$X_2 \cdot X_4$	0.27	3.14	0.091

Table 4.5. ANOVA for the coefficients of the model of concentration.

Coefficient	Parameter	Value	F-value	P-value
b_0	Constant	18		
b_1	X_1	$-2.7 \cdot 10^{-8}$	3.61	0.069
b_2	X_2	1.3	7.60	0.011
b_3	X_5	-1.6	25.80	<0.0001
b_4	$X_2 \cdot X_2$	-0.077	17.76	0.0003
b_5	$X_1 \cdot X_5$	$3.1 \cdot 10^{-9}$	6.44	0.0178

Discussion

Model of efficiency

In Figure 4.5 the predicted efficiency is plotted against the measured efficiency. The predicted efficiency is calculated using the model and the values of parameters set in the experiments. Figure 4.5 shows that the model predicts the efficiency well. The R-squared is 0.88 and the P-value is <0.0001. This means that the predicted efficiencies are in good agreement with the measured efficiencies and this correspondence is very significant.

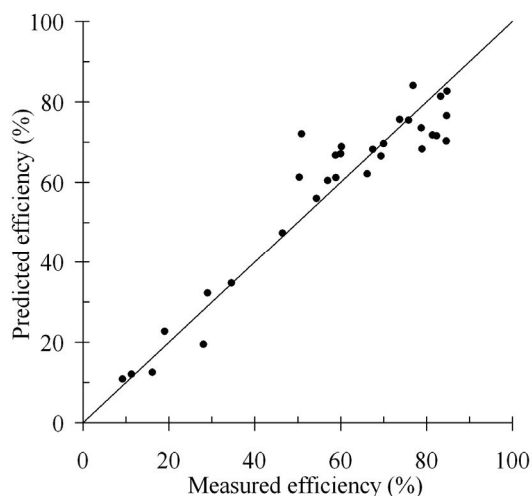


Figure 4.5. Parity plot of measured efficiency vs. predicted efficiency. The solid line represents a perfect match.

Optimums

In the response area two maxima could be predicted with efficiencies larger than 90%. At these high levels of efficiency the ultrasonic field was put off fast (60 s), to release the cells. The first maximum occurred at high power inputs (8W), low cell concentrations and ingoing flow rates between 8-10 L day⁻¹. The second maximum was acquired at low power inputs (4W), high cell concentrations and flow rates between 4-6 L day⁻¹.

The second maximum is of most interest for our application because the power consumption is low, the field is stable and the cell concentrations which could be harvested are in good accordance with cell concentrations reached in practice. Also, because the field is stable the cell experiences no shear stress. Therefore, only this second optimum will be discussed.

A surface plot was made to show the influence of the ingoing flow rate and the biomass concentration (Figure 4.6). Efficiencies higher than 90% were achieved at flow rates of 4-6 L day⁻¹ and high biomass concentrations. At these biomass concentrations high efficiencies are predicted, up to 92 % $\pm$ 12% at a biomass concentration of $3.3 \cdot 10^8$ cells mL⁻¹ and an ingoing flow rate of 5.0 L day⁻¹. At these settings, a concentration factor of 11 ± 2 could be predicted. Higher efficiencies can possibly be reached at higher biomass concentrations and by turning the field off more frequent.

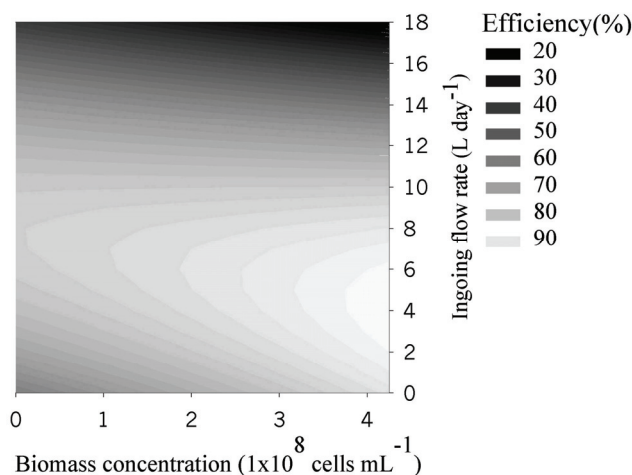


Figure 4.6. Surface chart of efficiency, biomass concentration vs. ingoing flow rate. The field was relieved each 60 s and the power input was 4 W.

Parameter influence

The ingoing flow rate had a lot of impact on the efficiency. At low values there is a positive effect on the efficiency, but at flow rates higher than 6.2 L day^{-1} the efficiency drops rapidly due to the negative square effect of the flow rate. The P-value of this square effect (b_2 coefficient) is very small. This means that this is an effect of a high significance. At ingoing flows higher than 10 L day^{-1} , the efficiency of the process dropped below 70%. This is also reported elsewhere (Hawkes and Coakley 1996; Spengler and Jekel 2000). At higher flow rates the ultrasonic field cannot capture cells fast enough and they are dragged into the return flow. At these higher flow rates, larger separation chambers should be used. Also the ingoing biomass concentration influenced the efficiency a lot and the interaction between ingoing flow rate and biomass concentration was highly significant. Biomass concentration determined the amount of cells that the electronic field could capture. At low power inputs, we achieved low efficiencies at low biomass concentrations. At these concentrations probably too small aggregates of algae are formed that sediment poorly. At very high biomass concentrations the ultrasonic field could not capture all the cells and the efficiency of the process decreased. This happened only during our process at high flow rates and biomass concentrations.

The ratio between harvest flow and ingoing flow had no influence on the separation efficiency. This is surprising, because if this ratio is too low, the separation device should fill with biomass and efficiency of the process should drop. But, within the area investigated here, the difference between those two flows did not significantly influence the separation efficiency.

Comparison of efficiency

Our maximum efficiency is comparable with maximum efficiencies reported in other papers. The efficiency of separation in our system was lower than reported for insect cells (Zhang et al. 1998) and yeast cells (Hawkes and Coakley 1996), but higher than for bacteria cells (Hawkes et al. 1997). This can be explained by two reasons. First, when the drag force is combined with the gravity force (Figure 4.7)

acting on the particle, the critical radius (r_c) can be calculated (Equation 3, according to Hawkes and Coakley, 1996).

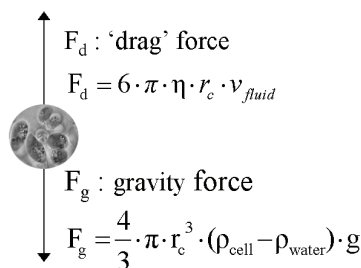


Figure 4.7. Forces acting on the particle.

$$r_c = [(9 \cdot \eta \cdot v_{\text{fluid}}) / (\rho_{\text{cell}} - \rho_{\text{fluid}}) \cdot g]^{0.5} \quad (3)$$

The critical radius is the radius of clumps at which the gravity force overcomes the Stokes' drag force that carries them to the return flow: the clumps become stationary and the liquid flows past. In Equation 3, η is the viscosity of the passing fluid, v_{fluid} the fluid flow in the resonator chamber, g the gravitational acceleration constant, ρ_{cell} the density of the cell and ρ_{fluid} the density of the fluid. Here, the fluid is assumed to be water. The critical radius is calculated using Stokes' law and combining this with the gravity force acting on the particle. It is valid at Reynolds numbers <1 . Table 4.6 summarizes the calculations for the different types of cells.

It was found that the critical radius for this alga is larger than those found for insect cells or yeast cells. Due to the smaller size of the alga, more cells are needed to form this critical radius and this is why algal cells are more easily dragged into the return flow and efficiencies reported are lower for these cells than found with both other types of cells.

Table 4.6. Critical radius and amount of cells needed for this critical radius for different types of cells calculated at an ingoing flow rate of 6 L day⁻¹. Parameters yeast cells (Kubitschek 1987), density bacteria (Kubitschek 1984), density algal cells (Kashyap 1998) and density insect cells: estimation.

	Insect cells	Yeast	Alga	Bacteria
Maximum efficiency (%)	>98	>99	93	84
r_c (μm)	35	41	59	45
Amount of cells needed for r_c	124	$5.5 \cdot 10^3$	$3.0 \cdot 10^4$	$7.1 \cdot 10^5$
Diameter of cells (μm)	14	4.6	3.8	1.0
Density of cells (kg m^{-3})	$1.15 \cdot 10^3$	$1.11 \cdot 10^3$	$1.05 \cdot 10^3$	$1.09 \cdot 10^3$

When bacteria are harvested, even more cells are needed and this is why reported efficiencies for bacteria are lower. Secondly, it takes time for the cells to reach the concentrated clumps in the pressure node planes. This time is dependent on the radiation force. In the meantime, cells are dragged upward with the flowing liquid and when the time is too long the cells are not captured in the ultrasonic field. The radiation force and thus the time to reach the concentrated clumps is strictly proportional with the volume of the cells. So, for smaller cells it takes a longer time to reach the clumps.

Both facts implicate that bacteria cells are harvested with the lowest efficiency, followed by algal, yeast and insect cells. In our system we measured the diameters and number of algal cells in the different flows. Figure 4.8 illustrates that indeed small algal cells are less easily captured by the field and that larger cells are preferably harvested.

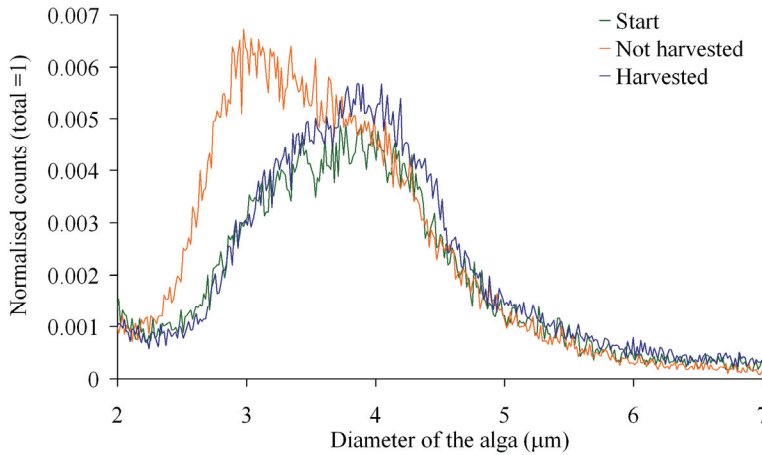


Figure 4.8. Normalized size distribution of the alga in the different flows.

Model of concentration

Figure 4.9 shows the measured concentration factor and the predicted concentration factor by the model. The R-squared for the model is 0.75. Figure 4.9 shows that there is some scattering. When calculating mass balances, it appeared that this scattering is predominantly caused by experimental errors in measuring harvest flow rates and the amount of cells in these harvest flows. Also, because the concentration of cells in the harvest flow was high, the sample had to be diluted. This caused an extra experimental error.

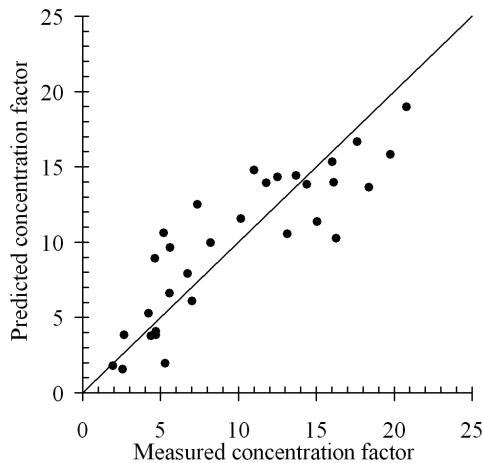


Figure 4.9. Parity plot of predicted concentration vs. measured concentration factor. The solid line represents a perfect match.

Parameter influence

Two parameters were of major importance: biomass concentration and the ratio between harvest and ingoing flow. It was found that at low biomass concentration and low harvest flows high concentration factors were reached (Figure 4.10). At low biomass concentration of the ingoing flow, it is easier to concentrate the cells because the concentration factor is calculated relative to the ingoing biomass concentration. For high concentration factors, the ratio between harvest and ingoing flow rate should be set as low as possible. Because the harvest flow is low, higher concentrations of cells in the harvest flow are attained.

The power input and the time frequency had no influence on the separation process. However, it is advised to put the power input low, because otherwise non-stable fields are created.

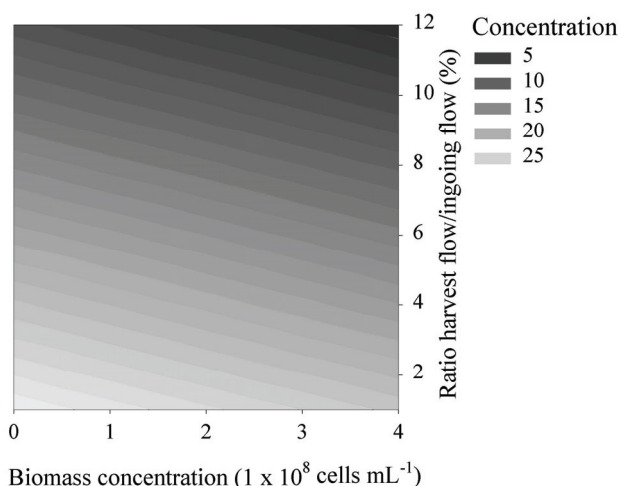


Figure 4.10. Surface plot of concentration factor, biomass concentration vs. ratio of harvest and ingoing flow rate. The flow rate was 8 L day^{-1} .

Optimization

The optimal concentration factor in the scope of area investigated was 20 ± 2.5 . This concentration was predicted at an ingoing flow rate of 8.4 L day^{-1} , biomass concentration of $1 \cdot 10^7 \text{ cells mL}^{-1}$ and a ratio between harvest and ingoing flow rate of 2.0 %. At these settings an efficiency of the process of $83\% \pm 12\%$ was calculated.

Efficiency and concentration

When looking at both models and the main parameters which influence the model are compared, it seems that there is a contradiction of parameter settings between the models to reach high values of efficiency and concentration factor. While the model of efficiency needs a high biomass concentration, this is the opposite for the model of concentration which predicts the highest factors at low biomass concentrations.

In practice, generally high biomass concentrations are harvested, which means lower concentration factors. At these concentrations, high efficiencies are more important than high concentration factors, because otherwise biomass is lost. After this first concentration step, other processes should be used to concentrate the biomass further.

Comparison of the new process with conventional systems

When the ultrasonic harvest process is compared with conventional processes as continuous centrifugation and membrane filtration, it is obvious that the power consumption is very high compared to those processes. Also the concentration factor (up to 20) is lower than with these systems (concentration factors up to 50 times). The power consumption of the ultrasonic process at a power input of 4 W, neglecting the energy costs used for cooling, in this small resonator is 345 kW day⁻¹. So, the costs of operation due to energy costs will be high. Nowadays, a larger system, which can handle flows up to 1000 L day⁻¹, is available. This device consists of six separation chambers which are cooled by a water bath. Further scaling up of the separation chambers itself is difficult due to temperature gradients that disturb the homogeneity of the field. But, due to the necessary cooling system, the energy costs of large scale ultrasonic harvesting systems will be very high. This means that on industrial scale, because of lower energy costs and higher concentration factors, centrifuges are a better system to use.

However, at lab- or pilot-plant scale, the new system has some major advantages. First, it never gets blocked with cells. Secondly, cells are still viable, because no shear stress is involved when using ultrasound of frequencies in the order of MHz. This means the harvested biomass can be used as inoculum or can still be investigated by analytical techniques. Thirdly, the occupation space of the complete system is very small. Also, when an organism excretes a high valuable secondary metabolite, this technique can be used as a retention system. The resonation chamber acts as a biological filter by rejecting the organisms and allows the solubilised product to pass. Higher biomass concentrations can be reached inside the bioreactor and the concentration of the desired product will rise. This application is already used in the case of insect cells.

Acknowledgements

This research was financially supported through a grant from the Programme Economy, Ecology and Technology (E.E.T.) by the Netherlands Department of Economic Affairs, the Department of Public Housing, Spatial Planning and Environmental Protection, and the Department of Education, Cultural Affairs and Sciences (K99005/398510-1010).

Nomenclature:

b_x	= regression coefficient
C_{in}	= concentration of cells in the ingoing flow (cells mL ⁻¹)
C_{return}	= concentration of cells in the return flow (cells mL ⁻¹)
Exp. SS%	= explained sum of squares
F	= F-value
F_d	= drag force (N)
F_g	= gravity force (N)
g	= gravitational acceleration constant (9.81 m s ⁻²)
n	= degrees of freedom
P-value	= probability value
r_c	= critical radius (m)
R^2	= R-squared
v_{fluid}	= fluid flow in the resonator (m s ⁻¹)
X_1	= biomass concentration (cells mL ⁻¹)
X_2	= ingoing flow rate (L day ⁻¹)
X_3	= time frequency (s)
X_4	= power input (W)
X_5	= ratio of harvest and ingoing flow rate
Y_1	= efficiency of the process (%)
Y_2	= concentration factor
ρ_{cell}	= density of the cells (kg m ⁻³)
ρ_{fluid}	= density of the fluid (kg m ⁻³)
η_{fluid}	= viscosity of the fluid (Ns m ⁻²)
ϕ_{in}	= ingoing flow rate (L day ⁻¹)
ϕ_{return}	= return flow rate (L day ⁻¹)
ϕ_{harv}	= harvest flow rate (L day ⁻¹)

Marine biotechnology in education: a competitive approach

Abstract

This paper describes the development of a practical, which is taught to third year biotechnology students. We wanted to motivate the students by making them responsible for a research project. Competition was added as a stimulus for interaction between the students. A virtual company called CaroTech employed the students for two weeks. They worked in groups of two persons and each group was responsible for a 0.8L flat panel photobioreactor. They had to produce as much β -carotene as possible using the marine alga strain *Dunaliella salina* in this photobioreactor. On the first day, students developed a strategy to obtain optimal algal growth rate. They putted this plan into practice the second day and while cultivating the organism, they developed a second strategy how and when to stress the alga to initiate β -carotene production. At the ninth day, the total amount of β -carotene was measured. To stimulate competition, the group that produced the most β -carotene obtained half a point bonus on the final practical mark. On the tenth day, each group presented their results and an evaluation of their chosen strategies to the CaroTech board. Most groups were successful in growing algae. In the second phase some groups failed to stress the alga. The best group produced more than two times β -carotene than the runner-up. The students were motivated by being responsible for their own results and the competitive approach. All students liked the practical and indicated that they learned a lot by following this practical.

Published as:

Bosma, R. and R. H. Wijffels (2003). Marine biotechnology in education: a competitive approach. *Biomolecular Engineering* 20(4-6): 125-131.

Introduction

A marine biotechnology course was developed for third year academic students. This course consisted of three different subjects. First, the bottlenecks in cultivation, the physiology and products of sponges were treated (Osinga et al. 2003). Secondly there was dealt with photobacteria producing hydrogen (Akkerman et al. 2002) and thirdly a module aimed at the cultivation of algae was developed consisting of introductory lectures followed by a practical. The lectures covered the principles of photosynthesis, calculation of light transfer in photobioreactors and the main characteristics of common photobioreactors (Janssen et al. 2003). This paper will deal with the development of the practical and the students' results. The practical was granted with one credit point (40 h).

Students were employed in a virtual company called CaroTech. The students were asked to produce as much β -carotene as possible using the marine alga *Dunaliella salina* in a flat panel photobioreactor. This organism accumulates β -carotene as response to stress conditions (Borowitzka et al. 1988). Students worked in groups of two persons and each group was responsible for a photobioreactor. The students developed strategies to grow the algae and to stress them using knowledge acquired from lectures or literature.

D. salina was chosen as the marine alga strain to use. This alga is easy to grow, the β -carotene production process has many variables, and a lot of literature is available about growth and stress conditions. A flat panel reactor was chosen as photobioreactor. Our practical scope was to enhance learning by motivation of the students. From theories on learning and instruction it is known that motivated students learn better, faster and also remember better (Schmidt and Moust 1998). Competition was included as extra motivation and to promote interaction between the students. In this paper we give an overview of the practical, the equipment and methods used, the results and an evaluation by the students.

Material and methods

Layout of the practical

Students were employed in a virtual company called CaroTech. The company name stands for Carotene & Technology. The students were informed that this company, one of the major β -carotene producers in the world, cultivated algae in open-pond systems. Their research and development (R&D) group is investigating new technologies such as a new cultivation system: the flat panel photobioreactor. This R&D group employed the students as production managers. After two weeks they had to report their results to the CaroTech board.

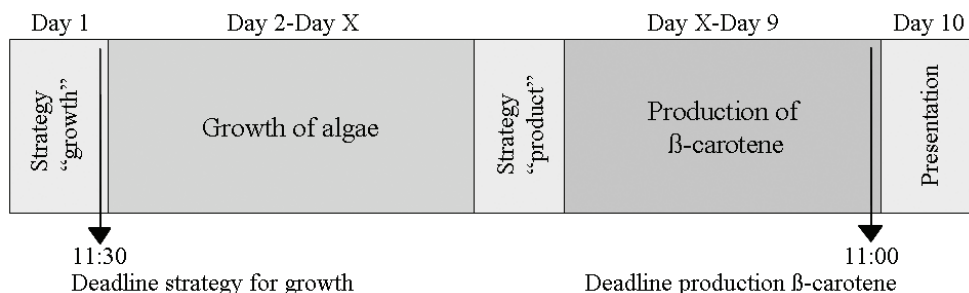


Figure 5.1. Time schedule

The students' goal was to produce as much β -carotene as possible within 9 workdays using the strain *D. salina* (Figure 5.1). To reach this goal, students developed the first day a strategy for biomass growth. In this strategy students had to select their growth conditions (temperature, light intensity, airflow and pH), medium recipe and choices had to be motivated. To imitate time limits, which companies usually have, students had only three hours to develop this strategy. Then, this "growth" strategy was reported to a research manager of CaroTech. The score for the strategy counted for 25% of the final mark of the practical. On the following day, groups started to cultivate the microalgae. They prepared media according to their plan, set up the conditions for growth and inoculated the reactor with 50 mL pure culture. Biomass growth was monitored by measuring optical density at 530 nm (OD_{530}).

While growing biomass, students had to develop a second strategy for applying stress on the algae. For this, forty journal papers were available at the practical. It was possible to search in a database containing these papers with the program Reference Manager 8.0. Different stress strategies (light, nutrient limitation, salt, temperature and pH stress) had to be discussed; students then had to choose and motivate their stress strategy (or a combination of strategies). In this plan they should also include and motivate the moment at which they wanted to apply stress on the microalgae. This strategy was reported to the R&D general manager. The score for the strategy counted for 25% of the final mark of the practical. At day 9 at 11:00 h, the culture volume was determined and the carotene content was measured. The group that produced the most β -carotene obtained a half point extra upon their final mark; the runner-up obtained a quarter point extra.

The tenth day was used to prepare presentations for the CaroTech board. This presentation contained students' strategies, results, discussion and recommendations. Students had a time limit of 10 min for their presentation. A computer with a video projector was available

for their presentations. The score for the presentation counted for 50% of the final mark of the practical.

Learning goals

After this course the student should be able to:

- design a production strategy for biomass growth;
- construct a strategy to produce β -carotene;
- cultivate marine algae and produce β -carotene;
- evaluate the chosen strategies and results.

Equipment

Armatures	: PRO _{max} [®] artnr. 0158161 (max. 150 W) PRO _{max} [®] artnr. 0158175 (max. 500 W)
Centrifuge	: Beckman CS15R with a S4180-rotor
Control valves	: Schott Produran 4
Light climate cabinet	: Fridina AR 7-6503 L
Light meter	: LiCor Li-192SA with light meter LI-250
Magnetic stirrers	: Framo [®] Geräte technik M20/1
Mass balance	: 2 x analytical balance Mettler AE 260 2 x precision mass balance Sartorius 68100P
Mass flow controllers	: Brooks control box 0154 Air : Brooks mass flow controller 5851S CO ₂ : Brooks mass flow controller 5850S
pH-meter	: Schott CG842
Reactor	: Plexiglas flat panel reactor
Spectrophotometer	: CECIL Instruments series 2000 type 2020
Vortex mixer	: LaboTech TMO1
Waterbath	: Julabo F25

Flat panel reactor

The flat panel photobioreactor was made of Plexiglas (dimensions 20x10x4 cm). The total volume of the reactor was 0.8 L. A water jacket was constructed at the front and back of the reactor to keep the culture at the desired temperature. The water jacket was made in such a way that water flowed freely out of the reactor to prevent pressure build up inside the water jacket. During this practical, the water bath temperature was fixed at 25 °C.

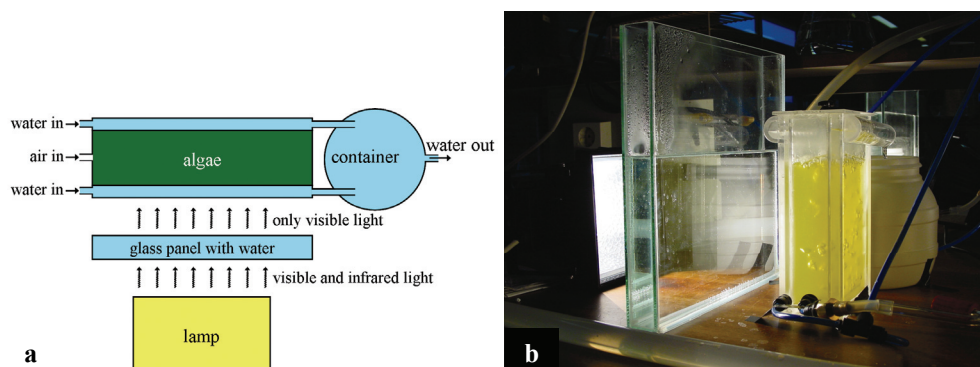


Figure 5.2. **a.** Schematic set-up (from the manual). **b.** Real student set-up.

Set-up

Figure 5.2 shows a schematic set-up from the manual and a practical set-up as used by the students. A glass panel with water could be placed between the lamp and the reactor to remove most infrared radiation and resulting heat.

Flow regulation

The airflow was enriched with carbon dioxide (4% v/v) and water-saturated in a Nalgene bottle of 20 L. A control box regulated the airflow to 15 L min^{-1} and carbon dioxide to 0.6 L min^{-1} . When the pressure inside the bottle exceeded 0.3 bar, a pressure valve released the over-pressure. From the bottle, carbon dioxide enriched air flowed into an air distributor and each group could regulate its flow with a control valve. As sparger, a round Plexiglas tube with 20 holes (diameter 1 mm) located at 60 and 120° was used to supply enriched air to the culture.

Dodecane extraction

One mL of algae sample was put into glass reagent tubes (10 mL) and the tubes were centrifuged (1750g, 5 min). The medium was decanted and 2 mL of dodecane (Sigma 22110-4) was added to the reagent tube. The reagent tube was mixed intensively with a vortex mixer for 5 min. Then, the reagent tubes were centrifuged again at the same conditions as above. The absorption of supernatant was measured with a spectrophotometer at 453 nm against dodecane as a blank. The amount of β -carotene inside the sample was determined using a calibration graph.

Inoculum

D. salina was used as strain in all experiments. The inoculum was grown aseptically in a light climate cabinet at 20 °C. Each group obtained 50 mL of this algal culture (OD₅₃₀ of 0.35) to inoculate their reactor.

Strategies

Table 5.1 shows the growth conditions as chosen by the students. Most groups kept the salt concentration inside the media at 2M. However, group 2, 3, 4 and 7 lowered the salt concentration because they read in articles (Borowitzka 1995; Miravalles and Leonardi 1999; Thakur and Kumar 1998) that this would benefit the alga. Most groups chose low light intensities to grow the alga (40-120 $\mu\text{mol m}^{-2}\text{s}^{-1}$) (Park et al. 1998; Webb and Melis 1995). Group 9 chose for a higher light intensity and higher temperature than the others did (25 °C), by removing the glass panel between the lamp and reactor, because they thought this combination would benefit biomass growth (Hejazi et al. 2002; Miravalles and Leonardi 1999).

Table 5.1. Growth conditions as chosen by the students

Group nr.	Light ($\mu\text{mol m}^{-2}\text{s}^{-1}$)			Other conditions
	I _{front}	I _{back}	I _{av} *	
1	146	70	104	
2	107	58	80	1.5 M NaCl
3	144	70	103	1.0 M NaCl 10 mM KNO ₃
4	135	53	90	1.5 M NaCl, pH 7.5
5	164	88	122	
6	129	62	93	
7	80	45	61	1.5 M NaCl, pH 7.5
8	49	23	35	
9	332	128	222	26 °C

* I_{av}: The average light intensity was calculated as $\frac{1}{2} \cdot (0.95 \cdot I_{\text{front}} + I_{\text{back}})$. The ingoing light intensity was multiplied with a factor 0.95 to correct for the thickness of the light meter.

During cultivation, students were allowed to adapt growth conditions. Based on elemental balance calculations some groups adapted their medium. Also some groups controlled pH and others changed light intensities during the growth phase (Table 5.2). After the growth phase, stress conditions were chosen to produce β -carotene. Some groups selected light as

the only stress condition; some chose salt and other groups used a combination of temperature, light and salt to stress the algae (Figure 5.3).

Table 5.2. Condition changes during the growth phase

Group	Changed conditions
1	At day 2, I_{front} was changed to $54 \mu\text{mol m}^{-2}\text{s}^{-1}$
2	At day 2, 5 mL trace elements* was added and pH was set to 8.0
3	At day 2, 5 mL trace elements* was added and pH was set to 8.0
4	-
5	At day 1, 0.31 mmol FeCl_3 was added. At day 3, 0.15 mmol FeCl_3 , 2 mL trace elements and 8 mmol KNO_3 were added
6	At day 3, I_{front} was changed to $180 \mu\text{mol m}^{-2}\text{s}^{-1}$ and 5 mL trace elements was added
7	At day 1, 44.5 mmol KNO_3 and 10 mL trace elements were added
8	At day 2 and day 4, I_{back} was changed to $40 \mu\text{mol m}^{-2}\text{s}^{-1}$
9	At day 2 an extra light was put at the other side of the reactor, so both sides had an I_{back} of $50 \mu\text{mol m}^{-2}\text{s}^{-1}$

Trace elements solution: $12.3 \text{ mmol Na}_2\text{EDTA} \cdot 2 \text{ H}_2\text{O}$, 4.66 mmol FeCl_3 , $42.0 \mu\text{mol CuSO}_4$, $60.6 \mu\text{mol ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $17.0 \mu\text{mol CoCl}_2 \cdot 6\text{H}_2\text{O}$, $366 \mu\text{mol MnCl}_2 \cdot 4 \text{ H}_2\text{O}$ and $1.04 \mu\text{mol NaMoO}_4 \cdot 2 \text{ H}_2\text{O}$.

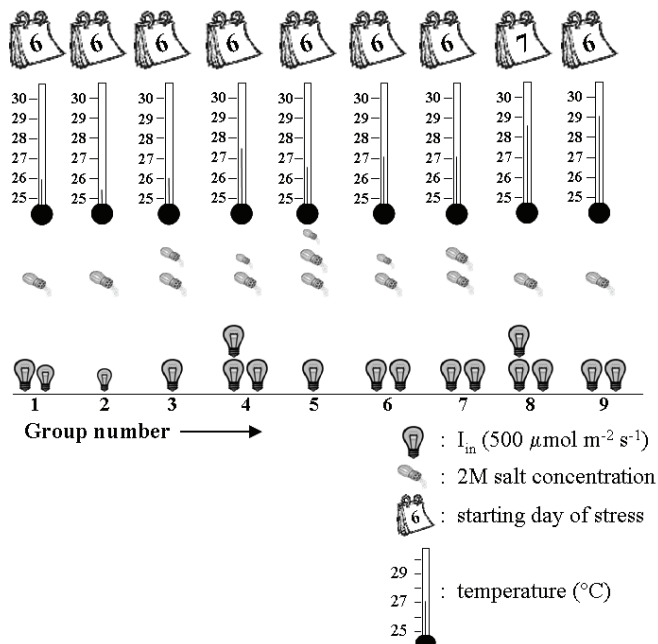


Figure 5.3. Stress conditions as used by the students

Results and Discussion

Biomass

Figure 5.4 shows the amount of biomass in the bioreactors at day 6. Group 9 produced most biomass after six days, followed by group 8, 3, 4 and 6. Group 9 grew organisms at 26 °C and a light intensity of $222 \mu\text{mol m}^{-2} \text{s}^{-1}$. Initially growth was low as cells were stressed, but after two days cells started to grow exponentially with a growth rate of 0.56 day^{-1} . Group 8 was runner-up (OD_{530} 0.98). They started with the lowest light intensity but at day 2 and 4 they observed that almost no light was falling through the culture. They increased the light intensity until $40 \mu\text{mol m}^{-2} \text{s}^{-1}$ was falling through the culture. The exponential growth rate was 0.54 day^{-1} .

Group 7 obtained the lowest biomass concentration (OD_{530} 0.32). They chose a low light intensity and did not change the light intensity when the biomass absorbed more light. The growth rate was 0.30 day^{-1} .

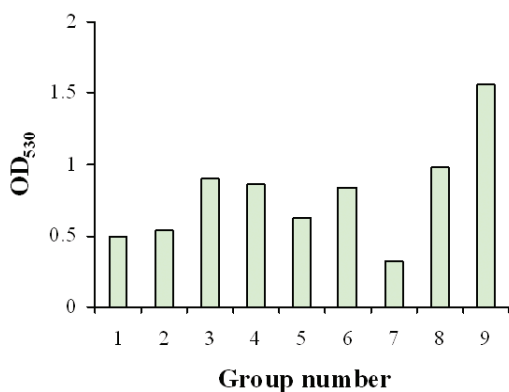


Figure 5.4. Amount of biomass inside the student's bioreactors at day 6.

Stress reaction

From day 6, students started to stress the algae. At day 9, the culture volume was determined and a sample, for measurement of the β -carotene content, was taken from their reactors. Figure 5.5 shows two practical set-ups at day 9. Group 9 tried to stress their dense culture with excessive light ($1000 \mu\text{mol m}^{-2} \text{s}^{-1}$) but Figure 5.5a shows that this group was not successful in applying stress to the culture. The culture was green, which means that the cells contained a lot of chlorophyll. Their culture was very dense, so light could not penetrate through the culture and in the centre of the reactor a dark zone existed. So, instead that the organisms felt stressed, they grew further exponentially.

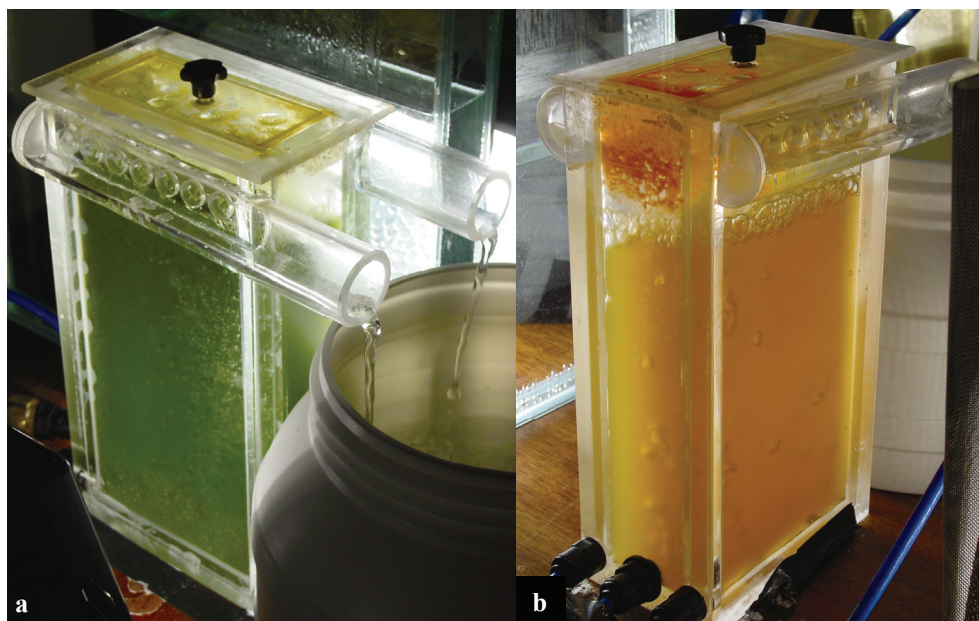


Figure 5.5. Student reactors at day 9. **a.** No stress was evoked (group 9).
b. Stress was successfully applied (group 7).

When stress is applied successfully, the culture should be orange due to β -carotene accumulation and a decrease in chlorophyll content should occur (Gomez-Pinchetti et al. 1992). At normal light levels (below $500 \mu\text{mol m}^{-2}\text{s}^{-1}$) β -carotene together with chlorophyll acts as a light harvesting pigment. However, at higher light intensities β -carotene is mostly localized at the sides of the cells in the form of globules and acts as a kind of ‘sun-screen’ to protect the cells from excessive light. Figure 5.5b shows that group 7 was successful in evoking stress on the organism. Microscopic observations showed that cells in this ‘stressed’ culture were round-shaped and had a size of $\pm 20 \mu\text{m}$ while non-stressed cells were green-colored and oval-shaped ($10 \times 5 \mu\text{m}$).

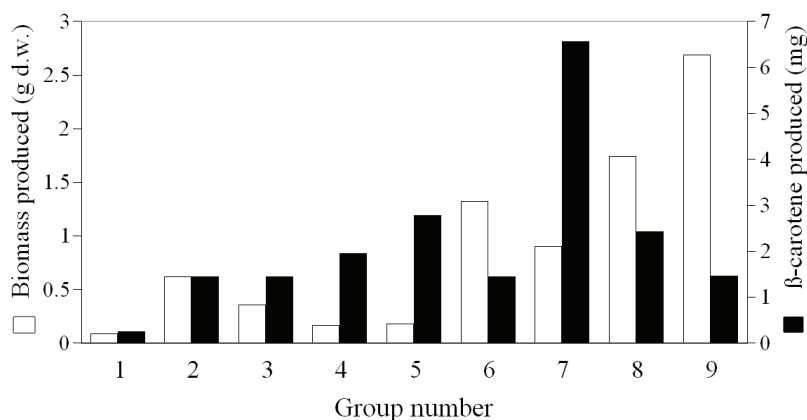


Figure 5.6. Total amounts of biomass and β -carotene produced.

Productivity was calculated by multiplying β -carotene content (mg L^{-1}) with the culture volume (L). Figure 5.6 shows total amounts of β -carotene as produced by the different groups. At most 6.5 mg β -carotene was produced by group 7. This group chose salt and light as stress conditions. At day 6, they had the lowest amount of biomass (Figure 5.4) but their stress strategy was very effective. Other groups (group 8 and 9) were less successful in stressing the alga. They produced most biomass (1.8 and $2.7 \text{ g L}^{-1} \text{ d.w.}$) but, by using only light as stress factor, they were not able to stress the cells.

Learning goals

The research managers discussed growth strategies with students. Most of these strategies were clear, but some students did not motivate their choices properly. Due to time limitation it was also difficult for them to find light intensities in dimensions which could be measured ($\mu\text{mol m}^{-2}\text{s}^{-1}$). However, all students were successful in growing algae (growth rates $0.30\text{-}0.56 \text{ d}^{-1}$).

From the fifth day, students gave the R&D general manager their strategy to stress the organism. At day 8 the general manager discussed the plan with them. Figure 5.3 shows the conditions that each group chose. In our opinion, most strategies were good, even discussing stress influence on the physiology of the alga, but one was marked unsatisfactory. This group failed to evaluate different stress conditions and to motivate their chosen conditions.

The board of CaroTech (five members) all agreed that the presentations were well done. Four groups recommended that the best growth condition was to start with a low light intensity and to increase light at higher biomass concentrations. Groups that obtained high

biomass concentrations, who wanted to stress algae with only light, reported that nutrient and salt stress are better stress techniques to use. In our opinion, the practical succeeded in achieving the learning goals.

Student experiences

Because this was the first time that this course was given, we asked students to judge the practical in the form of an inquiry. We asked them to fill in this inquiry the tenth day of the practical. Table 5.3 shows some answers. One of our own aims was to link theory and practice in a competitive way. Table 5.3 shows that most students thought we succeeded.

Table 5.3. Inquiry results (response 15 of 17 students)

Question	Mark*				
	1	2	3	4	5
Did we succeed to link theory and practice in a competitive way?	-	-	2	6	7
Did you like the practical?	-	-	-	6	9
Did you learn a lot during this practical?	-	-	2	9	4
What would you give as total mark for the practical?	1	-	2	7	5
Would you recommend this practical to other students?	-	-	1	5	9

* Answers were given on a scale of 1-5, where 1 is bad and 5 is a good mark.

- : No answers at this value.

Most students liked the competition, but one of them did not. Our observation was that competition had an advantage. On the first day, students hardly interacted. At the fourth day, they started looking at the reactors of other groups and discussed differences in results and conditions with each other.

In education it is very important that students like the courses, because this influences their motivation and thereby their ability to learn. This was asked to the students and Table 5.3 shows that all students enjoyed the practical. It also shows that, in the students' opinion, they learned a lot. The practical was highly appreciated by them and also they would recommend this practical to other students. Most students spent only 30 h on the practical, so we are considering including economics in the practical next year.

Nomenclature:

d.w.	= dry weight	(g L ⁻¹)
I	= light intensity	(μmol m ⁻² s ⁻¹)
I _{av}	= average light intensity	(μmol m ⁻² s ⁻¹)
I _{front}	= light intensity at the reactor front	(μmol m ⁻² s ⁻¹)
I _{back}	= light intensity at the reactor back	(μmol m ⁻² s ⁻¹)
OD ₅₃₀	= optical density at 530 nm	
R&D	= research and development	
r.p.m.	= rounds per minute	

General discussion

Towards increased microalgal productivity in photobioreactors

Abstract

Marine biotechnology is an exciting new area of biotechnology and there is much interest from industry to cultivate microalgae for the production of bulk products like lipids for biodiesel or as feedstock for industrial chemical processes. To achieve this it is essential to develop cultivation systems in which algae convert the light with a higher photosynthetic efficiency, to get microalgae with better production characteristics, to optimize the medium, to develop cheap harvesting methods and finally specialists should be educated to implement and develop the technology.

For the development of better cultivation systems, insight is needed in the production of algae in such a photobioreactor. Therefore we modeled an outdoor photobioreactor using the approach of light and growth integration. Here, these approaches are compared with the concept of light dilution to reflect on a photobioreactor design with a potential higher photosynthetic efficiency.

Also, a fast screening method for microalgae was developed. This method can be applied to select strains with better production characteristics, to optimize the medium, to compare cultivation conditions and to investigate the toxic effects of chemicals.

Apart from obtaining enhanced algal productivities a low-cost harvesting process is required. We studied if ultrasound could be used for a first concentration step and optimized its efficiency and concentration factor. However, due to the high energy demands of this process, it will not be used for bulk products but can only be commercially interesting for harvesting high-value secondary metabolites. For harvesting of bulk products, as first concentration step auto flocculation on demand is worth investigating.

Beside these research lines, more and better education for biochemical engineers should be developed to push microalgal biotechnology ahead.

Accepted for publication in adapted form in the International Sugar Journal as:

Bosma R, Vermuë MH, Tramper J and Wijffels RH. Towards increased microalgal productivity in photobioreactors.

Introduction

Microalgae have become a hot topic since they are seen as promising organisms for the large-scale production of bulk products like proteins, polysaccharides and lipids to be used as feedstock for industrial chemical processes and biofuel production. However, to make commercial production of such products with microalgae economically feasible, still many bottlenecks in the algal process should be overcome (Figure 6.1).

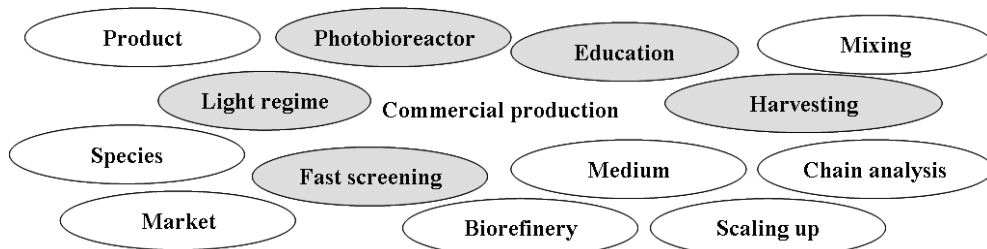


Figure 6.1. Bottlenecks to be overcome in order to achieve commercial production of microalgae in photobioreactors. The bottlenecks in gray areas are addressed in this thesis.

Currently the major costs are involved in production of the algal biomass and basically, the productivity of the microalgae in photobioreactors should increase to make algal production processes economically feasible. This productivity is directly coupled to efficient use of light by the microalgae and therefore the light regime in photobioreactors has been addressed in this thesis with emphasis on the conversion of light into biomass. In addition, a fast screening method was developed, which can be used to optimize the medium or select microalgae with better production characteristics. Apart from increased algal production, a cheap harvesting method is required and we investigated if ultrasound could be used to harvest microalgae. Besides these research lines, learning material and courses should be developed in the field of microalgal biotechnology that trains biochemical engineers to acquire specific knowledge and skills on microalgae and especially to design production processes in which light is required. This thesis covers the development of a practical in which students learned how to design a complex production process.

In this chapter we will reflect on the research addressed in this thesis and elaborate on possible strategies to push microalgal biotechnology forward.

Light as substrate for algae in photobioreactors

To reduce the costs of microalgal production much higher areal as well as volumetric productivities of the microalgae should be realized in the photobioreactor. This should be done by bridging the gap between the real and the theoretical maximum photosynthetic efficiency. The photosynthetic efficiency (PE) represents the amount of biomass formed per light energy (complete spectrum) provided. This paragraph starts with an introduction to light as substrate for cultivating microalgae and is followed by two different approaches how to reach high productivities per ground area. After that, existing state-of-the-art photobioreactors and innovative productions systems are discussed.

In photobioreactors, individual algal cells in dense cultures experience a fluctuating light environment. They experience high light intensities close to the reactor surface and because of absorption of light by the microalgae, the intensities experienced by the algae will decrease with increasing radial depth until eventually darkness is reached (dark zone) in the interior of the photobioreactor. Due to mixing, algae will travel from the reactor wall to the dark interior of the photobioreactor and back; this is defined as a light/dark cycle (Figure 6.2). The total time span of light/dark cycles can be in the order of milliseconds (ms) to seconds, depending on reactor size and mixing intensity (Grobbelaar 1991; Janssen et al. 2000; Janssen et al. 2001).

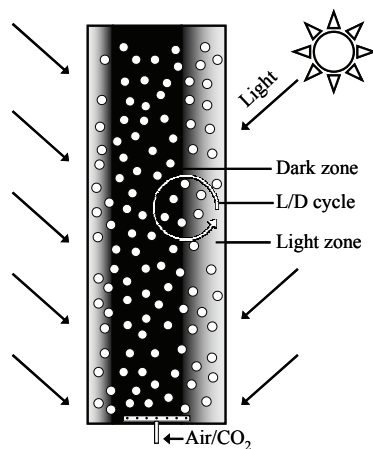


Figure 6.2. Schematic overview of a photobioreactor

Large commercial systems are required and generally sunlight has to be used as energy source to make these systems economically interesting. The light intensity of sunlight on a cloudless day can easily exceed $1500 \mu\text{mol m}^{-2}\text{s}^{-1}$, which is much more than the algae can handle. If the cultivation system, as in an open pond, is poorly mixed, the photosynthetic efficiency of the microalgae close to the reactor surface will be low. With increasing depth, light energy will be converted more efficiently, but in these regions not much light is available. We modeled the volumetric productivity in such a photobioreactor using two modeling approaches: growth and light integration (Bosma et al. 2007).

The growth-integrated approach assumes that the algae experience local light intensities and because of that, photosynthetic yields are calculated with local light intensities. Then, by multiplying absorbed light with photosynthetic yield, local production rates are obtained, which are integrated over the entire radius of the photobioreactor to get total production. At

the reactor wall, where local light intensities are highest (Figure 6.3A), photosaturation or photoinhibition occurs and photosynthetic yields are low (Figure 6.3B).

To obtain maximum productivities in photobioreactors, the incident light intensity should be decreased so that microalgae experience light intensities below the saturating light intensity. This reduction can be achieved in two ways; by light integration or by light dilution. If the light integration approach is used, it is assumed that intensive mixing ensures that the microalgae convert all absorbed light with a photosynthetic yield based on the average light intensity that they experience (Figure 6.3A). Especially at high light intensities above photosaturation level, high turbulence is advantageous because if mixing is fast enough, the algae may experience an average lower light intensity below the photosaturation level and the productivity will thus be increased (Park et al. 2000; Richmond et al. 2003; Terry 1986). This higher production is also predicted by models using complete light integration (Bosma et al. 2007).

Also, fast light/dark alternations, in the order of several milliseconds with a light/dark ratio of about 1:10, have been shown to enhance the algal growth to some extent (Janssen et al. 2001; Kok 1956; Qiang et al. 1998; Terry 1986). However, while beneficial effects are reported, complete light integration has never been shown in mass cultures and only partial light integration is achieved (Tredici and Zittelli 1998; Shen et al. 2009).

To achieve at least partial light integration, photobioreactors should be designed with a short optical path (0.5-1 cm) and the algae should experience short light/dark cycles (Qiang and Richmond 1996; Richmond 2004b; Richmond et al. 2003). Further, a compromise should be found between higher photosynthetic yields (resulting in higher productivities) and the energy costs of mixing.

Another way to increase the overall areal productivity is by diluting the amount of light that falls on a certain ground area by spreading it over a much larger illuminated area (Posten 2009; Schenk et al. 2008). To achieve this, several vertically mounted photobioreactors

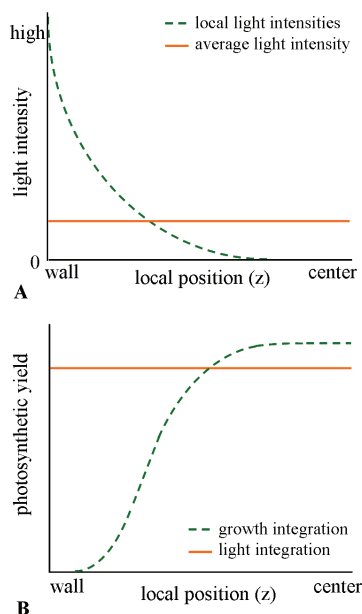


Figure 6.3

A. Light intensities at different radial positions in the photobioreactor for both modeling approaches

B. Photosynthetic yield at different positions in the photobioreactor for both modeling approaches

should be placed in north/south orientation in order to reflect the light between the photobioreactors (Figure 6.4). The light intensity that an alga receives from the incident intensity in this way is reduced to below the saturating light intensity, thereby increasing the biomass yield on sunlight.

This light dilution can also be achieved by collecting direct sunlight (sunlight having a specific direction) with lenses or parabolic mirrors combined with a solar tracking device. The collected sunlight can be distributed by optical fibers or light guidance systems over a larger area per volume, again reducing the light intensity that an alga receives below saturation (Janssen et al. 2003; Zijffers 2008). The major drawback of this system is that only direct sunlight can be collected by these systems (Netherlands 49%, Spain 75%) and the diffuse sunlight is wasted. In addition the re-distributing plates and optical fibers are expensive. This makes this system not interesting for commercial production of algal bulk products.

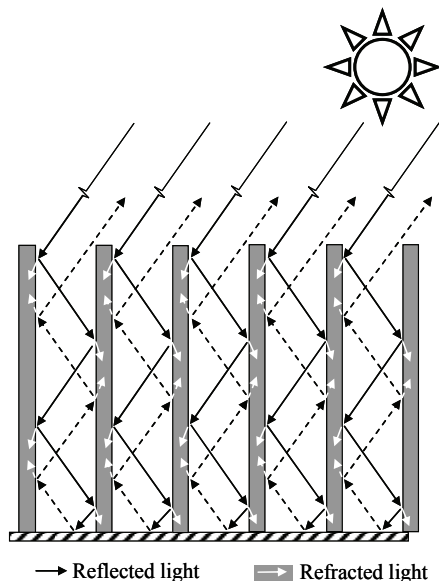


Figure 6.4. Schematic overview of light dilution

Commercial photobioreactors

To produce bulk products with microalgae sunlight should be used as light source. Artificial illumination is much too costly; only the energy cost for the illumination (based on LED technology) is already 23 € kg biomass⁻¹ (Appendix II), which is much more than the actual production costs of 5 to 10 € and 3 to 30 € kg biomass⁻¹ (Shen et al. 2009) that were reported for open ponds and photobioreactors, respectively. Theoretically, microalgae can maximally convert 9% of the absorbed sunlight (based on complete spectrum) into biomass (Chapter 1). Preferably, the photobioreactor is placed in countries close to the equator where much light is available. Photobioreactors should be designed to minimize reflection of light by the photobioreactor material and in addition a material should be chosen that has highest transmittance of PAR (Photosynthetic Active Region, 400-700 nm) light, so that the microalgae receive most light. However, transmission curves show that at least 10% will be lost on transmission and even more light will be lost when the angle of incidence of the sunlight gets higher than 45° (Pollet and Pieters, 2002). During the night

also biomass will be lost due to dark respiration of storage compounds in the microalgae. Night biomass losses (NBL) range from 2-14% (Doucha and Livansky 2006; Grima et al. 1995; Ogbonna and Tanaka 1996) and can be minimized by decreasing the culture temperature after sunset. In addition, energy is needed for maintenance of the cells, which is expected to be proportional to the biomass density of the culture and is known to be significant (Yang et al. 2000).

If we assume 10% loss of the PAR light due to transmission and 5% loss of biomass due to photorespiration during the night and 10% of the energy absorbed is used for maintenance, the maximum photosynthetic efficiency using sunlight for algal production in photobioreactors will be about 7 % as shown below:

Theoretical maximum photosynthetic efficiency	9%
10% of light lost by transmission	x 0.90
5% of biomass lost during the night	x 0.95
10% of energy used for maintenance	x 0.90
Maximum photosynthetic efficiency in photobioreactors:	7%

To reach this photosynthetic efficiency (PE), productivity should only be limited by light and other factors (nutrient limitation, temperature, growth inhibition by algal products or oxygen, loss of light due to photosaturation etc.) should be prevented.

Most of the commercial photobioreactors that have been developed so far are based on either the concepts of light integration or on light dilution. Table 6.1 shows the reported or calculated photosynthetic efficiencies reached in several existing photobioreactors and the calculated corresponding areal productivities based on daily natural sunlight data for Huelva in Southern Spain (Appendix I).

Open ponds show the lowest productivity because the algae convert the light with a low photosynthetic efficiency due to a long optical path, slow mixing and lack of control (temperature, pH).

The tubular reactor (Figure 6.5A) and the thin-layer pond (Figure 6.5B) have a higher efficiency due to a shorter optical path and higher turbulence combined with better control. However, light/dark cycles are still too long to obtain complete light integration and also gradients (pH, nutrients, oxygen) occur in both systems (Richmond 2004a). The biggest disadvantage of these systems is that oxygen produced in these systems by the microalgae can easily exceed 300% oxygen saturation, leading to growth inhibition of the algae and a severe loss of productivity (Aiba 1982; Tredici et al. 1991; Weissman et al. 1988). This can be solved by increasing liquid velocities, but only at the expense of high energy costs (Sierra et al. 2008).

Table 6.1. Areal productivities of commercial photobioreactors for Huelva, Spain (37°15'56" North) deduced from data provided in literature.

Reactor	Approach	Optical path (cm)	PE (%)	Areal productivity ton ha ⁻¹ yr ⁻¹	References
Open pond	Growth integration	30	0.9	27	(Jimenez et al. 2003a)
Thin-layer pond	Light integration	0.6	2.6*	78	(Doucha and Livansky 2006)
Tubular reactor	Light integration	5	3	90	(Tredici and Zittelli 1998) (Fernandez et al. 2001)
Flat panel	Light dilution	1-2	6**	179	(Posten 2009)
Theoretical maximum in photobioreactors			7	200	

* Photosynthetic efficiency (PE) measured **Estimated from (Zittelli et al. 2000), assumed that light was diluted to an uniformly two-sided illumination of 115 $\mu\text{mol m}^{-2}\text{s}^{-1}$.

Using the light integration approach for photobioreactor design leads to high demands of energy for mixing (334 W m⁻³), required to achieve short light/dark cycles (Janssen et al. 2003). In addition, Zijffers found that increased turbulence does not necessarily lead to higher observed efficiencies for *Chlorella sorokiniana* in a flat panel photobioreactor (optical path 1.25 cm) since the maintenance requirements of the high density culture of the microalgae will decrease the observed photosynthetic efficiency on light (Zijffers 2009). Also dissolved oxygen levels will be high and probably will limit productivity (Jimenez et al. 2003b). Altogether, it is better not to pursue the light integration approach in photobioreactor design, since this will lead to lower photosynthetic yields and thus high production costs of the biomass.

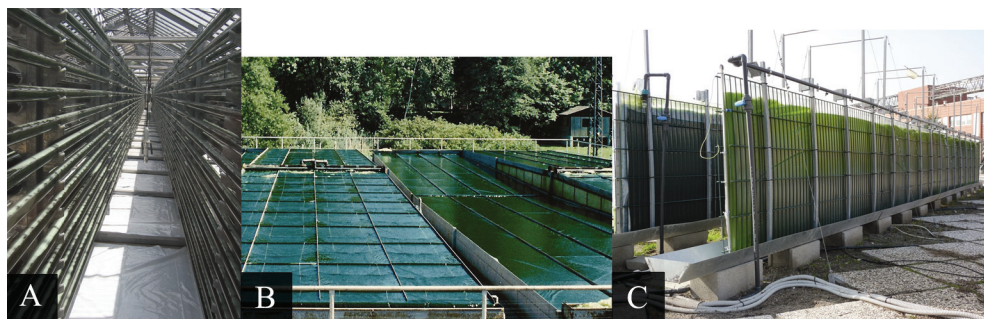


Figure 6.5. **A.** Tubular reactors in Ritschenhausen (GMBH). **B.** Thin-layered pond (courtesy of J. Doucha, Třeboň laboratory, Czech Republic) **C.** Flat panels, also described as green wall panel (courtesy of M.R. Tredici, University of Florence, Italy).

Innovative photobioreactors can better use the approach of light dilution to reduce the incident light intensity to $100 \mu\text{mol m}^{-2}\text{s}^{-1}$. In this case, the lower amount of light that is absorbed by the microalgae can be converted with a much higher photosynthetic yield (Chapter 2, Figure 2.6). Zittelli also showed that in a modular flat panel photobioreactor illuminated by a light intensity of $115 \mu\text{mol m}^{-2}\text{s}^{-1}$ in which *Nannochloropsis sp.* was cultivated, a PE of 6% could be reached (Zittelli et al. 2000). With this efficiency, an areal productivity of $179 \text{ ton ha}^{-1} \text{ yr}^{-1}$ is possible in Southern Spain (Table 6.1).

As building block, a thin-layered flat panel (optical path $< 2 \text{ cm}$) film can be used best. Advantage of flat panels is that they have, like other vertical systems, a high mass transfer (0.006 s^{-1}) and require less power (53 W m^{-3}) compared to tubular photobioreactors ($2400\text{--}3200 \text{ W m}^{-3}$) to achieve this mass transfer (Sierra et al. 2008). Photobioreactors with longer optical paths should not be used because the light is not used efficiently, resulting in voluminous reactors with low volumetric productivities and thereby high costs of downstream processing.

Total costs of the cultivation system should be less than 15 € m^{-2} to make production costs of energy products with microalgae commercially feasible (Schenk et al. 2008). In my opinion, thin-layered inflatable plastic films should be used to reduce the costs of the material needed. Typical examples of photobioreactors using these types of films are presently developed (Figure 6.6).

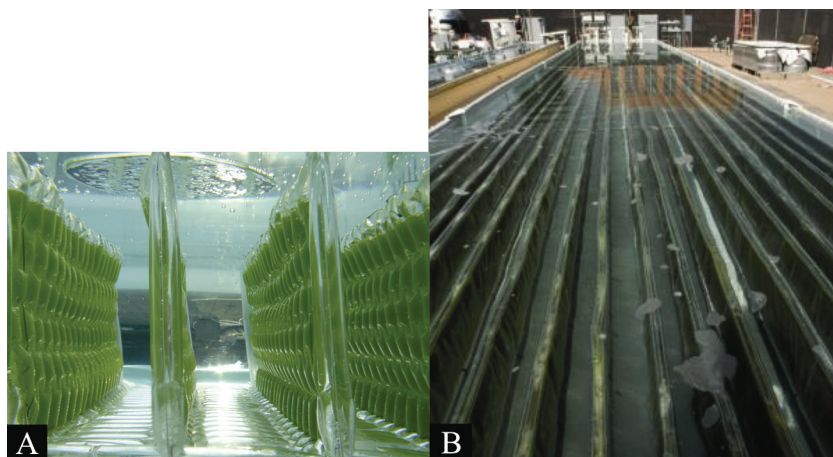


Figure 6.6. A. Flat panel photobioreactor of Proviron (courtesy of M. Michiels, Proviron, Belgium)
B. Flat panel photobioreactor of Solix Biofuels (courtesy of B. Willson, Colorado State University).

In the Proviron flat-panel type photobioreactor, the air pressure around the cultivation chambers is kept higher than the external air pressure to get support (patent EP 2 039 753 A1). The plastic modules are submerged in a large water basin for extra support and temperature control. In the Solix Biofuels photobioreactor the plastic bags are hung on a support device and like in the Proviron design the whole construction is placed in a water basin. The ground on which the cultivation is placed should be white-washed to reflect 64% of sunlight to otherwise shaded parts (Zijffers 2009).

Fast screening

For commercial production it is important to select the best strains and to grow them under optimal conditions in the best growth media. With high throughput screening methods, microalgae can be screened on desired product formation and on required stress conditions to obtain the highest amount of product per biomass (Schenk et al. 2008). We developed a screening method in which 24-wells plates are inoculated with microalgae that are subsequently grown under controlled conditions. Then, by measuring the optical density with a well reader in time using linear regression, specific growth rates of the microalgae can be determined. This bioassay was used to determine the growth inhibition effects of free fatty acids in *Monodus subterraneus* (Bosma et al. 2008).

It can also be used to study the effect of process conditions in a short time. Figure 6.7A for example, shows the effect of pH of the medium on the growth of *C. vulgaris*. To measure it, only one day was needed. Figure 6.7B shows the toxic effects of a disinfectant on the growth of *M. subterraneus*, determined in a time span of three days. These results show that a bioassay can be a powerful tool for optimization conditions and as troubleshooting device when commercially producing microalgae.

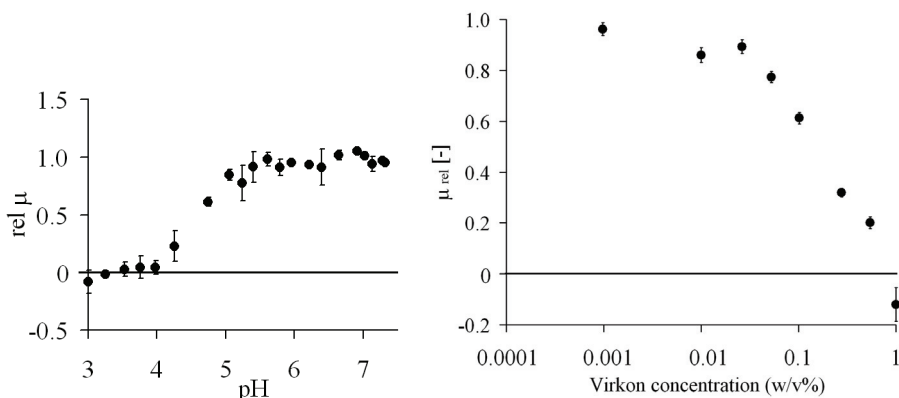


Figure 6.7. A. Relative growth rate of *C. vulgaris* (CCAP 211-118) in M8-A medium at different pH. B. Relative growth rates of *Monodus* at different disinfectant (Virkon[®]) concentrations (Bosma et al. 2008).

Medium optimization

I would like to propose to use high throughput growth measurements methods in combination with a genetic algorithm for rapid medium optimization. Often, algae are cultivated in media that were developed for a range of species and not optimized for a specific species resulting in suboptimal productivities (Kennedy and Krouse 1999). For example, several media are suggested for cultivation of *Chlorella* (Table 6.2). This table shows that nutrient concentrations vary widely; the macronutrients nitrogen and phosphate vary with a factor 10 and 35, respectively, and trace elements like boric acid, cobalt and aluminum are used in some media, while in other media they are omitted. Using media optimization techniques, essential nutrients can be identified and their optimal concentration for growth or product formation can be determined.

Table 6.2. Compositions of media used to cultivate *Chlorella vulgaris*

	BG-11	BBM	M8-A	Mod. Fitz	Sorokin/ Krauss
Macronutrients (mmol L⁻¹)					
N	17.6	2.9	29.7	5.0	12.4
P	0.2	1.7	6.9	0.3	9.2
S	0.3	0.4	2.2	0.6	4.2
Mg	0.3	0.3	1.6	0.5	4.1
Fe	0.03	0.02	0.49	0.03	0.2
Ca	0.24	0.17	0.09	0.13	0.3
Trace elements (μmol L⁻¹)					
Zn	0.8	30.7	11.1	1.0	0.31
Cu	0.3	6.3	7.3	0.0008	0.06
Mn	9.1	7.3	65.6	10.1	0.07
Co	0.2	1.8	-	0.6	0.02
Mo	1.6	4.9	-	0.6	0.05
B	46	185	-	50.1	1.84
Al	-	-	28.7	1.0	-
EDTA	2.3	171	27	32	1711
Salts (mmol L⁻¹)					
Na	18.0	3.5	2.9	5	1.7
K	0.4	1.3	35.1	0.5	21.5
Cl	0.5	0.8	0.3	0.3	0.5
Organics (mmol L⁻¹)					
Citric acid	0.06	-	-	-	-
Reference	(Andersen 2005)	(Mandalam and Palsson 1998)	(Widjaja et al. 2009)	(Richmond 2004)	

To optimize such a medium, efficient optimization methods like experimental designs and genetic algorithms are available. Experimental designs are systematical optimization tools that heavily rely on logic and statistics. They need a design and an optimization technique to describe the results of well-designed experiments (Kennedy and Krouse 1999; Haaland 1989). The design specifies the concentration ranges of the parameters (e.g. nutrients) that are chosen. These parameters are varied simultaneously and a response factor is chosen. Then, a mathematical model, depending on the chosen optimization technique, is used to optimize the parameters to the chosen response factor. When only a few parameters (e.g.

macronutrients) are optimized, this method has the advantage that the design space is modeled and insight is obtained how parameters interact and the chosen response factor can be predicted (Bosma et al. 2003; Bosma et al. 2007). However, mostly media contain many nutrients (>6) and these have to be optimized. Then this method is not so efficient because, still many experiments are needed to find the true optimum.

In those cases, a genetic algorithm (GA) can better be used. It is inspired on evolution and can deal with larger sets of parameters in a highly directed way and has proven to be a powerful tool for optimization studies in several scientific fields (Bäck and Schwezel 1993; Weuster-Botz 2000). A schematic overview of a GA to optimize a growth medium to obtain highest specific growth rate of a microalgae is given in Figure 6.8. First, the problem should be defined and the number of variable nutrients as well as a concentration range of these nutrients should be chosen; this defines the parameter space. From this parameter space a random set of media (first generation) is generated by the GA program. The algae are then cultivated in these media at defined conditions and the specific growth rates are determined. Media in which algae show a high growth rate are selected by the GA program to generate a new set of media via cross-over and mutation, after which the alga is cultivated in this new set of media. Again growth of the alga on the tested media is evaluated. If the media all show convergence to one optimal value for the growth rate, the optimum medium for growth is found. If not, the process of selection, crossover, mutation and evaluation of media is repeated until an optimal solution is found (Marteijs et al. 2003; Weusterbotz and Wandrey 1995).

The minimum amount of media (M) that should be tested in each population can be calculated if the probability (p_M) is defined, indicating the chance that each point in the variable space can be reached by crossover starting from the start population (Weuster-Botz 2000):

$$M > 1 + \log (1 - p_M^{1/L}) \cdot (\log 0.5)^{-1}$$

With: p_M 0.9999, total bit string (L) of 60 (variation of 12 nutrients simultaneously with 32 (binary code: 2^5 bits) concentration steps), it can be calculated that at least 21 media should be tested. The single-point crossover chance in a GA should be higher than 90% and the minimal mutation chance (p_m) should be more than the reciprocal value of the total bit string ($p_m > 1/L$) to prevent convergence to local optimums (Weuster-Botz 2000). Several media for bacteria, yeast and insect cells were already successfully optimized using GA's yielding improvements up to 87% compared to a control medium (Etschmann et al. 2004; Weusterbotz and Wandrey 1995). However, optimization of nutrients for algae with a genetic algorithm was not done before, because a reproducible screening method to determine specific growth rates was not available.

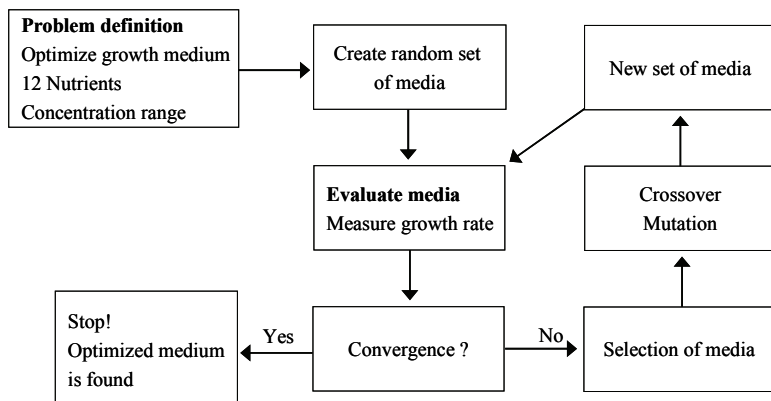


Figure 6.8. Schematic overview of a genetic algorithm to obtain an optimized growth medium.

Presently, we use a genetic algorithm and our developed bioassay to optimize the medium for *Chlorella vulgaris* SAG 211-11b to obtain highest specific growth rate of this alga by changing twelve nutrients simultaneously. We chose to measure the growth rate of the algae in 36 media per population, a crossover chance of 95% and a mutation rate of 2%. In the third generation, five media were already found in which the algae grew 75% faster than in M8-A medium (unpublished results).

Figure 6.9 shows that this technique is indeed potentially very powerful. In the first generation, the concentration of the nutrients is chosen randomly. This can be seen in the most left figures, there are few media (1-5) that have a certain concentration of that nutrient. In the second generation, for nitrogen and phosphate still no convergence takes place, because the concentration of these nutrients that is added to the media is still chosen over a large concentration range. For sulphate, some convergence can already be seen in the second generation, because more media are present that use a high concentration of sulphate. Finally, in the last generation (4), many media show convergence to a certain concentration of the main macronutrients (N, P, S).

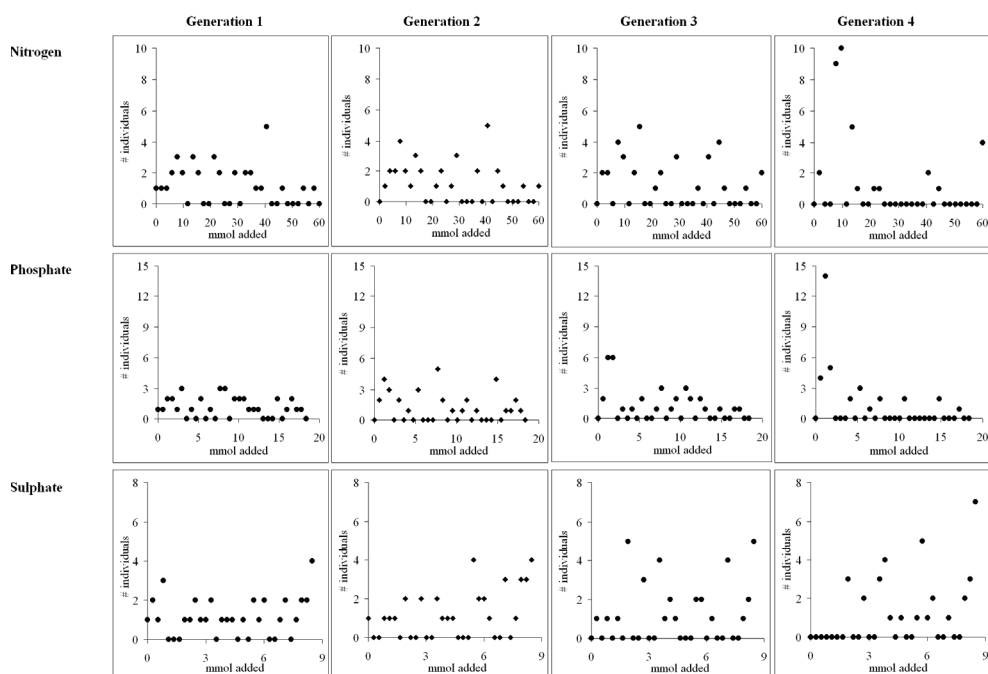


Figure 6.9. Amount of media per generation that contained a certain concentration of a nutrient.

Improved species

Wild-type microalgae kept in culture collections are merely used in commercial production systems. However, these strains often show suboptimal characteristics. To make a production process for bulk products commercially interesting, much higher productivities are needed and the cultivation process should be robust. Therefore, algal screening programs are needed that select on desired criteria by natural strain selection or genetically improved strains should be developed with these desired characteristics (Table 6.3).

Characteristics of new developed or newly discovered strains could be tested in bioassays or by other selective screening methods.

Table 6.3. Examples of desired characteristics of a commercial algal strain.

Desired characteristic	Advantage	Selection criterion
High specific growth rate	High algal productivity	Biomass formation in time
High photosynthetic yield	Higher efficiency	Biomass per amount of light
High product content	More product per algae	Product per dry weight
No wall growth	No internal wall cleaning needed	Wall growth in time
Inducible auto flocculant	Easy down-stream processing	Flocculation and zeta-potential
Extremophile	Species domination	Growth rate under extreme conditions

A robust production process is needed when cultivating microalgae commercially, because the costs of cleaning, sterilizing and starting up a photobioreactor are relatively high. Therefore, contamination by unwanted species should be prevented. A pure algal culture can be established via plating and/or using antibiotics (Andersen 2005; Kooistra et al. 1991; McCracken 1989). This culture can be used as inoculum for a closed photobioreactor in which contamination can be prevented. Also a hybrid system can be used in which algae precultured in closed systems are used to inoculate open systems in which secondary product formation is evoked by stressing the microalgae (Rodolfi et al. 2009). Another way to prevent contamination is to select algae that outcompete other organisms (extremophiles) or select species that excrete antibacterial substances. Several microalgae (including *Chlorella*, *Desmococcus* and *Scenedesmus*) are known to excrete such compounds (Ördög et al. 2004). When genes could be identified that are responsible for producing and excreting these antibacterial compounds, maybe also other algae can be genetically altered to do so.

Hyper producing mutants can be selected by quantitative flow cytometry and cell sorting (FCCS). This method allows processing of many cells (up to thousands cells s⁻¹) and therefore is ideal for screening purposes. The cells are analyzed on the fluorescence of desired products and hyper producing cells can be isolated with a flow sorter. However, most products have a low fluorescence and FCCS cannot be used. This can be solved by fluorescent dyes that bind specifically to the desired product like Nile Red (9-diethylamina-5Hbenzo[a]phenoxarine-5-one) that stains intracellular lipids. This seems therefore a promising approach to select hyper producing species (Chen et al. 2009; de la Jara et al. 2003; Mendoza et al. 2008).

While with bacteria and yeast, genetic modification is widely applied, the field of transgenic algae is still in its infancy (Walker et al. 2005). However, with complete genome sequences of algae being identified (Bowler et al. 2008) and the successful transformation of several microalgae, algal research groups and biotechnology companies are now beginning to apply genetic engineering technology to modify key metabolic pathways. Like with bacteria and yeast, genomic databases should be constructed that can be combined with biochemical and physiological information to construct genome-scale flux models for the primary metabolism of microalgae. This genome-scale models can be used to get better understanding of cellular metabolism, to develop metabolic engineering strategies and to design media and processes (Baart et al. 2007; Smid et al. 2005).

For the near future, probably known species will be altered to get desired characteristics (Walker et al. 2005). When this field is further developed, probably a few algae are selected as high producing host organisms in which genes can be inserted to produce or accumulate a desired product (Dijck van 1999). An algal strain that already has some desired characteristics is *Chlorella sorokiniana*. It has a relatively high growth rate (0.11 h^{-1}), can grow up to temperatures of $46.5\text{ }^{\circ}\text{C}$ and stills grows well at concentrations of $10\text{ }\%\text{CO}_2$, 100 p.p.m. NO and 225 p.p.m. SO_2 (Morita et al. 2000). At these extreme conditions, a mono-algal culture can easily be established and flue gas can be used as carbon dioxide source. However, a major disadvantage of this species is that it cannot grow on seawater. Marine microalgae can better be screened, because seawater is more abundant available and already prevents many contaminants to grow.

Harvesting

A low-cost harvesting process is required to make the commercial production of algal bulk products possible. Harvesting costs are significant because microalgae grown in cultivation systems are relatively diluted ($0.5\text{--}3\text{ g L}^{-1}$) compared to other microbial (yeast, bacterial) processes ($50\text{--}100\text{ g L}^{-1}$). In addition, unicellular microalgal cells are small, usually smaller than $20\text{ }\mu\text{m}$ and sometimes even under $5\text{ }\mu\text{m}$ in diameter. Harvesting of algae accounts for about 30% of the total costs in case of algal production in open ponds (Grima et al. 2003; Zittelli et al 2006), because as harvesting process centrifugation is used. A better approach would be to use a preconcentration step until $1\text{--}5\text{ }\%\text{ w/w}$ and then concentrate this further to an algal paste ($15\text{--}20\text{ }\%\text{ w/w}$) with centrifugation, which is required for further biomass processing (Benemann and Oswald, 1996). As first concentration step, induced aggregation of the cells (leading to larger and heavier particles) seems promising because this leads, according to Stokes' law, to enhanced sedimentation (Chapter 4, Figure 4.7). We investigated if ultrasound, which is based upon this principle, could be used as harvesting

process and optimized the harvesting efficiency and concentration factor (Bosma et al. 2003).

This harvesting method had an efficiency of 92% and maximally the ingoing biomass could be concentrated with a factor 20. It has as main advantages that it is a non-fouling technique, cells are still viable and potential interesting metabolites are not destroyed, because no shear stress is involved. Also, when an organism excretes a high valuable secondary metabolite, this technique can be used as a retention system. The resonation chamber acts as a biological filter by rejecting the organisms and allowing the solubilised product to pass. For a hybridoma cell line producing a monoclonal antibody, it was already shown that a perfusion process using a pilot-scale acoustic cell-retention device (200 L d⁻¹) is reliable and simple to operate (Dalm et al. 2005).

Table 6.4. Relative costs, resulting solid concentrate, energy input and the most important disadvantage (for biodiesel production) of different concentration or pre-harvesting methods. Adapted from Benemann and Oswald 1996.

Harvesting method	relative cost	obtained solid conc. (%)	energy input	main disadvantage
Centrifugation	10	>10	high	expensive
Filtration (cross flow)	4-6	2-6	high	optimal for large or colonial algae
Discrete sedimentation	0.5-1.5	4-6	low	species dependent
Ultrasound*	>10	1-3	very high	expensive, small scale only
Chemical flocculation	4-8	8-10	medium	expensive
Polymer induced flocculation	0.5-1	1-3	low	species dependent

* Based on Chapter 4

For commercial algal cultivation processes, ultrasound as primary harvesting process is too costly due to too high energy costs. Also other harvesting processes like flotation, centrifugation, sedimentation and filtration (Table 6.4) are too costly or inefficient to be used on commercial scale (Benemann and Oswald 1996; Danquah et al. 2009; Ryll et al. 2000; Shen et al. 2009).

It was reported that flocculation could be induced by a higher pH and that calcium and orthophosphate should be present in the medium to give flocculation of the microalgae. Calcium ions stick to the algal cells, neutralizing the negative charge of these algal cells and thereby increasing the chance that coagulation occurs (Sukenic and Shelef 1984). Orthophosphate probably is needed to get a double layer around the algae to obtain a less negatively charged layer around the cells, increasing the chance of coagulation. In

commercial systems, this flocculation could be induced by ceasing carbon dioxide supply causing the pH to rise. An advantage of this harvesting process is that after sedimentation of the microalgae, the medium might be recycled.

Auto flocculation, the spontaneous flocculation of microalgae without addition of any chemicals, seems another good alternative. In this process a polymer, produced by the algae themselves leads to flocculation. Forming of this polymer can probably be induced by nutrient limitation (Lee et al. 2009; Mancuso Nichols et al. 2009; Mishra and Jha 2009). These polymers can partly or completely bind to the polysaccharides that are present in the outer membrane of the algal cells. When binding occurs, the polymer can also bind to other algal cells and a network of polymers and algae can be formed, called a floc, which then leads to enhanced sedimentation, like in the ultrasonic harvesting process (Tenney and Verhoff 1973). Induced flocculation and polymer induced flocculation should be studied and tested on commercial scale to see if they can be used as first concentration step.

Education

Marine biotechnology is an exciting new area of biotechnology and therefore gained much interest from industry to cultivate microalgae for the production of bulk products like lipids for biodiesel or as feedstock for other industrial chemical processes. Research is still in an early stage and companies in the field of marine biotechnology start to develop. At present, there are a few applications and probably many will follow. Biochemical engineers are able to develop and build industrial plants for biochemical or microbial (bacteria, yeast) processes. However, they lack specific knowledge about microalgae and especially the design of production processes in which light is required. To obtain such specific knowledge, education of biochemical engineers in the field of marine biotechnology is needed.

At the moment, only a few courses at BSc and MSc level are available that can be used for training in the field of microalgal biotechnology. They are mostly at university level and cover the biology and taxonomy of microalgae. Almost no courses are available that teach people how to cultivate microalgae, engineer cultivation systems, and build and manage complete cultivation plants. To push microalgal biotechnology forward, it should become part of the curriculum of universities and business schools.

Since 2003 microalgal technology was addressed in the optional course marine and animal biotechnology for MSc student's biotechnology at Wageningen University. In this introductory course students obtained basic knowledge on marine biotechnology (Table 6.5). A practical was developed for this course (Bosma and Wijffels 2003).

In September 2008 within Wageningen University an MSc programme in biotechnology was initiated with emphasis on specialization in the field of marine biotechnology. This specialization includes molecular biology-oriented courses on bioinformatics, metabolic modeling and pathway analysis, and courses focusing on marine biotechnology. For this reason an advanced course was developed, with emphasis on cell biology of marine micro-organisms and design of photobioreactors. An overview of the current learning objectives of both courses focusing partly on microalgae is given in Table 6.5.

Table 6.5. Learning objectives MSc courses on marine biotechnology at Wageningen University

Introduction to marine biotechnology	Advanced course on marine biotechnology
Learn the basics of photosynthesis	Design of a photobioreactor for a algal product
Determine what limits productivity in algal cultivations	Construct a simplified metabolic network for microalgal growth
Describe state-of-the-art photobioreactors and discuss differences	Gain insight in new developments in the field of marine biotechnology
Describe different operating conditions and discuss how these affect algal productivity	Understand how metagenomic sequence analysis can provide novel leads for biotechnological exploitation
Learn how to cultivate algae in photobioreactors	Design production and research strategies for production of bioactive compounds from sponges
Learn how to develop a production strategy for an algal product	Quantify the maximal productivity of microalgae based bioprocesses
Describe commercial applications of microalgae	Identify biomarker components

For the advanced course, a distance learning module has been developed, covering a case study on photobioreactor design. In this module the students are asked to work out a mathematical model for the design of a photobioreactor for the production of a high value product from algae. This case study offers students the possibility to elaborate on design principles and constraints (Schaaf et al. 2003). The constructed model is used by the students to identify bottlenecks in an algal production process and to address future leads for algal productivity improvement.

In addition to the topics covered in the existing courses, more attention should be paid to educate students in down-stream processing and to the derivation of the algal biomass into useful products. It would be worthwhile to include in the education of specialists in algal technology the analysis of a complete production process in terms of energy, exergy and costs. Then, they can learn to identify bottlenecks and get new insights how a production process can be improved.

In addition to education at university level, also education in the field of marine biotechnology should be introduced at secondary school. Classroom experiments with microalgae can be used to show the process of photosynthesis and can form a bridge between physiology, ecology and biotechnology (Wunschiers and Lindblad 2002) making future algal biotechnologists already enthusiastic for this field.

To achieve increased microalgal productivity at full industrial scale we should not invest in further research and development of microalgal technology only, but also invest in adequate education of dedicated people.

Appendix I. Calculation of areal productivities

$$P_{\text{year}} = \frac{E_{\text{a,year}} \cdot \text{PE}}{CV_{\text{biomass}}} \quad \text{Equation 1}$$

P_{year}	= productivity of biomass per year per ha	[ton ha ⁻¹ year ⁻¹]
$E_{\text{A,year}}$	= total amount of sunlight (PVGIS, solar irradiation data)	[MJ ha ⁻¹ y ⁻¹]
PE	= photosynthetic efficiency of the photobioreactor	[%]
CV_{biomass}	= heat of combustion of biomass, here 22.9 (Morita et al. 2000)	[kJ g ⁻¹]

Appendix II. Cost of artificial lighting

Based on document 6322 635 57271 of Philips

GreenPower LED module HF 8727900 908312 00

Needed for algae	100	μmol m ⁻² s ⁻¹	(PAR)
Per LED module	10	μmol m ⁻² s ⁻¹	(PAR)
So needed per m ²	10	LED modules	
Per LED module	10	W	energy
so	100	W	energy per m ²
Continuous light	24	hr	
Energy needed	2.4	kWhr m ⁻² d ⁻¹	
Energy costs	0.15	€ kWhr ⁻¹	(several suppliers in the Netherlands)
Continuous light	0.36	€ m ⁻² d ⁻¹	energy costs
Yield	1.8	g biomass mol photons ⁻¹	(Zijffers 2009)
Amount of photons	8.64	mol d ⁻¹	
Max. produced	15.6	gram biomass m ⁻² d ⁻¹	
Implicating energy cost of	0.023	€ g ⁻¹	biomass
	23	€ kg ⁻¹	biomass

References

- Aiba S. 1982. Growth kinetics of photosynthetic microorganisms. *Advances in Biochemical Engineering* 23:85-156.
- Akkerman I, Janssen M, Rocha J, Wijffels RH. 2002. Photobiological hydrogen production: photochemical efficiency and bioreactor design. *International Journal of Hydrogen Energy* 27(11-12):1195-1208.
- Andersen RA. 2005. *Algal culturing techniques*: Academic Press. 578 p.
- Baart GJE, Zomer B, de Haan A, van der Pol LA, Beuvery EC, Tramper J, Martens DE. 2007. Modeling *Neisseria meningitidis* metabolism: from genome to metabolic fluxes. *Genome Biology* 8(7):20.
- Bäck T, Schwezel H-P. 1993. An Overview of Evolutionary Algorithms for Parameter Optimization. *Evolutionary Computation* 1(1):1-23.
- Becker EW. 1994. *Microalgae: biotechnology and microbiology*. Cambridge: Cambridge University Press.
- Benemann JR, Oswald WJ. 1996. Systems and economic analysis of microalgae ponds for conversion of CO₂ to biomass. Berkely: University of California. 215 p.
- Berg Hvd, Oudshoorn A, Tramper F, Keijzer T. 2001 High density cell cultures in perfusions. A perspective for vaccine production? *BIOforum*.
- Berg JM, Tymoczko JL, Stryer L. 2006. *Biochemistry*, Chapter 19: "The light reactions of photosynthesis". New York: Freeman. 1026 p.
- Bierau H, Perani A, Al-rubeai M, Emery AN. 1998. A comparison of intensive cell culture bioreactors operating with Hybridomas modified for inhibited apoptotic response. *Journal of Biotechnology* 62:195-207.
- Borowitzka MA. 1995. Microalgae as sources of pharmaceuticals and other biologically active compounds. *Journal of Applied Phycology* 7:3-15.
- Borowitzka MA. 1999. Commercial production of microalgae: ponds, tanks, tubes and fermenters. *Journal of Biotechnology* 70:313-321.
- Borowitzka MA, Borowitzka LJ. 1988. *Dunaliella*. Micro-algal Biotechnology. Cambridge: Cambridge University Press. p 27-58.
- Bosma R, Wijffels RH. 2003. Marine biotechnology in education: a competitive approach. *Biomolecular engineering* 20(4-6):125-131.
- Bosma R, van Spronsen WA, Tramper J, Wijffels RH. 2003. Ultrasound, a new separation technique to harvest microalgae. *Journal of Applied Phycology* 15(2-3):143-153.

-
- Bosma R, van Zessen E, Reith JH, Tramper J, Wijffels RH. 2007. Prediction of volumetric productivity of an outdoor photobioreactor. *Biotechnology and Bioengineering* 97(5):1108-1120.
- Bosma R, Miazek K, Willemsen SM, Vermuë MH, Wijffels RH. 2008. Growth inhibition of *Monodus subterraneus* by free fatty acids. *Biotechnology and Bioengineering* 101(5):1108-1114.
- Bowler C, Allen AE, Badger JH, Grimwood J, Jabbari K, Kuo A, Maheswari U, Martens C, Maumus F, Ottillar RP and others. 2008. The *Phaeodactylum* genome reveals the evolutionary history of diatom genomes. *Nature* 456(7219):239-244.
- Camacho FG, Gomez AC, Fernandez FGA, Sevilla JMF, Grima EM. 1999. Use of concentric-tube airlift photobioreactors for microalgal outdoor mass cultures. *Enzyme and Microbial Technology* 24:164-172.
- Cardozo KHM, Guaratini T, Barros MP, Falcão VR, Tonon AP, Lopes NP, Campos S, Torres MA, Souza AO, Colepicolo P and others. 2007. Metabolites from algae with economical impact. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 146(1-2):60-78.
- Chen W, Zhang C, Song L, Sommerfeld M, Hu Q. 2009. A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. *Journal of Microbiological Methods* 77(1):41-47.
- Coakley WT, Hawkes JJ, Sobanski MA, Cousins CM, Spengler J. 2000. Analytical scale ultrasonic standing wave manipulation of cells and microparticles. *Ultrasonics* 38:638-641.
- Cohen Z. 1994. Production Potential of Eicosapentaenoic acid by *Monodus subterraneus*. *Journal of the American Oil Chemists Society* 71(9):941-945.
- Cohen Z. 1999. Products from microalgae. *Handbook of Microalgal Mass Culture*. p 421-454.
- Dalm MCF, Jansen M, Keijzer TMP, Grunsven WMJv, Oudshoorn A, Tramper J, Martens DE. 2005. Stable hybridoma cultivation in a pilot-scale acoustic perfusion system: Long-term process performance and effect of recirculation rate. *Biotechnology and Bioengineering* 91(7):894-900.
- Danquah MK, Ang L, Uduman N, Moheimani N, Forde GM. 2009. Dewatering of microalgal culture for biodiesel production: Exploring polymer flocculation and tangential flow filtration. *Journal of Chemical Technology and Biotechnology* 84(7):1078-1083.
- de la Jara A, Mendoza H, Martel A, Molina C, Nordström L, de la Rosa V, Diaz R. 2003. Flow cytometric determination of lipid content in a marine dinoflagellate, *Cryptothecodinium cohnii*. *Journal of Applied Phycology* 15:433-438.
- Dijk van PWM. 1999. Made by Genetic Engineering: A series of articles to promote a better understanding of the use of genetic engineering. *Journal of Biotechnology* 67(1):77-80.
- Doblhoff-Dier O, Gaida T, Katinger H. 1994. A novel ultrasonic resonance field device for the retention of animal cells. *Biotechnology-Progress*. 10(4):428-432.
- Doucha J, Livansky K. 2006. Productivity, CO₂/O₂ exchange and hydraulics in outdoor open high density microalgal (*Chlorella sp.*) photobioreactors operated in a Middle and Southern European climate. *Journal of Applied Phycology* 18(6):811-826.

- Dubinsky Z, Falkowski PG, Wyman K. 1986. Light harvesting and utilization by phytoplankton. *Plant and Cell Physiology* 27(7):1335-1349.
- Etschmann MMW, Sell D, Schrader J. Medium optimization for the production of the aroma compound 2-phenylethanol using a genetic algorithm. 2004. Elsevier Science Bv. p 187-193.
- Evers EG. 1991. A model for light-limited continuous cultures: growth, shading, and maintenance. *Biotechnology and Bioengineering* 38:254-259.
- Fawley MW. 1984. Effects of light intensity and temperature interactions on growth characteristics of *Phaeodactylum tricornutum* (Bacillariophyceae). *Journal of Phycology* 20:67-72.
- Fernandez FGA, Sevilla JMF, Perez JAS, Grima EM, Chisti MY. 2001. Airlift-driven external-loop tubular photobioreactors for outdoor production of microalgae: assessment of design and performance. *Chemical Engineering Science* 56(8):2721-2732.
- Gill I, Valivety R. 1997. Polyunsaturated fatty acids. 1. Occurrence, biological activities and applications. *Trends in Biotechnology* 15(10):401-409.
- Gomez-Pinchetti JL, Ramazanov Z, Fontes A, Garcia-Reina G. 1992. Photosynthetic characteristics of *Dunaliella salina* (Chlorophyceae, Dunaliellales) in relation to beta-carotene content. *Journal of Applied Phycology* 4:11-15.
- Gordillo FJL, Jimenez C, Chavarri J, Niell FX. 2001. Photosynthetic acclimation to photon irradiance and its relation to chlorophyll fluorescence and carbon assimilation in the halotolerant green alga *Dunaliella viridis*. *Photosynthesis Research* 68(3):225.
- Grima EM, Pérez JAS, Camacho FG, Sevilla JMF, Fernández FGA, Cardona JA. 1995. Biomass and eicosapentaenoic acid productivities from an outdoor batch culture of *Phaeodactylum tricornutum* UTEX 640 in an airlift tubular photobioreactor. *Applied Microbiology and Biotechnology* 42(5):658-663.
- Grima EM, Sevilla JMF, Pérez JAS, Camacho FG. 1996. A study on simultaneous photolimitation and photoinhibition in dense microalgal cultures taking into account incident and averaged irradiances. *Journal of Biotechnology* 45:59-69.
- Grima EM, Camacho FG, Pérez JAS, Fernandez FGA, Sevilla JMF. 1997. Evaluation of photosynthetic efficiency in microalgal cultures using averaged irradiance. *Enzyme and Microbial Technology* 21:375-381.
- Grima EM, Belarbi H, Fernandez FGA, Medina AR, Chisti MY. 2003. Recovery of microalgal biomass and metabolites: process options and economics. *Biotechnology Advances* 20:491-515.
- Grobbelaar JU. 1991. The influence of light/dark cycles in mixed algal cultures on their productivity. *Bioresource Technology* 38:189-194.
- Grobbelaar JU, Kurano N. 2003. Use of photoacclimation in the design of a novel photobioreactor to achieve high yields in algal mass cultivation. *Journal of Applied Phycology* 15:121-126.
- Gröschl M. 1998. Ultrasonic separation of suspended particles - Part I: Fundamentals. *Acustica - acta acustica* 84:432-447.

-
- Gröschl M, Burger W, Handl B, Doblhoff-Dier O, Gaida T, Schmatz C. 1998. Ultrasonic separation of suspended particles - Part III - Application in Biotechnology. *Acustica - acta acustica* 84:815-822.
- Haaland PD. 1989. *Experimental Design in Biotechnology*. New York: Marcel Dekker Inc. 259 p.
- Han BP, Virtanen M, Koponen J, Straskraba M. 2000. Effect of photoinhibition on algal photosynthesis: a dynamic model. *Journal of Plankton Research* 22(5):865-888.
- Hancke TB, Hancke K, Johnsen G, Sakshaug E. 2008. Rate of O₂ production derived from pulse-amplitude-modulated fluorescence: Testing three biooptical approaches against measured O₂-production rate. *Journal of Phycology* 44(3):803-813.
- Hawkes JJ, Coakley WT. 1996. A continuous flow ultrasonic cell-filtering method. *Enzyme and Microbiology Technology* 19:57-62.
- Hawkes JJ, Limaye MS, Coakley WT. 1997. Filtration of bacteria and yeast by ultrasound-enhanced sedimentation. *Journal of Applied Microbiology* 82:39-47.
- Heel ACSv. 1964. *Inleiding in de optica*. 's Gravenhage: Nijhoff. 427 p.
- Hejazi MA, Lamerliere Cd, Rocha JMS, Vermuë M, Tramper J, Wijffels RH. 2002. Selective extraction of carotenoids from the microalga *Dunaliella salina* with retention of viability. *Biotechnology and Bioengineering* 79(1):29-36.
- Hoek Cvd, Mann DG, Jahns HM. 1995. *Algae: An introduction to phycology*. Cambridge University Press. 637 p.
- Hu Q, Guterman H, Richmond A. 1996. A flat inclined modular photobioreactor for outdoor mass cultivation of photoautotrophs. *Biotechnology and Bioengineering* 51(1):51-60.
- Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, Darzins A. 2008. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *Plant Journal* 54(4):621-639.
- Ikawa M, Hartshorne T, Caron LA, Iannitelli RC, Barbero LJ, Wegener K. 1984. Inhibition of growth of the green alga *Chlorella pyrenoidosa* by unsaturated fatty acids. *Journal - Association of Official Analytical Chemists* 61(12):1877-1878.
- Ikawa M, Sasner JJ, Haney JF. 1997. Inhibition of *Chlorella* growth by degradation and related products of linoleic and linolenic acids and the possible significance of polyunsaturated fatty acids in phytoplankton ecology. *Hydrobiologia* 356: 143-148.
- Janssen M, de Winter M, Tramper J, Mur LR, Snel JFH, Wijffels RH. 2000. Efficiency of light utilization of *Chlamydomonas reinhardtii* under medium-duration light/dark cycles. *Journal of Biotechnology* 78:123-137.
- Janssen M, Slenders P, Tramper J, Mur LR, Wijffels RH. 2001. Photosynthetic efficiency of *Dunaliella tertiolecta* under short light/dark cycles. *Enzyme and Microbial Technology* 29(4-5):298-305.
- Janssen M, Tramper J, Mur LR, Wijffels RH. 2003. Enclosed outdoor photobioreactors: light regime, photosynthetic efficiency, scale-up and future prospects. *Biotechnology and Bioengineering* 81(2):193-210.

- Jimenez C, Cossio BR, Labella D, Niell FX. 2003a. The Feasibility of industrial production of *Spirulina* (*Arthrospira*) in Southern Spain. *Aquaculture* 217(1-4):179-190.
- Jimenez C, Cossio BR, Niell FX. 2003b. Relationship between physicochemical variables and productivity in open ponds for the production of *Spirulina*: a predictive model of algal yield. *Aquaculture* 221(1-4):331-345.
- Kashyap S, Sundararajan A, Lu-Kwang J. 1998. Flotation characteristics of cyanobacterium *Anabaena flos-aquae* for gas vesicle production. *Biotechnology and Bioengineering* 60(5): 636-641.
- Kennedy M, Krouse D. 1999. Strategies for improving fermentation medium performance: a review. *Journal of Industrial Microbiology & Biotechnology* 23:456-475.
- Kilburn DG, Clarke DJ, Coakley WT, Bardsley DW. 1989. Enhanced sedimentation of mammalian cells following acoustic aggregation. *Biotechnology and Bioengineering* 34:559-562.
- Kirk JTO. 1994. Light and photosynthesis in aquatic ecosystems. Cambridge: Cambridge University Press. 509 p.
- Kok B. 1956. Photosynthesis in flashing light. *Biochimica et Biophysica Acta* 21:245-258.
- Kooistra WHCF, Boele-Bos SA, Stam WT. 1991. A method for obtaining axenic algal cultures using the antibiotic cefotaxime with emphasis on *Chladophoropsis membranacea* (Chlorophyta). *Journal of Phycology* 27:656-658.
- Kubitschek HE. 1984. Independence of buoyant cell density and growth rate in *Escherichia coli*. *Journal of Bacteriology* 158: 296-299.
- Kubitschek HE. 1987. Buoyant density variation during the cell cycle in microorganisms. *Critical Reviews in Microbiology* 14: 73-97.
- Lee AK, Lewis DM, Ashman PJ. 2009. Microbial flocculation, a potentially low-cost harvesting technique for marine microalgae for the production of biodiesel. *Journal of Applied Phycology* 21(5):559-567.
- Lehr F and Posten C. 2009. Closed photo-bioreactors as tools for biofuel production. *Current Opinion in Biotechnology* 20(3):280-285.
- Lu C, Acien-Fernandez FG, Guerrero EC, Hall DO, Grima EM. 2002. Overall assessment of *Monodus subterraneus* cultivation and EPA production in outdoor helical and bubble column reactors. *Journal of Applied Phycology* 14:331-342.
- Mancuso Nichols CA, Nairn KM, Glattauer V, Blackburn SI, Ramshaw JAM, Graham LD. 2009. Screening Microalgal Cultures in Search of Microbial Exopolysaccharides with Potential as Adhesives. *The Journal of Adhesion* 85(2):97-125.
- Mandalam RK, Palsson BO. 1998. Elemental balancing of biomass and medium composition enhances growth capacity in high-density *Chlorella vulgaris* cultures. *Biotechnology and Bioengineering* 59(5):605-611.
- Marteijn RCL, Jurrius O, Dhont J, de Gooijer CD, Tramper J, Martens DE. 2003. Optimization of a feed medium for fed-batch culture of insect cells using a genetic algorithm. *Biotechnology and Bioengineering* 81(3):269-278.

-
- McCracken MD, Middaugh RE, Middaugh RS. 1980. A chemical characterization of an algal inhibitor obtained from *Chlamydomonas*. *Hydrobiologia* 70:271-276.
- McCracken IR. 1989. Purifying algal cultures - a review of chemical methods. *Proceedings of the Nova Scotian Institute of Science* 38:145-168.
- Mendoza H, De la Jara A, Freijanes K, Carmona L, Ramos AA, De Sousa Duarte V, Serafim Varela JC. 2008. Characterization of *Dunaliella salina* strains by flow cytometry: a new approach to select carotenoid hyperproducing strains. *Electronic Journal of Biotechnology* 11(4):1-13.
- Miravalles AB, Leonardi PI. 1999. Optimization of culture conditions of an Argentine strain of *Dunaliella salina* (Chlorophyta), for the synthesis of beta-carotene. *Proc.WOCMAP-2* 502:153-157.
- Mishra A, Jha B. 2009. Isolation and characterization of extracellular polymeric substances from micro-algae *Dunaliella salina* under salt stress. *Bioresource Technology* 100(13):3382-3386.
- Mogee ME. 1993. Educating innovation managers - strategic issues for business and higher-education. *Ieee Transactions on Engineering Management* 40(4):410-417.
- Morita M, Watanabe Y, Saiki H. 2000. High photosynthetic productivity of green microalga *Chlorella sorokiniana*. *Applied Biochemistry and Biotechnology* 87:203-218.
- Myers RH, Montgomery DC. 1995. Response surface methodology, process and product optimization using designed experiments. New York: Wiley. p.728.
- Namikoshi M, Suzuki S, Meguro S, Kobayashi H, Mine Y, Kasuga I. 2002. Inhibitors of microtubule assembly produced by the marine fungus strain TUF98F139 collected in Palau. *Journal of Tokyo University of Fisheries* 88:1-6.
- Norton TA, Melkonian M, Andersen RA. 1996. Algal biodiversity. *Phycologia* 35(4):308-326.
- Ogbonna JC, Tanaka H. 1996. Night biomass loss and changes in biochemical composition of cells during light/dark cyclic culture of *Chlorella pyrenoidosa*. *Journal of Fermentation and Bioengineering* 82(6):558-564.
- Olaizola M. 2003. Commercial development of microalgal biotechnology: from the test tube to the marketplace. *Biomolecular engineering* 20(4-6):459-466.
- Ördög V, Stirk WA, Lenobel R, Bancírová M, Strnad M, van Staden J, Szigeti J, Németh L. 2004. Screening microalgae for some potentially useful agricultural and pharmaceutical secondary metabolites. *Journal of Applied Phycology* 16(4):309-314.
- Osinga R, Belarbi el H, Grima EM, Tramper J, Wijffels RH. 2003. Progress towards a controlled culture of the marine sponge *Pseudosuberites andrewsi* in a bioreactor. *Journal of Biotechnology* 100:141-146.
- Park DH, Ruy HW, Lee KY, Kang CH, Kim TH, Lee HY. 1998. The production of hydrocarbons from photoautotrophic growth of *Dunaliella salina*. *Applied Biochemistry and Biotechnology* 70-72:739-746.
- Park KH, Kim DI, Lee CG. 2000. Effect of flashing light on oxygen production rates in high-density algal cultures. *Journal of microbiology and biotechnology* 10(6):817-822.

- Pollet IV, Pieters JG. 2002. PAR transmittances of dry and condensate covered glass and plastic greenhouse cladding. *Agricultural and Forest Meteorology* 110(4):285-298.
- Posten C. 2009. Design principles of photo-bioreactors for cultivation of microalgae. *Engineering in Life Sciences* 9(3):165-177.
- Pulz, O. 2001. Photobioreactors: production systems for phototrophic microorganisms. *Applied Microbiology and Biotechnology* 57: 287-293.
- Qiang H, Guterman H, Richmond A. 1996. Physiological characteristics of *Spirulina platensis* (cyanobacteria) cultured at ultrahigh cell densities. *Journal of Phycology* 32(6):1066-1073.
- Qiang H, Richmond A. 1996. Productivity and photosynthetic efficiency of *Spirulina platensis* as affected by light intensity, algal density and rate of mixing in a flat plate photobioreactor. *Journal of Applied Phycology* 8(2):139-145.
- Qiang H, Zhengyu H, Cohen Z, Richmond A. 1997. Enhancement of eicosapentaenoic acid (EPA) and gamma-linolenic acid (GLA) production by manipulating algal density of outdoor cultures of *Monodus subterraneus* (Eustigmatophyta) and *Spirulina platensis* (Cyanobacteria). *European Journal of Phycology* 32:81-86.
- Qiang H, Zarmi Y, Richmond A. 1998. Combined effects of light intensity, light-path and culture density on output rate of *Spirulina platensis* (Cyanobacteria). *European Journal of Phycology* 33(2):165-171.
- Richmond A. 2004a. Handbook of microalgal culture: biotechnology and applied phycology. Blackwell publishing. p. 584.
- Richmond A. 2004b. Principles for attaining maximal microalgal productivity in photobioreactors: an overview. *Hydrobiologia* 512(1):33-37.
- Richmond A, Cheng-Wu Z, Zarmi Y. 2003. Efficient use of strong light for high photosynthetic productivity: interrelationships between the optical path, the optimal population density and cell-growth inhibition. *Biomolecular engineering* 20(4-6):229-236.
- Richmond A, Zou N. 1999. Efficient utilization of high photon irradiance for mass production of photoautotrophic micro-organisms. *Journal of Applied Phycology* 11(1):123-127.
- Rippka R, Deruelles J, Waterbury JB, Herdman M, Stanier RY. 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *Journal of General Microbiology* 111:1-61.
- Rodolfi L, Zittelli GC, Bassi N, Padovani G, Biondi N, Bonini G, Tredici MR. 2009. Microalgae for oil: Strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. *Biotechnology and Bioengineering* 102(1):100-112.
- Ryll T, Dutina G, Reyes A, Gunson J, Krummen L, Etcheverry T. 2000. Performance of small-scale CHO perfusion cultures using a acoustic cell filtration device for cell retention: Characterization of separation efficiency and impact of perfusion on product quality. *Biotechnology and Bioengineering* 69(4):440-449.

-
- Schaaf H van der, Vermuë M, Tramper J, Hartog R. 2003. "A design environment for downstream processes for Bioprocess-Engineering students". *European Journal of Engineering Education* 28(4):507-521.
- Schenk PM, Thomas-Hall SR, Stephens E, Marx U, Mussnug JH, Posten C, Kruse O, Hankamer B. 2008. Second Generation Biofuels: High-Efficiency Microalgae for Biodiesel Production. *Bioenergy Research* 20-43.
- Schmidt HG, Moust JHC. 1998. *Probleemgestuurd Onderwijs: Praktijk en Theorie*. Groningen: Wolters-Noordhoff bv. 297 p.
- Shen Y, Yuan W, Pei ZJ, Wu Q, Mao E. 2009. Microalgae mass production methods. *Transactions of the ASABE* 52(4):1275-1287.
- Sierra E, Acién FG, Fernández JM, García JL, González C, Molina EM. 2008. Characterization of a flat plate photobioreactor for the production of microalgae. *Chemical Engineering Journal* 138(1-3):136-147.
- Simopoulos AP. 1999. Essential fatty acids in health and chronic disease. *American Journal of Clinical Nutrition* 70(3):560S-569S.
- Simopoulos AP. 2004. Omega-6/omega-3 essential fatty acid ratio and chronic diseases. *Food Reviews International* 20(1):77-90.
- Smid EJ, van Enckevort FJH, Wegkamp A, Boekhorst J, Molenaar D, Hugenholtz J, Siezen RJ, Teusink B. 2005. Metabolic models for rational improvement of lactic acid bacteria as cell factories. *Journal of applied microbiology* 98(6):1326-1331.
- Spengler J, Jekel M. 2000. Ultrasound conditioning of suspensions - studies of streaming influence on particle aggregation on a lab- and pilot-plant scale. *Ultrasonics* 38:624-628.
- Spolaore P, Joannis-Cassan C, Duran E, Isambert A. 2006. Commercial Applications of Microalgae. *Journal Of Bioscience And Bioengineering* 101(2):87-96.
- Spruell JA. 1984. Response of algae and zooplankton to C-18 fatty acids of *Chlamydomonas Reinhardtii*. *Hydrobiologia* 114:9-12.
- Sukenik A, Shelef G. 1984. Algal autoflocculation - verification and proposed mechanism. *Biotechnology and Bioengineering* 26(2):142-147.
- Sushchik NN, Kalacheva GS, Gladyshev MI. 2001. Secretion of Free Fatty Acids by Prokaryotic and Eukaryotic Algae at Optimal, Supraoptimal, and Suboptimal Growth Temperatures. *Microbiology* 70(5):542-547.
- Sushchik NN, Kalacheva GS, Zhila NO, Gladyshev MI, Volova TG. 2003. A Temperature Dependence of the Intra- and Extracellular Fatty-Acid Composition of Green Algae and Cyanobacterium. *Russian Journal of Plant Physiology* 50(3):420-427.
- Suzuki L, Johnson CH. 2001. Algae know the time of day: Circadian and photoperiodic programs. *Journal of Phycology* 37:933-942.
- Tenney MW, Verhoff FH. 1973. Chemical and autoflocculation of microorganisms in biological wastewater treatment. *Biotechnology and Bioengineering* 15:1045-1073.
- Terry KL. 1986. Photosynthesis in modulated light: Quantitative dependence of photosynthetic enhancement on flashing rate. *Biotechnology and Bioengineering* 28(7):988-995.

- Thakur A, Kumar A. 1998. Effect of pH, temperature, salinity and nitrogen concentration on the growth and carotenoid content of *Dunaliella salina*. *Acta Botanica Hungarica* 41(1-4):393-397.
- Todorova A, Jüttner F. 1996. Nostocyclamide M: a cyanobacterial cyclic peptide with allelopathic activity from *Nostoc* 31. *Phycologica* 35(6):183-188.
- Torzillo G, Pushparaj B, Masojidek J, Vonshak A. 2003. Biological constraints in algal biotechnology. *Biotechnology and Bioprocess Engineering* 8:338-348.
- Trampl F, Sonnerhoff SA, Pui PWS, Kilburn DG, Piret JM. 1994. Acoustic Cell Filter for High-Density Perfusion Culture of Hybridoma Cells. *Bio-Technology* 12(3):281-284.
- Tredici MR, Carlotto P, Zittelli GC, Materassi R. 1991. A vertical alveolar panel (VAP) for outdoor mass cultivation of microalgae and cyanobacteria. *Bioresource Technology* 38:153-159.
- Tredici MR, Zittelli GC. 1998. Efficiency of sunlight utilization: Tubular versus flat photobioreactors. *Biotechnology and Bioengineering* 57(2):187-197.
- Velds CA. 1992. Zonnestraling in Nederland (Solar radiation in The Netherlands): KNMI. 166 p.
- Vonshak A, Torzillo G, Masojidek J, Boussiba S. 2001. Sub-optimal morning temperature induces photoinhibition in dense outdoor cultures of the alga *Monodus subterraneus* (Eustigmatophyta). *Plant Cell and Environment* 24:1113-1118.
- Vorum H, Brodersen R, Kragghansen U, Pedersen AO. 1992. Solubility of Long-Chain Fatty-Acids in Phosphate Buffer at pH 7.4. *Biochimica Et Biophysica Acta* 1126(2):135-142.
- Walker TL, Purton S, Becker DK, Collet C. 2005. Microalgae as bioreactors. *Plant cell Reports* 24(11):629-641.
- Webb MR, Melis A. 1995. Chloroplast response in *Dunaliella salina* to irradiance stress, effect on thylakoid membrane protein assembly and function. *Plant Physiology* 107:885-893.
- Weissman JC, Goebel RP, Benemann JR. 1988. Photobioreactor design: Mixing, carbon utilization, and oxygen accumulation. *Biotechnology and Bioengineering* 31:336-344.
- Weusterbotz D, Wandrey C. 1995. Medium optimization by genetic algorithm for continuous production of formate dehydrogenase. *Process Biochemistry* 30(6):563-571.
- Weuster-Botz D. 2000. Experimental design for fermentation media development: Statistical design or global random search? *Journal of Bioscience and Bioengineering* 90(5):473-483.
- Widjaja A, Chien CC, Ju YH. 2009. Study of increasing lipid production from fresh water microalgae *Chlorella vulgaris*. *Journal of the Taiwan Institute of Chemical Engineers* 40(1):13-20.
- Wijffels RH. 2008. Potential of sponges and microalgae for marine biotechnology. *Trends in Biotechnology* 26(1):26-31.
- Winkelmeier P, Glauner B, Lindl T. 1993. Quantification of Cytotoxicity by Cell-Volume and Cell-Proliferation. *Atla-Alternatives to Laboratory Animals* 21(2):269-280.

- Wozniac B, Dera J, Ficek D, Majchrowski R, Ostrowska M, Kaczmarek S. 2003. Modelling light and photosynthesis in the marine environment. *Oceanologia* 45(2):171-245.
- Wu JT, Chiang YR, Huang WY, Jane WN. 2006. Cytotoxic effects of free fatty acids on phytoplankton algae and cyanobacteria. *Aquatic Toxicology* 80(4):338-345.
- Wunschiers R, Lindblad P. 2002. Hydrogen in education - a biological approach. Pergamon-Elsevier Science Ltd. 1131-1140.
- Yamada N, Murakami N, Mormioto T, Sakakibara J. 1993. Auto-growth inhibitory substance from the fresh-water cyanobacterium *Phormidium tenue*. *Chemical and Pharmaceutical Bulletin* 41(10):1863-1865.
- Yang C, Hua Q, Shimizu K. 2000. Energetics and carbon metabolism during growth of microalgal cells under photoautotrophic, mixotrophic and cyclic light-autotrophic/dark-heterotrophic conditions. *Biochemical Engineering Journal* 6(2):87-102.
- Zhang J, Collins A, Chen M, Knyazev I, Gentz R. 1998. High-density perfusion culture of insect cells with a biosep ultrasonic filter. *Biotechnology and Bioengineering* 59(3):351-359.
- Zhang K, Kurano N, Miyachi S. 1999. Outdoor culture of a cyanobacterium with a vertical flat-plate photobioreactor: effects on productivity of the reactor orientation, distance setting between the plates, and culture temperature. *Applied Microbiology and Biotechnology* 52(6):781-786.
- Zijffers JWF, Janssen M, Tramper J, Wijffels RH. 2008. Design process of an area-efficient photobioreactor. *Marine Biotechnology* 10(4):404-415.
- Zijffers JWF. 2009. The Green Solar Collector: optimization of microalgal areal productivity. Wageningen, Wageningen University. 156 p.
- Zittelli GC, Tomasello V, Pinzani E, Tredici MR. 1996. Outdoor cultivation of *Arthrospira platensis* during autumn and winter in temperate climates. *Journal of Applied Phycology* 8:293-301.
- Zittelli GC, Pastorelli R, Tredici MR. 2000. A modular flat panel photobioreactor (MFPP) for indoor mass cultivation of *Nannochloropsis* sp. under artificial illumination. *Journal of Applied Phycology* 12:521-526.
- Zittelli GC, Rodolfi L, Biondi N, Tredici MR. 2006. Productivity and photosynthetic efficiency of outdoor cultures of *Tetraselmis suecica* in annular columns. *Aquaculture* 261:932-943.
- Zonneveld C. 1998. Photoinhibition as affected by photoacclimation in phytoplankton: a model approach. *Journal of Theoretical Biology* 193:115-123.

Summary

The biodiversity of microalgae is enormous and they represent an almost untapped resource. Many of these microalgae contain unique products from the classes of carotenoids, antioxidants, fatty acids, enzymes, polymers, peptides, toxins, sterols and more. There is a niche market for the high-value algal products in agriculture, pharmaceutical, cosmetic, textile and food industry as a source of livestock feed, vitamins, pigments or essential fatty acids. However, the large scale commercial production of bulk products from microalgae is still in its infancy. Many bottlenecks still need to be solved to turn microalgal biotechnology into a mature technology. This thesis addresses several of these bottlenecks: light regime, harvesting, development and application of a fast screening method and the training of people in the field of algal biotechnology.

Microalgae use photosynthesis to obtain energy for growth. If cultivated in photobioreactors, individual algal cells in dense cultures experience a fluctuating light regime. They experience high light intensities close to the reactor surface. Because light is absorbed by the microalgae, the light intensities that algae experience will decrease with increasing radial depth until eventually darkness is reached (dark zone) in the interior of the photobioreactor. Due to mixing, algae will travel from the reactor wall to the dark interior of the photobioreactor and back. Insight is needed how microalgae cope with this fluctuating light regime.

For this purpose, we studied the productivity of the microalga *Monodus subterraneus* under fluctuating light conditions and modeled the productivity of a pilot-plant bubble column placed outdoors (Chapter 2). Two extreme approaches to model the photobioreactor were chosen. Firstly, a model with growth integration in which it is assumed that microalgae can adapt immediately to local light conditions was used. Secondly, full light integration implicating that microalga can convert all absorbed light with a photosynthetic yield based on the average light intensity. Because temperature and light conditions in our photobioreactor changed during the day, kinetic parameters of the algae at any combination of temperature and light intensity were needed. These were determined in repeated-batch lab-scale experiments with an experimental design. The model was evaluated in an outdoor bubble column at different natural light conditions and different temperatures. Volumetric productivities in the bubble column were predicted and compared with experimental

volumetric productivities. It was found that the light integration model over-estimated productivity, while the model in which we assumed growth integration under-estimated productivity. Partial light integration occurred (47%) during the period investigated (half July - end October). The models described in Chapter 2 can be used to determine minimal and maximal volumetric productivities at any geographical location from kinetic data of the algae obtained in independent lab-scale experiments.

To obtain higher algal productivities in photobioreactors, fast screening methods are needed. These are necessary to select strains with better production characteristics, develop optimized media and investigate toxic effects of chemicals. We developed a screening method to determine the specific growth rate of microalgae in well-plates with good reproducibility (Chapter 3). This bioassay was used to investigate the growth inhibiting effects of free fatty acids on the microalga *Monodus subterraneus*. In microalgal cultivation, a high volumetric productivity is advantageous to decrease photobioreactor size and downstream processing costs. High volumetric productivities can be reached in flat panel photobioreactors with small optical paths, obtaining some light integration. Such photobioreactors have to be operated at high cell densities ($> 10 \text{ g L}^{-1}$) to obtain maximum productivities. However, at such high biomass concentrations, productivity decreases because microalgal growth is inhibited. Fatty acids and their oxidation products are often considered as algal growth inhibitors. It is known that fatty acids can be excreted in the medium by microalgae. We hypothesized that in high density cultures of *Monodus subterraneus*, free fatty acids cause the growth inhibition of this species. We found that growth of *Monodus subterraneus* was completely inhibited at a saturated concentration (96 μM) of palmitoleic acid (16:1 ω 9). But, the saturated fatty acid palmitic acid (16:0) and the mono-saturated oleic acid (18:1 ω 9) were much stronger inhibitors. Growth was inhibited for 50% already at concentrations of 0.4 μM 16:0 and 3 μM 18:1 ω 9, respectively. These fatty acids probably cause the growth inhibition in high cell density cultures of *Monodus subterraneus*.

To make production of microalgae with photobioreactors commercially interesting, not only the cost of cultivation of this microalgae should be taken into account, but also the cost of harvesting and the refining of the harvested microalgae into products. Conventional processes to harvest microalgae are centrifugation, membrane filtration and chemical-induced flocculation. We proved that an innovative harvesting method with ultrasound could also be used to harvest microalgae (Chapter 4). This separation process is based on gentle acoustically induced aggregation followed by enhanced sedimentation. We optimized the efficiency of harvesting and the concentration factor of this harvesting process. For this optimization, five parameters were modeled simultaneously by the use of

an experimental design. Efficiencies higher than 90% were reached at biomass concentrations of about 2 g L⁻¹ and flow rates of 4-6 L day⁻¹. At most, 92% of the organisms could be harvested and a concentration factor of 11 could be achieved at these settings. It was not possible to harvest this microalga at higher efficiencies due to its small size and its small density difference with water. Highest concentration factors (harvest flow/ingoing flow), up to 20, could be reached at low biomass concentrations and low harvest flows. Disadvantages of this harvesting process are the high power consumption, low efficiency and low concentration factor compared to conventional processes. However, for special applications on lab- or pilot-plant scale, the non-evasive character, which keeps the cells viable after harvesting, is an advantage of the ultrasonic harvesting process. Also, when the algae excrete a soluble high-value product, this system can be used as a biofilter.

In the field of microalgal biotechnology only a few courses about microalgae are available. They are mostly at university level and cover the biology and taxonomy of microalgae. Almost no courses are available that teach people how to cultivate microalgae, engineer cultivation systems or to build and manage a complete cultivation plant. Therefore we developed a practical meant to teach students the basics of cultivating algae and let them understand how different conditions (temperature, light, media) influence the algal growth and β -carotene accumulation (Chapter 5). The students were motivated by giving them responsibility. Competition was added as a stimulus for interaction between the students. Students had to produce as much β -carotene as possible using the marine alga strain *Dunaliella salina* in a flat panel photobioreactor. On the first day, they developed a strategy to obtain optimal algal growth rate. They brought this into practice during the second day. While cultivating the organism, they developed a second strategy how and when to stress the alga to initiate β -carotene production. At the ninth day, the total amount of β -carotene was measured. To stimulate competition, the group that produced most β -carotene obtained half a point bonus on their final practical mark. On the tenth day, each group presented their results and an evaluation of their chosen strategies. Most students were successful in growing algae. In the second phase some groups failed to stress the algae. The best group produced more than two times β -carotene than the runner-up. The students were motivated by being responsible for their own results and the competitive approach.

To convert microalgal production processes into a mature technology still many questions remain. In Chapter 6, we evaluated the research addressed in this thesis with emphasis on the photobioreactor, light regime, fast screening methods with possible applications, harvesting and education. Enhanced productivities of the microalgae in the photobioreactor should be achieved, which can be reached by the development of cultivation systems that

use the concept of light dilution. Research should focus on medium optimization, species screening and optimization of the amount of product per biomass. Also, genome-based metabolic flux models for the primary metabolism of microalgae should be developed to get better understanding of cellular metabolism and develop metabolic engineering strategies. Apart from obtaining enhanced algal productivities a low-cost harvesting process is required. We studied if ultrasound could be used as first concentration step and optimized its efficiency and concentration factor. However, due to the high energy demands of this process, it will not be used for bulk products but can only be commercially interesting for harvesting high-value secondary metabolites. For harvesting bulk products, as first concentration step auto-flocculation on demand is worth investigating. Beside these research lines, more and better education for biochemical engineers should be developed to push microalgal biotechnology ahead.

Samenvatting

Microalgen bezitten een grote biodiversiteit en ze zijn een potentiële bron van vele unieke producten waaronder carotenoïden, antioxidanten, vetzuren, enzymen, polymeren, peptiden, giftige stoffen etc. Er bestaat op dit moment een kleine markt op het gebied van hoogwaardige algenproducten zoals vitamines, kleurstoffen en essentiële vetzuren in de landbouw, farmacie, cosmetica en voedselindustrie. Om commerciële productie van bulkproducten met algen mogelijk te maken, moeten nog diverse onderzoeksvraagstukken opgelost worden. Dit proefschrift behandelt enkele onderwerpen van deze vraagstukken, waaronder lichtregime, het oogsten van de microalgen, de ontwikkeling en toepassing van een snelle screenmethode en de ontwikkeling van onderwijs op het gebied van de technologie rond microalgen.

Microalgen gebruiken fotosynthese om te groeien. Als ze in fotobioreactoren gegroeid worden, ondervinden individuele algen in een dichte cultuur fluctuerende licht condities. Ze ervaren hoge lichtintensiteiten bij de reactorwand en door absorptie van licht door de algen, lagere intensiteiten verder van de wand af, tot ze zich in het donkere midden van de reactor bevinden. Doordat de reactoren gemengd worden, zullen algen heen en weer bewegen tussen de reactorwand en het binnenste van de reactor en zullen ze dus steeds bloot gesteld worden aan wisselende lichtomstandigheden. Het is daarom belangrijk om kennis te vergaren hoe microalgen omgaan met de wisselende lichtomstandigheden. Daarom is de productiviteit van de microalg *Monodus subterraneus* in een bellenkolom van 65 liter, die buiten geplaatst was, bestudeerd (Hoofdstuk 2). Twee grensgevallen om de reactor te modelleren werden gekozen. Ten eerste, een model met groei-integratie, deze nam aan dat de microalgen zich onmiddellijk konden aanpassen aan locale lichtcondities. Ten tweede, volledige lichtintegratie; wij namen in dit model aan dat de microalgen al het geabsorbeerde licht konden omzetten met een fotosynthetische efficiëntie die gebaseerd was op de gemiddelde lichtintensiteit die de alg ondervindt in de fotobioreactor. Omdat in de reactor de hoeveelheid licht en de temperatuur niet gecontroleerd werden, varieerden deze continu en was het nodig om de fotosynthetische efficiëntie te weten bij elke combinatie van licht en temperatuur. Deze werden bepaald in herhaalde batchexperimenten op labschaal met behulp van een experimenteel ontwerp. Het model werd geëvalueerd in de buitenreactor. Het bleek dat het model met lichtintegratie de productiviteit overschatte, terwijl het andere model, waarin groei-integratie aangenomen wordt, de productiviteit onderschatte. Er werd

geconcludeerd dat er gedeeltelijk licht integratie (47%) plaatsvond gedurende de periode waarin gemeten werd (half juli – eind oktober). De modellen, die beschreven staan in Hoofdstuk 2, kunnen gebruikt worden om de minimale en maximale productiviteit van algen per reactorvolume op elke plek in de wereld te bepalen met de kinetische parameters van de algen die bepaald kunnen zijn in onafhankelijke labschaalexperimenten.

Om de productiviteit van algen in fotobioreactoren te verhogen, zijn snelle screenmethodes nodig om microalgen stammen te selecteren met betere productie-eigenschappen, om geoptimaliseerde media te ontwikkelen en de toxische of groeiremmende effecten van chemicaliën te onderzoeken. In dit proefschrift wordt een dergelijke snelle screenmethode beschreven waarin we de groei bepaalden van microalgen in microtiterplaten met een goede reproduceerbaarheid (Hoofdstuk 3). Deze screenmethode werd daarna gebruikt om de groeiremmende effecten van een vrij vetzuur op de alg *Monodus subterraneus* te onderzoeken. Wanneer algen gekweekt worden, is een hoge productiviteit per volume wenselijk om de fotobioreactor te verkleinen en daarnaast de oogstkosten te verlagen. Een hoge productiviteit kan behaald worden door vlakke-plaatreactoren te gebruiken met een korte optische weg. Zulke reactoren moeten bedreven worden bij hoge celconcentraties ($> 10 \text{ g L}^{-1}$) om een maximale productiviteit te verkrijgen. Maar, bij zulke hoge biomassaconcentraties gaat de productiviteit omlaag omdat de groei van de microalgen geremd wordt. Vetzuren en hun oxidatieproducten worden vaak genoemd als groeiremmers. Daarnaast kunnen vetzuren worden uitgescheiden door microalgen. Daarom veronderstelden wij dat in hoge dichtheidsculturen van *Monodus subterraneus* deze vetzuren de groeiremming veroorzaakten en onderzochten dit. Er werd gevonden dat de groei van deze alg compleet geremd wordt bij een verzadigde oplossing (96 μM) van palmitoleïne zuur (16:1 ω 9). Echter, het verzadigde palmitine zuur (16:0) en eenmalig onverzadigde oliezuur (18:1 ω 9) zijn veel sterkere groeiremmers. De groei wordt al voor 50% geremd bij vetzuurconcentraties van 0.4 μM 16:0 en 3 μM 18:1 ω 9. Wij denken dan ook dat deze twee vetzuren de groeiremming in hoge dichtheidsculturen van *Monodus subterraneus* veroorzaken.

Om productie van microalgen met fotobioreactoren commercieel interessant te maken, moet niet alleen naar de kosten van de cultivatie, maar ook naar de kosten van oogsten van de microalgen gekeken worden en het opwerken hiervan in bruikbare producten. Conventionele processen om algen te oogsten zijn centrifugatie, membraanfiltratie en chemische flocculatie. Er werd bewezen dat een nieuwe oogstmethode met ultrasoon geluid gebruikt kan worden om microalgen te oogsten (Hoofdstuk 4). Dit scheidingsproces is gebaseerd op het voorzichtig samenklonteren van de cellen onder invloed van ultrasoon geluid, waarna deze geklonterde cellen versnelt sedimenteren. In dit onderzoek werden de

efficiëntie en de hoeveelheid concentrering van de algen van het oogstproces geoptimaliseerd. Dit is gedaan door vijf parameters tegelijk te variëren en te modelleren met behulp van een experimenteel ontwerp. Efficiënties groter dan 90% werden verkregen bij biomassaconcentraties van ongeveer 2 g L^{-1} en een vloeistofsnelheid van $4\text{-}6 \text{ L dag}^{-1}$. Maximaal 92% van de microalgen kon geoogst worden en bij deze instelling werden de algen 11 keer geconcentreerd. Het was niet mogelijk om hogere efficiënties te behalen omdat de algen te klein waren en het dichtheidsverschil van deze algen met water te klein was. De hoogste hoeveelheid concentrering van de algen, een factor 20, werd bereikt bij een lage biomassaconcentratie en een lage vloeistofsnelheid van de oogststroom ten opzichte van de toevoersnelheid van de microalgen in de ultrasone kamer. Dit ultrasone oogstproces had als nadelen t.o.v. de conventionele processen dat het veel stroom gebruikte, dat het een relatieve lage efficiëntie behaalde en een lage concentratie van cellen. Maar, voor speciale applicaties op kleine schaal, heeft dit ultrasone oogstproces als voordeel dat het de cellen niet beschadigt en dat de cellen dus nog levend zijn nadat ze zijn geoogst. Ook kan het handig zijn om dit proces te gebruiken indien een oplosbaar hoogwaardig product door de algen wordt uitgescheiden, waarbij de ultrasone kamer als een soort biofilter wordt gebruikt.

Op dit moment zijn er op het gebied van microalgen technologie weinig mogelijkheden voor opleiding aanwezig. Deze zijn meestal op universiteitsniveau en gaan over het identificeren van microalgen. Er is bijna geen cursusmateriaal aanwezig waarin men leert hoe algen moeten worden gekweekt, hoe cultivatie systemen moeten worden ontworpen, hoe een dergelijk systeem moet worden gebouwd en vervolgens bedreven. Dit proefschrift behandelt de ontwikkeling van een practicum dat bedoeld is om studenten de basis van het kweken van algen te leren en het produceren van een product (β -caroteen) met deze algen door ze op verschillende manieren (temperatuur, medium, licht) te stressen (Hoofdstuk 5). De studenten werden gemotiveerd doordat ze zelf verantwoordelijk waren voor hun onderzoeksproject. Daarnaast werd een competitie-element toegevoegd om de studenten te stimuleren. De opdracht was om zoveel mogelijk β -caroteen te produceren binnen 2 weken met de microalg *Dunaliella salina*. Op de eerste dag moesten de studenten een strategie bedenken om de alg zo optimaal mogelijk te laten groeien. Deze strategie brachten ze de volgende dag in praktijk. Daarnaast moesten ze bedenken hoe en wanneer ze de algen moesten stressen om zoveel mogelijk β -caroteen te produceren. Op de 9^e dag werd de hoeveelheid β -caroteen in hun reactoren gemeten. De groep die het meeste β -caroteen geproduceerd had kreeg een half punt bonus op het eindcijfer. Op de 10^e dag hebben de studenten hun bevindingen en strategieën toegelicht met behulp van een presentatie. Bij de meeste studenten groeiden de algen goed, maar in de stressfase lukte het sommige

groepen niet om de algen β -caroteen te laten produceren. De beste studenten produceerden twee keer zoveel β -caroteen als de nummer twee. Het bleek dat de studenten gemotiveerd werden door zelf verantwoordelijk te zijn voor hun resultaten en door het competitieve element.

Om microalgen biotechnologie in een volwassen technologie te veranderen moet nog veel onderzoek gedaan worden. In hoofdstuk 6 worden de vraagstukken die besproken zijn in dit proefschrift geëvalueerd met nadruk op de fotobioreactor, lichtregime, snelle screenmethodes en mogelijke applicaties, oogsten en onderwijs. Een verhoogde productiviteit van de microalgen in de fotobioreactor moet bereikt worden door het ontwikkelen van cultivatiesystemen met het concept van lichtverdunning. Onderzoek dient zich te focussen op snelle screenmethodes die gebruikt kunnen worden om medium te optimaliseren, nieuwe algensoorten te screenen, algen op producten te screenen en de hoeveelheid product per microalg te optimaliseren. Daarnaast zullen metabole flux modellen van het primaire metabolisme van een alg gemaakt moeten worden om een beter inzicht te verkrijgen in het cellulaire metabolisme en om doelgericht gentechnieken te gebruiken. Verder dienen er nieuwe oogstprocessen ontwikkeld te worden om de oogstkosten van algen te beperken. Wij hebben ultrasoon geluid bestudeerd, maar doordat veel energie nodig is om dit proces te bedrijven is deze commercieel alleen interessant om hoogwaardige algenproducten te oogsten. Voor het oogsten van bulkproducten, is het onderzoeken van autoflocculatie als eerste concentratiestap waarschijnlijk de moeite waard. Naast de bovengenoemde onderzoekslijnen, zal er meer en beter onderwijs ontwikkeld moeten worden om de algen biotechnologie verder te helpen.

Publications

Bosma R, Miazek K, Willemsen SM, Vermuë MH, Wijffels RH. 2008. Growth inhibition of *Monodus subterraneus* by free fatty acids. *Biotechnology and Bioengineering* 101(5):1108-1114.

Bosma R, Zessen E van, Reith JH, Tramper J, Wijffels RH. 2007. Prediction of volumetric productivity of an outdoor photobioreactor. *Biotechnology and Bioengineering* 97(5):1108-1120.

Bosma R, Wijffels RH. 2003. Marine biotechnology in education: a competitive approach. *Biomolecular Engineering* 20:125-131.

Bosma R, Spronsen WA van, Tramper J, Wijffels RH. 2003. Ultrasound, a new separation technique to harvest microalgae. *Journal of Applied Phycology* 15:143-153.

Schroën CGPH, Nierstrasz VA, Bosma R, Kemperman GJ, Strubel M, Ooijkaas LP, Beftink HH, Tramper J. 2002. In situ product removal during enzymatic cephalixin synthesis by complexation. *Enzyme and Microbial Technology* 31:264-273.

Schroën CGPH, Nierstrasz VA, Bosma R, Kroon PJ, Tjeerdsma PS, DeVroom E, VanderLaan JM, Moody HM, Beftink HH, Janssen AEM, Tramper J. 2002. Integrated reactor concepts for the enzymatic kinetic synthesis of cephalixin. *Biotechnology and Bioengineering* 80(2):144-155.

Schroën CGPH, Nierstrasz VA, Bosma R, Dijkstra ZJ, VandeSandt EJAX, Beftink HH, Tramper J. 2002. Process design for enzymatic adipyl-7-ADCA hydrolysis. *Biotechnology Progress* 18:745-751.

Kemperman GJ, Gelder R de, Dommerholt FJ, Schroën CGPH, Bosma R, Zwanenburg B. 2001. Complexants for the clathration mediated synthesis of the antibiotic cephradine. *Green Chemistry, The Royal Society of Chemistry* 3:189-192.

Schroën CGPH, Nierstrasz VA, Moody HM, Hoogschagen MJ, Kroon PJ, Bosma R, Beftink HH. 2001. Modeling of the enzymatic kinetic synthesis of cephalixin -Influence of substrate concentration and temperature. *Biotechnology and Bioengineering* 73(3):171-178.

Nierstrasz VA, Schroën CGPH, Bosma R, Kroon PJ, Beftink HH, Janssen AEM, Tramper J. 1999. Thermodynamically controlled synthesis of cefamandole. *Biocatalysis and Biotransformation* 17:209-223.

Schroën CGPH, Nierstrasz VA, Kroon PJ, Bosma R, Janssen AEM, Beftink HH, Tramper J. 1999. Thermodynamically controlled synthesis of beta-lactam antibiotics. Equilibrium concentrations and side-chains properties. *Enzyme and Microbial Technology* 24:498-506.

Nierstrasz VA, Schroën CGPH, Bosma R, Kroon PJ, Beftink HH, Janssen AEM, Tramper J. 1997. Separation and analysis of beta-lactam antibiotics by high-performance capillary electrophoresis: Enzymatic synthesis, a case study. *Biotechnology Techniques* 11: 899-903.

Dankwoord

Met veel plezier ga ik naar mijn werk; de gezellige en open sfeer op de vakgroep en de uitdaging die ik als analist aangeboden kreeg om te promoveren hebben ervoor gezorgd dat mijn baan niet alleen mijn werk is maar (meestal) één van mijn favoriete bezigheden.

Dit proefschrift zou er niet zijn als jij, *René*, niet in mij geloofd had en had besloten mij eerst als analist en later als promovendus op het gebied van algen aan te nemen. Gedurende de afgelopen tien jaren, gaf jij me alle ruimte om mezelf te ontplooien. Jij hebt een leuke en goede werkomgeving gecreëerd. Als het aankomt op het gezellig afsluiten van een congresdag of een borrel/feestje op de vakgroep, kunnen wij ook altijd op jou rekenen, sommige avonden waren onvergetelijk. Jij bent een schoolvoorbeeld voor stelling nummer 4. Bedankt voor alles, maar vooral voor je vertrouwen! *Marian*, zonder jou had dit proefschrift hier niet gelegen. De laatste jaren ben jij mijn directe begeleider geweest en stond je deur altijd open voor mij. Jij bent degene die met mij meedenkt en mijn schrijven verbeterde door met geduld en toewijding nieuwe versies te corrigeren. Jouw enthousiasme werkt aanstekelijk, ik ben heel blij dat jij mij de laatste jaren begeleidt en dat onze samenwerking vanzelfsprekend gaat. *Hans*, toen ik bij Proceskunde kwam was jij de professor van een leuke sociale groep waar ik me direct goed bij voelde. Ik ben zeer verheugd dat jij mijn promotor wilt zijn en jouw deskundigheid en ervaringen heeft geleid tot een wetenschappelijk mooi proefschrift.

Pieter, jij bent mijn ideale kamergenoot. Jouw scherpe geest heeft menige zin in dit proefschrift omgebogen en je was altijd beschikbaar als ik even tot rust moest komen. Daarnaast is jouw humor onontbeerlijk voor een prettige werkdag. Ik ben ook blij dat je de culinaire vaardigheden van Maruschka met mij hebt willen delen. Ik hoop nog vele jaren samen met jou op één kamer te zitten! *Marcel*, op cruciale momenten heb jij bijgedragen aan verschillende delen van dit proefschrift. Je eerlijke wetenschappelijke kritiek liet me mijn onderzoek opnieuw bekijken. Jouw theoretische kennis heeft dit proefschrift tot een beter proefschrift gemaakt. *Barbi (Maria)*, de eerste jaren hebben wij samen gezellig op het lab gewerkt. Jouw aanstekelijke lach en positieve instelling maakten de afdeling, samen met de kerstmannen, tot een gezellige vakgroep. Ik ben blij dat je na een paar jaar

omzwerven weer in Nederland woont en dat we straks samen in een team gaan werken om AlgaePARC neer te zetten. *Sina*, jouw snelle geest, doelgerichtheid en ontembare feromonen hebben mij op verschillende manieren geprikkeld (natuurlijk niet op de manier waarop je nu denkt). Ik kijk uit naar onze samenwerking de komende jaren. *Jan-Willem*, wij werkten beide aan algen en hebben op congressen verscheidene kamers gedeeld. Het was leuk om iemand te hebben die ook met vlakke platen en algen werkte, wat naast effectief ook nog gezellig was. *Saskia*, jij bent een fantastische kamergenoot geweest; jouw enthousiasme en vrolijkheid waren aanstekelijk. Je flapte er gewoon alles uit, heerlijk!

Als ik bedenk waar mijn startpunt als onderzoeker ligt, dan is dat absoluut begonnen tijdens het Chemferm project. *Vincent*, jij zorgde ervoor dat ik verschillende vakken bij Proceskunde kon volgen. Jouw enthousiasme en hulp zijn onontbeerlijk geweest voor een gedegen technische ondergrond en jij hebt me een wetenschappelijke basis bijgebracht die essentieel is geweest voor mijn promotieonderzoek. *Rik*, jij bent voor mij een bijzonder iemand en altijd beschikbaar voor vragen, gezelligheid en (natuurlijk) een veiligheidsronde, bedankt. *Karin*, jij was één van de postdocs bij het Chemferm project. Samen met Vincent zorgde jij voor een goed onderzoek en een leuke sfeer.

Toen ik bij René op de vakgroep begon, was dat op het EET-project. Binnen dit project heb ik veel geleerd op het gebied van samenwerken en het heeft geresulteerd in twee artikelen. *Wim*, de eerste jaren hebben wij samengewerkt op het EET project. Jij groeide *Monodus* in kleine bellenkolommen en deze data hebben de basis gelegd voor het modelartikel. *Hans R*, wij hebben elkaar voor het eerst ontmoet bij mijn 1^e ISAP conferentie. Wij hadden een natuurlijke click en ik ben blij dat we samen binnen het EET-project hebben kunnen samenwerken. *Erik*, bedankt dat ik de data van de bellenkolom, die bij jullie op het dak stond, mocht gebruiken.

Tijdens het promotietraject hebben verscheidene studenten mij met het onderzoek geholpen. *Sybrand*, jij was mijn eerste student en samen met Wim probeerden wij de praktische opstelling te vervolmaken om *Monodus* te laten groeien. Jij hebt ervoor gezorgd dat de eerste tijd met veel tegenslagen toch een erg leuke periode werd! *Tom*, jij volgde Sybrand op en groeide *Monodus* bij verschillende temperaturen en licht intensiteiten. *Martijn*, uit jouw onderzoek bleek dat een beter medium voor *Monodus* ontwikkeld moest worden. Jouw gedrevenheid vergde vaak het uiterste van mij als begeleider. Het was mooi om te zien hoe jij daarna je eigen promotieonderzoek supersnel afrondde. *Marieke*, jij legde de basis voor het groei inhibitie werk dat in hoofdstuk 3 beschreven staat. Het was leuk om met jou samen te werken en om de bioassay te ontwikkelen. *Krystian*, you completed most of the practical work for chapter 3 and it was great to supervise a strange, intelligent person

as you. It was incredible how fast you improved during your thesis and it was a disappointment that you had to return to Poland. *Jeroen*, wij waren twee handen op één buik. Het was super om met jou samen te werken. Jij probeerde om groei inhibitie aan te tonen in een vlakke plaat reactor met *Monodus*, helaas gooide een infectie roet in het eten. *Marcel*, jij was de student die begon met medium optimalisatie en het protocol voor de bioassay verfijnde. Jouw werk heeft geleid tot de eerste stappen in medium optimalisatie met microalgen.

Marieke B, als het aankomt op het organiseren van borrels, labuitjes, stukjes en rare/gekke dingen; dan gaat dat absoluut het beste als jij erbij bent. Jij hebt mijn eerste jaren bij proceskunde opgeleukt. *Dorinde*, jij bent voor mij een vriendin op de vakgroep met wie ik altijd even gezellig kan drinken en lachen. Gedenkwaardig was de onwerkelijke voettocht door Tokyo, in de vroege ochtend samen met René. *Marieke K*, jij bent altijd vrolijk en zorgde voor een leuke sfeer bij proceskunde. Samen met jou een sketch maken was gezellig en resulteerde altijd in een leuk stukje. *Klaske*, met jou erbij gebeuren er altijd te gekke dingen op borrels en op feestje, zonder jou waren de laatste jaren zeker minder enerverend en gezellig geweest. *Jos*, vanaf het begin was jij altijd bereid om mij te helpen met praktische zaken op het lab. Jouw vriendelijkheid en werklust zijn een voorbeeld voor ons allen. *Martin*, jij hielp mij met het invullen van allerlei formulieren. Het is leuk om met jou koffie te drinken, practicum te geven of te lunchen. *Fred*, als de biocontroller weer eens niet deed wat ik wilde, zorgde jij ervoor dat hij dat even later wel weer deed. Mensen zoals jij, zorgen ervoor dat een technisch probleem geen onoverkomelijk probleem hoeft te zijn. Dat is fantastisch. *Gerrit*, jij hielp me altijd met een glimlach en een cynische opmerking met alle computerproblemen en programma's. *Remko*, in Afrika en Japan hebben wij heerlijk gewandeld. Vooral het kopen van een 'beslagen houten' kistje was een bijzondere ervaring. *Mathieu*, in Japan ben ik veel met jou op getrokken, ik herinner me nog goed de tempel-run-tempel dag samen met Sina. *Maria C*, when you were around in the Netherlands, people smiled more and dancing occurred naturally. You should come over more often to brighten our life. *Floor*, op verschillende momenten hebben wij leuke gesprekken gehad, jouw vriendelijkheid en betrokkenheid bij onze vakgroep siert je. *Amos*, it was great to work with you for half a year. You showed us how to sieve *Spirulina* and to prevent growth inhibition in high density cultivations. You were ever friendly and your encouragements meant much to me. *Carlos*, amigo, it was great to see you once in a while at a conference or when you were visiting our group. *Arno*, jij was een serieuze hard werkende kamergenoot en het verbaasde Pieter en mij niet dat je zo snel promoveerde.

Annette, het was plezierig om samen met jou en Packo het mariene practicum te geven. *Packo*, bedankt voor je wilde hypothesen, met jou erbij was een pauze nooit kleurloos. *Ronald O, Catrinus, Pieter O, Jeroen, Sebastiaan, Dirk, Mark, Detmer, Dominick M, Arjen, Tim, Koen*; met jullie heb ik leuke momenten beleefd tijdens het werk en zeker ook daarbuiten. *Hedy, Miranda, Joyce*, jullie waren altijd beschikbaar om mij te helpen met allerlei zaken. Secretaresses zoals jullie zijn onontbeerlijk voor het goed functioneren van een groep en jullie zorgen voor de extra gezellige noot.

Reinoud, Hans R, Hans M, André, Jan, Mees en Erik, jullie hebben het mogelijk gemaakt om vooruitstrevend onderzoek uit te voeren door soms (bijna) wonderen te verrichten en complexe onderzoeksofstellingen te bouwen. Jullie behulpzaamheid heb ik altijd erg op prijs gesteld.

Ontspanning zoek ik in tafeltennis. *Theo*, jij probeert continu mijn spel en aparte techniek bij te schaven wat zorgt voor extra plezier tijdens de wedstrijden, bedankt! *Michiel*, jij was een fijne teamgenoot, ik heb genoten van de tijd dat we samen in een team speelden. *Hans van G*, gezelligheid kent geen tijd met jou, die ene laatste (ééntje) kon er altijd nog bij. *Henk*, jouw topsportinstelling is een voorbeeld voor elke andere sporter, het was geweldig om een aantal seizoenen met jou in een team te spelen. *Hans de B en Pim*, de laatste twee jaar waren jullie mijn teamgenoten; ik heb genoten van onze seizoenen, de sportieve hoogtenpunten en de fantastische teamsfeer. Ik hoop nog jaren met jullie te tafeltennissen!

Tjarko, tweelingbroer, gedurende onze jeugd trokken wij altijd met elkaar op. Met iemand van dezelfde leeftijd die ook nog familie is door de verschillende fasen van het leven gaan is fantastisch. Dat jij trots op mij bent is ongeveer het beste wat een broer kan overkomen. *Margot*, jouw enthousiasme en vrolijkheid werkt aanstekelijk. Ik denk tóch dat jij fanatieker bent dan ik, stelling nummer 6 is zeker van toepassing op jou. *Jilles & Nolda*, jullie zijn twee geweldige mensen met een hart van goud! In jullie heb ik een tweede paar ouders gevonden die altijd geïnteresseerd zijn in ons leven. De steun die jullie Evelien en mij geven betekent veel voor ons. *Heit & Mem*, dankzij jullie heb ik een fantastische jeugd gehad en is het me nooit aan iets ontbroken. Jullie deelden veel tijd met ons en hebben mij gestimuleerd om mijn eigen leven te leiden. Ik ben blij twee zulke moderne ouders te hebben die met zoveel plezier in het leven staan en die dit plezier ook op mij hebben kunnen overbrengen, bedankt voor alles! *Jelle en Emy*, door de vreugde die jullie brengen in mijn leven, heeft deze een extra dimensie gekregen. *Evelien*, jij bent de zon in mijn leven, ik ben blij dat ik mijn ideale vrouw heb gevonden.

Rouke

Training activities

Discipline specific activities

Courses

Introduction to Process Engineering (WUR P053-200)

Biochemical Reactor design: fermentations (WUR P053-220)

Biochemical Reactor design: applied biocatalysis (WUR P053-221)

Bioreactor Design (VLAG, 2006)

Meetings

ISAP conference Montecatini Terme, Italy (1999)

ISAP Conference Almeria, Spain (2002)

Symposium Marine Biotechnology (VLAG, 2003)

IMBC conference St John's Newfoundland, Canada (2005)

ISAP Conference Galway, Ireland (2008)

General courses

Elementary course Didactics (OWU, 2000)

Intercultural Communication (OWU, 2003)

Scientific Writing (Language center, 2003)

Presentation skills (Language center, 2004)

Course Giving Lectures (OWU, 2004)

Skills for oral communication (OWU, 2006)

Optionals

PhD study tour South Africa (2000)

Symposium BDSL (2006)

PhD study tour Japan (2008)

12th NBC Ede, Netherlands (2008)

Curriculum Vitae

Rouke Bosma was born on 27 August 1973 in Sneek, the Netherlands. He went to primary school in Franeker and in this city he lived most of his childhood. In 1991, he passed secondary school (atheneum) at the “Rijkscholengemeenschap Simon Vestdijk” in Harlingen. He started his education at HBO Biochemistry at the “Hanzehogeschool” in Groningen. In his last study year, he investigated the enzymatic synthesis of phenylglycine and p-hydroxy-phenylglycine at DSM Andeno in Venlo. He graduated in July 1996 and started working as a technician at Process Engineering on the Chemferm project: “enzymatic antibiotic synthesis” at the Wageningen University. In July 1999, he started his permanent job as technician. On January 2000, his PhD started by doing research for the EET project entitled: “Duurzame co-productie van natuurlijke fijnchemicaliën en energie uit micro-algen”. At the end of 2003, this project was stopped, but Rouke continued his PhD, working on engineering aspects from algae. From 2008 on, he works as a researcher in the Algicoat project: “Coating Components firing the Economic Biorefinery of Algae”. At the end of this year he also became coordinator of the website www.algae.wur.nl. So, in the past nine years, on a part-time basis Rouke researched different aspects of microalgae, from which the results are described in this thesis.

The studies described in Chapter 2 and 4 were financially supported through a grant from the Programme Economy, Ecology and Technology (E.E.T. K99005/398510-1010) by the Netherlands' Department of Economic Affairs, the Department of Public Housing, Spatial planning and Environmental Protection, and the Department of Education, Cultural Affairs and Sciences'.

Publication of this thesis was financially supported by:
BioProcess Engineering, Wageningen University
VLAG

Printed by Ipskamp, Nijmegen

The Cover design was made with Andrea Mosaic v3.30, which was used to make a mosaic from a 400x magnified *Tetraselmis suecica* contrast microscope picture with persons closely related to Rouke.

Stellingen

1. De maximale theoretische fotosynthetische efficiëntie van microalgen kan met zonlicht niet bereikt worden (dit proefschrift).
2. De ideale fotobioreactor lijkt binnen handbereik, de ideale microalg niet (dit proefschrift).
3. Experimental designs zijn niet experimenteel, maar gebaseerd op wiskunde.
4. Informele communicatie stimuleert formele samenwerking.
5. Licht is voor de alg als voedsel voor de mens: overdaad schaadt.
6. Competitie geeft meedoen een extra dimensie.
7. Mobiele telefoons zijn heel soms handig.
8. Tijd = tijd, afspraak = afspraak. Het niet voldoen aan één van deze voorwaarden leidt tot irritatie.

Stellingen behorende bij het proefschrift:

Towards high productivities of microalgae in photobioreactors

Rouke Bosma

Wageningen, 5 Maart 2010

Propositions

1. The maximum theoretical photosynthetic efficiency of microalgae on sunlight cannot be achieved (this thesis).
2. The design of the ideal photobioreactor seems to be near; the design of the ideal microalga is still far away (this thesis).
3. Experimental designs are not experimental, but based on mathematics.
4. Informal communication encourages formal cooperation.
5. Light is for algae as food for man: moderation is key.
6. Competition gives participating an extra dimension.
7. Cell phones are seldom handy.
8. Time = time, appointment = appointment. Irritation will follow if one of these conditions is not met.

Propositions belonging to the thesis:

Towards high productivities of microalgae in photobioreactors

Rouke Bosma

Wageningen, 5 March 2010

