

# **Continuous Culture of *Botryococcus braunii* For Hydrocarbons Production**

**Simone Zenobi**

Submitted to the University of Exeter as a Thesis for the Degree  
of Master by Research (M. Res.) in Biosciences

January 2013

This thesis is available for Library use on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature: .....

## **ACKNOWLEDGMENTS**

I would like to first thank Prof. John Love for the opportunity to study at Exeter University and also for his patience, guidance and advice throughout the period of this MRes degree.

Many thanks also to Shell Global Solution UK for funding my research project. In particular many thanks to Dr David Parker and Prof. Robert Lee for giving me the opportunity to perform my experimental works at Shell Technology Centre Thornton - Chester.

My deepest gratitude to my colleague and friend Steve Brown for giving me training, advice, support, encouragement and a lot of help during all my time at Shell.

A special thanks also to Joao Diogo Gouveia for the supplying of the microalgal culture every time I needed.

I would like also to thank you all my colleagues at Biodomain department at Shell and in particular Louise Coppack for the initial training and several advices on my research project.

## ABSTRACT

*Botryococcus braunii* is a green, colonial microalga that can produce up to 75% of its dry weight as liquid hydrocarbons that can be converted easily in fuel. In order to cultivate *Botryococcus braunii* on commercial scale for the biofuel industry, new cultivation methods must be investigated. Until now, no studies have been performed on continuous culture of *Botryococcus braunii* under a photoperiod (cyclostat mode). The objective of this work is to investigate if the microalga can produce a constant amount of biomass and hydrocarbons in continuous culture under a light/dark cycle. *Botryococcus braunii* was grown in a stirred tank photobioreactor in continuous culture under a photoperiod of 12 h light and 12 h dark and using warm white LED light and red/blue LED light as light source with intensity of  $442 \mu\text{E m}^{-2} \text{s}^{-1}$ , and compared to continuous and batch culture. Results show that *Botryococcus braunii* in continuous culture under a photoperiod and in warm white LED light can produce a constant amount of dry biomass and hydrocarbons. Conversely *Botryococcus braunii* was not able to produce a constant amount of hydrocarbons and dry biomass in red/blue LED light.

## INDEX OF FIGURES

Fig. 1.1.....	p. 2
Fig. 1.2.....	p. 5
Fig. 1.3.....	p. 6
Fig. 1.4.....	p. 7
Fig. 1.5.....	p. 8
Fig. 1.8.....	p. 18
Fig. 1.9.....	p. 20
Fig. 1.10.....	p. 22
Fig. 2.1.....	p. 26
Fig. 2.2.....	p. 27
Fig. 2.4.....	p. 30
Fig. 3.1.....	p. 35
Fig. 3.2.....	p. 36
Fig. 3.3.....	p. 50
Fig. 3.4.....	p. 45
Fig. 3.5.....	p. 50
Fig. 3.6.....	p. 52
Fig. 3.7.....	p. 54
Fig. 3.8.....	p. 56
Fig. 3.9.....	p. 57

## INDEX OF TABLES

Table 2.1.....	p. 33
Table 3.1.....	p. 40
Table 3.2.....	p. 47

## INDEX OF ABBREVIATIONS

%	percentage
µm	micrometre
<i>B. braunii</i>	<i>Botryococcus braunii</i>
g l <sup>-1</sup>	grammes per litre
g l <sup>-1</sup> d <sup>-1</sup>	grammes per litre per day
GC – FID	gas chromatography – flame ionization detector
GC – MS	gas chromatography – mass spectrometry
mg g <sup>-1</sup>	milligrammess per gramme
mg l <sup>-1</sup>	milligrammes per litre
SE	standard error
µE m <sup>-2</sup> s <sup>-1</sup>	micro-Einstein per square metre per second
h	hour
D	dilution rate
µ	growth rate
Ln	natural logarithm
LED	light emitting diode

RB

red/blue

WW

warm white

## TABLE OF CONTENTS

Acknowledgements.....	i
Abstract.....	ii
Index of Figure.....	iii
Index of tables.....	iv
Index of Abbreviations.....	iv
Table of Contents.....	vi
Chapter 1: Introduction.....	p. 1
1.1 Biodiesel from Microalgae.....	p. 1
1.2 <i>Botryococcus braunii</i> .....	p. 3
1.3 Culture Condition in <i>Botryococcus braunii</i> .....	p. 9
1.4 Presence of Symbiotic bacteria in <i>Botryococcus braunii</i> cultures.....	p. 9
1.5 Methods for General Cultivation of Microalgal Cells.....	p. 9
1.6 Photobioreactors.....	p. 17
1.7 Light Sources for Bioreactors.....	p. 23
1.8 Aim of the project.....	p. 23
Chapter 2: Materials and Methods.....	p. 25
2.1 <i>Botryococcus Braunii</i> , culture conditions and equipment used.....	p. 25
2.2 Inoculum preparation .....	p. 31
2.3 Sample collection .....	p. 31
2.4 Absorbance of the algal cultures.....	p. 31
2.5 Dry Biomass estimation.....	p. 31
2.6 Chlorophyll content estimation.....	p. 31
2.7 Terpene analysis.....	p. 32
2.8 Ion Analysis.....	p. 32
2.9 Microscopy Analysis.....	p. 32
2.10 Summary of Experiments.....	p. 33

Chapter 3: Results.....	p. 34
3.1 <i>Botryococcus braunii</i> Culture in Batch Mode.....	p. 34
3.1.2 <i>Botryococcus braunii</i> in red/blue LED light.....	p. 37
3.1.3 <i>Botryococcus braunii</i> in Batch Mode: Red/Blue LED Light vs Warm White LED Light.....	p. 38
3.2 Continuous Culture of <i>Botryococcus braunii</i> in Chemostat and Cyclostat Mode.....	p. 41
3.2.1 Continuous Culture of <i>Botryococcus braunii</i> in Chemostat Mode.....	p. 41
3.2.2 Continuous Culture of <i>Botryococcus braunii</i> in Cyclostat Mode.....	p. 42
3.2.3 Comparison between Continuous Culture of <i>Botryococcus braunii</i> in Chemostat and Cyclostat Modes.....	p. 42
3.2.4 Comparison between Continuous Culture of <i>Botryococcus braunii</i> in Chemostat and Cyclostat Modes taking into account the biomass removed from the system.....	p. 46
3.3 Continuous Culture of <i>Botryococcus braunii</i> in Cyclostat Mode and Red Blue LED Light.....	p. 50
3.4 <i>Botryococcus braunii</i> in Warm White LED Light: a Comparison between the Continuous culture (Cyclostat mode) with the Batch Culture.....	p. 52
3.5 <i>Botryococcus braunii</i> in Red Blue LED Light: Cyclostat Mode vs Batch Mode	p. 54
3.6 Ion Analysis.....	p. 56
3.7 Microscopy Analysis.....	p. 57
 Chapter 4: Discussion.....	 p. 58
 Chapter 5: Conclusion.....	 p. 62
 References .....	 p. 63

## CHAPTER 1

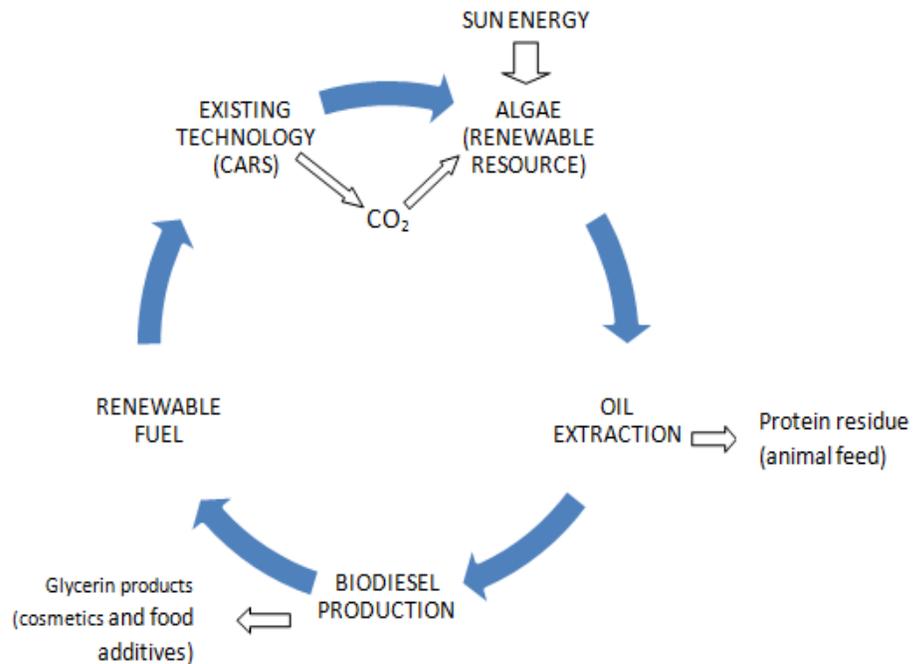
### INTRODUCTION

#### 1.1 Biodiesel from Microalgae.

Continued use of petroleum sourced fuels is now widely recognized as unsustainable because of depleting supplies and the contribution of these fuels to the accumulation of carbon dioxide in the environment (Chisti, 2007). Renewable, carbon neutral transport fuels are necessary for environmental and economic sustainability and energy security. Biodiesel derived from oil crops, waste cooking oil and animal fat is a potential renewable and carbon neutral alternative to petroleum fuels. Unfortunately, biodiesel from these sources cannot realistically satisfy even a small fraction of the existing demand for transport fuels (Chisti, 2007).

Microalgae appear to be the only source of renewable biodiesel that is capable of meeting the global demand for transport fuels. Microalgae are photosynthetic organisms that absorb carbon dioxide from the atmosphere; a main cause of the greenhouse effect (Figure 1.1). Oil productivity of many microalgae exceeds by 5-10 folds the oil productivity of the best producing oil crops (Chisti, 2007).

In addition, biofuel production through microalgae contributes to mitigating carbon dioxide emissions. The microalga *Botryococcus braunii* is particularly interesting from the perspective of biofuel production because it produces high levels of hydrocarbons (Brown *et al.*, 1969; Brown *et al.*, 1970) that can be readily converted to a practical biofuel (Hillen LW *et al.* 1982).



**Figure 1.1** Schematic representation of microalgae as source of renewable biofuel.

Algal oils which are used to produce mostly biodiesel and glycerin products. The fuel produced from renewable energy is used to power conventional transport vehicles. Carbon dioxide produced from the transport or energy sectors is used as a carbon source from algae; in this way there is a mitigation of carbon dioxide in the atmosphere.

## 1.2 *Botryococcus braunii*

*Botryococcus braunii* (Kutzing, 1849) is a planktonic, widespread, colonial microalga and a member of the *Chlorophyceae*. *B. braunii* is characterized by a considerable production of lipids which can reach up to 86 % of the algal dry weight (Brown, 1969). These lipid substances include hydrocarbons, i.e. highly reduced compounds comprising only carbon and hydrogen (Brown et al. 1970) and a number of specific ether lipids (Metzger *et al.*, 1991; Metzger and Largeau 1999). *B. braunii* is considered an ancient algal species dating back at least 500 MYA and it is one of the few organisms known to have directly contributed to the existing oil and coal shale deposits found on Earth (Derenne *et al.*, 1997; Mastalerz and Hower, 1996). Fossil *B. braunii* accounts for up to 1.4% of the total hydrocarbon content in industrially-exploited oil shales (Moldowan *et al.*, 1980).

Today, *B. braunii* is found in freshwater lakes, reservoirs, ponds, brackish waters and ephemeral lakes. Colonies vary in size from 30µm to 2 mm and consist of single or multiple cell clusters united by transparent strands. The cells are generally pyriform (7 x 13µm), are arranged around the periphery of the colonial cluster and are embedded within cups of an extracellular matrix that is impregnated by oil (Figures 1.2 and figure 1.3). Ultrastructural investigations show that the matrix consists of outer walls originating from successive cellular divisions, (Cohen, 1999).

*B. braunii* are classified into three different races, termed A, B, and L, that are differentiated generally on the basis of the characteristic hydrocarbons produced (Figure 1.4).

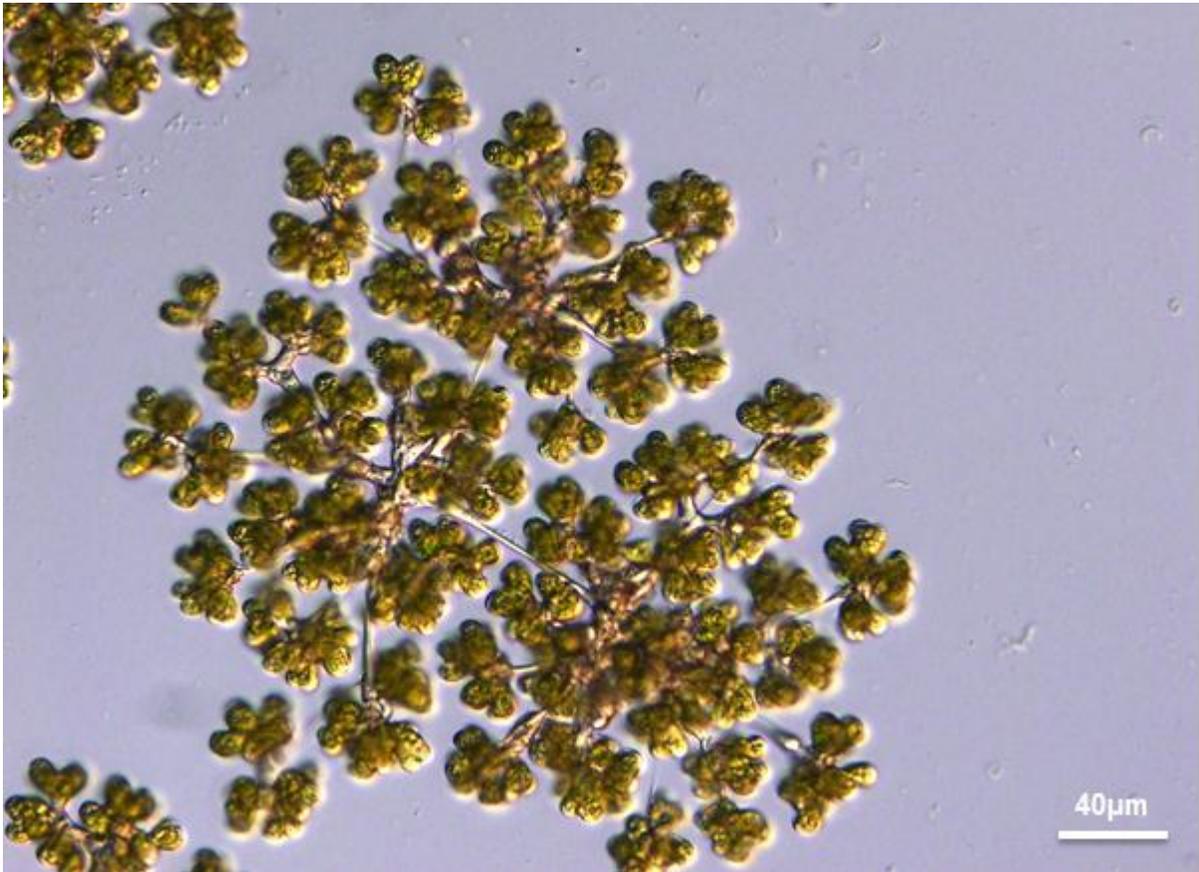
Race A predominantly produces C25 to C31, odd-numbered n-alkadienes and alkatrienes (two double bonds and three double bonds). These linear olefins can constitute up to 61% of the dry cell mass of the green active-state colonies (Gelpi *et al.*, 1970).

Race B typically produces triterpenoid hydrocarbons, C30–C37 Botryococcene (Metzger et al. 1985a) and C31 - C34 methylated squalenes (Huang and Poulter 1989a; Achitouv et al. 2004). In natural populations, botryococcene can constitute from 27 to 86% of the dry cell mass (Brown and Knights, 1969). Race L produces a single tetraterpenoid hydrocarbon, lycopadiene, C40H78 (Metzger and Casadevall 1987; Metzger et al. 1990). This hydrocarbon accounts for 2 to 8% of the dry biomass (Metzger and Casadevall, 1987).

For all *B. braunii* races, hydrocarbon productivity is optimal during the exponential phase of growth (Largeau *et al.* 1980; Metzger *et al.* 1985a, 1990). Thus, the production of hydrocarbons appears to be a normal feature of *B. braunii*. Similarly, ether lipid production was

shown to be maximal during the exponential and early deceleration stages of growth (Villareal-Rosales *et al.* 1992).

The hydrocarbons oils of *B. braunii* race B (mainly Botryococcene) are readily converted to starting materials for industrial chemical manufacturing and high quality fuels under standard hydrocracking/distillation conditions in yield approaching 97% (figure 1.5) (Hillen *et al.*, 1982).



**Figure 1.2** Colonies of *B. braunii*, strain Guadeloupe

Typically colonies of the *B. braunii* Guadeloupe strain have three-dimensional structures with a morphology resembling bunch of grapes.

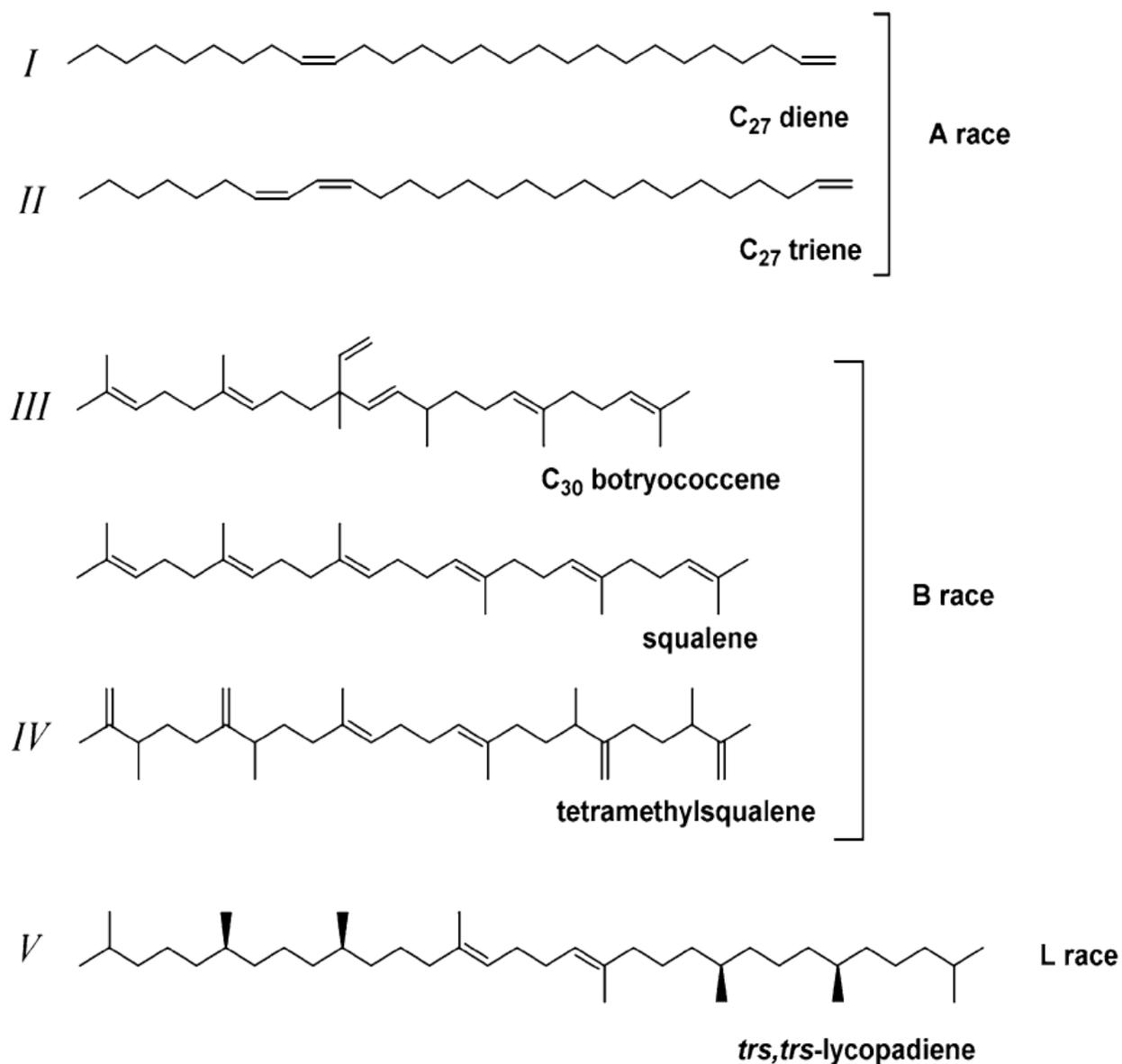
Images were acquired using an Olympus BX51 System Microscope with an attached DP71 digital CCD camera. Image processing and analysis software used was Cell F version 2.8 from Olympus.



**Figure 1.3** *B. braunii* strain Guadeloupe

The pyriform-shaped *B. braunii* cells are held together by a thick hydrocarbon matrix. Oil containing vesicles are clearly visible inside the cells.

Images were acquired using an Olympus BX51 System Microscope with an attached DP71 digital CCD camera. Image processing and analysis software used was Cell F version 2.8 from Olympus)

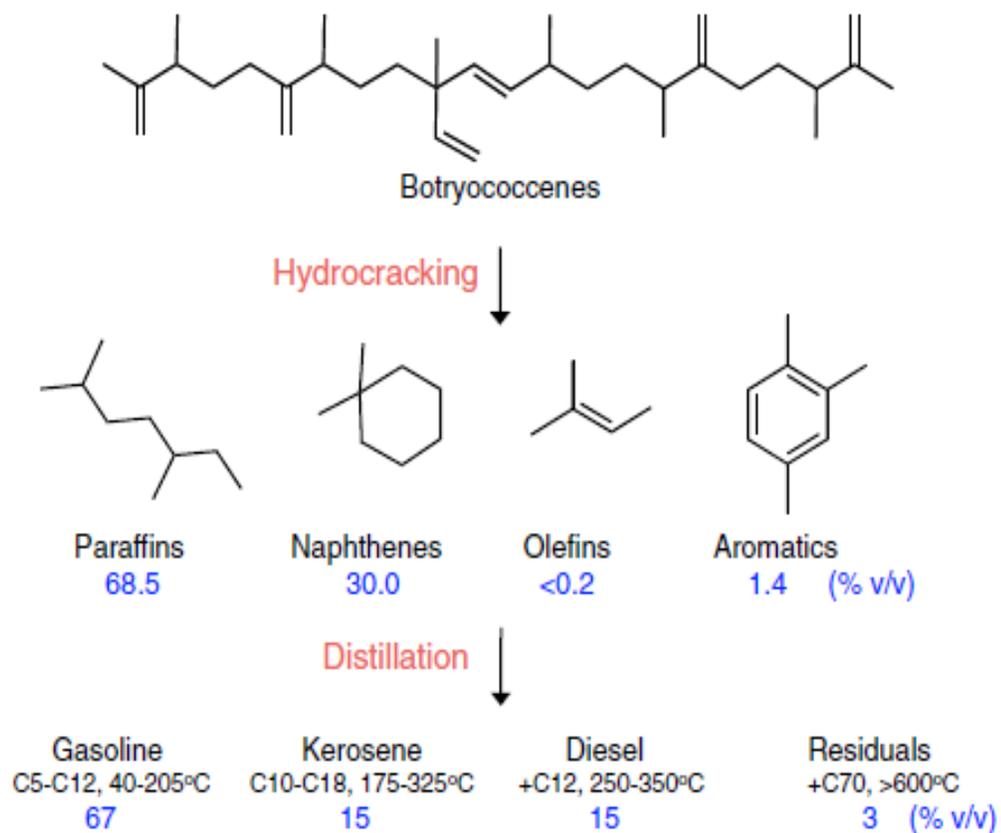


**Figure 1.4** Types of hydrocarbons produced by the three chemical races of *B. braunii*.

*B. braunii* synthesizes and secretes race-specific long chain hydrocarbons during normal growth:

I Knights *et al.* 1970; II: Villarreal-Rosales *et al.* 1992; III: Metzger and Casadevall 1983;

IV: Huang and Poulter 1989b; V: Metzger and Casadevall 1987



**Figure 1.5** Hydrocracking/Distillation of Botryococcene produces different type of fuels. Botryococcenes Hydrocracking generates Paraffins (68% v/v), Naphthenes (30%v/v), Olefins (<0.2%v/v), Aromatics (1.4%v/v); Distillation of these compounds produces Gasoline (67%v/v), Kerosene (15% v/v), Diesel (15% v/v) and some residual (3% v/v) (Niehaus et al. 2011).

### 1.3 Culture of *Botryococcus braunii*

Like all photosynthetic microorganisms, *B. braunii* requires CO<sub>2</sub>, light, inorganic nutrients and water to grow. A modified Chu-13 medium (Largeau *et al.* 1980; Metzger *et al.* 1991) has been found favorable for culturing of all three *B. braunii* races. In this culture medium, phosphate concentration is not a limiting factor for growth (Casadevall *et al.* 1985). By contrast, an increase in nitrate results in a longer exponential phase, but leads to a decrease in hydrocarbon production, even though the biomass increases (Casadevall *et al.* 1985).

It has been shown that CO<sub>2</sub> enrichment of the supplied air (2%-20%) has a little effect on the pH on the medium but increase biomass and hydrocarbon production (Ge *et al.* 2010; Chirac *et al.* 1985).

*B. braunii* can grow under a wide range of irradiance, from 68 to 822  $\mu\text{E m}^{-2}\text{s}^{-1}$ , but hydrocarbon synthesis is favored by light intensity ranging from 182 to 411  $\mu\text{E m}^{-2}\text{s}^{-1}$  (Cepak and Lukavsky 1994).

### 1.4 Presence of Symbiotic bacteria in *Botryococcus braunii* cultures

Unicellular microalgae generally grow in the presence of bacteria. These bacteria can stimulate algal growth by releasing substances such as vitamins and nitrogen derivatives. Some of these bacteria have been identified as *Pseudomonas* sp. and *Rizhobium* sp. (Rivas *et al.*, 2010).

Numerous bacteria were shown to exert considerable effect, either antagonistic or beneficial, on *B. braunii* growth yield and hydrocarbon production. Such effects were strongly dependent on the species involved as well as culture conditions. The presence of various microorganisms can influence the quantity of hydrocarbons produced and their level in the algal biomass. However, the chemical structure of the hydrocarbons' produced is not affected (Chirac *et al.*, 1985).

### 1.5 Methods for General Cultivation of Microalgal Cells

There are several ways of culturing microorganisms, however, in particular for microalgae-phytoplankton, three main methods are used batch culture, continuous culture and semi-continuous culture (Lavens and Sorgeloos, 1996).

## Batch Culture

Batch Culture is the most simple and common method for cultivation of microalgal cells. In batch culture systems, a limited amount of complete culture medium and algal inocula are placed in a culture vessel and incubated in favorable conditions of light and temperature. Agitation by shaking or by impeller mixing is necessary to ensure nutrient and gaseous exchange at the cell-water interface. Culture vessels can range from conical flask to an environmentally controlled fermenter. Algal cells grow better if supplied by either purging the flask with CO<sub>2</sub> enriched air (normally 5% v/v CO<sub>2</sub> in air) prior to culture and capped, or gassing the culture continuously with CO<sub>2</sub> enriched air.

Under the typical regime of a simple homogenous batch culture (closed system), where the nutrient supply is limited and nothing is added or removed during growth, the algae pass through different growth phases (Figure 1.6). The various phases represent the reaction of the algal population to the changes in environmental conditions and therefore depend on the inocula, the actual cultivation method, nutrient concentration, light intensity, temperature and other environmental parameters.

Phase 1 is the lag or induction phase (adaption), in which no increase in cell numbers is apparent. The condition of the inoculum has a strong bearing on the duration of the lag phase. An inoculum taken from a healthy exponentially growing culture is unlikely to have any lag phase when transferred to fresh medium under similar growth conditions. In general the length of the lag phase will be proportional to the length of time the inoculum has spent between stationary phase and death phase (Spencer, 1954).

Inoculum size is also important. If small inoculum is used, even cells taken when exponential growth is well established can show a long lag in fresh medium. A lag phase may also occur if the inoculum is transferred from one set of growth conditions to another.

Phase 2 is the exponential phase or log phase, in which cell multiplication is rapid and numbers increase in geometric progression. The cells start growing at a constant and maximum rate and this period is called log or exponential phase. The Exponential phase can be calculated by the equation:

$$dx / dt = \mu X \quad (1)$$

- X is the concentration of microbial biomass (g l<sup>-1</sup>)
- t is time (days)
- $\mu$  is the specific growth rate, in hours<sup>-1</sup> or in days<sup>-1</sup>

On integration equation (1) gives:

$$X_t = X_0 e^{\mu t} \quad (2)$$

- $X_0$  is the original biomass concentration
- $X_t$  is the biomass concentration after the time interval, t
- e is the base of the natural logarithm.

On taking natural logarithms equation (2) becomes:

$$\ln X_t = \ln X_0 + \mu t$$

A plot of the natural logarithm of biomass concentration against time should yield a straight line, where the slope is equal to  $\mu$  which represents the growth rate of the microbial culture:

$$\mu = (\ln X_t - \ln X_0) / t$$

During the exponential phase the nutrient are in excess and the organism is growing at its maximum specific growth rate,  $\mu_{max}$

The mean generation time or "doubling time" (g) is the average time required for all the components of the culture to double. This is calculated from the following equation:

$$g = \ln 2 / \mu$$

Phase 3 is one of linear or arithmetic growth that progress to a – phase of declining relative growth. In this phase, the growth rate decreases because some factors as exhaustion of nutrients, alteration of pH, reduction of the light intensity by self- shading and auto-inhibition. Auto- inhibition occurs when certain algae produce substances toxic to themselves during the course of their metabolism.

Phase 4 is the stationary phase in which cell numbers remain more or less stationary. During this phase the metabolism slows down as result of reduction in light intensity due to self-

shading and nutrient limitation. There is equilibrium between new cells generation and loss of biomass.

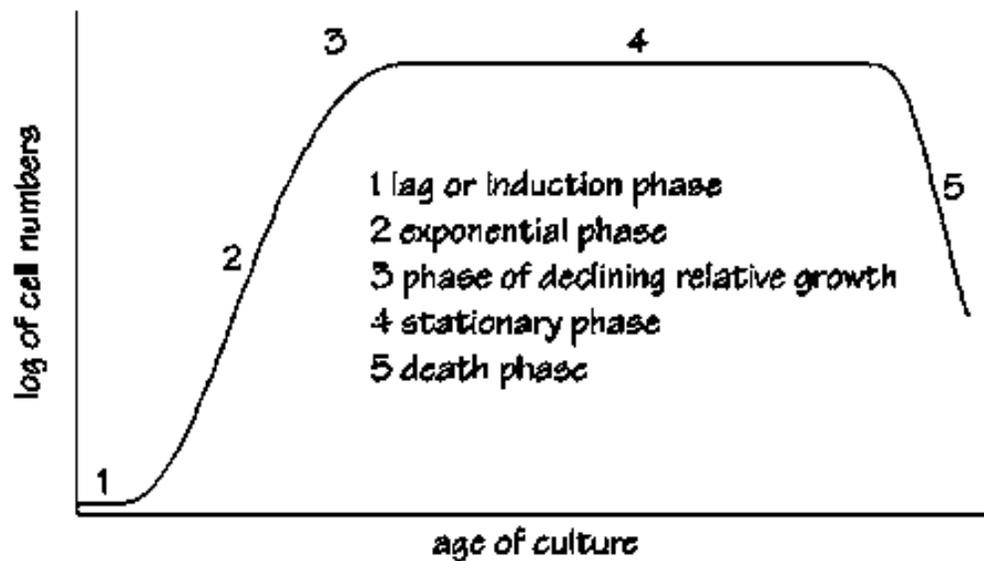
Finally, phase 5 is the death phase, in which complete breakdown of the algal population due to of absence of nutrients, poor light intensity and accumulation of toxic compounds in the media is observed (Lavens and Sorgeloos,1996).

### Continuous culture

The continuous culture technique allows a microbial culture to remain in exponential growth indefinitely and consequently ensures continuous production of new cells. Continuous culture techniques are proving to be exceptionally useful tools for studying the growth kinetics as well as the physiology and the biochemistry of microalgae. In continuous culture, a certain amount of fresh medium is added continuously to the vessel whilst an equal volume of culture (medium plus microorganisms) is removed (Figure 1.7).

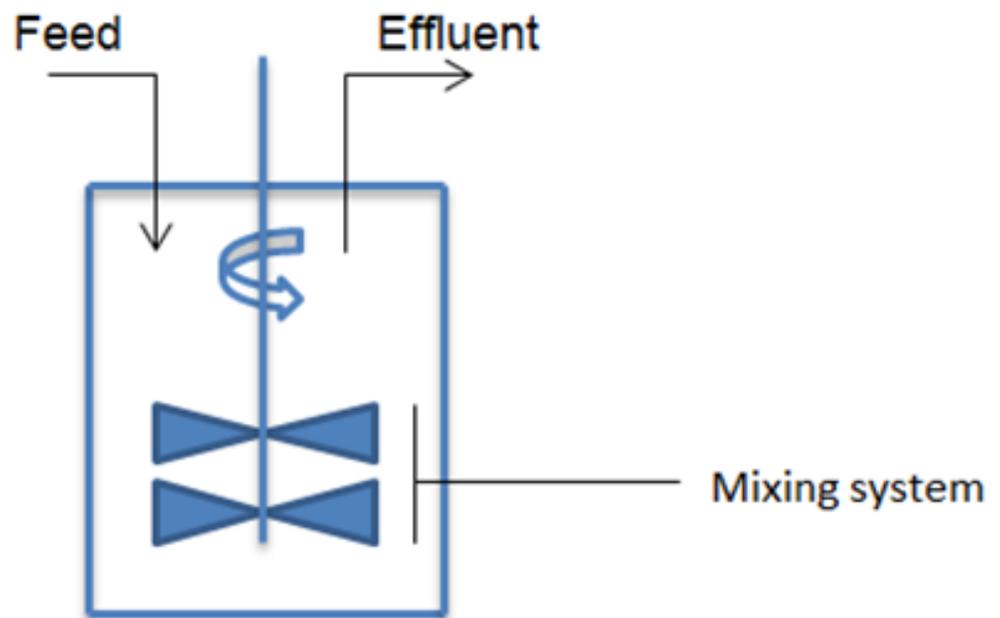
### Semi-continuous culture

In a semi-continuous culture a portion of the culture is harvested at regular intervals and replaced by an equal volume of medium. The approach differs from chemostat in that the fresh medium is added episodically rather than continuously.



**Figure 1.6** Schematic representation of growth phase on batch culture

1) Lag or induction phase, 2) exponential phase or log phase, 3) linear growth – phase of declining relative growth 4) Stationary phase, 5) Death phase. Diagram reproduced from the Manual on the Production and Use of Live Food for Aquaculture (Lavens and Sorgeloos, 1996).



**Figure 1.7** *Schematic representation of continuous system*

In a continuous system fresh medium is added continuously and an equal volume of culture is removed continuously to keep the culture volume constant. The mixing system is typical in a stirred tank bioreactor (paragraph 1.6).

To establish a constant culture, a certain amount of fresh medium is continuously added to vigorously growing algae cells whilst removing the same amount of culture; at some point the culture will reach a steady state. By definition a culture is in steady state when three total volume changes have been performed. For example three liters of culture is in steady state if nine liters of fresh medium has passed through the vessel.

In the steady state condition, microorganisms grow at a constant rate and in a constant environment meaning continuous production of cells of similar biochemical composition and physiological state.

If fresh medium is continuously added to the culture this leads to cultural dilution; the dilution rate (D) is calculated as follows:

$$D = F / V$$

- F is the medium flow rate (liters per day for algal cultures) expressed in  $\text{dm}^3\text{h}^{-1}$
- V is the volume of the culture vessel (usually liters)  $\text{dm}^3$ , kept normally constant
- D is expressed in  $\text{h}^{-1}$  or in  $\text{days}^{-1}$ .

The net change in cell concentration over a time period may be expressed as:

$$\text{dx/dt} = \text{growth} - \text{output} \quad \text{or} \quad \text{dx} / \text{dt} = \mu x - Dx$$

Under steady state conditions the cell concentration remains constant, no net change of biomass, thus

$$\text{dx/dt} = 0 \quad \text{and} \quad \mu x = Dx \quad \rightarrow \quad \mu = D$$

Under steady state the *specific growth rate* ( $\mu$ = defined as the increase in cell mass per unit time) of the population in continuous culture is determined by the dilution rate which is an experimental variable.

For success the medium flow rate (and consequently D) has to be chosen very carefully. If the medium is supplied at a rate higher than  $\mu$ , the cells are unable to divide rapidly enough to maintain a stable population and are eventually washed out of the growth vessel. There are two types of continuous cultures: the chemostat and the turbidostat.

In a chemostat (**chemical** environment is **static**) a flow of fresh medium is introduced into the culture at a steady, predetermined rate. This method adds a limiting vital nutrient (e.g. nitrate) at a fixed rate and in this way the growth rate and not the cell density is kept constant. Chemostats are largely used in research as they allow for maintenance of a specific growth rate at pre-determinate values. Culture parameters such as temperature, pH, light intensity and substrate concentration could be readily adjusted and studied at fixed specific growth rates.

Conversely, in a turbidostat (**turbidity static**-constant turbidity) the cell density is kept constant and the inflow rate of fresh medium is varied. The original turbidostat is a chemostat provided with a photoelectric cell for sensing the turbidity of the culture, adding medium when biomass concentration rises above a chosen level.

The turbidostat is particularly useful for operating under conditions that are unstable in the simple chemostat, such as high irradiance or presence of inhibitory substrates. On the other hand turbidity measurements are not reliable, except in short term cultures, for two main reasons: cell adhesion to the surface of the optical cell and its sensitivity to changes in cell or aggregate size (Microalgal Culture, Richmond, 2004). The turbidostat is particularly useful for slow growing algae and those with a complex cell cycle; for example *Haematococcus lacustris* has a different max specific growth rate at various stages in the cell cycle, resulting in complete washout of the culture in a fixed dilution rate chemostat (Lee and Ding, 1994).

When a microalgal continuous culture is subjected to alternating light and dark periods, chemostat principles are not totally valid. For algal cultures maintained on light/dark cycles, the instantaneous population growth rate  $\mu(t)$  is not instantaneously equal to the culture dilution rate  $D$ , as in steady-state chemostat. Instead, only the period-average growth rate  $\langle\mu\rangle_t$  is equal to  $D$  (Frisch & Gotham, 1977, 1979). In this case it is advised to use a cyclostat and apply the theory of Cyclostat growth (H. L. Frisch and I. J. Gotham, 1979; Ivan J. Gotham and G-Yull Rhee, 1982). By definition a cyclostat is a type of chemostat grown under a repeatably varying regimen of light or (rarely) temperature and it is therefore more appropriate to define the culture in terms of quasi-steady state condition.

## 1.6 Photobioreactors

A bioreactor is a system for growing cells or tissues in research or industry, normally comprising a cylindrical vessel made of stainless steel or glass. A bioreactor can be surrounded by a light source that is normally artificial light. Obviously, in the last case, the bioreactor's wall has to be transparent to the light and is called photobioreactor (abbreviated as PBR).

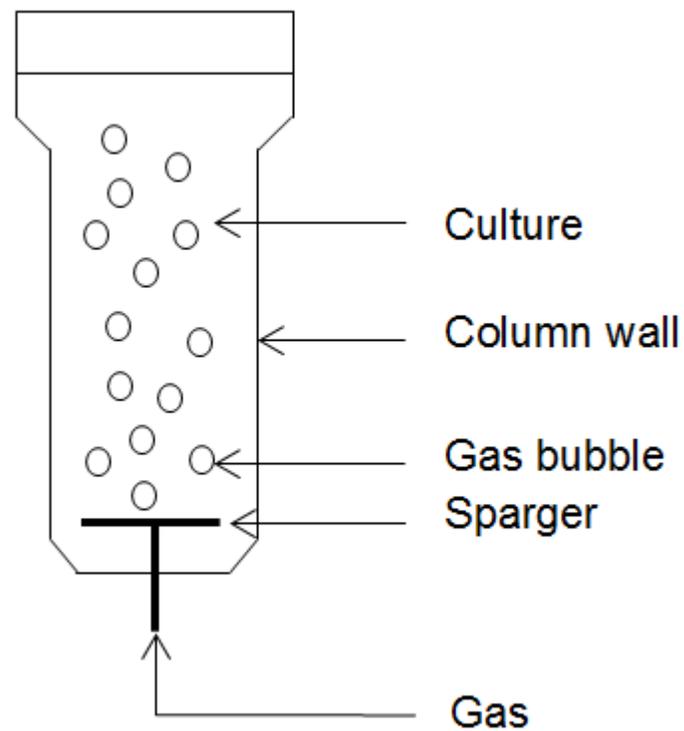
Consequently, photobioreactors are bioreactors in which phototrophs (microbial, algal or plant cells) are grown or used to carry out photobiological reaction (Tredici, 1999).

Although an open pond can be seen as a photobioreactor, normally the term of photobioreactor describes a closed system in which there is no direct exchange of gases and contaminants with the environment (Tredici, 1999). Photobioreactors are very useful for growing algae; first they enable high productivity, second, it is possible to control and monitor the environment parameters such as; light intensity, temperature, pH, oxygen concentration and carbon dioxide concentration (Handbook of microalgal culture, 2004).

There are several types of Photobioreactors for microalgal cultivation (Singh *et. al*, 2012): vertical or horizontal tubular PBRs (bubble column and airlift), helical type PBRs, stirred tank PBRs and flat pane PBRs. For research purposes, the most commonly used PBRs are vertical tubular PBRs (Bubble column and Airlift) and stirred tank PBRs. Consequently a brief description of them will be reported below.

#### Bubble column Photobioreactors

Bubble column reactors are cylindrical vessel with height greater than twice the diameter. It has advantage of low capital cost, high surface area to volume ratio, lack of moving parts, satisfactory heat and mass transfer, relatively homogenous culture environment, efficient release of O<sub>2</sub> and residual gas mixture. Mixing and CO<sub>2</sub> mass transfer is done through bubbling the gas mixture from sparger (Singh *et. al*, 2012), Figure 1.8.



**Figure 1.8** Schematic representation of Bubble column PBR.

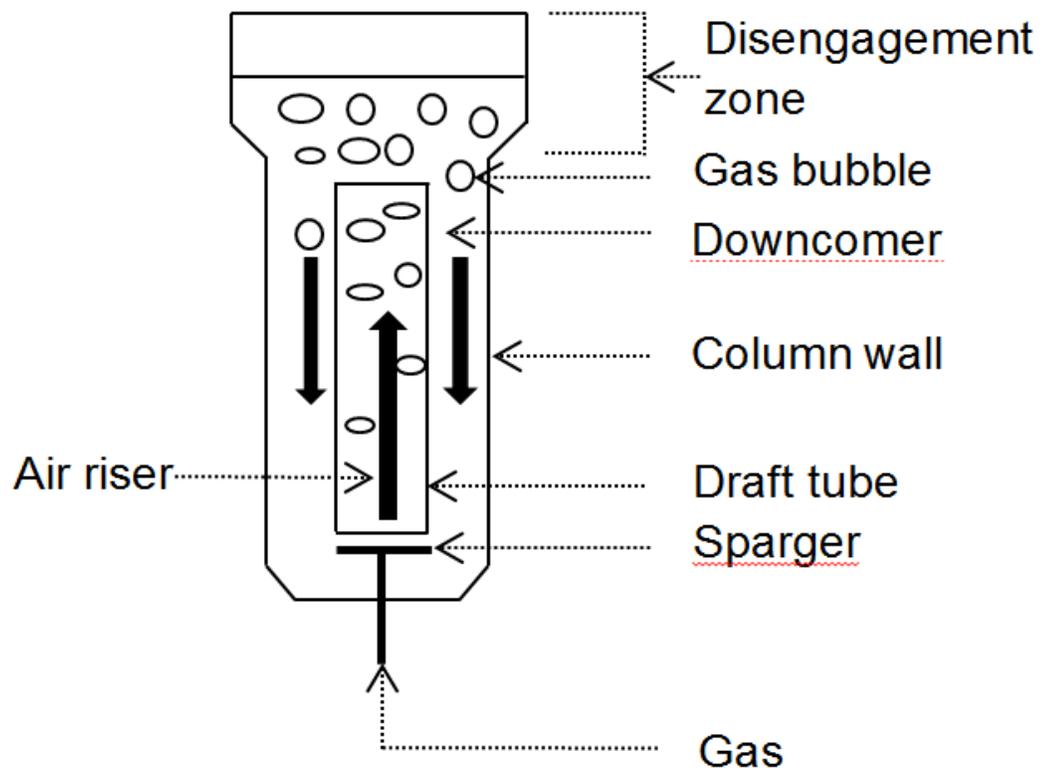
The introduction of gas takes place at the bottom of the column and causes a turbulent stream to enable an optimum gas exchange and culture mixing. Column wall is made out of transparent material in order to allow light penetration.

### **Airlift Photobioreactor**

Airlift PBRs are vessels with two interconnecting zones. One of the tubes is called the riser where gas mixture is sparged whereas the other region is called downcomer which does not receive the gas. Mixing is done by bubbling the gas through sparger in the riser tube without any physical agitation. The riser is similar to a bubble column where sparged gas moves upward randomly and haphazardly. The gas sparging decreases the density of the riser making the liquid move upward.

In the disengagement zone gas leaves the liquid and its performance depends upon design of this section and the operating conditions. The amount of gas which does not disengage in the disengagement zone gets trapped by liquid moving downward in the downcomer. Gas hold up in the downcomer has a significant influence in the fluid dynamics of the airlift reactor (Singh *et al.*, 2012), figure 1.9.

The main advantages of airlift PBRs are low shear rate, high capacity, good mixing, absence of mechanical agitators and ergonomic. Airlift bioreactors are very useful for large culture volumes.



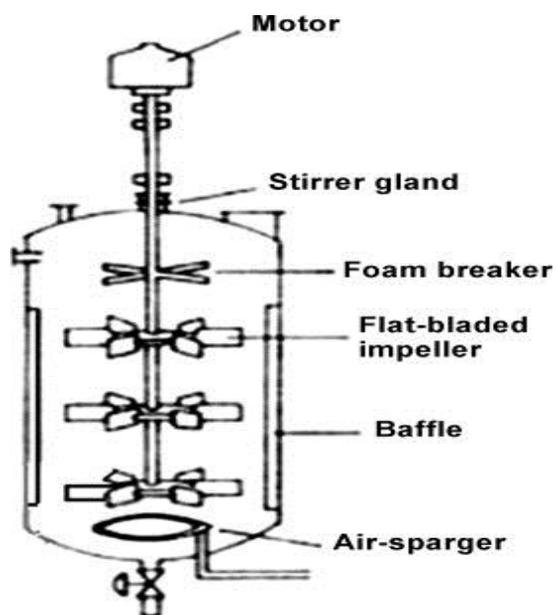
**Figure 1.9** Schematic representation of airlift photobioreactor.

Black arrows show fluid dynamic in the vessel. The introduction of gas takes place at the bottom of the column. Column wall is made out of transparent material in order to allow light penetration.

### **Stirred Tank Photobioreactors**

In stirred tank PBRs (STBs) reactors, mechanical stirrers (using impellers) are used to mix the reactor to distribute heat and materials (such as oxygen and substrates). This type of PBR is very useful in case working with small volume of microalgal culture is needed. Stirred Tank Bioreactors (STB) have the following functions ([www.rocw.raifoundation.org](http://www.rocw.raifoundation.org)): Homogenization, suspension of solids, dispersion of gas liquid mixtures, aeration of liquid and heat exchange.

STBs are provided with a baffle and a rotating stirrer is attached either at the top or at the bottom of the bioreactor. Baffles are usually flat vertical plates whose width is about one-tenth of the vessel diameter. Normally, 4-6 baffle plates are fitted to the inside vessel walls to aid mixing and mass transfer by increasing turbulence, preventing vortex formation and eliminating 'dead spaces'. In each vessel, the impeller is connected to an external motor, which drives the stirrer system. The effectiveness of agitation depends upon the design of the impeller blades, speed of agitation and the depth of liquid (Figure 1.10).



**Figure 1.10** Schematic representation of Stirred tank bioreactor (Singh *et al.*, 2012)

## 1.7 Light Sources for Bioreactors

A variety of artificial light sources can be used for plants growth including incandescent grow lights, fluorescent lights, high-pressure sodium lights and light –emitting diodes (LEDs). Studies have recently shown that illuminating plants with LEDs leads to higher biomass productivity per unit of irradiance (Massa *et al.* 2008). LEDs have several advantages over traditional forms of horticultural lighting; their small size, durability, long lifetime, cool emitting temperature, and the option to select specific wavelengths for a targeted plant response make LEDs more suitable for plant-based uses than many other light sources. (Massa *et al.* 2008).

In particular, it has been observed that using red LEDs to illuminate PBRs leads to excellent microalgal biomass production (Choul-Gyun Lee *et al.* 1994). Moreover reports show that a red and blue combination of LED light improves biomass production in plants (Brown *et al.* 1995).

Commercial-scale culture of microalgae is currently performed at scale in open outdoor ponds. Consequently despite the benefits that red-blue LED light may confer on algal biomass productivity, in PBRs it is often more realistic to use a light source that can simulate better the wavelengths and intensities of natural light. Hence the development of warm white LEDs that simulates natural light more accurately than red-blue LED combination.

## 1.8 Aim of the project.

The microalga *Botryococcus braunii* produces high quantity of hydrocarbons (Brown and Knights *et al.* 1970) that can be easily converted in biofuel (Hillen *et al.*, 1982). Consequently the goal of biofuel industry is continuously produce hydrocarbons from *B.braunii* that are ideally cultivated in open outdoor ponds. This goal can be achieved growing *B.braunii* in continuous culture. However, continuous culture of *B. braunii* requires solid knowledge pertaining to the growth rate and nutrient uptake that is best investigated in a closed PBR.

The aim of this project is to cultivate *B. braunii*, strain Guadeloupe in a PBR under a photoperiod and irradiance that mimic those in latitudes where *B.braunii* might be grown commercially; *i.e.* the equatorial / tropical region. Cell will be harvested on a regular basis for as long as possible, to ascertain the culture dynamics and nutrient requirements of this system.

Previous studies have been performed on continuous culture of *B.braunii* under continuous illumination (E. Casadevall, *et al.*, 1984; Sawayama, *et al.*, 1994), but currently there are no relevant studies of continuous culture under a photoperiod. In the end of this study, it will be possible to conclude if *B.braunii* strain Guadeloupe can grow in continuous culture under a photoperiod with high hydrocarbons production. This knowledge will benefit downstream processing applications and potentially inform less controlled culture systems, such as algal raceways or ponds.

## CHAPTER 2

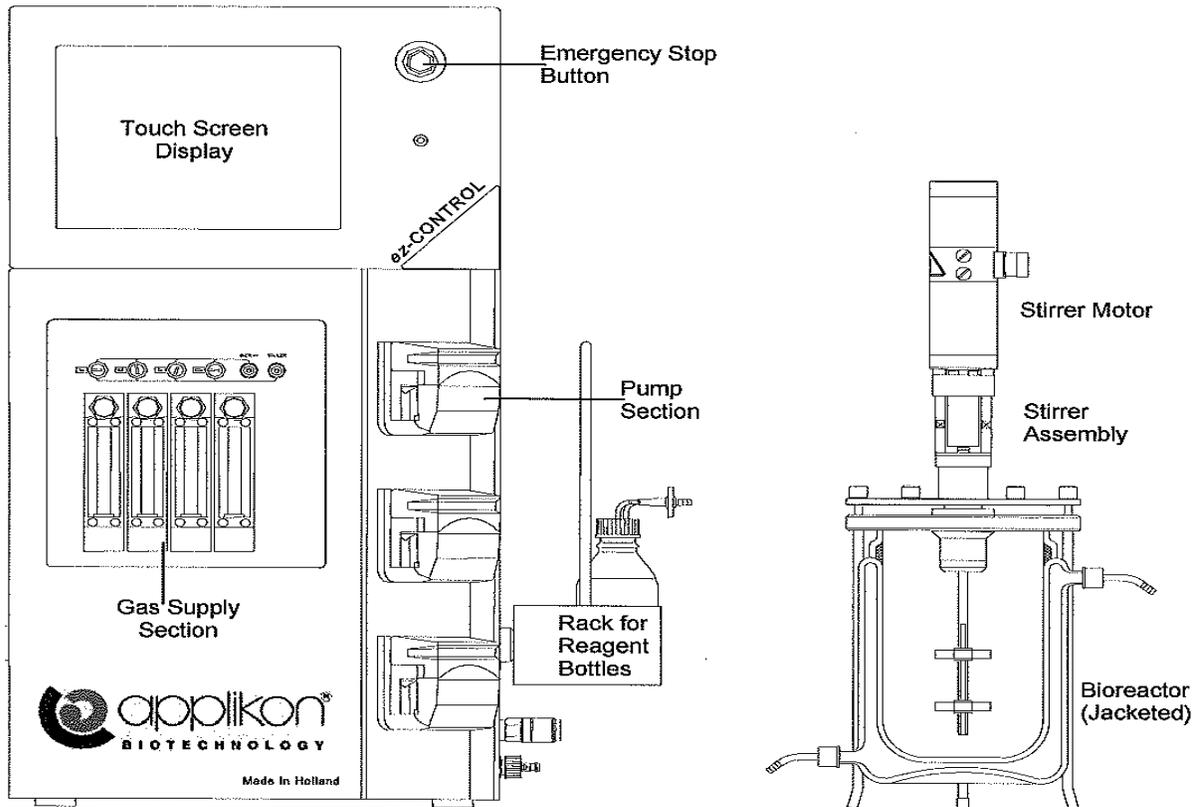
### MATERIALS AND METHODS

#### 2.1 *Botryococcus Braunii*, Culture Conditions and Equipment Used.

*Botryococcus braunii* race B, strain Guadeloupe, was obtained from Pierre Metzger (Laboratoire de Chimie Bioorganique et Organique Physique, Ecole Nationale Supérieure de Chimie de Paris), and cultured in modified Chu13 medium supplemented with vitamins, citric acid and sodium selenate (MCV): 400 mg l<sup>-1</sup> KNO<sub>3</sub>, 200 mg l<sup>-1</sup> MgSO<sub>4</sub>·2H<sub>2</sub>O, 200 mg l<sup>-1</sup> citric acid, 108 mg l<sup>-1</sup> CaCl<sub>2</sub>·2H<sub>2</sub>O, 104.8 mg l<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub>, 20 mg l<sup>-1</sup> Fe-Na<sub>2</sub>EDTA, 2.86 mg l<sup>-1</sup> H<sub>3</sub>BO<sub>3</sub>, 1.8 mg l<sup>-1</sup> MnSO<sub>4</sub>·4H<sub>2</sub>O, 1.1 mg l<sup>-1</sup> thiamine, 220 µg l<sup>-1</sup> ZnSO<sub>4</sub>·7H<sub>2</sub>O, 135 µg l<sup>-1</sup> cobalamin, 90 µg l<sup>-1</sup> CoSO<sub>4</sub>·7H<sub>2</sub>O, 80 µg l<sup>-1</sup> CuSO<sub>4</sub>·5H<sub>2</sub>O, 60 µg l<sup>-1</sup> Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 25 µg l<sup>-1</sup> biotin, 10 µl l<sup>-1</sup> H<sub>2</sub>SO<sub>4</sub>, 9.4 µg l<sup>-1</sup> Na<sub>2</sub>O<sub>4</sub>Se, pH 7.5 with NaOH.

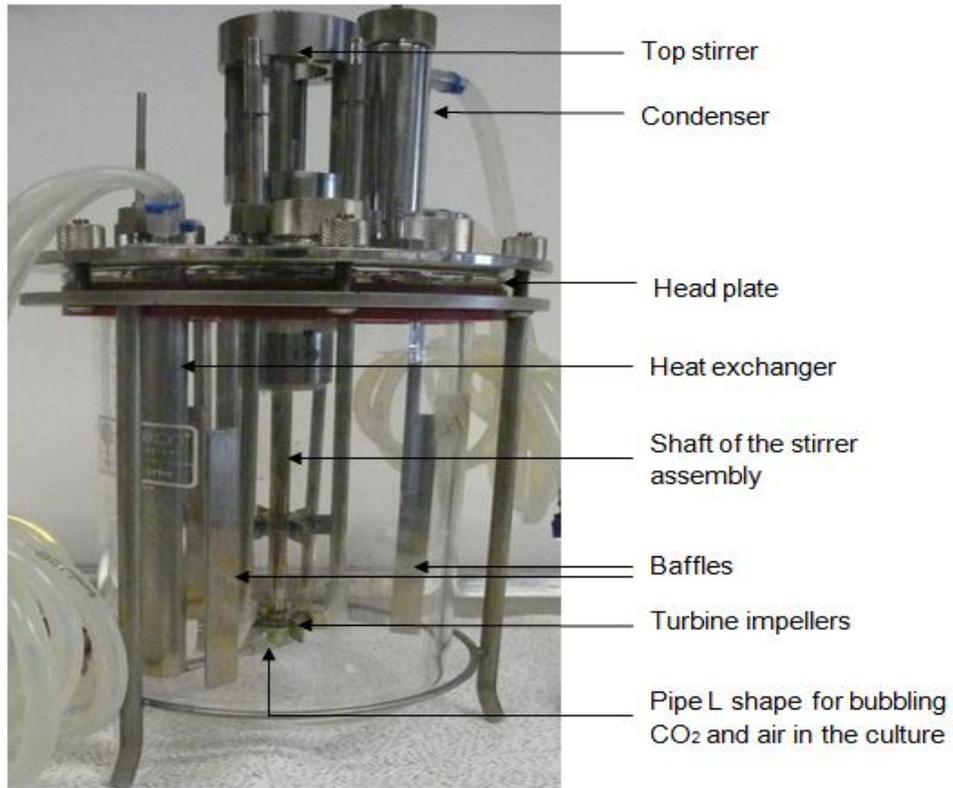
*B.braunii* was grown at 23°C with 5% of atmospheric CO<sub>2</sub>, mixing speed at 400 rpm, using an Applikon autoclavable Bioreactor System (Figure 2.1). Depending on the aim of the experiment, the bioreactor was illuminated by red blue LED light or warm white LED light with an intensity of 442 µE m<sup>-2</sup>s<sup>-1</sup>. The light : dark photoperiod was 12h : 12h or 24h : 0h. Some algal cultures were grown in batch mode and other in continuous mode depending on the aim of the experiment.

The Applikon autoclavable Bioreactor System (Figure 2.1), supplied from Applikon biotechnology, consists of the following parts. An autoclavable stirred tank bioreactor with the appropriate auxiliaries (Figure 2.2a and Figure 2.2b) like a stirrer assembly, sensors (oxygen probe, carbon dioxide probe, pH probe, temperature probe and level probe), baffles and an aeration assembly. An ez-control Bio Controller for measurement and control of process variables (pH, temperature, oxygen, carbon dioxide and liquid level in the vessel and stirrer speed) with corresponding controller outputs in order to keep process conditions on set point. The ez-control combines and support actuators like pumps and valve.

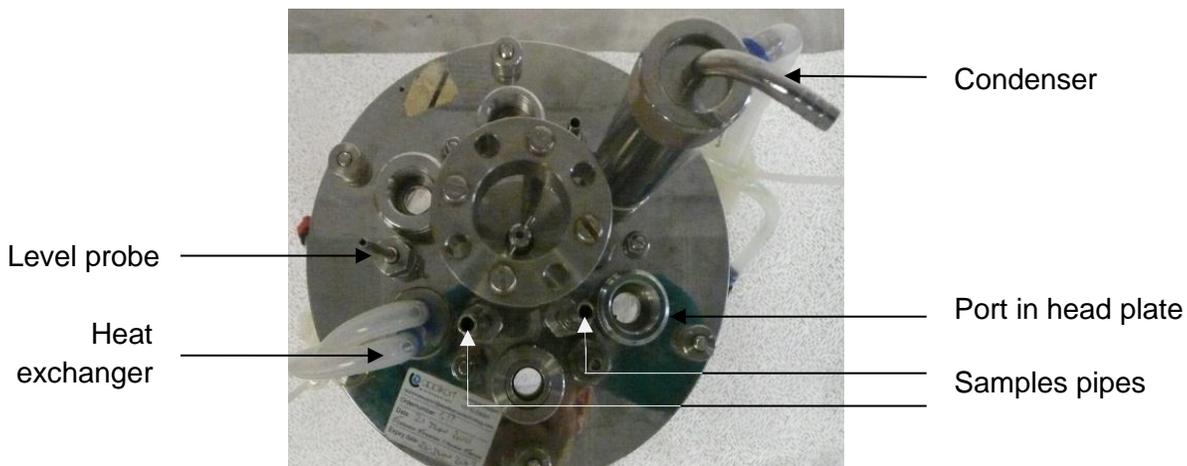


**Figure 2.1** Schematic representation of Applikon autoclavable Bioreactor System used for experiments.

On the left the ez-control Bio Controller and on the right the Stirred Tank bioreactor Jacketed; in this research work a bioreactor not Jacketed was used. Picture taken from Applikon Biotechnology Manual operator.



**Figure 2.2a** the assembled bioreactor



**Figure 2.2b** Head plate top view

For experiments was used a 3 l Dished bottom bioreactor, Applikon catalogue number Z611000310.

The autoclavable bioreactor is built in two parts: one glass vessel and on the top of it there is a metallic head plate where is possible to insert auxiliaries. Auxiliaries are devices that can be mounted in a head plate and protrude into the bioreactor.

The following auxiliaries were used:

A stirrer assembly which include a top stirrer assembly which is mounted at the central stirrer port in the bioreactor head plate and turbine impellers which fit to the shaft of the stirrer assembly. Then three baffles which are used to increase the mixing efficiency. Baffles are mounted in the head plate near the reactor wall in order to have an optimal mixing performance. One L-type sparger which is a pipe L shape used to bubble carbon dioxide and air inside bioreactor. This pipe was connected to the ez-control through a Peroxide-Cured Silicone Tubing L/S 25. The gas (air + carbon dioxide) was filter sterilized using a filter PTFE Membrane supplied from Pall (part number 12082).

An air outlet condenser which is mounted on the top of head plate and it is used to prevent culture evaporation. It is connected to the ez-control through Masterflex Peroxide-Cured Silicone Tubing L/S 25. Two Addition pipes for adding fluids to the reactor.

A liquid entry system to prevent back grown of microorganism into the medium container when the bioreactor operates in continuous culture. The liquid entry system uses a sterile gas flow to transfer the fresh medium to the reactor. In this way, direct contact between the culture and the medium storage container does not exist.

Two sample pipes; one was connected with a Peroxide-Cured Silicone Tubing L/S 25 to the Sample system supplied from Applikon to withdrawn samples and the other one was used to withdrawn algal culture in continuous mode.

A heat exchanger: connected to the ez-control through Masterflex Peroxide-Cured Silicone Tubing L/S 25, it is used to maintain the temperature constant.

A thermometer pocket: it allows inserting a temperature probe in the reactor. 5 sensors, including: Oxygen sensor (catalogue Number Z010023520), pH sensor (catalogue Number Z001023551), temperature probe (catalogue Number Z034150010) and level sensor (catalogue Number Z71205AF03), supplied from Applikon, were inserted in the bioreactor and connected and controlled from the ez-control.

The carbon dioxide sensor InPro5000 (Part Number 52206068), supplied from Mettler Toledo, was inserted as well in the bioreactor but was controlled with an external device, the Transmitter M400 supplied from Mettler Toledo.

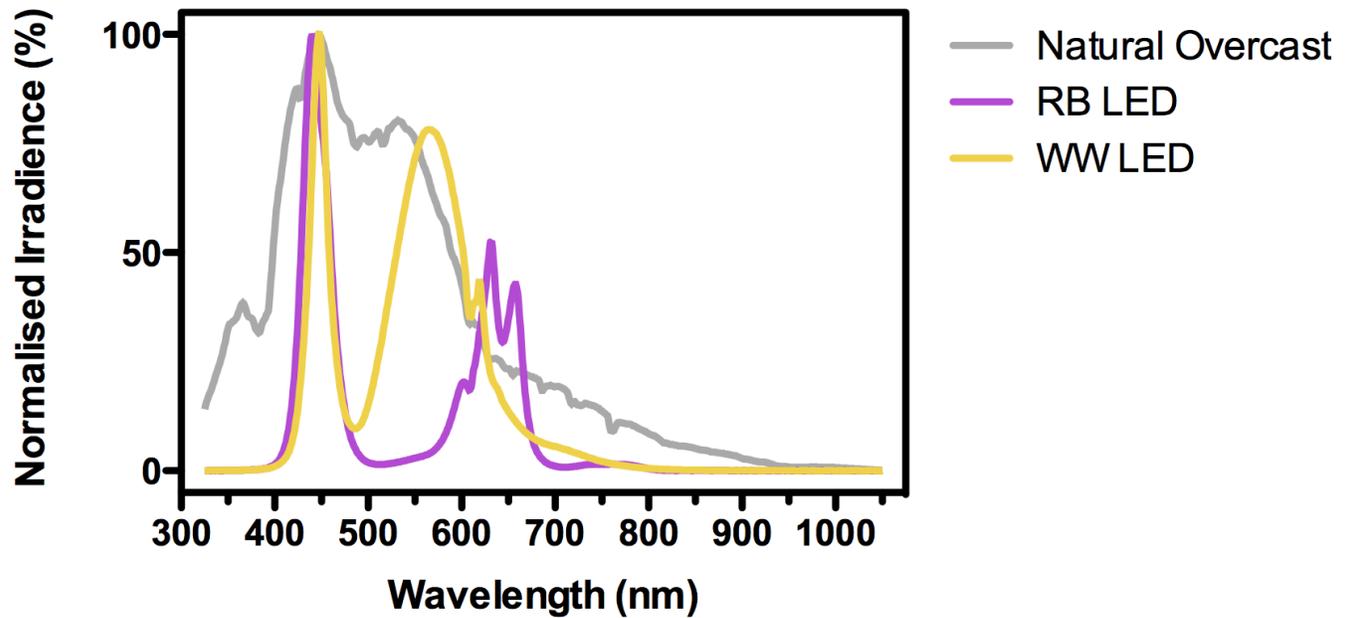
The following equipment were used for operating in continuous mode.

A peristaltic pump supplied from Masterflex (L/S Variable- Speed Console Drive With 10-Turn Speed Control and Remote Capabilities). A Masterflex L/S Pump heads – standard, (flow rate 0.001 to 6 mL/min). A Masterflex - Peroxide-Cured Silicone Tubing L/S 16 and L/S 13 size.

The pump and other accessories were used to pump fresh medium inside the reactor through the liquid entry system. An aquarium pump was used to pump air inside the liquid entry level.

The peristaltic pump of Applikon ez-control and a Masterflex - Peroxide-Cured Silicone Tubing L/S 14 were used for withdrawing medium from reactor.

The bioreactor was surrounded by a four panel box emitting LED light. Two different boxes were used: one emitting red and blue LED light and another emitting warm white led light. The LED light boxes were manufactured in the laboratory to have a maximum light intensity of  $884 \mu\text{E m}^{-2} \text{s}^{-1}$ . The light intensity was measured using Hansatech Quantitherm Light-Meter-Thermometer. The light spectrum of each led light box was determined using an ASD Handheld photo-radiometer (Figure 2.4).



**Figure 2.4** Light Spectra of three different light sources, natural light, red-blue LED light and warm white LED light. Emission spectrum is between 300nm and 1000nm.

The graphics compares the light spectrum of natural light with warm white LED light and red-blue LED light. The light spectra were determined using an ASD Handheld photo-radiometer

## 2.2 Inoculum Preparation:

A certain amount of algal culture of *B.braunii* race B (Guadeloupe) actively growing from a stock culture, was centrifuged to pellet symbiotic bacteria and dead algal cells, then sieved and washed with CHU medium. Washed algae were re-suspend in Chu medium and used to inoculate the bioreactor containing 2 l of sterilized fresh medium. The starting culture in the bioreactor had an optical density, measured at 680nm, approximately of 0.2.

## 2.3 Sample collection

The first sample was harvested immediately after the inoculation, then subsequent samples every 48 hours.

## 2.4 Absorbance of the algal cultures

The absorbance of 1 ml of algal culture was quantified at 680 nm ( $OD_{680}$ ) using a UV/Vis spectrophotometer (Thermo-Scientific, Genesys 10S UV-Vis).

## 2.5 Dry Biomass estimation

5 ml aliquots of *B. braunii* cultures were filtered onto pre-weighed 2.7  $\mu$ m GF/D glass-fibre membranes (Whatman). The filter was rinsed with 10 ml of deionized water, dried at 70°C for 24 h and biomass determined weighing the glass-fibre membrane again. The dry biomass comprised all insoluble material collected by the GF/D glass filter membrane which includes living, non living algae cells and terpenes. Terpenes can represent up to 86 % dry weight (Brown, 1969).

## 2.6 Chlorophyll content estimation

5 ml aliquots of *B. braunii* cultures were filtered onto 2.7  $\mu$ m GF/D glass-fibre membranes (Whatman). The filter was inserted in a glass vial and 10 ml of methanol was added. The GF/D filter was sonicated for 30 min and all the chlorophyll dissolved in the methanol. After sonication, the chlorophyll extract was centrifuged at 2,000xg for 30 min to pellet the cell debris and the remains of the glass-fibre membrane.

Chlorophyll absorbance was measured at 650 nm and at 665 nm using a UV/Vis spectrophotometer (Thermo-Scientific, Genesys 10S UV-Vis). The chlorophyll concentrations

were calculated according to the following formula (Hipkin and Baker, 1986): Total chlorophyll ( $\text{mg l}^{-1}$ ) =  $25.8 \cdot \text{Abs}_{650} + 4.0 \cdot \text{Abs}_{665}$

## 2.7 Terpene analysis

A glass vial was filled with 5 ml of *B. braunii* cultures and 5 ml of ethyl acetate (sigma), closed and shaken using a reciprocating shaker at  $250 \text{ strokes min}^{-1}$  for 30 min (Stuart reciprocating shaker SSL2). The mixture was centrifuged at 2000 g for 15 min to separate the aqueous phase from the organic phase. 1 ml of the oil phase was taken for terpene analysis. Terpene analysis was carried out using Gas Chromatography. The instrument used was an Agilent 7890 GC with a column, HP5 30 meter x 320micron x 0.25 microns. The carrier gas was helium set to a flow rate of 2.25mls per min (11.15psi). The oven was set up as follows: 65°C, hold for 0 minutes, increase temperature by 8°C per min to 320°C and hold for 7 minutes. The Injector was on-column with oven tracking and detector was flame ionization, temperature 345°C. The Injection volume was 1µl. Terpenes are quantified against external standards of squalene diluted in heptane.

## 2.8 Ion Analysis

5 ml of algal culture was filtered through a 2.7 µm GF/D glass-fibre membrane (Whatman). The medium that passed through the filter was used for cation and anion analysis. Before ion analysis, the sample was stored at -20 degree Celsius. Ion analysis was carried out using the ICS-3000 Reagent-Free™ Ion Chromatography (RFIC™) system, supplied from DIONEX.

## 2.9 Microscopy Analysis

2 ml of culture was taken for microscopy analysis in order to check contaminant level, lipid production and localization, colonies and cell shape. The instrument used for microscopy analysis was an Olympus BX51 System Microscope with attached DP71 digital CCD camera. The image processing and analysis software used was Cell F version 2.8 from Olympus. Microscopical techniques used are: Brightfield microscopy for algal cluster size analysis, Nomarski differential interference contrast (DIC) for morphological examination of algal clusters. (Produces distinctly shadowed relief images with a 3-D quality). Reflected fluorescence microscopy using Bodipy 505/515 for lipid localization.

## 2.10 Summary of Experiments

Five experiments (A-B-C-D-E), each one in triplicate, were performed to assess the behavior of *B.braunii* strain Guadeloupe in batch and in continuous mode (Table 2.1).

**Table 2.1** Summary table of all experiments performed

	BATCH MODE		CONTINUOUS MODE		
	Experiment A	Experiment B	Experiment C	Experiment D	Experiment E
Red blue light led	YES		YES		
Warm white light led		YES		YES	YES
Photoperiod 12h light : 12h dark	YES	YES	YES	YES	
Photoperiod 24h light : 0h dark					YES
Light intensity $221 \mu\text{E m}^{-2}\text{s}^{-1}$					YES
Light intensity $442 \mu\text{E m}^{-2}\text{s}^{-1}$	YES	YES	YES	YES	

Experiment A was performed in batch mode, red blue LED light with light emission intensity of  $442 \mu\text{E m}^{-2}\text{s}^{-1}$  in 12 hours light and 12 hours dark.

Experiment B was performed in batch mode, warm white LED light with light emission intensity of  $442 \mu\text{E m}^{-2}\text{s}^{-1}$  in 12 hours light and 12 hours dark.

Experiment C was performed in continuous mode, red blue LED light with light emission intensity of  $442 \mu\text{E m}^{-2}\text{s}^{-1}$  in 12 hours light and 12 hours dark.

Experiment D was performed in continuous mode, warm white LED light with light emission intensity of  $442 \mu\text{E m}^{-2}\text{s}^{-1}$  in 12 hours light and 12 hours dark.

Experiment E was performed in continuous mode, warm white LED light with light emission intensity of  $221 \mu\text{E m}^{-2}\text{s}^{-1}$  in 24 hours light and 0 hours dark

## CHAPTER 3 RESULTS

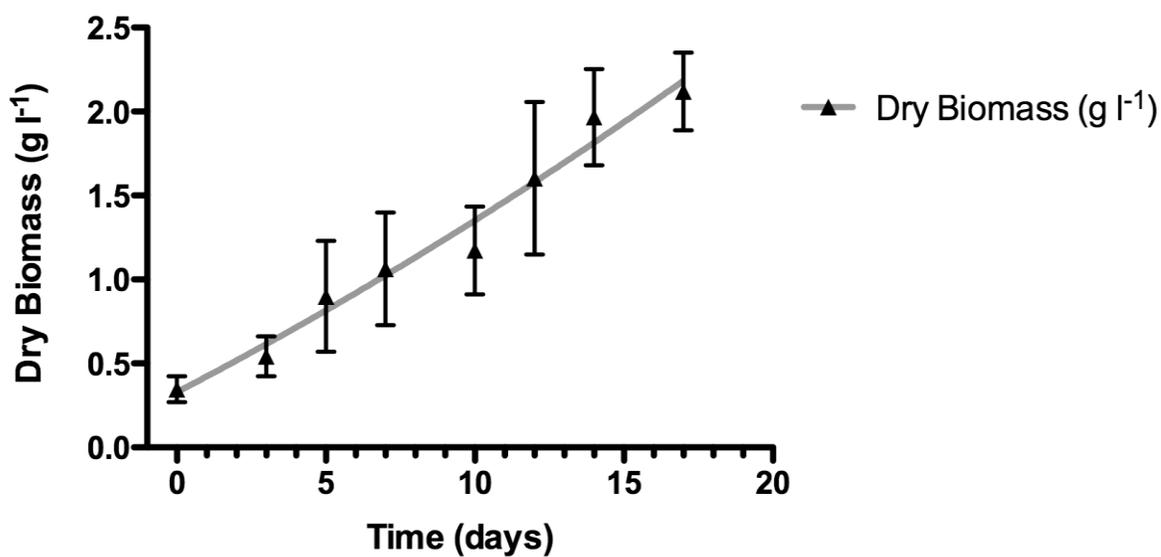
### 3.1 *Botryococcus braunii* Culture in Batch Mode

*B.braunii* was grown in batch mode under a photoperiod of 12 hours light and 12 hours dark (12L:12D) in two different light regimes; red blue LED light and warm white LED light. In both cases the light intensity was  $442 \mu\text{E m}^{-2} \text{s}^{-1}$ . The aim of the experiment was to determine growth and terpene production for *B.braunii* and analyse any observed differences between the two light regimes. Consequently dry biomass, optical density at 680nm, chlorophyll content and terpenes were measured and plotted against time expressed in days.

#### 3.1.1 *Botryococcus braunii* Batch Culture in Warm White LED Light

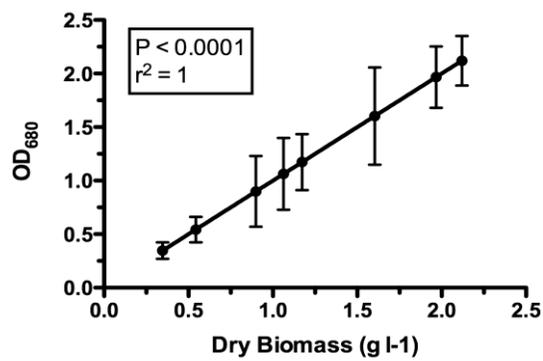
Measurements of dry biomass of *B.braunii* cultures in warm white LEDs light show that the microalgal culture grew with an average growth rate of  $0.034 \text{ day}^{-1}$  calculated between day 0 and day 17. Lag phase was not observed. Also, in 17 days the total dry biomass generated was, on average,  $1.78 \text{ g l}^{-1}$  and the total amount of terpenes calculated was, on average,  $0.61 \text{ g l}^{-1}$ . The average growth rate measured between day 14 and day 17 was  $0.046 \text{ day}^{-1}$ ; this means that the microalgal culture was still in linear growth phase at day 17 (Figure 3.1). This observation was confirmed from measurements of chlorophyll and optical density at 680nm ( $\text{OD}_{680}$ ) which both show a comparable increase between day 14 and day 17.

Dry biomass and  $\text{OD}_{680}$  of cultures grown in warm white LED light were plotted against each other and a positive linear correlation was observed between both types of measures (Figure 3.2a). Consequently dry biomass only was chosen to represent the growth of *B.braunii* as it facilitates future parameter analysis. The correlation between chlorophyll content and dry biomass and correlation between terpene content and dry biomass were also linear (Figure 3.2b and Figure 3.2c).

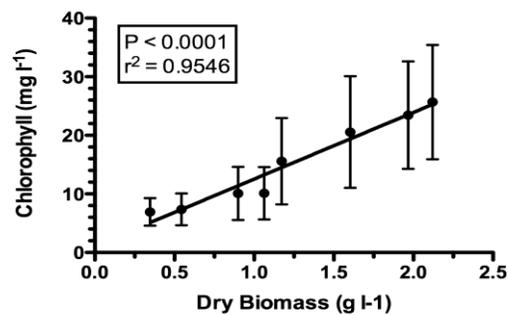


**Figure 3.1** *Botryococcus braunii* growth in warm white led light and batch mode  
*B.braunii* was grown over 17 days in warm white LED light with intensity of  $442 \mu\text{E m}^{-2} \text{s}^{-1}$  and a photoperiod of 12 hours light and 12 hours dark. Points represent the mean of three biological replicates with standard error bars shown. The curve represents the best fit of the data. The best fit curve was generated by Prism software.

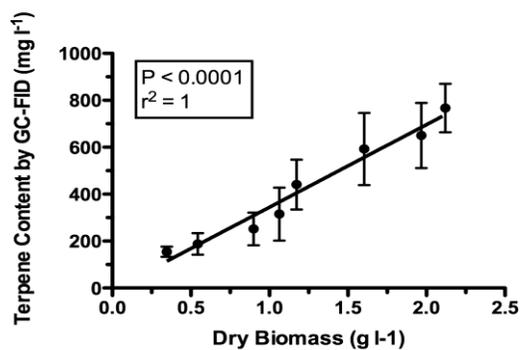
A



B



C



**Figure 3.2** Correlation between *Botryococcus braunii* growth parameters.

The correlation between dry biomass, OD<sub>680</sub> (A), chlorophyll content (B) and terpene content (C) and dry biomass is, in all cases, linear. *B.braunii* were cultured in warm white LED light (442  $\mu\text{E m}^{-2} \text{s}^{-1}$ ), in 12L:12D, in batch culture. Points represent the mean of 3 replicates ( $\pm$  standard error) and the curves represent the best fit of the data. The best fit curve was generated by Prism software.

### 3.1.2 *Botryococcus braunii* in red/blue LED light.

*B.braunii* in red/blue LED light was grown over 19 days and from optical density at 680nm and dry biomass measurements it was observed that the microalgal culture had a linear phase of 14 days (day 0 – day 14) with a growth rate of  $0.042 \text{ day}^{-1}$ .

The stationary phase was 3 days, from day 14 to day 17. In 17 days the total amount of dry biomass generated was, on average,  $1.36 \text{ g l}^{-1}$  (Figure 3.3a) and the total amount of terpene was, on average,  $220 \text{ mg l}^{-1}$  (Figure 3.3c); terpene concentration at day 0 was  $585.56 \text{ mg l}^{-1}$  and at day 17 terpene concentration was on average  $846.67 \text{ mg l}^{-1}$ . The difference between  $846.67 \text{ mg l}^{-1}$  and  $585.56 \text{ mg l}^{-1}$  gives  $261.11 \text{ mg l}^{-1}$ . After day 17 the growth rate of the *B.braunii* culture started to decrease.

Between day 0 and day 10 there was a linear and constant increase in chlorophyll content; the chlorophyll content rise stopped at day 10 whilst the dry biomass increased continuously till day 14. Between day 10 and day 14 the chlorophyll concentration was stable and after day 14 a sharply decrease of chlorophyll content was observed whilst the dry biomass was constant between day 14 and day 17. Chlorophyll content at day 14 was  $8.39 \text{ mg l}^{-1}$  and at day 19 (end of experiment)  $6.12 \text{ mg l}^{-1}$  (Figure 3.3b).

### 3.1.3 *Botryococcus braunii* in Batch Mode: Red/Blue LED Light vs Warm White LED Light.

It is interesting to highlight differences between the *B.braunii* culture grown in red / blue LED light and the *B.braunii* culture grown in warm white LED light in batch mode. *B.braunii* in warm white LED light had a linear phase that was longer than that of *B.braunii* culture in red blue LED light, with 17 days and 14 days respectively.

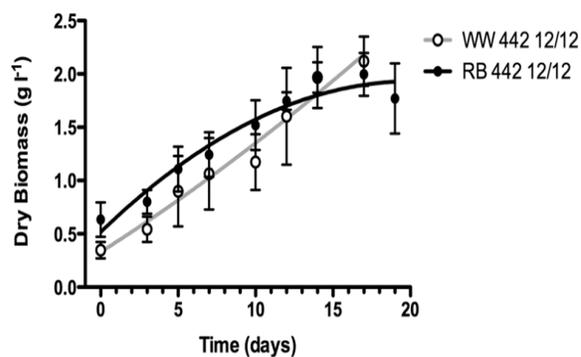
In red/blue LED light, *B.braunii* culture reached the stationary phase at day 14. No significant changes in terms of dry biomass were observed thereafter, just an average increase between day 14 and day 17 of  $0.02 \text{ g l}^{-1}$ . In warm white LED light the average dry biomass increase between day 14 and day 17 was  $0.15 \text{ g l}^{-1}$ ; *i.e.* the culture was still in linear phase after day 14, (figure 3.3a). The average growth rate ( $\mu$ ) calculated between day 0 and day 17 in red/blue LED light was  $0.041 \text{ day}^{-1}$  and in warm white LED light was  $0.034 \text{ day}^{-1}$ . The total biomass generated in 17 days in red/blue LED light was, on average,  $1.36 \text{ g l}^{-1}$  and in warm white LED light was, on average,  $1.78 \text{ g l}^{-1}$ .

The chlorophyll content of *B.braunii* cultured in warm white LED light increased continuously till day 17. Conversely in red/blue LED light the chlorophyll content increased till day 10 and at day 14 the chlorophyll decreased sharply till the end of the experiment. Furthermore in warm white LED light *B.braunii* produced more chlorophyll compared to algal cells in the red/blue LED light regime (figure 3.3b). The total amount of chlorophyll content generated in red/blue LED light regime during the exponential phase was  $4.37 \text{ mg l}^{-1}$  and chlorophyll generated in warm white LED light regime during the exponential phase was  $16.52 \text{ mg l}^{-1}$  (Figure 3.3b).

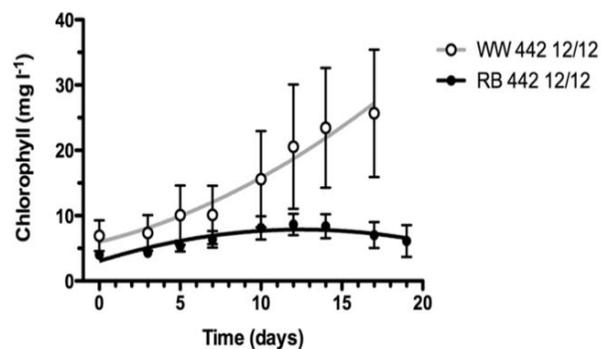
In 14 days *B.braunii* in red/blue LED light has generated an average amount of  $261 \text{ mg l}^{-1}$  of terpene and in warm white LED light the terpene production was, on average,  $496 \text{ mg l}^{-1}$  (Figure 3.3c).

Figure 3.3a displays a large discrepancy in the starting biomass, chlorophyll and terpene content at time zero between warm white LED light and red blue LED light. It depends on the different stock culture which was used to prepare the inoculum. In the warm white LED experiment, it was used an inoculum originated from a stock culture adapted to warm white LED conditions. In the red blue LED experiment an inoculum originated from a stock culture adapted to red blue LED conditions was used.

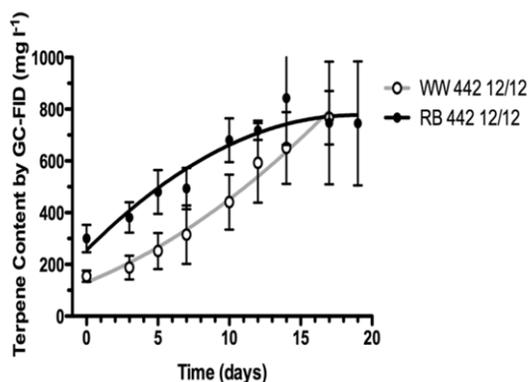
A



B



C



**Figure 3.3** *Botryococcus braunii* growth parameters

A comparison between *B.braunii* grown in warm white LED light ( $442 \mu\text{E m}^{-2} \text{s}^{-1}$ ), in 12L:12D, in batch culture and *B.braunii* grown in red/blue LED light ( $442 \mu\text{E m}^{-2} \text{s}^{-1}$ ), in 12L:12D in batch culture. (A) Dry biomass production in red blue LED light and in warm white LED light, (B) Chlorophyll content in red/blue LED light and in warm white LED light, (C) Terpene content in red/blue LED light and in warm white LED light. Points represent the mean of 3 replicates ( $\pm$  standard error) and the curves represent the best fit of the data. When error bars are not seen, they are masked by the symbol. The best fit curve was generated by Prism software.

The following table summarize the differences between *B.braunii* in red/blue LED light and in warm white light.

Yields During Linear Growth	Red/Blue LED	Warm White LED
Duration of Linear Phase	14 days	>17days
Dry Biomass (g l <sup>-1</sup> )*	1.36*	1.78*
$\mu$ (day <sup>-1</sup> )**	0.041**	0.034**
Total Chlorophyll Produced in linear phase (mg l <sup>-1</sup> )***	4.37***	16.52***
Total Terpene Produced in linear phase (mg l <sup>-1</sup> )****	261****	612****

**Table 3.1** Difference between *B.braunii* grew in batch mode in red blue LED light and warm white light

\*Average dry biomass calculated as difference between dry biomass at day 0 and at day 17 in warm white LED light and as difference between dry biomass at day 0 and at day 14 in red/ blue LED light.

\*\* Average growth rate ( $\mu$ ) calculated using the formula:  $\mu = (\ln X_t / X_0) / t - t_0$

\*\*\*In warm white LED light the average chlorophyll produced is calculated as difference between chlorophyll content at day 0 and at day 17. In red blue LED light the average chlorophyll produced is calculated as difference between chlorophyll content at day 0 and at day 14.

\*\*\*\*In warm white LED light the average terpene produced is calculated as difference between terpene content at day 0 and at day 17. In red blue LED light the average terpene produced is calculated as difference between terpene content at day 0 and at day 14; terpene concentration at day 0 was 585.56 mg l<sup>-1</sup> and at day 14 terpene concentration was on average 846.67 mg l<sup>-1</sup>. The difference between 846.67 mg l<sup>-1</sup> and 585.56 mg l<sup>-1</sup> gives 261.11mg l<sup>-1</sup>

### 3.2 Continuous Culture of *Botryococcus braunii* in Chemostat and Cyclostat Mode

Continuous culture of *B.braunii* in warm white LED light was grown first in continuous illumination (chemostat mode) and then under a photoperiod of 12 hours light and 12 hours dark (cyclostat mode).

#### 3.2.1 Continuous Culture of *Botryococcus braunii* in Chemostat Mode.

*B.braunii* was grown in continuous illumination in warm white LED light regime with light intensity of  $221 \mu\text{E m}^{-2}\text{s}^{-1}$ . The microalgal culture was started in batch mode and switched to chemostat mode after 7 days. The aim of the experiment was to determine if the microalgal culture could maintain a constant production of biomass and terpenes in chemostat conditions. Growth parameters and the terpene content were measured and plotted against the time expressed in days.

Analyses of growth parameters show that there was a linear increase of dry biomass, chlorophyll and terpenes between day 0 and day 7 that is consistent with the culture in batch mode. At day 7 the culture was switched to chemostat mode with a constant dilution rate of  $0.05 \text{ day}^{-1}$  and the dry biomass was  $1.46 \text{ g l}^{-1} \pm \text{SE } 0.37$ . An increase of dry biomass was observed from day 7 and day 14 (dry biomass =  $1.71 \text{ g l}^{-1} \pm \text{SE } 0.53$ ). Between day 14 and end of experiment (at day 18) a decrease of dry biomass was observed. Dry biomass at day 18 was  $1.56 \text{ g l}^{-1} \pm \text{SE } 0.44$ .

Chlorophyll content increased continuously from day 7 (chlorophyll content =  $8.37 \text{ mg l}^{-1} \pm \text{SE } 3.9$ ) till day 9 (chlorophyll content =  $9.6 \text{ mg l}^{-1} \pm \text{SE } 5$ ); after day 9 the chlorophyll content started to decrease sharply till day 18 (end of the experiment), chlorophyll content day 18 was  $4.8 \text{ mg l}^{-1} \pm \text{SE } 2.19$ .

Terpene content increased constantly from day 7 (terpene content =  $508 \text{ mg l}^{-1} \pm \text{SE } 145$ ) till day 11 (terpene content =  $648 \text{ mg l}^{-1} \pm \text{SE } 164$ ), then terpene content remained almost constant (no significant changes observed) till the end of the experiment; terpene content end of experiment was  $673 \text{ mg l}^{-1} \pm \text{SE } 180$ .

### 3.2.2 Continuous Culture of *Botryococcus braunii* in Cyclostat Mode

*B.braunii* was grown in a photoperiod of 12 hours light and 12 hours dark using warm white LED light as light source with intensity of  $442 \mu\text{E m}^{-2} \text{s}^{-1}$ . The aim of the experiments was to determinate if the microalgal culture can maintain a constant and continuous production of biomass and terpene in cyclostat mode. Consequently dry biomass, optical density at 680nm, chlorophyll content and terpene were measured and plotted against time expressed in days.

The microalgal culture was started in batch mode and then after 7 days in batch mode, the culture was switched to cyclostat mode, with a dilution rate  $D$  of  $0.0625 \text{ day}^{-1}$ ; the microalgal culture was fed only during the light phase. In batch mode (0 - 7 days) a linear increase of all growth parameters was observed; differences between growth parameters were observed after day 7, when the culture was switched to cyclostat mode.

An increase of dry biomass and terpene was observed between day 7 (dry biomass =  $1.38 \text{ g l}^{-1} \pm \text{SE } 0.32$ ; terpene content =  $440 \text{ mg l}^{-1} \pm \text{SE } 17$ ) and day 14 (Dry biomass =  $1.67 \text{ g l}^{-1} \pm \text{SE } 0.51$ ; terpene content =  $620 \text{ mg l}^{-1} \pm \text{SE } 198$ ).

After day 14, the dry biomass and terpene remained almost constant (with a tiny decrease) till the end of the experiment. Dry biomass at day 19 was  $1.62 \text{ g l}^{-1} \pm \text{SE } 0.48$ . Terpene concentration at day 19 was  $601 \text{ mg l}^{-1} \pm \text{SE } 315$ .

The chlorophyll content continuously increased from day 7 (chlorophyll content =  $5.8 \text{ mg l}^{-1} \pm \text{SE } 1.03$ ) to day 12 (chlorophyll content =  $7.94 \text{ mg l}^{-1} \pm \text{SE } 3.01$ ), then the chlorophyll content remained constant till day 14, but straight after the chlorophyll content started to decrease sharply till end day 19 (end of experiment); at day 19 chlorophyll content was  $5.18 \text{ mg l}^{-1} \pm \text{SE } 3.01$ .

### 3.2.3 Comparison between Continuous Culture of *Botryococcus braunii* in Chemostat and Cyclostat Modes.

The first difference that it was observed between the chemostat mode experiment and the cyclostat mode experiment was the growth rate between day 0 and day 7 (the time that cultures were in batch): in chemostat mode the growth rate was  $0.07 \text{ day}^{-1}$  and in cyclostat mode the growth rate was double ( $0.14 \text{ day}^{-1}$ ). That difference in growth rate is not related to the different initial dry biomass between the two experiments, but to the fact the *B.braunii* in a photoperiod 12L:12D shows often higher growth rate than in continuous illumination (Qin, 2005). Furthermore

several experimental results during this investigation work have shown that there is no correlation between growth rate and initial starting biomass.

Between day 7 and day 14 an increase of dry biomass were observed in both experiments even if between day 12 and 14 in cyclostat mode the increase of dry biomass was not significant (Figure 3.8); the growth rate calculated between day 7 and day 14 in chemostat mode was  $0.022 \text{ day}^{-1}$  and the growth rate calculated between day 7 and day 14 in chemostat mode was  $0.028 \text{ day}^{-1}$ .

A decrease in dry biomass was observed between day 14 and the end of the experiment (day 18 for chemostat mode and day 19 for cyclostat mode) both in chemostat mode and in cyclostat mode. The decrease of dry biomass in chemostat mode was, on average,  $0.15 \text{ g l}^{-1}$  and in cyclostat it was, on average,  $0.05 \text{ g l}^{-1}$ , Figure 3.4a.

Some differences were observed, between the chlorophyll content of *B.braunii* cultures in chemostat mode and *B.braunii* culture in cyclostat mode.

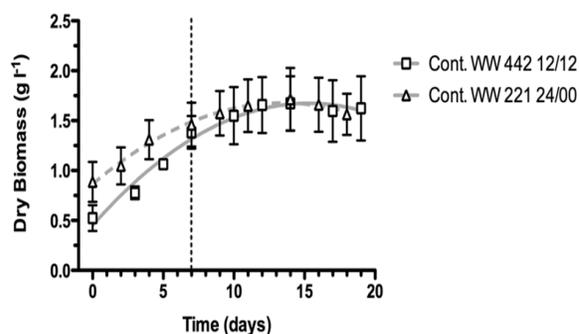
In chemostat mode *B.braunii* produces higher level of chlorophyll content than in cyclostat mode. However the chlorophyll content started to drop in chemostat mode at day 9 while in cyclostat mode the decrease started at day 14. Furthermore the decrease of chlorophyll was higher in chemostat mode than in cyclostat mode; chlorophyll content in chemostat mode at day 9 was  $= 9.6 \text{ mg l}^{-1} \pm \text{SE } 5$  and at end of experiment the chlorophyll content was  $4.8 \text{ mg l}^{-1} \pm \text{SE } 2.19$ . Chlorophyll content at day 14 in cyclostat mode was  $7.89 \text{ mg l}^{-1} \pm \text{SE } 3.64$  and chlorophyll content at end of experiment was  $5.18 \text{ mg l}^{-1} \pm \text{SE } 3.01$ , Figure 3.4b.

The amount of terpene produced either in chemostat mode or in cyclostat mode between day 7 and end of experiment was very similar; in chemostat mode the terpene quantity calculated in the bioreactor between day 7 and end of experiment (day 18), on average was  $165 \text{ mg l}^{-1}$ , and in cyclostat mode the terpene quantity calculated in the bioreactor between day 7 and end of experiment (day 19), on average was  $161 \text{ mg l}^{-1}$  (Figure 3.4c).

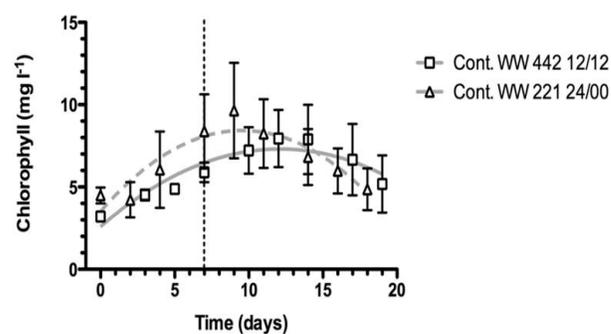
Figure 3.4a displays a discrepancy in the starting biomass, chlorophyll and terpene content at time zero between *B.braunii* grown in warm white LED light ( $442 \mu\text{Em}^{-2}\text{s}^{-1}$ ) 12L:12D (cyclostat mode) and *B.braunii* grown in warm white LED light ( $221 \mu\text{Em}^{-2}\text{s}^{-1}$ ), 24L:0D (chemostat mode). This difference in initial biomass is due to the different stock culture which was used to prepare the inoculum. In warm white LED light ( $442\mu\text{Em}^{-2}\text{s}^{-1}$ ) 12L:12D experiment an inoculum originated from a stock culture adapted to warm white LED and light intensity at  $442\mu\text{Em}^{-2}\text{s}^{-1}$  with photoperiod 12L:12D was used. In warm white LED light ( $221\mu\text{Em}^{-2}\text{s}^{-1}$ ) 24L:0D

experiment an inoculum originated from a stock culture adapted to warm white LED and light intensity at  $221\mu\text{Em}^{-2}\text{s}^{-1}$  with photoperiod 24L:0D was used.

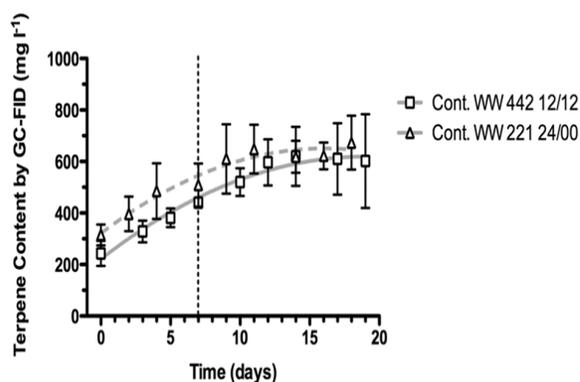
A



B



C



**Figure 3.4** *Botryococcus braunii* growth parameters

A comparison between *B. braunii* grown in warm white LED light (442  $\mu\text{E m}^{-2} \text{s}^{-1}$ ), in 12L:12D, in continuous culture (cyclostat mode) and *B. braunii* grown warm white LED light (221  $\mu\text{E m}^{-2} \text{s}^{-1}$ ), in 24L:0D, in continuous culture (chemostat mode). (A) Dry biomass production in cyclostat mode and chemostat mode, (B) Chlorophyll content in cyclostat mode and chemostat mode, (C) Terpene content in cyclostat mode and chemostat mode. Points represent the mean of 3 replicates ( $\pm$  standard error) and the curves represent the best fit of the data. When error bars are not seen, they are masked by the symbol. The best fit curve was generated by Prism software.

### 3.2.4 Comparison between Continuous Culture of *Botryococcus braunii* in Chemostat and Cyclostat Modes taking into account the biomass removed from the system.

An estimation of the biomass removed from the bioreactor (in chemostat and cyclostat mode) can be obtained from the constant dilution rate and biomass yield. The estimation of the biomass removed from the biomass was used to determine the total biomass production in chemostat mode and in cyclostat mode.

The total amount of biomass generated by bioreactor ( $B_T$ ) was calculated as follows:

$$B_T = (B_R + B_H) - B_I \quad (1)$$

Where,

$B_R$  is the biomass measured in the reactor at the end of experiment

$B_H$  is the biomass removed (estimated) from bioreactor in continuous culture

$B_I$  is the initial biomass measured at the beginning of the experiment.

The total biomass productivity  $B_P$  is calculated as follows:

$$B_P = B_T / \text{time (days)} \quad (2)$$

It is also possible to estimate the amount of chlorophyll and the amount of terpene removed from the bioreactor and consequently estimate the total chlorophyll production and the total terpene production, rearranging equation (1) and (2).

From calculations it was observed that *B.braunii* grown in cyclostat mode displayed a higher total biomass production, higher total chlorophyll content and higher total terpene production compared to *B.braunii* in chemostat mode (Table 3.2).

	<b>Cyclostat</b>	<b>Chemostat</b>
<b>Total Biomass production (g l<sup>-1</sup> day<sup>-1</sup>)</b>	0.193	0.145
<b>Total Chlorophyll production (mg l<sup>-1</sup> day<sup>-1</sup>)</b>	0.69	0.51
<b>Total Terpene production (mg l<sup>-1</sup> day<sup>-1</sup>)</b>	67.13	60.98

Table 3.2 Total biomass/chlorophyll / terpene production in *B.braunii* cyclostat mode and in *B.braunii* chemostat mode

Table shows the estimation of the total biomass production, total chlorophyll production, and total terpene production in cyclostat mode and in chemostat mode.

### 3.3 Continuous Culture of *Botryococcus braunii* in Cyclostat Mode and Red Blue LED Light

*B.braunii* was grown in a cyclostat under a photoperiod of 12 hours light and 12 hours dark, in red/ blue LED light. The aim of the experiments was to assess if the microalgal culture can maintain a constant and continuous production of biomass and terpene in cyclostat mode, using red blue LED light as light source instead of warm white LED light. Consequently dry biomass, optical density at 680nm, chlorophyll content and terpene were measured and plotted against time expressed in days.

The microalgal culture was started in batch mode and then after 7 days in batch mode, the culture was switched in cyclostat mode, with a dilution rate  $D$  of  $0.0625 \text{ day}^{-1}$ ; the microalgal culture was fed only during the light phase. In batch mode, a linear increase of all growth parameters was observed. Differences between growth parameters were observed after day 7, when the culture was switched to cyclostat mode, at day 7.

In red/blue LED light between day 7 and day 11 there was an increase in dry biomass, from  $1.3 \text{ g l}^{-1} \pm \text{SE } 0.07$  to  $1.46 \text{ g l}^{-1} \pm \text{SE } 0.43$ . Chlorophyll content increased as well after day 7; at day 7, chlorophyll content was  $6.26 \text{ mg l}^{-1} \pm \text{SE } 2.3$  and at day 11 it was  $7.42 \text{ mg l}^{-1} \pm \text{SE } 3.41$ . Between day 7 and day 11 an increase of terpene content was observed: from  $373 \text{ mg l}^{-1} \pm \text{SE } 90$  to  $585 \text{ mg l}^{-1} \pm \text{SE } 135$ .

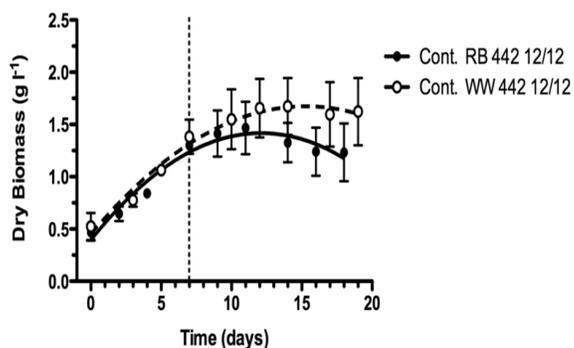
After day 11 the dry biomass started to decrease continuously till day 18 (end of the experiment). At day 18, dry biomass was  $1.23 \text{ g l}^{-1} \pm \text{SE } 0.47$ , Figure 3.5a. A constant decrease between day 11 and end of experiment it was observed also for chlorophyll and terpene; chlorophyll content at day 18 was  $5.51 \text{ mg l}^{-1} \pm \text{SE } 3.26$  and terpene content at day 18 was  $461 \text{ mg l}^{-1} \pm \text{SE } 114$ .

When comparing the continuous *B.braunii* culture in cyclostat mode and red blue LED light with the continuous *B.braunii* culture in cyclostat mode and warm white LED light two main differences were observed.

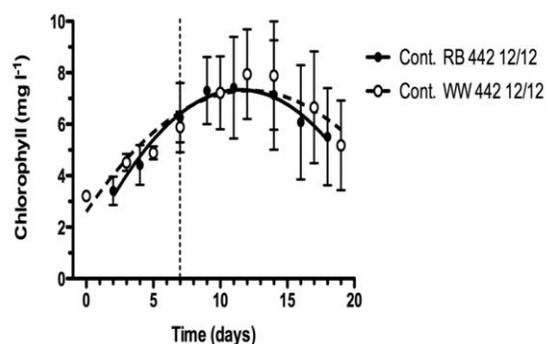
First, in warm white LED light, the dry biomass and terpene increased between day 7 and day 14, then remained almost constant from day 14 to the end of the experiment. Conversely, in red blue LED light the dry biomass and terpene increased between day 7 and day 11 then decreased from day 11 to the end of the experiment (day 18) (Figure 3.5a and Figure 3.5c).

The chlorophyll content of the *B.braunii* culture in cyclostat mode and in red blue LED light was very similar to the chlorophyll content of the *B.braunii* culture in cyclostat mode and in warm white LED light (Figure 3.5b).

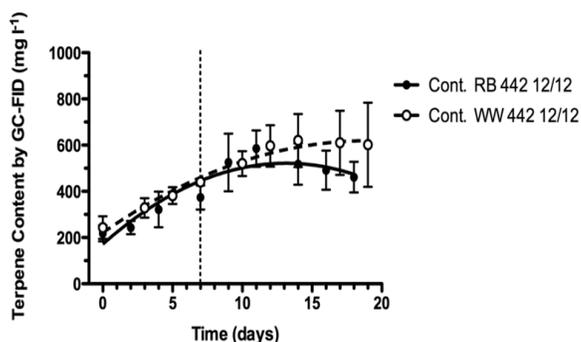
A



B



C



**Figure 3.5** *Botryococcus braunii* growth parameters in continuous culture (cyclostat mode).

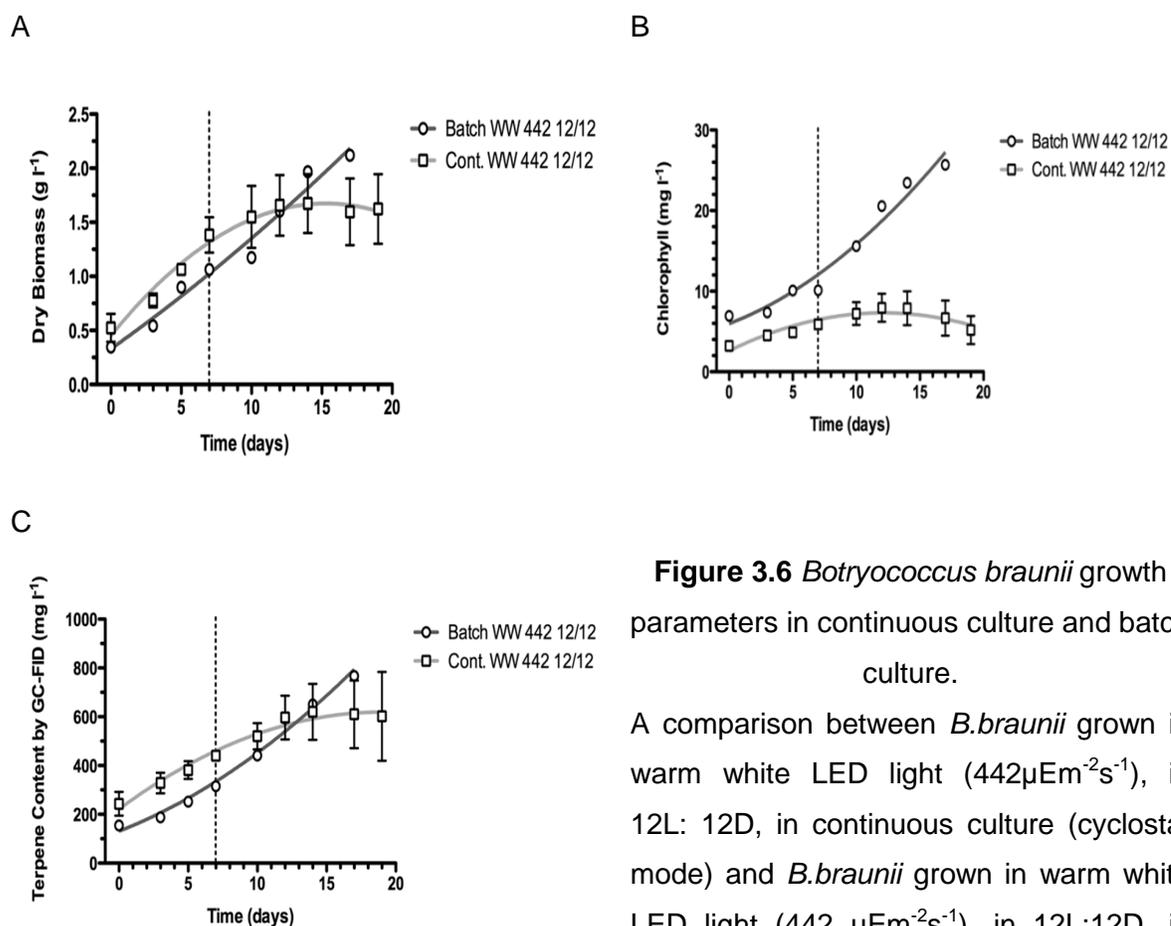
A comparison between *B. braunii* grown in warm white LED light ( $442 \mu\text{Em}^{-2}\text{s}^{-1}$ ), in 12L:12D, in continuous culture (cyclostat mode) and *B. braunii* grown red/blue LED light ( $442 \mu\text{Em}^{-2}\text{s}^{-1}$ ), in 12L:12D, in continuous culture (cyclostat mode). (A) Dry biomass production in red/blue LED light and warm white LED light, (B) Chlorophyll content in red/blue LED light and warm white LED light, (C) Terpene content in in red/blue LED light and warm white LED light. Points represent the mean of 3 replicates ( $\pm$  standard error) and the curves represent the best fit of the data. The best fit curve was generated by Prism software. When error bars are not seen, they are masked by the symbol.

### 3.4 *Botryococcus braunii* in Warm White LED Light: a Comparison between the Continuous culture (Cyclostat mode) with the Batch Culture

In terms of dry biomass production the *B.braunii* culture in batch mode was able to continuously produce biomass and terpenes with a linear increment from day 0 to day 17 (end of experiment) as discussed in 3.1.1. In particular from day 7 to day 17 the dry biomass increase, on average, by 100 %; when *B.braunii* was in cyclostat culture, the dry biomass increase, on average, by 17% from day 7 to day 17 (Figure 3.6a).

*B.braunii* in batch mode produced more chlorophyll than *B.braunii* in cyclostat mode. In batch mode the chlorophyll production had a linear rise till the end of experiment. The chlorophyll content of *B.braunii* in batch mode from day 7 to day 17 increased on average, by 150%. In comparison, in cyclostat mode the chlorophyll content is lower: the chlorophyll content of *B.braunii* in batch mode from day 7 to day 17 increased, on average, of 11% and between day 17 and day 19 (end of experiment) a decreased, on average, of 21% in chlorophyll content was observed (Figure 3.6b).

The terpene content in batch mode increased, on average, of 105% from day 7 to day 17; in cyclostat mode the terpene content increased, on average, of 37% from day 7 to day 17, (Figure 3.6c)



**Figure 3.6** *Botryococcus braunii* growth parameters in continuous culture and batch culture.

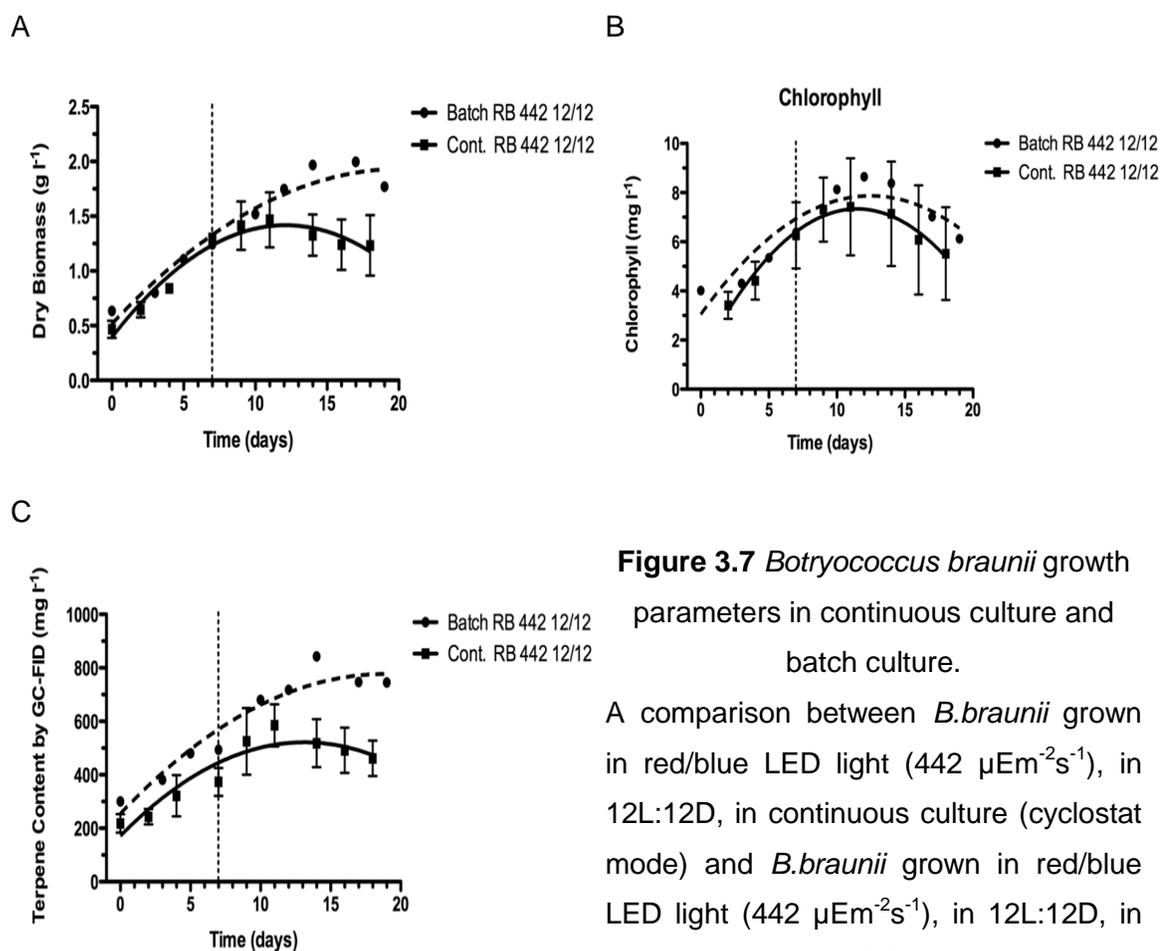
A comparison between *B. braunii* grown in warm white LED light ( $442\mu\text{Em}^{-2}\text{s}^{-1}$ ), in 12L: 12D, in continuous culture (cyclostat mode) and *B. braunii* grown in warm white LED light ( $442\mu\text{Em}^{-2}\text{s}^{-1}$ ), in 12L:12D, in batch culture. (A) Dry biomass production in batch culture and in continuous culture, (B) Chlorophyll content in batch culture and in continuous culture, (C) Terpene content in batch culture and in continuous culture. Points represent the mean of 3 replicates ( $\pm$  standard error) and the curves represent the best fit of the data. The best fit curve was generated by Prism software. When error bars are not seen, they are masked by the symbol. The dotted vertical line shows when the appropriate culture was switched in continuous culture.

### 3.5 *Botryococcus braunii* in Red Blue LED Light: Cyclostat Mode vs Batch Mode.

*B.braunii* in batch mode and red blue LED light produced more dry-biomass than *B.braunii* in cyclostat mode and red blue LED light, Figure 3.7a. There was a small difference in dry biomass production between *B.braunii* in batch mode and *B.braunii* in cyclostat mode; the increase of dry biomass between day 7 and day 19 in batch mode was, on average,  $0.54 \text{ g l}^{-1}$  and in cyclostat mode there was a decrease in dry biomass, on average, of  $0.07 \text{ g l}^{-1}$  between day 7 and day 18.

Significant differences were not observed in term of chlorophyll content between batch mode and cyclostat mode, Figure 3.7b. Both in batch mode or cyclostat mode, there was an increase of chlorophyll content from day 7 to day 11 in cyclostat mode and from day 7 to day 12 in batch mode; straight after a decrease of chlorophyll content was observed both in batch mode and in cyclostat mode. The chlorophyll content in batch mode decreased, on average, of  $0.26 \text{ mg l}^{-1}$  between day 7 and day 19 and in cyclostat mode the chlorophyll content decreased, on average of  $0.76 \text{ mg l}^{-1}$  between day 7 and day 18.

There was no difference in terpene production between batch mode and cyclostat mode; in batch mode the increase of terpene between day 7 and day 19 was, on average,  $94 \text{ mg l}^{-1}$  and in cyclostat mode the increase between day 7 and day 18 was, on average,  $88 \text{ mg l}^{-1}$  (Figure 3.7c).



**Figure 3.7** *Botryococcus braunii* growth parameters in continuous culture and batch culture.

A comparison between *B.braunii* grown in red/blue LED light ( $442 \mu\text{Em}^{-2}\text{s}^{-1}$ ), in 12L:12D, in continuous culture (cyclostat mode) and *B.braunii* grown in red/blue LED light ( $442 \mu\text{Em}^{-2}\text{s}^{-1}$ ), in 12L:12D, in batch culture. (A) Dry biomass production in batch culture and in continuous culture, (B) Chlorophyll content in batch culture and in continuous culture, (C) Terpene content in batch culture and in continuous culture. Points represent the mean of 3 replicates ( $\pm$  standard error) and the curves represent the best fit of the data. The best fit curve was generated by Prism software. The dotted vertical line shows when the appropriate culture was switched in continuous culture.

### 3.6 Ion Analysis

The amount of sodium, magnesium, calcium, sulphate (Figure 3.8a) and potassium (Figure 3.8b), remained almost constant, with no significant changes, from the beginning of each experiment to the end of each the experiment.

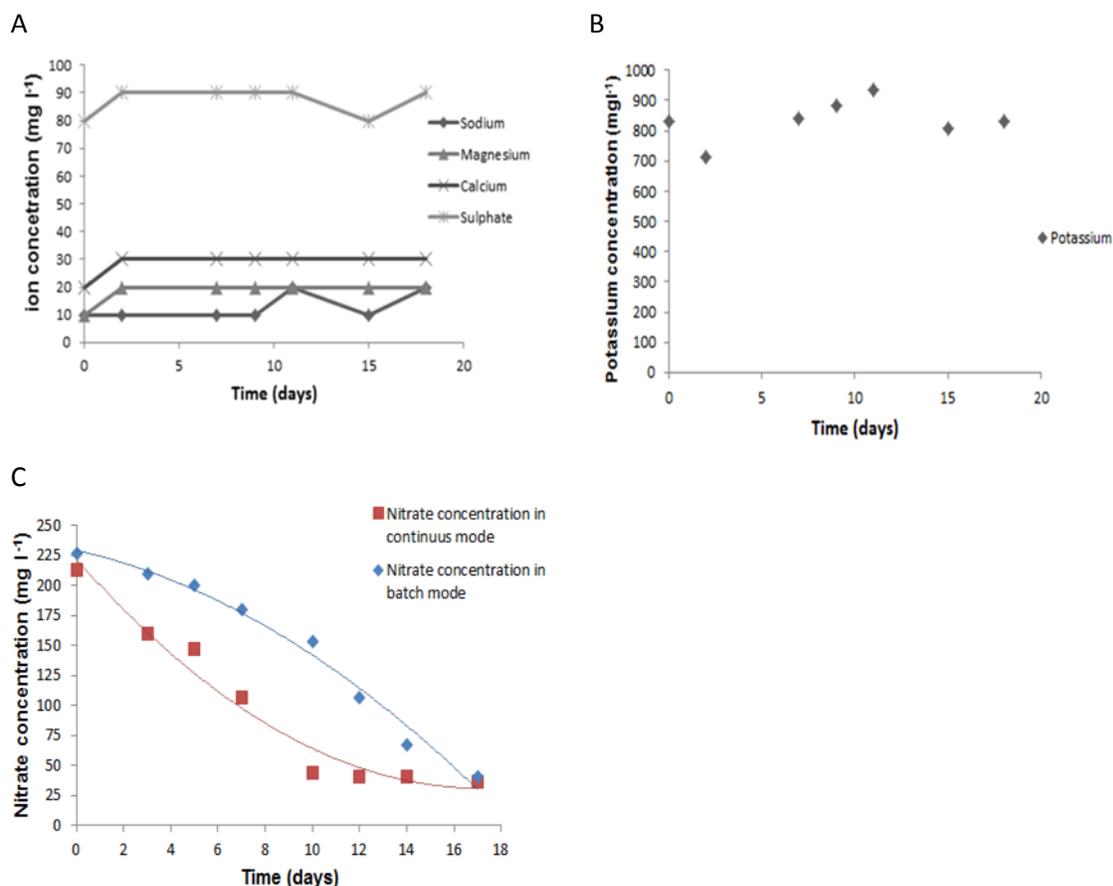
The amount of nitrate measured at the beginning of each experiment was different from the amount of nitrate measured at the end of each experiment.

The amount of nitrate measured at the beginning of each experiment was, on average, 200 mg l<sup>-1</sup>. From day 0 to day 7 a decrease of nitrate concentration, on average, by 50% was observed in each experiment in continuous culture. Between day 7 and day 10 the decrease of nitrate concentration was in each continuous culture, on average, 50%. After day 10 the amount of nitrate remained constant till the end of the experiment (day 18) (Figure 3.8c).

Probably the nitrates stabilised approximately at 50 mg l<sup>-1</sup> in the continuous experiments almost immediately upon switching to continuous mode because the nitrate reaches a balance between the nitrate which enters in the bioreactor, the nitrate which is adsorbed by the biomass and the nitrate which leaves the bioreactor.

In all batch cultures the nitrate concentration decrease from day 0 to day 7 was, on average, 20% and between day 7 and day 10 the decrease of nitrate concentration was, on average, 17%. It was observed a linear decrease of nitrate concentration from day 10 to day 17 (end of experiment) in each batch culture (Figure 3.8c).

The decrease of ion nitrate between day 7 and day 10 is higher in continuous culture than in batch culture due to the dilution effect which removes ion nitrate from culture medium.



**Figure 3.8** Ion concentrations in *B. braunii* culture medium

(A) Concentration of sodium, magnesium, calcium, sulphate, remained constant (with no significant changes) during the experiments. The graphic is representative of all experiments performed.

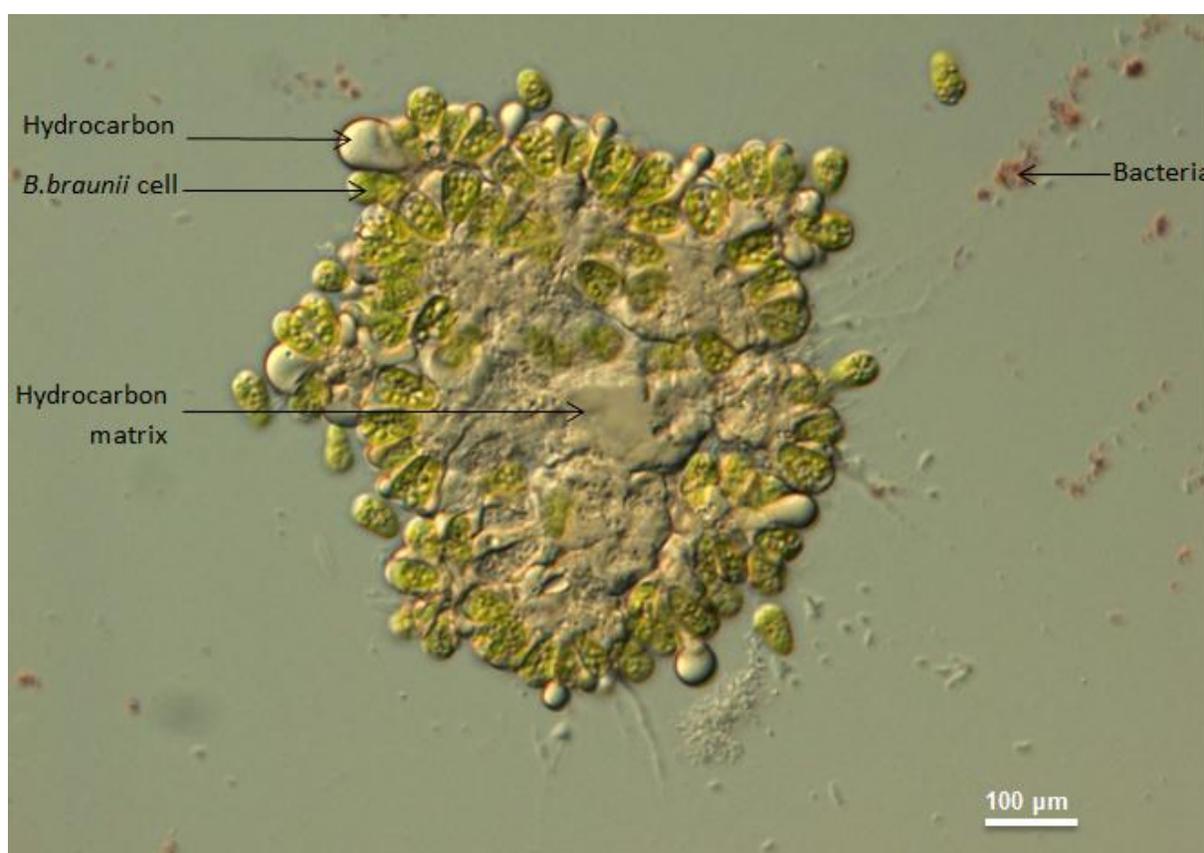
(B) Potassium concentration was variable during the experiment, but it remained in the range 700 mg l<sup>-1</sup> - 900 mg l<sup>-1</sup>. The graphic is representative of all experiments performed.

(C) Nitrate concentration in continuous culture decreased sharply from day 7 to day 10. After day 10, ion nitrate remained constant (with not significant changes). Nitrate concentration in batch mode started to decrease at the end of linear phase and beginning of stationary phase. The graphic is representative of all experiments performed both in batch culture and continuous culture.

Points represent the mean of 3 replicates ( $\pm$  standard error) and the curves in (C) represent the best fit of the data. When error bars are not seen, they are masked by the symbol.

### 3.7 Microscopy Analysis

From microscopy analysis, it was observed a medium contaminant level, (presence of bacteria colonies in the culture medium) lipid external localization and pyriform-shaped cells held together by a thick hydrocarbon matrix, Figure 3.9. Microscopy results were similar, with no significant differences, for each experiment.



**Figure 3.9** Image of 7 day old mature of *Botryococcus braunii* strain Guadeloupe. Pyriform-shaped cells are held together by a thick hydrocarbon matrix. Colonies of bacteria are visible. Images were acquired using an Olympus BX51 System Microscope with an attached DP71 digital CCD camera. Image processing and analysis software used was Cell F version 2.8 from Olympus.

## CHAPTER 4

### DISCUSSION

Microalgae have a clear potential to be used as a source for the production of renewable energy. In order to utilize microalgae for this purpose, some cultivation methods are investigated. In particular the microalga *Botryococcus braunii* is well known for its ability to produce hydrocarbons which have been loosely described as equivalent to the “gas-oil fraction of crude oil” (Hillen *et al.*, 1982).

In this investigation work, studies were performed in order to analyse how *B.braunii* responds to different light conditions in batch culture and how the microalga responds in continuous culture both chemostat mode and cyclostat mode.

The aim of this research work was to study in small scale the biomass and terpenes productivity of *B.braunii* in continuous culture, but in order to design the experiment in continuous culture it was important to investigate growth parameters of *B.braunii* in batch culture in different light conditions. Consequently *B.braunii* was grown in batch culture first in red/blue LED light and then in warm white LED light.

Results in batch culture show that *B.braunii* in red/blue LED light produced less terpenes and chlorophyll than *B.braunii* in warm white LED light. In addition *B.braunii* in red/blue LED light had a shorter life cycle than *B.braunii* in warm white LED light. However significant differences were not observed in biomass production between *B.braunii* in red/blue LED light and *B.braunii* in warm white LED light. Similar results were found in the green microalga *Scenedesmus* sp. LX1; in fact this alga illuminated with red / blue LED light produced less lipids than when illuminated with warm white LED light (Li X *et al.* 2010).

In batch culture, the light colour (red/blue LED light or warm white LED light) has an effect on lipid productivity; stimulating all photosynthetic pigments (with warm white LED light) can lead to a more efficient metabolism (e.g. more lipids production). Also the light colour effect the life cycle length of *B.braunii*, the chlorophyll content and a direct relationship between dry biomass and terpene productivity was observed. Furthermore, results can confirm previous studies that *B.braunii* is a slow growing microalga (Sheehan *et al.*, 1998) with average growth rate of  $0.041\text{day}^{-1}$  in red /blue LED light and average growth rate of  $0.034\text{ day}^{-1}$  in warm white LED light, both values calculate during the linear phase as neither a lag phase nor an exponential phase were detected. Lack of lag phase was also found in previous studies when *B.braunii* was grown in batch culture under air-lift condition (Casadevall *et al*, 1984).

By definition a continuous culture is in steady state growth (balanced growth) when at least three total volume changes have been performed; for example two litres of culture is in balanced growth if at least six litres of fresh medium has passed through the bioreactor's vessel. The fact that *B.brauni* has a slow growth rate means that for running just one experiment in continuous culture can take up to 60 days which is the time needed to 2 litres of *B.braunii* culture for being in steady state growth using for example a dilution rate of  $0.05 \text{ day}^{-1}$ . Consequently, for collecting enough results in different experimental conditions in a reasonable time, it was decided to grow *Botryococcus braunii* in continuous culture for 10 days.

Our results of *B.braunii* continuous culture in chemostat mode in warm white LED light shown that the cultures during the time in continuous can still produce dry-biomass and terpenes despite continuous dilution and continuous harvest; this is exactly what expected. Conversely the chlorophyll in the culture content constantly decreased during the time in continuous mode. This was an unexpected result as the chlorophyll content in warm white LED light should be proportional to dry biomass like shown in our experiment in batch mode.

*B.braunii* culture in warm white LED was switched to cyclostat mode on day 7 and between day 7 and day 12 there was increase of chlorophyll content and dry biomass with growth rate ( $\mu$ ) of  $0.036 \text{ day}^{-1}$ . This means that the dilution rate (D) applied was low and the culture continued to grow in linear phase. However, by definition, the steady state growth can be achieved either starting from an initially low D value or a high D value. After day 12 the *B.braunii* culture reached a status similar to a balance growth; in fact no significant changes of dry biomass and terpenes concentration were observed. Conversely, the chlorophyll content started to decrease constantly from day 14 till end of experiment (day19) even if dry biomass and terpenes were almost constant.

Although there was no significant difference in dry biomass production between chemostat mode and cyclostat mode, a higher decrease in dry biomass was observed in chemostat mode. Also algal cells in 24 h light daily (chemostat mode) shown brown colour at the end of cultivation compared with 12 h light (cyclostat mode). Therefore, it seems that the 12 h light and 12 h dark cycle is a better light regime for *B.brauni* continuous culture. This observation was confirmed from previous studies on *B.brauni* in batch culture (Qin, 2005). Conversely, marine microalgae like *Chlorella* and *Nannochloropsis* (grown in continuous culture system using tubular photobioreactor, at the same light intensity used in our work), shown that biomass productivity is higher when using a 24 h photoperiod compared to a 12:12 h photoperiod (James *et al.*, 1990).

In chemostat mode a D of 0.05 (= medium flow rate of 100ml per day) per day was used. In cyclostat mode a D of 0.0625 (= medium flow rate of 125ml per day) was used. In terms of medium flow rate the difference between chemostat and cyclostat is less than 10 % of the total culture volume. Consequently using a different D for both systems does not interfere on biomass productivity. More importantly, light is the most significant factor affecting biomass productivity, as shown in previous studies (Qin, 2005).

In cyclostat mode, it is very important to choose a dilution rate which has to be the closest to the period –average growth rate  $\langle \mu \rangle$ ; experimental results in the laboratory showed that the best dilution rate (D) for the cyclostat was 0.0625. The best dilution rate maintains the culture in steady state (balanced growth). For the chemostat the best dilution rate was 0.05.

Chemostat and Cyclostat are two different ways of growing *B.braunii* in continuous culture. In chemostat the growth rate is in each instant equal to the culture dilution rate (D), in cyclostat mode only the period –average growth rate  $\langle \mu \rangle$  is equal to D. Consequently, even if two identical dilution rates (D) were chosen for both experiments (cyclostat and chemostat), a dilution rate can never be the same for cyclostat and chemostat

Our results of *B.braunii* in continuous culture and warm white LED light confirmed previous studies which demonstrated that photosynthesis of algae is time-dependent (De Roos and Flik, 1985). A common result was observed between the *B.braunii* continuous culture in warm white LED light in chemostat mode and the *B.braunii* continuous culture in cyclostat mode in warm white LED light. After few days that the continuous culture started, a constant decrease of chlorophyll content was observed; this observation leads to observe a relationship between the dilution effect and the chlorophyll content in *B.braunii* continuous culture. In fact in *B.braunii* batch culture and warm white LED light (absence of dilution effect) a decrease of chlorophyll content was not observed. The dilution effect, in continuous culture, leads also to a higher decrease in nitrate concentration in the culture medium in compare to the culture medium in batch culture. In continuous culture, decrease in nitrate concentration, in the cell-free culture broth, also depends on low dilution rates or high irradiance (both conditions were present in our experimental condition) on the bioreactor surface as shown in studies on *Haematococcus pluvialis* (M.C. Garcia-Malea *et al.*, 2006). To a decrease of nitrate concentration follows a decrease of chlorophyll content (Richardson *et al.*, 1969). Other studies have shown that in *B.braunii* batch culture there is a relationship between lipid accumulations and decrease of chlorophyll (Healey, 1973; Fogg, 1965; Richardson *et al.*, 1969).

However future work is needed in order to investigate the reason of chlorophyll content decrease in continuous culture of *B.braunii*, i.e. investigate if the continuous culture is a stress factor for *B.braunii*, or keeping *B.braunii* longer in continuous culture in order to determine if the decrease of chlorophyll content was just a temporary status or adding extra amount of nitrate to the culture medium in order to observe if there is any increase in chlorophyll content. Considering that light intensity is a factor that affect *B.braunii* growth (Qin, 2005), further studies in continuous culture are needed in different light intensity.

Differently the *B.braunii* continuous culture in red / blue LED light and cyclostat mode after an initial increase of all growth parameters between day 7 and day 11 due to low dilution rate, a constant decrease of all growth parameter (terpenes concentration included) was observed from day 11 to day 18 (end of experiment). This observation and all our results lead to two considerations. The first one is that a decrease of chlorophyll content is a common factor in red/blue LED light both in continuous culture and a batch culture; consequently our results shows that the light colour, in photobioreactor, has an effect on chlorophyll content in *B.braunii* independently from which culture methods used, batch or continuous. Second, the *B.braunii* culture in warm white LED light has a more efficient metabolism (e.g. more terpene and dry biomass production) than *B.braunii* culture in red/blue LED light independently from the culture condition used (batch or continuous). Consequently it is better performed future studies on continuous culture of *B.braunii* using warm white light as only light source.

As observed above *B.braunii* grew better in both continuous culture or in batch culture under warm white LED light compared to red-blue LED light. This observation may be attributed to the fact that warm white LEDs generate a broader illumination spectrum than the combination of Red and Blue LEDs (Figure 2.4). Consequently, algal grown in warm white LEDs can harvest a greater amount of energy through chlorophyll and all accessory pigments (e.g. phycocyanin, xanthophyll and carotenes); accessory pigments boost energy received by photosynthesis. In red-blue LED light only the chlorophyll is involved in the photosynthetic process (McCree and Keith J, 1981).

Studies has shown that the continuous culture in turbidostat mode (in which medium input and harvest are controlled by dilution of the culture to a constant optical density), is particularly useful for slow growing algae (Lee and Ding, 1994); considering that *B.braunii* is a slow growing alga, future works in using a turbidostat mode instead of chemostat mode or cyclostat mode (where the max specific growth rate is variable due to light / dark cycle) should be taken in consideration.

## CHAPTER 5

### CONCLUSION

Continuous culture of *B.braunii* in a stirred tank photobioreactor under a light/dark cycle 12L:12D and in warm white LED was the best way for obtaining a constant production of dry biomass and hydrocarbons in compare to continuous culture of *B.braunii* under continuous illumination and in warm white LED and in a stirred tank photobioreactor.

It is also possible to grow *B.braunii* in continuous culture in red/blue LED light and in a light/dark cycle 12L:12D, but dry biomass and hydrocarbons production did not remain constant during the experiment. When operating in continuous culture, determining the right dilution rate (D) is extremely important to increase yields of dry biomass and hydrocarbons in *B.braunii*.

Consequently the microalga *B.braunii* could be able to grow in continuous culture and in open ponds for biofuel production on commercial scale.

## REFERENCES

AIBA SHUICHI and OGAWA TAKAHIRA (1977). Assessment of Growth Yield of a Blue-green Alga, *Spivulina platensis*, in Axenic and Continuous Culture, Journal of General Microbiology, 102, 179-182

ALONSOA DIEGO LOÂ PEZ, BELARBIB EL-HASSAN, FERNANDEZ-SEVILLAB JOSÊ M., RODRIÂGUEZ-RUIZA JUAN, EMILIO MOLINA GRIMA (2000). Acyl lipid composition variation related to culture age and nitrogen concentration in continuous culture of the microalga *Phaeodactylum tricornutum*, Phytochemistry 54

BOROWITZKA MICHAEL A. (1999) Commercial production of microalgae" ponds, tanks and fermenters tubes, Journal of Biotechnology 70 313-321.

BROWN CHRISTOPHER S., SCHUERGER ANDREW C., SAGER JOHN C. (1995) Growth and Photomorphogenesis of Pepper Plants under Red Light-emitting Diodes with Supplemental Blue or Far-red Lighting. J. AMER. SOC. HORT. SCI. 120(5):808-813

BROWN A.C., KNIGHTS B.A., CONWAY E., MIDDLEDITCH B.S., (1970) Hydrocarbons from the green form of the freshwater alga *Botryococcus braunii* Phytochemistry, Volume 9, Issue 6, Pages 1317-1324

BROWN A.C, KNIGHTS B.A., CONWAY E., (1969) Hydrocarbon content and its relationship to physiological state in the green alga *Botryococcus braunii*. Phytochemistry, Volume 8, Issue 3, March 1969, Pages 543-547

CARNACHO FERNANDO, MOLINA EMILIO, MARTINEZA MA. EUGENIA, SANCHEZC SEBASTIAN AND GARCIA FRANCISCO (1990) Continuous culture of the marine microalga *Tetraselm is sp.* - productivity analysis, Aquaculture, 90 75-84.

CASADEVALL E., DIF D., LARGEAU C., GUDIN C., CHAUMONT D. and DE SANTIT O. (1985). Studies on Batch and Continuous Cultures of *Botryococcus braunii*: Hydrocarbon Production in Relation to Physiological State, Cell Ultrastructure, and Phosphate Nutrition. *Biotechnology and Bioengineering*, Vol. XXVII, Pp. 286-295

CEPAK, V. & LUKAVSKY, J. (1994a) The effect of high irradiances on growth, biosynthetic activities and the ultrastructure of the green alga *Botryococcus braunii* strain Droop 1950/807-1. *Arch Hydrobiol Suppl*, 101, 1-17.

CEPAK, V. & LUKAVSKY, J. (1994b) The Effect of High Irradiances on Growth, Biosynthetic Activities and the Ultrastructure of the Green-Alga *Botryococcus-Braunii* Strain Droop 1950/807-1. *Archiv Fur Hydrobiologie*, 115-131.

CHIRAC, C., CASADEVALL, E., LARGEAU, C. & METZGER, P. (1985). Bacterial Influence upon Growth and Hydrocarbon Production of the Green-Alga *Botryococcus-Braunii*. *Journal of Phycology*, 21, 380-387.

CHISTI YUSUF (2007) Biodiesel from microalgae beats Bioethanol, *Trends in Biotechnology* Vol.26 No.3.

COHEN ZVI (1999) *Chemicals from Microalgae*. Taylor and Francis Ltd,

CUNNINGHAM A. and MAAS P. (1978), Time Lag and Nutrient Storage Effects in the Transient Growth Response of *Chlamydomonas reinhardtii* in Nitrogen-limited Batch and Continuous Culture. *Journal of General Microbiology* 104, 227-231.

DAYANANDA C., SARADA R., BHATTACHARYA SILA, RAVISHANKAR G.A. (2005) Effect of media and culture conditions on growth and hydrocarbon production by *Botryococcus braunii*. *Process Biochemistry* 40 3125–3131.

DE ROOS A.M. AND FLIK B.J.G., (1985) Modelling time-series of photosynthesis and comparisons with the fluorescence yield in *Chlorella vulgaris*: a study of adaptation, inhibition and recovery. J. Plankton Res. 7 (5): 665-677.

DI TERMINI ILARIA, PRASSONE ANNALISA, CATTANEO CLAUDIA, ROVATTI MAURO (2011), On the nitrogen and phosphorus removal in algal photobioreactor Ecological Engineering 37 976–980.

EROGLU ELA, MELIS ANASTASIOS (2010) Extracellular terpenoid hydrocarbon extraction and quantitation from the green microalgae *Botryococcus braunii* var. *Showa*. Bioresource Technology 101 2359–2366.

ETHIER S., WOISARD K. , VAUGHAN D. , WEN Z., (2011) Continuous culture of the microalgae *Schizochytrium limacinum* on biodiesel-derived crude glycerol for producing docosahexaenoic acid, Bioresource Technology 102 88–93.

FAY P. and KULASOORIYA S. A. (1973), A Simple Apparatus for the Continuous Culture of Photosynthetic Micro-Organisms. Br. Phycol. J. 8:51-57,

FOGG, G. E. (1975), Biochemical pathways in unicellular plants. In Photosynthesis and productivity in different environments, International Biological Programme Vol. 3, Cambridge University Press, pp. 437-457

FRISCH H.L. AND GOTHAM I. J. (1979) . A Simple Model for Periodic Cyclostat Growth of Algae. I. Math. Biology 7, 149-169

GARCIA-MALEA M.C., ACIEN F.G., FERNANDEZ J.M., CERON M.C., MOLINA E., (2006) Continuous production of green cells of *Haematococcus pluvialis*: Modeling of the irradiance effect Enzyme and Microbial Technology 38 981–989.

GOTHAM IVAN J. AND RHEE G-YULL (1982), Effects of Nitrate and Phosphate Limitation on Cyclostat Growth of Two Freshwater Diatoms. Journal of General Microbiology 128, 199-205.

HEALEY F. P., (1973), Inorganic Nutrient Uptake and Deficiency in Algae Critical Reviews in Microbiology, Vol. 3, No. 1: Pages 69-113

HEIJNEN J.J., TERWISSCHA VAN SCHELTINGA A.H. AND STRAATHOF A.J. (1992), Fundamental bottlenecks in the application of continuous bioprocesses. Journal of Biotechnology, 22 3-20.

HERBERT D., ELSWORTH R. and TELLING R. C. (1956), The Continuous Culture of Bacteria; a Theoretical and Experimental Study J. gen. Microbiol. 14, 601-622

HILLEN, L. W., POLLARD, G., WAKE, L. V. AND WHITE, N. (1982), Hydrocracking of the oils of *Botryococcus braunii* to transport fuels. Biotechnol. Bioeng., 24: 193–205 doi: 10.1002/bit.260240116

JAMES CHARLES M. and AL-KHARS A.M. (1990), An Intensive Continuous Culture System Using Tubular Photobioreactors for Producing Microalgae, Aquaculture, 87 381-393 381.

KOJIMA EIICHI and ZHANG KAI (1999). Growth and Hydrocarbon Production of Microalga *Botryococcus braunii* in Bubble Column Photobioreactors. Journal of bioscience and bioengineering vol. 87, no. 6, 811-815.

LEE CHOUL-GYUN AND PALSSON BERNHARD (1994). High-Density Algal Photobioreactors Using Light-Emitting Diodes Biotechnology and Bioengineering, Vol. 44, Pp. 1161-1167

LEE S.J., KIM S.-B., KIM J.-E., KWON G.-S., YOON B.-D. (1998), Effects of harvesting method and growth stage on the flocculation of the green alga *Botryococcus braunii*. Letters in Applied Microbiology 27, 14–18

LEE, Y.-K. and DING, S.-Y. (1994), Cell Cycle and Accumulation Of Astaxanthin In *Haematococcus Lacustris* (Chlorophyta). Journal of Phycology, 30: 445–449. doi: 10.1111/j.0022-3646.1994.00445.x

LIX, HUYI, YANG J (2010) , Effect of LED's red/blue light on the growth characteristic and lipid production of *Scenedesmus* sp. LX1. Huan Jing Ke Xue Feb; 31 (2):513-9.

MASSA GIOIA D., KIM HYEON-HYE and WHEELER RAYMOND M., MITCHELL CARY A. (2008). Plant Productivity in Response to LED Lighting. Hortscience Vol. 43(7)

METZGER P., LARGEAU C. (2005). *Botryococcus braunii*: a rich source for hydrocarbons and related ether lipids. Appl Microbiol Biotechnol

MIRÓN ASTERIO S., CARMEN M. GARCIA C., CONTRERAS GÓMEZA A., GARCIA CAMACHO F., MOLINA GRIMA E. , CHISTI Y. (2003). Shear stress tolerance and biochemical characterization of *Phaeodactylum tricornutum* in quasi steady-state continuous culture in outdoor photobioreactors. Biochemical Engineering Journal 16 287–297.

MORENO JOSE', ANGELES VARGAS M., OLIVARES HECTOR, RIVAS JOAQUIN GUERRERO MIGUEL G. (1998), Exopolysaccharide production by the cyanobacterium *Anabaena* sp. ATCC 33047 in batch and continuous culture, Journal of Biotechnology 60 175–182.

MOLDOWAN, J. M. & SEIFERT, W. K. (1980) First discovery of botryococcene in petroleum. Journal of Chemical Society Chemical Communications, 19, 912-914.

QIN JIAN, (2005), Bio - Hydrocarbons from Algae: impacts of temperature, light and salinity on algal growth. A report for the Rural Industries Research and Development Corporation. Publication No. 05/025

REBOLLOSO FUENTES M.M., GARCIA SANCHEZ J.L., FERNANDEZ SEVILLA , ACIEN FERNANDEZ F.G., SANCHEZ PEREZ J.A., E. MOLINA GRIMA (1999), Outdoor continuous culture of *Porphyridium cruentum* in a tubular photobioreactor: quantitative analysis of the daily cyclic variation of culture parameters. Journal of Biotechnology 70 271–288.

RICHARDSON B., ORCUTIT D. M., SCHWERTNER H. A., MARTINEZ CARA L., WICKLINE HAZEL E. (1969), Effects of Nitrogen Limitation on the Growth and Composition of Unicellular Algae in Continuous Culture, Applied Microbiology, P. 245-250 Vol. 18, No. 2.

SAWAYAMA S., INOUE S., YOKOYAMA S. (1994). Continuous culture of hydrocarbon-rich microalga *Botryococcus braunii* in secondarily treated sewage. Appl. Microbial Biotechnology

SHEEHAN, J., CAMOBRECO V. , DUFFIELD J. , GRABOSKI M., SHAPOURI H., (1998) Life Cycle Inventory of Biodiesel and Petroleum Diesel for Use in an Urban Bus U.S. government printing office: 1998-675-299.

VAN GULIK W.M., TEN HOOPEN H.J.G., HEIJNEN J.J. (2001), The application of continuous culture for plant cell suspensions. Enzyme and Microbial Technology 28 796–805.

WATSON T. G., (1969), Steady State Operation of a Continuous Culture at Maximum Growth Rate by Control of Carbon Dioxide Production. J. gen. Microbiol. 59, 83-89.

WHITBY G. E. and MURRAY R. G. E. (1980), Defined Medium for *Aquaspirillum serpens* VHL Effective in Batch and Continuous Culture. Applied and Environmental Microbiology, P. 20-24 Vol. 39, No. 1.

WILSON G. (1980), Continuous Culture of Plant Cells Using the Chemostat Principle. Advances in Biochemical Engineering Volume 16, pp 1-25

WOLF FRED R. (1983) *Botryococcus braunii*: An Unusual Hydrocarbon-Producing Alga. Applied Biochemistry and Biotechnology 8, 249-260

YANG SULING, WANG JUN, CONG WEI, CAI ZHAOLING and OUYANG FAN (2004). Utilization of nitrite as a nitrogen source by *Botryococcus braunii*. Biotechnology Letters, (2004).

ZHILA N.O., KALACHEVA G. S., and VOLOVA T. G. (2005) Effect of Nitrogen Limitation on the Growth and Lipid Composition of the Green Alga *Botryococcus braunii*. Russian Journal of Plant Physiology, Vol. 52, No. 3, pp. 311–319.

