International Ph.D. Program in Environmental Sciences and Engineering

Cycle XXIII

University of Cagliari	Universidade de Aveiro	University of Pushchino	Ecole Nationale de l'Industrie Minerale

Analysis of the growth of microalgae in batch and semi-batch photobioreactors

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Academic Year 2010 – 2011

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Acknowledgements

I would like to thank some of the people who along the way have had faith in me during these years of my Ph.D. course.

First of all I would like to express my sincere gratitude to my research advisor, Prof. Giacomo Cao, for his valuable guidance and encouragement without whom this research work would have not been possible and to have allowed me to be part of his dawning research group in the field of microalgae investigation.

A special acknowledgment to my tutor, Ing. Alessandro Concas, for his precious time, encouragement and guidance through this project and assistance in successfully completing this project.

Many other people have contributed directly and indirectly to this work and I sincerely appreciate the support they have provided me with in pursuing this research.

I would especially to thank the generous help that I have received from Prof. Riccardo Tombolini for his support and guidance in getting started with the research, particularly to have supported me in the laboratory work during the first year.

Thanks to all my fellow engineer, biologist and chemist colleagues for providing valuable assistance and help in the daily laboratory operations and for having stood the green presence of microalgae inside the laboratory as well as my presence in the office.

Finally, I would like to extend my gratitude to all my family for its moral support. Two last special thanks are to my best Canadian friend, Andrew Bell, for his fundamental help during all these years in improving my English (I hope that your teaching is clear in this work) and to my French friend Laurent and his wonderful family for their special and valuable friendship ("merçi beaucoup pour l'amitié que vous m'avez demonstrez pendant tous ces années").

G.A. Lutzu

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List of acronyms

AA = Arachidonic Acid

AC = Caen-ALGOBANK

ADP = Adenosine Diphosphate

ANACC = Australian National Algae Culture Collection

AP = Areal Productivity

ATCC = American Type Culture Collection

ATP = Adenosine Triphosphate

ACOI = Coimbra Collection of Algae

BWM = Brackish Water Medium

CCAC = Culture Collection of Algae at the University of Cologne

CCALA = Culture Collection of Autotrophic Organisms

CCAP = Culture Collection of Algae and Protozoa

CCPC = Canadian Phycological Culture Centre

COM = Commission of the European Communities

Cyt b6f = Cytochrome b6f Complex

DHA = Docosahexaenoic Acid

DO = Dissolved Oxygen

EABA = European Algae Biomass Association

EEA = European Environmental Agency

EPA = Eicosapentaenoic Acid

EU = European Union

Fd = Ferredoxin

FID = flame ionization detector

FNR = Ferredoxin-NADP Reductase

FP = Flate-Plate photobioreactor

GHG = Greenhouse Gases

GLA = γ -Linolenic Acid

GW = Global Warming

IMSL = International Mathematics and Statistics Library

IPCC = Intergovernamental Panel on Climate Change

ISP = Illuminated Surface Productivity

KTM = Kolkwitz Triple Modified

LHCI = Light Harvest Complex I

LHCII = Light Harvest Complex II

NADPH = Nicotinamide Adenine Dinucleotide Phosphate

NCBI = National Center of Biotechnology Information

NIES = National Institute for Environmental Studies

OD = optical density

PBRs = Photobioreactors

PC = Plastocyanin

PCC = Pasteur Culture Collection of Axenic Cyanobacterial Strains

PE = Photosynthetic Efficiency

PI = Photosystem I

PII = Photosystem II

PQ = Plastoquinone

PUFA = Polyunsaturated Fatty Acids

RFA = Renewable Fuel Agency

RNA = ribonucleic acid

RuBisCo = Ribulose 1-5 Bisphosphate Carboxylase Oxygenase

SAG = Sammlung von Algenkulturen Collection Gottingen

SCCAP = Scandinavian Culture Collection of Algae and Protozoa

TAGs = Triacylglycerols

UTEX = Collection at the University of Texas

VP = Volumetric Productivity

Chapter 1.

Introduction

Global warming (GW) induced by increasing concentrations of greenhouse gases (GHG) in the atmosphere has become today an important environmental concern. The major anthropogenic sources of GHG are transportations, energy sectors and agriculture which are responsible in European Union (EU) for more than 20%, 60% and 9% of emissions, respectively (EEA 2004, 2007).

A worldwide problem has become the depletion of petrochemical fuels and the continuous rise in oil prize that call us to make a global effort in order to find alternative energetic sources.

Presently many options are being studied and implemented in practice to meet the sustainability goals agreed under the Kyoto Protocol (1992) with different degrees of success. Wind, geothermal, solar (either thermal or photovoltaic), hydroelectric, ocean wave, carbon sequestration and bio fuels energy are been developed as more sustainable alternative energy sources compared with the combustion of fossil fuels (Dewily and Van Langenhove, 2006; Schiermeier *et al*., 2008). The use of fossil fuels is now widely accepted as unsustainable, due to depleting resources and the accumulation of GHG in the environment that have already exceeded dangerously high thresholds. For this reason, in order to achieve environmental and economics sustainability fuel production processes are required that are not only renewable but also capable of sequestrating atmospheric CO_2 so the development of CO_2-neutral fuels is one of the most urgent challanges facing our society (Sasi *et al*., 2011).

One important goal for the gradual replacement of fossil fuels by renewable energy sources, as a measure for transportation emissions reduction, is the use of biofuels which are seen as real contributors to reach those goals, particularly in the short term.

Today the most common biofuels are biodiesel and bio-ethanol, which can replace diesel and gasoline, respectively. In EU biodiesel represent 82% of total biofuels production (Bozbas, 2008) and is still growing in Europe, South America and United States, based on political and economic objectives.

The first generation of biofuel production systems (starch- and sugar-based ethanol production crops) demonstrated the feasibility of generating liquid transportation fuels from renewable sources, but at initially low energy-conversion efficiencies and high cost. Plants that produce high levels of cellulose and hemicellulose biomass (which can be converted into sugars using advanced enzyme catalysts) are being developed as second generation biofuel production systems. These biofuel crops do not compete directly with food production, require less agronomic inputs and have lower

environmental impacts than first generation biofuels. However since vegetable oil produced by crops of first generation may also be used for human consumption, this can lead to an increase in price of food-grade oils, causing the cost of biodiesel to increase and preventing its usage. Morevor the use of biodiesel from second generation crops may also be advantageous since the land requirements for biofuels production may lead to a competition with arable land and biodiversity loss, due to the cutting of existing forests and the use of potential invasive crops that may disrupt the biological integrity of local ecosystems and important ecological areas (Scarlat *et al*., 2008; RFA, 2008).

Although biofuels are still more expensive than fossil fuels their production is increasing in countries around the world also encouraged by policy measures and biofuels targets for transport (COM, 2006).

A transition to a third generation biofuels, such as microalgae, is than needed since low-cost and profitable biodiesel should be produced from low-cost feedstocks in order not to compete with edible vegetable oils and should have lower environmental impacts. Thus transition can also contribute to a reduction in land requirements due to their higher energy yields per hectare as well as to their non-requirement of agricultural land.

Concerning potential feedstock microalage are among the more interesting possibilities currently being investigated and implemented at pilot scale or even at industrial scale. Their use as a possible solution to the problem of GW is desirable since this group of fast-growing unicellular organisms shows several advantages which make them one of the most promising and attractive renewable sources for a fully sustainable and low-carbon economy portfolio. Between their advantages: widespread availability, absent (or very reduced) competition with agricultural land, utilization of cheap and abundant nutrient sources (sunlight, carbon dioxide, water), high oil and biomass yields, high quality and versatility of the by-products, generation of biomass for biofuel production with concomitant CO_2 sequestration and suitability for wastewater treatments and other industrial plants (Vilchez *et al*., 1997; Olguín, 2003; Mulbry *et al*., 2008; EABA, 2012).

The high potentiality of algae based biofuels is confirmed by the number of recent papers available in the literature related to the use of microalgae in the energy sector (Usui and Ikenouchi, 1997; Borowitzka, 1999; Kargi and Ozmihçi, 2004; Chisti, 2007), by the growing investments of private companies (Solazyme, Ocean Nutrition Canada,

Cellana, AlgaeLink) and governments (US Dep. Energy, 2010) in algae-related research activity as well as by the increasing number of filed patents (Burton and Cleeland, 2008; Wu and Xiong, 2009; Cao and Concas, 2010; Parsheh *et al*., 2010; Rispoli *et al*., 2011).

Despite this growing interest, the current microalgae-based technology is still not widespread since it is affected by technical and economic constraints that hinder its full scale-up (Chen *et al*., 2011). Therefore, great R&D efforts are currently undertaken to produce biodiesel at competitive costs and with the required quality starting from microalgae feedstock. In particular given the potential benefits of microalgae, their cultivation should be studied and optimized to make them competitive as fuel producing systems in the global market (Debska *et al*., 2010).

The main technical barriers are related to the fact that photosynthetic efficiency, growth rate and lipid content of microalgae are still low if compared to the rate of fuel demand of the transportation market. In order to overcome such drawback, scientific community is moving on three main directions. The first one is the identification of cultivation conditions and photobioreactors configurations that maximize lipid productivity and CO_2 fixation by means of a reduced number of known microalgae (Yoo *et al*., 2010; Yeh *et al*., 2011). The second main research line is targeted to the identification of new microalgae strains which are intrinsically characterized by high growth rates and high lipid content (de la Vega *et al*., 2011). Finally the most attractive scientific challenge to face this problem is the genetic manipulation of existing strains in order to increase their photosynthetic efficiency and/or to regulate their metabolism in order to achieve an abundant production of lipids coupled with high biomass accumulation (León-Bañares *et al*., 2004).

Coherently to the research lines above reported, these topics have been focused in this work with a double aim.

The first is to investigate for the first time in the literature the potentiality of a relatively unknown marine strain, *Nannochloris eucaryotum*, for the viable production of biofuels and high value-added products at the industrial scale by means of a process which uses flue gases as CO_2. The effect of medium composition and nutrient starvation on the growth kinetics of this microalga is investigated using batch photobioreactors with the aim to gain useful information for the process optimization. The determination of nutrient levels in the medium, or any restriction associated with them, which are capable to affect the growth rate of cells during cultivation, represents a first step

towards the increase of cultures productivity, and hence the improvement of the economics of microalgae-derived fuel production.

The second aim is to evaluate the potential use of a fresh-water strain, *Chlorella vulgaris,* for mass cultivation in batch and continuous photobioreactors by coupling the use of pure CO_2 and an enriched medium that could improve the algal biomass productivity. The target of this activity is strictly connected to the possibility of optimize the CO_2 capture and bio-oil production. In fact when trying to transpose this technology at industrial scale, one of the main concerns is the management of huge quantities of waste gases. Separating the CO_2 from the other gases which constitute the flue gas can reduce the quantity of gases to be managed up to 90% thus simplifying the system operability of photobioreactors.

Chapter 2.

Literature review

2.1 Microalgae

Algae are recognised to be as one of the oldest life-forms appared on the Earth about 3.5 billions of years ago (Falkowski and Raven, 1997). They are considered as ancestors of primitive plants (thallophytes), i.e. lacking roots, stems and leaves, have no sterile covering of cells around the reproductive cells and have chlorophyll *a* as their primary photosynthetic pigment (Lee, 1980). Algae structures are primarily for energy conversion without any development beyond cells, and their simple development allows them to adapt to prevailing environmental conditions and prosper in the long term (Falkowski and Raven, 1997).

Prokaryotic cells (cyanobacteria) lack membrane-bound organelles (plastids, mitochondria, nuclei, Golgi bodies and flagella) and are more similar to bacteria rather than algae. Eukaryotic cells, which comprise of many different types of common algae, do have these organelles that control the functions of the cell, allowing it to survive and reproduce. Eukaryotes are categorised into a variety of classes mainly defined by their pigmentation, life cycle and basic cellular structure (Khan *et al*., 2009). The most important classes are: green algae (Chlorophyta), red algae (Rhodophyta) and diatoms (Bacillariophyta).

As will be explained more in detail in section 2.6, algae can either be autotrophic or heterotrophic. The former require only inorganic compounds such as CO_2, salts and a light energy source for growth while the latter are non photosynthetic therefore require an external source of organic compounds as well as nutrients as an energy source. Some photosynthetic algae are mixotrophic, i.e. they have the ability to both perform photosynthesis and acquire exogenous organic nutrients. Autotrophic algae are considered photosynthetic oxygenic autotrophs because they use light energy to convert CO_2 absorbed by chloroplasts into Adenosine Triphosphate (ATP), the usable energy currency at cellular level, which is then used in respiration to produce energy to support growth. Depending on species and type of algae, this energy can be in the form of lipids as well as carbohydrates. It is the lipid that can be easily converted into a suitable industrial fuel source.

2.2 Photosynthesis

Photosynthesis is a very complex process carried out by green plants and algae. These organisms are able to harness the energy contained in sunlight, and via a series of oxidation-reduction reactions, produce O_2 (which is release in the environment as

"bi-product") and carbohydrates, as well as other compounds, which may be utilized for energy (Kruse *et al.,* 2005) as well as the synthesis of other compounds such as lipids and proteins (Karube *et al.* 1992).

$CO_2 + H_2O + \text{light energy} \rightarrow (CH_2O)_n + O_2$

This equation is the net result of two different processes: the first, which is often referred to as the "light reaction", involves the splitting of water in an oxidative process that requires light and have the function to generate reducing agents, ATP and Nicotinamide Adenine Dinucleotide Phosphate (NADPH), to be used in the second phase (the so called "dark or indipendent-light reaction") of carbon assimilation by using atoms of carbon supplied by CO_2.

Briefly, there are two functionally separate sites of photon absorption, coupled in tandem by a chain of redox carrier molecules (photosystem II (PSII), plastoquinone (PQ), plastocyanin (PC), cytochrome b6f complex (Cyt b6f), photosystem I (PSI), ferredoxin (Fd), ferredoxin-NADP reductase (FNR) and ATP synthase). The photon absorption elicits a charge separation of at two reaction sites, PSII and PSI. This is depicted in Figure 1 as the so-called ''Z-scheme'' of photosynthesis.

The electron flow away from the chlorophyll molecules draws electrons from water.

This whole complex of photon capturing mechanisms, charge separation, generation of metabolic energy, and reducing capability, and the water splitting system is embedded in the lipid membrane of flattened sac-like structures present in the chloroplast, known as thylakoids.

The electrons pumped by the two reaction centres eventually give rise to the production of the NADPH used in the process of carbon assimilation. At the same time protons are pumped across the membrane into the inner cavity of the thylakoid (the lumen). This sets up a charge gradient. On their return, the protons spin a molecular rotor, which gives rise to the synthesis of ATP, the biological energy currency.

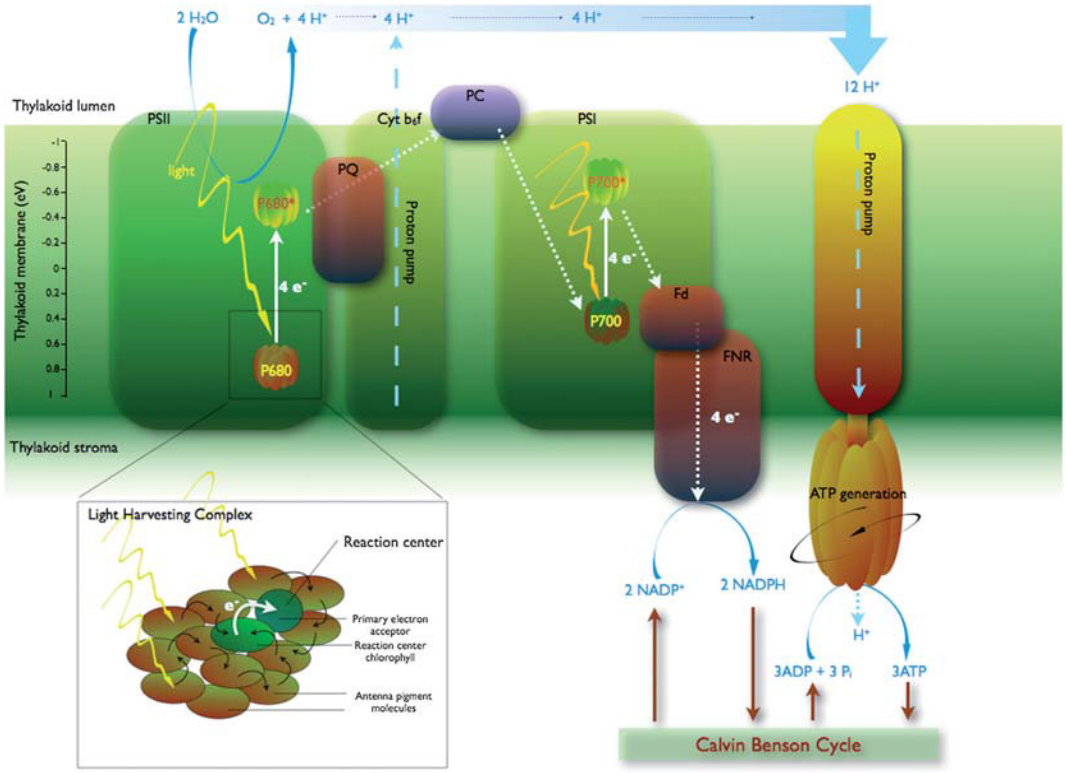

Figure 1. Illustration of the light reactions of photosynthesis (the so-called Z-scheme). The major functional units are represented as oval shapes; photosystem II (PSII), plastoquinone (PQ), plastocyanin (PC), cytochrome b6f complex (Cyt b6f), photosystem I (PSI), ferredoxin (Fd), ferredoxin- NADP reductase (FNR) (in order of electron transport chain) and ATP synthase. P680 and P700, refer to the reaction centres of photosystem II (PSII) and I (PSI) respectively, the asterisk (*) indicates the excited state. The inset shows a schematic close-up of the light harvesting complex (LHC) (Williams and Laurens, 2010).

Finally, the ‘‘reducing power’’ (NADPH) and energy (ATP) produced by the light reaction are used in the enzymatic ‘‘light-independent’’ part of photosynthesis to enable the incorporation of CO_2 into organic material and its subsequent reduction. This process is regulated by a series of enzymes such as ribulose 1-5 bisphosphate carboxylase oxygenase, commonly known by the shorter name of RuBisCO. The carboxylation by means of RuBisCO represents the Calvin cycle which leads to the end to the production of a glucose molecule.

The "light reaction" may be written as:

$$12\ H_2O + \text{light} \rightarrow 6O_2 + 24\ H^+ + 24e^-$$

The oxidation of water is accompanied by a reduction reaction resulting in the formation of NADPH.

This reaction is illustrated below

$$NADP^+ + H_2O \rightarrow NADPH + H^+ + O$$

The NADPH formation reaction is linked or coupled to yet another reaction resulting in the formation of a highly energetic compound, ATP. As this reaction involves the addition of a phosphate group to a compound called, Adenosine Diphosphate (ADP) during the light reaction, it is called photophosphorylation. The light energy, which is captured, is stored in the form of chemical bonds of compounds such as NADPH and ATP. The energy contained in both NADPH and ATP is then used to reduce CO_2 to glucose, a type of sugar ($C_6H_{12}O_6$).

2.3 General considerations for culturing microalgae

Culturing requirements are species specific, but some media are "broad" with respect to meeting the nutritional/culturing needs of groups of microalgae. Successful culturing entails formulating the medium and environmental conditions to meet the target algae's requirements for optimal growth. Temperature, light, pH (Goldman, *et al.,* 1982), salinity (for marine strain) and mixing, as well as nutrient quantity and quality are the parameters of interest to obtain optimal growth.

Moving from the laboratory to large scale is not just "doubling" the batch. It does not work for brewing and it does not work for growing algae. One problem is that the laboratory algae may have been grown under "unbalanced growth" conditions. It is essential to develop standards or standardized ranges that parallel the conditions that will exist in the larger scale cultivation unit in the laboratory. Range requirements for nitrogen, phosphorus and carbon, quality and quantity of light, temperature, salinity, and mixing or turbulence with respect to a particular species must be carefully established before moving out of the laboratory.

CO_2 bubbling can physically damage cells and, unless filtered with a 0.2 μm filter unit there is a chance of bacterial or viral contamination. Bubbling does increase the surface area exposure to CO_2 and removes the excess O_2 produced. If there is not sufficient algae biomass to utilize the CO_2, the higher concentration of CO_2 can lower the pH.

Beijerinck, Bold Basal, BG11 are some of the most common media recommended for freshwater algae in Chlorophyceae (CCAP). Optimal growth requires optimal nutrient availability, temperature and light intensity. Optimal in this case means most advantageous to the specific algae, since each species has specific growth requirements.

In addition to the algae species selection, Mata *et al*. (2010) summarizes some of the points made by Maxwell *et al*. in 1985. The first consideration related to the importance of water, its chemistry and its availability. The second is the ease of access/cost to carbon and mineral nutrients (N and P in particular). This makes the use of sewage effluent (source of N and P) coupled with CO_2 (source of C) sequestration an attractive solution.

2.4 Nutrients requirement and their effect on the growth

Microalgae need some essential macronutrients (carbon, nitrogen, phosphorus, sulphur, calcium, magnesium) for their growth while micronutrients requirement is limited to small amount of some elements such as iron, boron, manganese, copper, molybdenum, vanadium, cobalt, nickel, selenium and in same case silicon (Such and Lee, 2003). Some of these nutrients can be easily found in nature bound in minerals while others are supplied by bacteria metabolism.

It is important to develop a balanced medium for optimum microalgae cultivation and CO_2 fixation.

Nitrogen and phosphorous are considered elements key to algal metabolism then they must be found in the media in which algae are grown. The researchers asserted that balancing the nutrients based on the elemental composition of the biomass should be the basis for effective medium design (Mandalam and Palsson, 1998). However, some nutrients need to be present in excess. For example, phosphorus must be supplied in excess because the phosphates react with metal ions (Chisti, 2007).

Phosphorous is most often limited in nature because it is effectively bound in sediment. This element, in the form of orthophosphate, is generally considered the main limiting nutrient in freshwater aquatic ecosystems: that is, if all the phosphorous are used, autotrophic growth will cease, no matter how much nitrogen is available (Barsanti and Gualtieri, 2006).

On the other hand, in nature nitrogen is not necessarily limiting because bacteria are fixing nitrogen and supplying the algae with a constant nitrogen source.

Nutrient deficiencies and excess nutrients, both, can cause physiological and morphological changes in microalgae since they can inhibit some of the vital metabolic pathways.

Applying stress in the form of limited nutrients (especially N or P) can increase lipid percentages within the biomass, as can been see from Figure 2. However, this stress application also affects the growth rate and thus may lower overall lipid production.

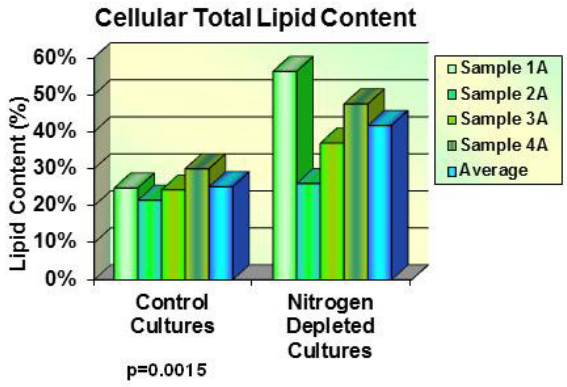

Figure 2. Effect of nitrogen depletion on microalgal total lipid content.

There are three main different situations of nutrient supply: nutrient-sufficient, nutrient-limited and nutrient-deficient. The first case should be evident, whereas the difference between the latter two cases may be subtle. Nutrient limitation occurs when cells are grown in an environment of a constant, but insufficient, supply of a limiting nutrient, to which the cells generally adapt. Nutrient deficiency is characterized by the culture's reliance on endogenous reserves because there are no nutrients in the environment (Rodolfi *et al.* 2009, Dragone *et al.* 2011).

The effect of nitrogen and phosphorus concentrations on microalgae growth has been extensively addressed in the literature (Przytocka Jusiak, 1976; Shifrin and Chisholm, 1981; Piorreck, 1984; Jeanfils *et al.* 1993; Tam and Wong, 1996; Kilham *et al.*, 1997; Mandalam *et al.*, 1998; Martinez *et al.* 1999; Illman *et al.* 2000; Kirpenko, 2001; Xu *et al.* 2001; Mohapatra *et al.* 2002; Leonardos and Geider, 2004; Sassano *et al.* 2004, 2007; Soletto *et al.* 2005; Li *et al.* 2008a; Qu *et al.* 2008; Bilanovic *et al.* 2009; Celekli and Balci, 2009; Converti *et al.* 2009; Debska *et al.*, 2010; Hu and Zhou, 2010; Li *et al.* 2010; Bhola *et al.,* 2011; Feng *et al.* 2011; Lin and Lin, 2011).

In particular, industrial and agricultural wastewater and secondary sewage treated effluent can be used as medium source of nitrogen and phosphorus (Prathima Devi *et al.* 2012). Actually, although nitrogen and phosphorous are elements key to algal growth, they are also serious pollutants in many waterways. Municipal sewage, industrial, and agricultural wastewaters contain carbon, nitrogen, and phosphorus to varying degrees. Agricultural wastewater often has higher levels of nitrogen and

phosphorus than municipal sewage, and the composition of industrial wastewaters depends on the type of industry that creates them and might even include wastewater loaded with heavy metals that would be of little value in promoting algal growth. Algae can thrive in nitrogen- and phosphorus-rich conditions common to many wastewaters, and this feature may be harnessed to not only remove, but also capture these important nutrients in order to return them to the terrestrial environment as agricultural fertilizer. This tackles the matter of eutrophication in the aquatic environment where the wastewater is eventually returned (Pittman *et al*., 2011).

2.4.1 Carbon

A number of microalgal species have been shown to be able to utilize carbonates such as Na_2CO_3 and $NaHCO_3$ for cell growth (Goldman and Graham, 1981; Novak and Brune, 1985; Chen and Johns, 1991; Ginzburg 1993; Merrett *et al*., 1996; Huertas *et al*., 2000b; Lin *et al*., 2003; Hongjin and Guangce, 2009; Šoštaric *et al*., 2009; Hu and Zhou, 2010, Kim *et al*., 2010, Romanenko *et al.*, 2010; Yeh *et al.,* 2010). Some of these species typically have high extracellular carbo-anhydrase activities (Huertas *et al*., 2000a), which is responsible for the conversion of carbonate to free CO_2 to facilitate CO_2 assimilation. This mechanism is directly related to the pH of the medium (Azov, 1982). In addition, the direct uptake of bicarbonate by an active transport system has been found in several species (Colman and Rotatore, 1995; Merrett *et al*., 1996). Adoption of carbonate-utilizing strains for CO_2 fixation could be advantageous in two aspects: 1) as only a limited number of microalgal species thrive in media containing high concentration of carbonate salts, species control (i.e., preventing wild-type microalgal species from contaminating the cultivation system) is relatively simple; 2) most of these species have high pH optimum (in the range of 9 to 11) further simplifying species control (Ginzburg, 1993).

2.4.2 Nitrogen

Ammonia, urea and nitrate are often selected as the nitrogen source for the mass cultivation of microalgae (Xu *et al*., 2001; Li *et al*., 2008a). The choice of the suitable source of nitrogen depends on strain considered since metabolic pathways related to nitrogen are species-specific. Although ammonium and urea are often used in mass cultivation owing to the relatively low-cost, selecting proper nitrogen source for each

algal species is important in improving biomass and oil productivity (Li *et al*., 2008b).

Urea and nitrate were found to be better than ammonia for the growth and lipid accumulation in *Chlorella* sp., *Chlorella vulgaris*, *Neochloris oleoabundans* and *Scenedesmus rubescens* (Tam and Wong, 1996; Liu *et al*., 2008; Li *et al*., 2008b; Hsieh and Wu, 2009; Pruvost *et al*., 2009; Lin and Lin, 2011). In contrast, for *Ellipsoidion* sp. the ammonium has been demonstrated to produce higher biomass and lipid content than those of urea and nitrate (Xu *et al*., 2001).

In general, lipid productivity and content are inversely correlated with each other; and stress conditions, e.g. deprivation or limitation of nitrogen (or of phosphate, to a lesser extent), limit cell growth while increasing lipid content (Rodolfi *et al*., 2009; Khozin-Goldberg and Cohen, 2006). Nitrogen limitation has been observed to result in lipid content increase in many *Chlorella* strains such as *Chlorella emersonii* (63%), *Chlorella minutissima* (56%), *Chlorella vulgaris* (57.9%), *Chlorella luteoviridis* (28.8%), *Chlorella capsulata* (11.4%), and *Chlorella pyrenoidosa* (29.2%) as well as others microalgae strains (Shifrin and Chisholm, 1981; Reitan *et al*., 1994; Stephenson *et al*. 2010, Mutlu *et al*. 2011, Yeh and Cheng, 2011). It has been reported that, under nitrogen deficient conditions, many other strains show increase in their lipid content and modification on fatty acids composition (Griffiths *et al*. 2011). *Neochloris oleoabundans* cells accumulate lipids in a range 25–54% with 80% triglycerides component (Tornabene *et al*., 1983; Kawata *et al*., 1998; Pruvost *et al*., 2011). It has also been reported that the triglycerides accumulated in *Nannochloris* sp. under nitrogen deficient conditions could be 2.2 times of that in nitrogen sufficient cultures (Yamaberi *et al*., 1998, Takagi *et al*., 2000).

The general principle is that when there is insufficient nitrogen for the protein synthesis required by growth, excess carbon from photosynthesis is channelled into such storage molecules as triacylglycerols or starch (Scott *et al*. 2010).

2.4.3. Phosphorus

Phosphorus is an important limiting nutrient in many ecosystems (such as lakes, rivers, and estuaries), and also one of the most likely to limit the rate of phytoplankton production. Microalgae use phosphorus for their metabolism in form of polyphosphate. Recent reviews on polyphosphate metabolism in photosynthetic

organisms have been made by Yang and Finnegan (2010), Seufferheld and Curzi (2010) and Yao *et al.* (2011).

Even if with a little extent compared to the larger results on nitrogen, phosphorus starvation can also enhance microalgal biomass and lipid productivity, as reported for *Monodus subterraneus* (Khozin-Goldberg and Cohen, 2006), and produce changes in fatty acids composition as reported for *Phaeodactylum tricornutum* and *Dunialella tertiolecta* (Siron *et al.*, 1989).

2.4.4 Other elements

Iron, sulphur, magnesium, and other elements are also indispensable for the growth of microalgae.

Iron is involved in electron flow from H_2O to nicotinamide adenine dinucleotide phosphate (ADP in its oxidized form) (Roden and Zachara, 1996). Some investigations have been addressed to the effect of iron on microalgae growth. High iron concentrations have been show to enhance cell growth (Sung *et al.*, 1998) and induce lipid accumulation (Liu *et al.*, 2008) in *Chlorella* strains; this suggests that some metabolic pathways may be modified upon exposure to high levels of that oligoelement in the medium.

Sulphur is an essential component of two amino-acids, cysteine and methionine. In its absence, protein biosynthesis is impeded and the photosynthetic system PSII repair cycle is blocked (Zhang *et al.,* 2002).

Magnesium is required as essential element in the core of the tetrapyrrolic ring which is the base of the chlorophyll molecule.

Some trace metals play key roles in (non-cyclic) photosynthetic electron transport (Raven *et al.*, 1999). For instance, manganese is essential for O_2 evolution, and calcium has an important role in the thylakoid lumen in facilitating H_2O dehydrogenation and O_2 evolution.

2.5 Effect of temperature, pH and light on the growth

2.5.1 Temperature

Temperature is one of the major factors that regulate cellular, morphological and physiological responses of microalgae (Dauta *et al.*, 1990; Hosono *et al.*, 1994; Mayo and Noike, 1996; Mayo, 1997; Hirata *et al.*, 1997; Martinez *et al.*, 1999; Carvalho

and Malcata, 2003; de Castro Araujo *et al*., 2005; Kitaya *et al*., 2005; Shi *et al*., 2006; Cho *et al*., 2007; Colla *et al*., 2007; Durmaz *et al*., 2007; Converti *et al*., 2009).

Higher temperatures generally accelerate the metabolic rates of microalgae, whereas low temperatures lead to inhibition of microalgal growth (Muñoz *et al*., 2006). In suitable temperature condition, the enzymes in microalgal cells possess the highest activity. Although algae may be able to grow at a variety of temperatures (Chinnasamy *et al*., 2009), different species show different optimal temperatures which are specific to each strain (Renaud *et al*., 2002; Cho *et al*., 2007). For example, the optimum temperature range for *Nannochloropsis*, *Tetraselmis*, and *Isochrysis* species were found to be 19-21, 19-21 and 24-26°C, respectively (Abu-Rezq *et al*., 1999). For many species, optimal growth temperatures of 15–26°C have been observed with maximum cell densities obtained around 23°C (Ono and Cuello, 2003). However, optimal temperatures are also influenced by other environmental parameters, such as light intensity and the distance between cultivation apparatus and artificial illumination system.

The control of temperature is a key factor for culturing microalgae outdoors (Torzillo *et al.,* 1991). Actually, temperature can vary depending on the geographic region of cultivation (Ramos de Ortega and Roux, 1986). Seasonal and even daily fluctuations in temperature can interfere with algae production. Temperatures can reach as high as 30°C higher than ambient temperature in a closed photobioreactor without temperature control equipment. For this reason, evaporate cooling or shading techniques are employed frequently to inhibit temperatures of that magnitude (Suh and Lee, 2003). On the other hand, some "thermophiles" *Chlorella* species are reported to be tolerant to high temperature (Hanagata *et al*., 1992; Sakai *et al*., 1995; Loseva *et al*., 1998).

2.5.2 pH

The pH is a fundamental parameter which regulates cell metabolism and biomass formation (Goldman *et al*., 1982). Each strain of microalgae has a narrow optimal range of pH (Mayo and Noike, 1994, 1996; Mayo *et al*., 1997; Alyabyev *et al*., 2011) and most microalgal species are favoured by neutral pH. However, there are some extremophilic species which dwell in environments that are characterized by very low or high pH-values (acidophiles or alkalophiles). For example, between microalgae which show growth under alkaline conditions there is *Spirulina platensis*

with an optimum pH around 9 (Hu *et al.*, 1998; Qiang *et al.*, 1998) while *Chlorococcum littorale* thrive well with an acidic pH around 4 (Kodama *et al.*, 1993; Schnackenberg *et al.*, 1996). *Galdieria sulphuraria* (Barbier *et al.*, 2005) and *Chlamydomonas acidophila* (Cuaresma *et al.*, 2011) have been reported to be also resistant to pH 0 and 1.5-2.5, respectively.

The pH of the medium is linked to the concentration of CO_2 and pH increases steadily in the medium as CO_2 is consumed during flow downstream in a cultivation system (Suh and Lee, 2003). The pH affects mainly the liquid chemistry of polar compounds and the availability of nutrients such as iron, organic acids and even CO_2 (Coleman and Colman, 1981; Lee and Pirt, 1984).

There is a complex relationship between CO_2 concentration and pH in microalgal photobioreactor related to the chemical equilibrium among chemical species such as CO_2, H_2CO_3, HCO_3^- and CO_3^{2-} (Livanski and Bartos, 1986). The chemical equilibrium between these forms is pH dependent with CO_2 the predominant form at lower pH below 7 and CO_3^{2-} predominant above pH 10. Rapid growth of algae can, with the assimilation of CO_2 as the C source, cause the pH to rise.

Increasing CO_2 concentrations can lead to higher biomass productivity, but will also decrease pH, which can have an adverse effect upon microalgal physiology. By contrast, microalgae have been shown to cause a rise in pH to 10–11 because of CO_2 uptake (Oswald *et al.*, 1988). This increase in pH can be beneficial in open ponds for instance for neutralization of pathogens in microalgal wastewater treatment, but can also inhibit microalgal growth.

2.5.3 Light

In photosynthetic cultures, the light energy is used by the cells either for maintenance purposes or formation of new biomass (Pirt, 1986). Consequently, the biomass productivity and the cell growth rate are directly linked to the light energy available, which varies from day to night.

The effect of light intensity on growth kinetics and biomass accumulation (Dauata *et al.*, 1990; Ogbonna *et al.*, 1995; Qiang *et al.*, 1998; Lu *et al.*, 2001; Olguin *et al.*, 2001, Carvalho and Malcata, 2003; Yun and Park, 2003; Leonardos and Geider, 2004; You and Barnett, 2004; Kitaya *et al.*, 2005; Chen and Chen, 2006; Yeh *et al.*, 2010; Pedrosa Bezerra *et al.* 2011; Shu *et al.*, 2011; Amini Khoeyi *et al.*, 2012; Ruangsomboon, 2012) as well as the effect of illumination cycles (hours of light and

hours of dark) on biochemical composition (Shifrin and Chisholm, 1981; Ogbonna and Tanaka, 1996; Ma *et al*., 1997, Janssen *et al*., 2001; Ratchford and Fallowfield, 2003; Meseck *et al*., 2005; Umorin and Lind, 2005; Jacob-Lopes *et al*., 2009; Hodaifa *et al*., 2011; Seyfabadi *et al*., 2011; Sforza *et al*., 2011) have been characterized on different microalgae strains such as *Chlorella vulgaris*, *Chlorella pyrenoidosa*, *Nannochloropsis salina*, *Dunaliella tertiolecta, Monodus subterraneus, Pavlova lutheri, Spirulina platensis, Chaetoceros muelleri, Porphyridium cruentum, Euglena gracilis, Tetraselmis chui, Scenedesmus obliquues* and *Botryococcus braunii.*

2.6 Modality of cultivations

2.6.1. Photoautotrophy

Phototrophic cultivation occurs when the microalgae use light (such as sunlight or artificial light provided by lamps) as the energy source and inorganic carbon (e.g., CO_2) as the carbon source to form chemical energy through photosynthesis (Huang *et al.*, 2010). This is the most commonly used cultivation condition for microalgae growth (Gouveia *et al*., 2009; Gouveia and Oliveira, 2009; Illman *et al*., 2000; Yoo *et al.*, 2010) and currently is the only method which is technically and economically feasible for large-scale production of algae biomass for non-energy production (Borowitzka, 1997). Under phototrophic cultivation there is a large variation in the lipid content of microalgae depending on the type of microalgae species. Normally a nitrogen-limiting or nutrient-limiting condition is used to increase the lipid content in microalgae (Mata *et al.*, 2010). As a result, achieving higher lipid content is usually at the expense of lower biomass productivity. Thus, lipid content is not the sole factor determining the oil-producing ability of microalgae. Instead, both lipid content and biomass production need to be considered simultaneously. Hence, lipid productivity, representing the combined effects of oil content and biomass production, is a more suitable performance index to indicate the ability of a microalga with regard to oil production. The major advantage of using autotrophic cultivation to produce microalgal oil is the consumption of CO_2 as carbon source for the cell growth and oil production. However, when CO_2 is the only carbon source, the microalgae cultivation site should be close to factories or power plants which can supply a large quantity of CO_2 for microalgal growth.

2.6.2. Heterotrophy

Some microalgae species can not only grow under phototrophic conditions, but also use organic carbon under dark conditions, just like bacteria. The situation when microalgae use organic carbon as both the energy and carbon source is called heterotrophic cultivation (Chojnacka and Marquez-Rocha, 2004).

Although microalgae can utilize light efficiently, photoautotrophic growth is often slow because of light limitation at high cell densities on a large scale (Wen and Chen, 2003) or "photoinhibition" due to excessive light. In view of these disadvantages associated with photoautotrophic cultivation, heterotrophic growth of microalgae for biomass production should be favourably considered (Chen, 1996; Miao and Wu, 2006). Heterotrophic cultivation offers several advantages over photoautotrophic cultivation including elimination of problems associated with limited light that hinder high cell density in large scale photobioreactors during phototrophic cultivation (Huang *et al*., 2010) allowing much simpler scale-up possibilities since smaller reactor surface to volume ratio's may be used (Eriksen, 2008), good control of the cultivation process, and low-cost for the harvesting of biomass because of higher cell density obtained (Chen and Johns, 1991).

In heterotrophic culture, both cell growth and biosynthesis of products are significantly influenced by medium nutrients and environmental factors. Carbon sources are the most important element for heterotrophic cultivation of microalgae. Moreover, heterotrophic microalgae might utilize carbon sources as acetate, glucose, ethanol, glycerol, sucrose, lactose, galactose, mannose and fructose depending on microalgal species (Yokochi *et al*., 1998, Liang *et al*., 2009). Liu *et al*. (1999) compared several carbon sources and concluded that glucose was preferred. Than, in order to lower the production cost of microalgal oil as biodiesel, cheaper carbon sources should be considered.

In this process microalgae are grown in stirred tank bioreactors or fermenters. These systems provide a high degree of growth control and also lower harvesting costs due to the higher cell densities achieved (Wen and Chen, 2003; Chen and Chen, 2006). The set-up costs are minimal, although the system uses more energy than the production of photosynthetic microalgae because the process cycle includes the initial production of organic carbon sources via the photosynthesis process (Chisti, 2007).

Higher biomass production and productivity could be obtained from using heterotrophic cultivation. The feasibility for large-scale biodiesel production based on

heterotrophic cultivation of *Chlorella protothecoides* was outlined by Lee *et al.* (2007) and Xiong *et al.* (2008). This microalga shows higher lipid content during heterotrophic growth and a 55% increase in lipid content was obtained by changing the cultivation condition from phototrophic to heterotrophic (Miao and Wu, 2006). The highest lipid productivity (3700 mg L^{-1} d^{-1}) was reported by Xiong *et al.* (2008) using a 5-L fermentor operated with an improved fed-batch culture strategy. Hence, they concluded that heterotrophic cultivation could result in higher production of biomass and accumulation of high lipid content in cells.

Other studies also suggest higher technical viability of heterotrophic production compared to photoautotrophic methods in either open ponds or closed photobioreactors for the cultivation of *Crypthecodinium cohnii* (de Swaaf *et al.*, 2003) and *Galdieria sulphuraria* (Graveholt *et al.*, 2007). Using heterotrophic growth gives much higher lipid productivity, as the highest lipid productivity from heterotrophic cultivation is nearly 20 times higher than that obtained under phototrophic cultivation. However, the sugar based heterotrophic system frequently suffers from problems with contamination since it is difficult to prevent bacteria proliferation. Actually, open ponds and raceway ponds are usually operated under phototrophic cultivation conditions since, compared to other types of cultivation, the contamination problem is less severe when using heterotrophic growth (Mata *et al.*, 2010).

2.6.3. Mixotrophy

Mixotrophic cultivation is when microalgae undergo photosynthesis and use both organic compounds and inorganic carbon (CO_2) as a carbon source for growth. This means that the microalgae are able to live under either phototrophic or heterotrophic conditions, or both (Zhang *et al.*, 1999). Microalgae assimilate organic compounds and CO_2 as a carbon source, and the CO_2 released by microalgae via respiration will be trapped and reused under phototrophic cultivation (Mata *et al.*, 2010). The ability of mixotrophs to process organic substrates means that cell growth is not strictly dependent on photosynthesis, therefore light energy is not an absolutely limiting factor for growth (Andrade *et al.*, 2007) as either light or organic carbon substrates can support the growth (Chen *et al.*, 1996). Examples of microalgae that display mixotrophic metabolism processes for growth are the cyanobacteria *Spirulina platensis* and the green alga *Chlamydomonas reinhardtii* (Chen *et al.*, 1996). The

photosynthetic metabolism utilises light for growth while aerobic respiration uses an organic carbon source (Zhang *et al*., 1999). Growth is influenced by the media supplement with glucose during the light and dark phases; hence, there is less biomass loss during the dark phase (Andrade *et al*., 2007). Growth rates of mixotrophic algae generally compare favourably with cultivation of photoautotrophic algae in closed photobioreactors but are considerably lower than for heterotrophic production. Successful production of mixotrophic algae allows the integration of both photosynthetic and heterotrophic components during the diurnal cycle. This reduces the impact of biomass loss during dark respiration and decreases the amount of organic substances utilised during growth. These features infer that that mixotrophic production can be an important part of the microalgae-to-biofuels process. It should be pointed out that, compared with phototrophic and heterotrophic cultivation, mixotrophic cultivation is rarely used in microalgal oil production.

2.7. Production systems.

Microalgae cultivation can be done in open-culture systems and/or in highly controlled closed-culture systems called photobioreactors (PBRs). Comparitions between the two type of cultivation system are reported in Table 1.

2.7.1 Open ponds

Open ponds are the most widely used systems for large-scale outdoor microalgae cultivation (Borowitzka, 1993) and most commercial microalgal cultivation is presently carried out, with few exceptions, in open systems (Richmond, 1999).

There are many types of open cultivation systems which vary in size, shape, material used for construction, type of agitation and inclination (Tredici, 2004). Algal cultures can be defined (one or more selected strains), or are made up of an undefined mixture of strains. However, to date only a few species of microalgae (e.g *Spirulina* sp., *Chlorella* sp., *Dunaliella salina*) have been found to be able to be grown successfully at a commercial scale in open ponds (Tredici and Materassi, 1992; Borowitzka, 1993; Parsheh *et al*., 2010).

Many different designs have been suggested for pond construction but only four major pond design have been developed and operated at a large-scale: a) unstirred

Table 1. Comparison between open ponds and photobioreactors

Parameter	*Open systems*	*PBRs*
Contamination risk	High	Low
Sterility	None	Achievable
Species control	Difficult	Easy
Area/Volume ratio	Low	High
Water losses	High	Low
CO_2 losses	High	Depends on pH, alkalinity
O_2 inhibition	Low	Problematic
Mixing	Very poor	Uniform
Light utilization efficiency	Poor	High
Temperature control	Difficult	Less difficult
Evaporation of growth medium	High	Low
Hydrodynamic stress on algae	Very low	Low-High
Process control	Complicated	Less complicated
Maintenance	Easy	Difficult
Yield	Low	High
Population (algal cell) density	Low	High
Biomass concentration	Similar in both	3-5 times in PBRs
Constructions costs	Low	High
Weather dependance	High	Low
Overheating problems	Low	High
Dissolved oxygen concentration	Low	High
Scale-up	Difficult	Difficult

ponds (lakes and natural ponds), b) inclined ponds, c) central pivot ponds and d) raceway ponds (Figure 3). Generally, these cultivation systems are less expensive to build and operate, more durable than large closed reactors and with a large production capacity when compared with closed systems. On the other hand, the production through ponds requires more extensive land areas despite being cheap since it uses very low amount of CO_2 of the air and are more susceptible to contaminations from other organisms such as mushrooms, bacteria and protozoa and also to weather conditions, not allowing control of water temperature, evaporation and lighting. They also show low photosynthetic efficiency (Morita *et al*., 2001b) due to low CO_2 (since atmosphere only contains 0.03% (v/v) CO_2 it is expected that mass transfer limitation could slow down the cell growth of microalgae) and sunlight available only at the pond surface. According to Richmond (2004) ponds use more energy to homogenize nutrients and the water level cannot be kept much lower than 15 cm (or 150 L m^{-2}) for the microalgae to receive enough solar energy to grow. In these open-culture systems nutrients can be normally provided through runoff water from nearby land area or by channelling the water from sewage/water treatment plants. The water is typically kept

Figure 3. Example of open cultivation systems: a) natural unstirred pond (Wikipedia, 2011), b) small pond for *Spirulina* culture, Asia (Wikipedia, 2011), c) Open raceway-type culture ponds of Earthrise in California, US (Spirulina.org.uk), d) Paddle wheel of a raceway pond (Department of Chemical Engineering, Tehran Polytechnic, Iran)

in motion by paddle wheels or rotating structures, and some mixing can be accomplished by appropriately designed guides.

Profitable production of microalgae, at present, is limited to comparatively few production plants producing high value health foods such as ß-carotene by *Dunaliella salina* and proteins and carotenoids by *Chlorella sp., Spirulina platensis, Haematococcus pluvialis* (Jimenez *et al*., 2003; Borowitzka, 2005; Del Campo *et al*., 2007). Most of these plants are located in South East Asia, Australia and USA, mainly Hawaii, California, Texas and Arizona (Borowitzka and Borowitzka, 1990; Benemann, 1992; Richmond, 1992).

2.7.2 Photobioreactors

To overcome some constraints related to ponds efficiency, closed PBRs have been proposed, which not only possess higher photosynthetic efficiency, but also temperature control of the culture medium, since temperature normally increases with

the exposure to the sunlight (Morita *et al*., 2001c) and allow for the use of external contamination control. The critical design requirement in the PBR design is the illumination surface area per unit volume and a high surface area to volume ratio (S/V ratio) is required to have an efficient PBR (Ogbonna and Tanaka, 1997).

Further, despite several research efforts for the design and operation of many PBRs, devising and developing suitable apparatus, cultivation procedures and algal strains susceptible of undergoing substantial increases in efficiency for the use of solar energy and CO_2 are the major challenges for the industrial microalgal culturing.

Figure 4. Example of closed cultivations systems: a) tubular horizontal photobioreactor (Bioprodukte-steinberg.de), b) column photobioreactor (Bioenergy Noe, 2012), c) inclined column photobioreactor (algaebiodiesel.com), d) flate pannel photobioreactor (Wijffels, 2007), e) BIOCOIL (heatingoil.com)

Accordingly, there is no 'best reactor system' to achieve maximum productivity with minimum operation costs, irrespective of the available biological and chemical systems (Carvalho *et al*., 2006). Selection of the PBR depends on the ability to maximize the productivity and photosynthetic efficiency.

Design and operation of suitable microalgal biomass production systems have been discussed and patented extensively in the literature (Molina Grima *et al*. 2000; Hirabayashi *et al*., 2002; Scragg, 2002; Carvalho *et al*., 2006; Lopez *et al*., 2006; Levin, 2007; Lewnard and Wu, 2008; Oyler, 2008; Ugwu *et al*. 2008; Hsieh *et al*. 2009b; Kayama and Kadowaki, 2009; Lin, 2009; Slavin, 2009; Briassoulis *et al*. 2010; Dimanshteyn, 2010; Erb and Peterson, 2010; Haley, 2010; Hu, 2010; McNeff, 2010; Melkonian and Podola, 2010; Seebo 2010; Woerlee and Siewers, 2010; Dhale, 2011; Edelson, 2011; Hulatt *et al*. 2011; Kassebaum and Kassebaum, 2011; Katoch and Katoch, 2011; Lin, 2011; Zhang *et al*., 2011) with recent reviews comprehensively presenting several types of closed bioreactors for the production of microalgae based on transport phenomena and process engineering methodological approaches (Janssen *et al*., 2003; Choi *et al*., 2003; Hankamer *et al*., 2007; Kunjapur and Eldridge, 2010).

Between the numerous types of enclosed PBRs suitable for large-scale cultivation that have been designed in an attempt to best control the growth factors of microalgae there are four main categories which are summarized in the Figure 4: 1) tubular/horizontal, 2) column/vertical, 3) flat plate or flat panel, 4) helical/tubular (BIOCOIL).

Table 2. Prospects and limitations of different photobioreactors

Culture systems	***Advantages***	***Disadvantages***
Tubular PBRs	Large illumination surface area, suitable for outdoor cultures, fairly good biomass productivities, relatively cheap	Gradients of pH, dissolved oxygen and CO_2 along the tubes, fouling, some degree of wall growth, requires large land space
Vertical-column PBRs	High mass transfer, good mixing with low shear stress, low energy consumption, high potentials for scalability, easy to sterilize, readily tempered, good for immobilization of algae, reduced photoinhibition and photo-oxidation	Small illumination surface area, their construction require sophisticated materials, shear stress to algal cultures, decrease of illumination surface area upon scale-up
Flat-plate PBRs	Large illumination surface area, suitable for outdoor cultures, good for immobilization of algae, good light path, good biomass productivities, relatively cheap, easy to clean up, readily tempered, low oxygen build-up	Scale-up require many compartments and support materials, difficulty in controlling culture temperature, some degree of wall growth, possibility of hydrodynamic stress to some algal strains

Each type of PBR has advantages and disadvantages (Table 2) in terms of potential efficiency of sunlight utilization, effective mass transfer of O_2 and CO_2, easiness of cleaning and scalability.

The main parameter used to compare the photosynthetic productivities of different type of PBRs is the photosynthetic light conversion efficiency (PE) which is defined as the percentage of light energy recovered as microalgal biomass to the total light energy received by the cultivation system (Watanabe and Hall, 1996a). PE should be used in conjunction with volumetric productivity when evaluating systems operated under similar climactic conditions (Tredici and Zitelli, 1998).

2.7.2.1 Tubular / horizontal PBRs

Tubular configuration is considered as one of the most suitable types for outdoor mass cultures. Most of these outdoor cultivation systems are usually constructed with either glass or plastic tube of small diameter, often mounted as parallel loops on a rigid scaffold.

They can be in form of horizontal / serpentine (Tredici and Materazzi, 1992; Molina Grima *et al*., 2001), vertical (Pirt *et al*., 1983), near horizontal (Henrard *et al*., 2011), conical (Norsker, 2002) or inclined PBRs (Vunjak-Novakovic *et al*., 2005). Re-circulation, aeration and mixing of the cultures in these PBRs are usually done by air-pump or preferably by airlift systems.

Tubular PBRs are very suitable for outdoor microalgae mass cultures since they have large illumination surface area. On the other hand, one of their major limitations is poor mass transfer. It should be noted that mass transfer (oxygen build-up) becomes a problem when tubular FBRs are scaled up. For instance, some studies have shown that very high dissolved oxygen (DO) levels are easily reached in tubular photobioreactors (Torzillo *et al*., 1986; Richmond *et al*., 1993; Molina Grima *et al*., 2001).

Normally they consist of straight, coiled or looped transparent tubing arranged in various ways for maximizing sunlight capture. Properly designed completely isolate the culture from potentially contaminating external environments, hence, allowing extended duration monoalgal culture.

Also, photoinhibition is very common in outdoor tubular PBRs. When a tubular reactor is scaled up by increasing the diameter of tubes, the illumination surface to volume ratio would decrease. On the other hand, the length of the tube can be kept as short as possible while a tubular PBR is scaled up by increasing the diameter of the tubes. In this case, the cells at the lower part of the tube will not receive enough light for cell growth (due to light shading effect) unless there is a good mixing system.

Also, it is difficult to control culture temperatures. Actually, although they can be equipped with thermostat to maintain the desired culture temperature, this could be very expensive and difficult to implement. It should also be noted that adherence of the cells to the walls of the tubes is common. Furthermore, long tubular PBRs are characterized by gradients of oxygen and CO_2 transfer along the tubes. The increase in pH of the cultures would also lead to frequent re-carbonation of the cultures, which would consequently increase the cost of algal production.

Consequently, even if this reactor type is quite effective it is too expensive and needs too much auxiliary energy for pure biofuel production.

2.7.2.2 Tubular / helicoidal (BIOCOIL)

In 1999 Borowitzka described a particular type of tubular reactor, a cylindrically shaped helical tubular design known as BIOCOIL (Robinson *et al*., 1988) which was supposedly the most promising design at that time. Considerations around tubular PBRs lead to the conclusion that diameter of tubes and position of PBRs to respect to the light could improve the PE. The solar collector tubes are generally 0.1 m or less in diameter. Tube diameter is limited because light does not penetrate too deeply in the dense culture broth that is necessary for ensuring a high biomass productivity of the PBR (Chisti, 2007). Instead of being laid horizontally on the ground, the tubes may be made of flexible plastic and coiled around a supporting frame to form a helical coil tubular PBR (Chisti, 2007). However, there has been a limited discussion of the BIOCOIL during the last 20 years suggesting that its potential should be longer addressed (Chrismadha and Borowitzka, 1994; Watanabe *et al*., 1995, 1998; Watanabe and Hall, 1995, 1996a, 1996b; Watanabe and Saiki, 1997; Rorrer and Mullikin, 1999; Borodin *et al*., 2000, 2002; Morita *et al*., 2000a, 2000b, 2001a, 2001b, 2002; Lu *et al*., 2001, 2002, Travieso *et al*., 2001; Nerantzis *et al*., 2002; Scragg *et al*., 2002; Acién Fernández *et al*., 2003; Hall *et al*., 2003a; Watanabe, 2004; Carlozzi and Pinzani, 2005; Moheimani, 2005; Perner-Nochta *et al*., 2007; Fan *et al*., 2008; Soletto *et al*., 2008; Concas *et al*., 2009,2010; Briassoulis *et al*., 2010; Kong *et al*., 2010; Westerhoff et al., 2010; Moheimani *et al*., 2011). Watanabe and Hall (1996a) noted that the design has radiation losses in the central area of the reactor. The authors attempted to improve it by constructing a laboratory-scale cone-shaped helical tubular reactor. It should be noted that little has been reported on the ability of the cone-shaped design to be scaled up. This design supposedly increased the

illuminated surface area while covering the same area on the ground as a regular tubular or FP reactor (Watanabe and Hall, 1996b). The biomass productivity obtained for *Spirulina platensis* and *Chlorella* sp. HA-1 were 15.9 g L^{-1} d^{-1} and 21.5 g L^{-1} d^{-1} which correspond to a PE of 6.83% and 5.67%, respectively (Watanabe and Hall, 1996a; Watanabe and Saiki, 1997). A maximum photosynthetic productivity of 34.4 g L^{-1} d^{-1} corresponding to a greater PE of 8.67% was obtained for *Chlorella sorokiniana* cultivated in a conical COIL feded with 10% CO_2 (Morita *et al*., 2000a). Soletto *et al*. (2008) reached a PE of 9.4% during a fed-batch cultivation of *S. platenis* by changing the feeding rate of CO_2 and light intensity at the values of 0.44 g L^{-1} d^{-1} and 125 µmol m^2 s^{-1}, respectively.

2.7.2.3 Column/vertical PBRs

Vertical-column PBRs are compact, low-cost and easy type of reactors to operate monoseptically and appear to best satisfy the design considerations at least at laboratory scale. There are two main types of vertical reactors: air-lift reactors and bubble column reactors. In these types of reactors, mixing energy is provided by the gas intake, thus combining aeration and dispersion. Generally, the reaction volume is sparged from the bottom.

They have been reported to be very promising for large-scale cultivation of microalgae since can attain a final biomass concentration and specific growth rate that are comparable to values typically reported for narrow tubular PBRs (Merchuk and Mukmenev, 2000). Vertical air-lift reactors improve gas exchange, liquid flow and exposure of cells to light (Camacho *et al*. 1999). Airlift reactors circulate the culture without moving parts or mechanical pumping, which reduces the potential for contamination and for cell damage due to shear. The air-lift driven tubular PBR both circulated the fluid through the loop and stripped oxygen from the culture (Spolaore *et al*. 2006).

Some bubble column PBRs are equipped with either draft tubes or constructed as split cylinders. In the case of draft tube PBRs, intermixing occurs between the riser and the down comer zones of the PBR through the walls of the draft tube. Their main advantages include: high mass transfer, good mixing with low shear stress, low energy consumption, high potentials for scalability, easy to sterilize, readily tempered, good for immobilization of algae, reduced photoinhibition and photo-oxidation. Their main limitations include: small illumination surface area required

sophisticated materials for their construction, shear stress to algal cultures, decrease of illumination surface area upon scale-up.

2.7.2.4 Flat plate PBRs

Another kind of system configuration which allows having a large illumination surface area is represented by flat-plate (FP) PBRs (Sierra *et al*., 2008). In 1985 Samson and Leduy developed a flat reactor equipped with fluorescence lamps following the pioneering work of Milner (1953). A year later, Ramos de Ortega and Roux (1986) developed an outdoor FP reactor by using thick transparent PVC materials. As time went on, extensive works on various designs of vertical alveolar panels and FP reactors were reported (Tredici and Materassi, 1992; Zhang *et al*., 2002; Hoekema *et al*., 2002; Nebdal *et al*., 2010). Generally, FP PBRs are made of transparent materials in order to maximize the utilization of solar light energy. Accumulation of dissolved oxygen concentrations in this type of PBRs is relatively low compared to horizontal tubular PBRs. It has been reported that with FP PBRs, high photosynthetic efficiencies can be achieved (Richmond, 2000). Between the main advantages in utilizing FP PBRs there are: large illumination surface area thus making them suitable for outdoor cultures, good capacity of algae immobilization, good light path, good biomass productivities, relatively cheap, easy to clean up, readily tempered, low oxygen build-up. Main limitations of these PBRs are: the scale-up require many compartments and support materials, a difficulty in controlling culture temperature, some degree of wall growth, and possibility of hydrodynamic stress to some algal strains.

2.7.3 Comments on different photobioreactor systems

Some authors claimed that the PE is significantly higher in tubular PBRs compared to FP PBRs because their curved surface resulted in the spatial dilution of light. Although some authors have claimed that FP reactors may have greater PE (Janssen *et al*. 2003) the results of Tredici *et al*. are convincing and it appears that PE is a drawback for FP reactors. Another drawback for FP reactors is that cell damage may occur because of the high stress resulting from aeration, a problem that has never been reported in tubular reactors. However, FP PBRs have advantages over other closed reactors. In FP PBRs, the oxygen path is much shorter than in tubular reactors

(Sierra *et al*. 2008). A shorter oxygen path results in FP concentrations than horizontal PBRs (Ugwu *et al*. 2008). Power consumption is another important criterion for comparison among reactor types. FP reactors consume less power than tubular reactors to achieve similar or greater mass transfer capacity (Sierra *et al*. 2008).

The comparisons between tubular and column reactors, made by Sánchez Mirón *et al*. (1999), lead to the significant conclusion that tubular reactors have very limited possibility for commercial scale applications, whereas column reactors do have potential. Bubble column reactors performed better than tubular reactors because they are supposedly more suited for scale-up, require less energy for cooling because of the low surface to volume ratio, and overall outperform tubular reactors throughout the year. Under high light intensity, vertical reactors experience less photoinhibition, and under low light intensity, a vertical orientation captures more reflected light (Sánchez Mirón *et al*. 1999). A vertical orientation also requires less land area (Camacho *et al*. 1999). Molina Grima *et al*. (2001) asserted that, for tubular reactors, a two layered loop with the lower set of tubes displaced horizontally in between the upper set of tubes maximizes efficiency of land use.

2.8 Microalgal productivity

It should be noted that the objective of the PBR photosynthetic production process of microalgal biomass is to obtain simultaneously the reduction of input energy and the achievement of high photosynthetic production. Also, these closed PBRs may be located indoors or outdoors, although outdoor location is more common due to the ease of using free sunlight. Comparison of performances achieved by PBRs and open ponds may not be easy, as the evaluation depends on several factors, among which the algal species cultivated and the method adopted to compute productivity.

In order to evaluate productivity in algae production units three parameters are common used (Scragg, 2002; Richmond, 2004):

- Volumetric productivity (VP): productivity per unit reactor volume (expressed as g L^{-1} d^{-1}),
- Areal productivity (AP): productivity per unit of ground area occupied by the reactor (expressed as g m^2 d^{-1}),
- Illuminated surface productivity (ISP): productivity per unit of reactor illuminated surface area (expressed as g m^2 d^{-1}).

These productivities vary with type of the system. For example, the productivity (mg L^{-1} d^{-1}) values of 370, 400–700 and 900 have been recorded for tubular, shallow and coiled outdoor tubular ponds, respectively, compared with 510 mg L^{-1} d^{-1} obtained for the indoor reactor (Chisti, 2007; Del Campo *et al.*, 2007). Although microalgae production efficiency is often mentioned in the literature, no consensus was observed on how to calculate it.

The cost of biomass production in PBRs may be one order of magnitude higher than in ponds. While in some cases, for some microalgae species and applications it may be low enough to be attractive for aquaculture use, in other cases, the higher cell concentration and the higher productivity achieved in PBRs may not compensate for its higher capital and operating costs.

Generally, in any PBR design, the system productivity in continuous operating mode is obtained by multiplying the steady-state biomass concentration by the dilution rate used. These are related to the average irradiance inside the photobioreactor, which in turn is a function of the irradiance on the reactor surface, operational variables such as fluid-dynamics and dilution rate along with the pigment content (Molina Grima *et al.* 2000; Fernandez *et al*. 2003; Hu *et al.* 2008).

Of several geometries of PBRs mentioned above the most efficient one is reported to be tubular type, which should maximize the use of solar light, to avoid large areas of shade and facilitate the diffusion of CO_2 along with the control of temperature. In this kind of configuration microalgae are maintained in circulation with turbulent flow to avoid the sedimentation and to reduce deposit in the walls of the tubes (Chisti *et al*. 2007).

Further, time-dependent changes in the culture medium temperature in every season have been predicted (Morita *et al*. 2001c) using a heat balance model of the conical helical tubular PBR previously established (Illman *et al*. 2000). Using these results, the energy required to maintain the temperature of culture medium within an appropriate range as well as the maximum and minimum culture medium temperatures has been predicted for several sites with different climate characteristics. This helps to examine the possibilities for the combinations of the microalgae used for practically higher photosynthetic production of microalgal biomass, with less operating energy consumption throughout the year at various sites. A large difference in photosynthetic productivity was caused by the difference in ambient temperature in each site, if temperature control of the culture medium was

not maintained. This helped to get practically higher photosynthetic production with less operating energy consumption throughout the year, using a combination of various strains that had different characteristics relative to temperature.

As stated by Richmond (2004) despite closed systems offer no advantage in terms of areal productivity, they largely surpass ponds in terms of volumetric productivity (8 times higher) and cell concentration (about 16 times higher). On the other hand, despite their advantages it is not expected that PBR have a significant impact in the near future on any product or process that can be attained in large outdoor raceway ponds. PBRs suffer from several drawbacks that need to be considered and solved. Their main limitations include: overheating, bio-fouling, oxygen accumulation, difficulty in scaling up, the high cost of building, operating and of algal biomass cultivation, and cell damage by shear stress and deterioration of material used for the photo-stage.

Accordingly, the choice of the most suitable system is situation-dependent, dictated by both the available species of algae and the final intended purpose. The need of accurate control impairs the use of open-system configurations, so focus has shifted mostly on closed systems.

In conclusion, PBRs and open ponds should not be viewed as competing technologies because in the opinion of researchers the real competing technology in the future will be the genetic engineering and to this field is addressed the attention of the scientific community (Gressel, 2008).

2.9 Application of microalgae

2.9.1 Biodiesel production

As the demand for diesel increases worldwide there is an increasing interest in sources other than crude oil for producing diesel fuel. Renewable fuel sources include, but are no limited to, plant oil such as corn, rapeseed, canola, soybean and algal oils, animal fats such as inedible tallow, fish oils and various waste streams such as yellow and brown greases and sewage sludges.

In Table 3 biodiesel productivities from common seed plants and microalgae are compared.

It can be seen how microalgae show a clear advantage in terms of land use because of their higher biomass productivity and oil yield. It should be also noted that although

the oil contents are similar between seed plants and microalgae there are significant variations in the overall biomass productivity and resulting oil yield and biodiesel productivity with a clear advantage for microalgae.

The common feature of these sources is that they are composed of glycerides and free fatty acids. Both of these classes of compounds contain aliphatic carbon chains having formed about 8 to 24 carbon atoms. The aliphatic chains can be fully saturated or mono, di or poly-unsaturated.

Table 3. Comparition between plants and microalgae biodiesel productivities*

Plant sources	***% seed oil content (wt in biomass)***	***Oil yield (L oil/ha year)***	***Land use (m^2 year/Kg biodiesel)***	***Biodiesel productivities (Kg biodiesel/ha year)***
Corn/Maize (*Zea mays* L.)	44	172	66	152
Hemp (*Cannabis sativa* L.)	33	363	31	321
Soybean (*Glycine max* L.)	18	636	18	562
Jatropha (*Jatropha curcas* L.)	28	741	15	656
Camelina (*Camelina sativa* L.)	42	915	12	809
Canola/Rapeseed (*Brassica napus* L.)	41	974	12	862
Sunflower (*Helianthus annus* L.)	40	1070	11	946
Castor (*Ricinus communis* L.)	48	1307	9	1156
Palm oil (*Elaeis guineensis*)	36	5366	2	4747
Microalgae (low oil content)	30	58700	0.2	51927
Microalgae (medium oil content)	50	97800	0.1	86515
Microalgae (high oil content)	70	136900	0.1	121104

*Mata *et al.*, 2010

Fatty acids are the main components of lipids and represent the chemical skeleton for biofuel production.

Research regarding biofuel from microalgae is mainly devoted to the production of biodiesel. Table 4 shows a comparison of biodiesel quality from microalgal oil and common diesel fuel.

Table 4. Comparison of biodiesel quality from microalgal oil and common diesel fuel$

Properties	***Biodiesel from microalgal oil***	***Diesel fuel***
Density Kg L^{-1}	0.864	0.838
Viscosity Pa·s	5.2 x 10^{-4} (40°C)	1.9 x 10^{-4} (40°C)
Flash point °C	65-115*	75
Solidifying point °C	-12	-50 -10
Cold filter plugging point °C	-11	-3.0 (-6.7 max)
Acid value mg KOH g^{-1}	0.374	0.5 max
Heating value MJ Kg^{-1}	41	40-45
HC ratio	1.18	1.18

* Based on data from multiple sources available in literature

$ Source: Department of Biological Sciences and Biotechnology, Tsinghua University, Beijing , China (2004)

Biodiesel is produced by a transesterification process in which triglycerides react with a monoalcohol and a catalyst (Chisti, 2007). Thus biodiesel production is extremely dependant on intracellular lipids content.

Microalgae can have lipid contents exceeding 80% by weight of dry biomass (Rodolfi *et al.*, 2009) although this is usually in the range of 15–35% and is dependent upon algae strains and growth conditions (Chisti, 2007). In Table 5 the common range of lipid content for some of the most studied microalgae is reported.

Under optimal conditions of growth, algae synthesize fatty acids principally into glycerol-based membrane lipids, which constitute about 5–20% of their dry content weight (Hu *et al.*, 2008). Unlike the glycerolipids found in membranes, the triacylglycerols (TAGs) do not perform a structural role but instead serve primarily as a storage form of carbon and energy.

Thus, one of the key criteria for selection of microalgae strains for biodiesel feedstock production is a high intracellular lipid and TAG content. Total lipids are composed of neutral lipid in the form of energy reserve bodies, as well as glyco-and phospholipids in the structural membranes. Neutral lipids are typically the major constituents of algal lipid-oil in aging or stressed cultures, mainly in the form of TAGs (Hu *et al.*, 2008; Chen *et al.*, 2008).

In this context, not all microalgae are suitable. As a matter of fact, even though some microalgae (such as *Chlorella* sp., *Botryococcus braunii*, *Nannochloris* sp., *Nannochloropsis* sp., *Schizochytrium* sp., *Nitzchia* sp., *Parietochloris incisa*) have been claimed to possess up to 30% lipids (Chisti, 2007; Satyanarayana *et al.,* 2011), the same microalgae species may present much lower percentages, depending on environmental and operational conditions applied for culturing (González-Fernández *et al*. 2010).

Additionally, not all intracellular lipids are suitable for biodiesel production (Chisti, 2007). Therefore, biodiesel production from algae not only lies on the extraction of lipids but also in the finding of high lipid content algae species (Demirbas and Demirbas, 2011).

Exploitation of microalgae for not only biodiesel production but also bioenergy generation (biomethane, biohydrogen), or combined applications for biofuels production and CO_2-mitigation, by which CO_2 is captured and sequestered, has been investigated with a growing interest from the first ‘90s and are still under research

Table 5. Mean lipid content of major investigated microalgae strains

Microalgae species	***Lipid content (% $_{w/w}$ DW)***	***Reference***
Botryococcus braunii	25.0-75.0	Meng *et al*., 2009
Chaetoceros calcitrans	14.6-16.4	Mata *et al*., 2010
Chaetoceros meulleri	30.8	Rodolfi *et al*., 2009
Chlorella emersonii	29.0-63.0	Illman *et al*., 2000
Chlorella minutissima	31.0-57.0	Illman *et al*., 2000
Chlorella pyrenoidosa	2.0	Mata *et al*., 2010
Chlorella prototechoides	50.3-57.8	Xiong *et al*., 2008
Chlorella sorokiniana	20.0-22.0	Illman *et al*., 2000
Chlorella vulgaris	28.0-58.0	Scragg *et al*., 2002
Chlorella sp.	28.0-32.0	Chisti, 2007
Chlorococcum sp.	19.3	Rodolfi *et al.,* 2009
Crypthecodinium cohnii	20	Meng *et al*. 2009
Cylindrotheca sp.	16.0-37.0	Meng *et al*. 2009
Dunaliella primolecta	23.0	Chisti, 2007
Dunaliella salina	5.0-25.0	Mata *et al*., 2010
Dunaliella tertiolecta	60.6-67.8	Takagi *et al*. 2006
Ellipsoidion sp.	27.4	Rodolfi *et al*., 2009
Haematococcus pluvialis	25.0	Mata *et al*., 2010
Isochrysis galbana	7.0-40.0	Mata *et al*., 2010
Isochrysis sp.	27.4	Rodolfi *et al*., 2009
Monallanthus salina	> 20.0	Chisti, 2007
Monodus subterraneus	39.3	Li *et al*. 2008a
Nannochloris sp.	29.9-40.3	Takagi *et al*., 2000
Nannochloropsis oculata	22.7-29.7	Chiu *et al*., 2009
Nannochloropsis sp.	35.9	Rodolfi *et al*., 2009
Neochloris oleabundans	15.9-56.0	Gouveia *et al*., 2009
Nitzschia laevis	69.1	Li *et al*., 2008a
Nitzschia sp.	45.0-47.0	Chisti, 2007
Ocystis pupilla	10.5	Mata *et al*., 2010
Parietochloris incisa	62.0	Li *et al*., 2008a
Pavlova lutheri	35.5	Rodolfi *et al*., 2009
Pavlova salina	30.9	Rodolfi *et al*., 2009
Phaeodactylum tricornutum	18.0-57.0	Mata *et al*., 2010
Porphyrodium cruentum	9.5	Rodolfi *et al*., 2009
Scenedesmus obliquus	11.0-55.0	Mata *et al*., 2010
Scenedesmus quadricauda	18.4	Rodolfi *et al*., 2009
Scenedesmus sp.	21.1	Rodolfi *et al*., 2009
Schyzochytrium sp.	50.0-77.0	Chisti, 2007
Skeletonema costatum	21.1	Rodolfi *et al*., 2009
Skeletonema sp.	31.8	Rodolfi *et al*., 2009
Spirulina maxima	4.1	Gouveia and Oliveira, 2009
Spirulina platensis	4.0-16.6	Mata *et al*., 2010
Tetraselmis sp.	14.7	Rodolfi *et al*., 2009
Tetraselmis suecica	15-23	Chisti, 2007
Thalassiosira pseudonana	20.6	Rodolfi *et al*., 2009

(Scragg *et al*., 2003; Miao and Wu 2004; Kruse *et al.,* 2005; Tsukahara and Sawayama 2005; Xu *et al*., 2006; Chisti 2007; Huntley and Redalje 2007; Li *et al*., 2007; Ono and Cuello 2007; Marker *et al*., 2009, Sayre, 2009; Wu and Xiong, 2009; Neto, 2010; Pruvost *et al*., 2011; Satyanarayana *et al*., 2011). Only a few microalgal strains are produced commercially (e.g. *Spirulina*, *Chlorella*, *Dunaliella,*

Haematococcus and *Nannochloropsis*) and some of the dominating microalgae species in biodiesel investigation field includes *Phaeodactylum tricornutum, Botryococcus braunii*, *Dunaliella tertiolecta* (Lee *et al.,* 2009), *Chlorella sp.* (Wu *et al.*, 2009), *Scenedesmus* sp., *Tetraselmis sp.* (Lee, 1997), as reported in Figure 5.

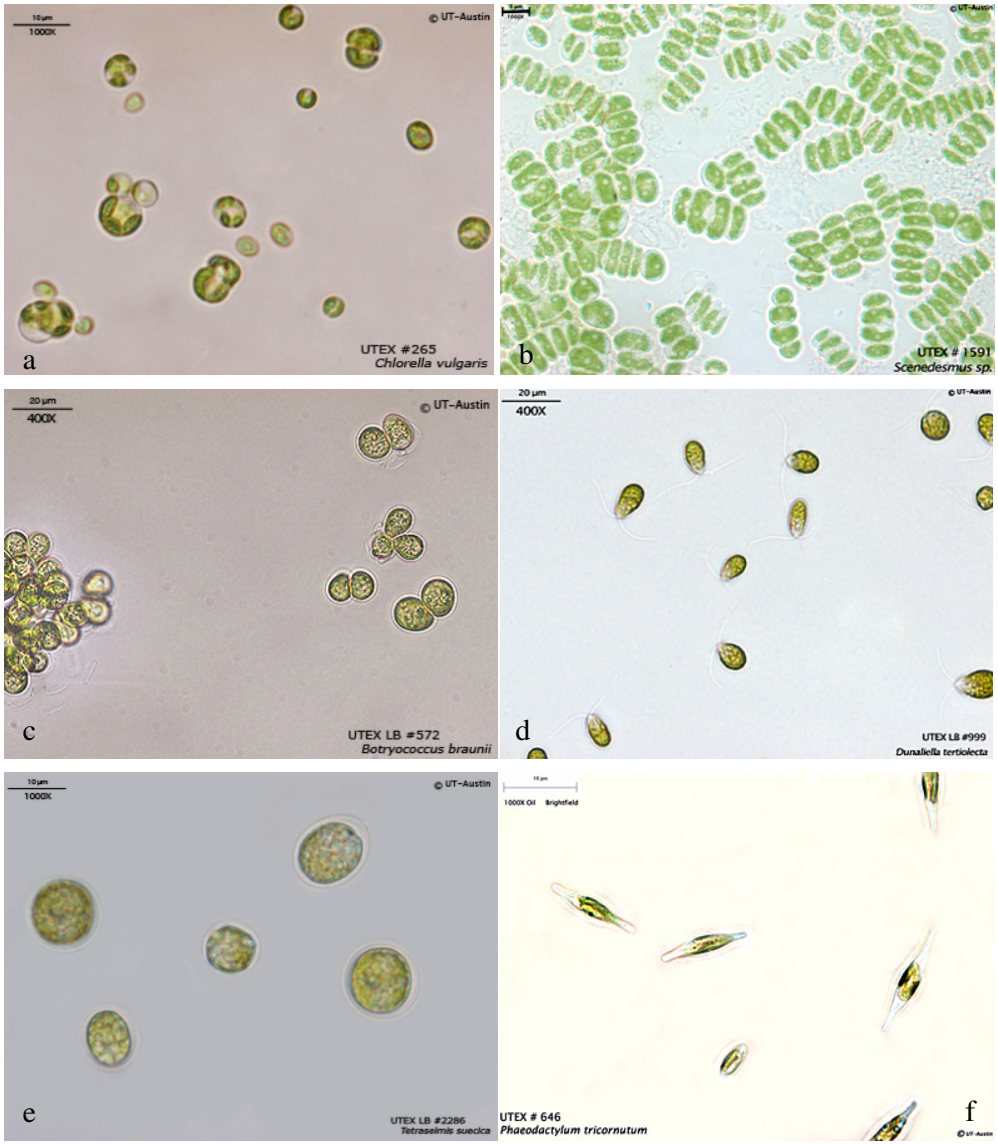

Figure 5. Some of the dominating microalgae species used in biodiesel investigation field. A) *Chlorella* sp., b) *Scenedesmus* sp., c) *Botryococcus braunii*, d) *Dunaliella* sp., e) *Tetraselmis* sp., f) *Phaeodactylum tricornutum.* All the strains here reported are depositated in the Culture Collection of Algae at the University of Texas, Austin (http://web.biosci.utexas.edu/utex/default.aspx).

These strains are probably not the best strains for the production of biodiesel. For this reason is essential to continue a screening for new strains or modify the strains such that optimal production of lipids for biodiesel becomes feasible.

The interest on biodiesel production from microalgae is also attested by some of the reported top companies in algae fuel industry: Algenol, A2BE Carbon Capture,

Aurora BioFuels, Inc., Aquaflow bionomics corporation, Blue Marble Energy, Cellana, Community Fuels, GreenShift, International Energy, LiveFuels, OilFox Argentina, Organic Fuels, OriginOil, PetroSun Biofuls, Petroalgae, Sapphire Energy, Seambiotic, Solix Biofuels, Solazyme (www.oilgae.com).

2.9.2 CO_2 fixation

Generally, phototrophic microalgal growth requires a supply of CO_2 as a carbon source. Usual sources of CO_2 for microalgae include: a) atmospheric CO_2; b) CO_2 from industrial exhaust gases (e.g. flue gas and flaring gas); 3) CO_2 chemically fixed in the form of soluble carbonates (e.g. $NaHCO_3$ and Na_2CO_3). The tolerance of various microalgal species to the concentration of CO_2 is variable; however, the CO_2 concentration in the gaseous phase does not necessarily reflect the CO_2 concentration to which the microalga is exposed during dynamic liquid suspension, which depends on the pH and the CO_2 concentration gradient created by the resistance to mass transfer.

One of the most attractive features of microalgal biomass production is the potential to fix CO_2 from the atmosphere or combustion flue gas. Atmospheric CO_2 levels [0.03% (v/v)] are not sufficient to support the high microalgal growth rates and productivities needed for full-scale biofuel production. Flue gases from power plants, which are responsible for more than 7% of the total world CO_2 emissions from energy use (Kadam, 1997), contains CO_2 at concentrations ranging from 5 to 15% (v/v) (Maeda *et al*., 1995) providing a CO_2-rich source for large-scale production of microalgae and a potentially more efficient route for CO_2 bio-fixation. Therefore, to use a flue gas emission from an industrial process unit (e.g. from fuel-fired power plants) as a source of CO_2 for the microalgae growth is envisioned to have a great potential to diminish CO_2 and to provide a very promising alternative to current GHG emissions mitigation strategies. Owing to the cost of upstream separation of CO_2 gas, direct utilization of power plant flue gas has been considered in microalgal biofuel production systems (Lackne, 2003).

Chemical analysis has shown that algal biomass consists of 40% to 50% carbon, which suggests that about 1.5 to 2.0 kg of CO_2 is required to produce 1.0 kg of biomass (Sobczuk *et al*., 2000). In 1994 the delivered cost of CO_2 was $40 to $60 per day (Becker, 1994), making algae production costly. The current emphasis on sequestering carbon from available sources such as industrial waste gas is both

economically and politically attractive. According to previous studies, the supply of carbon to microalgal mass culture systems is one of the principal difficulties and limitations that must be solved (Benemann, 1987, Oswald, 1988, Tapie and Bernard, 1988). The principal point of all considerations relating to the CO_2 budget is that, on the one hand, CO_2 must not reach the upper concentration that produces inhibition and, on the other hand, must never fall below the minimum concentration that limits growth (Rados *et al.*, 1975). These maximum (inhibition) and minimum (limitation) concentrations vary from one species to another and are not yet adequately known, ranging from 2.3×10^{-2} M to 2.3×10^{-4} M (Rados *et al.*, 1975, Lee and Hing, 1989). Fortuitously, the benefits of flue gas injection on microalgal growth were observed to be greater than the growth impacts solely attributed to inhibition of photorespiration by high CO_2 concentrations, with 30% increase in biomass productivity. This was attributed to the presence of nutrient (sulphur and nitrate) in the flue gas (Douskova *et al.*, 2009).

2.9.3 Microalgae CO_2 tolerant

A series of experiments have been carried out in the last twenty years in order to test the possibility of growing microalgae by capturing CO_2 emitted by fired power plants. Although CO_2 concentrations vary depending on the flue gas source (Kadam, 2001), 10%-20% (v/v) is typically assumed. For this reason several species have been tested under CO_2 concentrations of over 15% both using simulating and real flue gas concentrations (Ono and Cuello, 2003).

Simulating flue gas concentrations can be obtained in laboratory experiments by mixing at different flow rates filtered compressed air and pure CO_2 provided by pumps and cylinders, respectively. Many attempts have been successfully carried out, depending on strains used and cultivation conditions, with a range of CO_2 concentrations between atmospheric air and 15-20% (v/v).

One of the genus most studied, *Chlorella* sp., was widely cultivated with 15% (v/v) CO_2 concentrations since this value is recognized close to real flue gas emitted by different kind of power plant installations (Watanabe *et al.*, 1992; Negoro *et al.*, 1993; Yun *et al.*, 1997; Keffer and Kleinheinz, 2002; Lee *et al.*, 2002; Yue *et al.*, 2005; Chiu *et al.*, 2008; Chinnasamy *et al.*, 2009; Sasi *et al.*, 2011; Bhola *et al.*, 2011).

The direct use of power plant flue gas has been also considered for CO_2 sequestration systems (Benemann *et al*., 1987, Aresta *et al*., 2005). *Tetraselmis suecica* was cultivated using actual flue gas from an electric power plant (Laws and Berning, 1991), flue gas produced from a boiler was used to cultivate *Tetraselmis* sp., *Phaeodactylum* sp. and *Nanncohloropsis* sp. in Electric Power Company of some Japanese cities (Negoro *et al*., 1992,1993; Hamasaki *et al*., 1994; Kurano *et al*., 1995; Maeda *et al*., 1995; Matsumoto *et al*., 1995, 1997), two strains of *Dunaliella parva* and *tertiolecta* were tested with flue gas emitted by a combustion turbine generator on the roof of MIT's Cogeneration Power Plant at Cambridge-Boston (Vunjak-Novakovic *et al*. 2005), *Euglena gracilis* was tested with flue gas from a power plant (Chae *et al*., 2006), a series of experiments were carried out by Aquaculture Inc. in conjunction with the University of Hawaii to test 20 microalgae strains and 5 kind of flue gas emitted from a propane-fired boiler system (Nakamura *et al*. 2008). Korean researchers evaluated three microalgae, *Botryococcus braunii*, *Chlorella vulgaris* and *Scenedesmus* sp. for their carbon fixation ability to determine which organism to select for use with high levels of CO_2 for the production of biodiesel (Yoo *et al*., 2010). All the strains were submitted to real flue gas emitted by heating generator burning liquefied petroleum. The study found that the *C. vulgaris* grew in up to 10% (v/v) CO_2 with no negative effects. However, these researchers concluded that *Scenedesmus* sp. was the best of the three with regards to CO_2 mitigation due to its better CO_2 fixation ability. Also *Chlorella* sp. was tested using flue gas in Korean and Turkish facilities (Lee *et al*., 2002, Şen *et al*., 2005) and in Czech Republic municipal incinerators (Doucha *et al*., 2007, Douskova *et al*., 2009, 2010). A complex treatment of agricultural waste (including anaerobic fermentation of suitable waste, cogeneration of the obtained biogas and growth of microalgae consuming the CO_2 from biogas and flue gas) was verified for *Chlorella* sp. under field conditions in a pilot-scale photobioreactor (Kastanek *et al*., 2010).

The advantage of utilizing flue gas directly is the reduction of the cost of separating CO_2 gas. Since power plant flue gas contains a higher concentration of CO_2 (Kikkinides *et al*. 1993) identifying high CO_2 tolerant species is important. One of the highest CO_2 tolerant species is *Euglena gracilis.* Growth of this species was enhanced under 5%-45% concentration of CO_2 even if the best growth was observed with 5% (v/v) CO_2 concentration. However, the species did not grow under greater than 45% CO_2 (Nakano *et al*., 1996). Hirata *et al.* (1996a, b) reported that *Chlorella* sp. UK001

could grow successfully under 10% (v/v) CO_2 conditions. It is also reported that *Chlorella* sp. can be grown under 40% (v/v) CO_2 conditions (Hanagata *et al*., 1992). Furthermore, Maeda *et al*., (1995) found a strain of *Chlorella* sp. T-1 which was tested under a wide range of CO_2 concentrations (from 0.03% to 100%). When precultivated in 50% (v/v) CO_2 enriched medium this strain could grow slowly under 100% (v/v) CO_2, although the maximum growth rate occurred under a 10% concentration. Hanagata *et al*., (1992) have screened five green freshwater microalgae for tolerance to high CO_2 concentrations. Between them, *Scenedesmus* was found to be better able to tolerate very high CO_2 concentrations than *Chlorella*. However *Scenedesmus* could grow under 80% (v/v) CO_2 conditions while was completely inhibited by 100% (v/v) CO_2 and the maximum cell mass was observed in 10-20% (v/v) CO_2 concentrations. *Chlorococcum littorale* was found to grow with 60% (v/v) CO_2 but no growth was observed between 70-100% (v/v) even when the seed culture was grown at 60% (v/v) CO_2 (Kodama *et al*., 1993). Some attempts were made to test the ability of different *Chlorella* genus to grow in a range of CO_2 concentrations 0.03-70%. *Chlorella pyrenoidosa* was grown until 50% (v/v) CO_2 but showed its better growth with 15% (v/v) (Tang *et al*., 2011), *Chlorella* ZY-1 showed its optimum between 10-20% (Yue and Chen, 2005) and *Chlorella* KR-1 have an optimum at 15% (v/v) (Sung *et al*., 1999a, b).

In relation to the possibility of growing microalgae under pure CO_2 the first strain discovered to have this ability was the thermoacidophilic *Cyanidium caldarium* (Seckback *et al*., 1971a, b, Woodward *et al*., 1992), which was able to grow in low pH environment along with *Galdieria partita*. Watanabe *et al.* (1992) tested the ability of *Chlorella* sp. HA-1 to grow by using increasing concentrations of CO_2. They show that at the pure CO_2 condition HA-1 could not grow even if the strain still shows a green colour at the end of the experiments. Sergeenko *et al.* (2000) outlines for the first time to have successfully cultivated *C. vulgaris* in batch cultures with pure CO_2. This strain was previously cultivated in a 5% CO_2 enriched medium. Recently *Chlorella minutissima* was tested in a wide range of CO_2 concentrations (from 0.03% to 100%). It was found that this strain is able to better tolerate concentration until 40% (v/v) but after 60% (v/v) the growth is strongly reduced (Papazi *et al*., 2008). A new unknown isolated strain collected from the Johannesburg Zoo Lake was tested at 5, 10, 25, 50 and 100% (v/v) CO_2 by varying the flow rate at

20, 50 and 100 ml min^{-1}, respectively. The best yield of biomass was obtained with 100% (v/v) CO_2 at the flow rate of 50 ml min^{-1} (Kativu *et al.*, 2010).

2.9.4 Wastewater treatment

Since large scale cultivation of microalgae implies the consumption of huge amounts of nutrients, the economic feasibility of the process could be seriously compromised when fresh water is employed and synthetic reagents are used as main source of macronutrients. A possible solution to this drawback is to harness costless resources to produce the macronutrients and the water needed to perform large scale cultivation. In particular seawater can be used instead of fresh water due to its costless availability in huge amounts. Moreover, wastewater could be used as inexpensive source of nitrogen and phosphorus while at the same time flue gases could be exploited to provide the suitable CO_2 supply (Craggs *et al.*, 1997; Kim and Jeune, 2009). Besides allowing the economic viability of the process, the exploitment of such costless resources could have a positive impact on important environmental concerns since it contributes to the reduction of water pollution and consumption. Furthermore the recycling of flue gases, results in lower CO_2 emissions thus contributing to increase the environmental sustainability of industries that use fossil fuels for power generation (Zeiler *et al.*, 1995). In this scenario, the combination of the three roles of microalgae – CO_2 fixation, wastewater treatment and biofuel production – has the potential to maximize the impact of microalgal biofuel production systems.

The involvement of microalgae in aquaculture systems where cells growth is combined with biological cleaning and wastewater treatment seems to be an advantageous and promising tool for microalgal biomass production coupled with greenhouse gas mitigation (CO_2 uptake) and biodiesel production (Yun *et al.*, 1997; Kumar *et al.*, 2010b). In this way domestic wastewater can be seen a residue of interest for biofuels production from microalgae (Rawat *et al.*, 2011; Wu *et al.*, 2012). Microalgae ponds can be used in secondary or tertiary treatment. This could allow the nutrition of cells by using wastewater as sources of organic compounds, mainly nitrogen and phosphorous (Craggs *et al.*, 1997) which are the first cause of eutrophication in the aquatic environment representing a serious problem for receiving water bodies (Mulbry *et al.*, 2008).

Actually, although nitrogen and phosphorous are elements key to algal growth, they are also serious pollutants in many waterways. Algae can thrive in nitrogen- and phosphorus-rich conditions common to many wastewaters (Pittman *et al*., 2011), and this feature may be harnessed to not only remove, but also capture these important nutrients in order to return them to the terrestrial environment as agricultural fertilizer.

Use of microalgae for municipal wastewater treatment in ponds is well established (Woertz *et al*., 2009), and algae based treatment of dairy and piggery waste also has been investigated (An *et al*. 2003; Craggs *et al*., 2004; Kebede-Westhead *et al*., 2006; Travieso *et al*., 2006; Mulbry *et al*., 2008).

Additionally, microalgae can been also used for the treatment of industrial wastewater (Hodaifa *et al*., 2008; Muñoz *et al*., 2008), the removal of heavy metal from effluents and acid mine drainage (Das *et al*., 2009) and mitigate the effects of sewage effluent such as those originating from water treatment or fish aquaculture (Muller-Fuega, 2000).

Wastewater production by human activity is ubiquitous, and the continuous increase in world population, particularly in developing countries, makes it an inexhaustible resource. Because the use of microalgal biomass grown in domestic wastewaters as a human and animal food supplement faces toxicological problems, intensive research is being carried out on the promotion of lipid accumulation for further biodiesel production (Burton and Cleeland, 2008; Theodore and Wardle, 2009).

Generally, the use of wastewater could reduce nutrient addition for nitrogen and phosphorous by approximately 55% (Yang *et al*., 2011). Aslan and Kapdan (2006) used *Chlorella vulgaris* for nitrogen and phosphorus removal from wastewater with an average removal efficiency of 72% for nitrogen and 28% for phosphorus. Other widely used microalgae cultures for nutrient removal are *Chlorella* (Yun *et al*., 1997; Lee *et al*., 2001; de-Bashan *et al*., 2008), *Scenedesmus* (Martìnez *et al*., 2000, Ruiz Marin *et al*., 2010), *Spirulina* (Olguín *et al*., 2003; Kumar *et al*., 2010a), *Chlamidomonas* (Kong *et al*., 2010) species. Nutrient removal capacities of *Nannochloris* (Jimenez-Perez, 2004), *Botryococcus brauinii* (An *et al*., 2003) as well as the screening of other microalgae with such capabilities (Sydney *et al*., 2011) have also been investigated.

2.9.5 High-value added products

High-value added products from microalgae comprise an important market in which compounds such as β-carotene, astaxanthin (Wang and Chen, 2008), polyunsaturated fatty acids (PUFAs) such as Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA), and polysaccharides such as ß-glucane dominate (Reyes Suarez *et al*. 2008). Some of these fine chemicals play an important role for human nutrition while others can be also utilise in the pharmaceutical, neutracetical and cosmetic market (Pulz and Gross 2004; Spolaore *et al*. 2006, Mata *et al.,* 2010). Normally the production of these bioactive compounds demands the use of monocultures in order to better control productivity and production efficiency. For these reasons cultivation systems such as large-scale PBRs have been preferred to open ponds.

2.9.5.1 Fatty acids

Microalgae are considered as potential sources of PUFAs of more than 18 carbons (Hirabayashi *et al*., 2003; Thomas and Kumaravel; 2011) since they are commonly found in fish originating from microalgae consumed in oceanic environments (Certik and Shimizu, 1999). Between microalgal PUFAs of particular interest there are γ -Linolenic acid (GLA) (Sajilata *et al*., 2008), Arachidonic acid (AA), EPA and DHA. However, currently, DHA is the only algal PUFA commercially available (Wu *et al*., 2010). Indeed, even if the potential industrial production of EPA has been demonstrated for some species such as *Monodus subterraneus, Nannochloropsis* sp., *Crypthecodinium cohnii***,** *Porphyridium purpureum, Phaeodactylum tricornutum* and *Isochrysis galbana,* (Qiang *et al*., 1997; Chini Zitelli *et al*., 1999; Jiang *et al*., 1999; Wen and Chen, 2003) no purified algal oil is currently economically competitive with other sources (Belarbi *et al*., 2000).

2.9.5.2 Pigments and carotenoids

Among the hundreds of known carotenoids, only very few are used commercially: β-carotene (Hosseini Trafeshi and Shariati, 2006), astaxanthin (Chang *et al*., 2008; Miao *et al*., 2008) and, of lesser importance, lutein (Cerón *et al*., 2008), xanthophill (Zhang, 2009), zeaxanthin and lycopene (Del Campo *et al*., 2000). Their most important uses are as natural food colorants (e.g., orange juice) and as additive for animal feed (poultry, fish). Carotenoids also have applications in cosmetics. The

nutritional and therapeutic relevance of certain carotenoids is due to their ability to act as provitamin A, that is, they can be converted into vitamin A (García-González *et al.*, 2005; Pangestuti and Kim, 2011).

2.9.6 Human nutrition

Microalgae for human nutrition are nowadays incorporated into pastas, snack foods, candy bars or gums and beverages in different forms such as tablets, capsules and liquids. Owing to their diverse chemical properties, they can act as a nutritional supplement or represent a source of natural food colorants (Borowitzka, 1999; Apt and Behrens, 1999; Rodriguez-Garcia *et al*., 2008). The commercial applications are dominated by three main strains: *Spirulina, Chlorella* and *Dunaliella salina*. In particular, *Spirulina* is used in human nutrition because of its high proteins content and its excellent nutritive value with health-promoting effects (Desmorieux, *et al*., 1999; Rangel-Yagui *et al*., 2004).

2.9.7 Cosmetic field

Some microalgal species, mainly *Spirulina* and *Chlorella,* are established in the skin care market (Stolz and Obermayer, 2005). Microalgae extracts can be mainly found in face and skin care products (e.g., anti-aging cream, refreshing or regenerant care products, emollient and as an anti-irritant in peelers) as well as in sun protection and hair care products.

2.10 Genetic engineering

One of the most intriguing tools to improve the photosynthetic efficiency and biomass productivity of microalgae as well as the economics of biofuel production is the genetic engineering (Chisti, 2007). This emerging technology could enhance fuel production in a variety of ways including the increase of cellular lipid content and improving temperature tolerance of algae to reduce cooling expenses (Chisti, 2008). In addition, genetic engineering could increase algal cells tolerance to light saturation, photoinhibition and photooxidation (Chisti, 2007).

Genetic engineering is regarded as an attractive solution that could improve productivity and economics, but it is affected by some constraints which led scientific community to be critical regards to its applicability. Actually, it will require long-

term research and funding as well as overcoming regulations against the release of genetically modified organisms (Rodolfi *et al*., 2009).

Among the reasons to be wary of genetic engineering could be considered the following: first, increases in lipid content and other valuable cellular components are inherently constrained by cellular metabolism; second, genetically modified algae may have a variety of detrimental effects on the environment; third, genetically modified algae are not as fit as natural strains and thus unlikely to overcome competition without the aid of other agents (Pulz and Gross, 2004).

Nevertheless, genetic engineering has tremendous potential and has already achieved successes in the laboratory (León-Bañares *et al*., 2004). For example, Mussgnug et al. (2005, 2007) described experiments that altered the so called "light harvesting complexes" (LHCs) which have the ability to capture solar energy and control the flow of the excitation energy to the photosynthetic reaction centres. They also facilitate the dissipation of light energy as heat or fluorescence when irradiation exceeds photosynthetic capacity. This second trait is especially undesirable in algal bioreactors because it reduces efficiency. To resolve this issue, the authors used RNA technology to create a mutant of *C. reinhardtii* that significantly down regulated the amount of LHCI and LHCII complexes. Their experiments, which were successful, also showed that the reduction was permanent, something that had not previously been reported in literature. The strain of mutant microalga resulted in a decrease in dissipation of captured light energy, an increase in photosynthetic quantum yield, and reduced sensitivity of the system to photoinhibition.

Anastasios Melis, professor of plant and microbial biology at the University of California-Berkley, and his students are working to genetically modify green algae to enhance their capacity to generate hydrogen. They are manipulating genes to reduce the amount of chlorophyll in the chloroplast. The goal is to make individual cells absorb less sunlight, so that more light can penetrate deeper into the algal culture and let more cells use the sunlight to make hydrogen.

In particular, Melis and co-workers focused their attention on altering the optical characteristics of microalgae in order to improve solar-to-biofuels energy conversion efficiency in mass culture under bright sunlight conditions. This objective was achieved by genetically truncating the size of the LHC arrays in *C. reinhardtii* that serve to absorb sunlight in the photosynthetic apparatus. They found that 132 Chl molecules (37 for PSII and 95 for PSI) is the smallest Chl antenna size that will

permit assembly of the photosystems in chloroplasts. Such Chl antenna size configuration of the photosystems would compromise the competitive ability and survival of the cells in the wild. However, it would enable efficient solar-to-product conversion by the cells in mass culture, leading to high rates of biomass accumulation and hydrocarbon production by these microorganisms (Masuda *et al*., 2003; Polle *et al*., 2003; Tetali *et al*. 2007; Mitra and Melis, 2008; Melis, 2009).

Although the application of genetic engineering to improve biofuel production in algae is in its infancy, significant advances have been made in the development of genetic manipulation tools by many private companies such as: Synthetic Genomics (La Jolla, CA) which is engineering microalgae to continuously excrete oils from the cells and which will allow the non-invasive extraction of algal oils, Solazyme (South San Francisco, CA), Sapphire Energy (San Diego, CA), Phycal (Highland Heights, OH;), Seambiotic (Israel) and TransAlgae (Israel).

2.11 Chlorophyta

The Chlorophyta, one of the 10 recognized Algal Divisions, are commonly known as the green algae. They have green chloroplasts that are not masked by other pigments and both chlorophyll *a* and *b* are present. In addition they have β- and γ-carotene and several xanthophylls. These characteristics are very similar to higher plants and this similarity may be of significance when investigating green algae nutrient requirements. Starch is the polysaccharide storage product. Green algae as a group range in body type from non-motile single cells, to flagellates, and to colonial multicellular complexes.

Plant evolutionists believe that land plants evolved directly from a class of green algae, the Trentepohliophyceae. In addition to this class, Division Chlorophyta contains nine other Classes: Prasinophyceae, Ulvophyceae, Cladophorophyceae, Briopsidophyceae, Zygnematophyceae, Klebsormidiophyceae, Dasycladophyceae, Charophyceae, and Chlorophyceae. Most of the Order Chlorophyceae within the Class Chlorophyceae lives in fresh water. There are about 355 genera that include 2650 species in the Order.

During the past decades extensive collections of microalgae have been created by researchers in different countries. Some examples are: the freshwater microalgae collection at the University of Coimbra (ACOI) in Portugal considered one of the world's largest, having more than 4000 strains and 1000 species, the Sammlung von

Algenkulturen Collection at the University of Gottingen (SAG) and the Culture Collection of Algae at the University of Cologne (CCAC) in Germany, the Pasteur Culture Collection of Axenic Cyanobacterial Strains (PCC) and the Caen-ALGOBANK (AC) in France, the Culture Collection of Algae and Protozoa (CCAP) in Scotland, the Culture Collection of Autotrophic Organisms (CCALA) in Czech Republic, the Scandinavian Culture Collection of Algae and Protozoa at the University of Copenhagen (SCCAP) in Denmark, the Collection at the University of Texas (UTEX) and the American Type Culture Collection (ATCC) in USA, the Canadian Phycological Culture Centre (CPCC) in Canada, the National Institute for Environmental Studies (NIES) in Japan and the Australian National Algae Culture Collection (ANACC). These collections attest to the large variety of different microalgae available to be selected for use in a broad diversity of applications, such as value added products for pharmaceutical purposes, food crops for human consumption and as energy source.

To the Class of Chlorophyceae belong the two strains objects of this study: *Chlorella vulgaris* and *Nannochloris eucaryotum.*

Both species, which are genetically related, are unicellular nonmotile coccoid (round) cells, typically 2 to 12 μm. They can live in freshwater, seawater or on soil and are easy to grow, making them useful in physiological and biochemical laboratory studies.

While *Chlorella vulgaris* is a well studied and documented specie starting form 50's (Tamiya, 1957), *Nannochloris eucaryotum* is a relatively new isolated strain (Whilhem and Wild, 1982).

2.11.1 Nannochloris eucaryotum

Nannochloris eucaryotum was identified for the first time in 1982 by Wilhem *et al.* and named *Nanochlorum eucaryotum.* In that year they published two articles in the journal "Zeitschrift für Naturforschung" showing the characteristics of this new isolated marine microalgae. They outlined its unusual metabolic and physiological behaviour despite its particular reduced size and genetic sequence. These findings were also confirmed two years later by Zahn (1984). In particular Wilhem *et al.* focused their attention on its growth in different conditions of salinity. In 1987 Geisert *et al.* investigated the effect of nutrients content on the profile of its

carotenoids with particular emphasis on the production of sporopollenin (a heteropolymer made up of fatty acids, phenols and carotenoids).

In 1988 two articles, Oed *et al*. and Sargent *et al*. pointed out how the classification of this microalgae inside the genus *Nanochlorum* was unclear. Actually this strain showed a high similarity of its genetic sequences to these of the genus *Chlorella* and for this reason for many years it was considered as a substrain of that genus. On the other hand, it was hard to explain its metabolism since some of the genetic sequences inside the *Chlorella* genus were not found inside its genes. These findings lead the researchers to believe that *Nanochlorum* was a real new strain probably filogenetic related in the past to the genus *Chlorella*. By considering all these informations, Menzel and Wild in 1989 assessed a comparative investigation of some *Nannochloris* species and clearly found out that *Nanochlorum eucaryotum* belonged to a new genus. Starting to 1989 the name of this strain has been accepted as *Nannochloris eucaryotum* even if little modifications of its name has been used as synonyms such as *Nanochlorum eucayotum* (Derenne *et al*., 1992a, b), *Nanochlorum eukaryotum* (Schreiner *et al*. 1995) and *Nannochloris eucaryota* (Tschermack-Woess, 1999). Currently four synonims of this strain (*Nannochloris eucaryotum, Nanochlorum eucayotum*, *Nannochloris eucaryota, Nannochloris eucaryota)* are accepted as reported in the official taxonomy browser of the National Center of Biotechnology Information (NCBI). In 2004 Henley *et al*. carried out a complete phylogenetic analysis of the '*Nannochloris*-like' algae with particular emphasis on the strain *Picochlorum oklahomensis* genus and specie. They found out that *Nannochloris eucaryotum* showed a high genetic similarity to the genus *Picochlorum* and for this reason a new synonym *Picohlorum eucaryotum* was selected for the strain. According to the NCBI and to the algaebase, the largest algae database in the world with about 130.000 names of strains, the authority names currently accepted for this strain are *Nannochloris eucaryotum* and *Picochlorum eucaryotum.* This strain is reported to be characterized by a high adaptability to extreme environmental conditions such as high salinity, low irradiance, elevated CO_2 and decreased O_2 levels (Geisert *et al*., 1987).

Over the last thirty years, from its discovery onwards, only 25 articles have been published on the specie *Nannochloris eucaryotum* (including all its variants of name). Amongst them, 15 are focused on the field of taxonomy, philogenetics and genetics trying to clarify the exact identity of this microalgae and its position inside the genus. Few articles have provided some informations about its growth kinetics (Wilhem *et*

al., 1982a,b; Zahn, 1984; Huesemann *et al.*, 2003), carotenoids content (Geisert *et al.*, 1987); constituents of its outer wall (Derenne *et al.*, 1992a,b; Krienitz *et al.*, 1999), life cycle (Tschermack-Woess, 1999; Yamamoto and Nozaki, 2001), effect of a produced substance on the cell lysis of a red tide organism (Perez, 2001; Perez and Martin, 2001) and marine ecology (Li *et al.*, 2008).

It should be pointed out that, except the papers of Wilhem *et al.*, 1982, Geisert *et al.* 1987 and Huesmann *et al*, 2003, in the literature there are not informations about the optimization of *Nannochloris eucaryotum* culture conditions, its nutrients requirement, lipid content, fatty acid composition and cultivation in batch photobioreactors.

2.11.2 Chlorella vulgaris

As cosmopolitan genus, the growth physiology relative to major nutrient elements N, P, K, Mg, and S of *Chlorella* species was studied in the late 50's using a synchronous culture technique. The life cycle of these microalgae was studied by Hase *et al.* (1957) which divided it into seven stages and investigated the role played by nutrients deficiency on growth retardation and stages division.

C. vulgaris has been extensively studied for various purposes, such as the production of biomass as a source of valuable chemicals or heath foods, aquaculture feed (Amini Khoeyi *et al.,* 2011), wastewater treatment (Sydney *et al.*, 2011), CO_2 uptake in photobioreactors (Hulatt *et al.* 2011). Sasi *et al.* (2011) have developed a photobioreactor to optimize the growth rate of *C. vulgaris* by outlining that light intensity, CO_2 concentration and the application of a dark phase are important growth limiting factors. This strain seems to be an ideal candidate due to its easy cultivation in a relatively low-priced media without the necessity of utilizing very specific compounds (Šoštaric *et al.,* 2009). It is also known as one of the fastest growing microalgae (Becker, 1994) with an average lipid content of 14–30% by weight of dry biomass (Illman *et al.*, 2000; Spolaore *et al.*, 2006) and a reasonable amount (14-22%) of triglycerides (Becker, 1994). Studies looking at algae to be used for animal feed found those harvested in the late-logarithmic growth phase were 30-40% protein, 10-20% lipids, and 5-15% carbohydrates. At other growth phases, these percentages can vary considerably.

For all these reasons *C. vulgaris* is commonly used for large scale algal production (Hulatt *et al*., 2011). That *Chlorella* is a good choice for biodiesel production is also a

conclusion reached by Mata *et al.* (2010) in their extensive review of microalgae and biodiesel production. They found lipid content measured as percent dry weight biomass ranged from 5.0% to 58.0%, lipid productivity as mg L^{-1} d^{-1} from 11.2 to 40.0, and biomass productivity as g L^{-1} d^{-1} from 0.02 to 0.20 for *C. vulgaris*. This strain is also reported to grow in heterotrophic and mixotrophic (combining auto- and heterotrophic) conditions as well as the typical autotrophic condition.

Chapter 3.

Effect of medium composition

on the growth of *Nannochloris eucaryotum*

in batch photobioreactors

3.1 Introduction

A supply of secure, equitable, affordable and sustainable energy is vital to future prosperity (Hall *et al*., 2001). Approximately 30% of final consumer energy is used for transport and therefore is mainly derived by fossil fuels. Thus, CO_2 emissions produced to meet this demand account for substantial amounts of total global emissions (IPCC). For this reason, today there is a rising interest to biofuels as one of most attractive resource of energy, with particular emphasis on transportation. Their environmental benefits, i.e., no net increased release of carbon dioxide and very low sulfur content that could be cut down by 10%, represent the main advantages to be considered (Cadenas and Cabezndo, 1998; Huang *et al*., 2010). By taking their renewable origin into account, biofuels have a significant economical potential as compared to non-renewable fossil fuels whose market prices will definitely increase in the future (Huang *et al*., 2010). However, their high cost and the controversial matter of land competition for food are the main limitations to widespread their commercialization. Actually, biofuels may become a viable alternative and survive in the market, if and only if they could economically compete with standards ones. It is also well known that the end cost of biofuels mainly depends on the market price of the feedstock that is responsible of 60-75% of biofuels total cost (Mata *et al*., 2010). This percentage depends upon of the cost of the fats and oils used in its production, which could be minimized by using cheaper oils from non-edible sources (Vasudevan and Fu, 2010).

Process engineering to produce bio-oils from microalgae is an emergent area for industrial practice with great promise in order to partially replace petroleum-derived fuels and biofuels from oil crops (Chisti, 2007). Industrially scaling-up of microalgal oil production has the potential to reduce the arable land necessary worldwide to replace the current fuel demand using biofuels (Sheehan *et al*., 1998). However, limitations of organism survival, growth and lipid content, carbon dioxide enrichment, light penetration, seasonability, harvest and biosafety of transgenic microalgae are the main barriers to the industrial production of microalgae-derived bio-oils (Francisco *et al*., 2010). Therefore, great R&D efforts are currently undertaken to produce biodiesel at competitive costs and with the required quality starting from microalgae feedstock. In particular, as recently pointed out by Debska *et al*. (2010), given the potential benefits of microalgae, their cultivation should be studied and optimized to make them competitive as fuel producing systems in the global market. Along these lines, a novel

process that makes use of CO_2 from industrial plants and microalgae cultivation from which bio-oil may be extracted has been recently patented (Cao and Concas, 2008, 2010). The process allows also the production of green coal, as well as useful compounds in the food, biomedical, cosmetic, and bio-technical industry.

Nowadays more than 30.000 different strains of microalgae have been isolated. In spite of this high number, only few of them are potentially exploitable at the industrial scale. Among these ones, the small unicellular marine eukaryotic green alga *Nannochloris eucaryotum* (Menzel and Wild, 1989) [also known as *Nannochlorum eucaryotum* (Wilhelm and Wild, 1982) or *Picochlorum eucaryotum* (Henley *et al*., 2004)] shows a high adaptability to extreme environmental conditions such as high salinity, low irradiance, elevated CO_2 and decreased O_2 levels (Geisert *et al*., 1987). It has been also found that the lipid content of *Nannochloris* genus can be increased up to about 56% (Negoro *et al.*, 1991) Thus, such microalga species represents a suitable candidate for large-scale biomass production, including its potential exploitation in the patented process (Cao and Concas, 2008, 2010).

Along these lines, the key factor is represented by the full operability of large photobioreactors capable of high biomass productivity. In this regard, it is well known that one of the major factors affecting the growth rate of microalgae is the culture medium being used. Although exhaustive broad-scale optimization studies are prohibitively time-consuming and costly, condition-specific medium analysis serves as a preliminary step to process improvement (Debska et al., 2010). The effect of medium composition and nutrient starvation on the growth of *N. eucaryotum* was then investigated for the first time in this work using batch photobioreactors with the aim to gain information useful for the process optimization. It should be noted that, to the best of our knowledge, these aspects have not been addressed in the literature as far as this microalga strain is concerned. The results here reported may then help the design and development of *N. eucaryotum*-based processes. In fact, the determination of nutrient levels in the medium, or any restriction associated with them, which are capable to affect the growth rate of cells during cultivation, represents a first step towards the increase of cultures productivity, and hence the improvement of the economics of microalgae-derived fuel production (Debska *et al*., 2010).

3.2 Materials and methods

3.2.1 Microorganism

The marine algal strain *Nannochloris eucaryotum* (strain N° 55.87) obtained from the Sammlung von Algenkulturen at the University of Göttingen (SAG), Germany, was considered in this work. Stock cultures were propagated and maintained in Erlenmeyer flasks with a Brackish Water Medium (BWM) whose composition is reported in Table 6. Other culture conditions were temperature of 25°C, a photon flux density of 98 μmol m^{-2} s^{-1} provided by four 15 W white fluorescent tubes, and a light/dark photoperiod of 12 h. Flasks were continuously shaken at 100 rpm (Universal Table Shaker 709, ASAL Srl, Cernusco sul Naviglio, (MI), Italy).

3.2.2 Culture conditions

Growth experiments were conducted in 150-ml Erlenmeyer flasks and 250-ml Pyrex bottles in contact with atmospheric air via open culture conditions and stirring. Cultures were maintained at room temperature and under a photon flux density of 84 μmol m^{-2} s^{-1} provided by three 15 W white fluorescent tubes and a light/dark photoperiod of 12 h. The culture media volumes were 75 ml and 200 ml for Erlenmeyer flasks and bottles, respectively. Both flasks and bottles were agitated by a magnetic stirrer at 500 rpm by means of magnetic PFTE stir bars (6 mm diameter and 30 mm length). Flasks, bottles, and magnetic stir bars, as well as culture media were sterilized in autoclave at 121°C for 20 min prior to microalgae inoculation. The initial pH of the culture medium was adjusted to 7.0 by using 1 M NaOH or 1 M HCl aqueous solutions depending on the culture medium. The initial cell concentration in each experiment was about 0.1 g L^{-1}.

3.2.3 Culture media

The culture media were prepared by considering the following media and components:

- Natural seawater (Mediterranean sea, lat. 39° 11' N - long. 09° 10' E) centrifuged at 4000 rpm for 15 min (Thermo Fisher Scientific Inc. Waltham, MA, USA) and then filtered by means of 0.45 μm filter (Millipore Corporation, Bedford, MA, USA);
- De-ionized water;
- Soil extract;

- Macronutrients aqueous solution (KNO_3 10 g L^{-1}; K_2HPO_4 1 g L^{-1}; $MgSO_4 \bullet 7H_2O$ 1 g L^{-1});
- Micronutrients aqueous solution (H_3BO_3, 2.86 g L^{-1}; $MnCl_2 \cdot 4H_2O$, 1.81 g L^{-1}; $ZnSO_4 \cdot 7H_2O$, 0.222 g L^{-1}; $CoCl_2 \cdot 6H_2O$, 0.035 g L^{-1}; $CuSO_4 \cdot 5H_2O$, 0.08 g L^{-1}; $Na_2MoO_4 \cdot 2H_2O$, 0.230 g L^{-1}; EDTA-Na_2, 29.754 g L^{-1}; $FeSO_4 \cdot 7H_2O$, 24.9 g L^{-1})
- Vitamin B_{12}.

Microalgae growth was investigated in six different culture media, hereafter named A to F, which represent modifications of BWM. The composition of each culture medium is reported in Table 6. It should be noted that the amount of inorganic and organic compounds dissolved in the natural seawater as well as in the soil extract was not determined.

Table 6: Composition of culture media investigated in this work[a]

Component	units	BMW	A	B	C	D	E	F
KNO_3	[g L^{-1}]	$2.0 \cdot 10^{-1}$	-	-	-	$2.0 \cdot 10^{-1}$	$2.0 \cdot 10^{-1}$	$2.0 \cdot 10^{-1}$
K_2HPO_4	[g L^{-1}]	$2.0 \cdot 10^{-2}$	-	-	-	$2.0 \cdot 10^{-2}$	$2.0 \cdot 10^{-2}$	$2.0 \cdot 10^{-2}$
$MgSO_4*7H_2O$	[g L^{-1}]	$2.0 \cdot 10^{-2}$	-	-	-	$2.0 \cdot 10^{-2}$	$2.0 \cdot 10^{-2}$	$2.0 \cdot 10^{-2}$
H_3BO_3	[g L^{-1}]	$2.86 \cdot 10^{-3}$	-	-	-	$2.86 \cdot 10^{-3}$	$2.86 \cdot 10^{-3}$	$2.86 \cdot 10^{-3}$
$MnCl_2*4H_2O$	[g L^{-1}]	$1.81 \cdot 10^{-3}$	-	-	-	$1.81 \cdot 10^{-3}$	$1.81 \cdot 10^{-3}$	$1.81 \cdot 10^{-3}$
$ZnSO_4*7H_2O$	[g L^{-1}]	$2.22 \cdot 10^{-4}$	-	-	-	$2.22 \cdot 10^{-4}$	$2.22 \cdot 10^{-4}$	$2.22 \cdot 10^{-4}$
$CoCl_2*6H_2O$	[g L^{-1}]	$3.5 \cdot 10^{-5}$	-	-	-	$3.5 \cdot 10^{-5}$	$3.5 \cdot 10^{-5}$	$3.5 \cdot 10^{-5}$
$CuSO_4*5H_2O$	[g L^{-1}]	$8.0 \cdot 10^{-5}$	-	-	-	$8.0 \cdot 10^{-5}$	$8.0 \cdot 10^{-5}$	$8.0 \cdot 10^{-5}$
$Na_2MoO_4*2H_2O$	[g L^{-1}]	$2.3 \cdot 10^{-4}$	-	-	-	$2.3 \cdot 10^{-4}$	$2.3 \cdot 10^{-4}$	$2.3 \cdot 10^{-4}$
EDTA-Na_2	[g L^{-1}]	$2.98 \cdot 10^{-2}$	-	-	-	$2.98 \cdot 10^{-2}$	$2.98 \cdot 10^{-2}$	$2.98 \cdot 10^{-2}$
$FeSO_4*7H_2O$	[g L^{-1}]	$2.49 \cdot 10^{-2}$	-	-	-	$2.49 \cdot 10^{-2}$	$2.49 \cdot 10^{-2}$	$2.49 \cdot 10^{-2}$
Vitamin B_{12}	[µg L^{-1}]	5	-	-	-	-	-	5
Soil extract	[ml L^{-1}]	30	-	-	32	-	30	-
Seawater	[ml L^{-1}]	469	1000	503	487	469	455	469

[a] Amounts of inorganic and organic compounds added with soil extract or seawater have not been taken into account.

3.2.4 Biomass concentration and pH measurements

The growth of microalgae was monitored through daily measurements of the culture media optical density (*OD*) (Genesys 20 spectrophotometer, Thermo Fisher Scientific Inc. Waltham, MA, USA) at 560 nm wavelength (D_{560}) with 1 cm light path. The biomass concentration (*X)* was calculated from OD measurements using an *X* vs. *OD* calibration curve. The latter one was obtained by gravimetrically evaluating the biomass concentration of known culture medium volumes which were previously

centrifuged at 4000 rpm for 15 min and dried at 105°C for 24 h. pH was daily measured by pHmeter (KNICK 913, Bodanchimica S.r.l., Cagliari, Italy). For the sake of reproducibility, each experimental condition was investigated at least in triplicate. The average and standard deviation values of the experimental results were calculated using OriginPro 6.1®.

3.3 Results and discussion

3.3.1 Effect of Brackish Water Medium constituents.

N. eucaryotum was batch cultured to investigate the effect of the BWM constituents in 150-ml Erlenmeyer flasks using the six different media described in the experimental section (Table 6). The comparison among the different culture media was performed in terms of growth rate of microalgal cells. The latter one was defined by assuming that the rate of increase of microalgal cell mass is a function of the cell mass only, according to the well know *Malthus' law* (Bailey and Ollis, 1986):

$$\frac{dX}{dt} = \mu X \qquad (1)$$

where X represents the cell mass concentration [g L^{-1}], t is the time [h], and μ the growth rate [h^{-1}], which is typically a function of nutrients concentration, pH, temperature, light, and culture conditions. The average growth rate $\overline{\mu}$ is defined as follows:

$$\overline{\mu} = \frac{1}{\tau}\int_{o}^{\tau} \mu \, dt \qquad (2)$$

where τ [h] is the total time of cultivation. By integrating Eq. (1) with the initial condition $X = X_0$ at $t = 0$, and using Eq. (2), the following relationship between the microalga mass concentration and the average growth rate is obtained

$$\overline{\mu} = \frac{1}{\tau}\ln\left(\frac{X_\tau}{X_0}\right) \qquad (3)$$

where X_τ is the biomass concentration at $t = \tau$, which was set equal to 360 h in this work. Results of *N. eucaryotum* growth in the different culture media investigated are summarized in Figure 6. It can be observed that this microalga grows very slowly in the medium *A*, *B*, and *C*, while a significantly averaged higher growth rate is obtained when medium *D*, *E*, and *F* are used, respectively. The slight lower growth rate measured for the case of medium *B* can be related to the reduced concentration of the

nutrients contained in this medium, i.e., inorganic salts dissolved in the seawater, with respect to medium *A*. On the other hand, the use of medium *C* obtained by adding soil extract to medium *A* slightly improved the growth rate, which significantly increases when the inorganic salts solution is added to the culturing broth (medium *D*). The comparison between media *D*, *E*, and *F* reveals that the soil extract addition (medium *E*) slightly contributes to the increase of the growth rate of *N. eucaryotum*, while the effect of vitamin B_{12} (medium *F*) is negligible.

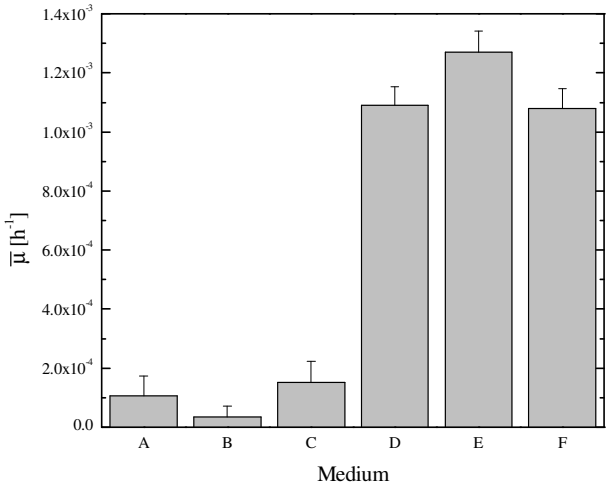

Figure 6. Growth rate of *N. eucaryotum* in different culture media: A, seawater; B, diluted seawater; C, soil extract enriched diluted seawater; D, soil extract- and vitamin-depleted BWM; E, vitamin-depleted BWM; F, soil extract-depleted BWM. The average and standard deviation values of each datum were obtained from three independent cultures.

According to the findings presented above, only results obtained using media *D*, *E*, and *F* will be considered in the sequel. Figure 7 shows the time evolution of pH during the cultivation of *N. eucaryotum* in the media *D*, *E*, and *F*, respectively. It can be clearly seen that the presence of soil extract and vitamin B_{12} does not significantly affect the culture pH, which continuously increases during the microalgae growth up to about 8.6. The analysis of the experimental results reported in Figures 6 and 7 shows that under the adopted experimental conditions the growth rate of *N. eucaryotum* is influenced mostly by the inorganic salts concentration while the effect of the other nutrient sources, such as soil extract or vitamin B_{12}, is small or negligible, respectively.

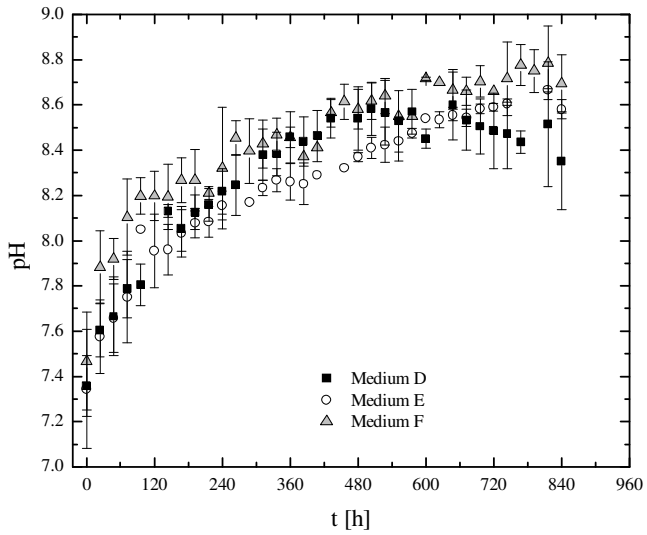

Figure 7. pH time profile measured during *N. eucaryotum* cultivation (D, soil extract- and vitamin-depleted BWM; E, vitamin-depleted BWM; F, soil extract-depleted BWM). The average and standard deviation values of each datum were obtained from three independent cultures.

3.3.2 Effect of the initial concentration of potassium nitrate

A series of batch experiments in 250-ml bottles was carried out with the medium *E* (cf. Table 6) to evaluate the effect of nitrogen starvation on the growth of *N. eucaryotum* by varying its initial content in the culture medium. More specifically, the initial concentration of potassium nitrate in medium *E* was reduced in three different experiments from $N_0 = 2\ 10^{-1}$ g L^{-1} to one half and one fourth of N_0 while the initial concentration of the other nutrients were maintained at the values reported in Table 6. The results of this study are shown in Figure 8, where it can be seen that growth curves without lag phase were obtained regardless the initial concentration of nitrate. It can be also observed that for the case $N = N_0$ the selected microalga exponentially grows up to the end of the cultivation time (t = 840 h). Since microalga growth is clearly responsible of nitrogen depletion, it can be stated that in this case the growth rate is not significantly affected by the diminishing nitrate concentration. On the other hand, a different behavior is shown by *N. eucaryotum* when N is reduced to 1/2 and 1/4 of N_0. Specifically, the growth curve presents an inflection point after about 360 h of cultivation for both the experiments. In the case of $N = 1/2\ N_0$, the growth rate decreases after 360 h so that the growth curve approaches a stationary phases when t equals about 720 h.

When the initial concentration of nitrate was reduced to 1/4 of N_0, the growth curve reaches a stationary phases at *t* equals about 420 h. However, a slight increase in biomass concentration follows the stationary phase. This finding may be explained by recalling that microalgae cells can grow using the organic matter produced by the old died cells as nitrogen source.

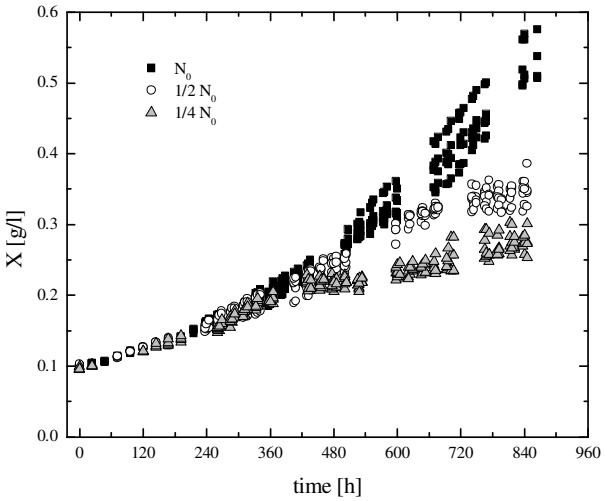

Figure 8. Biomass concentration (X) versus cultivation time for three cultures of *N. eucaryotum* at different initial nitrate concentration (N_0 corresponds to the initial concentration of potassium nitrate in medium E, i.e. 2 10^{-1} g L^{-1}).

At the end of the stationary phase living cells might change their metabolism to reduce the nitrogen requirements, thus making the organic nitrogen sufficient for their growth. A similar result was obtained by Debska *et al*. (2010) during cultivation of *Chlorella vulgaris*. They concluded that *C. vulgaris* can utilize internal reserves of nitrogen, hence maintaining growth after external sources are depleted.

Figure 9 shows the effect of the initial nitrate concentration on medium pH during *N. eucaryotum* growth.

No significant difference in the pH time profiles was observed during the exponential growth (cf. Figure 9) for the different nitrate concentrations.

However, for the cases N = 1/2 N_0 and N = 1/4 N_0 a decrease of pH can be seen once the culture reaches the stationary phase. This is probably due to the increase of dissolved CO_2 related to its lower consumption by microalgae during such growth phase.

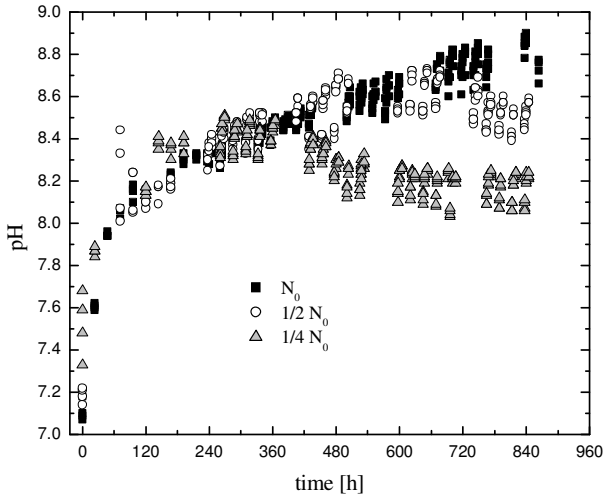

Figure 9. pH of the medium versus cultivation time for three cultures of *N. eucaryotum* at different initial nitrate concentration (N_0 corresponds to the initial concentration of potassium nitrate in medium E, i.e. 2 10^{-1} g L^{-1}).

3.3.3 Effect of the initial concentration of potassium phosphate

With the aim to investigate the effect of phosphorus starvation *N. eucaryotum* was cultivated in medium E by reducing its initial concentration of phosphate to 1/2 and 1/4 of P_0, while the initial concentration of other nutrients remains the same of those ones reported in Table 1 for each experiment. The results of this investigation in terms of biomass concentration as a function of time are shown in Figure 10. For all the initial phosphate concentrations the growth curves do not present lag phase. It can be also seen that the growth of *N. eucaryotum* do not significantly change when the initial concentration of phosphate is reduced to 1/2 of P_0. This result reveals that the selected microalga is less sensitive to phosphorus content than to nitrogen one. Indeed, microalgal biomass exponentially grows for the entire length of the cultivation. On the other hand, when the initial P content was further reduced to 1/4 of P_0, the cells mass concentration exponentially increased only during the first 360 h of cultivation. A stationary phase was then reached, followed by an approximately linear increase of biomass with time.

The cells growth occurring at the end of the stationary phase can be related to the ability of microalgae to change their nutrients requirements. In fact, the rate of phosphorus consumption depends on its concentration in the medium as well as the corresponding intracellular content (Kaplan *et al.*, 1986). Therefore, the stationary phase can be regarded as a period during which microalgal cells are getting adapted to a low phosphorus content environment. It should be also mentioned that the

microalgae can grow at the expense of their internal phosphorus reservoirs (Martinez *et al.,* 1999).

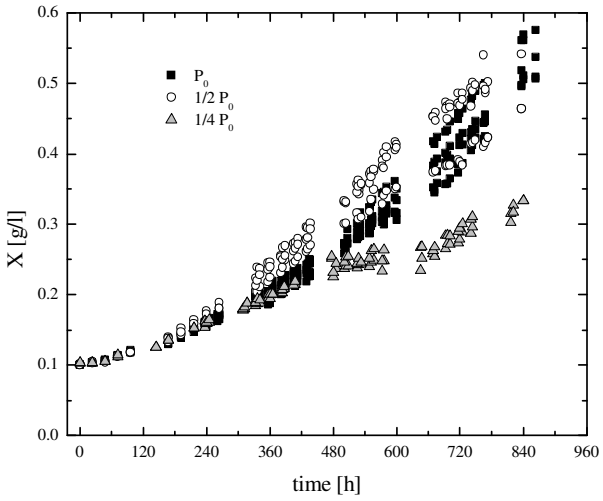

Figure 10. Biomass concentration (X) versus cultivation time for three cultures of *N. eucaryotum* at different initial phosphate concentration (P_0 corresponds to the initial concentration of potassium phosphate in medium E, i.e. 2 10^{-2} g L^{-1}).

3.3.4 Effect of pH

A series of batch experiments were performed to investigate the effect of pH on growth of *N. eucaryotum*. The latter one was cultivated in 200 ml of medium *E* contained in Pyrex bottles. pH was measured six times per day and adjusted to the desired value by means of additions of 0.5 M NaOH and HNO_3 aqueous solutions. These experiments were conducted for 360 h since it was previously shown that during this time interval the microalgal growth is not significantly affected by nutrients concentration. The remaining experimental conditions are the same as those ones reported in the experimental section. In order to make a comparison in terms of growth rate of microalgal cells the integral method (Fogler, 2004) was adopted. Specifically, by assuming that μ remains constant during the exponential growth phase, Eq. (1) can be integrated with the initial condition $X = X_0$ at $t = 0$ to give the following relationship between the microalgae mass concentration and time:

$$\ln\left(\frac{X}{X_0}\right) = \mu t \qquad (4)$$

The temporal profile of biomass concentration can be then linearly fitted through Eq. (4) to obtain the growth rate μ. The experimental data along with the linear fitting results for each value of pH investigated are reported in Figure 11. It can be seen that the growth rate μ increases as the pH of the medium increases. Specifically, the growth rate was $9.85\pm0.54\bullet10^{-4}$ h^{-1}, $2.03\pm0.03\bullet10^{-3}$ h^{-1}, and $2.86\pm0.06\bullet10^{-3}$ h^{-1}, when pH was controlled at 6.60±0.67, 8.29±0.34, and 8.47±0.53, respectively. The regression coefficient was higher than 0.9, thus confirming that during the investigated time interval the variation of nutrients concentration and of light absorbance taking place do not affect the growth rate significantly. This finding can be explained by recalling that the carbon dioxide dissolved in the culture medium is the main nutrient of microalgae since it is photo-synthetically converted to biomass. Since phase and chemical equilibria of CO_2 with water states that the total concentration of inorganic carbon dissolved in the culture media increases as pH increases, the results shown in Figure 11 can be explained by considering the availability of inorganic carbon for the microalgae growth which increases as pH is augmented.

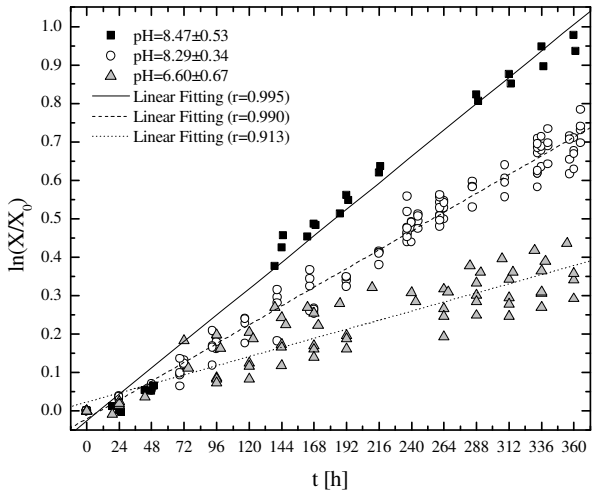

Figure 11. Left-hand side of equation (4), i.e., $ln(X/X_0)$, versus cultivation time at different pH of the medium. The average and standard deviation values of each datum were obtained from three independent cultures.

It should be mentioned that the finding described above is substantially different from the one obtained by Geisert *et al*. (1987). In fact, these authors found that the optimal pH for *N. eucaryotum* growth was in the range between 5 and 7, even though cell growth was found significantly also at pH equal to 4 and 9, respectively. It is important to note that pH have a very strong effect on microalgae growth. A possibile

explanation of such experimental result might be due to the fact that alkaline pH might favour the enzymatic activity of RuBisCO. This enzyme is involved in the first major step of carbon fixation during the photosynthesis, a process by which atmospheric CO_2 is converted to energy-rich molecules such as glucose. In particular alkaline pH and Mg^{2+} are needed to favour the formation of the so-called carbamate (a molecule of lysine plus a molecule of CO_2 which represent the active site of the RuBisCo). As microalgae grow by photosynthesis there is an increase of the pH in the culture medium and the more alkaline the pH is the more the activity of RuBisCo is favoured in order to fix CO_2 and produce sugars (Lodish *et al.*, 2000).

3.4 Concluding remarks

In this work, the effect of medium composition and nutrient starvation on the growth of *N. eucaryotum* in batch photobioreactors was investigated for the first time. A modified Brackish Water Medium (BWM) and atmospheric air (0.03% v/v CO_2) were used. Specifically, the algae growth was first analyzed by using a culture medium where some of the BWM components (inorganic nutrients, soil extract, and vitamin B_{12}) have been eliminated. Then, the starvation effect in terms of nitrate and phosphate concentrations was addressed. It was found that when only natural seawater is used as cultivation medium, the growth was inhibited. On the other hand, the algae growth did not result significantly affected by neither soil extract removal nor vitamin B_{12} addition. To study the effect of starvation in terms of nitrate and phosphate contents, the latter ones were reduced by two and four times with respect to the BWM composition, respectively. The obtained results show that the algae growth is affected by the nitrate concentration reduction. The same results holds true also only when the phosphate concentration is reduced four times. Finally, the effect of pH medium on growth rate is investigated.

Although the presented results are based on lab-scale mechanically agitated flasks and bottles experiments, they may provide useful information of the effects of medium composition on *N. eucaryotum* growth in view of its exploitation to large-scale photobioreactors. In particular, during future stages of process development, economic and operational studies will have to be performed by taking advantage of the results obtained in this work.

Indeed, in order to make the microalgal-derived biofuels economical with respect to conventional ones, the cost of microalgae cultivation should be reduced as much as possible. Along this line, the most intuitive processing option would be to cultivate microalgae in natural waters without any nutrients addition. Moreover, marine strains would be preferred since seawater is cheaper and easier to be obtained with respect to fresh water. This is particularly true in arid or semi-arid regions, which are the most suitable areas for microalgae cultivation due to their higher temperature and radiation.

However, the case of *N. eucaryotum* investigated in this work demonstrates that the target above is not guaranteed. The choice of marine microalgae may not be sufficient to prevent the need to enrich the culture medium with inorganic nutrients. On the other hand, it is demonstrated that some of medium nutrients that are typically prescribed for laboratory cultivation of microalgae may have a small or negligible effect on algae

growth. This consideration makes the elimination of expensive substances, such as vitamins, quite reasonable when developing microalgae-based processes at the industrial scale.

Finally, the high tolerance and growth rate this microalga strain shows at high pH may favor the combination of CO_2 sequestration and biomass production. In fact, it is well known that CO_2 absorption in aqueous solution increases as the pH solution increases. Therefore, an alkaline aqueous solution can be used in absorption processes to capture CO_2 emitted for instance by power plants. The liquid phase obtained from the CO_2 absorption process can be then exploited for the microalgae cultivation. The high pH and total inorganic carbon concentration of this medium determine high growth rate of *N. eucaryotum*, which, in turn, gives rise to a higher production of microalgal biomass to be fed in the subsequent biofuels production process.

Chapter 4.

Nannochloris eucaryotum growth in batch photobioreactors: kinetic analysis and use of 100% (v/v) CO_2

4.1 Introduction

The production of biofuels from renewable resources is well known to be highly critical to guarantee a sustainable economy and face global climate changes (Cheng and Timilsina, 2011). In recent years, microalgae have been recognized to be a promising alternative source for biofuel-convertible lipids (Halim *et al.*, 2011). In fact, when compared to first generation biofuels, microalgae are characterized by higher growth rates and larger bio-oil productivities. In addition, cultivation of microalgae does not compromise arable lands (Chisti, 2007) thus avoiding "food for fuel" concerns. Moreover, biological fixation of CO_2 can be carried out much more effectively by using autotrophic microalgae rather than terrestrial crops (Usui and Ikenouchi, 1997; Borowitzka, 1999; Chisti, 2007). For these reasons, the potential use of microalgae as renewable feedstock for the massive production of liquid biofuels is receiving a rising interest mostly driven by the global concerns related to the depletion of fossil fuels supplies and the increase of CO_2 levels in the atmosphere (Olguin, 2003; Mulbry *et al.,* 2008). The high potential of algae based biofuels is confirmed by the number of recent papers available in the literature (Francisco *et al*., 2008; Huang *et al*., 2010; Demirbas, 2011; Gong and Jiang, 2011; Li *et al*., 2011; Mallick *et al*., 2011; Phukan *et al*., 2011; Sasi *et al*., 2011; Singh *et al*., 2011;) on the subject, the growing investments of private companies (Torrey, 2008) and governments (Sheehan *et al*., 1998) as well as the increasing number of filed patents (Mata *et al*., 2010). Despite such interest, the current microalgae-based technology is still not widespread since it is characterized by technical and economic constraints that might hinder its full scale-up. In particular, the main barriers are related to the extensive land's areas needs as well as the estimated high costs of the operating phases of microalgae cultivation, harvesting and lipid extraction (Chen *et al*., 2011). Thus, one of the main targets of the scientific community is to identify, and/or create, new microalgae strains which are intrinsically characterized by high biomass productivity and lipid content as well as the capability of capturing CO_2 from flue gases (Cao and Concas, 2008, 2010; Mazzuca Sobczuk and Chisti, 2010). In such contest the most attractive scientific challenge is the genetic manipulation of existing strains with the aim of increasing their photosynthetic efficiency (Tetali *et al*., 2007; Mitra and Melis, 2008; Melis, 2009) and/or regulate their metabolism (Dorval

Courchesne *et al.*, 2009; Radakovits *et al.*, 2010) in order to achieve an abundant production of lipids coupled with high biomass accumulation. A further goal is to suitably exploit process engineering techniques to identify the operating conditions of photobioreactors (i.e. light supply, mass transfer, culture media etc.) that maximize lipid productivity and CO_2 fixation as well as the economic viability of the technique (Concas *et al.*, 2010). In particular, one of the most impacting cost items is related to the need of a continuous replenishment of macronutrients (mainly CO_2, nitrogen and phosphorus) during algal cultivation (Jiang *et al.*, 2011). In fact, as rule of thumb, about 1.8 kg of CO_2, 0.33 kg of nitrogen and 0.71 kg of phosphate are consumed to produce 1 kg of microalgal biomass (Amaro et al., 2011; Yang et al., 2011). Since large scale cultivation of microalgae implies the consumption of huge amounts of such macronutrients, the economic feasibility of the entire process could be seriously affected by the erroneous evaluation of their depletion kinetics. Therefore, in view of industrial scaling-up, the effect of nutrients concentration in the medium on biomass composition and productivity should be quantitative evaluated. In addition, changes in nutrients concentration can result in conflicting effects on the process economics. For instance, a decrease of nitrogen concentration in the cultivation broth typically results in higher lipid contents counteracted by lower growth rates. This inverse relationship between biomass productivity and lipid content makes the process optimization in terms of lipid productivity not straightforward. Therefore, since nutrients concentration and supplies are among the most controllable factors in microalgae cultivation, at least the main macronutrients (i.e. nitrogen and phosphorus) uptake rates need to be quantitatively evaluated for the microalgae strains candidate to industrial exploitation. This way, macronutrients concentrations might be precisely controlled during cultivation. Hence, biomass production can be optimized with respect to the required process end-products by means of suitable growth kinetics and broth composition. Moreover, the exploitation of costless feedstock such as seawater and flue gas as sources of micronutrients and CO_2, might greatly improve the economic feasibility of the microalgae-based technology while simultaneously producing a positive impact on important environmental concerns such as water and air pollution. In addition, marine strains capable to survive under elevated CO_2 concentration might represent suitable candidate for the industrial cultivation of microalgae for biofuels production and CO_2 capture. Among such strains the unicellular marine eukaryotic green alga *Nannochloris eucaryotum*

(Menzel and Wild, 1989) (also known as *Nanochlorum eucaryotum* (Wilhelm and Wild, 1982) or *Picochlorum eucaryotum* (Henley *et al*., 2004), shows high adaptability to extreme environmental conditions such as high salinity, low irradiance and elevated CO_2 levels (Geisert *et al*., 1987). It has been also found that the lipid content of strains belonging the same genus (i.e. *Nannochloris*) can be close to about 50% (Negoro *et al*., 1991). While these aspects make this microalgae strain a suitable candidate for large-scale biofuel production and CO_2 capture, it is important to note the lack of information available in the literature about its growth kinetics and lipid content. Thus, such strain seems to be worthy of further and deeper investigations. Along the lines of our recent work (Lutzu *et al.,* 2012) on this subject, the growth kinetics of *N. eucaryotum* in batch photobioreactors is quantitatively investigated in this work (Concas *et al*., 2012) with the aim of determining useful kinetic parameters which might be used for process engineering and its optimization. In particular, the Monod's model for multiple nutrients limitation is adopted to quantitatively describe the growth of this microalga as a function of nitrogen and phosphorus concentrations. The maximum growth rate, the half saturation constants and yields coefficients for nitrate and phosphate uptake are also determined by suitably fitting the experimental data (Lutzu *et al.,* 2012). The reliability of the obtained parameter values is than tested by suitably predicting new experimental data. Finally, the possibility of using 100% (v/v) CO_2 gas as carbon source in a semi-batch photobioreactor is also investigated in this work with the aim of verifying the capability of *N. eucarytoum* of capturing CO_2 from sources characterized by high concentration values of this gas. Lipid content and fatty acid composition is also evaluated in order to assess the potential exploitability of *N. eucaryotum* as feedstock for biofuel production. It should be noted that, to the best of our knowledge, all these aspects have not been addressed in the literature as far as this microalga strain is concerned.

4.2 Materials and methods

4.2.1 Microorganism

The marine algal strain *Nannochloris eucaryotum* (strain N° 55.87) obtained from the Sammlung von Algenkulturen at the University of Göttingen (SAG), Germany, was investigated in this work. Stock cultures were propagated and maintained in Erlenmeyer flasks with a Brackish Water Medium (BWM) (SAG, 2009) under incubation conditions of 25°C, a photon flux density of 98 µmol m^{-2} s^{-1} provided by four 15 W white fluorescent tubes and a light/dark photoperiod of 12 h was assured. Flasks were continuously shaken at 100 rpm (Universal Table Shaker 709).

4.2.2 Culture conditions

The experimental data used to fit the kinetic parameters of the Monod's equation shown in the next section were obtained in a recent work (Lutzu *et al.,* 2012) from which the description of the experimental set up and procedure, also used to obtain novel data in this work, might be seen. In addition, the possibility of exploiting 100% (v/v) CO_2 gas as carbon source was evaluated using the photobioreactor whose schematic representation is reported in Figure 12. It consists of a cylindrical glass photobioreactor (9.5 cm diameter and 21 cm height) with a volumetric capacity of 1.5 L and operated in semi batch mode (i.e. batch mode for the liquid phase and continuous mode for the gas one). The reactor was then filled with 1 L of growth medium and then mechanically stirred at 400 rpm through a rotating blade powered by an electrical engine (GZ high power overhead stirrer). Cultures were maintained at 25°C by a thermostatic bath (GD120 series) and illuminated by a photon flux density of 100 µmol m^{-2} s^{-1} provided by eight 11 W white fluorescent bulbs with a light/dark photoperiod of 12 h. A gas constituted by pure CO_2 (100% v/v) from a cylinder was continuously supplied through suitable spargers at a flow rate of 40 ml min^{-1}. The inlet pressure of CO_2 was equal to 1.6 bar.

4.2.3 Culture medium

The culture medium to perform the experiments with pure CO_2 was prepared by considering the following components and proportions:

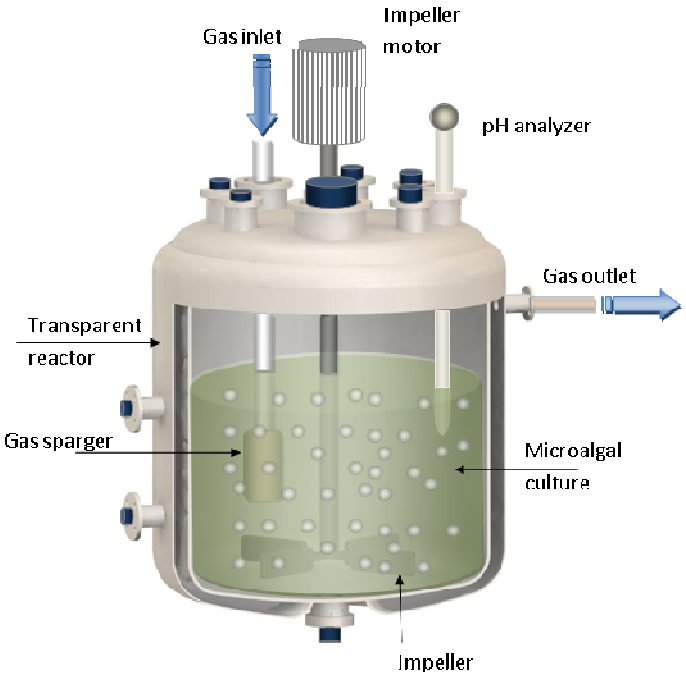

Figure 12. Schematic representation of the semi-continuous photobioreactor used for cultivating *N. eucaryotum* under high CO_2 concentration levels.

- natural sea water (Mediterranean sea, lat. 39° 11' N - long. 09° 10' E) centrifuged at 4000 rpm for 15 min (Thermo Fisher Scientific Inc. Waltham) and then filtered by means of 0.45 µm filter, 455 ml L^{-1};
- de-ionized water, 450 ml L^{-1};
- soil extract (SAG, 2009), 30 ml L^{-1};
- macronutrients aqueous solution (KNO_3 10 g L^{-1}; K_2HPO_4 1 g L^{-1}; $MgSO_4 \cdot 7H_2O$ 1 g L^{-1}), 20 ml L^{-1};
- micronutrients aqueous solution (SAG, 2009) 5 ml L^{-1}.

The volumetric and chemical composition of the growth medium is reported in Table 7. Cultivations were then performed with different initial concentrations of nitrates (N_0) and phosphates (P_0).

4.2.4 Biomass concentration and pH measurements

The growth of microalgae was monitored through daily measurements of the culture media optical density (OD) (Genesys 20 spectrophotometer, Thermo Fisher Scientific Inc. Waltham) at 560 nm wavelenght (D560) with 1 cm light path. The biomass concentration (X) was calculated from OD measurements using an X vs. OD

calibration curve (Lutzu *et al.,* 2012). The latter was obtained by gravimetrically evaluating the biomass concentration of known culture medium volumes which were previously centrifuged at 4000 rpm for 15 min and dried at 105°C for 24 h. pH was daily measured by pHmeter (KNICK 913). For the sake of reproducibility, each experimental condition was investigated at least in duplicate.

Table 7. Volumetric and chemical composition of the culture medium used in this work

Component	***units***	***(N_0, P_0)***
Volumetric composition		
De-ionized water	ml L^{-1}	450
Seawater	ml L^{-1}	455
Soil extract	ml L^{-1}	30
Macronutrients aqueous solution	ml L^{-1}	20
Micronutrients aqueous solution	ml L^{-1}	5
Resulting chemical composition		
KNO_3	g L^{-1}	$2.0 \cdot 10^{-1}$
K_2HPO_4	g L^{-1}	$2.0 \cdot 10^{-2}$
$MgSO_4 \cdot 7H_2O$	g L^{-1}	$2.0 \cdot 10^{-2}$
H_3BO_3	g L^{-1}	$2.86 \cdot 10^{-3}$
$MnCl_2 \cdot 4H_2O$	g L^{-1}	$1.81 \cdot 10^{-3}$
$ZnSO_4 \cdot 7H_2O$	g L^{-1}	$2.22 \cdot 10^{-4}$
$CoCl_2 \cdot 6H_2O$	g L^{-1}	$3.5 \cdot 10^{-5}$
$CuSO_4 \cdot 5H_2O$	g L^{-1}	$8.0 \cdot 10^{-5}$
$Na_2MoO_4 \cdot 2H_2O$	g L^{-1}	$2.3 \cdot 10^{-4}$
EDTA-Na_2	g L^{-1}	$2.98 \cdot 10^{-2}$
$FeSO_4 \cdot 7H_2O$	g L^{-1}	$2.49 \cdot 10^{-2}$

4.2.5 Fatty acids methyl esters analysis

After lipid extraction the total amount of saponifiable lipids and fatty acid composition of extracted lipid was determined after transesterification with methanol-acetyl chloride. Gas chromatographic analysis was carried out according to EEC N° 2568/91 (2011) using a flame ionization detector (FID) (Thermo Trace Ultra, GC-14B) and a RTX-WAX column T (fused silica, 0.25 mm x 60 m x 0.25 µm) maintained at 180ºC. Helium was used as carrier gas at a flow rate of 1 ml min^{-1}.

4.3 Model equations and parameters evaluations

The relevant material balance used to quantitatively describe the growth of microalgal cells in the batch photobioreactor used in this work (Lutzu *et al.,* 2012) to obtain kinetic parameter is reported as follows:

$$\frac{dX}{dt} = \mu X \tag{1}$$

where X represents the cell mass [g L^{-1}], t is the time [h], and μ the growth rate [h^{-1}], which is typically a function of nutrients concentration, pH, temperature, light and other culture conditions. With the aim of evaluating the kinetic parameters of nitrogen and phosphorus uptake by *N. eucaryothum*, the Monod's model for multiple nutrients limitation was adopted to simultaneously take into account the effect of nitrogen and phosphorus concentration on *N. eucaryotum* growth (Bailey and Ollis, 1986):

$$\mu = \mu_0(CO_2, T, I, pH)\prod_{i=N,P}\frac{C_i}{K_i + C_i} \quad i = N, P \tag{2}$$

where μ_0 can be regarded as the maximum growth rate under the temperature level T, the light intensity I, the CO_2 mass transport and the pH conditions of the adopted experimental set-up while K_i represents the half saturation constant. Mass concentration C_i (g L^{-1}) of nitrogen and phosphorus in the medium may be related to the biomass concentration X under batch operation through the following relationship:

$$C_i = C_{0,i} - Y_i(X - X_0) \quad i = N, P \tag{3}$$

where $C_{0,i}$ is the initial concentration of the limiting nutrients, and Y_i the yield coefficient of nitrogen and phosphorus. On the basis of Eqs. (1), (2), and (3), the values of five parameters (μ_0, K_N, K_P, Y_N, Y_P) are needed to quantitatively interpret the experimental results. The strategy adopted to fit the above mentioned kinetic parameters is illustrated in what follows. By assuming μ as a constant during the phase of exponential growth, Eq. (1) can be integrated with the initial condition $X = X_0$ at $t = 0$ to give the following relationship between the microalgae mass concentration and time:

$$\ln\left(\frac{X}{X_0}\right) = \mu t \tag{4}$$

Experimental data obtained in the case where the exponential growth took place without being affected by nutrient or light limitation phenomena (i.e. $\mu = \mu_0$), are then

linearly fitted through Eq. (4) in order to obtain the value of μ_0. While maintaining fixed the above reported value of μ_0, the kinetic parameter K_N and Y_N were evaluated by coupling the numerical integration of Eqs. (1), (2), and (3) with a non-linear fitting of the experimental data related to the case where nitrogen limitation phenomena took place. Numerical integration was performed using standard IMSL (International Mathematics and Statistics Library) routines. Finally, by maintaining fixed the fitted values of μ_0, K_N and Y_N, the kinetic parameters K_P and Y_P were obtained by non-linearly fitting the experimental data obtained in case where phosphorus limitation phenomena took place. The reliability of the fitted parameters were then evaluated by successfully predicting novel experimental results obtained in this work when nitrogen and phosphorus starvation phenomena occurred both simultaneously or separately, albeit at different concentration levels with respect to the experimental data used during the fitting procedure.

4.4 Results and discussions

A series of batch experiments were carried out recently (Lutzu *et al.,* 2012) to evaluate the effect of the initial concentration of nitrogen (N_{init}) and phosphorus (P_{init}) on the growth of *N. eucaryotum* by varying the initial content of potassium nitrate and potassium biphosphate in the culture medium. The initial concentrations of nitrogen and phosphorus in the "base case" experiment will be hereafter indicated as N_0 and P_0, respectively (cf. Table 7). It can be observed from Figure 13 that, for the case where $N_{init} = N_0$ and $P_{init} = P_0$, *N. eucaryotum* grows exponentially with time up to the end of the cultivation period. Since microalgae growth causes nutrients depletion, it can be stated that in this case the growth rate does not seem to be significantly affected by the diminishing nitrates and phosphates concentrations.

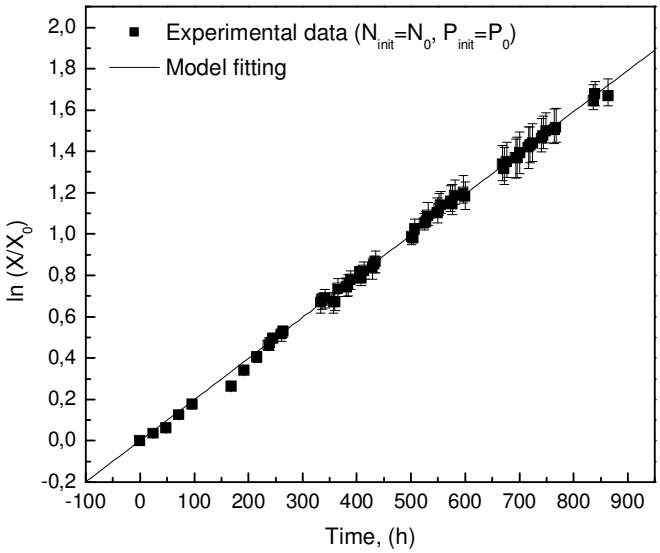

Figure 13. Comparison between model results and experimental data (Lutzu *et al.,* 2012) in terms of cells mass as a function of time to obtain the *N. eucaryotum* maximum growth rate (μ_0).

Thus, the experimental data obtained for the case $N_{init} = N_0$ and $P_{init} = P_0$ can be linearly fitted through Eq. (4) as it may be seen from Figure 13, by means of a constant growth rate equal to 0.00199 h^{-1} under the selected experimental conditions. This finding confirms that in this case the growth rate is not significantly affected by the diminishing nitrogen and phosphorus concentrations as well as by the decreasing light intensity inside the medium due to microalgae absorbance. In addition, during

this experiment (Lutzu *et al.,* 2012), the medium pH varied from the initial value of 7.0 to 8.8.

Thus, the growth rate does not appear to depend on pH in the above mentioned range. The fitted value of μ can be then regarded as the maximum growth rate μ_0 (cf. Eq. (2)) under the temperature, light intensity, CO_2 transfer and pH conditions available in the case where $N_{init} = N_0$ and $P_{init} = P_0$.

The effect of initial nitrogen concentration was also investigated (Lutzu *et al.,* 2012) by reducing it to one half and one fourth of N_0, while maintaining constant the initial phosphate concentration.

From the experimental data reported in Figure 14, it clearly appeared that for $N_{init} = 1/2\ N_0$ growth curve approached a stationary phase after about 720 h thus indicating the occurrence of nitrogen starvation phenomena.

Thus by maintaining fixed the above reported value of μ_0, the kinetic parameter K_N and Y_N were evaluated with the proposed model by fitting the experimental data where it is assumed that C_P is much greater than K_P, since such phosphorus does not limit the algae growth under these conditions, as it may be seen from Figure 13.

Model results are compared with experimental data in Figure 14. The best fitting value for the half saturation constant K_N was equal to 5.2 10^{-4} g_N L^{-1} while the corresponding value of the nitrogen yield Y_N was 5.9 10^{-2} $g_N/g_{biomass}$.

As far as the effect of phosphorus depletion on the growth kinetic of *N. eucaryotum* is concerned, the experimental data reported in Figure 15 clearly show that when the initial content of P was reduced to $1/4\ P_0$, the cells mass concentration increased only during the first 400 h of cultivation. Then a stationary phase was reached up to 700 h of cultivation.

This fact indicates that for $P_{init} = 1/4\ P_0$ phosphorus becomes a limiting nutrients after a specific culture time. Hence, by assuming that C_N is much greater than K_N under these experimental conditions and maintaining fixed the values of μ_0 already obtained, the kinetic parameters K_P and Y_P were obtained by non-linearly fitting the experimental data for the case when $N_{init} = N_0$ and $P_{init} = 1/4\ P_0$ in the time interval 0-700 h. Model results are compared with experimental data in Figure 15.

In particular, the best fitting value of 2.5 10^{-5} g L^{-1} is obtained for the half saturation constant K_P while the corresponding value of 6.0 10^{-3} $g_P/g_{biomass}$ is obtained for the phosphorus yield Y_P. With the aim of testing the predictive capability of the adopted

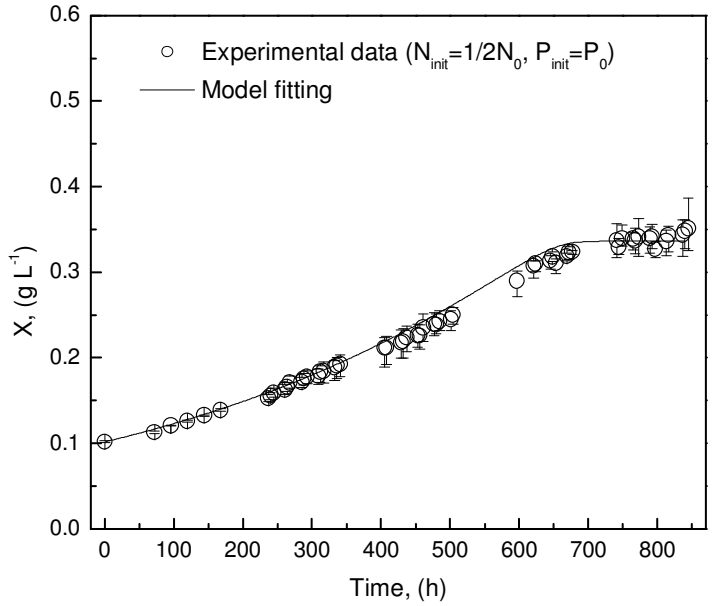

Figure 14. Comparison between model results and experimental data (Lutzu *et al.,* 2012) in terms of cells mass as a function of time to obtain the half-saturation constant (K_N) and yield coefficient (Y_N) for nitrates uptake by *N. eucaryotum.*

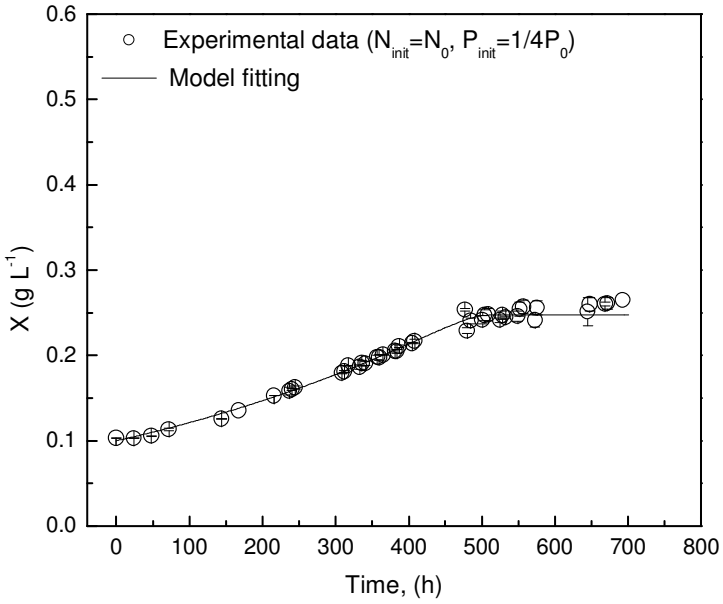

Figure 15. Comparison between model results and experimental data (Lutzu *et al.,* 2012) in terms of cells mass as a function of time to obtain the half-saturation constant (K_P) and yield coefficient (Y_P) for biphosphates uptake by *N. eucaryotum.*

growth model as well as the reliability of the fitted parameters, numerical simulation of new experimental runs where only the initial nitrogen was further reduced (i.e N_{init} = *1/4* N_0 and P_{init} = P_0) and only the initial phosphorus concentration was halved (i.e.

N_{init} = N_0 and P_{init} = *1/2* P_0) were performed. Figure 16 illustrates the comparison between experimental data and model results which were obtained by maintaining fixed the corresponding parameters obtained through the fitting procedure described above. To further test the predictive capability of the model when both the initial nitrogen and phosphorus concentrations are simultaneously reduced, new experimental data have been obtained in this work for the case where N_{init} = *1/2* N_0 and P_{init} = *1/2* P_0, following the procedure described in the literature (Lutzu *et al.,* 2012). Experimental results are compared with model predictions in Figure 17, from which it can be seen that also in this case the model permits to predict the culture

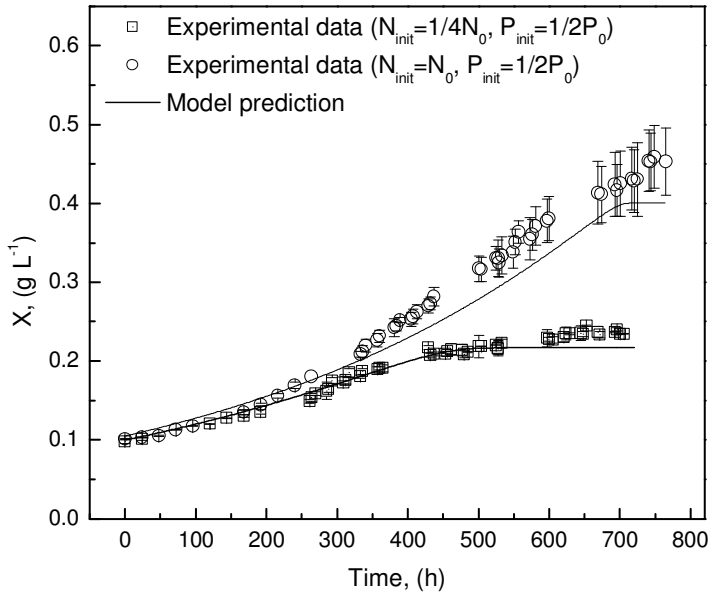

Figure 16. Comparison between model predictions and experimental data (this work) in terms of cells mass as a function of time.

behavior at varying initial nitrate and phosphate concentrations in the medium with a reasonable accuracy. Experimental results are compared with model predictions in Figure 17, from which it can be seen that also in this case the model permits to predict the culture behavior at varying initial nitrate and phosphate concentrations in the medium with a reasonable accuracy. It should be mentioned however, that the so called phenomenon of "diaouxic growth" that occurred after prolonged culture times

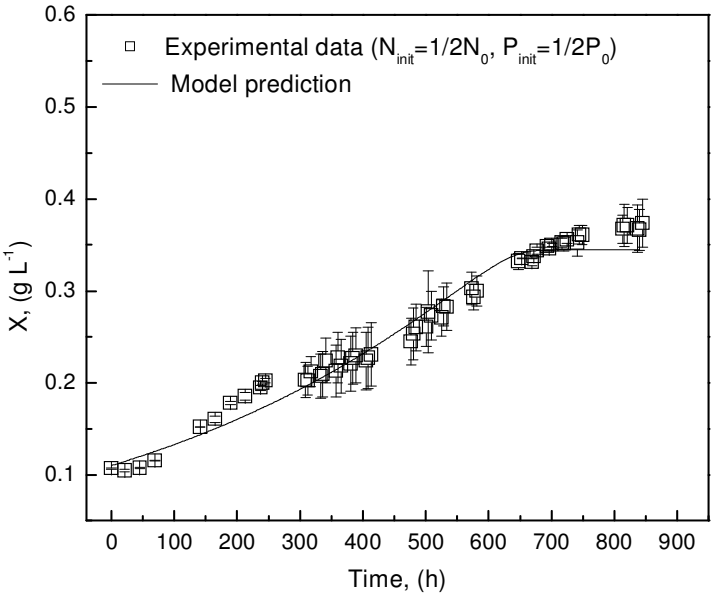

Figure 17. Comparison between model predictions and experimental data (this work) in terms of cells mass as a function of time.

(Lutzu *et al.,* 2012) was not simulated in this work. Subsequently, the effect of high CO_2 concentration on the growth of *N. eucaryotum* in batch photobioreactors was also investigated in this work. Specifically, specific experiments were carried out where CO_2 (100% v/v) was continuously bubbled at a flow rate of 40 ml min^{-1} into the growth medium when $N_{init} = N_0$ and $P_{init} = P_0$. To this aim the semi-batch photobioreactor shown schematically in Figure 12 was used.

From Figure 18 it can be observed that, under the above mentioned conditions, microalgae start growing with a modest lag phase, which probably indicates the intrinsic affinity of *N. eucaryotum* for high dissolved CO_2 concentration in the growth medium. Moreover, when comparing the experimental results of Figure 15 with the corresponding ones (i.e. $N_{init} = N_0$ and $P_{init} = P_0$) obtained in our previous work (Lutzu *et al.,* 2012) using CO_2 available in the atmosphere, a higher initial growth rate can be observed. Such behavior is due to the higher availability of dissolved CO_2 which results in the increase of the specific growth rate μ_0 *(*CO_2*, pH, I)*, thus suggesting that its dependence upon dissolved CO_2 concentration should be also taken into account through Monod's type kinetics. In fact CO_2 is the main macronutrient for triggering photosynthesis in microalgae. On the contrary, a stationary phase is attained after about 350 h of cultivation when the biomass concentration was about 0.35 g L^{-1}, while using CO_2 from the atmosphere microalgae keep growing almost exponentially up to 840 h of cultivation (Lutzu *et al.,* 2012). Once the steady state was attained, the possibility to operate the photobioreactor in fed-batch mode was evaluated.

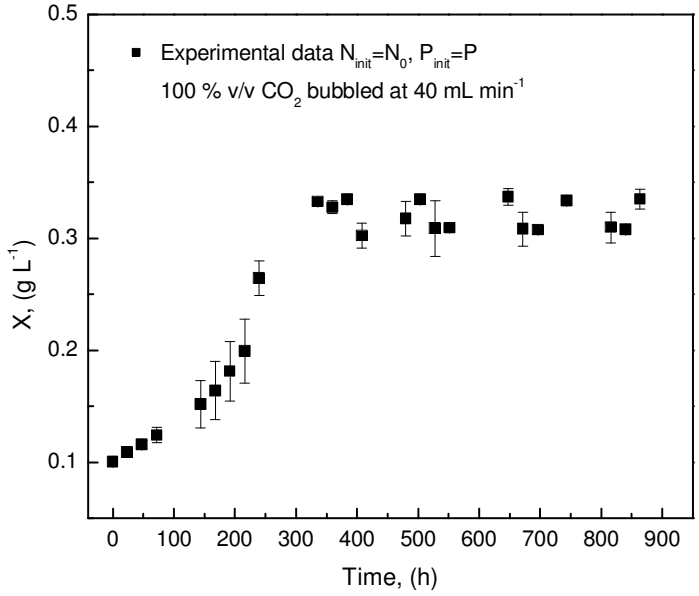

Figure 18. Growth of *N. eucaryotum* in the batch photobioreactor depicted in Figure 12 in terms of cells mass as a function of time. Culture conditions: 100% (v/v) CO_2, aeration rate = 40 ml min^{-1}, agitation speed = 400 rpm and 25°C.

In fact starting from the 16th day of culture, 150 ml of culture were withdrawn every 5 days and then replaced by an equal volume of fresh medium, thus imposing a dilution rate (D) (Novick and Szilard, 1950; Fogler, 2006) of about 0.0015 h^{-1}. As shown in Figure 18, after each withdrawal, the biomass concentration decreases and then starts increasing as a result of nutrient availability and the diminished concentration of toxic catabolites. In particular, 4 cycles of withdrawal and replacement with fresh medium were performed and, after 5 days from each withdrawal, the biomass always reached the concentration corresponding to the steady state. Such behavior demonstrates that the photobioreactor can be suitably operated in fed-batch mode while assuring the culture stability with a dilution ratio (D) of 0.0015 h^{-1}. By indicating with X_s the microalgae concentration at the steady state, i.e. 0.35 g L^{-1}, the potential biomass productivity (P_b) (Mazzuca Sobczuk and Chisti, 2010) was evaluated, through the equation $P_b = D \cdot X_s$, to be about 12.6 mg L^{-1} d^{-1}. It should be noted that, given the high growth rate observed during the initial phase, higher dilution rates could be probably used while guaranteeing reactor stability. This could allow us to obtain higher biomass productivities. Moreover, it is worth noting that such result is obtained under

extreme operating conditions such as elevated CO_2 levels and low pH (cf. Figure 19) at which very many of the algal strains investigated so far in the literature have been shown to grow with strongly reduced capability (Papazi *et al*., 2008) or not to grow at all (Watanabe *et al*., 1992). Figure 19 shows the pH evolution during the experiment. It can be observed that when the culture is started, pH drops to the value of 5.32, as a result of the CO_2 inlet. Although such low value of initial pH, microalgae start growing exponentially while pH increases as a result of the photosynthetic activity. According to Geisert *et al*. (1987) this behavior confirms that *N. eucaryotum* could survive under very low pH values. In fact, even though the optimal pH for *N. eucaryotum* is in the range between 5 and 7, cell growth can take place at pH equal to 4 and 9, respectively (Geisert *et al.,* 1987).

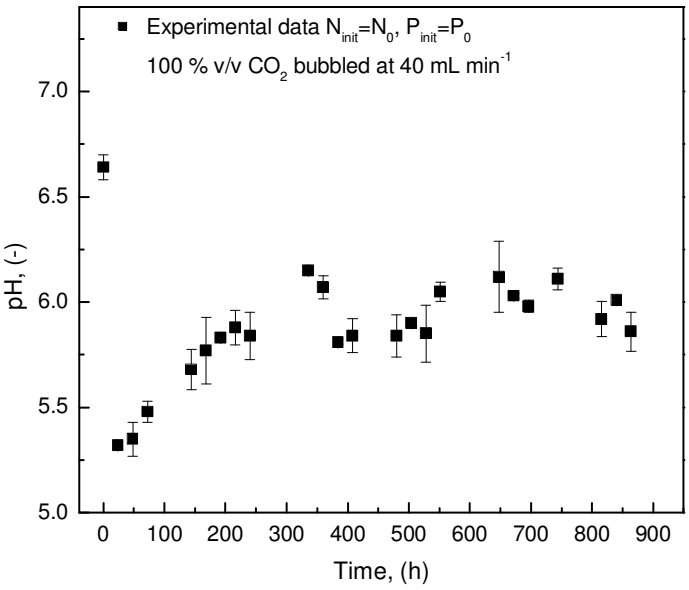

Figure 19. pH evolution as a function of time during the growth of *N. eucaryotum* in the batch photobioreactor depicted in Fig. 1. Culture conditions: 100% (v/v) CO_2, aeration rate = 40 ml min^{-1}, agitation speed = 400 rpm and 25°C.

Such result is very important in view of the utilization of such strain to capture CO_2 from sources where its concentration is quite high. In fact, such microalgae grows not only at low pH but also at a higher rate during the initial growth phase with respect to the corresponding one observed when lower CO_2 levels are used. It is worth noting in passing that under the above mentioned experimental conditions the lipid content of *N. eucaryotum* is evaluated to be 16.2% $wt/wt_{biomass}$. While details of the extraction

procedure will be reported in a subsequent publication (Rossi *et al*., 2012), it should be noted that the cumulative amount of fatty acid methyl esters having carbon numbers from C16 to C18 is about 71.2% wt/wt. Thus it can be stated that, at least from a qualitative point of view (Damiani *et al*., 2010), lipids extracted from *N. eucaryotum* could be suitably exploited for the production of biodiesel.

4.5 Concluding remarks

In this work the Monod's growth model for multiple nutrients limitation was adopted in order to evaluate the kinetic parameters related to the growth of *N. eucaryotum* under the experimental conditions of our recent work (Lutzu *et al.,* 2012). The maximum growth rate was evaluated to be 1.99 10^{-3} h^{-1}. Half saturation concentrations for nitrate (K_N) and phosphate uptake (K_P) were evaluated as 5.4 10^{-4} $g_N\,L^{-1}$ and 2.5 10^{-5} $g_P\,L^{-1}$, respectively. Yield factors for nitrogen (Y_N) and phosphorus (Y_P) resulted to be 5.9 10^{-2} $g_N/g_{biomass}$ and 6.0 10^{-3} $g_P/g_{biomass}$, respectively. Predictive capability of the adopted growth model along with the fitted kinetic parameters was also tested with good results. It is worth noting that these results represent a first step for developing useful mathematical models to simulate and optimize the growth of *N. eucaryotum* in large-scale photobioreactors. Subsequently, the possibility to grow *N. eucaryotum* in a semi batch photobioreactor fed with a gaseous stream of pure (100% v/v) CO_2 was experimentally demonstrated for the first time in this work. The strain showed a good adaptability to high concentrations of dissolved CO_2 as well as to low pHs thus being potentially useful for the CO_2 capture from flue gases. Finally, although the potential biomass productivity is not high, the lipid content of *N. eucaryotum*, grown under elevated CO_2 levels, is relatively good (i.e. 16.16% wt/wt) and the FAMEs composition of the extracted oil is in compliance with the European regulation for quality biodiesel. This aspect represents an interesting result since the oil extracted from the majority of microalgal strains is characterized by FAMEs composition that is not suitable for the production of biodiesel through simple transesterification processes. Although further analyses should be performed to evaluate the potential exploitability of *N. eucaryotum* as feedstock for biofuels production, the obtained results allow to state that, at least from a qualitative point of view, the oil extracted from this strain seems to be suitable for the production of biodiesel. On the other hand, the low biomass productivity might severely affect its exploitability at the industrial level. For these reasons the optimization of operating conditions should be performed by means of suitable mathematical models where the kinetic parameters obtained in this work are needed in order to achieve this target.

Chapter 5.

On the growth of high CO_2 acclimated *Chlorella vulgaris* in acidic media

5.1 Introduction

Literature review reported in the Chapter 2 suggests that, in view of the industrial scaling-up of microalgae cultivation, the current technology today available should be optimized in terms either of selected algal strains or design/operating parameters (Sierra *et al*., 2008). While the creation of new microalgal strains intrinsically characterized by high lipid productivities is an ambitious goal which can be achieved through genetic manipulation of existing strains (Dorval Courchesne *et al*., 2009; Radakovits *et al*., 2010), the optimization of design and operating parameters may be accomplished by exploiting suitable process engineering techniques. To this aim, suitable mathematical models, that are capable of quantitatively describing the influence of the crucial operating parameters (i.e. photobioreactors geometry, heat and mass transfer conditions, growth medium composition, pH etc.) on microalgae growth and lipid accumulation, should be developed. Several mathematical models of microalgae growth within photobioreactors have been proposed in the literature. So far, the basic characteristics of algal kinetics have been taken into account (Cornet *et al.*, 1995; Acién Fernández *et al*., 1997; Zonneveld *et al*., 1997; Molina Grima *et al*., 1999; Camacho Rubio *et al*., 1999; Wu and Merchuk, 2001; Molina Grima *et al.*, 2001; Li *et al*., 2003; Berenguel *et al*., 2004; Pruvost *et al*., 2008). In particular, most mathematical models available in the literature were capable of quantitatively describing the evolution of biomass concentration as a function of light density distribution within the culture (Cornet *et al*., 1995; Acién Fernández *et al*., 1997). Other modeling efforts have been devoted to quantitatively describe the production of photosynthetic oxygen and the corresponding consumption of dissolved carbon dioxide within the culture (Camacho Rubio *et al*., 1999), the pH evolution (Berenguel *et al*., 2004), the mass transfer phenomena (Molina Grima *et al*., 1999) and the influence of hydrodynamic regime on light conversion (Pruvost *et al*., 2008). Recently, also the effect of cell size distribution on the nutrient uptake capacity of microalgae has been simulated by means of suitable population balances (Concas *et al*., 2010). However, in spite of the large number of mathematical models appearing in the literature, to the best of our knowledge, no comprehensive models, which simultaneously account for all the above mentioned phenomena taking place, have been proposed. In particular very few models were able to quantitatively describe the evolution of pH during photosynthetic growth of microalgae (Camacho Rubio *et al.*, 1999). Moreover the few works which have

attempted to model the pH evolution during microalgal growth were steady state models and were based on the erroneous assumption that only the inorganic carbon species affect the solution pH (Camacho Rubio *et al.*, 1999) while the effects of other major ions in solution were neglected. Nevertheless, the quantitative description of pH evolution during microalgal growth is very important since it can influence photosynthetic phenomena in a number of ways. In fact pH can affect the distribution of carbon dioxide species and carbon availability, alter the speciation (and thus the availability) of macro- and micronutrients, and at extreme levels potentially provoke direct physiological effects (Chen *et al.*, 1994). Moreover, in microalgal cultures, $[H^+]$ is recognized to be a non-competitive inhibitor near neutral conditions, but at too low or too high pHs, it can inhibit photosynthetic growth and substrate utilization rates (Mayo, 1997). Furthermore pH can affect the enzymatic activity of intra- and extra-cellular carbonic anhydrase thus influencing the carbon capture mechanism of some microalgal strains (Rigobello-Masini *et al.*, 2003).

Therefore the quantitative description of pH evolution in microalgal cultures is a key goal in order to properly control and optimize photobioreactors. Indeed pH variations are not only a fundamental indicator of the evolution of photosynthetic activity but can, in turn, strongly influence the growth kinetics of microalgae. In particular this is of crucial importance when high CO_2 concentrated gases, such as flue gases, are used as carbon source. In fact in this case the medium's pH can reach very low values that might inhibit microalgae growth. On the other hand, the potential exploitation of costless feedstock such as flue gases as source of CO_2 is one of the main targets of scientists and technicians operating in this field. In fact the use of flue gases as carbon source or microalgae might greatly improve the economic feasibility of the microalgae-based technology while simultaneously producing a positive impact on significant environmental concerns such as air pollution, climate changes. Thus the correct evaluation of the effect of pH is critical also for assuring the possibility of exploiting/capturing CO_2 from flue gases through microalgae. For the above mentioned reasons, microalgae strains capable to survive under elevated CO_2 concentration might represent suitable candidate for the industrial cultivation of microalgae for biofuels production and CO_2 capture. Among such strains the unicellular eukaryotic fresh green alga *Chlorella vulgaris* is characterized by high growth rates (Radakovits *et al.*, 2012), coupled with a significant lipid content (Mallick *et al.*, 2011; Sasi *et al.*, 2011). Moreover *C. vulgaris* is tolerant to high-

temperatures and toxic compounds such as NO_x and SO_x (Ho *et al.*, 2011) and is capable to grow in inexpensive media such as wastewaters (Chinnasamy *et al.*, 2009). Finally, according to Baba and Shiraiwa (2012) *C. vulgaris* is one among that strains which are capable of developing suitable molecular mechanisms that allows its adaptation to extremely high CO_2 concentrations. For all these reasons this microalgae is potentially one of the more useful strains for biofuels production and CO_2 capturing from flue gases.

In the light of the foregoing the goal of the present chapter is to describe the growth of *C. vulgaris,* previously acclimated to high CO_2 concentrations, under acidic conditions in semi batch photobioreactors fed with pure CO_2 (100% v/v), to provide experimental data in order to validate a rigorous and comprehensive mathematical model that have been developed by our research group (Concas *et al.* 2012b). Model results and experimental data in terms of dry biomass concentration and pH were successfully compared, thus demonstrating the validity of the proposed model as well as its predictive capability.

Particularly, the focus of this work is to investigate the growth kinetics of *C. vulgaris* in enriched media to provide useful experimental data in order to validate the mathematical model. This will permit to properly simulate cell growth and pH evolution during microalgae growth within the photobioreactor. However model results are not shown since the target of the present work is focused only on the experimental analysis of microalgae behavior under high CO_2 concentrations.

5.2 Materials and methods

5.2.1 Microorganism and culture medium

The fresh water algal strain *Chlorella vulgaris*, provided by Prof. Torzillo of the Centro per lo Studio dei Microorganismi Autotrofi di Firenze, Italy, was investigated in this work. Stock cultures were propagated and maintained in Erlenmeyer flasks with a Kolkwitz Triple Modified medium (KTM-A) under incubation conditions of 25°C, a photon flux density of 98 µmol m^{-2} s^{-1} provided by four 15 W white fluorescent tubes, and a light/dark photoperiod of 12 h. Flasks were continuously shaken at 100 rpm (Universal Table Shaker 709).

5.2.2 Strain acclimation to high CO_2 concentrations

Acclimation of *C. vulgaris* to high CO_2 concentrations was carried out in a 6 L helical tubular photobioreactor coupled with a degasser system, as shown in Figure 20. The light collector of the photobioreactor consisted of 66 m transparent polyurethane tubing arranged around a circular metal frame. It was internally illuminated by tree 60W white fluorescent lamps providing a light intensity of 100 µmol m^{-2} s^{-1} for a light-dark photoperiod of 12 h. Liquid circulation in the light collector was assured by a peristaltic pump. The degasser unit was a 1 L bubble column which allowed removing photosynthetic oxygen by exposing the broth to atmosphere. Pure CO_2 (100% v/v) was continuously bubbled in the growth medium by means of a flowmeter (Rotameter FL-3207C, OMEGA Eng. Ltd.) at a flow rate of 30 ml min^{-1}. The growth medium consisted of KTM-A. Once the culture reached the stationary growth phase the photobioreactor was operated in fed-batch mode by assuring a suitable dilution rate for about 230 days. Such a long cultivation time was assured in order to trigger permanent changes in the strain to extremely high CO_2 concentrations. The withdrawals made during the operation in fed-batch mode were used as inoculum for the experiments performed in the semi-batch continuously stirred photobioreactors described in what follows.

5.2.3 Culture conditions

The possibility of exploiting 100% (v/v) CO_2 gas as carbon source for growing high CO_2 acclimated strain of *C. vulgaris* in a continuously stirred system. A schematic

Figure 20. Schematic representation of a 6 L helical tubular photobioreactor (BIOCOIL). It consists of a 4.5 L light-receiver photostage coupled with a 1.5 L degasser system. The circulation and illumination of culture medium are provided by a peristaltic pump and tree 60W white fluorescent lamps, respectively.

representation of the photobioreactor is shown in Figure 12. It consists of a cylindrical glass photo-bioreactor (9.5 cm diameter and 21 cm height) with a volumetric capacity of 1.5 L and operated in semi batch mode (i.e. batch mode for the liquid phase and continuous mode for the gas one). The reactor was filled with a volume equal to 1 L of growth medium and then mechanically stirred at 400 rpm through a rotating blade powered by an electrical engine (GZ high power overhead stirrer). Cultures were maintained at 25°C by a thermostatic bath (GD120 series) and illuminated by a photon flux density of 84 µmol m^{-2} s^{-1} provided by eight 11 W white fluorescent bulbes with a light/dark photoperiod of 12 h. A gas constituted by pure CO_2 (100% v/v) from a cylinder was continuously supplied through suitable spargers at a flow rate of 40 ml min^{-1}. The inlet pressure of CO_2 was equal to 1.6 bar.

5.2.4 Culture Medium

C. vulgaris was cultured in 1L of modified Kolkwitz medium (KTM-A) containing (g L^{-1}): KNO_3, 2.5; KH_2PO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.27; $CaCl_2 \cdot 2H_2O$, 0.04; $NaHCO_3$, 1 and 1 ml of A5 micronutrients solution as well as 1 ml of E.D.T.A.Na_2-

Fe solution. The micronutrients solution contained (g L^{-1}): H_3BO_3, 2.86; $MnCl_2 \cdot 7H_2O$, 1.81; $ZnSO_4 \cdot 7H_2O$, 0.222; $CoCl_2 \cdot 6H_2O$, 0.035; $CuSO_4 \cdot 5H_2O$, 0.080; $Na_2MoO_4 \cdot 2H_2O$, 0.230. As far as the E.D.T.A.Na_2-Fe solution, it contained (g L^{-1}): 29.754 g E.D.T.A.-Na_2 and 24.9 g $FeSO4 \cdot 7H_2O$.

5.2.5 Biomass and pH measurement

The growth of microalgae was monitored through spectrophotometric measurements of the culture media optical density (OD) (Genesys 20 spectrophotometer, Thermo Fisher Scientific Inc. Waltham) at 560 nm wavelenght (D_{560}) with 1 cm light path. The biomass concentration *X* (g L^{-1}) was calculated from OD measurements using the *X* vs. *OD* calibration curve shown in Equation (1). The latter was obtained by gravimetrically evaluating the biomass concentration of known culture medium volumes which were previously centrifuged at 4000 rpm for 15 min and dried at 105°C for 24 h.

$$X \text{ (g } L^{-1}) = 0.538 * D_{560} \quad (1)$$

pH was daily measured by pHmeter (KNICK 913). For the sake of reproducibility, each experimental condition was investigated at least in duplicate. The average and standard errors values of the experimental results were calculated using OriginPro 8.

5.2.6 Design of the experiments

C. vulgaris growth was performed with different initial concentrations of total dissolved inorganic nitrogen ($N_{tot,l}$), phosphorus ($P_{tot,l}$) and carbon ($C_{tot,l}$) as shown in the Table 8. In order to evaluate the effect of pure CO_2 on the microalgae growth, by varying the concentrations of the three main macronutrients, three different versions of KTM were prepared. With regards to the bicarbonate, nitrate and phosphate content it is that of the best case in KTM-A. Nitrate and phosphate content are doubled while maintaining constant bicarbonate content in KTM-B to respect KTM-A and bicarbonate content is halved while nitrate and phosphate content are maintained constant in KTM-C to respect KTM-A, respectively.

Table 8. Composition of KTM investigated in this work

Component	units	KTM-A	KTM-B	KTM-C
KNO_3	[g L^{-1}]	2.5	5	2.5
KH_2PO_4	[g L^{-1}]	$0.5 \cdot 10^{-1}$	1	$0.5 \cdot 10^{-1}$
$NaHCO_3$	[g L^{-1}]	$0.5 \cdot 10^{-1}$	$0.5 \cdot 10^{-1}$	$0.25 \cdot 10^{-1}$
$MgSO_4*7H_2O$	[g L^{-1}]	$2.7 \cdot 10^{-1}$	$2.7 \cdot 10^{-1}$	$2.7 \cdot 10^{-1}$
$CaCl_2*2H_2O$	[g L^{-1}]	$4.0 \cdot 10^{-2}$	$4.0 \cdot 10^{-2}$	$4.0 \cdot 10^{-2}$
EDTA-Na_2-Fe	[ml L^{-1}]	1	1	1
A5	[ml L^{-1}]	1	1	1

5.3 Results and discussions.

5.3.1 Adaptation of C. vulgaris to pure CO_2 in the BIOCOIL

In order to adapt *C. vulgaris* to grow with pure CO_2, 600 ml of *C. vulgaris,* cultivated in KTM-A under atmospheric air, were used as inoculum to start the growth in the BIOCOIL using the base case medium. The inoculum concentration, which represents 1/10 of the total volume (6 L), was equal to 0.1 g L^{-1}.

Figure 21a shows the biomass concentration of *C. vulgaris* in the BIOCOIL photobioreactor. It can be seen that microalgae start growing with a much reduced lag phase, which probably indicates the intrinsic affinity of *C. vulgaris* for high dissolved CO_2 concentration in the growth medium. Subsequently, after two weeks the culture reaches the stationary phase when the biomass concentration is about to 2 g L^{-1}. Starting from then the microalgae still continue to grow maintaining a biomass concentration around that value with small fluctuations which are due to the operation of the photobioreactor in fed-batch mode. Actually, once the steady state was attained, the possibility to operate the BIOCOIL in fed-batch mode was evaluated. In fact starting from around the 40^{th} day of culture, an aliquot of culture (about 150-200 ml) was withdrawn and then replaced by an equal volume of fresh medium. It should be pointed out that the withdrawal of a known amount of culture medium and its replacement not occurred at the same interval time. Since withdrawals were not executed at a specific interval time, it was not possible to impose a specific dilution rate. Anyway, as shown in Figure 21a, after a series of withdrawals, the biomass concentration decreased and then started increasing as a result of nutrient availability and the diminished concentration of toxic catabolites. In particular, after about 5-7 days from each withdrawal, the biomass always reached the concentration corresponding to the steady state. Such behavior demonstrates that the BIOCOIL can be suitably operated in fed-batch mode while assuring the culture stability for a long period of time. This represents a very interesting result if we consider that in the literature there are not examples of photobioreactors operated consecutively in fed-batch mode for 7 months. Actually, only Borowitzka (1999) outlines to have successfully grown a wide range of marine microalgae (including *Tetraselmis* sp., *Isochrysis galbana*, *Phaeodactylum tricornutum* and *Chaetoceros* sp.) as well as *Spirulina* sp. at high productivities in pilot scale BIOCOILs of up to 700 L for periods greater than 4 months in semi-continuous culture.

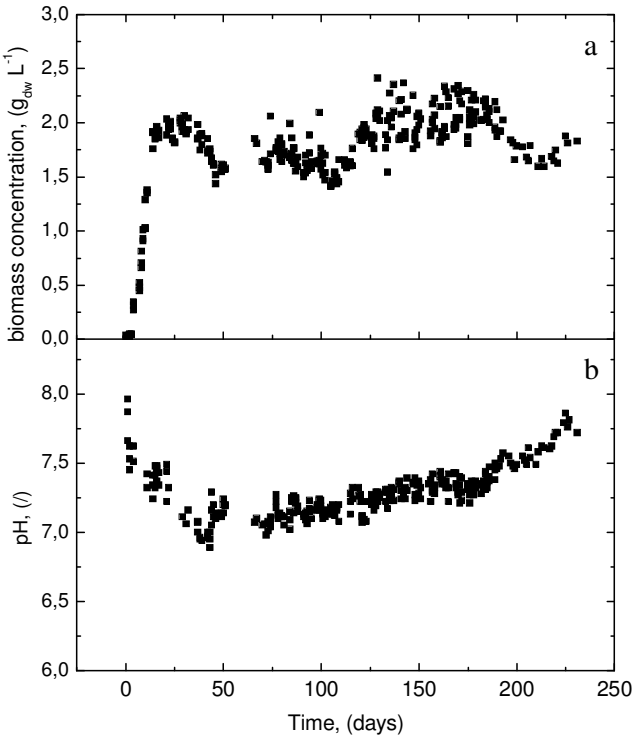

Figure 21. Biomass concentration (a) and pH (b) evolution during the seven month cultivation of *Chlorella vulgaris* in the helical tubular photobioreactor (BIOCOIL). Culture conditions: 100% (v/v) CO_2, aeration rate = 30 ml min^{-1}, mixing 35 rpm and 25°C.

The pH evolution during the cultivation of *C. vulgaris* in the BIOCOIL is reported in the Figure 21b. It can be seen that at the beginning the pH drops as a consequence of CO_2 inlet which force the cells to live in an environment where the pH becomes more acidic. Microalgae cells need some time (the extent depends on the strain considered) to adapt their metabolic apparatus to the "stressful" situation represented by the acidification of the culture medium due to the feeding with pure CO_2 (Collins and Bell, 2006; Collins *et al.*, 2006). Once the enzymatic apparatus has been modulated to face the acidic environment, microalgae start to grow and the pH slowly starts to increase following the photosynthetic activity of the cells. This experimental result confirms that high CO_2 acclimated *C. vulgaris* may represent a suitable candidate for biofuels production through exploitation of flue gases as carbon source.

5.3.2 Effects of nutrient concentrations and pH on the growth of C. vulgaris

The effects of nutrient concentrations and acidic pH on the growth kinetics of *C. vulgaris* were quantitatively evaluated in this work by cultivating this strain, previously acclimated to high CO_2 concentrations, in a semi-batch stirred tank photobioreactor (cfr. Figure 12). In the base case experiment the operating conditions shown in the Materials and Methods section were adopted. Further experiments were then carried out to evaluate the effect of the initial concentration of dissolved inorganic carbon, nitrogen and phosphorus on the growth of *C. vulgaris* by varying the initial content of sodium bicarbonate, potassium nitrate and potassium biphosphate in the culture medium. During each experiment, pure CO_2 (100% v/v) was continuously bubbled at a flow rate of 40 mL min^{-1} into the growth medium.

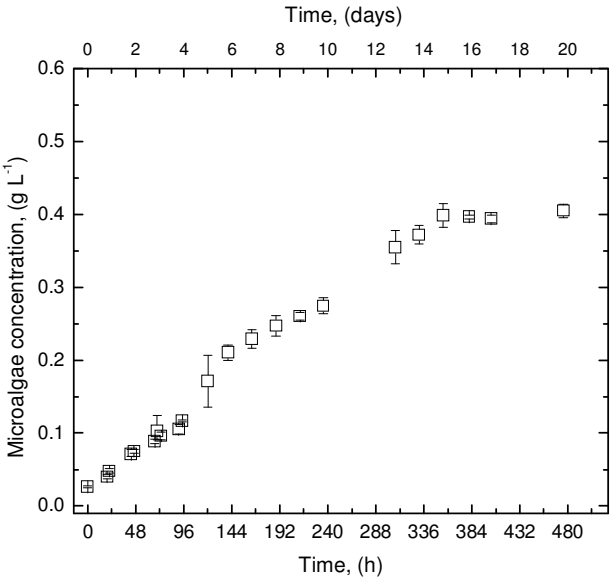

Figure 22. Biomass concentration as a function of time when KTM-A is used for culturing *C. vulgaris* (initial concentration of sodium bicarbonate, potassium nitrate and potassium biphosphate are $0.5 \cdot 10^{-1}$, 2, $0.5 \cdot 10^{-1}$ g L^{-1}, respectively).

From Figures 22 and 23 it can be observed that, under the base case conditions (KTM-A), the culture starts growing without showing a significant lag phase despite the high CO_2 concentrations and the low pH reached by the medium when gas bubbling started. This is probably due to the fact that *C. vulgaris* was

previously adapted to grow under high dissolved CO_2 concentrations. This behavior is consistent with the assumption made by some authors on the fact that a prolonged exposition to high CO_2 concentrations may probably trigger changes of the metabolism and the cellular structure of microalgae (Giordano *et al.*, 2005). In fact Baba and Shiraiwa (2012) recently recognized that high CO_2 concentrations may trigger the expression of specific genes that may underlie changes in the carbon capture mechanism (CCM) of several strains of microalgae as well as changes of their structural anatomy and in the redistribution of certain cellular organelles (Bozzo *et al.*, 2000). A comparison of the functional states in CO_2-susceptible and CO_2-tolerant species was carried out in several works (Pesheva et al., 1994; Iwasaki et al., 1996). An increase in the PSI activity was found in CO_2-tolerant *Chlorococcum littorale* during its adaptation to high CO_2 concentration. In this case, the transition state of the PS was observed during the first day following CO_2 increase from 0.03 to 40%. A similar increase in CO_2 concentrations did not induce the state transition and completely blocked the growth of CO_2-susceptible specie *Stichococcus bacillaris*. These results seems to demonstrate that the tolerance to high CO_2 levels could be correlated with a reduced PSI activity and cell inability to maintain the energetic of photosystems and also that the susceptibility to CO_2 stress depends on different microalgae strains considered. Although high CO_2 inducible mechanisms in microalgae at the molecular level are still not well understood, the capability of *C. vulgaris* to adapt to high levels of CO_2 is well confirmed also by the present results. In fact, from Figure 22 it can be shown that culture starts to grow almost exponentially until about 150 h when the decelerating growth took place. After 300 h of cultivation the culture reaches a kind of "plateau" when the biomass concentration was about 0.400 g L^{-1}. The stationary phase is probably reached due to the synergic effects of catabolic products accumulation and insufficient penetration of light rather than for the exhaustion of nutrients. In fact initial concentrations of macro and micronutrients were high enough to sustain the exponential growth of *C. vulgaris* for a period of time longer than the one observed in the experiment.

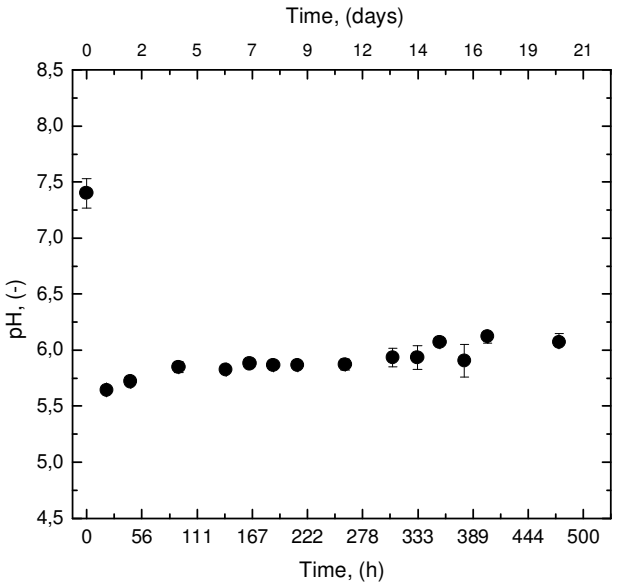

Figure 23. pH as a function of time when KTM-A is used for culturing *C. vulgaris* (initial concentration of sodium bicarbonate, potassium nitrate and potassium biphosphate are $0.5 \cdot 10^{-1}$, 2, $0.5 \cdot 10^{-1}$ g L^{-1}, respectively).

Figure 23 shows the pH evolution during the experiment. It can be observed that when the culture is started, pH drops to the value of about 5.6, as a result of the CO_2 inlet. Although such low value of pH, the culture starts growing and subsequently pH increases slightly as a result of the photosynthetic activity which determines the consumption of CO_2 and the use of $[H^+]$ as substrate by microalgae. According to Goldman and Graham (1981) and Mayo (1994) such behavior confirms that *C. vulgaris* could survive under very low pH values and high CO_2 concentrations. In fact, even though the optimal pH for *C. vulgaris* is in the range between 6 and 8, cell growth can take place at pH equal to 4 and 10, respectively (Mayo, 1994).

Figures 24 and 25 show the biomass concentration and pH of *C. vulgaris* when the concentration of nitrate and phosphate are doubled in the medium while maintaining constant all the other macronutrients (KTM-B). It can be seen that, under these experimental conditions, microalgae growth seems to have the same metabolic behavior for the first part of the growth curve since it shows a kind of plateau after about 250 h, which is similar to that obtained with KTM-A. What is evident is that doubling the concentrations of nitrate and phosphate permit to obtain a final biomass

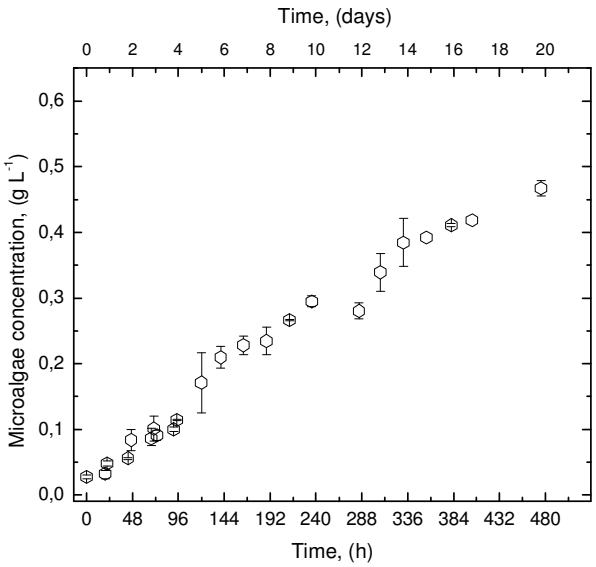

Figure 24. Biomass concentration as a function of time when KTM-B is used for culturing *C. vulgaris* (initial concentration of sodium bicarbonate, potassium nitrate and potassium biphosphate are $0.5 \cdot 10^{-1}$, 5, 1 g L^{-1}, respectively).

concentration of 0.470 g L^{-1} which is higher compared to that one obtained whit the concentrations of nitrate and phosphate in the base case (0.400 g L^{-1}). It can be also seen that, while the biomass concentration with KTM-B seems potentially continue to increase after 500 h of cultivation (cfr. Figure 25), the growth with KTM-A appears to have reached a stable concentration of 0.400 g L^{-1}. This difference is probably due to the fact that the larger availability of nitrate and phosphate in KTM-B allow the cells to sustain photosynthetic activity with higher rates to respect the cells in KTM-A. It should be also noted how the use of pure CO_2 with KTM-B seems no to have particular effect on microalgae growth. Actually, as shown in Figure 24, the culture starts growing without showing a significant lag phase despite the use of pure CO_2. Figure 25 shows the low pH reached by the medium when 100% (v/v) CO_2 is bubbled. It can be seen how the evolution of pH is similar to that one shown in Figure 23 obtained for KTM-A, thus confirming the possibility for *C. vulgaris* to survive under very low pH.

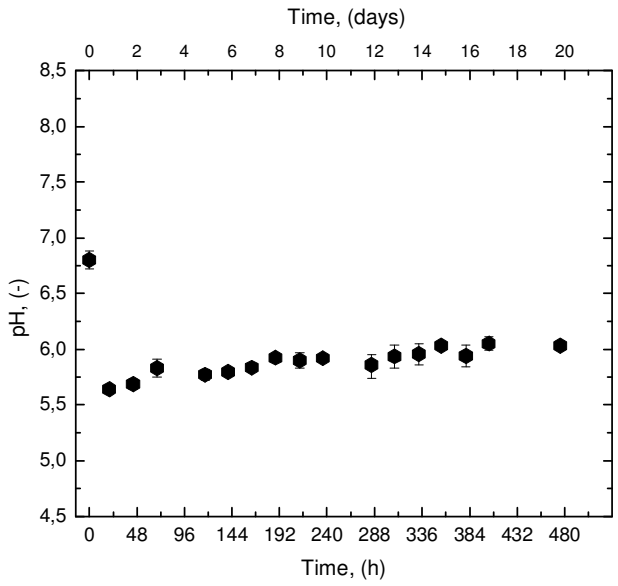

Figure 25. pH as a function of time when KTM-B is used for culturing *C. vulgaris* (initial concentration of sodium bicarbonate, potassium nitrate and potassium biphosphate are $0.5 \cdot 10^{-1}$, 5, 1 g L^{-1}, respectively).

In Figure 26 the biomass concentration when *C. vulgaris* is cultured with KTM-C is reported. It can be observed how the reduction of carbon content in the medium leads to a reduction of microalgae biomass concentration. Actually it can be noted that culture starts to grow almost exponentially until about 100 h when the decelerating growth took place. After 220 h of cultivation the culture reaches a plateau when the biomass concentration was about 0.3 g L^{-1}. This value is clearly lower if compared to biomass concentration reached when carbon content is doubled in the culture medium 0.4 g L^{-1}. As above mentioned, the acclimation of microalgae to high CO_2 concentrations may provoke significant changes in the carbon uptake mechanisms of microalgae. In particular it is reasonable to assume that strains acclimated to high CO_2 conditions are more sensitive to the reduction of initial carbon concentration. Thus, for high CO_2 acclimated cells, the halving of the initial carbon concentration may have determined the starting of a carbon starvation phenomena leading to a strong inhibitory effect on the mechanisms used to uptake and concentrate carbon inside the cells.

Also in this case, the inlet of pure CO_2 to the medium where the carbon content has been halved seems no to have negative effect on the growth since microalgae shows a much reduced lag phase.

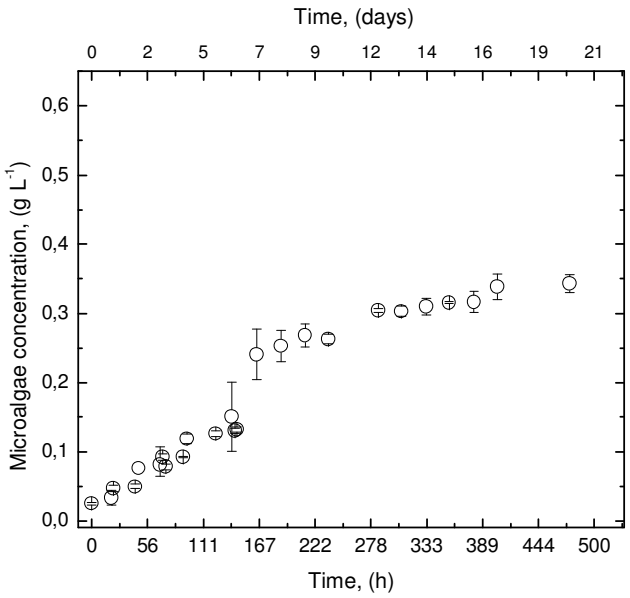

Figure 26. Biomass concentration as a function of time when KTM-C is used for culturing *C. vulgaris* (initial concentration of sodium bicarbonate, potassium nitrate and potassium biphosphate are $0.25 \cdot 10^{-1}$, 2.5, $0.5 \cdot 10^{-1}$ g L^{-1}, respectively).

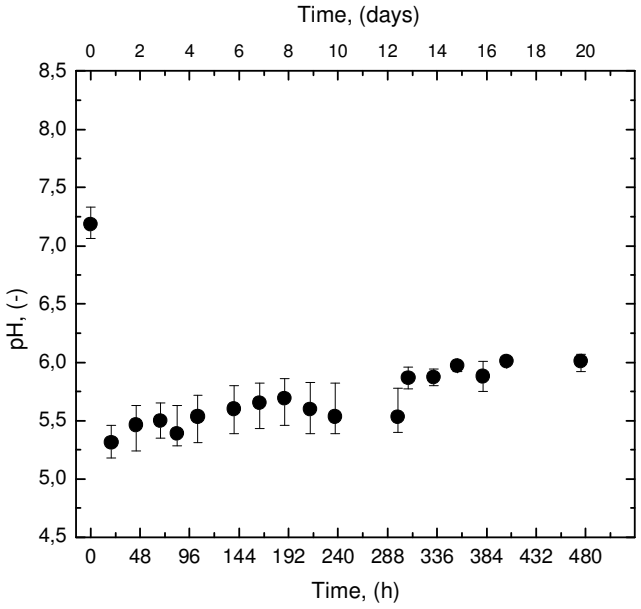

Figure 27. pH as a function of time when KTM-C is used for culturing *C. vulgaris* (initial concentration of sodium bicarbonate, potassium nitrate and potassium biphosphate are $0.25 \cdot 10^{-1}$, 2.5, $0.5 \cdot 10^{-1}$ g L^{-1}, respectively).

Finally in the Figure 27 the pH evolution during the experiment with KTM-C is reported. It can be observed that the culture shows the same behavior seen for KTM-A

and –B. Actually, pH drops at the beginning to the value of about 5.5, as a consequence of the culture medium acidification, due to the inlet of the CO_2.

Than the culture starts growing producing the subsequent increase of pH as a result of the photosynthetic activity which, in turn, determines the consumption of CO_2 and the use of $[H^+]$ as substrate by microalgae, leading to the basification of the medium.

From Figures 23, 25 and 27 it can be seen how the pH of the medium changes during the culture time, with a common initial phase characterized by the reduction of pH as a consequence of the pure CO_2 inlet. The addition of CO_2-enriched gas induces new equilibrium states between gaseous CO_2 and dissolved forms of inorganic carbons (dissolved CO_2, H_2CO_3, HCO_3^- and CO_2^{2-}) which results in a pH decrease in the medium. However, as shown for the three media used, the decrease of the pH at the early culture time seems not to affect the algal growth because pH is resumed to the physiological range for the growth of *C. vulgaris* during the long linear growth period.

The dependence of microalgal growth and productivity on the CO_2 concentration has been rather well investigated, especially in the range of low (atmospheric) and slightly elevated (2-15%) CO_2 concentrations (Yun *et al.*, 1996; Yue *et al*., 2005; Chiu *et al*., 2008). The effect of extremely high CO_2 concentrations on photosynthesis, growth and cell metabolism has been less studied (Watanabe *et al*., 1992; Maeda *et al*., 1995; Papazi *et al*., 2008; Kong *et al*., 2010). In the literature examples are found where *C. vulgaris* was able to grow in the presence of high CO_2 concentrations after being gradually adapted to grow in a medium with increasing concentration of CO_2 (Yun *et al*., 1996). On the other hand, Hanagata *et al*. (1992) showed that *Scenedesmus* sp. was able to grow when the level of CO_2 in the gas was increased up to 80%. This strain was totally inhibited when the concentration was increased up to 100% (v/v), even if the growth was resumed when the concentration was returned to 20%. These results demonstrate that the mechanism of inhibition due to the growth in the presence of high concentration of CO_2 is reversible. They also confirm that this mechanism is not the same for all the microalgae strains and that further research should be addressed to clear this point. Actually, the mechanism of inhibition of algal growth by high concentration of CO_2 has been extensively investigated in cyanobacteria while little is known about the principle of inhibition in eukaryotic microalgae as well as the metabolic nature of the adaptation.

In this work *C. vulgaris* was able to grow in the presence of pure CO_2 without being progressively adapted to grow in a medium with gradually increased concentration of

CO_2. Another example of *Chlorella* strain which was able to grow with pure CO_2, without have to be precultivated with 100% (v/v) CO_2, is given by Murakami *et al*. (1998). They characterized the growth of a new isolated strain, *Chlorella* sp. UK001, and reported that it was able to grow with almost constant high growth rate under CO_2 concentrations ranging from the atmospheric level to 40% (v/v) in air without to gradually acclimation.

In our experiment, air-adapted cells of *C. vulgaris* were directly inoculated in the helical tubular photobioreactor where 100% (v/v) CO_2 was supplied. This strain was able to grow both in the BIOCOIL (cfr. Figure 20) and in the batch photobioreactor (cfr. Figure 12) without showing inhibition phenomena. The growth in the BIOCOIL was characterized by a little lag phase (due to the acclimation of the metabolic apparatus to high CO_2) while the growth in the batch photobioreactor not showed lag phase at all.

When cultivated under 100% (v/v) CO_2 in the BIOCOIL photobioreactor the lipid content of *C. vulgaris* was evaluated to be 17.50% $wt/wt_{biomass}$. This result can not be compared with lipid contents of other microalgae strains cultivated under pure CO_2 since there are not examples of lipid content obtained under these experimental conditions. On the other hand, the obtained results are consistent with the typical ones reported in the literature for *C. vulgaris* cultivated with normal medium without nitrogen starvation (Murakami *et al*., 1998; Illman *et al*., 2000).

Finally it should be also pointed out that, in order to be exploited as a fuel, the microalgae should be characterized by a high calorific value. Microalgae grown under normal conditions showed high relatively calorific values between 18 and 21 KJ g^{-1} (Changdong and Azevedo, 2005). Several studies report that the calorific value of *C. vulgaris* biomass can be increased by growing them under low nitrogen conditions (Illman *et al*., 2000; Scragg *et al*., 2002). In our case the calorific value of *C. vulgaris* biomass was found to be 18.64 KJ g^{-1} and this value is consistent with the results obtained by Bhola *et al*. (2011) and Illman *et al.* (2000), which reported calorific values equal to 17.44 KJ g^{-1} and 18 KJ g^{-1}, respectively.

Thus it can be stated that, at least from a qualitative point of view, by taking into consideration its lipid content and calorific value *C. vulgaris* could be suitably exploited for the production of biodiesel.

5.4 Concluding remarks

In this work the growth kinetics in semi batch photobioreactors fed with 100% (v/v) CO_2 of a fresh water strain, *C. vulgaris*, was investigated in order to provide experimental data to validate model results of a rigorous and comprehensive developed mathematical model (not shown).

This strain showed a good adaptability to high concentrations of dissolved CO_2 as well as to low pHs thus being potentially useful for the CO_2 capture from flue gases.

Actually, *C. vulgaris* was successfully adapted to grow with pure CO_2 by cultivating it in a 6 L helical tubular photobioreactor (BIOCOIL). With the experimental conditions adopted, the BIOCOIL was operated consecutively for seven months maintaining an almost constant biomass concentration of about 2 g L^{-1} and the pH in a range between 6 and 7.

Microalgae adapted in the BIOCOIL to pure CO_2 were used to investigate the growth kinetics of *C. vulgaris* in acidic culture media fed with pure CO_2. The experimental results showed that *C. vulgaris* was able to grow under 100% (v/v) CO_2 without revealing inhibition phenomena as confirmed by the almost total absence of lag phase during the early phases of cultivation. The biomass concentrations reached at the end of cultivation time, about 20 days (480 h), were 0.400 g L^{-1}, 0.470 g L^{-1} and 0.340 g L^{-1} when three different culture media KTM-A, KTM-A and KTM-A were used, respectively. The best result was obtained when the content of nitrate and phosphate were doubled (KTM-B) while the lowest biomass concentration was reached when the carbonate content was halved (KTM-C) with respect to the base case (KTM-A), respectively. In all the media *C. vulgaris* starts to grow without showing lag phases thus confirming its ability to survive in the presence of elevated CO_2 concentrations.

While these experiments in batch photobioreactors were carried out by using *C. vulgaris* adapted to 100% (v/v) CO_2, this strain was also able to grow in the BIOCOIL photobioreactor without pre-adaptation to pure CO_2 thus demonstrating that *C. vulgaris* has the intrinsic capability to modulate its metabolic and genetic apparatus to face the high level of CO_2 as well as the low pHs due to the inlet of pure CO_2 in the culture media.

By taking into considerations the ability of *C. vulgaris* to grow under pure 100% (v/v) and with acidic pHs, this CO_2-tolerant microalgae could represent an ideal candidate in view of the utilization of industrial emissions containing CO_2 to culture microalgae.

Chapter 6.

Conclusions and suggestions

for further research

The growth kinetics of two microalgae strains, *Nannochloris eucarytoum* and *Chlorella vulgaris,* was investigated under 100% (v/v) CO_2 in order to verify the possibility to use flue gases emitted by industrial power plants as carbon sources for performing microalgal mass cultivation and to gain information useful for the biodiesel process optimization.

The first one, the marine strain *N. eucaryotum*, was characterized for the first time in the literature with regards to its response to the use of pure CO_2 as well as to the effect of medium composition and nutrient starvation on its growth kinetics.

As far as the fresh water strain *C. vulgaris*, it was demonstrated its potential exploitability for the management of huge amounts of waste gases since it was successfully cultivated in batch and fed-batch photobioreactors under pure CO_2 and acidic media.

It was found that both *N. eucaryotum* and *C. vulgaris* are able to tolerate extreme elevated CO_2 concentrations thus making them two real candidates for the mass cultivation when flue gases from industrial installations are used.

Major conclusions of this work include the followings:

1. The growth of *N. eucaryotum* does not result significantly affected by neither removal nor addition of some of the nutrients that are typically prescribed for laboratory cultivation. This consideration makes the elimination of expensive compounds, such as vitamins, quite reasonable when developing microalgae-based processes at the industrial scale.
2. The growth of *N. eucaryotum* is affected by the reduction of nitrate concentration as well as by the decrease of phosphate concentration even if with a little extent. The results about the effect of the medium composition on the growth, even if obtained through lab-scale experiments, represent useful information in view of *N. eucaryotum* exploitation in large-scale photobioreactors.
3. When atmospheric CO_2 was supplied, the pH first slightly decreased and subsequently increased as a result of microalgae growth thus making alkaline the aqueous culture medium. This strain showed also a good tolerance to high pHs thus confirming that it could be potentially useful for the CO_2 capture from flue gases since alkaline aqueous solution can be used in absorption processes to capture CO_2 emitted for instance by power plants.

4. *N. eucaryotum* was experimentally demonstrated, for the first time, to growth in a semi batch photobioreactor fed with a gaseous stream of pure (100% v/v) CO_2. The strain showed a good adaptability to high concentrations of dissolved CO_2 as well as to low pHs.

5. The choice of a marine strain, such as *N. eucaryotum*, allows the use of natural seawater for producing the growth medium. This aspect has positive impact on the technology viability since seawater it is cheaper and easier to be obtained with respect to fresh water. On the other hand, experimental results demonstrate that the use of only natural seawater may not be sufficient to prevent the need to enrich it with inorganic nutrients.

6. When grown under high CO_2 levels *N. eucaryotum* shows a relatively good average lipid content (i.e. 16.16% wt/wt) as well as a FAMEs composition of the extracted oil which is in compliance with the European regulation for quality biodiesel. These obtained results (although further analyses should be performed to enhance lipid productivity) allow to state that, at least from a qualitative point of view, the oil extracted from this strain seems to be suitable for the production of biodiesel.

7. *Chlorella vulgaris* showed a good tolerance to high concentrations of dissolved CO_2 as well as to acidic pHs thus being potentially useful for the CO_2 capture from flue gases. Actually, this strain was demonstrated, for the first time in the literature, to be able to continuously grow for seven months in BIOCOIL photobioreactor using 100% (v/v) CO_2.

Suggestions for further research which arise from this work include the followings:

8. When compared to other microalgae strains, *N. eucarytoum* is characterized by a relatively low biomass productivity which might affect its exploitability at the industrial scale. For these reasons the optimization of operating conditions should be performed by means of suitable mathematical models by taking into consideration the kinetic parameters obtained in this work.

9. Since large scale cultivation of microalgae implies the consumption of huge amounts of macronutrients, the economic feasibility of the process could be seriously compromised when fresh water is employed and synthetic reagents are used as source of macronutrients. A possible solution to this drawback is to harness costless resources to produce the macronutrients and the water needed to perform large scale cultivation. In particular seawater can be used instead of fresh water due to its costless availability

in huge amounts. Moreover, wastewater could be used as inexpensive source of nitrates and phosphates while flue gases could be exploited to provide the suitable CO_2 supply. Besides allowing the economic viability of the process, the exploitment of such costless resources could have a positive impact on important environmental concerns since it contributes to the reduction of water pollution and consumption. Furthermore the recycling of flue gases, results in lower CO_2 emissions thus contributing to increase the environmental sustainability of industries that use fossil fuels for power generation.

10. Since it was demonstrated that *Chlorella vulgaris* was able to continuously grow in BIOCOIL photobioreactor using 100% (v/v) CO_2 at laboratory scale, a possible development of this line could be the setup of a BIOCOIL outside in order to verify the photosynthetic efficiency of this strain by the direct use of solar radiation coupled with the use of carbon dioxide emitted by a combustion engine (i.a. current generator).

11. The possibility to genetically modify the genes involved in the photosynthetic and metabolic apparatus of these microalgae (*C. vulgaris* and *N. eucaryotum*) should be investigated to the aim of enhancing their photosynthetic efficiency and biomass productivity.

12. The scientific community is currently focused on the exploitability at industrial level of a few number of well known microalgae strains, that represent only the 5% of the thousands strains reported in the main algae databases. This means that the researchers have the possibility to address their attention on hundreds of unstudied strains in order to verify their photosynthetic efficiency, biomass productivity, CO_2 tolerance and lipid content. A possible winning choice could be to focus this activity on those strains which are genetically related at least at genus level. This should permit to study new microalgae which have in common with the known strains crucial genes and enzyme involved in the main metabolic pathways responsible for biodiesel production.

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