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Novel technologies for microalgae cultivation and subsequent lipid extraction

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Table of contents

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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

World economy is strictly linked to the availability of fossil fuels, which nowadays meet the world's growing energy demand. However, the intensive exploitation of fossil fuels as main source of energy is currently recognized to be not sustainable due to the continuous depletion of available resources as well as to their contribution to environmental pollution and greenhouse gases emissions (Ahmad et al., 2011). Moreover 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.

Currently, 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, the production of renewable sources of energy such as biofuels is recognized to be critical to fulfill a sustainable economy and face global climate changes (Cheng and Timilsina, 2011). Therefore, biofuels deriving from feedstocks such as plants, organic wastes or algae could help to reduce the world's oil dependence (Naik et al., 2010).

In fact, biomass feedstocks are intrinsically renewable since they are produced through a natural process, i.e. photosynthesis that is continuously replenished by sunlight. Moreover, biofuels would mitigate global warming problems since all the CO2 emitted during their burning can be fixed by plants used as biomass feedstock, through photosynthetic mechanisms. On the other hand, first and second generation biofuels are characterized by several drawbacks which can limit their exploitation as alternative source of energy.

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 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. 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. 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.

Morevor the use of biodiesel from second generation crops may also be advantageous since they do not require arable lands and do not affect biodiversity deriving from 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). However the main drawback of second generation biofuels is that they cannot be produced at a rate which coud meet the growing energy demand of the transopration sector. This is due to the fact that they are produced from feedstocks such as wastes or agricultural residues whose production is constrained by the original productive process from which they come. Morevoer second generation biofuels process have not still attained the economic sustainability.

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). Another 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). A futher 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). Finally the identification of novel techniques to improve lipid extraction from microalgae is one of the main target to be achieved in order to make the technology economically sustainable.

Along these lines the present PhD activity has been focused on two different lines of research which share the common target of identifying suitable strategies to increase the lipid productivity of the current microalgal technology. Specifically, in the first line of activity, a novel cell disruption technique for the enhancement of lipid extraction yields from C. Vulgaris is proposed. In the second line of activity the exploitation of iron-based strategies to increase lipid synthesis in C. Vulgaris, is investigated.

Chapter 2.

Literature review

2.1 Microalgae

Microalgae are microorganisms living in sea or fresh water that convert sunlight, water, carbon dioxide and inorganic salts to algal biomass through photosynthesis. Several microalgae are exceedingly rich in oil, which can be extracted and subsequently converted to biodiesel using existing technologies (Chisti, 2008). When compared to crops used for first generation biofuels, microalgae display superior biomass growth rates. Moreover, the corresponding oil content is higher than the one of terrestrial crops since it can exceed 80% of the dry weight of biomass. For these reasons the oil productivity of microalgae exceeds that one of terrestrial crops even 10 -100 times (Chisti, 2008). Microalgae, differently from crops, are cultured in aquatic environments. For these reasons, cultivation of microalgae can be carried out in less extended and lower-quality lands, thus avoiding the exploitation of arable ones. In addition, cultivation of microalgae might be coupled with the direct bio-capture of CO_2 emitted by industrial activities. Therefore, 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. From a conceptual point of view the process shown in Figure 1.1 can be carried out for producing biofuels and capturing CO_2 through microalgae.

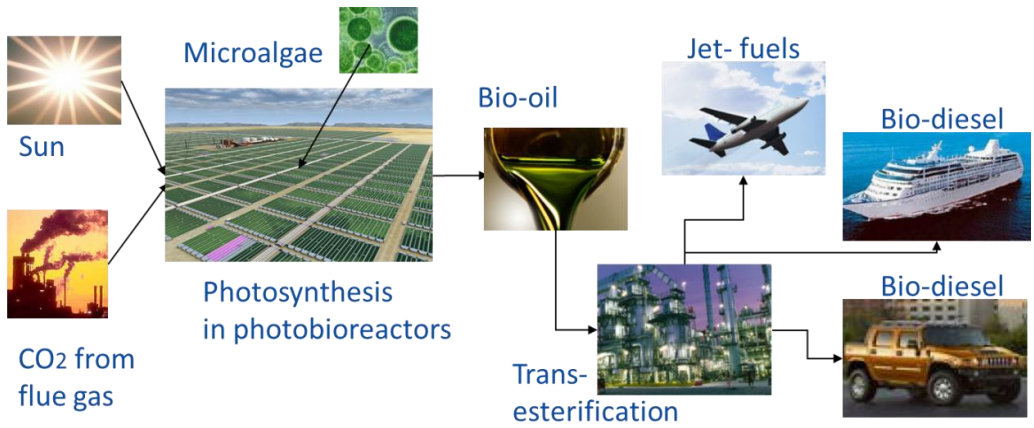


Figure 1.1 Conceptual scheme for the production of biofuels and CO_2 capture through microalgae.

Despite the apparent simplicity of the process, its implementation to the industrial scale is still not widespread since it is characterized by technical and economic constraints that might hinder its full scale-up. Moreover, the complexity of the biological phenomena involved during the algal growth further complicates the optimization of the

process through the classical process engineering techniques. In what follows the biochemical phenomena involved during microalgae growth and lipid accumulation are briefly discussed.

2.2 Photosynthesis and lipid production in microalgae

The process exploited by microalgae for converting sunlight energy and inorganic compounds into the energy-rich molecules constituting the microalgal biomass is called photosynthesis. A simplified scheme of the photosynthetic phenomena occurring in vegetal cells is shown in Figure 1.2.

Basically, the photosynthetic process can be divided into two sets of reactions: the light-dependent (light) reactions and the light-independent (dark) reactions. The first ones, which convert the energy of light into chemical energy, take place within the thylakoid membranes of the chloroplasts, whereas the dark reactions, which use the produced chemical energy to fix CO2 into organic molecules, occur in the stroma of the chloroplast. During the light reactions, the energy transported by incident photons is captured by specific pigments and then used to "split water" into molecular oxygen, two H+ ions and into one pair of electrons, respectively. The energy of light is thus transferred to these electrons and is, finally, used to generate adenosine triphosphate (ATP) and the electron carrier nicotinamide adenine dinucleotide phosphate (NADPH). These two compounds carry the energy and the electrons generated during the light reactions to the stroma, where they are used by the enzymatic dark reactions related to the Calvin cycle to synthesize sugars from CO2. The main sugar synthesized during the Calvin cycle is glyceraldehyde 3-phosphate (G3P). Therefore the net result of the photosynthesis is the conversion of light, water and CO2 into G3P and molecular oxygen.

The synthesized G3P finally passes into the cytosol where it will be involved as intermediate in the central metabolic pathways of the cell that lead to the production of several macromolecules among which starch, proteins and sugars. In the chloroplast also free fatty acids are synthesized starting from G3P. Fatty acids, along with G3P, are then transferred to the endoplasmatic reticulum where they are further converted into nonpolar storage lipids, such as triacylglycerides (TAGs), through a number of enzymatic reactions. Finally, TAGs are packaged into oil bodies that bud off into the cytosol (Sakthivel et al., 2011).

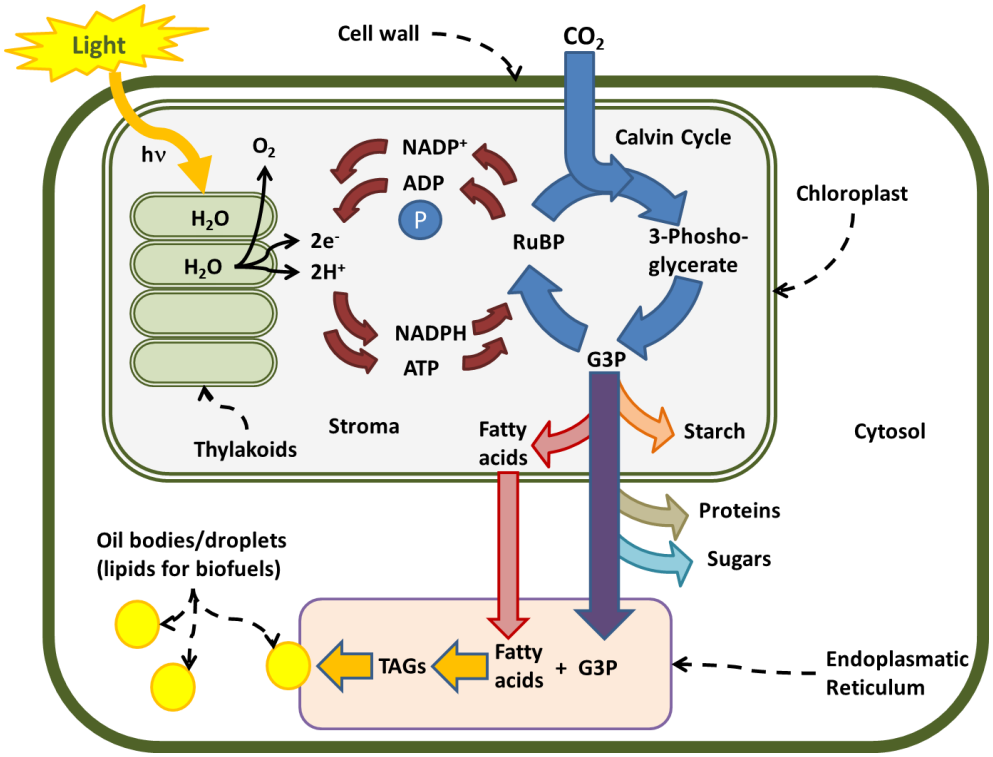


Figure 1.2 Simplified scheme of photosynthesis in microalgae and lipid production metabolic pathways (adapted from Yonghua, 2012).

These oil bodies have a fatty acid composition comparable to vegetable oils and thus can be extracted from the microalgae cell and subsequently converted to useful biofuels (Klok et al., 2013). Specifically, oils from algae can yield biodiesel through transesterification, and gasoline (petrol) or jet fuels through distillation and cracking, respectively (Georgianna and Mayfield, 2012). When compared with first generation biomass feedstocks, microalgae have been found to contain higher concentrations of lipids. The average lipid content varies between 1 and 70% while under specific operating conditions certain species can reach 90% of oil weight by weight of dry biomass (Mata et al., 2010). Depending on the specific strain considered, microalgae can be characterized by high biomass growth rates which, coupled with the intrinsic high lipid content, can lead to very high oil productivity. Table 1.1 shows lipid content as well as lipid and biomass productivities of different microalgae species.

From Table 1.1 it can be observed that volumetric lipid productivity of microalgae is extremely variable depending upon the specific strain considered, and goes from 0.01 to 3,67 goil L-1 day-1. However, it is worth noting that lipid productivity can be strongly affected by the specific culturing conditions adopted, i.e. growth medium composition, light regime, photobioreactor configuration and operation mode etc.

Consequently, it can be argued that, by suitably choosing the best performing strains, very high volumetric productivities of lipids can be achieved by using algae.

Table 1.1 Biomass productivities, lipid content and lipid productivities of different microalgae species

Strain	Biomass productivity (g/L/day)	Lipid content (% biomass)	Lipid productivity (mg/L/day)	Reference
Botryococcus braunii	0,35	17,9	61,8	(Orpez et al., 2009)
Botryococcus braunii	0,29	17,9	51,4	(Orpez et al., 2009)
Botryococcus braunii	0,03	36,1	12,3	(Sydney et al., 2011)
Botryococcus braunii*	0,02	50,0	10,0	(Mata et al., 2010)
Botryococcus braunii	0,04	22,0	9,5	(Dayananda et al., 2007)
Chaetoceros calcitrans	0,04	39,8	17,6	(Rodolfi et al., 2009)
Chaetoceros muelleri	0,07	33,6	21,8	(Rodolfi et al., 2009)
Chaetoceros muelleri*	0,07	33,6	21,8	(Mata et al., 2010)
Chlamydomonas reinhardtii	2,00	25,3	505,0	(Kong et al., 2010)
Chlorella*	0,00	37,5	18,7	(Mata et al., 2010)
Chlorella emersonii*	0,04	44,0	30,2	(Mata et al., 2010)
Chlorella protothecoides	7,30	50,3	3671,9	(Xiong et al., 2008)
Chlorella protothecoides	4,10	43,0	1763,0	(Cheng et al., 2009)
Chlorella protothecoides	4,85	36,2	1214,0	(Mata et al., 2010)
Chlorella protothecoides	2,02	55,2	1115,0	(Xu et al., 2006)
Chlorella pyrenoidosa*	3,27	2,0	65,4	(Mata et al., 2010)
Chlorella sorokiniana IAM-212	0,23	19,3	44,7	(Rodolfi et al., 2009)
Chlorella sp.	0,00	32,6	110,0	(Hsieh and Wu, 2009)
Chlorella sp.	0,08	66,1	51,0	(Hsieh and Wu, 2009)
Chlorella sp.*	1,26	29,0	42,1	(Mata et al., 2010)
Chlorella vulgaris	0,35	42,0	147,0	(Feng et al., 2011)
Chlorella vulgaris	0,35	42,0	147,0	(Feng et al., 2011)
Chlorella vulgaris	0,20	18,4	36,9	(Rodolfi et al., 2009)
Chlorella vulgaris	0,15	23,0	35,0	(Liang et al., 2009)
Chlorella vulgaris	0,17	19,2	32,6	(Rodolfi et al., 2009)
Chlorella vulgaris	0,09	34,0	31,0	(Liang et al., 2009)
Chlorella vulgaris*	0,11	31,5	25,6	(Mata et al., 2010)
Chlorella vulgaris	0,10	22,0	22,0	(Liang et al., 2009)
Chlorococcum sp.	0,28	19,3	53,7	(Rodolfi et al., 2009)
Chlorococcum sp.*	0,28	19,3	53,7	(Mata et al., 2010)
Crypthecodinium cohnii*	10,00	35,6	3555,0	(Mata et al., 2010)
Dunaliella primolecta*	0,09	23,1	20,8	(Mata et al., 2010)
Dunaliella salina*	0,28	15,5	116,0	(Mata et al., 2010)
Dunaliella sp.*	0,00	42,3	33,5	(Mata et al., 2010)
Dunaliella tertiolecta*	0,12	43,9	52,6	(Mata et al., 2010)
Ellipsoidion sp.	0,17	27,4	47,3	(Rodolfi et al., 2009)
Euglena gracilis*	7,70	17,0	1309,0	(Mata et al., 2010)
Haematococcus pluvialis*	0,06	25,0	13,8	(Mata et al., 2010)
Isochrysis galbana*	0,96	23,5	225,6	(Mata et al., 2010)
Isochrysis galbana	0,17	22,3	38,0	(Su et al., 2007)

Isochrysis galbana	0,12	14,3	17,2	(Su et al., 2007)
Isochrysis sp.	0,14	27,4	37,8	(Rodolfi et al., 2009)
Isochrysis sp.*	0,13	20,1	37,8	(Mata et al., 2010)
Isochrysis sp.	0,17	22,4	37,7	(Rodolfi et al., 2009)
Monallanthus salina*	0,08	21,0	16,8	(Mata et al., 2010)
Monodus subterraneus	0,19	16,1	30,4	(Liang et al., 2009)
Monodus subterraneus*	0,19	16,0	30,4	(Mata et al., 2010)
Nannochloris sp.*	0,34	38,0	68,7	(Mata et al., 2010)
Nannochloropsis	0,17	29,2	49,7	(Rodolfi et al., 2009)
Nannochloropsis sp.	0,21	29,6	61,0	(Rodolfi et al., 2009)
Nannochloropsis sp.	0,20	24,4	48,2	(Rodolfi et al., 2009)
Nannochloropsis sp.	0,17	21,6	37,6	(Rodolfi et al., 2009)
Neochloris oleabundans	0,31	40,0	125,0	(Li et al., 2008)
Neochloris oleabundans	0,63	15,0	98,0	(Li et al., 2008)
Neochloris oleabundans	0,15	28,0	37,8	(Gouveia et al., 2009)
Neochloris oleabundans	0,03	52,0	14,4	(Gouveia et al., 2009)
Neochloris oleoabundans*	0,00	47,0	112,0	(Mata et al., 2010)
Pavlova lutheri	0,14	35,5	50,2	(Rodolfi et al., 2009)
Pavlova salina	0,16	30,9	49,4	(Rodolfi et al., 2009)
Phaeodactylum tricornutum	0,24	18,7	44,8	(Rodolfi et al., 2009)
Porphyridium cruentum	0,37	9,5	34,8	(Rodolfi et al., 2009)
Scenedesmus obliquus*	0,37	33,0	122,8	(Mata et al., 2010)
Scenedesmus quadricauda	0,19	18,4	35,1	(Rodolfi et al., 2009)
Scenedesmus sp.	0,26	21,1	53,9	(Rodolfi et al., 2009)
Scenedesmus sp.*	0,15	20,4	47,4	(Mata et al., 2010)
Scenedesmus sp.	0,21	19,6	40,8	(Rodolfi et al., 2009)
Skeletonema costatum	0,08	21,1	17,4	(Rodolfi et al., 2009)
Skeletonema sp.	0,09	31,8	27,3	(Rodolfi et al., 2009)
Skeletonema sp.*	0,09	22,6	27,3	(Mata et al., 2010)
Spirulina maxima*	0,23	6,5	15,0	(Mata et al., 2010)
Spirulina platensis*	2,18	10,3	224,5	(Mata et al., 2010)
Tetraselmis sp.*	0,30	13,7	43,4	(Mata et al., 2010)
Tetraselmis suecica	0,28	12,9	36,4	(Rodolfi et al., 2009)
Tetraselmis suecica*	0,22	15,8	31,7	(Mata et al., 2010)
Tetraselmis suecica	0,32	8,5	27,0	(Rodolfi et al., 2009)
Thalassiosira pseudonana	0,08	20,6	17,4	(Rodolfi et al., 2009)

* average values are reported

In particular, from Figure 1.3 it can be observed that oil yields per area (areal productivity) of microalgae could greatly exceed the one of the best oilseed crops. This aspect clearly determines up to 20 times smaller land areas to produce the same amount of biofuels (for example biodiesel) through microalgae.

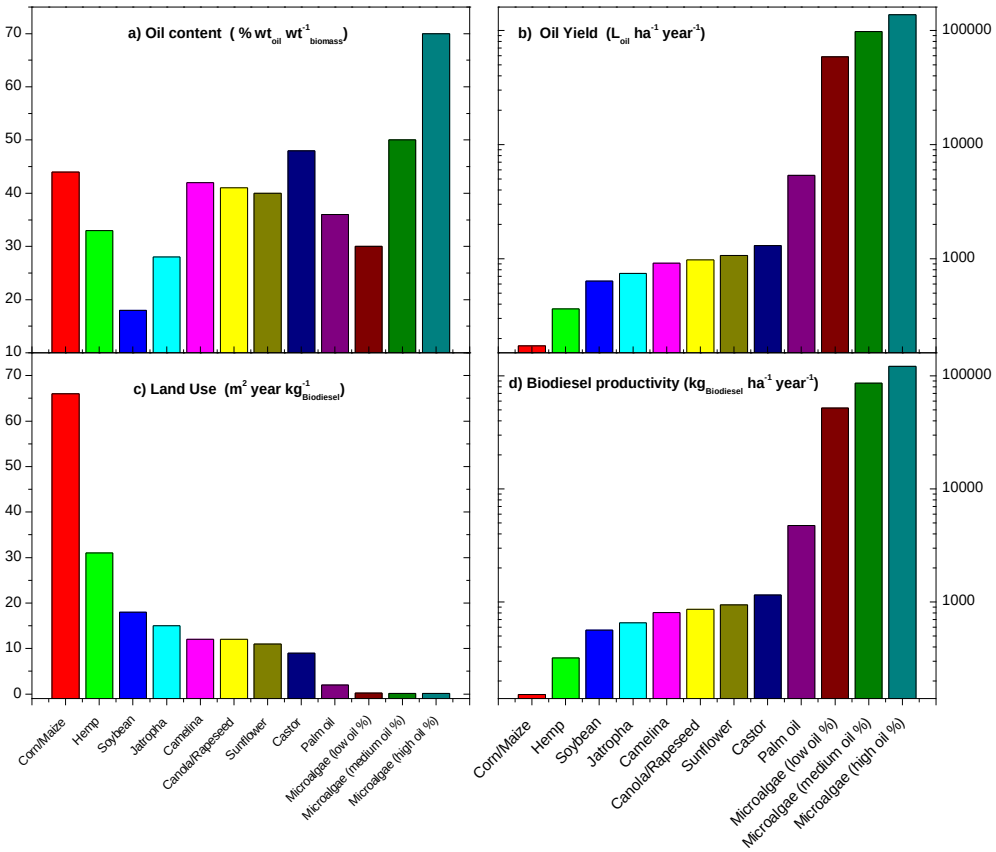


Figure 1.3 Comparison of microalgae with oil crops in terms of biodiesel productivity and land's area needs (adapted from Mata et al., 2010).

Furthermore, since the cultivation of microalgae is carried out in open ponds and photobioreactors which can be located in marginal lands that are unsuitable for conventional agriculture, the competition with arable ones is drastically reduced. In addition, microalgae are not directly involved in the human food supply chain, thus eliminating the food versus fuel dispute that represents the main drawback related to first generation biofuels (Ahmad et al., 2011). Further advantages of microalgae over higher plants as feedstock for biofuel production are summarized as follows:

- microalgae are able to double their biomass in very short times (4 – 24 h) thus allowing harvesting cycles of 1-10 days which are much shorter as compared with those ones of crop plants, i.e. only once or twice for each year (Schenk et al., 2008; Ahmad et al., 2011);
- microalgae grow in aquatic media, while less water is needed with respect to terrestrial crops (Rodolfi et al., 2009);
- microalgae display larger light capture and conversion efficiency than crop plants which leads to reduce fertilizer and nutrient inputs thus resulting in less waste and pollution (Schenk et al., 2008). Moreover, fertilizers suitable for

microalgae cultivation (especially nitrogen and phosphorus) can be obtained from wastewaters (Concas and Cao, 2011; Rodolfi et al., 2009);

- cultivation of microalgae might be coupled with the direct bio-capture of CO2 emitted by industrial activities that use fossil fuels for energy generation (Concas et al., 2012);
- microalgae can be used for producing valuable co-products or by-products such as biopolymers, proteins, carbohydrates, vitamins, antioxidants, PUFAs etc. (Ahmad et al., 2011);
- cultivation of microalgae does not require the use of herbicides or pesticides (Rodolfi et al., 2009);
- oil content of microalgae can be further increased by adopting specific operating conditions during their growth (i.e. nitrogen starvation, etc.)

Ultimately, when compared to first and second generation biofuels, microalgae are characterized by a more sound environmental sustainability and economic viability (Quinn et al., 2011). For these reasons, the potential exploitation of microalgae as renewable resource for the production of liquid biofuels is receiving a rising interest (Olguin, 2003; Mulbry et al., 2008).

2.3 Parameters affecting microalgae growth

It is well known that algae growth in batch cultures proceeds according to the five main phases depicted in Figure 1.4 and described in what follows (Jalalizadeh, 2012):

- A lag phase, where a growth delay takes place when cultivation starts due to physiological adjustments of the inoculum to changes in nutrient concentration, light intensity and other culture conditions;
- An exponential phase, where cells grow and replicate exponentially with time, as long as all the conditions affecting algae growth are optimized, i.e. nutrients and light availability, optimal temperature and pH, etc.
- A linear growth phase, where biomass concentration grows linearly as a function of time;
- A stationary growth phase, where the biomass concentration remains constant as a result of the reduced availability of nutrients and light that lead the death rate to equal the growth one;

- A decline or death phase, where the decrease in the concentration of nutrients and/or the accumulation of toxic waste products lead the death rate to overcome the growth one.

Such a growth behavior can be well described by the mass balance for microalgae biomass reported in what follows:

$$\frac{dX}{dt} = (\mu - k_d) X$$

where X is the microalgal biomass concentration (mass/volume), μ is the specific growth rate (1/time) and k_d is the specific mass loss rate (1/time) which accounts for all the phenomena that are responsible of biomass depletion, i.e. cell catabolism, apoptosis, lysis, etc. The term ($\mu - k_d$) is the net growth rate.

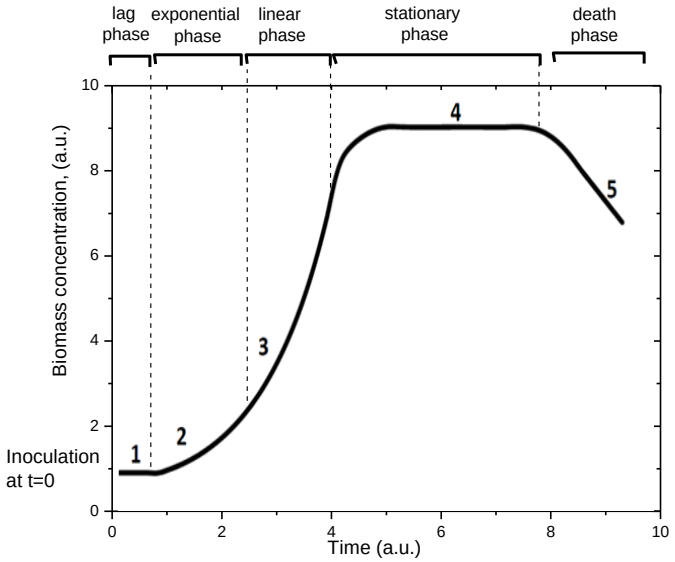


Figure 1.4 Schematic representation of biomass growth in a batch culture (adapted from Jalalizadeh, 2012).

While k_d is usually considered to be constant, the growth rate μ depends upon several factors which can affect microalgae growth. Among them, light, nutrient concentration, pH and temperature (T) are quite important. From a mathematical point of view the influence of each parameter on the growth rate μ can be expressed as follows:

$$\mu = \mu_{\max} \cdot g(I_{av}) \cdot h(S) \cdot f(pH) \cdot \psi(T)$$

where μ_{max} is the maximum growth rate which can be achieved under optimal growth conditions for the specific strain considered, I_{av} is the light intensity available during cultivation and S the generic substrate concentration. The functions expressing the

influence of each parameter on the specific growth rate will equal to 1 when the parameter value is optimal for microalgae growth. In what follows the effects of each operating parameter is analyzed and discussed.

2.3.1 Effect of light

Light is essential for the phototropic growth of microalgae. Spectrum, intensity and photoperiod of light influence microalgae growth. Photosynthetically active radiation (PAR) designates the spectral range (wave band) of solar radiation from 400 to 700 nm that microalgae are able to use during the process of photosynthesis. It should be noted that photons at shorter wavelengths (<400 nm) carry a very high energy content that can damage microalgal cells, while at longer wavelengths (> 700 nm) the energy carried does not allow photosynthesis to take place. Therefore, if we denote by I□ (□molphotons m-3 s-1 or □E m-3 s-1) the intensity of light at the generic wavelength □ (m), the total intensity I exploitable by algae for phototrophic growth can be calculated as follows:

$$I(t) = \int_{400\,(\mathrm{nm})}^{700\,(\mathrm{nm})} I_{\lambda}(t,\lambda)\, d\lambda$$

When light penetrates in an optically dense medium such as a microalgal culture it experiences attenuation phenomena due to absorption by the medium as well by the pigments of microalagal cells. Such effect is usually represented by Lambert-Beer's law:

$$I(r,t) = I(0,t) \cdot \exp(-k_a \cdot r \cdot X)$$

where r is the path length traveled by the light ray within the culture, ka is the extinction coefficient and X is the biomass concentration. Thus, the light intensity reaching a microalgal cell depends upon its position with respect to the light direction. While it can be difficult to identify the light intensity reaching each single cell in a culture, it is simpler to evaluate the average light irradiance in the culture vessel. For this reason the light dependent kinetics of microalgae growth are usually expressed with reference to the averaged light intensity which can be calculated as follows (Sevilla and Grima, 1997):

$$I_{av}(t) = \frac{\int_V I(r,t) \cdot dV}{V}$$

where V is the culture volume. The effect of average light intensity on microalgae growth and photosynthesis has been extensively studied in the literature. Typically, the

concentration of microalgae in solution increases with the intensity of light up to a certain level (saturation intensity), beyond which a further increase of light intensity does not provoke the increase of algal growth rate that remains almost constant (cf. Figure 1.5a).

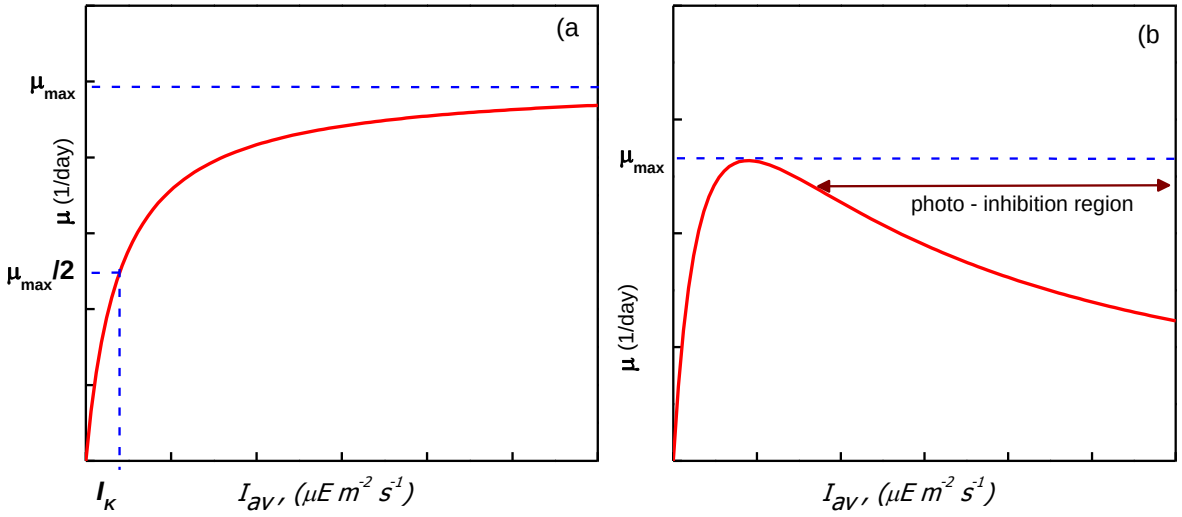


Figure 1.5 Effect of light intensity on growth rate without (a) and (b) with photo-inhibition (adapted from Chisti, 2007)

Such a behavior is quantitatively well described by the classical saturation kinetics:

$$\mu = \mu_{\max} \cdot g\left(I_{av}\right) = \mu_{\max} \cdot \frac{I_{av}}{I_K + I_{av}}$$

where IK represents the half saturation constant for light intensity. On the other hand, when light intensities overcome a certain threshold, a damage of the microalgae photosystem can take place, which ultimately results in a decrease of the growth rate as shown in Figure 1.5b (Grima et al., 1999). In Table 1.2 a list of suitable equations for the specific growth rate as a function of light intensity which are also able to quantitatively *describe photo – inhibition phenomenon, is reported.*

Table 1.2 Specific growth rate expressions available in the literature as a function of light intensity (cf. Sevilla and Grima, 1997)

Kinetic model
$\mu = \mu_{\max} \cdot g\left(I_{av}\right) = \mu_{\max} \cdot \frac{I_{av}^{n}}{I_K^{n} + I_{av}^{n}}$
$\mu = \mu_{\max} \cdot g\left(I_{av}\right) = \mu_{\max} \cdot \exp\left(1 - \frac{I_{av}}{I_{\max}}\right)$

$$\mu = \mu_{\max} \cdot g\left(I_{av}\right) = \mu_{\max} \cdot \frac{I_{av}}{I_{\max}} \cdot \exp\left(1 - \frac{I_{av}}{I_{\max}}\right)$$

$$\mu = \mu_{\max} \cdot g\left(I_{av}\right) = \mu_{\max} \cdot \frac{I_{av}}{I_K + I_{av} + I_{av}^2 / K_I}$$

Such expressions have been successfully used to simulate experimental data obtained either in batch or turbidostat mode with optically thin cultures (Sevilla and Grima, 1997).

2.3.2 Effect of nutrients

The medium where microalgae grow basically consists of water enriched by macro (C, N, P, S) and micro (Mg, Zn, Fe, K, Na etc.) nutrients as well as by the CO_2 transferred from the gas phase (i.e flue gas or air). Besides CO_2, whose role in photosynthesis has been already discussed in paragraph 1.3.1, nitrogen and phosphorous are key elements for algae metabolism. Their suitable balancing in the growth medium is thus critical for an effective process design (Mandalam and Palsson, 1998). Ammonia, urea and nitrate are often selected as nitrogen source for the mass cultivation of microalgae. Although ammonia and urea are often used in mass cultivation owing to their relatively low-cost, selecting proper nitrogen source for each algal species is important in improving biomass and oil productivity (Li et al., 2008). Urea and nitrate were found to be better nutrients than ammonia for the growth and lipid accumulation when considering Chlorella sp., Chlorella vulgaris, Neochloris oleoabundans and Scenedesmus rubescens (Li et al., 2008; Hsieh and Wu, 2009). On the contrary, for different strains, the use of ammonia has been demonstrated to provoke higher biomass and lipid content than urea and nitrate (Xu et al., 2001).

It should be noted that the optimal concentration of nitrogen to be assured in the growth medium depends upon two counteracting effects. Specifically, while a high availability of nitrogen typically leads to a high biomass productivity, a decrease of nitrogen concentration in the cultivation broth typically results in higher lipid contents counteracted by lower growth rates. Such behavior depends upon the fact that, under starvation conditions, nitrogen concentration is not enough for activating the metabolic pathways leading to protein synthesis required by growth so that the excess of carbon coming from photosynthesis is channeled into storage molecules such as triacylglycerides or starch (Scott et al., 2010).

This inverse relationship between biomass productivity and lipid content makes the choice of the suitable nitrogen concentration not straightforward since a trade-off value should be assured in order to maximize lipid productivity (Concas et al., 2013). When considering phosphorus, microalgae are capable of metabolizing it mainly in polyphosphate form. Orthophosphate is generally considered the main limiting nutrient for freshwater strains but also in this case its optimal concentration depends upon contrasting effects. In fact, phosphorus starvation can result in higher lipid productivity, as reported for Monodus subterraneus, while may provoke changes in fatty acids composition for Phaeodactylum tricornutum and Dunialella tertiolecta (Liu et al., 2007). For all these reasons the preparation of the culture broth is a critical step for the entire process of biofuels production through microalgae. Moreover, the need of a continuous replenishment of macronutrients during algal cultivation is one of the most impacting cost item of the entire process. 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. 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 (Concas et al., 2013). Since nutrients concentration and supplies are among the most controllable factors in microalgae cultivation, at least the main macronutrients uptake rates need to be quantitatively evaluated for the microalgae strains candidate to industrial exploitation. In this way, macronutrients concentrations might be properly 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. Monod equation is the most common kinetic model used for describing the relationship between the microalgae growth rate and the concentration of the limiting nutrient:

$$\mu = \mu_{\max} \cdot h(S) = \mu_{\max} \cdot \frac{[S]}{K_S + [S]}$$

where Ks (g/L) is the half saturation constant and [S] (g/L) the substrate concentration. When multiple nutrients limitation take place, the Monod model can be written as follows:

$$\mu = \mu_{\max} \prod_{j=1}^{Nutrients} \frac{[S_j]}{K_{S,j} + [S_j]}$$

On the other hand, the use of the Monod model is limited to the case where no luxury uptake of nutrients and nutrient storage phenomena take place. However, the last ones are common phenomena in microalgae and lead to a temporal uncoupling between growth rate and dissolved nutrient concentrations (Jalalizadeh 2012). Therefore, when intracellular storage of nutrients takes place, the cell quota of the limiting nutrient, expressed as the total amount of nutrient contained within the cell per cell weight, better describes the nutritional status than does the concentration in solution. Ultimately, the growth rate of algae is more dependent on the internal cellular concentrations than on the external ones (Richmond, 2008). Under such conditions the Droop model (Droop, 1983), which is capable to relate growth rate to the internal cell quota, may properly simulate microalgae growth kinetics. The Droop model can be written as:

$$\mu = \mu_{\max} \cdot \left(1 - \frac{q_{\min}}{q} \right)$$

where q is the internal cell quota of the limiting nutrient (gnutrient/gcell) while qmin is the minimal internal cell quota below which algae growth does not take place.

2.3.3 Effects of pH

The time evolution of medium's pH during algal growth is a significant indicator of how well are evolving photosynthetic processes. In fact, as algae grow, dissolved CO2 is consumed by photosynthesis and, consequently, pH increases. However, pH variation not only represent a fundamental indicator of the evolution of photosynthetic activity but can also, in turn, strongly affects the growth kinetics of microalgae influencing the distribution of carbon dioxide species and carbon availability causing direct physiological effects (Cornet et al., 1995; Chen and Durbin, 1994). Moreover, in microalgal cultures, the hydrogen ion is recognized to be a non-competitive inhibitor near neutral conditions, while it can limit photosynthetic growth and substrate utilization rates at very low or very high pH levels (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 (Concas et al., 2012). In order to evaluate the dependence of growth rate upon pH the following expression has been proposed by Mayo (1997):

$$\mu = \mu_{\max} \cdot f(pH) = \frac{\mu_{\max} \cdot \left[H^+ \right]}{\left[H^+ \right] + K_{OH} + \left[H^+ \right]^2 / K_H}$$

which states that [H+] can be considered as a non-competitive substrate when the medium pH is high, while displays an inhibition effect when the pH of the medium is

low (Mayo, 1997). The resulting dependence of growth rate from pH is depicted in Figure 1.6.

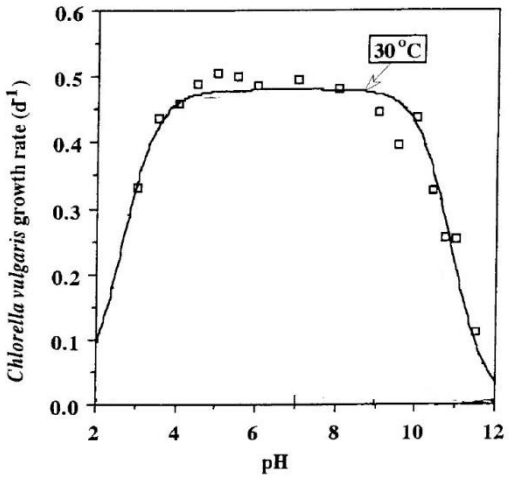


Figure 1.6 Effect of pH on the growth of Chlorella vulgaris at 30°C (adapted from Mayo, 1997).

As it can be observed from Figure 1.6, the kinetic model proposed by Mayo (1997) well describes the fact that each strain of microalgae has a relatively narrow optimal range of pH and most microalgal species are favoured by neutral pH.

2.3.4 Effect of temperature

Temperature is one of the main factors which regulate cellular, morphological and physiological responses of microalgae (Mayo, 1997; Durmaz et al., 2007). High temperatures generally accelerate the metabolic rates of microalgae, whereas low ones lead to inhibition of microalgal growth (Munoz and Guieysse, 2006). Under optimal temperature condition, the enzymes of microalgal cells show the highest activity. The optimal temperature range for microalgal growth depends on the specific strain considered but in general, it typically goes from a minimum of 5°C to a maximum of 35°C (Abu-Rezq et al., 1999).

As far as the kinetic dependence of growth rate from temperature is concerned, different relationships have been proposed in the literature. One of the most used equation, according to an Arrhenius type dependence, is reported as follows (Mayo, 1997):

$$\mu = \mu_{max} \cdot \psi(T) = \mu_{max} \cdot \frac{A \cdot \exp\left(-E_1 / R \cdot T\right)}{\left[1 + K \cdot \exp\left(-E_2 / R \cdot T\right)\right]}$$

where A and K are pre-exponential factors (-), E1 and E2 are the activation energies (J/mole), R is universal gas constant and T is the absolute temperature (K). This equation well interprets experimental data such as those ones reported as an example in Figure 1.7.

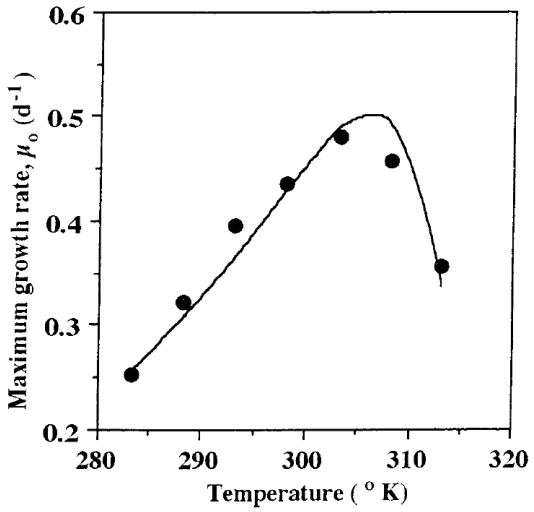


Figure 1.7 Temperature dependency of the growth rate for Chlorella vulgaris (adapted from Mayo, 1997).

The control of temperature is a key factor for cultivating microalgae outdoors. Actually, temperature can vary depending upon the geographic region of cultivation. Seasonal and even daily fluctuations in temperature can interfere with algae production. The internal temperature in photobioreactors can reach values that are 30°C higher than ambient one if suitable temperature control equipment is not used. To overcome this problem evaporation, cooling or shading techniques are successfully employed.

2.4 Production of biodiesel from microalgae

Microalgae cultivation systems are very different from those ones typically used for producing biomass feedstock for first and second generation biofuels. This is mainly due to the fact that, while biomass used for first and second generation biofuels consists mainly of terrestrial crops, the life of the microalgae and their proliferation occurs in liquid environments. Therefore, when compared to terrestrial crops, the production of microalgae requires specific cultivation, harvesting and processing techniques which should be correctly implemented to the aim of viably produce biodiesel (Mata et al., 2010).

Basically, the current processes for biodiesel production from microalgae involve distinct operating steps where cells are grown, separated from the growing media, dried (or disrupted) and finally undergone to lipid extraction processes. Once extracted, microalgal lipids are processed through technologies similar to the existing ones for the production of biodiesel or other biofuels starting from first generation biomass feedstocks. While different biofuels can be produced from microalgae in this work we will focus on the production of biodiesel. Figure 1.8 shows a schematic representation of the process for the CO2 capture and biodiesel production through microalgae. As it can be seen, the process starts with the CO2 capture and its conveying in the cultivation system where microalgae grow exploiting the sunlight and the nutrients suitably provided. Then, it follows the biomass harvesting, downstream processing and oil extraction to supply the biodiesel production unit.

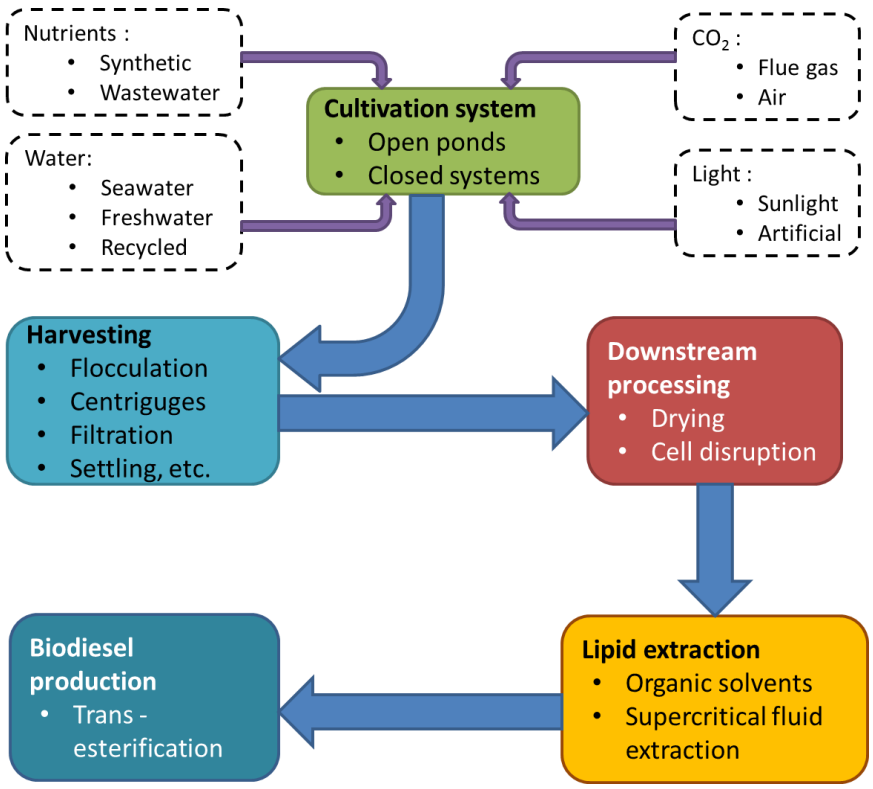


Figure 1.8 Schematic representation of the "algae to biodiesel" process.

As reported in Figure 1.8, cultivation of microalgae can be performed in open systems (ponds, raceways, lakes) or in closed ones, i.e. photobioreactors. Whatever the system being used, a suitable source of CO_2 must be supplied to microalgae. To this aim, atmospheric air (0.03 %v/v of CO_2), flue gas (9-15% of CO_2) or pure concentrated CO_2 (100%v/v) can be used. Atmospheric air as CO_2 source, significantly simplifies the lay-

out and the operation of the plant while, because of the lower CO_2 concentration in air, high volumes of air are required in order to sustain microalgae growth at an acceptable rate. This can result in very large cultivation systems that require a high land availability. On the contrary, when flue gases are used as carbon source, lower flow rates of gases should be pumped into the cultivation system for supplying the necessary amounts of carbon to sustain microalgae growth. Moreover, the use of costless feedstocks such as flue gases as source of CO_2 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 and climate changes. For this reason the potential exploitation of CO_2 from flue gases is one of the main targets of scientists and technicians operating in this field (Concas and Cao, 2011; Francisco et al., 2010). However, the use of flue gas as carbon source might raise specific concerns related to the toxicity of some of its constituents with respect to algae. For this reason the flue gas should be pre-treated before feeding it in the cultivation system. A further challenge in the carbon capture through microalgae is the use of pure concentrated CO_2 (100 %v/v) obtained from flue gas. In this case in fact, beside the lesser volumes of photobioreactors that are needed, the potential poisoning effects provoked by other compounds in flue gas (NOx, SOx etc.) could be reduced thus increasing the net growth rate of microalgae.

Besides CO_2, several micro and macro nutrients must be supplied to the culture in order to sustain microalgal growth. While the importance of the nutrients has been already discussed in paragraph 2.2, it is noteworthy to underline that the exploitation of costless feedstocks such as wastewaters as sources of macronutrients, might greatly improve the economic feasibility of the microalgae-based technology while simultaneously producing a positive impact on important environmental concerns such as water pollution. In fact , wastewaters, even if pre-treated, may contain residual concentration of nitrogen and phosphorus which are capable to sustain microalgal growth (Concas and Cao, 2011). In particular, industrial and agricultural wastewater and secondary sewage treated effluent can be used as source of nitrogen and phosphorus (Devi et al., 2012). For this reason the operation step of medium preparation can involve a pre-mixing with wastewater.

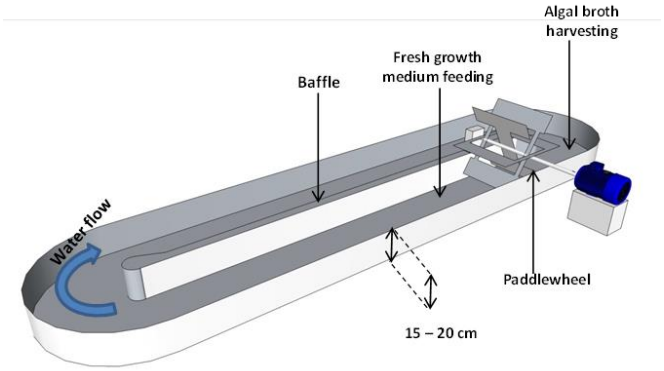
Finally, in order to suitably cultivate microalgae, large amounts of water must be available. Depending upon the specific microalgal strain to be cultivated, freshwater or seawater can be used for preparing the growth medium. The use of marine strains would

be convenient since seawater can contain suitable concentrations of micronutrients whose purchase could be so avoided. Moreover, seawater would be available in large amounts without affecting water resources to be exploited for other uses. Since water consumption might be significant, the recycling of the exhaust growth medium at the outlet of the cultivation systems should be always considered in an optimized flow-sheet.

Finally water and nutrients are dosed and mixed within a suitable pre-mixing unit. The obtained mixture is then filtered, sterilized and finally pumped into the cultivation systems.

2.5 Cultivation of Algae in open ponds

Different designs have been proposed for open ponds, natural or artificial ones, operating at large scale. Typical examples are the unstirred ponds (lakes and natural ponds), the inclined ones, central pivot and the raceway ponds. Among the others, the most widespread typology of open pond is the so called "raceway pond". It basically consists of open channels where a paddlewheel is used to drive the flow, while algae are kept suspended in water around a racetrack. Baffles in the channels guide the flow in order to minimize space. Raceways are typically made by concrete but can also simply dug into the soil and waterproofed with a plastic liner to prevent the liquid filtration through the ground. These systems are usually operated in a continuous mode, where the fresh medium (containing macro and micro nutrients) is fed in front of the paddlewheel and algal broth is harvested behind it after being circulated through the loop (Singh and Sharma, 2012). The raceways (cf. Figure 1.9) are characterized by low water depths of about 15-20 cm in order to assure a suitable light penetration along the hydraulic section thus avoiding dark zones where microalgae can't grow. At such depths biomass concentrations of 1 g L-1 can be achieved and productivities ranging from 15 to 25 g m-2 day-1 are possible (Schenk et al., 2008).



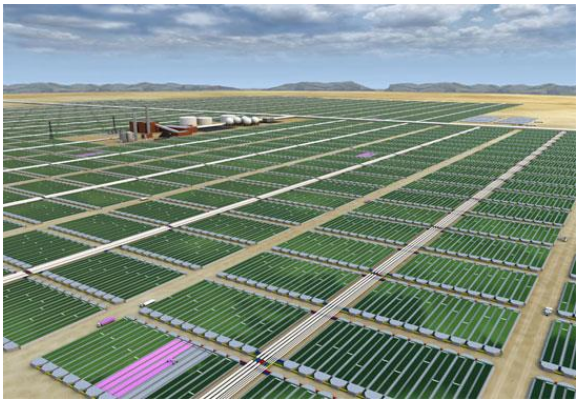


Figure 1.9 Scheme of a single raceway pond and photography of raceway pond farm (adapted from Rapier 2012).

In general, these cultivation systems are less expensive to build and simpler to operate than closed ones. For this reason they are currently considered as the most cost effective way for the massive production of microalgae at a large scale. However, open ponds display several limitations. In particular, when compared to close systems, open raceways are characterized by a lower productivity that is the result of a number of factors. Evaporative losses can lead to changes in the ionic composition of the growth medium thus potentially provoking negative effects on culture growth such as iper-salinity, nutrient's precipitation etc. Changes in temperature, photoperiod deriving from seasonal variation cannot be suitably controlled in open ponds (Rawat et al., 2012). These systems are more susceptible to contaminations by competing organisms such as mushrooms, bacteria and protozoa. Furthermore, since atmospheric carbon dioxide is used as carbon source, its transfer rate is very low and consequently carbon starvation phenomena could take place. Finally, sunlight is available only at the surface of the pond and hence, in the deeper strata of the liquid bulk, light limitation phenomena can arise. Improved mixing and bubbling the air at the bottom of the ponds by means of suitable spargers can minimize impacts of both CO2 and light limitation but in general the productiviy of these systems is very low whereby large areas of land may be required to meet the desired output of cultivation (Rawat et al., 2012).

To overcome limitations related to open system and in the meantime keeping their low operating cost, the potential use of closed raceway ponds are currently under study. These systems consist essentially of an open pond covered by a transparent or translucent barrier which turns it into a greenhouse (Singh and Sharma, 2012). This configuration prevents the microalgae to be contaminated by competing bacteria and allows a better control of crucial operating parameters such as temperature, evaporation

etc. Moreover, by using closed raceways the amount of CO_2 provided can be increased since the gas bubbled at the bottom cannot escape to the atmosphere.

2.6 Closed systems (photobioreactors)

Photobioreactors (PBR) are closed systems having no direct exchange of gases and contaminants with the environment where culture broth and microalgae are exposed to a photonic energy flux which triggers photosynthetic phenomena hence allowing biomass growth. Since they are closed reactors the crucial operating parameters such as temperature, pH, nutrient concentration, light intensity distribution, mixing, gas mass transfer rate can be suitably controlled and optimized. As a result photobioreactors typically have higher biomass productivities than open ponds. On the contrary, photobioreactors are more expensive and complicated to operate than open ponds. A qualitative comparison between photobioreactors and open ponds is summarized in Table 1.3.

Ideally, a photobioreactor for production of biomass should catch all sunlight available, dilute and distribute it uniformly in the growth medium where algae are suspended in such a way that all the caught light energy can be suitably exploited by algae for biomass formation. For this reason a critical design parameter of photobioreactors is the illumination surface area per unit volume. Typically, a high illuminated surface area to volume ratio (SVR) results in a higher light availability in the liquid bulk and consequently in higher volumetric productivities of the systems

The surface to footprint ratio (SFR) is another critical design parameter. Higher values of SFR correspond to a larger areal productivity of the photobioreactor and consequently the lesser is the land's area needed for producing the required output of microalgal biomass. Different types of photobioreactors are currently under study and development with the aim of reaching the more suitable configuration where SVR and SFR are maximized.

Table 1.3 Comparison between open ponds and photobioreactors (adapted from Lutzu, 2012)

Parameter	***Open systems***	***Photobioreactors***
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	1 g L^{-1}	3- 5 g L^{-1}
Constructions costs	Low	High
Weather dependence	High	Low
Overheating problems	Low	High
Dissolved oxygen concentration	Low	High
Scale-up	Difficult	Difficult
Surface to volume ratio, SVR (m^2/m^3)	< 4	<100
Surface to footprint ratio, SFR (m^2/m^2)	1	<10

2.6.1 Vertical tubular photobioreactors

The classical configuration of vertical tubular photobioreactor is the bubble column. It is basically a cylinder with radius of up to 0.2 m and height of up to 4 m. The height to diameter ratio is typically kept greater than 2 in order to maximize the SVR ratio. The CO2 is provided to the algae by bubbling the gas from the bottom upwards through suitable spargers. While allowing a better CO2 mass transfer, the bubbles flow provides also the suitable mixing degree without provoking significant shear stresses on microalgae. Moreover, the gas flow enables the effective removal of photosynthetic O2 produced by algae which, if accumulated in the liquid, can inhibit the growth. The height constrain of these columns (< 4m) depend upon the gas transfer limitations and

the strength of the transparent materials used to construct the columns. Since CO2 supply and O2 removal is optimized, in such type of reactors algal growth is often limited by other parameters such as light (Wang et al., 2012). A schematic representation of different types of vertical tubular photobioreactors is shown in Figure 1.10.

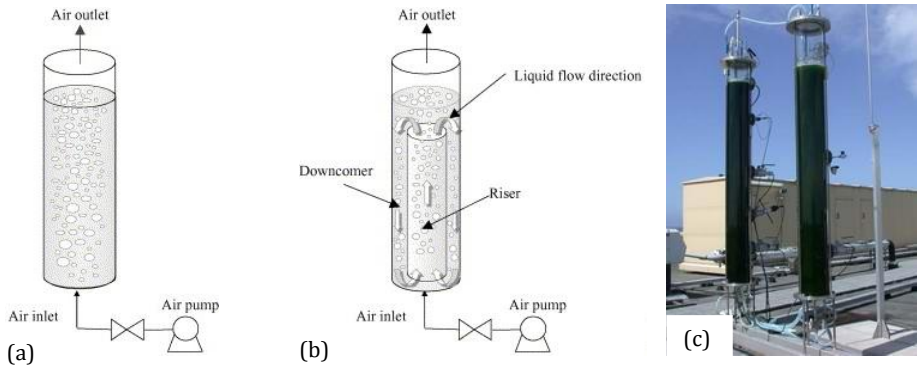


Figure 1.10 Schematic representation of bubble column (a) and airlifit (b) photobioreactors and picture (c) of an industrial bubble column photobioreactor (adapted from Krichnavaruk et al., 2005).

A specific configuration of vertical tubular photobioreactors is the so called airlift reactor. It consists of a vessel with two interconnecting zones (i.e. the riser and the downcomer). The gas flow is introduced at the bottom of the riser and carries the liquid upward. At the top of the column liquid/gas separation takes place in the freeboard regime thus allowing the removal of accumulated photosynthetic oxygen. Subsequently, the degassed liquid falls downward in the downcomer. Mixing is therefore guaranteed by aeration and liquid circulation. This system allows a better exposure of microalgal cells to light radiation than classical bubble columns as well as an effective mixing and degassing of the liquid. Airlift PBR configurations may include an internal loop airlift, split column airlift and external loop airlift.

2.6.2 Flat panel photobioreactors

Flat panels (cf. Figure 1.11) are parallelepiped shaped photobioreactors having a minimal light path and a large illumination surface area (SVR) which can reach values of up to 40 m-1 (Singh and Sharma, 2012). The thickness of plate is the crucial parameter in the design of flat panels because it determines the surface area/volume ratio and the length of light path (Wang et al., 2012). They can be made from transparent materials like glass, plexiglass, polycarbonate etc. The CO2 is provided by

bubbling the gas from one side of the panel through suitable perforated tubes. Mixing of the liquid is assured by the gas flow or by rotating the photobioreactor through a motor (Singh and Sharma, 2012).

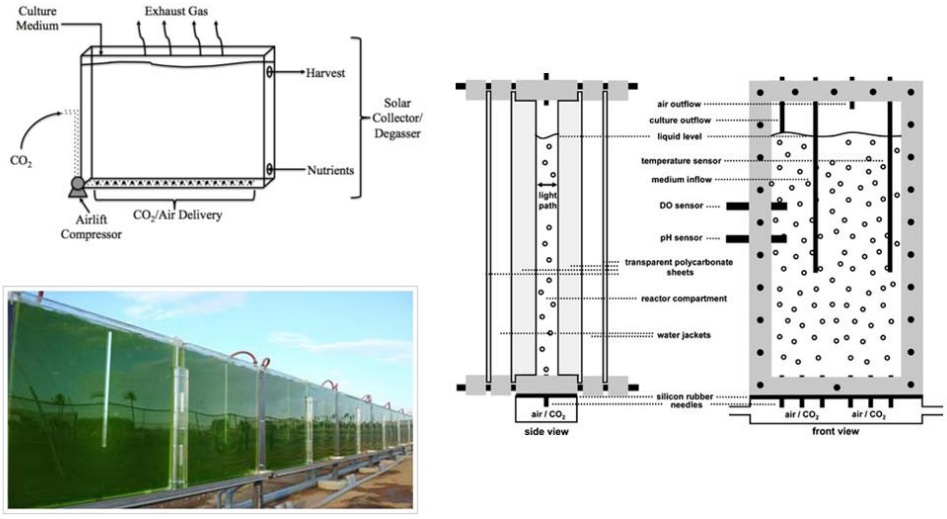


Figure 1.11 Schematic representations and picture of flat panel photobioreactors (adapted from Carter, 2012 and Zijffers et al., 2010).

Major limitations of conventional flat panels are the difficulties of controlling the liquid flow and the relatively high construction costs. To overcome these problems vertical alveolar panels, made in plexiglass, were proposed (Tredici and Materassi, 1992). These systems allows to obtain a high surface-to-volume ratio of about 80 m-1. A good biomass productivity can be achieved by using these alveolar panels as well as a good mixing degree and suitable mass transfer rates. Moreover, the manufacturing costs of these reactors are quite low. However, critical operating parameters such as temperature and light penetration should still be optimized in such a PBR (Wang et al., 2012).

2.6.3 Horizontal tubular photobioreactors

Horizontal tubular reactors typically consist of arrays of transparent thin tubes built in different patterns (i.e. straights, loop or serpentine shaped etc). The arrays of tubes can be arranged in parallel or in series and then placed horizontally on the ground. Horizontal placement of these tubes results in a better angle for incident light compared to vertical tubular reactors, allowing for more efficient light harvesting (Wang et al., 2012). Moreover, the tubes are preferably oriented towards the sunlight in order to

maximize the light capture and the ground under the tubes can be covered with white plastic sheets in order to increase the albedo. In fact, a high albedo results an increase of the total light received by the tubes. Typically, these tubes are less than 0.1 m in diameter since otherwise the light does not suitably penetrate in the less exposed zones of dense cultures. However, larger diameters may be used when suitable regimes of turbulence of the fluid are employed in order to assure the movement of algae from the illuminated part of the tube to the dark one and vice-versa. Prolonged exposure to light in the illuminated part of the tube can trigger photo-inhibition phenomena while a long time exposure to darkness can inhibit photosynthesis. Furthermore, the tubes should not be longer than 80 m in order to avoid the accumulation of photosynthetic oxygen in the culture and a too high increase of pH as algae grow (Concas and Cao, 2011). Besides the tubes, which act as solar collectors, the horizontal photobioreactors include the following components: the harvesting unit to separate algae from the suspension, a degassing column for gas exchange and cooling (or heating) and a circulation pump (Wang et al., 2012). In Figure 1.12, a specific configuration of horizontal photobioreactors is shown.

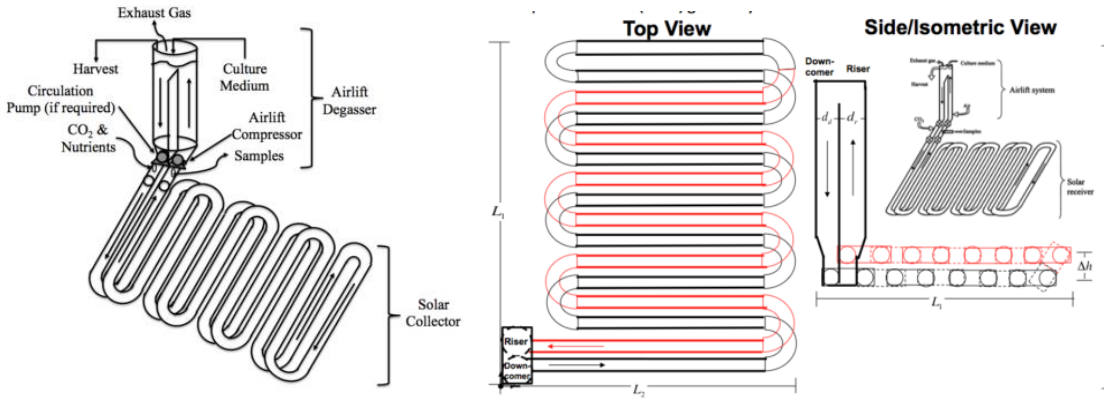


Figure 1.12 Schematic representations of horizontal (serpentine type) photobioreactors (adapted from Carter, 2012).

In the degassing device air or CO2 enriched air is injected in order to strip dissolved oxygen and at the same time provide the CO2 to algae culture. In the degasser also the feeding of fresh medium can be carried out. Typically horizontal photobioreactors are capable to capture light better than other photobioreactor thus potentially assuring higher productivities. On the other hand, just this characteristic can cause the onset of photo-inhibition phenomena as well as the accumulation of high amounts of heat. Thus,

expensive temperature control systems such as heat-exchangers are often required during large scale cultivation of algae. Furthermore, long tubular PBRs are characterized by gradients of oxygen, CO2 and pH 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. Finally, it should be noted that adherence of the cells to the walls of the tubes is common. This results in a progressive fouling of the tubes and a consequent worsening of light penetration in the culture.

2.6.4 Advantages and drawbacks of different types of photobioreactors

Each photobioreactor configuration described above is characterized by specific advantages and drawbacks that should be considered when designing the cultivation system. A summary of the main features of these three configurations is shown in Table 1.4.

Table 1.4 Prospects and limitations of different photobioreactors (adapted from Lutzu, 2012)

Culture systems	***Advantages***	***Disadvantages***
Horizontal 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, require 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 requires many compartments and support materials, difficulty in controlling culture temperature, some degree of wall growth, possibility of hydrodynamic stress to some algal strains

2.7 Harvesting of microalgae

At the outlet of the photobioreactor, the bulk culture medium is characterized by a water content ranging from 99.5% to 99.9% and thus the harvesting of microalgal biomass is basically carried out through a dewatering/concentrating phase. Such operating phase is usually accomplished through a two-step process (Pragya et al., 2013). The first step, where biomass is concentrated to 2%–7% dry weight, is called bulk harvesting. In the second one, called thickening, the algal slurry is further concentrated to about 15-25 % in order to obtain better manageable slurry in the subsequent operations of lipid extraction (Pragya et al., 2013). Thickening is more energy intensive than bulk harvesting (Chen et al., 2011). Either bulk harvesting or thickening can be performed by means of different techniques which will be briefly summarized in what follows.

2.7.1 Gravity sedimentation and fllucculation

This technique is typically used for the bulk harvesting of microalgae and refers to a process by which microalgal cells settle to the bottom of a liquid under the action of gravity and subsequently form a sediment which can be easily harvested. Sedimentation is performed through thickeners and clarifiers that are similar to those ones used in standard wastewater treatment plants. It is an energy efficient method while the separation yield depends upon several factors such as microalge cells size as well their tendency to aggregate. Since the relatively small diameter of cells (5-50 □m) and the colloidal character of microalgal suspensions, gravity settling is typically a very slow process (settling rates of 0.1-2.6 cm h-1) which requires large tanks in order to give an effective solid/liquid separation. Fortunately, sedimentation rate can be enhanced by the addition of flocculants to the system. The latter ones are chemicals which improve the rate of sedimentation of the microalgae by aggregating the dispersed microalgal cells into larger colonies which can settle down faster. Common inorganic flocculants are aluminum- and iron-based metal salts. However, metallic salts are quite expensive and require an acidic pH as well as a high dosage to provide an adequate result. On the other hand, cell apoptosis can be induced by the addition of aluminum salts. Residual metal salts after harvesting may negatively affect both the medium recycling and the quality of the desired products. For this reason organic flocculants or polyelectrolytes which are cationic polymers that physically link cells together are generally preferred. Such flocculants are better tolerated by algae, require a lower dosage and do not affect

medium recycling. Among the different types of organic flocculants available on the market, the most used are chitosan and grafted starch (Kim et al., 2013). After flocculation the microalgae settle down faster and can be harvested from the bottom of the settler. Another technique for increasing the settling rate of algae is the auto-flocculation. In this case chemicals such as carbonates and hydroxides (NaOH) are added to induce physiochemical reaction between algae and promote auto-flocculation due to carbonates precipitation when pH rises as a result of the photosynthetic phenomena.

A further technique is the bio-flocculation which consists in culturing microalgae with another microorganism that promotes sedimentation. Example is the use microbial flocculants for harvesting mass culture of Chlorella vulgaris from Paenibacillys sp. AM49 (Richmond, 2008; Chen et al., 2011).

2.7.2 Centrifugation

Centrifugation is a process that involves the use of the centrifugal force for the stratification of algal culture into regions with different solid concentration that are subsequently separated by draining the less concentrated phase (supernatant). Centrifugation can also be followed by sedimentation to separate the supernatant. This technique allows an effective separation of microalgae in a relatively short time. According to Pragya et al. (2013) about 80%–90% microalgae can be recovered within 2–5 min. For these reasons centrifugation is one of the most preferred methods for harvesting of algal biomass. On the other hand, this method is high energy consuming thus potentially being able to negatively affect the CO2 balance of the process. Energy requirement consumption for various types of centrifuge is estimated to range from 0.3 – 8 kWh m-3 (Alabi et al., 2009). Some authors claimed that centrifugation process needs about 48.8% of the total energy consumption during algal biofuel production. For all these limitations alternative methods for algae harvesting are currently under study and development.

2.7.3 Filtration

Filtration can be used to concentrate microalgal cells. The technique is based on the use of specific filters such as screens, micro-strainers or membranes through which the algal suspension is passed. Microalgae or microalgae colonies are retained by the filter depending on the difference between the cell size and mesh dimension of the filter. The conventional filtration processes are suitable for the harvesting of microalgae having a relatively large (>70µm) cell size such as Spirulina. It cannot be used for microalgae specie having diameters lower than 30 µm such as Scenedesmus, Dunaliella and Chlorella. Different filtration techniques can be used. Micro-strainers are rotating filters with fine mesh screens. They are simple to operate, require low investment and have high filtration ratios. Other methods of filtration include dead-end filtration, vacuum or pressure filtration and cross-flow filtration.

In dead end filtration the fluid flows perpendicularly to the filter and the trapped particles start to build up a "filter cake" on the surface of the membrane which reduces the efficiency of the filtration process until the filter cake is washed away in back flushing. Dead-end filtration of large amounts of algal broth can only be accomplished using packed bed filters (made from sand) and its application is limited to the removal of algae culture having low solid concentration due to the rheological properties of microalgae which produce compressible cakes and hence clog the filters.

To overcome such limitation dead hand filtration vacuum filters can be used. They are able to recover large amounts of microalgae, although they are less effective when applied to organisms approaching bacterial dimensions. A recovery of 80% to 90% of freshwater algae is achievable with vacuum tangential flow filtration. Tangential-flow filtration is widely used to decrease filter or membrane fouling and performs more efficiently than does dead-end filtration. In cross-flow filtration, backwashing and ventilation of the algae medium can help control the fouling and recover flux (Pragya et al., 2013).

Ultrafiltration is another technique that is capable to concentrate an algal suspension up to 150-fold (from 1 to 154.85 g/L) under conditions of pulsated air scouring combined with backwashing (Kim et al., 2013). Filtrations are basically simple but potentially very expensive depending on specific operating parameters such as filter pore size, algae aggregation rate, microalgae specie, filter materials etc (Greenwell et al., 2010). Energy consumptions range from 0.2-0.88 kWh m-3 to 0.1-5.9 kWh m-3 for vacuum or pressure filtration, respectively (Alabi et al., 2009).

2.7.4 Flotation

Laboratory experiments have shown that also flotation is suitable for harvesting small, unicellular algae. The separation of suspended microalgae from the liquid bulk phase is achieved through the use of air or gas bubbles which flow upwards within a flotation tank or basin. The small bubbles adhere to the suspended microalgae and then carry them to the liquid surface where they may be removed by a skimming device (Pragya et al., 2013). Microalgal cells with a diameter from 10–30 μm to 500 μm are preferred for effective flotation. Typically, the flotation efficiency depends on the size of the bubbles: nanobubbles (< 1 μm), microbubbles (1–999 μm), and fine bubbles (1–2 mm). As a rule of thumb, the smaller is the bubble size the longer is their longevity and the larger is their carrying capacity due to the increased surface area-to-volume ratio. Moreover, small bubbles rise slowly and thus can more easily adhere to microalgal cells and more stably transport them to the water surface than large bubbles (Kim et al., 2013). Depending on the bubble size, flotation can be carried out through dissolved air, dispersed air or electrophoresis. In dissolved air flotation the water stream is pre-saturated with pressurized air. The pressurized air is then released at atmospheric pressure in a flotation tank or basin. The released air forms tiny bubbles of 10–100 μm in size. On the contrary, dispersed air floatation is achieved by injecting air bubbles into the water through an air injection system and a high speed mechanical agitator. In this way bubbles having diameter ranging from 700 to 1500 μm can be produced. In both dissolved and dispersed air flotation, flocculants can be added to increase the microalgae separation yield. Electrophoresis techniques exploit the hydrogen bubbles generated by electrolysis of water for transporting microalgal flocs to the water surface. Moreover, since microalgal cells have a negatively charged surface, the application of an electric field can cause algae to migrate towards the positively charged anode where they can be harvested (Pragya et al., 2013). The major benefit of approaches based on electrophoresis is that no chemical addition is required, however, the high power requirements and electrode costs do not make for an appealing harvesting method, especially for large-scale applications (Christenson and Sims, 2011)

2.7.5 Final consideration about harvesting microalgae

It is worth noting that, according to some authors, harvesting alone, is one of the most expensive steps of the overall process of microalgae production since it accounts for

20%–30% of the total production cost (Rawat et al., 2011). Therefore, in order to assure the economic sustainability of the process, efficient and inexpensive harvesting methods should be developed and subsequently adopted at the industrial scale. Moreover, the correct choice of the technique for dewatering the microalgal culture is critical in order to reduce water consumption. In fact, the exhaust liquid growth media separated during this operating phase should be recycled for preparing the fresh growth medium. In Table 1.5 a comparison made by Christenson and Sims (2011) between the advantages and limitations of the main techniques for microalgae harvesting is reported.

Finally, it is worth noting that also suitable combinations of the methods described above can be exploited in order to increase harvesting efficiency. In figure 1.13 two possible combinations of different harvesting techniques proposed by Rios et al. (2013) are shown.

Table 1.5 Comparison of mechanical harvesting methods for algae (adapted from Christenson and Sims, 2011)

Method	Solids concentration after harvesting	Recovery yields	Major benefits	Major limitations
Centrifugation	12-22%	> 90%	Reliable, high solids conc.	Energy intensive, high cost
Tangential filtration	5-27%	70-90%	Reliable, high solids conc.	Membrane fouling, high cost
Gravity sedimentation	0.5-3%	10-90%	Low cost	Slow, unreliable
Dissolved air flotation	3-6%	50-90%	Proven at large scale	Flocculants usually required

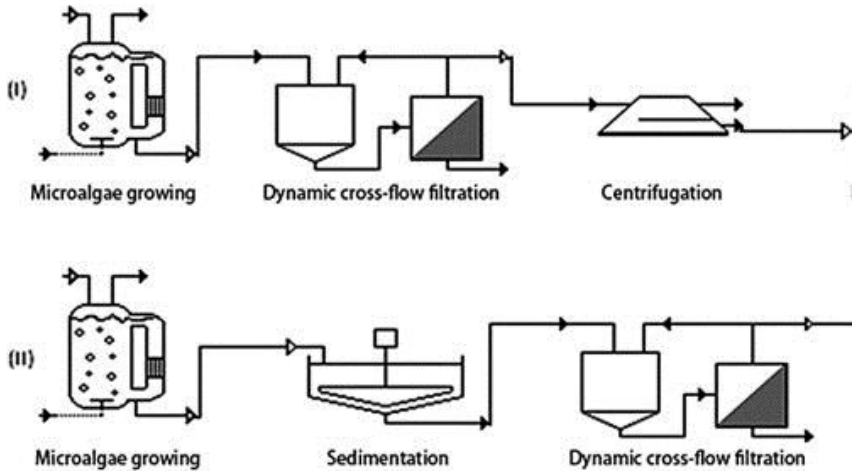


Figure 1.13 Diagrams of two possible concentration paths: from photobioreactor to (I) final concentrated biomass passing through dynamic microfiltration followed by centrifugation and to (II) final concentrated biomass passing through pH induce flocculation sedimentation and dynamic microfiltration (adapted from Rios et al., 2013).

2.8 Downstream processing

The harvested biomass slurry is characterized by a solid content ranging from 5 to 15 % dry weight. Lipid extraction can be performed both from wet and dry biomass. Depending upon the specific lipid extraction route adopted, specific pre-treatments should be carried out. In particular, if lipid extraction will be carried out from dry biomass, a drying/dehydration pre-treatment will be necessary, while, if the wet route is chosen, microalgae must be undergone to a specific pre-treatment aimed to break their cell wall (cf. Figure 1.14).

Since, after harvesting microalgal biomass is perishable, it must be processed rapidly. In what follows the main techniques for performing drying and cell disruption will be summarized.

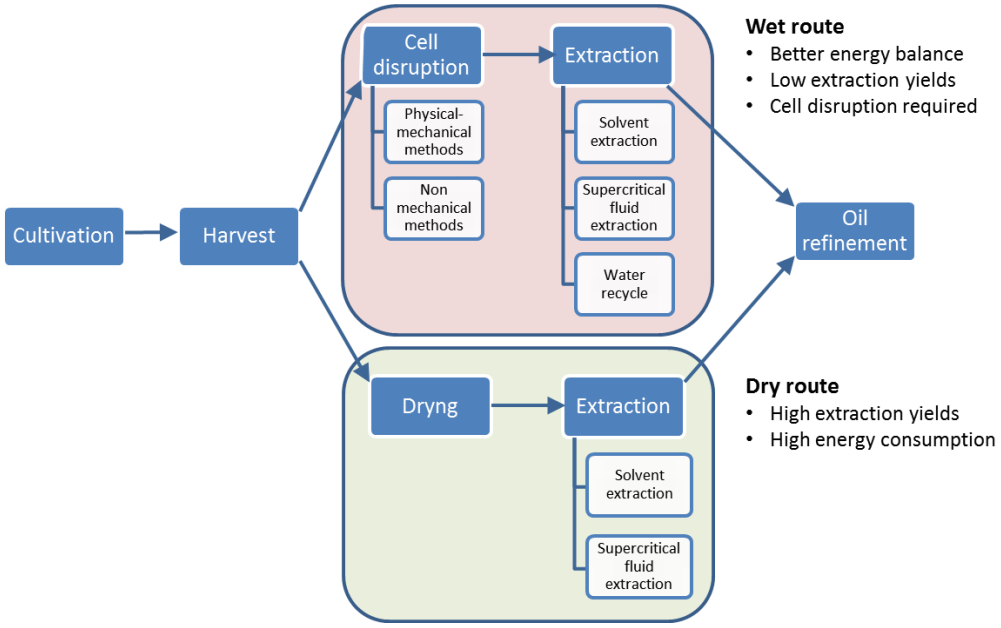


Figure 1.14 Comparison of dry and wet route of lipid extraction (adapted from Kim et al., 2013).

2.8.1 Drying methods

The target of the drying process is to extend the viability of the desired product and prevent the degradation of the harvested biomass slurry. It can be performed basically by sun drying, spray drying, solar drying, drum drying, fluidized bed drying, freeze drying and refractive window technology (Brennan and Owende, 2010). Other techniques include flash drying, and rotary dryers. The selection of the drying process depends on the scale of operation and the use for which the product is intended. Sun drying is a method based on natural evaporation of water. It is a slow drying technique which depends on weather conditions and requires large evaporation basins. On the

other hand, it is the cheapest way for drying microalgae since it consumes low energy. Spray drying is a technique of producing a dry biomass from slurry by rapidly drying it with a hot gas. All spray dryers use some type of atomizer or spray nozzle to disperse the liquid or slurry into a controlled drop size spray. This method is quite expensive but can be used for the extraction of higher value products from microalgae (Brennan and Owende, 2010). Freeze-drying, also known as lyophilization, is a dehydration process which works by freezing the algal biomass at -20°C and then reducing the surrounding pressure to allow the frozen water in the material to sublimate directly from the solid to the gas phase. While allowing good oil extraction yields, freeze drying is relatively expensive and thus is rarely used for large scale operations (Brennan and Owende, 2010). In drum drying the wet biomass is applied as a thin film to the surface of a heated drum, and the dried biomass solids are then scraped off with a knife. Drum drying is fast and efficient but is both cost and energy intensive.

Since it involves high energy consumptions, drying is one of the main costs of the whole process of oil production from algae. It can represent 70-75% of the processing cost. Thus, while allowing subsequent good extraction yields, drying as a pre-treatment process is not an economical process. For this reason drying is often skipped and the wet route is generally preferred. An improvement of the economic sustainability of the drying step can be achieved by suitably exploiting the energy of flue gas at the outlet of the emission source for heating the wet biomass (Concas and Cao, 2011).

2.8.2 Cell disruption methods

Cell disruption is an essential pretreatment when lipid extraction is carried out directly from wet biomass. In fact, lipid extraction from wet biomass is characterized by low yields due to the immiscibility of water with organic solvents typically used for dissolving lipids from algae. Therefore, when solvent extraction is applied to a wet biomass, the microalgal cells tend to remain in the water phase due to their surface charges and thus they cannot contact the organic solvent phase which is able to extract lipids (Kim et al., 2013). On the other hand, this phenomenon can be prevented by suitably breaking the cell wall of microalgae and thus facilitating the release of intracellular lipids from the microalgal cellular matrix. Once released from the algal cell, lipids pass to the organic phase from which they can be extracted by evaporating the solvent. The cell disruption is therefore a method for breaking the cell wall of algae.

Since microalgae have a cell wall, which is a thick and rigid layer composed of complex carbohydrates and glycoproteins with high mechanical strength and chemical resistance, this operating step can require high energy inputs. Cell disruption techniques can be conceptually divided into physical or mechanical methods and non-physical methods.

2.8.3 Physical mechanical methods

The physical or mechanical methods include high pressure homogenization, ball milling, microwaving, ultra-sonication and cavitation. The process of high pressure homogenization is a mechanical method which consists in pumping the cell suspension to a high pressure through a narrow opening of a valve before the cell suspension is released into a chamber of a lower pressure (Halim et al., 2012). High pressure homogenizers can greatly enhance the availability and the extraction of pigments from the cells.

On the other hand, it can cause high energy consumptions which can be up to 750 kWh/dry ton for 4-7 wt% solids. Ball milling is a very simple cell disruption technique that breaks cells by means of spheres made of quartz or metal that are shaken within a closed container filled with the target cells. The cells are disrupted by collision or friction with the spheres. This method is very simple and rapid but is hard to scale up and requires extensive cooling systems for preventing thermal degradation of lipids (Kim et al., 2013).

Microwaves break cells using the shocks generated by high-frequency electromagnetic waves (about 2500 MHz). This method has been successfully applied for disrupting vegetal cell walls and subsequently extracting lipids. The main limitation deriving from using microwaves at large scale is the high energy consumption (about 70 KW). Ultra sonication exploits the cavitation phenomena induced by ultrasounds (18-50 kHz) in a liquid. Cavitation leads to the formation and the immediate implosion of cavities (microbubbles) in the liquid. Such implosions result in the production of shockwaves which can disrupt the cell wall of microalgae thus allowing the release of intracellular lipids. The main advantage of this method is the high yields of cell disruption. On the other hand, the main limitations are the high energy consumptions which ranges from 60 to 120 MJ/kgwet biomass and the low scalability deriving from the fact that cavitation occurs only in small regions near the ultrasonic probes (Kim et al., 2013). Finally, a relatively new technique for cell disruption is the electroporation.

The latter one consists in the increase of the electrical permeability of cells by applying a pulsed electromagnetic field. It is usually applied in molecular biology for introducing specific substance into a cell, such as drugs or piece of DNA as well as for extracting intracellular compounds. No permanent effects are detected in the cells. However, if a very strong intensity of the electric field is applied cell wall can be destroyed thus allowing the subsequent extraction of lipids. Electroporation is very simple and low energy consuming since in the few studies carried out on microalgae an energetic input of about 36 kWh/m3 was sufficient for disrupting algal cells (Lee et al., 2012).

2.8.4 Non mechanical methods

The main non-mechanical methods for microagal cell disruption are osmotic shocks, enzymatic hydrolysis and physico-chemical methods. Osmotic shock is caused by a sudden change in the solute concentration which provokes a rapid change in the movement of water across the cell wall.

This results in the creation of a pressure gradient between the inner and the outside of the cells which can disrupt the cell envelopment. Both hyper-osmotic shocks and hypo-osmotic shocks can be used for breaking the cell wall. When the salt concentration is higher in the exterior, the cells suffer hyper-osmotic stress. As a result, the cells shrink since the inner cell fluids diffuse outwards, and a damage is caused to the cell walls. On the contrary, hypo-osmotic stress takes place when the salt concentration within the cell is lower than the exterior one. In this case water permeates into the cells which consequently swell or burst if the stress is too high. Osmotic shock is a relatively cheap and simple method for disrupting algal cell walls. On the other hand it produces high amounts of saline wastewater which must be treated (Kim et al., 2013). Enzymatic hydrolysis exploits specific enzymes to lyse algal cell walls. Specific enzymes such as papaine, pectinase, snailase, neutrase, lipase and alcalase can react with the cellulose and phospholipids of the cell wall converting them into glucose, fatty acids and glycerol respectively (Young et al., 2011). This way enzymatic reactions can break the cell wall, thus facilitating the subsequent phase of lipid extraction. Even though the enzymatic hydrolysis can lead to high yields of cell disruption, the cell lysing enzymes are still cost prohibitive and thus unsuitable for massive production.

Physicochemical methods include alkaline and acid hydrolysis through NaOH, HCl, and H2SO4. They are typically carried out within autoclaves at a temperature of about 90 °C (Sathish and Sims, 2012). Among the others, chemicals such as lysine acetone,

methanol, dimethyl sulfoxide (DMSO) and organic acids can be used for lysing the cell wall of algae. Despite the chemical methods have high cell-disruption performances, they show some significant limitations. The chemicals must be continuously supplied and this aspect greatly affects the economic sustainability of the method when large scale production is considered. Furthermore acids and alkalis can corrode the surface of reactors and attack target products (i.e. lipids) of the microalgal cell. Therefore, physic-chemical methods must be coupled with a mechanical pre-treatment aimed to reduce the chemical usage (Kim et al., 2013).

2.8.5 Comparison between main techniques for cell disruption

A comparison between the different techniques can be performed in term of disruption efficiency. The following parameter defined by Halim et al. (2012) as average disruption yield L can be used for comparing different techniques:

$$L = \left(1 - \frac{C}{C_0}\right) \cdot 100\ \%$$

where C/C0 is the average ratio between the number of intact (not disrupted cells) cells before and after disruption procedure. By comparing the main techniques summarized above in terms of such parameter, the result shown in Figure 1.15 was obtained:

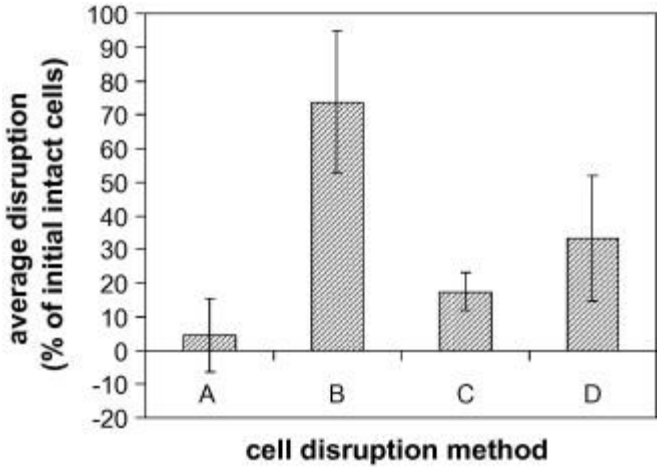


Figure 1.15 Comparison of the different cell disruption methods. Average disruption (± standard deviation) of each method is reported. Average disruption is expressed as % of initial intact cells. A: ultrasonication. B: high-pressure homogenization. C: bead beating. D: sulfuric acid treatment (adapted from Halim et al., 2012).

2.9 Lpid extraction

The feasibility of biofuel production from algae cultivation depends basically on their content of lipids (Jones et al., 2012). Amounts and typology of lipids in microalgae vary from strain to strain. However, the lipid categories are basically divided into neutral lipids (e.g., triglycerides, cholesterol, polyunsaturated fatty acids) and polar lipids (e.g. phospholipids, galactolipids). Triacylglycerols (TAGs) as neutral lipids are the most useful precursors for the production of biodiesel (Kirrolia et al., 2013). However, as shown in Figure 1.16, TAGs are contained within the microalgae cells, surrounded by a rigid cell wall, and thus extraction is needed for being suitably exploited. To this aim, dewatered biomass is processed in a pre-treatment step aimed to dry the biomass or alternatively to break the cell wall of microalgae to facilitate the subsequent step of lipid extraction. Therefore, depending on the specific pre-treatment adopted, microalgal biomass to undergone lipid extraction can assume one of the following physical states: disrupted concentrate or dried powder. Several methods for lipid extraction from microalgae are currently under investigation at the laboratory scale but only solvent extraction appears to be the viable way for an effective lipid extraction at the industrial scale. Typically, solvent extraction is carried out by contacting microalgal biomass with an eluting solvent which extracts TAGs and fatty acids out of the cells (Halim et al., 2011). The most suitable solvents for extracting lipids from microalgae are the organic ones and supercritical carbon dioxide. In what follows we will focus on these two techniques.

2.9.1 Organic solvent extraction

This technique is based upon the fact that when a microalgal cell is contacted with a non-polar organic solvent, such as hexane or chloroform, a static film of solvent surrounding the algal cell is formed as a result of the interactions between solvent molecules and the cell wall constituents (cf. Figure 1.16). The film thickness depends also upon hydrodynamics parameters such as stirring speed, solvent flow rate and cell diameter. Subsequently, the organic solvent diffuses through the cell membrane into the cytoplasm and interacts, through van der Waals type bindings, with the neutral lipids by forming organic solvent-lipids complexes. The latter ones, driven by a concentration gradient, counter-diffuses across the cell wall towards the static organic solvent film surrounding the cell and then towards the bulk organic solvent. As a result, the neutral

lipids are extracted out of the cells and remain dissolved in the non-polar organic solvent.

This method permits the extraction of free standing lipid globules that float in the cytoplasm. However, some lipids bodies are linked via hydrogen bonds to the proteins of the cell membrane. The van der Waals interactions promoted by non-polar organic solvents are not strong enough to break the above bonds which anchor lipid bodies to the cell membrane. On the contrary, polar solvents such as methanol ethanol or isopropanol, are capable of breaking such bonds. For this reason organic polar solvents are often used in synergy with non-polar ones for extracting lipids from microalgae. Once the lipid bodies are undocked from the membrane, they form complexes with the organic solvents and counter-diffuse towards the solvent bulk outside the cell driven by concentration gradients (Halim et al., 2012).

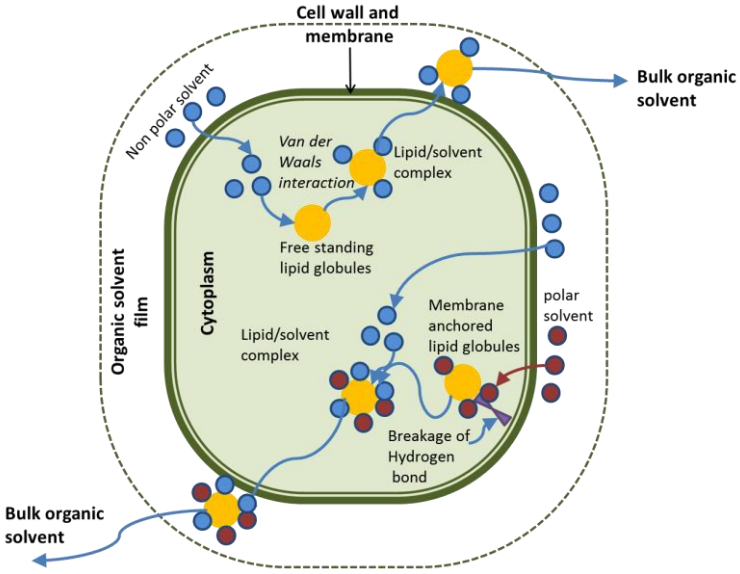


Figure 1.16 Simplified scheme of the main phenomena involved in lipid extraction through organic solvents (adapted from Halim et al., 2012).

Therefore for an effective extraction of lipids, mixtures of polar and non-polar solvent are often used. Useful combinations of non-polar/polar solvents are hexane/isopropanol, chloroform/methanol, hexane/ethanol etc. As shown in Figure 1.17 the two kinds of solvents are typically added simultaneously with a volumetric ratio which is established by means of specific experimental trials. If extraction is performed on wet biomass, the cell debris should be removed by means of a suitable liquid solid separation such as centrifugation. Once cell debris is removed, biphasic separation is induced by further adding suitable amounts of polar solvent and water.

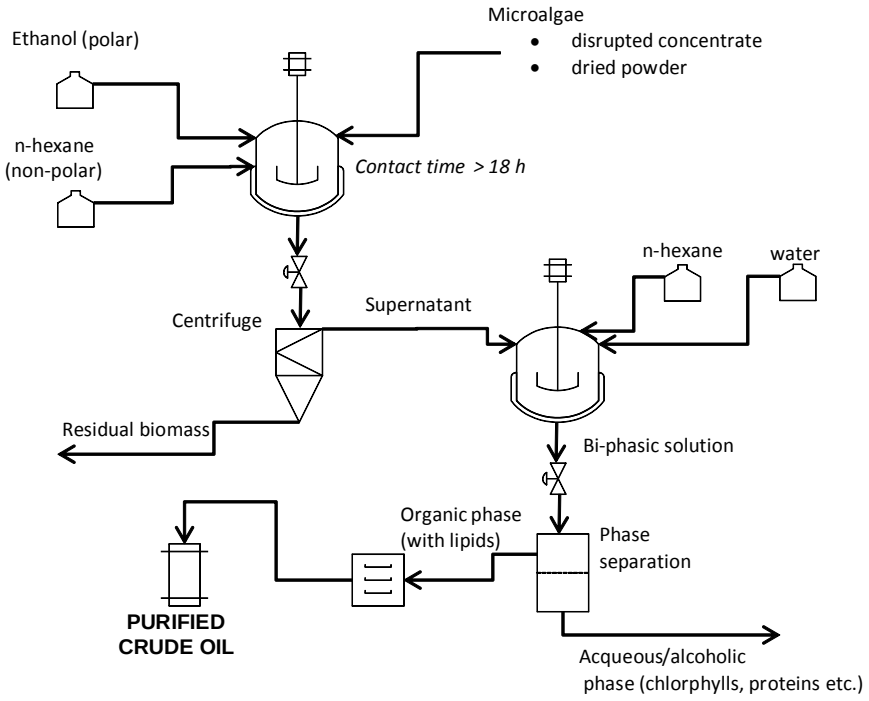


Figure 1.17 Scheme of a classical solvent extraction process.

After biphasic separation, neutral and polar lipids are concentrated in the organic phase which is a mixture of non-polar (e.g. hexane) and polar (e.g. ethanol) solvents and the aqueous/alcoholic phase which is a mixture of water and polar organic solvent (for example ethanol) where other cellular molecules such as chlorophylls, proteins and carbohydrates have been transferred. Therefore, biphasic separation allows not only the removal of residual water but also non-lipid contaminants from the mixture of organic solvents and lipids. The organic phase is then decanted and evaporated to yield purified crude lipids, which can be then fractionated and transesterified to produce biodiesel. Since the evaporation phase could be energy consuming, volatile solvents are typically preferred in order to reduce time and cost of the evaporation step. Moreover, specific solvents such as for example chloroform are effective but highly toxic and their use should be avoided. For this reason the most suitable combination of non-polar/polar solvents appear to be a mixture of hexane and ethanol (Fajardo et al., 2007).

The main operating parameter affecting the extraction yield is the contact time t (h). In particular, according to Halim et al. (2011), the lipid extraction process is observed to follow a first order kinetics which results in the following relationship between the cumulative amount of lipid extracted in the organic solvent me (glipid/gdried microalgae) and time t:

$$m_e = m_{s,0}\left(1 - e^{-kt}\right)$$

where $m_{s,0}$ is the amount of lipid originally present in the cells ($g_{lipid}/g_{dried\ microalgae}$) and k is the lipid mass transfer coefficient from the cells into the organic solvent (min^{-1}). Such kind of relation indicates that the cumulative mass of extracted lipids increases with time until a plateau is reached. Typically, extraction time depends on the specific algal strain. Extracting 90% of lipids may need up to 12 hours depending upon the value of the mass transfer coefficient k. The latter one is found to be a function of other operating parameters such as temperature T (°C), solvent/biomass ratio s/b ($mL_{solvent}/g_{biomass}$) and stirring speed ω (rpm). The higher is the value of these parameters the higher is the value of k. However, usual values of T range from 25 to 60 °C, stirring speed ω can vary from 500 to 800 rpm while the solvent/biomass ratio can vary from 5 to 250 ($mL_{solvent}/g_{biomass}$) (Fajardo et al., 2007; Lee et al., 1998).

The main limitations of the solvent extraction techniques are the need of a continuous supply of expensive solvents since not all of the organic ones can be recycled. Moreover, the high toxicity of solvents arises environmental and safety concerns.

2.9.2 Supercritical CO2 extraction

Supercritical Fluid Extraction (SFE) is the process of separating one component (for example lipids) from another (for example microalgae) using supercritical fluids as extracting solvent. A supercritical fluid is any substance at a temperature and pressure above its critical point (Tc, Pc), where distinct liquid and gas phases do not exist. Under such thermo-baric conditions supercriticial fluids behave like solvents thus becoming a suitable substitute of organic solvents in a range of industrial and laboratory processes. The capability of behaving like a solvent depends on the fact that supercritical fluids have density similar to the one of liquids while their viscosity and diffusivity are closer to the ones of gases. For this reason the solubility approaches that of the liquid phase while penetration and diffusion into a solid matrix is facilitated by the gas-like transport properties. As a consequence, the rates of extraction and phase separation can be significantly faster than that one for conventional extraction processes.

Among the various fluids, supercritical carbon dioxide ($SCCO_2$) is becoming an important solvent due to its low toxicity, its low flammability and its lack of reactivity which result in a low environmental impact. Moreover, its relatively low critical pressure (7.39 MPa) results in low compression cost, while its modest critical temperature (31.1 °C) permits a successful extraction of thermally sensitive lipid

fractions without degradation. The principle through which lipids can be extracted from microalgae through $SCCO_2$ is similar to the one schematically shown in Figure 1.16 for the extraction with organic solvent. First, $SCCO_2$ rapidly diffuses into the algal cell and then lipid bodies dissolve in the supercritical fluid by forming lipid-CO_2 complexes through van der Waals interactions. Subsequently, the so formed complexes counter-diffuse from the inner of the cell towards the static film and then towards the bulk driven by concentration gradients. While the phenomena on the base of lipid extraction are very similar to the ones involved in the organic solvent extraction, by using supercritical fluids the extraction rate can be up to ten times faster. Nevertheless, the properties of $SCCO_2$ can be altered by suitably tuning pressure and temperature for performing a selective extraction. Since $SCCO_2$ is unable to interact with either polar lipids or neutral lipids a polar modifier, often referred to as co-solvent, is added to CO_2. The target is to improve the affinity of the resulting fluid for polar lipids and lipid complexes. Common polar modifiers are methanol ethanol, toluene and methanol–water mixture. A simplified scheme of the $SCCO_2$ extraction process is shown in Figure 1.18. It involves the use of a source of pure CO_2 which, in the case where algae are cultivated near a coal-fired power station, can be conveniently obtained from the scrubbed flue gas of the station. The microalgal biomass, in the form of disrupted concentrate or dried powder, is placed in a packed bed previously filled with suitable packing elements. The CO_2 along with the co-solvent is heated through heat exchangers and compressed through compressors until the desired supercritical conditions of temperature and pressure are achieved.

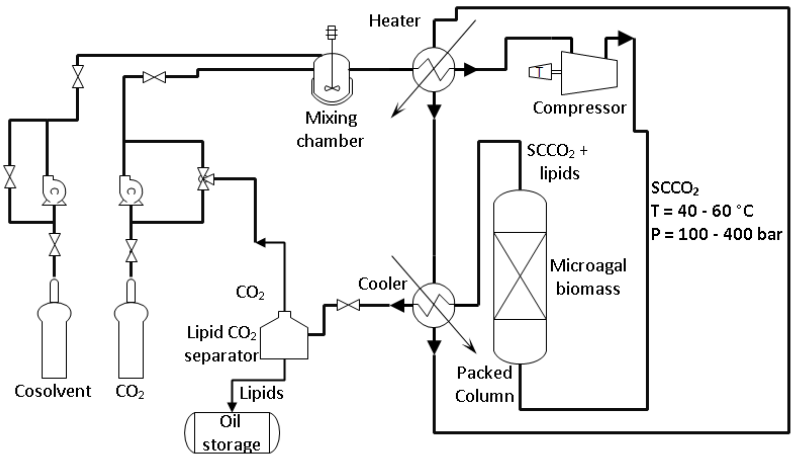


Figure 1.18 Scheme of the SCCO2 extraction of microalgal lipids.

The $SCCO_2$ is then pumped into the column from the bottom and flows upwards through the packed bed thus contacting microalgae and desorbing lipids according to the mechanisms depicted above. Therefore lipids enter in the bulk $SCCO_2$ flow which subsequently leaves the packed column to enter in the so called 'blow down vessel'. Here the pressure is reduced until CO_2 returns to the gaseous state. The lipids then precipitate down in a collection vessel while the gaseous CO_2 flows upwards. The CO_2 is then collected for being recirculated. As such, SFE-derived crude lipids are free from any extraction solvent and do not need to undergo an extraction solvent removal step (Halim et al., 2012).

Also for the case of supercritical lipid extraction, the process is observed to follow a first order kinetics. The time evolution of the cumulative amount of extracted lipids during the process can be described by mathematical relationship similar to the one reported for organic solvent extraction. However, in this case the mass transfer coefficient is a function of different operating parameters (Halim et al., 2012) such as the extraction pressure, the temperature, the concentration of co-solvent and the SCCO2 flow rate. Typical range of operating pressure for extracting lipids from algae is from 200 to 450 bar. The extraction temperatures can vary from 40 to 60°C and the SCCO2 flowrate range is 0.4 - 500 l/min (Andrich et al., 2005; Cheung, 1999; Mendes et al., 2003; Sajilata et al., 2008). The SCCO2 extraction technique is a very promising method for extracting lipids from microalgae since it can assure high extraction yields in relatively short times. Moreover, no concerns related to solvent toxicity can arise. Unfortunately, the main limitations of this method are the high energy consumption related to the operating phases of fluid heating and compression as well as the potentially high costs of investment.

2.9.3 Conversion of microalgal lipids for the production of biodiesel

Once extracted the algal crude oil must be further processed to be used as fuel. Depending on the specific post-processing technology, different kind of biofuels can be produced. However, in this work we will focus only on the production of biodiesel which can be obtained through the well-known process of trans-esterification. The need of further processing the oil depends on the fact that its viscosity is too high for its exploitation in internal combustion engines. Hence, to produce a useful biodiesel, the viscosity of microagal oil must be reduced. The most common method to produce biodiesl from vegetal oil is just the transesterification alcoholysis. It consists in the reaction between triglycerides of the oil with methanol. Such reaction leads to the

formation of methyl esters of fatty acids (FAMEs) that are biodiesel and glycerol (cf .Figure 1.19).

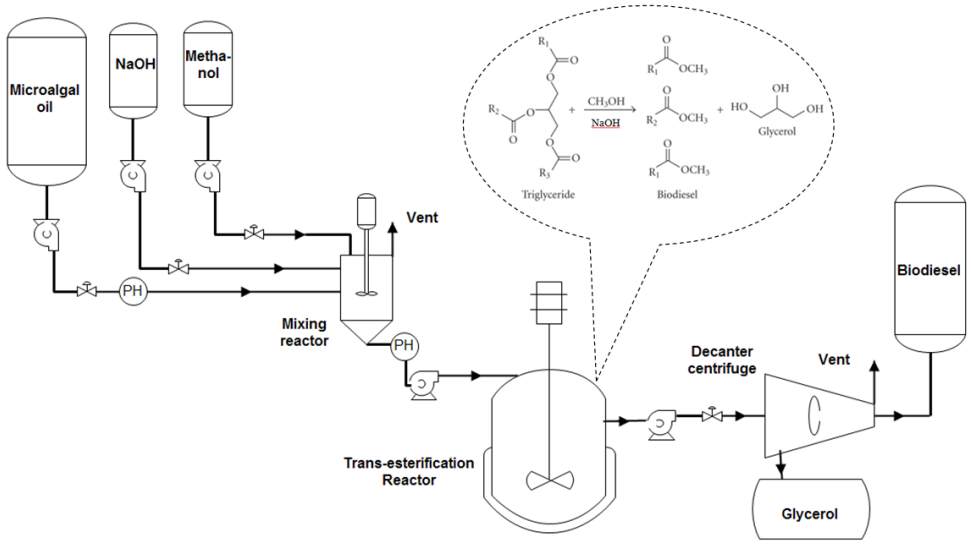


Figure 1.19 Scheme of the trans-esterification process.

The reaction can be catalyzed by acids, alkalis and lipase enzymes but usually alkalis such as sodium and potassium hydroxide are used since they are able to speed up the reaction about 4000 times than acids. Alkali-catalyzed trans-esterification is carried out at approximately 60 °C under atmospheric pressure. Under these conditions, reaction takes about 90 min to complete (Chisti, 2007). When trans-esterification reaction is completed, the two phases (glycerine and esters) are separated by gravimetric methods such as decanting and centrifuging. It has been reported that the achievable conversion of algal triglycerides to biodiesel is about 98% (Amin, 2009) and the produced biodiesel can be compatible with conventional petroleum derived diesel (cf. Table 1.6).

Table 1.6 Comparison of properties of biodiesel, diesel fuel and ASTM standard (adapted from Amin, 2009)

Properties	**Biodiesel from microalgae**	**Diesel fuel**	**ASTM standard**
Density (kg L^{-1})	0.864	0.838	0.86-0.90
Kinematic viscosity at 40°C (mm^2 s^{-1})	5.2	1.9-4.1	3.5-5.0
Flash point (^{O}C)	115	75	Min 100
Solidifying point (^{O}C)	-12	-50-10	-
Cold filter plugging point (^{O}C)	-11	-3.0 (max. -6.70)	Summer max 0; Winter max -15; Max 0.5
Acid value (mg KOH g^{-1})	0.374	Max 0.5	-
Heating value (MJ kg^{-1})	1.81	1.81	-

The bio-diesel produced can thus be used in the internal combustion engines.

2.10 Concluding remarks

The production of biofuels from renewable feedstocks is recognized to be critical to fulfill a sustainable economy and face global climate changes. When compared to first and second generation biofuel feedstocks, microalgae are characterized by higher growth rates and lipid content which result in larger bio-oil productivities. Moreover, cultivation of microalgae can be carried out in less- and lower-quality lands, thus avoiding the exploitation of arable ones. In addition, cultivation of microalgae might be coupled with the direct bio-capture of CO_2 emitted by industrial activities that use fossil fuels for energy generation. Ultimately, when compared to first and second generation biofuels, microalgae are characterized by a greater environmental sustainability and economic viability. For these reasons, the potential exploitation of microalgae as renewable resource for the 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. The high potential of algae based biofuels is confirmed by the number of recent papers available in the literature on the subject. In spite of such interest, the existing microalgae-based technology for CO_2 sequestration and biofuels production is still not widespread since it is affected by economic and technical constraints that might limit the development of industrial scale production systems. In particular, the main obstacles are related to the extensive land's areas needed as well as the estimated high costs of the operating phases of microalgae cultivation, harvesting and lipid extraction. Therefore, in view of industrial scaling-up, the current technology should be optimized in terms of selected algal strains as well as design/operating parameters. The latter target may be accomplished by exploiting suitable process engineering techniques. Along these lines significant efforts are currently in progress around the world. In the light of what above, the present chapter has been aimed to present the recent achievements related to th engineering aspects connected with the use of microalgae for biofuels production and CO_2 capture from flue gases. It has been shown that, cultivation of microalgae can be performed in closed ones, i.e. photobioreactors, or in open systems. The first ones can be bubble columns, airlift, flat panel and horizontal tubular photobioreactors. While photobioreactors are characterized by high biomass productivity, a better process control and lower contamination risks, if used for producing low value products, such as biofuels, they have still not attained economic levels of production. Significant efforts are currently being performed to decrease the operating and capital costs linked to the construction

and the operation of photobioreactors for producing biofuels. The major achievements in this direction are those related to the use of very low cost materials for the construction of photobioreactors, the use of flue gases and wastewaters as source of macronutrients and finally the use of engineered algae that are characterized by extremely high oil productivity, thus allowing the compensation of high operating costs with potentially large incomes. Also the valorization of microalgal compounds separated by lipids, such as chlorophylls, pigments and proteins might improve the economical sustainability of photobioreactors-based processes. On the other hand, open raceways, which are less expensive, show several drawbacks such as low oil productivity, high risks of contamination, high losses of water due to evaporation, scarce process control and high susceptibility to different weather conditions. To overcome limitations related to open system and in the meantime keeping their low operating cost, the potential use of closed raceway ponds are currently under study. These systems essentially consist of an open pond covered by a transparent or translucent barrier which turns it into a greenhouse. This configuration prevents the microalgae to be contaminated by competing bacteria and allows a better control of crucial operating parameters such as temperature, evaporation, etc.

Another bottleneck of the process is related to the harvesting step of microalgae. Essentially, harvesting can be performed by centrifugation, filtration, flotation and flocculation. It is worth noting that harvesting alone, can be the most expensive step of the overall process of microalgae production. Therefore, in order to assure the economic sustainability of the process, efficient and inexpensive harvesting methods should be developed and subsequently adopted at the industrial scale. In this regard the most promising processes for microalgae harvesting are those ones which combine different technologies such as for example microfiltration followed by centrifugation or pH-induced flocculation sedimentation and dynamic microfiltration. Also auto-flocculation and bio-flocculation are promising techniques. However they are still in embryonic phase and their potential use at the industrial scale for harvesting large amounts of microalgae still need to be investigated.

Since biomass drying after harvesting and before the lipid extraction, is very expensive, the direct extraction from wet biomass seems to be the only economically feasible way to recover lipids from microalgae. Therefore the downstream process of cell disruption represents an essential pretreatment when lipid extraction is carried out directly from wet biomass. Among the techniques which can be realistically applied at the industrial

scale, the most efficient ones for cell disruption are high-pressure homogenization, bead beating, and sulfuric acid treatment.

Lipid extraction is typically carried out by contacting microalgal biomass with an eluting solvent which extracts triacylglycerols and fatty acids out of the cells. The most suitable solvents for extracting lipids from microalgae are the organic ones and supercritical carbon dioxide ($SCCO_2$). The main limitation related to the use of organic solvents is their high cost and the need of their continuous supply since not all of them can be suitably recycled. Moreover, the toxicity of organic solvents arises environmental and safety concerns. On the other hand the extraction technique based on the use of $SCCO_2$ is a very promising method for extracting lipids from microalgae since it can assure high extraction yields in relatively short times. Moreover, no concerns related to solvent toxicity can arise. Unfortunately, the main limitations of this method are the high energy consumption related to the operating phases of CO_2 heating and compression as well as the potentially high costs of investment.

Chapter 3.

A novel cell disruption technique to enhance lipid extraction from microalgae

3.1 Introduction

The current microalgae-based technology for CO2 sequestration and biofuels production, albeit extremely promising, is still affected by economic and technical constraints that limit the development of industrial scale production systems. In particular, one of the main bottlenecks of the process is represented by the estimated high costs and low yields of the operating phases wherein lipids are extracted from microalgae cells (Concas et al., 2010; Concas et al., 2012; Concas et al., 2014). In fact, algal triacylglycerols, which are the most useful precursors for the production of biodiesel, are typically contained as lipid droplets within the microalgae cytoplasm surrounded by a rigid cell wall and thus have to be extracted in order to be suitably exploited for producing biofuels (Kirrolia et al., 2013). Several methods for lipid extraction from microalgae are currently under investigation at the laboratory scale but solvent extraction appears to be, so far, the only viable way for performing lipid extraction at the industrial scale (Chisti, 2007). Typically, solvent extraction is carried out by contacting microalgal biomass with an organic eluting solvent which diffuses through the cell wall/membrane into the cytoplasm and interacts, through van der Waals type bindings, with the neutral lipids by forming organic solvent-lipids complexes. The latter ones, driven by a concentration gradient, counter-diffuses across the cell wall towards the bulk solvent from which they can be collected to be further processed (Halim et al., 2011; Halim et al., 2012). Solvent extraction of algal lipids can be performed starting from both wet and dry microalgal biomass and, depending upon which option is chosen, specific pre-treatments should be carried out. In particular, if lipid extraction is carried out from dry biomass, a drying/dehydration pre-treatment is necessary. On the other hand, the drying step is typically characterized by high energy requirements and therefore the wet extraction is usually preferred in order to assure process feasibility and viability (Xu et al., 2011; Chen et al., 2012). However, a pre-treatment aimed to break the cell walls of microalgae is mandatory when lipid extraction is carried out directly from wet biomass. In fact, lipid extraction from untreated wet biomass is characterized by low yields due to the immiscibility of water with the organic solvents. Therefore, when solvent extraction is applied to wet biomass, the microalgal cells tend to remain in the water phase due to their surface charges and thus they cannot contact the organic solvent phase which is able to extract lipids (Kim et al., 2013). Fortunately, this phenomenon can be prevented by breaking the cell wall of microalgae to provoke the release of intracellular lipids into the extracting mixture, thus

facilitating the access of solvent to lipids. Therefore, once released from the algal cell, lipids are able to pass to the solvent phase from which they can be collected after evaporation of the solvent. Cell disruption is then considered a method for breaking the cell wall of algae. Since microalgae are characterized by a cell wall, which is a thick and rigid layer composed of complex carbohydrates and glycoproteins with high mechanical strength and chemical resistance, this operating step might require high energy inputs. The main cell disruption techniques can be conceptually categorized into physical and chemical methods. The formers include high pressure homogenization, ball milling, microwaving, ultra-sonication, electrocoagulation and hydrodynamic cavitation (Florentino de Souza Silva et al., 2014; Grimi et al., 2014; Keris-Sen et al., 2014; Wang et al., 2014). Recently, also thermolysis, osmotic shocks, laser treatments and electroporation have been proposed as viable physical methods for disrupting algal cells with the aim of lipids extraction (Kim et al., 2013; McMillian et al., 2013; Lee et al., 2012). Most of the physical disruption methods are very difficult to scale up (Wang et al., 2014) and might involve high energy consumption since they are based on the continuous supply of thermal, electrical or mechanical energy inputs until the cell wall is broken. In particular, energy consumptions ranging from 33 MJ kg-1 for hydrodynamic cavitation to 529 MJ kg-1 for high pressure homogenizers have been reported in the literature (Lee at al., 2013). If one considers that the average energy obtainable through combustion of microalgae is estimated to be about 29 MJ kg-1, it is apparent that the adoption of physical disruption methods would lead to an energetic imbalance which in turn might strongly undermine the economic sustainability of the microalgae based technology for producing biofuels (Lee et al., 2013). On the other hand, chemical methods for cell disruption rely on selective interaction of a chemical with the components of cell wall and are basically represented by enzymatic hydrolysis (Young et al., 2011) and chemical lyses. The latter ones include alkaline and acid hydrolysis through NaOH, HCl, and H2SO4 but also organic chemicals such as lysine acetone, methanol and dimethyl sulfoxide (DMSO) can be used to lyse the cell wall of algae (Sathish and Sims, 2012). When compared with physical methods, the chemical methods are less energy consuming while often showing higher yields of cell-disruption and furthermore, are simpler to scale-up. However, even these methods still show some significant limitations. In particular, chemicals must be continuously supplied and this aspect might greatly affect the economic sustainability of the technology when large scale production systems are considered. Moreover, exhaust disrupting solution should

be treated before being disposed as liquid waste. Furthermore acids and alkalis might corrode the equipment surface and attack the valuable products (i.e. lipids) of the microalgal cell, thus vanishing the whole process (Kim et al., 2013).

For these reasons, novel techniques are needed to perform cell disruption by means of physic-chemical methods. These techniques should be characterized by low energy consumptions, high disruption yields and should use low cost and safe disruption reactants. Moreover, operating parameters such as reactant concentration, contact time, operating temperature etc., should be suitably tuned so that disruption yields can be maximized while degradation of target products, costs of reagents and production of liquid wastes could be minimized. Along these lines, a simple and low energy consuming technique for cell disruption, based on the use of low toxicity and cheap reactants such as H2O2 and FeSO4, is proposed and investigated in this work. The effect of reactants concentration and contact time on the amount and the quality of extracted lipids is also investigated. This way, the optimal set of operating conditions which allow maximizing lipid extraction yields while minimizing lipid degradation and costs for reactant purchase has been identifie

3.2 Materials and methods

3.2.1 Microorganism

The fresh water algal strain *Chlorella vulgaris* (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 (KTM-A) medium 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).

3.2.2 Culture medium

C. vulgaris was cultured in a modified Kolkwitz growth medium (KTM-A) containing 2.5 g L^{-1} of KNO_3, 0.5 g L^{-1} of KH_2PO_4, 0.27 g L^{-1} of $MgSO_4 \cdot 7H_2O$, 0.04 g L^{-1} of $CaCl_2 \cdot 2H_2O$, 1 g L^{-1} of $NaHCO_3$ and 1 ml of EDTA-Na_2-Fe solution as well as 1 ml of micronutrients solution. The latter one contained 2.86 g L^{-1} of H_3BO_3, 1.81 g L^{-1} of $MnCl_2 \cdot 7H_2O$, 0.222 g L^{-1} of $ZnSO_4 \cdot 7H_2O$, 0.035 g L^{-1} of $CoCl_2 \cdot 6H_2O$, 0.080 g L^{-1}

of $CuSO_4 \cdot 5H_2O$, and 0.230 g L^{-1} of $Na_2MoO_4 \cdot 2H_2O$. As far as the EDTA.Na_2-Fe solution is concerned, it contained 29.754 g L^{-1} of EDTA.-Na_2 and 24.9 g L^{-1} of $FeSO_4 \cdot 7H_2O$.

3.2.3 Culture condition

Growth of *C. vulgaris* was carried out under high CO_2 concentrations in a 6 L helical tubular photobioreactor coupled with a degasser system, as described in the literature (Concas et al., 2010). Briefly, the light collector of the photobioreactor consisted of 66 m transparent polyurethane tubing arranged around a circular metal frame. It was internally illuminated by three 60W white fluorescent lamps providing a light intensity of 100 µE 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 to remove 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}. Once the culture reached the stationary growth phase the photobioreactor was operated in fed-batch mode. The withdrawals made during the operation in fed-batch mode as shown in Figure 3.1 were used for the cell disruption experiments.

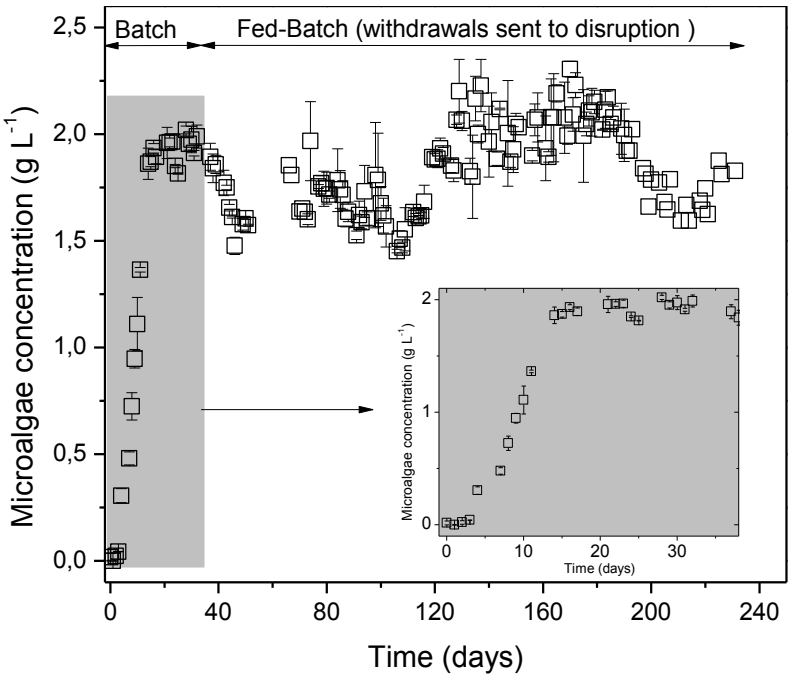


Figure 3.1 Growth of the microalgal culture in helical tubular photobioreactor

3.2.4 Biomass and pH measurements

The growth of microalgae was monitored through spectrophotometric measurements (Genesys 20 spectrophotometer, Thermo Fisher Scientific Inc. Waltham) of the culture media optical density (OD) at 560 nm wavelenght (D_{560}) with 1 cm light path. The biomass concentration C_b (g_{dw} L^{-1}) was calculated from OD measurements using a suitable C_b vs. OD calibration curve which was obtained by gravimetrically evaluating the biomass concentration of known culture medium volumes that were previously centrifuged at 4000 rpm for 15 min and dried at 105°C for 24 h. The pH was daily measured by pH-meter (KNICK 913).

3.2.5 Cell disruption

Once the culture in the photobioreactor reached the stationary growth phase, microalgae were first harvested and then centrifuged to obtain a concentrated pellet of wet biomass characterized by a water content of about 90 %wt/wt. The exact weight of dry biomass contained in the wet pellets was evaluated by means of the suitable calibration line shown in Figure 3.2, which was obtained by gravimetrically evaluating the wet weight of biomass obtained after centrifugation at 4000 rpm for 15 min and its corresponding dry weight after drying at 105°C for 24 h.

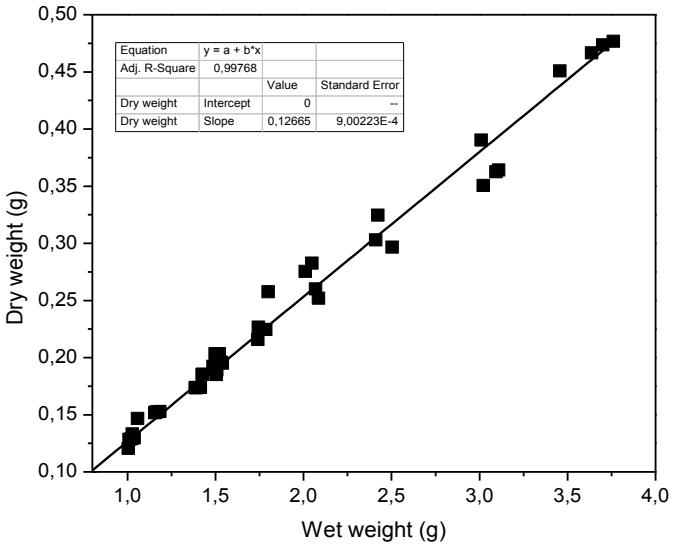


Figure 3.2 Calibration line showing the correlation between wet weight of biomass pellet subjected to disruption and the corresponding dry weight content

Thus, wet pellets containing known amounts of dry biomass were subjected to the cell disruption procedure which consisted of contacting them with known volumes of a disrupting solution within a falcon flask that was then sealed and continuously shaken at 300 rpm for suitable times at room temperature. The amounts of wet biomass and disrupting solution that were contacted were suitably chosen in order to assure a fixed weight ratio equal to 8 gsol/gdwt between disrupting solution and biomass dry weight. Two different disrupting solutions were tested. The first one consisted of an aqueous solution of H2O2, while in the second one the disruption agent was the Fenton's reactant, i.e. an aqueous solution of H2O2 and FeSO4. In both cases, during the experiments, different disruption agent concentrations and contact times were tested in order to identify the corresponding values which were able to maximize the lipids extracted in the subsequent operating steps. Specifically, when only H2O2 was considered as disruption agent, the corresponding concentrations ranges from 0 to 1.5 mol L-1 and contact times ranging from 0 to 5 min were investigated. On the other hand, when the Fenton's reactant was investigated, the concentration of FeSO4 in the disruption solution was kept constant at 0.025 mol L-1 while the H2O2 concentration was varied in the range between zero to 6 mol L-1. In this last case, contact times ranging from 0 to 5 min were evaluated. The operating conditions adopted are summarized in Table 3.1.

Disruption reactant	**Contact time range (min)**	**H_2O_2 concentration range (mol L^{-1})**	**[$FeSO_4$] concentration (mol L^{-1})**
H_2O_2	0 – 5.5	0 – 1.5	0
H_2O_2+$FeSO_4$	0 – 5	0 – 6	0.025

Table 3.1 Operating conditions investigated during the cell disruption experiments

It should be noted that, once the desired contact time is elapsed, the disruption reaction was suddenly stopped by diluting 1/10 the reacting mixture with ethanol so that the concentration of disruption reactants, and thus the reaction rate, were dramatically lowered up to values very close to zero. In fact, according to Wu et al. (2010) the cell disruption reaction rate is proportional to the product of the concentrations of hydroxyl radicals generated by H2O2 and the concentration of organic compounds constituting

the cell wall (glycanes, pectine etc.). Therefore, when the concentrations of hydroxyl radicals and microalgae cells, were reduced ten times through dilution, the related reaction rate was correspondingly decreased of about 100 times, and therefore the reaction can be actually stopped.

3.2.6 Lipid extraction

Neutral lipid extraction was performed directly on the wet disrupted biomass according to a method that represents a slight modification of the one proposed by Fajardo et al. (2007). The method consists firstly of diluting 1/10 the mixture of wet-disrupted biomass and disruption solution with ethanol (96% v/v) while assuring the contact for 18 hours under continuous stirring. As mentioned above, this step allowed also stopping the disruption reaction. The resulting hydro-alcoholic solution was then subjected to centrifugation at 4000 rpm for 10 min in order to separate solid residuals (i.e. pieces of broken cells, organelles, etc.) from the supernatant liquid where lipids were transferred. The lipid-rich supernatant was then suitably stored while the residual solid was further contacted with ethanol for 1 h under stirring in order to extract residual lipids remained in the solid phase. After centrifugation, the supernatant resulting from this step was separated and then mixed with the supernatant obtained from the first centrifugation step to obtain the so called "extracted crude oil" solution. Subsequently, a biphasic system was formed by adding 0.67 mL of de-ionized water and 0.6 mL of n-hexane to each mL of the extracted crude oil solution. This way, the purified lipids were transferred to the hexane phase while most impurities, such as for example carotenoids, chlorophylls, proteins etc., remained in the aqueous phase. The two phases were then separated and lipids were recovered from the hexane phase through evaporation. The percent weight of lipids extracted from the dry biomass was obtained as the ratio between the weight of lipid obtained and the original dry weight of microalgae which was subjected to the extraction process.

3.2.7 Fatty acid methyl esters analysis.

The fatty acid methyl esters (FAMEs) composition of extracted lipids was determined according to the European regulation/commission regulation EEC n° 2568 (1991) after transesterification with methanol-acetyl chloride is performed. To this aim gas chromatographic analysis was carried using a flame ionization detector (FID)

(Thermo Trace Ultra, GC-14B) and a RTX-WAX column T (fused silca, 0.25 mm x 60 m x 0.25 µm) maintained at 180 °C. Helium was used as carrier gas at a flow rate of 1 ml min-1.

3.3 Results and discussion

The evolution of microalgae concentration during cultivation of C. Vulgaris in the BIOCOIL photo bioreactor fed with pure CO2 is shown in Fig.1. It can be observed that, such strain is capable to grow effectively despite the high concentration of dissolved CO2. In fact , after a prolonged exponential growth of 15 days, the culture reached the stationary phase when the biomass concentration was about 2 g L-1. Once the steady state was attained, the photobioreactor was operated in fed-batch mode. In fact, starting from the 40th day of culture, suitable amounts of culture were periodically withdrawn and then replaced by an equal volume of fresh medium. As shown in Fig. 1, after each withdrawal, the biomass concentration decreased and then started to increase as a result of the higher nutrient availability and the diminished concentration of toxic catabolites. The wet biomass harvested during each withdrawal cycle was centrifuged and then subjected to the different disruption experiments. Subsequently, the wet biomass was subjected to the lipid extraction procedure in order to verify the effects of the disruption treatment on the amount of lipids which could be extracted from algae. As mentioned above, the contact time and the concentration of disrupting reactant were suitably varied in order to identify their corresponding values which allowed maximizing the extracted lipids. In Figure 3.3a, the effect of contact time variation on the amounts of lipids extracted from C. Vulgaris when cell disruption was performed through a disruption solution containing 0.29 mol L-1 of H2O2, is shown. It can be observed that when no disruption treatment was performed, i.e. the contact time was zero, extracted lipids were about the 7 %wt/wt by dry weight of biomass. However, when the cell disruption treatment was carried out for one minute, the extracted lipids have shown to increase to about 7.9 %wt/wt. Moreover, when the contact time was further augmented, the amount of lipids extracted was correspondingly increased until a maximum value of 9.2 %wt/wt was achieved for a 4 min prolonged disruption treatment. Ultimately, the more prolonged was the contact time the higher the extent of "disruption reaction" until it was stopped by means of dilution with ethanol. In other words, by augmenting the contact time, a growing number of cells was disrupted thus leading to an higher amount of lipids released in solution. However, when the contact time was prolonged over 4 min, a decrease of the extracted lipids was observed. Such

phenomenon was probably due to the fact that, over 4 min, the residual H2O2 in solution started to attack and degrade the lipids transferred in the bulk of the disrupting mixture, thus leading to a reduction of the corresponding amounts which were collected in the subsequent step of solvent extraction.

A similar behavior could be observed when cell disruption was performed under growing concentrations of H2O2 while keeping fixed the contact time at the value of 4 min (cf. Figure 3.3b). Specifically, by increasing the concentration of H2O2 from zero to 0.29 mol L-1, the extracted lipids correspondingly augmented due to a more effective cell disruption. However, when the concentration of H2O2 was further augmented, a reduction in the extracted lipids was observed (cf. Figure 3.3b).

Such a behavior was probably the result of two conflicting phenomena triggered by the high concentrations of H2O2. In fact from one side, the high concentration of H2O2, probably led to an higher rate of the disruption reaction which in turn resulted in higher amounts of lipids available in shorter times. On the other hand, once the intracellular material was released in the bulk solution, the residual concentration of H2O2 was probably still high and thus capable to attack and degrade lipids before the reaction could be suitably interrupted by dilution with ethanol.

It is then apparent that, if the two operating parameters investigated, i.e. contact time and concentration of disruption reactant, are tuned at correspondingly low values, the cell wall disruption cannot be effectively completed while, if their values are at the high side, the extracted lipids can be degraded, thus thwarting the disruption procedure.

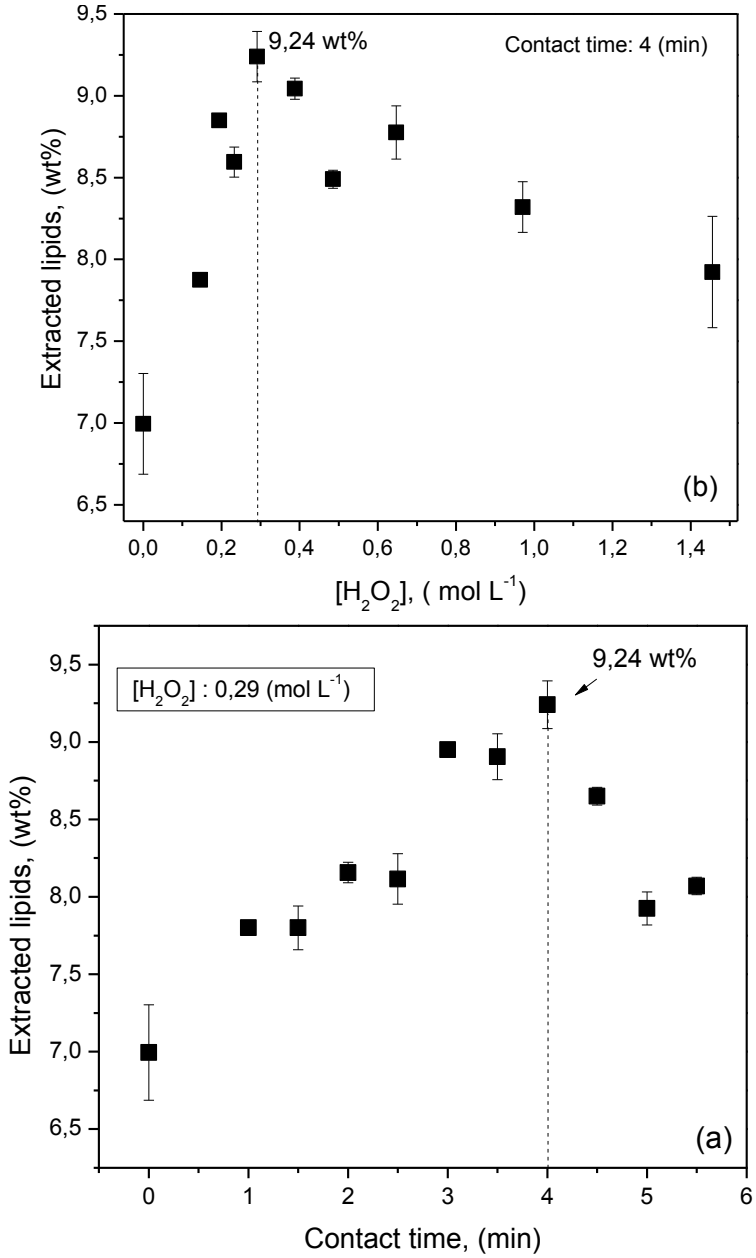


Figure 3.3 Lipid extracted from wet biomass subjected to cell disruption treatments performed with (a) an aqueous solution containing 0.5 mol L-1 of H2O2 and 0.024 mol L-1 of FeSO4 by varying the contact time up to 5 min, and (b) aqueous solutions containing concentrations of FeSO4 kept fixed to 0.024 mol L-1 and H2O2 concentrations up to 6 mol L-1 while maintaining the contact time at 3 min.

Therefore, such parameters should be suitably set to values that maximize extraction while simultaneously minimizing the lipid degradation phenomena. For the case so far illustrated, i.e. when only H2O2 is considered as disruption reactant, the ideal contact time and concentration of disruption reactant appear to be equal to 4 min and 0.29 mol L-1, respectively.

As far as the specific reactive mechanisms involved during the cell wall disruption process, only some assumptions may be formulated. Among them, the most realistic one is that H_2O_2 might generate the Fenton's reaction shown in Figure 3.4a with the Fe^{2+} ions which are present in the liquid solution. In fact $FeSO_4$ was used to prepare the growth medium which constitutes the liquid phase of the wet biomass subjected to disruption. According to Wu et al. (2010), the reaction between H_2O_2 and Fe^{2+} ions can produce hydroxyl radicals (•OH) which in turn may attack and degrade the organic compounds constituting the cell wall according to the simplified mechanism shown in Figure 3.4a and 4b. Probably, such a reaction occurs preferentially in specific zones of the cell wall constituted by organic compounds that are easily oxidized by •OH radicals. In fact, as confirmed by the microscopic analysis shown in Figure 3.4b, rupture takes place in certain areas of the cell wall and leads to the release of the intracellular material, including lipids, in the liquid bulk of the disrupting solution. Once transferred in the liquid bulk, even lipids might be attacked by hydroxyl radicals, as schematically shown in Figure 3.4c, thus generating degradation products such as for example lipid peroxides (González et al., 2012). The extent to which such undesired reaction proceeds depends upon the residual concentrations of H_2O_2 and Fe^{2+} as well as upon the time elapsed before ethanol is added in order to stop it (cf. Figure 3.4d). For this reason both H_2O_2 concentration and contact time should be suitably tuned. In fact, if such parameters are set to correspondingly low or high values, disruption cannot be completed or lipids might be degraded according to the mechanisms described above, respectively. In both cases the final result consists in the lowering of the amounts of lipids which can be collected in the solvent extraction step (cf. Figure 3.4e).

Further experiments, where the disruption was carried using the Fenton's reactant, i.e. a mixture of $FeSO_4$ and H_2O_2, were performed. The goal of these experiments was to drive forward the Fenton's reaction shown in Figure 3.4a, thus by increasing "ad hoc" the Fe^{2+} concentration in solution.

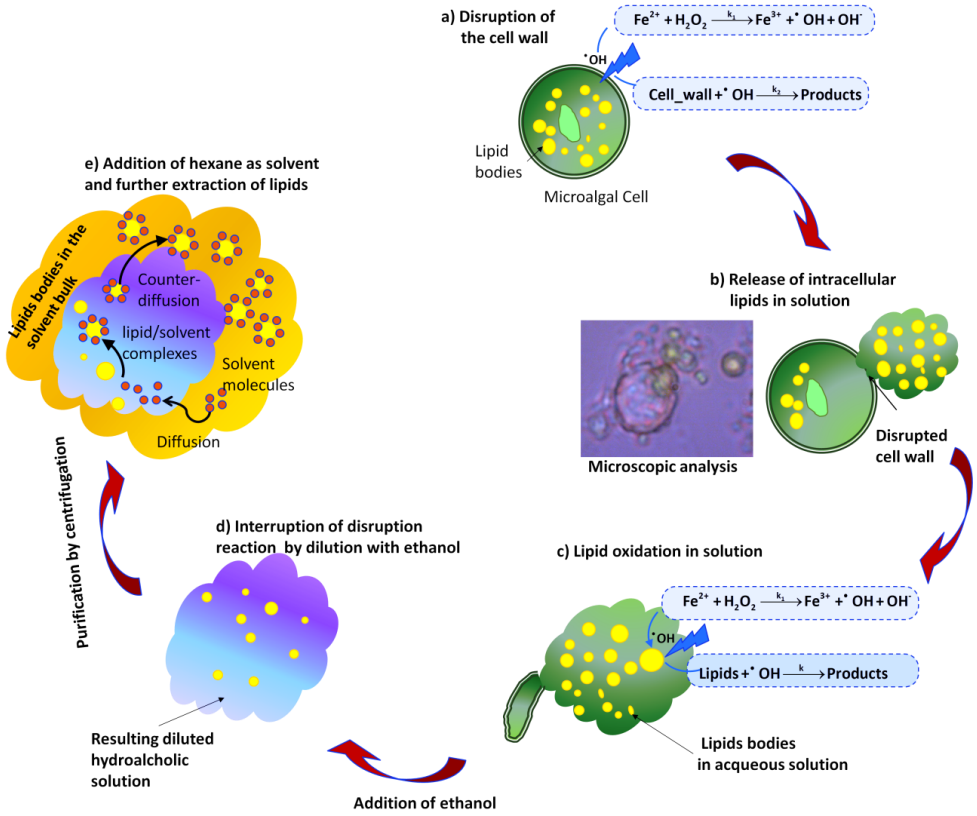


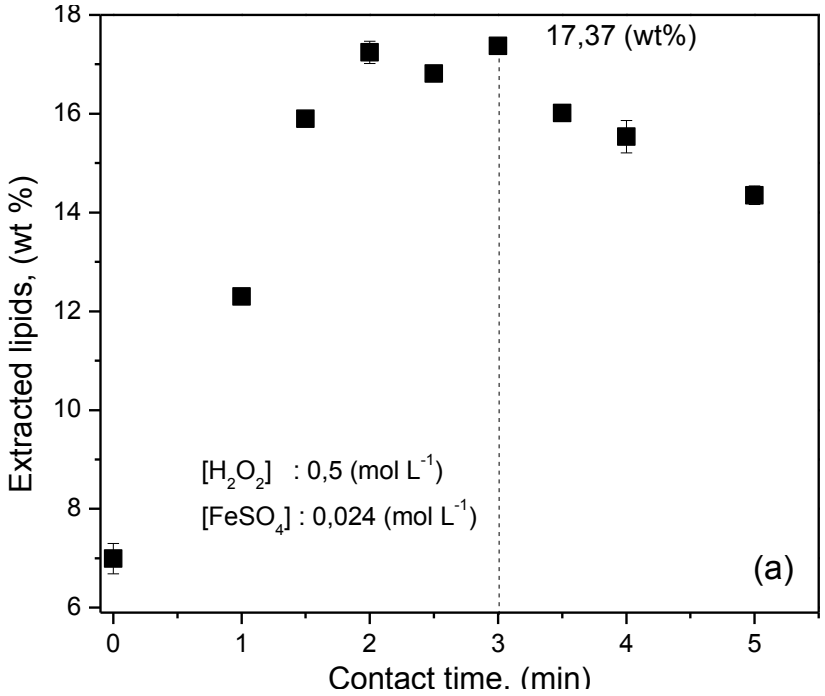
Figure 3.4. Scheme of the mechanism assumed to influence cell disruption and lipid extraction yields.

The effect of the contact time variation on the amount of extracted lipids when using a disruption solution containing 0.5 mol L-1 of H2O2 and 0.024 mol L-1 of FeSO4 is shown in Figure 3.5a. A similar behavior to the one already observed during the corresponding experiments performed with only H2O2 was found. Specifically, the amount of extracted lipids was increased as the contact time was augmented up to a certain value and then started decreasing after an optimum was reached. The mechanisms underlying such behavior are probably the same ones proposed to explain the results obtained when using only H2O2. However, in this case the amount of lipids extracted under the optimal contact time was dramatically increased, i.e more than doubled, with respect to the case when no disruption was performed (i.e. absence of contact time). In fact, a maximum value of extracted lipids of 17.34 %wt/wt was achieved when the contact time equal to 3 min was assured. In Figure 3.5b the effects resulting from the use of growing concentrations of H2O2 on the extracted lipids are displayed. In such experiments the concentration of FeSO4 was kept fixed at 0.024 mol L-1 while the concentration of H2O2 was varied within the range between zero and 6 mol L-1 corresponding to the range of [H2O2]/[FeSO4] molar ratios between zero and

250 $mol_{H_2O_2}\ mol^{-1}_{FeSO_4}$. The contact time was instead kept fixed at the value of 3 min. From Figure 3.5b it can be observed that when H2O2 concentration was increased up to 0.5 mol L-1, the extracted lipids were correspondingly increased.

However, when the H2O2 concentration was further augmented, a reduction of the extracted lipids was observed. This behavior is probably due to the mechanisms already depicted in Figure 3.4c namely, when disruption is carried out under higher concentrations of H2O2, cell wall disruption is achieved in a very short time and thus the lipids transferred in solution can be quickly oxidized by the hydroxyl radicals produced as a result of the high residual concentration of Fenton's reactants still present in solution.

While the latter phenomenon can lead to a pronounced lipid degradation for H2O2 concentrations greater than 1.2 mol L-1, on the other hand, when disruption is performed under the optimal H2O2 concentration of 0.5 mol L-1, i.e. using a [H2O2]/[FeSO4] molar ratio of 21 $mol_{H_2O_2}\ mol^{-1}_{FeSO_4}$, the amount of extracted lipids is dramatically higher (17.34 %wt/wt) than the corresponding one obtained without disruption (7 %wt/wt).



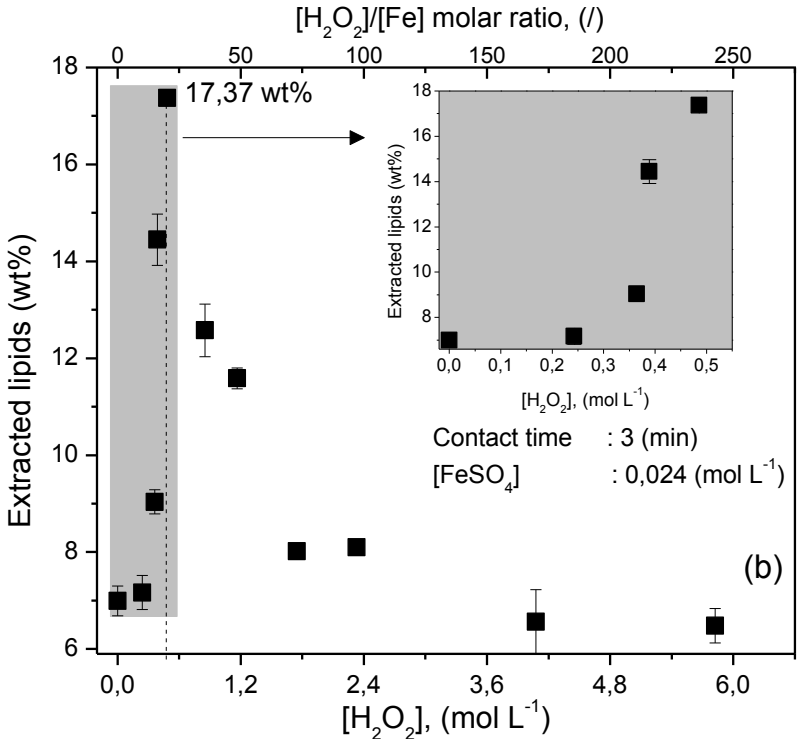


Figure 3.5 Lipid extracted from wet biomass undergone to cell disruption treatments performed (a) with an aqueous solution containing 0.5 mol L-1 of H2O2 and 0.024 mol L-1 of FeSO4 by varying the contact time between 0 and 5 min, and (b) aqueous solutions containing concentrations of FeSO4 kept fixed to 0.024 mol L-1 and H2O2 concentrations ranging from 0 to about 6 mol L-1 while keeping fixed the contact time at 3min.

In the synoptic chart reported in Figure 3.6 results obtained under optimal operating conditions in terms of weight percentage of lipid extracted for unit weight of dry biomass depending upon the different disruption techniques are summarized. It can be observed that the use of H2O2 resulted in the increase of extracted lipids from 6.9 to 9.2 %wt/wt with respect to the case when no disruption was preformed.

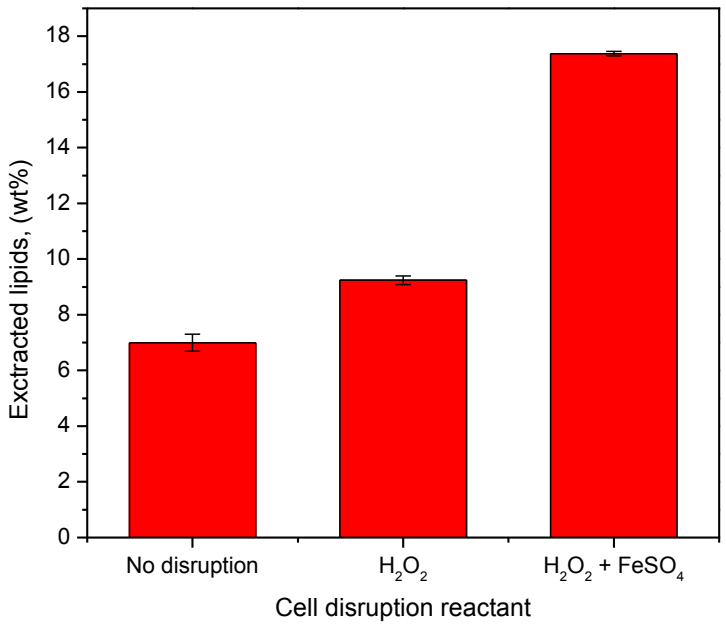


Figure 3.6 Comparison between the weight percentages of lipids extracted from microalgae subjected to the investigated disruption techniques under optimal operating conditions

Moreover, when Fenton reactant was used as disruption agent, the extracted lipids were more than doubled with respect to the case when no disruption was carried out. In fact the percentage of extracted lipids increased from 6.9 to 17.34 %wt/wt.

In order to verify whether the disruption treatments might have affected the quality of the extracted lipids, the content of fatty acid methyl esters (FAMEs) obtained after transesterification of lipids was analyzed. Such investigation was aimed also to verify the potential exploitability of the extracted lipids for producing biodiesel. About 95 %wt/wt of FAMEs obtained from lipid extracted from undisrupted biomass was identified, while 88 %wt/wt and 90%wt of the FAMEs obtained from biomass disrupted through H2O2 and H2O2 +FeSO4, respectively were identified. The comparison among FAMEs profiles is reported in Table 3.2 in terms of weight percentage of each fatty acid with respect to the total amount of FAMEs identified.

Carbon number	Fatty acid name	No disruption (%wt)	Disruption with H_2O_2 (%wt)	Disruption with H_2O_2 + $FeSO_4$ (%wt)
C14:0	Myristic	0.6 ± 0.23	1.28 ± 0.01	0.59 ± 0.1
C16:0	Palmitic	9.1 ± 0.1	30.45 ± 1.35	29.88 ± 0.59
C16:1	Palmitoleic	2.07 ± 0.02	8.52 ± 0.31	18.37 ± 0.07
C17:0	Heptadecanoic	6.26 ± 0.73	10.83 ± 0.27	1.38 ± 0.14
C17:1	Heptadecenoic	0.33 ± 0.07	ND	1.02 ± 0.09
C18:0	Stearic	1.35 ± 0.27	2.94 ± 0.05	2.89 ± 2.07
C18:1	Oleic	4.99 ± 0.21	14.12 ± 2.74	13.48 ± 0.11
C18:2	Linoleic	8.38 ± 0.88	15.51 ± 0.65	8.51 ± 0.31
C18:3	Linolenic	42.32 ± 0.45	15.72 ± 1.29	16.32 ± 1.53
C20:0	Arachidic	2.05 ± 0.33	0.63 ± 0.07	2.82 ± 2.57
C22:0	Behenic	1.19 ± 1.19	ND	ND
-	Other polyunsat.	21.37 ± 1.12	ND	4.75 ± 0.02

Table 3.2 Fatty acid methyl esters profile of lipids extracted after using the different cell disruption techniques investigated.

It can be observed that FAMEs obtained from not-disrupted biomass displayed an high content of linolenic acid (C18:3) of about 42 %wt and other polyunsaturated acids having more than 2 double bonds of about 21 %wt. On the contrary, a low content of total saturated (20 %wt/wt), monounsaturated (7.4 %wt/wt) and linoleic (8.4 % wt/wt) acids, which are the most useful fatty acids for producing biodiesel, was observed. The above characteristics make the lipids extracted from undisrupted biomass not-suitable for biodiesel production since the high degree of unsaturation of FAMEs leads to a high tendency of biodiesel to oxidize and degrade due to the action of air, light, heat, trace metals, etc. (Islam et al., 2013; Kaur et al., 2012; Chisti, 2007). On the contrary, when the cell disruption pre-treatment was performed, whatever the reactant used, such drawbacks were significantly reduced. In fact, linolenic acid content was more than halved when disruption was carried out either by means of H2O2 (15,7 %wt/wt) or H2O2 + FeSO4 (16.3 %wt/wt), respectively. As far as the other polyunsaturated acids are concerned, their relative content was dramatically reduced to 4.75 %wt/wt when disruption was carried out using H2O2 + FeSO4 while it was even below the instrument detection limits when adding H2O2 only. Furthermore, the cumulative weight percentage of saturated acids was 46 %wt/wt for disruption with H2O2 and 37 %wt

when H2O2+FeSO4 was used, thus significantly higher with respect to the corresponding ones measured when no disruption was performed. Also the cumulative percentage of monounsaturated acids was higher when disruption was taken into account, i.e. 22.64 %wt/wt for the H2O2-disrupted microalgae and 32.88 %wt/wt for the case when H2O2+FeSO4 was used, respectively. Moreover, linoleic acid, which is useful for producing biodiesel, was increased after disruption since a weight percentage of 22.64 %wt/wt was found in the FAMEs from H2O2-disrupted microalgae while a percentage of 32.88 %wt/wt was obtained using H2O2+FeSO4. In summary, when dividing the fatty acids in the two macrocateogories shown in Figure 3.7, it can be observed that the adoption of the disruption pretreatment leads to the increase of desired fatty acids and a simultaneous decrease of the undesired ones.

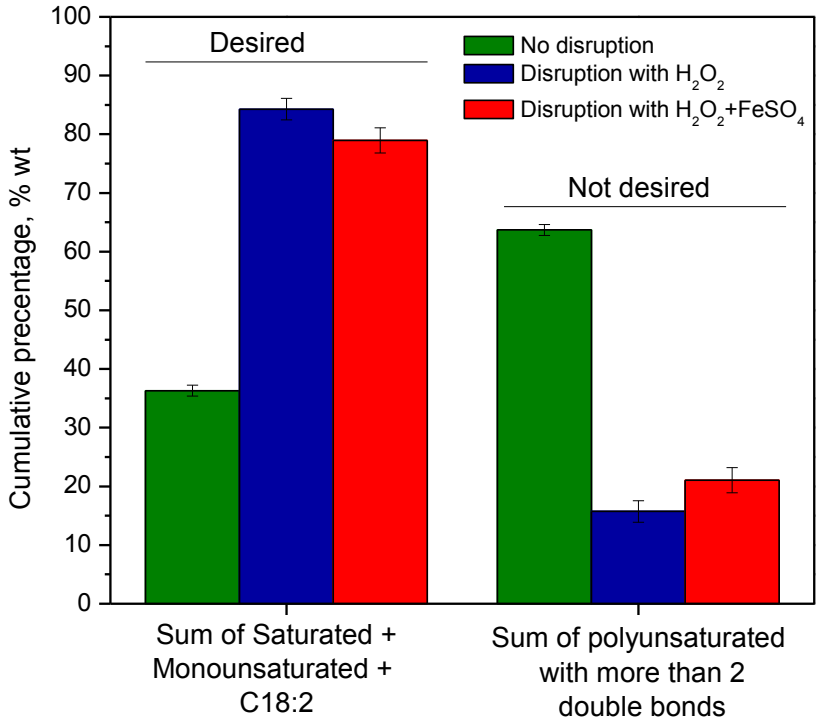


Figure 3.7 Comparison between the cumulative weight percentages of macrocategories of FAMEs related to their usefulness for producing biodiesel and obtained from microalgae subjected to the investigated disruption techniques.

Ultimately, in addition to the achievement of higher lipid extraction yields, the cell disruption pretreatment, whatever the reactant used, leads to the improvement of the final biodiesel quality (cf. Figure 3.7). While the chemical mechanisms underlying such results have to be still investigated, one possible explanation is that free radicals

produced by disrupting reactants might have oxidized double-bonded compounds into oxygenated functional groups like ketones and aldheydes, thus leading to a net decrease of the amount of polyunsaturated fatty acids. A similar mechanism was proposed by Komolafe et al. (2014) to explain the net reduction of polyunsaturated fatty acids deriving from the use of ozone as disrupting agent. However, all the hypotheses above need to be confirmed through specific experiments and analyses. Work is on the way along these lines.

Ultimately, it can be stated that, given the experimental results so far obtained as well as considering the extreme simplicity, the low cost of employed reactants and the modest energy consumption related to proposed technique, the investigated technique is very promising in view of its industrial transposition. Moreover, to the latter purpose, process scale-up might be quite simple since the disruption reactions could be carried out in the same reactor where lipid extraction is performed and thus specific equipments might not be required.

Chapter 4.

Investigation of the iron on the growth rate and lipid accumulation of Chlorella vulgaris in batch photobioreactors

4.1 Introduction

The potential exploitation of microalgae as renewable resource for the production of biofuels is receiving a rising interest mostly driven by the global concerns related to the depletion of fossil fuels supplies and the increase of CO2 levels in the atmosphere. In spite of such interest, the existing microalgae-based technology for CO2 sequestration and biofuels production is still not widespread since it is affected by economic and technical constraints that might have limited the development of industrial scale production systems (Jacob-Lopes and Franco, 2013).

Therefore, in view of industrial scaling-up, the current technology should be optimized in terms of lipid productivities as well as design/operating parameters. The identification of the optimal design and operating parameters that allow the existing strains to increase their lipid content while maintaining an higher growth rate, may be accomplished by exploiting suitable process engineering techniques (Concas et al., 2010; Concas et al., 2012). Among them, the most widespread one consists of the induction of nitrogen starvation phenomena in the culture (Sharma et al., 2012;). In fact, under starvation conditions, nitrogen concentration is not enough for activating the metabolic pathways leading to protein synthesis required by algal growth so that the excess of carbon due to photosynthesis is channeled into storage molecules such as triacylglycerides or starch (Scott et al., 2010). While from on hand, such phenomenon can lead to increase the lipid content, on the other one it results in lower growth rates of microalgae since fundamental proteins cannot be synthesized. Beside nitrogen starvation, several methods are currently being investigated for the induction of lipid biosynthesis in microalgae. Specifically these techniques are based on cultivating algae under extreme pH and temperature conditions, high radiation, osmotic stress, and high heavy metals concentration (Sharma et al., 2012). All these methods have in common process conditions that lead the microalgal cells to use the carbon assimilated through photosynthesis for synthesizing lipids rather than proteins or other structural molecules. In fact, lipids in the form of triacylglycerides provide a storage function that enables microalgae to endure adverse environmental conditions (Sharma et al., 2012). However, the side effect of all the techniques above is the lowering of microalgae growth rate. Therefore, similarly to what happen with nitrogen starvation, while from one side the lipid content of microalgae is increased, on the other hand the growth rate is correspondingly reduced and thus the global lipid productivity achieved is similar to the one which might be obtained by cultivating algae under normal conditions. For this

reason the identification of suitable operating conditions that allow to increase at the same time both lipid content and biomass growth rate is one of the main challenges in the field of biofuels production through microalgae.

Among the micronutrients which can improve microalgae growth rate, iron is well known to be one of the most important. In fact, a large number of studies confirmed that iron is one of the main limiting factors for microalgae cultivation. Such component is vital for microalgae metabolism, since it represents a constituent of the cytochrome b6-f complex which is an enzyme found in the thylakoid membrane of chloroplasts of green algae that mediates the transfer of electrons from Photosystem II to Photosystem I.

Moreover, iron limitation affects the synthesis of phycocyanin and chlorophyll. Finally, the redox properties of iron are critical for nitrogen assimilation and fixation, photosynthesis, respiration and DNA synthesis. Ultimately, iron limitation can result in the reduction of the rate of CO2 fixation and inorganic nitrogen assimilation of phytoplankton by limiting the light reactions of photosynthesis (Buitenhuis and Geider, 2010).

Liu et al. (2008) have shown that an increasing of bio-available iron concentration in the growth medium can lead to a simultaneous increase of both lipid content and growth rate of a marine strain of Chlorella. Specifically when the initial iron concentration in the growth medium is increased from 0 to 1.2· 10-5 mol L-1, a corresponding increase in the lipid content from 7.8 to 57% by weight of dry biomass could be observed.

While such results have not been so far quantitatively confirmed in the literature, from a qualitative point of view a similar behavior has been observed by Ruangsomboon (2012) when considering Botryococcus braunii. Specifically, it was observed that, while biomass growth rate was not significantly affected by initial iron concentration, the corresponding lipid content markedly increased from 22% to 35% by dry weight when the initial iron concentration is correspondingly augmented from 9 to 27 mg L-1.

Similar results were obtained by Ruangsomboon et al. (2013) when considering the green alga Scenedesmus dimorphus whose lipid content greatly increased when the initial iron concentration was augmented from 9 to 47 mg L-1. Specifically, a maximum lipid content of about 24.7 % was observed for Scenedesmus dimorphus when it was cultivated under the maximum initial concentration of iron considered (i.e. 47 mg L-1). Baky et al. (2012) reported that the accumulation of total lipids shows an increasing trend when Fe3+ concentrations in solution was augmented up to 20 mg L-1. Recently, Mata et al. (2013) demonstrated that by increasing the Fe concentration in the culture

medium 10 times with respect to the base case, the maximum lipid productivity of Dunaliella tertiolecta increased to almost the double, correspondingly. Finally, Yeesang and Cheirsilp (2011) reported that high level of iron improved lipid accumulation in four different strains of microalgae. Ultimately, the experimental results summarized above seem to confirm that when the initial iron concentration is increased within a specific range, a simultaneous augmentation of growth rate and lipid content can be observed for specific strains. While these results are promising in the light of the microalgae technology optimization, on the other hand, to the best of our knowledge, no exhaustive explanation on how iron can influence the lipid biosynthesis in microalgae has been so far provided. Moreover, such a lack of understanding seems to have limited the development of iron based strategies to improve bio-oil yields, and hence their potential application at the industrial scale for the production of biofuels through microalgae. For these reasons further and deeper investigations about the effect of iron on lipid accumulation in microalgae are required. In this regard, while the identification of the phenomena involved needs an extremely accurate experimental research, the optimization of design and operating parameters for the application of the iron-based strategy to the industrial scale, may be accomplished by exploiting suitable process engineering techniques. Consequently, the goal of the present work is to propose an experimental investigation on the growth of microalgae and their lipid accumulation as a function of iron concentration in solution. Therefore, specific experiments were performed with a strain of C. vulgaris, where the iron concentration in solution was suitably changed. It is worth noting in passing that, to the best of our knowledge, such experimental investigation deals for the first time with a freshwater strain of C. Vulgaris.

4.2 Materials and methods

4.2.1 Microorganism, culture conditions and culture medium

The fresh water algal strain Chlorella vulgaris (Centro per lo Studio dei Microorganismi Autotrofi, CNR, Florence, Italy) was considered in this work. Unialgal stock cultures were propagated and maintained in Erlenmeyer flasks with a Kolkwitz Triple Modified (KTM-A) medium under incubation conditions of 25°C, a photon flux density of 98 □E 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).

Growth experiments were performed in Erlenmeyer flasks and Pyrex bottles under axenic conditions. The culture media volumes were 250 mL and 1 L for flasks and bottles, respectively, which were agitated by a magnetic stirrer at 300 rpm using 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. Either flasks or bottles were stoppered by means of cotton plugs wrapped in cotton gauze during cultivation in order to prevent external contamination while at the same time assuring atmospheric CO2 diffusion within the culture. Algae were cultured at room temperature and under a photon flux density of 100 µE m-2 s-1 provided by six 11 W white fluorescent tubes and a light/dark photoperiod of 12 h. The initial cell concentration in each experiment varies from 0.065 to 0.088 g L^{-1}.

The culture medium consist of a modified Kolkwitz medium (KTM-A) containing 2.5 g L^{-1} of KNO_3, 0.5 g L^{-1} of KH_2PO_4, 0.27 g L^{-1} of $MgSO_4 \cdot 7H_2O$, 0.04 g L^{-1} of $CaCl_2 \cdot 2H_2O$, 1 g L^{-1} of $NaHCO_3$ and 1 mL of micronutrients solution The latter one contained 2.86 g L^{-1} of H_3BO_3, 1.81 g L^{-1} of $MnCl_2 \cdot 7H_2O$, 0.222 g L^{-1} of $ZnSO_4 \cdot 7H_2O$, 0.035 g L^{-1} of $CoCl_2 \cdot 6H_2O$, 0.080 g L^{-1} of $CuSO_4 \cdot 5H_2O$, and 0.230 g L^{-1} of $Na_2MoO_4 \cdot 2H_2O$. Iron was supplied in chelated form by adding to the culture medium suitable volumes from a solution containing 29.75 g L^{-1} of $Na_2EDTA \cdot 2H_2O$ and 24.90 g L^{-1} of $FeSO_4 \cdot 7H_2O$, respctively. Specifically, C. vulgaris was cultivated in the above specified medium supplemented with $FeSO_4 \cdot 7H_2O$ at the iron concentration levels equal to 0.0, $1.8 \cdot 10^{-1}$, $4.5 \cdot 10^{-1}$ and 1.8 mol m^{-3} or 0.0, 10.0, 25.0 and 100 g m^{-3} corresponding to $Na_2EDTA \cdot 2H_2O$ concentrations of 0.0, $1.6 \cdot 10^{-1}$, $4.0 \cdot 10^{-1}$ and 1.6 mol m^{-3}, respectively.

4.2.2 Biomass and pH measurement

The growth of microalgae was monitored through spectrophotometric measurements (Genesys 20 spectrophotometer, Thermo Fisher Scientific Inc. Waltham) of the culture media optical density (OD) at 560 nm wavelenght (D_{560}) with 1 cm light path. The biomass concentration C_b (g_{dw} L^{-1}) was calculated from OD measurements using a suitable C_b vs. OD calibration curve which was obtained by gravimetrically evaluating the biomass concentration of known culture medium volumes that were previously centrifuged at 4000 rpm for 15 min and dried at 105°C for 24 h. The pH was daily measured by pH-meter (KNICK 913). For the sake of reproducibility, each experimental condition was repeated at least twice. The average and standard errors values of the experimental results were calculated by taking advantage of OriginPro 8 software.

4.2.3 Lipid extraction

In order to evaluate the lipid content of C. vulgaris, the microalgae were first harvested and then centrifuged to obtain a wet biomass pellet characterized by a humidity of about 90 %wt/wt. Lipid extraction was performed directly on wet biomass. The method proposed by Molina Grima et al. (1994) was adopted for extracting lipids from microalgae through direct saponification. Briefly it consists in contacting suitable amounts of wet micro-algal biomass with an extraction/saponification solution prepared by dissolving 2.16 g of KOH in 100 mL of ethanol (96% purity). Specifically, 1 g of wet biomass was contacted for 8 hours with 6 mL of the solution above in a stirred flask at room temperature. Unsaponifiables were then separated by five extractions with 2 mL of hexane. In order to shift the equilibrium distribution of unsaponifiables to the hexane phase, 1 mL of water was added. The hydroalcoholic phase containing soaps, was then acidified by adding HCl in a 1:1 volumetric ratio in order to obtain a pH of about 1. The lipids obtained were then recovered through eight extractions with 2 mL hexane and subsequently weighted. By dividing the weight of lipids obtained and the initial dry weight of the biomass which underwent the extraction procedure, the lipid content of microalgae, q_ℓ was evaluated. It is worth noting here that when microalgae were cultivated in absence of dissolved iron, their lipid content q_ℓ^0 at the start of the cultivation was equal to the corresponding one measured at the end, q_ℓ^f.

4.3 Results and discussion

The effect of iron concentration on the growth kinetics of C. vulgaris and its lipid content was investigated in this work. Specific experiments were carried out by cultivating C. vulgaris in batch stirred flasks where the initial concentration of dissolved iron was suitably changed. In particular, the growth and lipid accumulation kinetics in absence of iron was first investigated. Subsequently, further experiments were carried out to evaluate the effect of the initial concentration of dissolved inorganic iron on the growth of C. vulgaris by varying the initial concentration of $FeSO_4 \cdot 7H_2O$ and $Na_2EDTA \cdot 2H_2O$, while keeping fixed their molar ratio (i.e. $[FeSO_4.7H_2O]/[Na_2EDTA \cdot 2H_2O]$). In Figure 4.1 the time evolution of total biomass concentration obtained when cultivating C. vulgaris in absence of dissolved iron is shown.

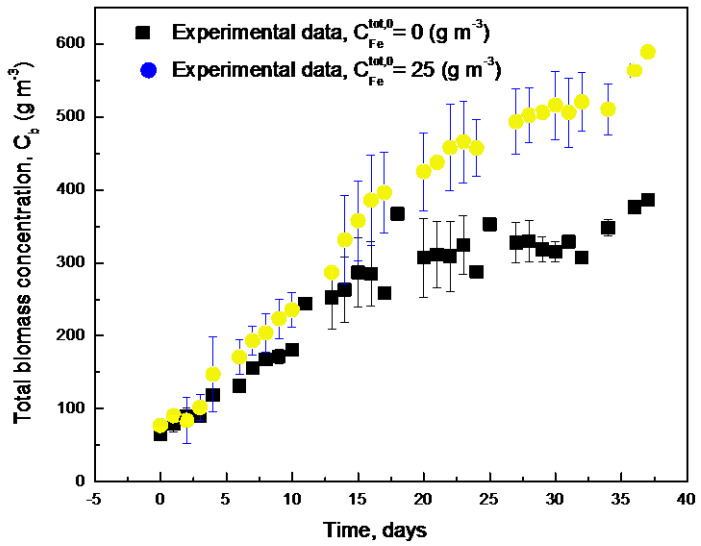


Figure 4.1 Comparison between model results (fitting) and experimental data in terms of total biomass concentration, which accounts for both non lipidic and lipidic fraction, as a function of time when the initial concentration of iron in solution is equal to zero and 25 g m-3.

In particular, it can be observed that the culture starts growing without showing a significant lag phase despite the absence of iron in solution. Probably, microalgae can grow also when iron concentration in solution is zero by exploiting the intracellular reservoir of iron, namely the initial iron cell quota. Therefore, the initial intracellular content of iron was high enough to permit Chlorella cells to grow and duplicate for a specific time interval, as shown in Figure 4.1. In fact, it can be observed that culture

grows until about 10 days, while after 20 days of cultivation it reaches a sort of "plateau" when the biomass concentration is about 320 g m-3.

According to the model propsed by Concas et al. (2014), it can be observed from Figure 4.2-a, such stationary phase is reached due to the consumption of the available intracellular iron. In particular, since the uptake of iron is zero because no iron is added to the solution, the iron cell quota correspondingly decreases. Therefore, when the iron cell quota reaches the minimum value that allows microalgae growth, the culture stops growing (cf. Figure 4.2-a).

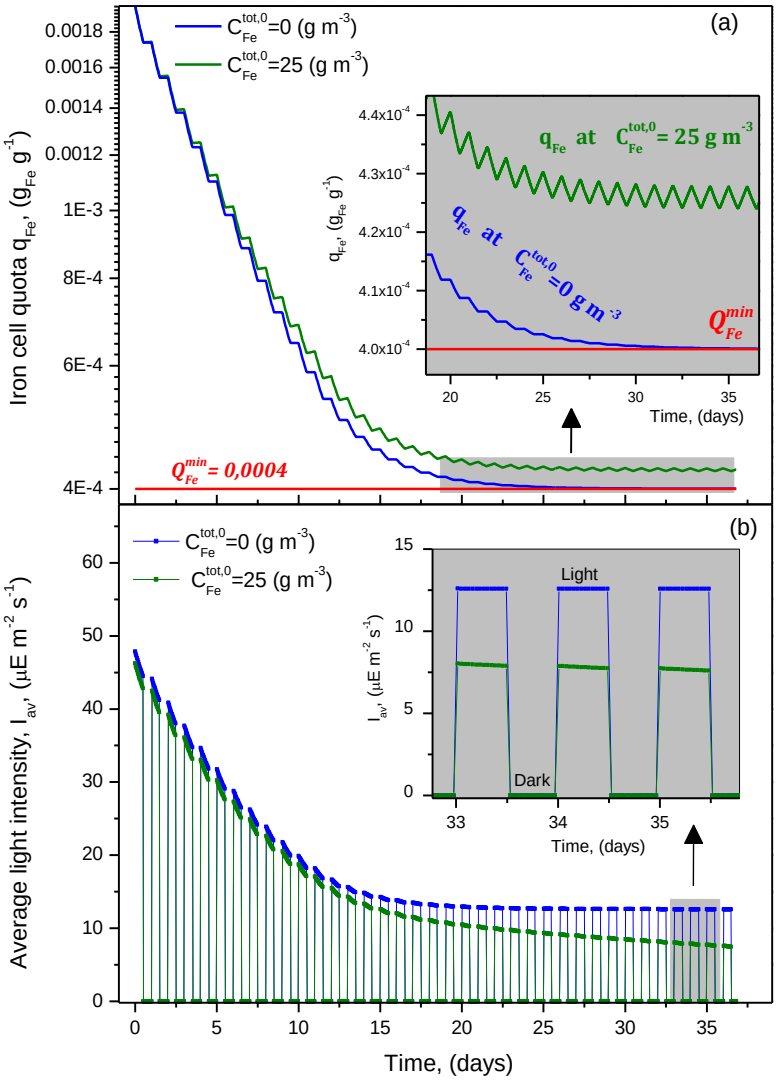


Figure 4.2 Time evolution of iron cell quota (a) and average light intensity (b) for the cases where $C_{Fe}^{tot,0} = 0$ and $C_{Fe}^{tot,0} = 25\ g\ m^{-3}$, as simulated by the proposed model (adapted from Concas et al., 2014).

It is worth noting that the experiment carried out in absence of dissolved iron permits to suitably evaluate the initial cell quota of iron. In fact, since no iron was present in solution and growth ends when the cell quota is equal to Q_{Fe}^{min}, from a simple material balance related on iron it follows that $q_{Fe}^{0} = (C_x^f \cdot Q_{Fe}^{min})/C_x^0$, where C_x^0 and C_x^f are the initial and the final concentrations of the non lipidic biomass concentration, respectively. The obtained value of the initial iron cell quota where $C_{Fe}^{tot,0} = 0$ and $C_{Fe}^{tot,0} = 25\ g\ m^{-3}$.

In order to evaluate the effect of iron on microalgae growth rate and lipid accumulation further experiments were performed by setting the initial concentration of $FeSO_4 \cdot 7H_2O$ equal to $4.5 \cdot 10^{-1}$ mol m^{-3} and the initial concentration of $Na_2EDTA \cdot 2H_2O$ to $4.0 \cdot 10^{-1}$ mol m^{-3}, thus assuring a molar ratio between iron and EDTA equal to 1.12:1. The corresponding initial concentration of total iron in solution, i.e. $C_{Fe}^{tot,0}$, was thus 25 gFe m-3. From the analysis of experimental data shown in Figure 4.1 it can be observed that under such operating conditions the culture keeps growing during the whole investigated time interval. Consequently, the biomass concentration at the end of the experiment is almost doubled with respect to the corresponding one observed in the case of absence of iron. Such a behavior is due to the fact that microalgae can prevent the decrease of their iron cell quota by taking advantage of iron available in solution. In fact, according to the model propsed by Concas et al. (2014), the cell quota remains thus always greater than the minimum value Q_{Fe}^{min} as a result of the uptake of iron from solution (cf. Figure 4.2-a). In Figure 4.1, the comparison the experimental data related to the case where $C_{Fe}^{tot,0}$ is equal to 25 gFe m-3, are shown.

According to Concas et al (2014), the change of the slope of the growth curve shown in Figure 4.1, is probably due to the fact that, under iron-replete conditions, at the start of the experiment, nitrogen becomes the main limiting nutrient and thus the value of the growth rate is dictated by nitrogen cell quota (cf. equation 14). Such inferences are confirmed by the model propsed by Concas et al. (2014) where it demonstrated that, as the culture grows, its optical density increases and consequently, as shown in Figure 4.2-b, the light intensity that is available for microalgae, decreases. As a result the carbon specific photosynthetic rate decreases in such a way that when very low values are reached, the culture becomes light-limited instead of nitrogen-limited and consequently the variation law of the growth rate changes. This phenomenon provokes

the change in the slope of the growth curve related to the experiment with $C_{Fe}^{tot,0} = 25\ g\ m^{-3}$ observed in Figure 4.1 after 15 days of cultivation.

From Figure 4.3 it can be seen that also the final lipid content is well fitted by the proposed model when total initial iron concentration in solution is equal to 25 g_{Fe} m^{-3}.

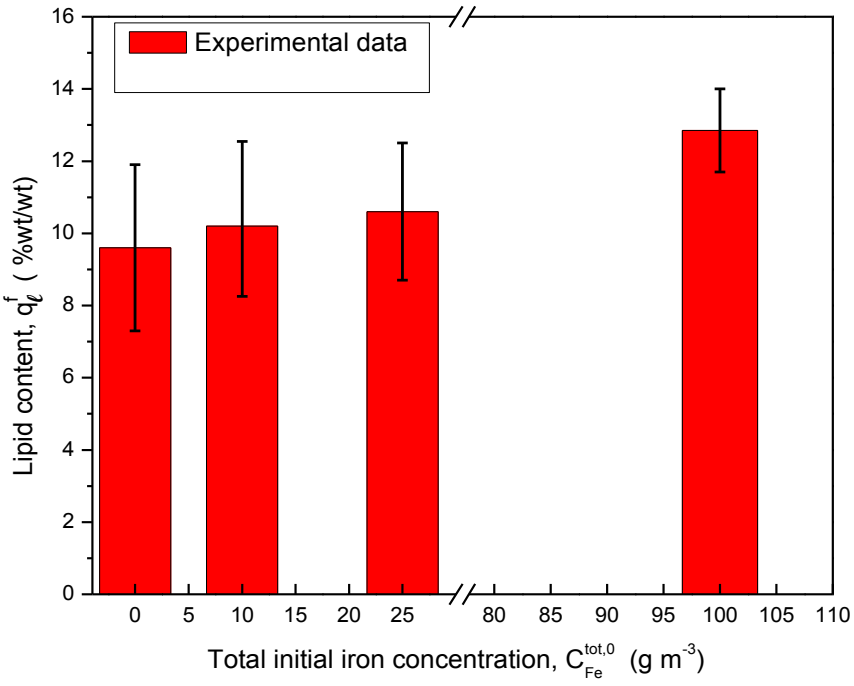


Figure 4.20 Comparison between model results (fitting/prediction) and experimental data in terms of final lipid content of microalgae as a function of the total initial iron concentration in solution.

Moreover, the experimental data confirm that total lipid content increases when the iron concentration in solution is augmented. Specifically, the lipid content increased from 9.6% to 10.6% by dry weight when the total initial iron concentration in solution was increased from 0 to 25 g_{Fe} m^{-3}, respectively. These results are qualitatively consistent with the ones obtained by Liu et al. (2008) with a marine strain of chlorella vulgaris. In fact also in this case a simultaneous increase of growth rate and lipid content was observed when iron concentration in solution was augmented. However, from a quantitative point of view, a less pronounced increase of lipid content is observed in our work with respect to the corresponding one obtained by Liu et al. (2008), where the lipid content increased from 0 to about 57% when the initial iron concentration was augmented from 0 to $6 \cdot 10^{-1}$ g m^{-3}, respectively. The differences with the present results are probably due to several reasons. First, all data published by Liu et al. (2008) refer to

a marine strain of C. vulgaris, while in the present work we took advantage of a freshwater strain. Moreover, different growth media were used and different illumination conditions were adopted. In particular, it is worth noting that, in the work by Liu et al. (2008), $FeCl_3$ was used as source of iron instead of $FeSO_4$. Thus, the iron addition is coupled to the presence of chloride species which is well known to provoke oxidative stress that is, in turn, capable to trigger lipid accumulation phenomena in microalgae. Therefore, the effect on lipid accumulation due to the presence of chloride species was probably superimposed to the one related to iron when considering the experiments performed by Liu et al. (2008).

Figure 4.5 shows the evolution of iron-related species as simulated by Concas et al. (2014) when $C_{Fe}^{tot,0}$ is set equal to 25 g m-3. In this figure, log scale is adopted for the time axis to allow the reader to better appreciate the dynamics of iron speciation phenomena taking place during the first 0.3 days of the experiments. In fact since speciation reactions of iron in presence of EDTA (Figure 4.4) are characterized by high rates they are almost completed within about 0.3 days.

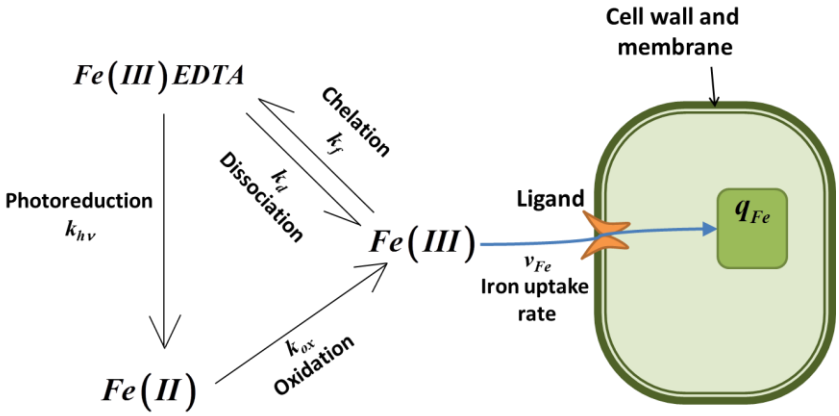


Figure 4.4 Schematic representation of Fe complexation, oxidation, chelation and algal uptake phenomena occurring in solution, adapted from Hudson et al. 1990 and Shaked et al. 2005.

As it can be observed, after this period of time, the most of iron in solution is bonded to EDTA which serves as a buffering agent. At the same time, the useful form of iron for algae, i.e. Fe(III) reaches a concentration equal to about 0.06 mol m-3 and subsequently does not vary significantly. This is due to the fact that Fe(III) concentration is much higher than the minimum needed by algae to grow. Consequently the uptake of iron by algae does not result in a lowering of Fe(III) concentration which might be appreciated

at the scale adopted. However, when zooming on a more detailed scale (cf. grey box in Figure 4.5), the reduction of Fe(III) concentration due to iron uptake by algae, can be appreciated. It is worth mentioning here that such high iron concentrations have been adopted in order to trigger lipid accumulation.

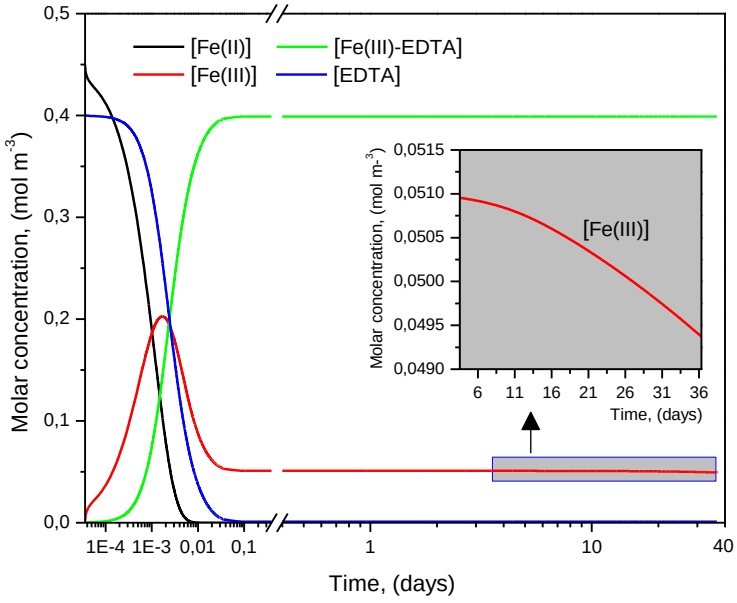


Figure 4.5. Time evolution of different iron species in solution as simulated by the proposed model for the case where $C_{Fe}^{tot,0} = 25\ g\ m^{-3}$ (adapted from Concas et al., 2014).

In fact while the iron starvation phenomena can be avoided by using much lower concentrations of iron, in order to provoke the oxidative phenomena that are on the base of lipid accumulation very high concentrations of iron are needed.

To confirm the results so far obtained, further experiments were performed using total iron concentration equal to 10 gFe m-3 and 100 gFe m-3, respectively. The corresponding initial EDTA concentrations were set in order to assure always the same initial molar ratio between iron and EDTA. The obtained experimental data are shown in Figure 4.3 for the case of final lipid content and in Figure 4.6 in terms of biomass concentrations as a function of time.

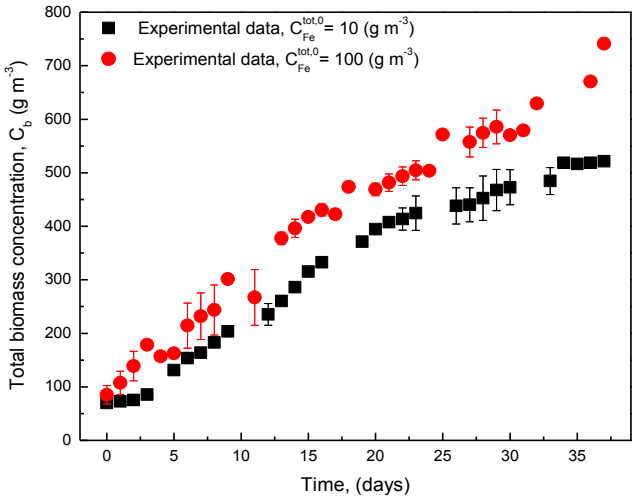


Figure 4.6 Comparison between model results (prediction) and experimental data in terms of total biomass concentration, which accounts for both non lipidic and lipidic fraction, as a function of time when the initial concentration of total dissolved iron in solution is equal to 10 and 100 g m-3, respectively.

As it can be seen, such experiments well confirm the ones obtained under iron cocnetrations of 0 and 25 g m-3 respectively both in terms of biomass concentration and final lipid content. In particular from Figure 4.6 one can observe that, under such operating conditions, microalgae grow in a similar way. In fact the biomass concentration achieved at the end of the experiment carried out when using an iron concentration of 100 g m-3 was clearly higher than the corresponding one obtained when using lower iron concentrations. Ultimately, when the iron concentration is augmented beyond 10 g m^{-3}, a slight increase in the total biomass growth rate is also observed. Such a behavior is consistent with the data reported in the literature (Liu et al., 2008; Yeesang and Cheirsilp, 2011; Mata et al., 2013; Ruangsomboon et al., 2013), where it is shown that when iron is added beyond a certain value a slight increase in the growth rate can be detected while, on the contrary, the lipid content results to be clearly augmented.

While the proposed model well captures such experimental behavior, the phenomena involved in the iron-induced lipid accumulation in chlorella are still unclear. On the basis of the literature data, only some hypotheses can be formulated about the mechanism that underlies the increased lipid accumulation deriving from the augmentation of iron concentration in solution. A first hypothesis is based on the fact that, under illumination, iron can promote the generation of hydroxyl and superoxide

radicals that may subject the cells to a significant oxidative stress (Fujii et al., 2010). Nevertheless, several micro algae have the ability to produce relatively high amounts of storage lipids as a response to oxidative stress (Sakthivel et al., 2011). In particular it has been recently demonstrated that specific intracellular content of lipids of chlorella vulgaris is positively correlated to the intracellular concentration of free radicals in a power law fashion (Menon et al., 2013). Thus the increased concentration of iron in solution might have led to an increased production of free radicals, both outside and inside the cell, which, in turn, might have stimulated the microalgal cells to synthesize more lipids. In addition, as discussed for soybeans seeds by Plank et al. (2001), iron can play an important role in activating the enzyme Acetyl-CoA carboxylase (ACCase) which may, in turn, catalyze the rate-limiting step in the bio-synthesis of fatty acids. While specific experiments should be performed to confirm such effects of iron on microalgae, a similar mechanism might be assumed to occur in Chlorella vulgaris. Moreover, the two mechanisms discussed above are probably acting simultaneously. In addition, according to Menon et al. (2013), also the high concentration of radicals, which is somehow related to the presence of iron, is capable to enhance the activity of ACCase in C. vulgaris by accelerating the irreversible carboxylation of acetyl-CoA to malonyl-CoA. However, all the hypotheses above need to be confirmed through specific experiments. Work is on the way along these lines.

Chapter 5.

Conclusions and suggestions

for further research

The existing microalgae-based technology for CO2 sequestration and biofuels production still not widespread since it is affected by economic and technical constraints that might have limited the development of industrial scale production systems. Therefore, in order to be viable scaled, the current technology should be optimized in terms of lipid productivities of existing strains. Such a target can be achieved either by stimulating the lipid synthesis of microalgae or, by increasing the yields of the lipid extractive step of the process. Along these lines the present PhD activity has been focused on two different lines of research which share the common target of identifying suitable strategies to increase the lipid productivity of the current microalgal technology. Specifically, in the first line of activity a novel cell disruption techniques for the enhancement of lipid extraction yields from C. Vulgaris have been investigated. In the second line of activity, the possibility to exploit iron-based strategy to increase lipid synthesis in C. Vulgaris was investigated. The identification of the optimal design and operating parameters that allow microalgae to increase their lipid content while maintaining an higher growth rate, may be accomplished by exploiting suitable process engineering techniques. Among them, the most widespread one consists of the induction of nitrogen starvation phenomena in the culture. Beside nitrogen starvation, several methods are currently being investigated for the induction of lipid biosynthesis in microalgae. These techniques are based on cultivating algae under extreme pH and temperature conditions, high radiation, osmotic stress, and high heavy metals concentration. However, the side effect of all the techniques above is the lowering of microalgae growth rate. For this reason the identification of suitable operating conditions that allow to increase at the same time both lipid content and biomass growth rate is one of the main challenges in the field of biofuels production through microalgae. Among the micronutrients which can improve microalgae growth rate, iron is well known to be one of the most important. In fact, iron limitation can result in the reduction of the rate of CO2 fixation and nitrogen assimilation of microalgae by limiting the light reactions of photosynthesis. Moreover, recent results reported in the literature seem to confirm that when the initial iron concentration is increased within a specific range, a simultaneous augmentation of growth rate and lipid content can be observed for specific

strains. While these results are promising in the light of the microalgae technology optimization, to the best of our knowledge, no comprehensive explanations accounting simultaneously for all the phenomena taking place during lipid accumulation in microalgae when varying iron concentration, have been so far proposed in the literature. For this reason further experimental activity has been carried out in this work in order to clarify mechanisms underlying the iron-induced lipid accumulation in C. vulgaris. The obtained results will be exploited to develop an iron-based strategy for the production of biofuels through microalgae.

Another method to increase the bio-oil production trough microalgae is based on the optimization of lipid extraction yields by taking advantage of cell disruption pre-treatments. From a close examination of the technologies currently available to increase lipid extraction yields form microalgae, a special attention should be paid to the cell disruption pre-treatments which are aimed to break the algal cell wall and facilitate the extraction of lipids contained within the cell. Among the technologies for cell disruption so far investigated at the laboratory-scale, the most relevant and worth of mentioning are: mechanical methods (French press, lyophilization, bead-beating grinding), physical methods (microwaves, gasification, ultrasonication, osmotic shock, supercritic CO2), and chemical methods (soxhlet, fermentation, solvents) methods. However, the above mentioned techniques require high energy inputs and thus potentially affect the economic feasibility of the entire biofuels production process when applied at the industrial scale. For this reasons novel techniques for cell disruption, which are characterized by low energy requirements as well as high disruption yields, are needed.

The goal of the first line of activity of the present work is the development of a novel cell disruption techniques for the improvement of lipid extraction from microalgae, an innovative low energy consuming technique for cell disruption, based on the use of low toxicity reactants, has been investigated. A brief summary of the research activity is reported in what follows. C. vulgaris was cultivated in photobioreactors operated in semi-batch mode in BIOICOIL photobioreactor. Once the culture reached the stationary growth phase, microalgae were harvested and centrifuged. The obtained biomass,

characterized by a water content of about 90%, was then subjected to cell disruption. To this aim, suitable amounts of water solutions containing different concentrations of H2O2 or Fenton reactant were contacted for different times with known amounts of wet biomass. Subsequently, disrupted cells were subjected to the lipid extraction procedure proposed in the literature, which allowed to evaluate the weight percentage of extracted lipids per unit weight of biomass on a dry basis. In the cell disruption experiments, both disruption agent concentration and contact time were suitably varied in order to identify the corresponding values which were able to maximize the extracted lipids. Experimental results have shown that the use of H2O2 resulted in the increase of extracted lipids from 6.9 to 9.2 %g/gdw with respect to the case where no disruption was previously preformed. Moreover when Fenton was used as disruption agent the extracted lipids were more than doubled with respect to the case where no disruption was carried out. In fact the percentage of extracted lipids increased from 6.9 to 17.4 %g/gdw. Thus the use of Fenton reactant as disruption agent might double lipid productivity of C. Vulgaris. The goal of the second line of activity regarding the development of an iron base strategy to improve lipid productivity of C. Vulgaris in batch photobioreactors. To this aim, specific experiments were performed with C. Vulgaris, where iron concentration in solution was suitably changed. The obtained experimental results confirm the positive effect of growing iron concentrations on lipid productivity of C. Vulgaris. In fact when iron concentration in the growth medium was augmented within a suitable range, a simultaneous increase of growth rate, fatty acid content and final biomass concentration could be observed. Thus, the proposed protocol for cultivating C. Vulgaris under high iron concentrations, might represent a useful tool to optimize iron-based strategies to improve the lipid productivity of microalgal cultures.

*As far the techniques based on the use of iron to improve lipid accumulation in microalgae the f*uture activities will be focused on: testing the proposed cultivation method on other microalgae strains; taking advantage of suitable experimental design tools in order to identify the optimal set of operating parameters. *As far the novel cell disruption technique is concerned, f*uture

activities will be aimed to further optimize the operating parameters; testing of new solvents.

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