Universidade do Minho
Escola de Engenharia

Adriana Salomé Amaro Machado

Development of an organic culture medium for *Chlorella vulgaris*

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Professor Doutor António Augusto Martins de Oliveira Soares Vicente
e da
Doutora Joana Gabriela Laranjeira da Silva
e supervisão de
Mestre Hugo Galvão Caiano Pereira

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ABSTRACT

The commercial value of microalgal biomass can be explored for various applications, such as human nutrition. *Chlorella vulgaris,* is one of the few species that are currently approved as food ingredient and supplements. In recent years, trends in the food industry have led to an increased demand for natural, healthy, sustainable and quality assured food. A growing niche market is the production of organic food products, due to the increasing awareness of customers nowadays.

This work aims at developing and establishing an effective organic culture medium for industrial production of autotrophic and heterotrophic microalgal biomass.

Initially, a preliminary study in 1.5 L airlift reactors was performed to determine the most promising organic substrates (OS). Starting with 2 mmol L^{-1} of nitrates and ammonia in the final growth medium, the highest biomass productivities were obtained in OS.4 (0.033 g L^{-1} d^{-1}) and OS.1 (0.028 g L^{-1} d^{-1}). After the elemental analysis of the OS was performed, a culture medium was developed to meet the nutrient needs of *Chlorella*. Once the culture medium was optimized with the ideal concentration of each solution, it was tested at autotrophic pilot and industrial scale, namely, 125 L green wall panels and a 10 m^3 tubular photobioreactor. Obtained results revealed that both organic and inorganic culture media displayed the same growth pattern. Interestingly, organic microalgae biomass presented good physicochemical properties with a significantly higher content of proteins compared to the control inorganic medium.

Since heterotrophic cultivation is a more suitable approach to "seed" (inoculum) the autotrophic production photobioreactors, a heterotrophic medium was optimized using an organic carbon source (molasse). Following the optimization of sucrose inversion in molasse at 45 ° C, pH 5.8 and 0.015 mg mL^{-1} of enzyme, the carbon source was optimized by testing different concentrations. From the study conditions, 5 g L^{-1} of glucose showed higher biomass productivity (0.07 g L^{-1} h^{-1}) but at 10 g L^{-1} glucose the growth was inhibited. Afterwards, the N concentration was optimized using the previously optimized autotrophic growth medium. In this context, different N concentrations were tested (1, 5 and 10 mmol L^{-1} of N). The highest volumetric productivities were obtained at 5 and 10 mmol L^{-1} of N (0.023 ± 0.002 and 0.025 ± 0.001 g L^{-1} h^{-1} respectively).

Following the optimization of the C and N concentrations a fed-batch growth on a 7 L bench top fermenter denoted that heterotrophic growth can be used for inoculum production. In this experiment, approximately 13.1 g L^{-1} of biomass dry weight were achieved, however, further

optimization of the fed-batch operation will enable a significant improvement of the final biomass productivities.

KEYWORDS

Organic biomass; *Chlorella vulgaris*; Autotrophic; Heterotrophic; Biochemical composition

Resumo

O valor comercial da biomassa algal pode ser explorado para várias aplicações, como na nutrição humana. *Chlorella vulgaris*, é uma das poucas espécies que atualmente são aprovadas para suplementos e ingrediente alimentar. Nos últimos anos, as tendências na indústria alimentar conduziram a um aumento crescente de alimentos naturais, saudáveis, sustentáveis e de qualidade assegurada. Um nicho de mercado em crescimento é a produção de produtos alimentares biológicos, devido a um aumento de consciencialização dos consumidores de hoje.

Este trabalho tem como objetivo desenvolver e estabelecer um meio de cultura biológica eficaz para produção industrial de biomassa microalgal autotrófica e heterotrófica.

Inicialmente, foi realizado um estudo preliminar em reatores airlift de 1,5 L para determinar quais os substratos biológicos mais promissores (OS). Começando com 2 mmol L^{-1} de nitratos e amónia no meio de cultura, as maiores produtividades em biomassa foram obtidas no OS.4 (0,033 g L^{-1} d^{-1}) e OS.1 (0,028 g L^{-1} d^{-1}). Após a análise elementar dos OSs, foi desenvolvido um meio para atender às necessidades nutricionais da *Chlorella*. Assim que o meio de cultura foi otimizado com a concentração ideal de cada solução, foi testado autotroficamente em escala piloto e industrial, nomeadamente green wall panels de 125 L e um fotobiorreactor tubular de 10 m^3. Os resultados obtidos revelaram os meios de cultura biológicos e não biológicos exibiram o mesmo padrão de crescimento. Curiosamente, a biomassa biológica apresentou boas propriedades físico-químicas com um conteúdo significativamente maior de proteínas em comparação com o controlo.

Sendo o cultivo heterotrófico a abordagem mais adequada para produção de "semente" (inóculo) nos fotobiorreactores em autotrofia, um meio heterotrófico foi otimizado usando como fonte de carbono (melaço). Após a otimização da inversão de sacarose no melaço a 45 ° C, pH 5,8 e 0,015 mg mL^{-1} de enzima, a fonte de carbono foi testando a diferentes concentrações. Nas condições em estudo, 5 g de L^{-1} de glucose apresentaram maior produtividade de biomassa (0,07 g L^{-1} h^{-1}), mas a 10 g de L^{-1}, o crescimento foi inibido. Posteriormente, a concentração de N foi otimizada usando o meio de crescimento autotrófico previamente otimizado. Neste contexto, foram testadas diferentes concentrações de N (1, 5 e 10 mmol L^{-1}). As produtividades volumétricas mais elevadas foram obtidas a 5 e 10 mmol L^{-1} (0,023 ± 0,002 e 0,025 ± 0,001 g L^{-1} h^{-1}, respetivamente).

Após a otimização das concentrações de C e N, um crescimento fed-batch num fermentador de bancada de 7 L indicou que o crescimento heterotrófico pode ser usado para a produção de inóculo. Nessa experiência, obtiveram-se aproximadamente 13,1 g L^{-1} de peso seco

em biomassa, no entanto, uma maior otimização em fed-batch permitirá uma melhoria significativa das produtividades finais da biomassa.

PALAVRAS-CHAVE

Biomassa biológica; *Chlorella vulgaris*; Autotrófica; Heterotrófico; Composição bioquímica

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

List of General Nomenclature

A – Absorbance

ANOVA – Analysis of variance

CGF - *Chlorella* growth factor

DW – Dry weight

EU – European Union

FAME – Fatty acid methyl esters

FAO - Food and Agriculture Organization

GW – Green Wall

HMF - 5-hydroxymethylfurfural

IFOAM - International Federation of Organic Agriculture Movements

LOD - Limit of Detection

LOQ - Limit of Quantification

m – Mass

MS – Mineral substrate

Mw – Molecular weight

OD – Optical density

OS – Organic substrate

OP – Organic production

PID – Proportional integral derivative

PBR - Photobioreactor

PUFA - Polyunsaturated fatty acid

rpm – Rotations per minute (r/min)

R^2 - Correlation Coefficient

P-value - Significance Value

μ - Specific growth rate (h^{-1} or d^{-1})

SD – Standard Deviation

t - Time

T - Temperature

λ – Wavelength

1. CONTEXTUALIZATION

Microalgae are unicellular microorganisms with enormous interest for different industrial applications, including the fixation of carbon dioxide (CO_2). These microorganisms present several benefits when compared to traditional terrestrial crops: i) they can reduce the anthropogenic effects when associated with flue gas, using CO_2 as a source of inorganic carbon, as well as the nitrogen and phosphorus (pollutants) present in wastewaters; ii) microalgae do not require arable land for growth and do not compete with food demand (Batista et al., 2013; Clarens et al., 2009); iii) as unicellular microorganisms, they display significantly higher photosynthetic efficiencies and consequently higher productivities than traditional crops.

In addition, the commercial value of microalgal biomass can be explored for various applications, such as: human and animal nutrition, fertilisers, biofuels, and as a source of bioactive molecules with wide application for nutraceutical, cosmeceutical, pharmaceutical and biomedical applications. Microalgae also bring positive effects for human health due to their composition rich in proteins, polyunsaturated fatty acids, antioxidants, pigments, among others. Certain strains are considered as functional/super foods that can be directly consumed or incorporated in conventional foods enhancing their nutritional content. (Spolaore et al., 2006) Since 1960, in Japan began the large-scale cultivation of *Chlorella vulgaris,* one of the few species that are currently approved as food ingredient and supplements (Chen et al., 2016).

In recent years, trends in the food industry have led to an increased demand for natural, healthy, quality assured food, even though if the production costs are higher. A growing niche market is the production of organic food products, due to the increasing awereness of customers nowadays; so why not certify organic microalgae? The market volume of organic food products has increased worldwide and more than quadrupled in the last 15 years (Willer & Lernoud, 2016). However, the concept of quality has expanded beyond the chemical and organoleptic characteristics of products, and embrace the ethical values and the environmental impact of the mode of production (FAO/WHO, 2001).

On the other side of these positive outcomes are some challenges that need to be overcome. Among them, stand out the lack of specific information on microalgae organic production, lack of support and investment of government for this type of production and research. Additionally, not all microalgae species grow adequately in the media prepared from these organic substrates. It is therefore important to optimize the nutritional requirements of nitrogen, phosphorus and

micronutrients for *Chlorella vulgaris* in the right quantities for an adequate production of organic biomass.

1.1 The company Secil / Algafarm

The work developed for this dissertation was carried out at Cimentos Maceira e Pataias (CMP), Secil group, a leading cement producer in Portugal, located in Pataias, Alcobaça. Inaugurated in 1930, Secil group works in complementary areas such as concrete, prefabricated concrete products, hydraulic lime, plasters, claddings, fibreboard, quarrying, among others. Semapa is a holding company quoted on Euro Next Lisbon, and holds the entire share capital of Secil since May 2012.

Being one of the main cement producing companies in Portugal, Secil operates internationally in Angola, Tunisia, Cape Verde, The Netherlands, Brazil and Lebanon. With an annual output of cement of 8 million tons (SECIL, 2016).

The increasing concentration of greenhouse gases in the atmosphere has been a growing concern not only for the scientific community but also for the general population. In this context, industry has been seeking solutions and developing technologies to reduce these emissions, without this leading to very high costs. Secil is a company that always strives to innovate, always having a high environmental awareness. The company started in 2007 a microalgae project, where the main goal was to capture CO_2 from the flue gas of the cement plant in Pataias.

In 2013, an industrial microalgae production plant, named Algafarm, was established close to the cement factory. This unit has been growing in the last years to become the largest closed photobioreactors (PBR) system in Europe, with a total production volume of 1300 m^3. The production unit operates in autotrophy system but the process for heterotrophy has been recently established. In addition to several tubular reactors and fermenters, they also have state of the art laboratories that monitor all production processes, since the inoculation of the cultures to the final powder product. The products depending on the microalga are commercialized with Allma brand, for human consumption, aquaculture, cosmetics and other biotechnological purposes (Silva, 2016).

Proof of quality assurance of the institute are the current certifications given by APCER: ISO 9001, OHSAS 18001, ISO 14001 and ISO 22000.

Figure 1 Aerial view of Algafarm.

1.2 Research aims

The main objective of this dissertation was to develop an effective organic culture medium for autotrophic and heterotrophic industrial production of microalgal biomass. Therefore, alternative organic sources of sugar, nitrogen, phosphorus and micronutrients were assessed to be used in the cultivation of *Chlorella vulgaris.* In addition, the maximum biomass productivity using organic substrates respecting the European Community standarts regarding the biological certification was also an important aim of this work. Finnaly, to compare the effect of inorganic and organic production methodologies, the biomass biochemical compostition (ash, lipids, fatty acids, C-H-N composition) was determined.

1.3 Thesis outline

The present dissertation is organized in 5 chapters, as follows.

In Chapter 1, it presents a contextualization of the theme, a brief description of the history, vision and work developed by Secil / Algafarm and finally the main objectives of this work and the manuscript's structure is also described.

Chapter 2 reviews the concept of microalgae and their importance for industry, as well as the cultivation of *Chlorella vulgaris* and its possible applications. The main differences in

autotrophic and heterotrophic growth and the challenges encountered in this area are also dealt with.

Chapter 3 describes the methodology used in the laboratory, the materials, main instruments, and reagents required for the techniques described and the experimental procedure. The statistical tools used for data analysis are also presented, along with the tests used for the growth curve.

In Chapter 4 it is possible to find the results obtained from the standard protocols presented in the previews chapter, ogether with a discussion of those results.

The final considerations of the work are presented in Chapter 5, as well as suggestions and recommendations for future work.

2. INTRODUCTION

2.1 Microalgae

Phycology or algology is the field of science that studies algae. By definition algae are thallophytes (plants with no roots, stems or leaves), that contain chlorophyll *a* as the main photosynthetic pigment (Canter-Lund, H., & Lund, 1995). They are primary producers of the marine ecosystem's food chain, producing organic material and oxygen from sunlight (source of energy), CO_2 (carbon source) and water (Lee, 2008). Their chloroplasts contain prokaryotic DNA and can have four types of chlorophyll, *a*, *b*, *c* (*c*1 and *c*2) and *d* (Figure 2). For photosynthesis to occur, the photosynthetic pigments must absorb the energy of a photon of a given wavelength and then use that energy to start the photochemical phase of photosynthesis.

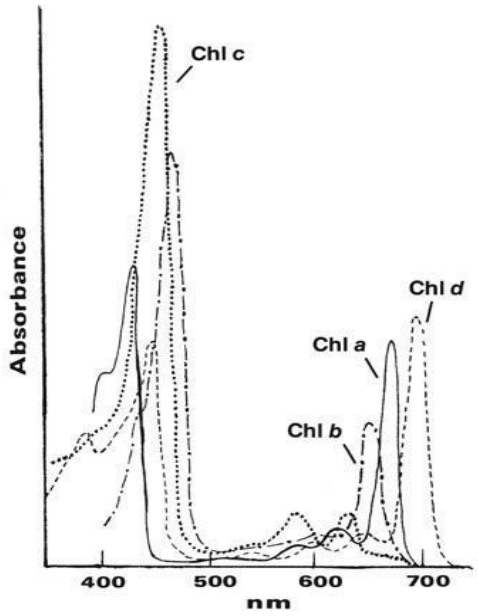

Figure 2 The absorption spectra of chlorophylls a, b, c, and d. (Wilcox & Graham, 2000)

Algae and microalgae can be found in salt, fresh or brackish water and are able to inhabit surface of snow, rocks, deserts, on tree trunks with associations of lichens, and in more extreme environments like hot springs (Lee, 2008). The term microalgae refer to microscopic eukaryotic algae (1-500 μm) and aerobic photosynthetic bacteria (cyanobacteria). Most of the phytoplankton are microalgae that serves as food for zooplankton (copepods, brine shrimp and rotifers), crustaceans and molluscs. There are approximately 40,000 species (Barsanti L, 2006) that can

be distinguished in phytoplankton (living free in the water column) and benthic communities (microalgae that grow fixed on any surface, such as rocks, skeletons of dead coral, animals, wrecks etc.).

2.1.1 Taxonomy

There is a wide diversity of microalgae and it is difficult to find a definite division between authors, but they differ essentially in the main reserve substance and coloration. According to Lee (2008), they can be organized in the following major groups (Figure 3):

Group 1 - Prokaryotes.

Cyanobacteria or blue-green algae are the only prokaryotic algae more related to bacteria, (with no organized nucleus 2 μm) than with eukaryotic algae (20 μm). These photosynthetic microorganisms to produce elemental oxygen in the primitive atmosphere, using water as an electron donor. They have thylakoids, two photosystems (PSI and PSII) and various pigments (Canter-Lund & Lund, 1995). Some species of this group have the capacity to produce toxins (cyanotoxins), namely microcystins. They are common constituents of phytoplankton, such as, *Arthrospira platensis (Spirulina).*

Group 2 Eukaryotic algae with chloroplasts surrounded only by the two membranes of the chloroplast envelope.

Green algae (*Chlorophyta*) are mainly composed of freshwater genus (90%), such as *Chlorella* and *Scenedesmus,* while only about 10% are considered marine species, such as *Tetraselmis*. They differ from the remaining eukaryotic microalgae since they store starch in chloroplasts where as the others use the cytoplasm insted.

Red algae (Rhodophyta division) are among the oldest groups of eukaryotic algae capable of living in waters with pH below 5 and in deeper saline waters. These aflagellated cells have as their main reserve substance florid starch and photosynthetic pigment, phycobiline. Agar-agar is obtained from these algae (Lee, 2008).

Group 3 Eukaryotic algae with chloroplasts surrounded by one membrane of chloroplast endoplasmic reticulum.

Euglenophyta are green in color and grow in freshwater habitats, such as puddles, lakes and rivers, particularly contaminated by decaying organic matter. Euglenoids are a large group of flagellated (e.g. genus Euglena), where the reserve substance is paramylon. Chloroplasts contain chlorophyll *a* and *c* and other pigments, which are probably derived from the capture of green algae (Lee, 2008).

Group 4 - Eukaryotic algae with chloroplasts surrounded by two membranes of chloroplast endoplasmic reticulum.

The phylum of Heterokontophyta, includes the following classes: *Chrysophyceae* (golden-brown algae), *Xanthophyceae* (yellow-green algae) and *Phaeophyceae* (brown algae). The brown color results from the dominance of the fucoxanthin over chlorophylls *a*, *c1* and *c2*. Their storage product is usually chrysolaminarin. Brown algae have a higher commercial interest as a source of alginato and iodine.

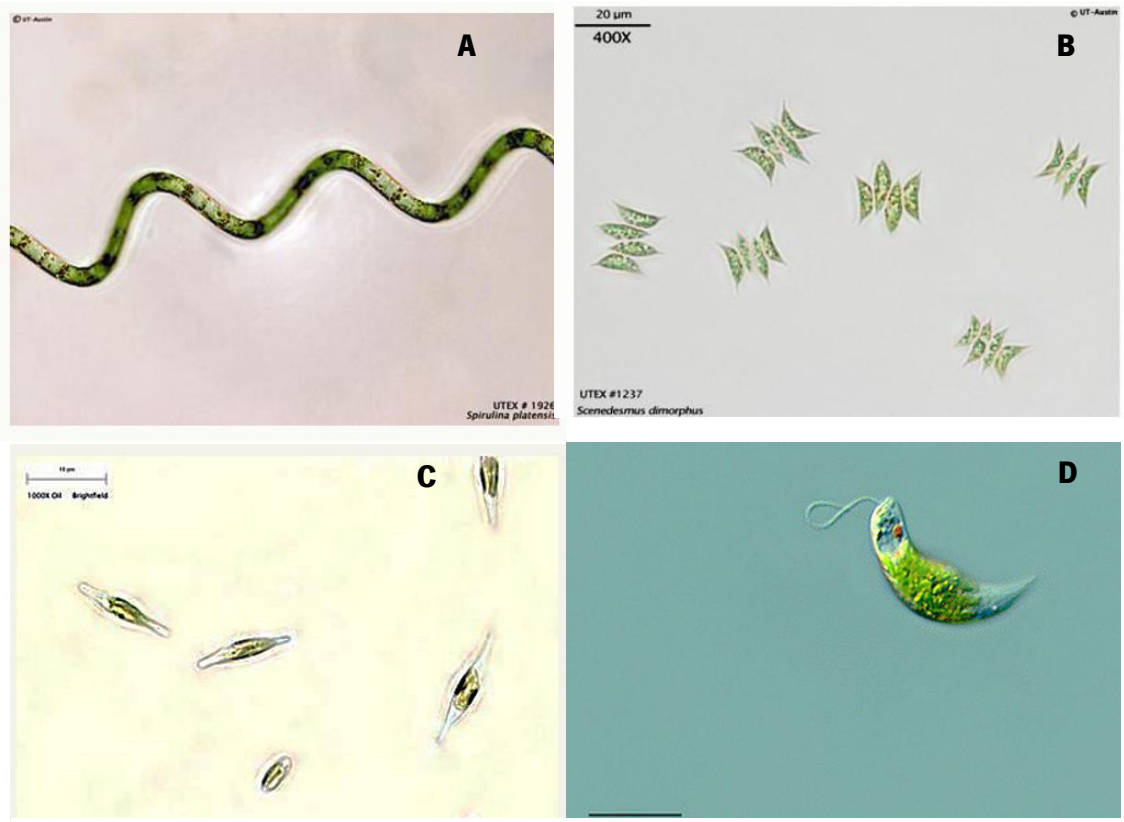

Figure 3 Microscopic image: from UTEX collection A) *Spirulina platensis*, B) *Scenedesmus dimorphus*, C) *Phaeodactylum tricornutum* (from www.fotolog.com/proyectoagua) and D) *Euglena viridis.*

2.2 Microalgae aplications

This heterogeneous group of microorganisms are used in several applications such as bioremediation, feed (aquaculture and livestock), human nutrition and biofuels (Olaizola, 2003). In addition, microalgae can offer several important high-value bioactive compounds, including dyes (from pigments), biopolymers, biofertilizers, bioinsecticides, among others.

Autotrophic microalgae can capture CO_2 releasing oxygen and contributing to minimizing the greenhouse effect. Thus, the flue gas from industrial chimneys can be used to supply the microalgae cultures directly. Moreover, they can also be used for the bioremediation of urban and industrial wastewater, removing or transforming existing pollutants (Spolaore, *et al.*, 2006).

Nowadays, there are few microalgae approved for human consumption, *Spirulina* and *Chlorella* currently dominate that market, and are sold in over 20 countries worldwide (Kent et al., 2015). These microalgae have high quality proteins (including essential amino acids), carbohydrates (polysaccharides such as, starch, glucose and cellulose), lipids (PUFAs, omega-3 and omega-6), as well as essential vitamins, chlorophyll and β-carotene pigments, functioning as antioxidants and anti-inflammatories. They are available in different forms, such as tablets, capsules and liquids and can be incorporated into pastas, dairy products, snack foods, candy bars, gums and beverages. Recently, in 2014, a company in Spain (Marino, 2015) got the approval of their marine microalgae (*Tetraselmis chuii*) for human consumption.

Several microalgae can be used in both terrestrial (*e.g.* poultry) and aquatic animal feed. In fact, the main market of microalgae biomass in the last decades was the aquaculture sector. Microalgae are the base of the food chain, they are mainly used to feed the zooplankton essential for an adequeate nutrition of fish larvae (Guedes & Malcata, 2012). Aquaculture is a growing industrial sector that allows to maintain the regular consumption of fish and, at the same time, it avoids the excessive capture of wild specimens. The most frequently used genus for these purposes are *Chlorella, Tetraselmis, Nannochloropsis, Spirulina* and *Isochrysis* (Gouveia et al., 2008).

In the last decade, the interest in microalgal biofuels triggered the field of microalgae biotechnology. Microalgae are currently considered a suitable feedstock to produce different biofuels, namely, biodiesel, bioethanol, bio-oil and biogas. (Zeng, Danquah, *et al.*, 2011) Nevertheless, although a large effort is being carried out by the scientific community, the current production costs of microalgal biomass restrain the development and commercialization of microalgal-based biofuels.

Some microalgae produce high value products such as, pigments (*e.g.* β-carotene, astaxanthin), vitamins, polyunsaturated fatty acids (e.g. eicosapentaenoic and docasahexaenoic acids), β-glucans and phycobiliproteins, which have a wide application for nutraceutical, cosmeceutical, biomedical and pharmaceutical applications (Kent et al., 2015). Recent reports revealed the several biological activities of certain microalgal strains, such as, antioxidant, anti-inflammatory, anti-cancer and neuroprotective properties (Custódio et al., 2012; Pereira et al., 2012).

It is noteworthy that abiotic factors (light intensity, temperature, pH, nutrients concentration and CO_2) are known to be key for the growth of microrganisms, affecting directly their chemical composition and consequently their final applications (Figure 4) (Reitan I., *et. al.*, 1997). For example, a target biochemical property (*e.g.* lipids) can be enhanced by exposing cultures to specific environmental conditions. (Liang, *et al.*, 2009)

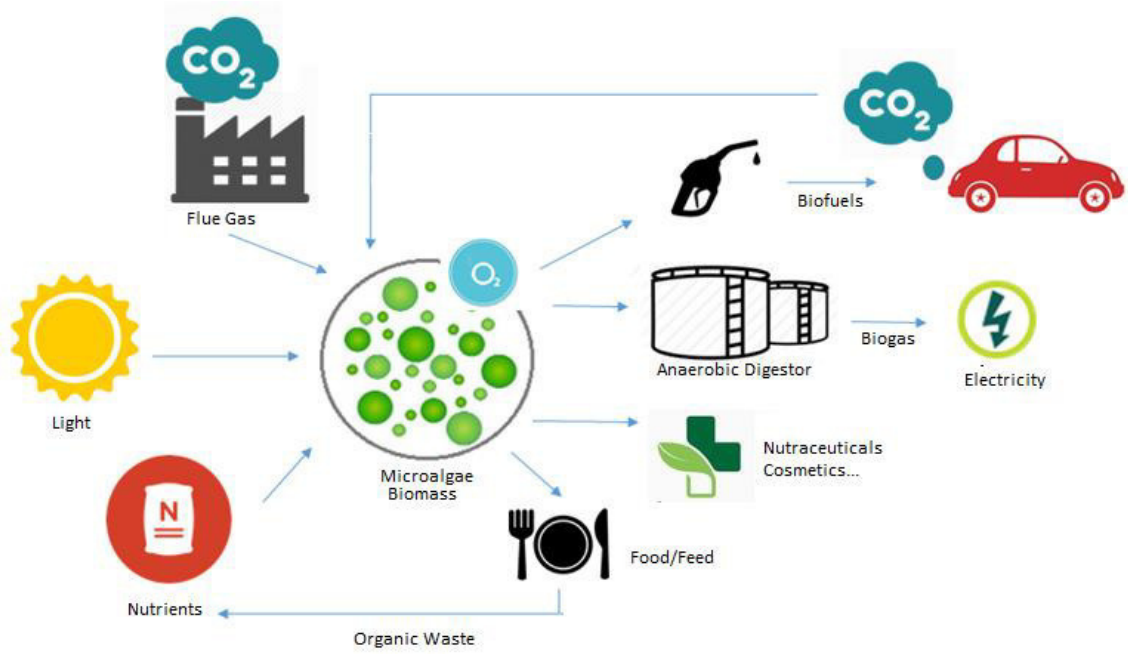

Figure 4 Main inptus and outputs in microalgae cultivation.

2.3 Chlorella vulgaris

The first photosynthetic microorganism to be isolated in a pure culture was the microalga *Chlorella vulgaris* (Beyerinck, 1890). The name *Chlorella* derives from the greek name *chloros* (green) and the latin diminutive suffix *ella* (small). This unicellular planktonic microorganism has a spherical shape with 2-5 μm length and is best adapted to grow in fresh water. Cells are non-flagellated and cultures can be grown in autotrophic, mixotrophic and heterotrophic conditions. *Chlorella* strains are known to present a high protein content (50-60 %) andother interesting components, such as as carotenoids (lutein/zeaxanthin). However, low lipid productivity and low fatty acid contents are commonly detected. The green coloration is due to the predominance of chlorophyll *a* and *b* in relation to carotenoids and xanthophylls (Liang et al., 2009; Prokop et al., 2015).

Table 1 Taxonomic classification of *Chlorella vulgaris*

Phylum	Chlorophyta
Class	Trebouxiophyceae
Order	Chlorellales
Family	Chlorellaceae
Genus	Chlorella
Specie	*Chlorella vulgaris*

Japan was the first country to introduce *Chlorella* sp. (Figure 5) in the market as a commodity food supplement with the name "*Chlorella* growth factor" (CGF) (Chen et al., 2016). CGF is a water-soluble extract composed of a variety of substances including essential amino acids, peptides, proteins, vitamins (also vitamin A precursor), and nucleic acids. Some studies indicate that the consumption of *Chlorella* can pose some heatlh benefits that can strengthen the immunity system and reduce blood pressure. For pregnant women may be useful as a resource of natural folate and iron and reduces the risk of associated anemia (Kent et al., 2015; Tang & Suter, 2011).

Nowadays, *Chlorella* is an important genus for microalgal industry due to to the easy cultivation and his specific growth rates, which allow high cellular concentrations in exterior cultures, with an estimated total production around 2000 ton $year^{-1}$ (Batista, et al., 2013).

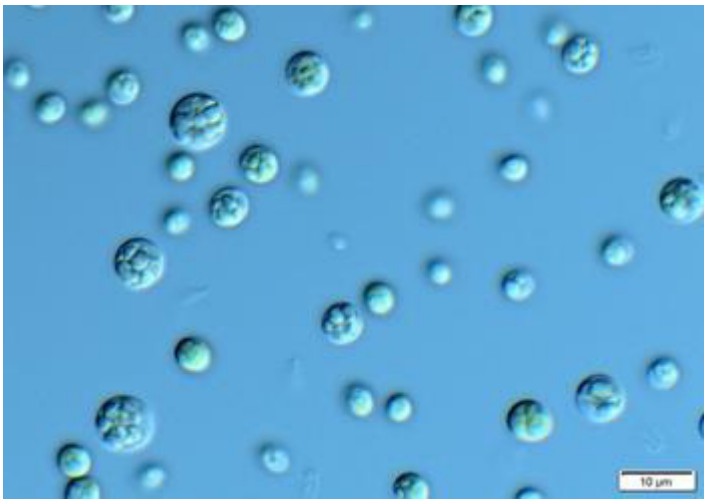

Figure 5 Microscopic view of *Chlorella vulgaris* in heterotrophic growth.

2.4 Nutritional needs

The key for successful microalgae growth is the selection of an adequate culture medium able to supply the adequate amounts of C (organic or inorganic), N, P, Fe and micronutrients that ensure the effective growth of cultures. The culture medium always tries to mimic as much as possible the natural environment from which the strains are derived, but this varies from strain to strain, trophic route and the purpose of cultivation (maintenance, optimal conditions, stress conditions).

Macronutrients (N and P) are the elements supplied in higher concentrations. Nitrogen is important for protein synthesis and can be supplied in the form of ammonium/ammonia, nitrate, aminoacids, or urea. P is used in every reaction in cells that require ATP (adenosine triphosphate) and in nucleic acids synthesis (Zeng & Lu, 2011).

The micronutrients also play an essential role (e.g. B, Co, Cu, Mn, Mg, Zn), providing all the trace metals needed for growth. In addition, for autotrophic growth, Fe is essential for the photosynthesis process, ensuring also N fixation (Kong et al., 2014).

Photoautotrophic growth is the most commonly used trophic route by microalgae and can provide several advantages. For example, they can be produced using natural energy sources (CO_2

and sun light as carbon and energy sources, respectively). However, lower cellular concentrations and long cultivation periods are observed in this light dependent process (Liang et al., 2009).

The most common syntetic media for freshwater microalgae are Bold's Basal Medium (Bischoff and Bold 1963) and BG-11 medium, while F/2 in 1975 and Walne in 1970 are mostly used in marine cultures. The synthetic media currently available are sometimes variations of each other, occurring at the level of nutrient concentrations to be added. The media may further have the addition of vitamins (Rawat et al., 2012). At Algafarm, the medium chosen is F/2, a medium which has undergone some adaptations and that is used in growing in and outdoors.

Although most microalgae grow exclusively by photosynthesis, some species are also heterotrophic and use extracellular organic carbon when a light source is not available. Sugars (glucose) and other organic compounds (glycerol, and acetate) are the main C sources, however, the costs are usually higher when compared with all other nutrients (Liang et al., 2009). This aerobic process presents an easier path for scaling-up process, with better quality inoculum. Biomass and lipid productivities are significantly higher than those obtained under autotrophic growth, reaching final dry weights between 20 and 100 g L^{-1} (Liang et al., 2009). In the other hand, the risk of contamination is higher.

Mixotrophic cultivation uses simultaneously inorganic (for example CO_2) and organic compounds as carbon source. Althoug it is not suitable for all strains the productivities obtained can be higher than heterotrophic process (Bhatnagar et al., 2011). Mixotrophic can join autotrophic and heterotrophic advantages but can not overcome the cost of the process, either the contamination risk.

2.5 Organic production

The production costs in microalgal industry lead to the development of a range of novel attractive healthy foods, prepared from microalgae biomass. An innovative product that has to cover unmet needs, bringing nutritional advantages and environmental aspects that appear to be the most in influencing factors for consumers when purchasing organic food over conventional products (Samman et al., 2008). Therefore, the use of microalgae for organic niche market is growing and is a priority area in algal biotechnology to balance the cost microalgae production system, with environmental and health-related beneficial effects (Batista et al., 2013).

For consumers, food is not only determinant for health condition but also for quality of life in a cultural, social and psychological holistic perspective. The quality concept has expanded beyond the chemical and organoleptic characteristics of the products, encompassing a broader concept - integrated value of the product – encompassing the environmental impact of the mode of production on the efficiency of energy, pollution and animal welfare. More and more consumers of organic products are a conscious and informed elite, who are prepared to pay more for the products because of the credibility of their origin from their labels (FAO, 2017).

The organic market in Europe has increased by 7.6 percent in 2014 (Figure 6). Some countries in Central Eastern Europe have reached high shares of organic agricultural lands, even though there are still large discrepancies among European countries where consumers have less purchasing power.

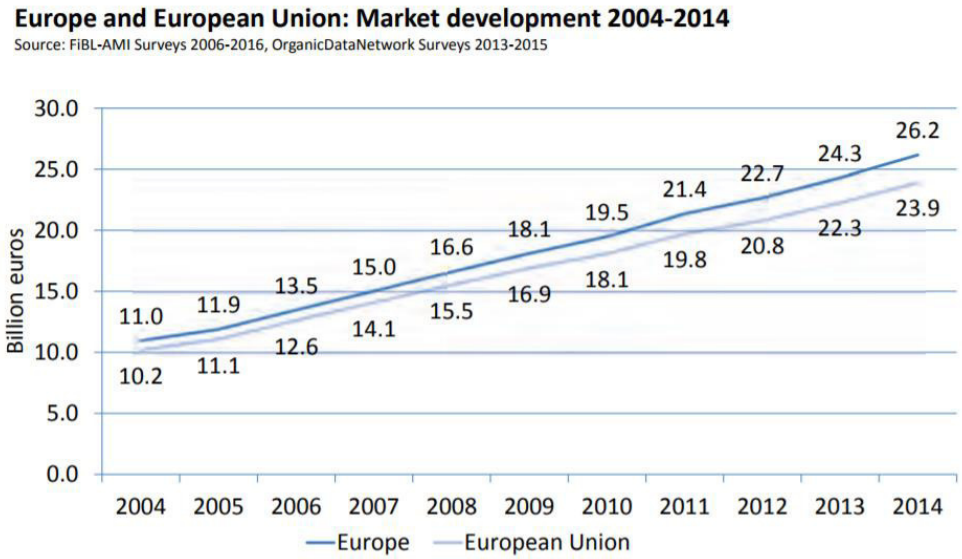

Figure 6 Growth of organic retail sales in Europe and the European Union, 2004-2014 Source: FiBL-AMI Surveys/IFOAM.

The countries that lead the world market for organic agricultural products are the United States (27.1 billion Euros), Germany (7.9 billion Euros) and France (4.8 billion Euros). In 2014, the leading country worldwide, with the highest per capita consumption was Switzerland (221 Euros), followed by Luxembourg (164 Euros) (Willer & Lernoud, 2016). In the U.S., the volume sales of products with an organic claim on the package grew 13.1 % in 52 weeks (Nielsen Global Survey, 2016). Global market trends and prospects for organic products indicate that the market is growing faster than production, and domestic supply cannot meet the current demand. The

number of public and private standards tends to increase by obstructing global trade in organic products.

Organic production systems are based on specific and precise standards of production that aim at achieving optimal agroecosystems which are socially, ecologically and economically sustainable (FAO/WHO, 2001).

The organic production method therefore plays a double role, on one hand, supplies a specific market which responds to consumers' demand for organic products and, on the other hand, supplies public goods which contribute to the protection of the environment and animal welfare as well as to rural development.

The only international organization for of the organic world started in 1972, IFOAM - International Federation of Organic Agriculture Movements, currently headquartered in Germany. In Portugal, the Portuguese Organic Agriculture Association (Agrobio) was founded in 1985.

According to IFOAM, organic farming is based on the following principles: i) Principle of **health**: Organic farming must maintain and improve soil quality, as well as the health of plants, animals, humans and the planet as one and indivisible. Ii) Principle of **ecology**. The natural cycles of ecosystems must be respected. Iii) Principle of **fairness**. Must be based on fair relations with the environment and life opportunities. iv) Principle of **care**. Must be managed in a precautionary way and responsible manner to protect the environment, health and well-being of present and future generations.

In 1991, the European Community published the first Regulation laying down the rules governing the organic production of plant products (EC No 2092/91) and animal products (Reg. No 1804/99). These regulations were repealed by Council Regulation (EC) Regulation No. 834/2007 of 28 June (CEE, 2007).

Reg. (EC) No 834/2007 is about organic production and labelling. Reg. (EC) No 889/2008 complements with laying down detailed rules for the implementation of previous regulation, concerning biological production, labelling and control.

The European logo (Figure 7) allows the identification of organic products by the consumers.

Below the current symbol there is usually a code, as follows: PT-BIO-000 where "PT" is the ISO code for the country where the controls take place, "BIO" is a term link with the organic production such as "ORG" "BIO" or "ECO" and "000" is the reference number of the control authorities composed in 1 to 3 digits. (European Commission.)

Figure 7 In left: EU organic logo, usually named "Euro-leaf". In right: the old design amending on 24th March of 2010.

The rules have the objective of avoiding any risks of contamination or mixing by substances or products not in compliance with the organic production rules. Articles 5, 9 and 12 of EC Reg. 834/2007 (CEE, 2007), include: no genetically modified plants or other genetically modified organisms (GMOs) may be used. Before the storage of organic products, suitable cleaning (recorded by operators) should be carried out. Only cleaning and disinfection products authorized for use in organic production may be used in plant production. Organic products and production areas shall be clearly identifiable all the times. For that, storage areas of biological raw materials and products should be physically separated from non-biological products.

Regarding the production of microalgae, the legislation is still unclear. Article 6°-A, Chapter 1A, Reg. (EC) No 889/2008 says that only applies *mutatis mutandis* to the production of all multicellular marine or phytoplankton algae and microalgae intended to feed aquaculture.

Certification is important for the consumer, who feels safe when buying organic products and for the producers because it allows to demonstrate that the product meets the necessary requirements, putting it in competitive advantage.

Private inspection and certification bodies such as SATIVA are approved in Portugal by the Ministry of Agriculture, Fisheries and Forestry. The conversion period shall commence when the operator notifies the (Ministry of Agriculture - General Directorate of Agriculture and Rural Development). This period is generally around 2 years for the annual crops and during the same the products cannot be labelled as organic.

2.5.1 Organic cultivation medium

The organic culture media takes a preponderant role in the growth performance of microalgal cultures. Reg. (EC) No 889/2008, show a list of raw materials that can be used for

organic farming. The culture medium can be composed of feedstocks from the following origin (Table 2): manure, compound of mushroom crops, earthworm droppings, sawdust, tree bark, wood ash, natural phosphate, crude potassium salts, vinasse, calcium carbonate, magnesium sulfate, elemental sulfur, solo powder, clays, among others. These and other natural resources are known to be rich in many nutrients.

Table 2 The most common organic source of nutrients (Rodrigues, 2009)

Nutrient	Sources
Nitrogen	Silkworm cocoons, hazelnut shells, vegetables, dry fish, feathers, coffee, dry blood
Potassium	Pea pods, banana and lemon peels, hardwood ashes (oak), maize, pig manure, granite powder
Phosphorus	Phosphate rocks, residues of sugar processing
Calcium	Eggshells, limestone
Magnesium, sulfur and iron	Leaves ground, luzerna, organic matter from algae

From these substrates and other by-products of local food companies it is possible to combine and formulate a medium that is in full conformity with the conditions and regulations for the organic farming sector established by the EU. Meaning that for processed products that means that at least 95 % of the agricultural ingredients are organic.

The biggest disadvantages for the application of organic medium is the higher costs of organic substrates and their preparation, the higher turbidity which can affect photosynthesis and the variation in composition due to the manufacturing techniques or the raw materials.

2.6 Heterotrophic organic production

Since heterotrophic cultivation allows high inoculum concentration in a shorter time, it can reaveal to be a suitable source for the organic production of the “seed” (inoculum). In this contex, molasses offers great promise as inexpensive organic substrate.

Molasses is a by-product of the sugar cane refining process, that has been extensively as a feed stock in fermentation proceses. The main sugars present (45-50%) are glucose, fructose and sucrose, containing also 2-5 % polysaccharides (dextrin, pentosans, polyuronic acids) (Najafpour & Shan, 2003).

Enzymes are involved in the complete conversion of sugars (Figure 8). A primary use is in the production of sweetener and reduction of dough viscosity to improve the texture and appearance of bread. MAXINVERT® contains ß-fructofuranosidase (invertase) extracted from a selected strain of *Saccharomyces cerevisiae* (baker's yeast). Invertase catalyses the hydrolysis reaction of sucrose (saccharose) in fructose and glucose. Enzymatic hydrolysis is a more controlled and sustainable process leading to a purer end-product. At the optimum pH and temperature conditions of pH 4.5 and above 60 °C, sugar inversion takes place within 8 to 16 hours depending on the syrup concentration the enzyme dosage and the targeted conversion rate (typically from 66% for "medium sugar inversion" to 97% for "total sugar inversion") (Information Sheet, *n.d.*).

This enzyme is Kosher approved, Halal approved and suitable for organic production.

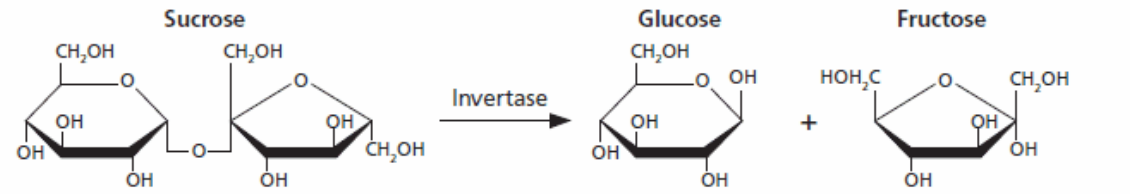

Figure 8 Enzimatic reaction on the hydrolysis of a disaccharide (sucrose).

2.7 Large scale biomass production

There are two types of autotrophic large-scale production systems, open ponds and closed reactors.

There are four main types of **open systems**: shallow big ponds, water tanks, circular ponds and the raceway ponds (Figure 9D). They all provide a direct contact between the culture and atmosphere, characterizing themselves for being a large system, but with low biomass concentration, low control capacity, high contamination risk and they are only valid for extreme conditions tolerant strains (Fonseca et al., 2015). Nevertheless, the capital and operational costs are significantly lower than those of closed systems.

On the other hand, **closed PBR,** are usually smaller systems such as tubular PBR where the culture is separated from atmosphere. They can appear in several forms, such as, horizontal or vertical tubes (Figure 9B and 9C), plastic bags, green wall panels (GWP, Figure A), among others. PBR display higher biomass productivities, higher control capacity of culture conditions and

a lower contamination risk. These systems are valid for most strains and are very indicated to produce monocultures. The biggest disadvantaged is the higher capital and operational costs (Fonseca et al., 2015).

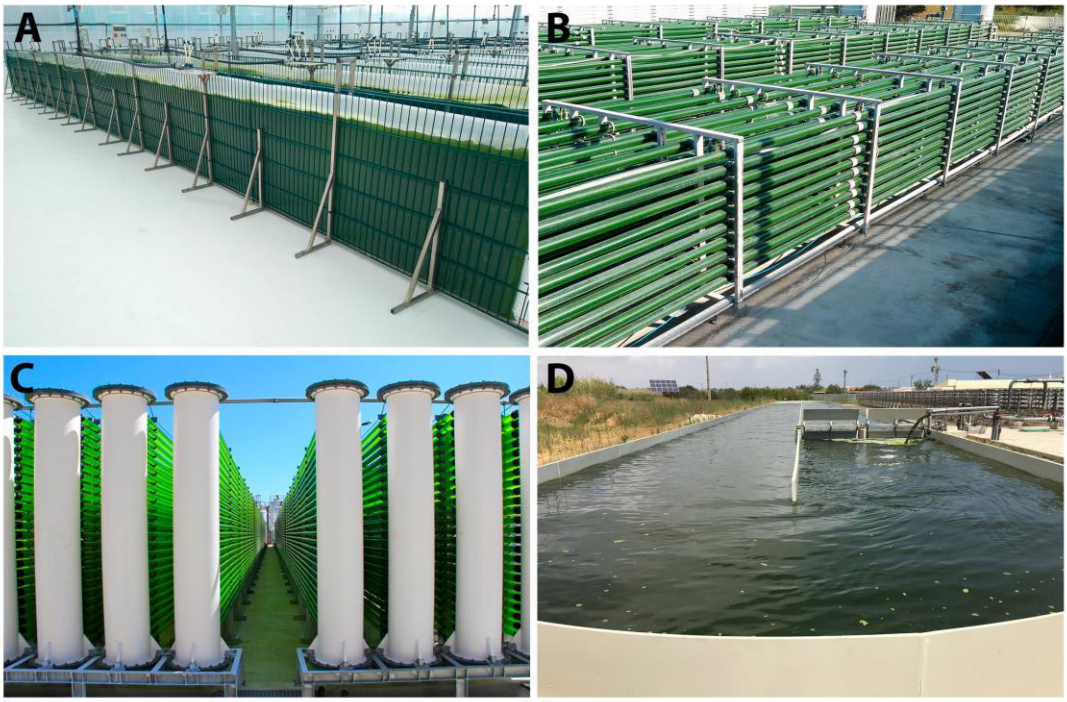

Figure 9 Examples of microalgal closed cultivation systems are as follows: (A) green-wall panels; (B and C) tubular PBRs from ALGAFARM); (D) pilot raceway pond, Necton, S.A, Portugal.

Industrial production of heterotrophic microalgal biomass, is commonly achieved in large-scale fermenters (Figure 10). Most microalgae ventures that commercialize DHA oil obtained from microalgal biomass, operate using this industrial system, as recently demonstrated by Solazyme Bunge Renewable (SB) Oils. This company holds four 650 m^3 fermenters that enable a mass production of microalgal biomass and oil for different industrial applications. Recently, Algafarm as implemented this approach at their facilities, using heterotrophic biomass produced in 200 L and 5000 L fermenters as inoculum to seed the autotrophic production reactors, to overcome the long and costly autototrophic scale-up.

Figure 10 Industrial fermenters with a working volume of 200 L (A) and 5000 L (B). The facilities are located at Algafarm (Pataias, Alcobaça).

MATERIALS AND METHODS

The experimental procedure was subdivided in four distinct phases:

Phase 1. Optimization of organic culture medium in laboratory.

Phase 2. Pilot-scale production of organic biomass.

Phase 3. Heterothropic growth on organic culture medium.

Phase 4. Biochemical profile of microalgae cultures produced with inorganic and organic media.

The following experiments described in the present work were performed at the facilities of CMP (Secil Group, Portugal), between the 15th of March and 15th of August 2017. Phase 4 was performed at the Marbiotech research group of the Centre of Marine Sciences (CCMAR, University of Algarve) between the 24th and 28th of July 2017.

2.8 Microalgae strain and inoculum preparation

Chlorella vulgaris was obtained from UTEX culture collection. To have reproducible results, all autotrophic experiments were carried out using a heterotrophic culture as inoculum.

The master cell bank of *Chlorella* was stored in a liquid nitrogen container. Each cryovial was transferred to 50 mL Erlenmeyers supplemented with an in-house heterotrophic culture medium, at 200 rpm and 28 °C. The later inoculum was transferred to a 500 mL Erlenmeyer under the same conditions, which was later used to inoculate a 7 L bench-top fermenter (New Brunswick BioFlo®/CelliGen®115; Eppendorf AG, Hamburg, Germany). The 7 L fermenter was maintained at 28 °C and the pH was kept at 6.5 by the addition of ammonia (24% m/m). The fermenter was operated in fed-batch mode as a solution of glucose was fed to the culture whenever the sugar was depleted in the medium. Cultures obtained from this fermenter were used to inoculate all the autotrophic reactors used in the present work.

2.9 Solubility tests of organic substrates

The organic substrates (OS) used during this study had the following origin:

OS.1 Substrate from manioc and other vegetables;

OS.2 Composting of raw materials such as:

- Horticultural products;
- Selected foods' leftovers in restaurants, canteens and similar establishments;
- Forest waste (trunks, branches, foliage) and green waste (flowers, grass, pruning, etc.); Meat / fish, fruit / vegetables, dairy and baking products which are no longer fit for human consumption;

OS.3 Composting 2;

OS.4 Organic matter from animal waste;

OS.5 Organic matter from animal waste 2.

To assess the solubilization of granular OS, the granulates were dissolved at increased concentrations (1, 5, 50, 100 and 300 g L^{-1}) in 1 L tap water and stirred for at least 30 minutes. To assess the effect of temperature in the solubilization, the experiments were conducted at ambient temperature and 60 °C (in duplicate). The non-soluble fibres and residues were filtered using a 1 mm mesh and then, the filtrate was centrifuged (Hermle z400k) at 4500 G for 15 min. All samples were stored at -20 °C for the determination of ammonia, nitrates and micronutrients.

2.10 Autotrophic Growth

2.10.1 Screening for macronutrients in organic substrates

Initially, 5 substrates were selected from 9 solutions of different raw materials (Annex B). Using the five most promising organic substrates (organic substrate 1, 2, 3, 4 and 5) an assay was carried out in triplicate to evaluate the growth of cultures. Cultivation was performed in 1.5 L airlift reactors (Figure 11), with gentle and constant aeration (filtered at 0.2 µm). Experiments were carried out at ambient temperature (~25 °C) under constant light exposure (24 hours, ~100 µmol photons m^{-2} s^{-1}). The fertilizers were diluted to reach a final concentration of 2 mmol L^{-1} of nitrates and ammonia in the final growth medium (defined form the solubilization assays), and later

autoclaved for 40 minutes at 121°C. The control medium (inorganic) used in all experiments was the implemented autotrophic medium in Algafarm, an Guillard's F2 medium adaptedtion with local water (2 mmol L^{-1} nitrates). The initial concentration of *C. vulgaris* was about 0.17 g L^{-1}. The concentration of nitrates and ammonia in the growth medium and growth performance of cultures were evaluated every two days.

Figure 11 Cultures in1.5 L photobioreactors.

2.10.2 Optimization of micronutrients solution

To optimize the solution of oligoelements a mineral substrate (MS) was used. Cultivation was performed in 1.5 L airlift reactors in triplicate to assess the growth of cultures. The MS were diluted to reach a final concentration of 0.1, 1, 10 and 100 g L^{-1}.

To these conditions a solution was added only with potassium nitrate (2 mmol L^{-1}) and potassium monophosphate (0.18 mmol L^{-1}) and 16.5 µl of Fe chelated with EDDHSA. To the control medium in addition to the previous solution was added 60 µl of a solution of trace elements used in Algafarm. The pH, growth performance of cultures was evaluated every two days.

2.10.3 Pilot-scale production of organic biomass

The organic medium for this assay had also to be prepared on a large scale. Thus, OS.1 (25 kg), OS.2 (30 kg) and OM (4 kg) were placed in 200 L of water at room temperature. The prepared medium was put inside a big net bag in solubilization for 2 h. Inside the solubilization tank (Figure 12) there was an air-lift type aeration that facilitated mass transfer. Subsequently, with the aid of a pump, the medium was transferred to another tank passing through a 2mm filter; and finally, 50, 20, 10, 1 and 0.2 μm.

Figure 12 On the right the solubilization tank with the net inside. On the left: the filter of 20 μm used.

Once the concentration of inorganic medium was optimized the pilot-scale production was performed in 125 L GWP in triplicate. The organic medium was supplied to complete 2 mmol L^{-1} of N and 1.4 ml of Fe chelated solution at each GWP. The inoculation was made with seed cultures from fermentation growth. The air-lift system was cleaned and remained in the disinfection tanks with sodium hypochlorite (200 mg L^{-1}) throughout the time that was not used (Figure 13). The water was chemically treated, and the culture media was sterilized (autoclaving) or filtered. The compressed air introduced in the airlift system was filtered at 0.2 μm.

The desired pH in the culture was kept by the introduction of CO_2 in the culture system. The temperature was controlled with a sprinkler-like irrigation system that cools the system and preventing it from reaching values above 30 °C.

Figure 13 "Green Wall Panel" photobioreactor used for scale-up.

Finally, a preliminary growth assay was performed in an industrial 10 m^3 tubular PBR. In this context, a 1000 L GWP was used to inoculate the industrial PBR, followed by the addition of 91 L of the organic culture previously optimized (Figure 14).

Figure 14 Addition of biological medium to an industrial PBR (10000 L)

2.11 Heterotrophic growth

The fermentation assays were conducted in 250 mL Erlenmeyer flask with 100 mL of culture on an orbital shaker, set at 200 rpm and 28 °C. The inoculum came from the 7 L bench-top fermenter previously mentioned. Two samples were taken per day during the test, where the growth

performance, pH and sugar (glucose and sucrose) concentration were monitored two times per day.

2.11.1 Optimization of carbon source

The molasses stock solution had 131 g L^{-1} of glucose after enzymatic inversion. To determine the best carbon source (molasse) concentration to be used, 1, 5 and 10 g L^{-1} of glucose was tested in triplicate from the molasses solution, which had been previously submitted to the enzymatic hydrolysis. Another condition tested was the use of unconverted molasses with 5 g L^{-1}. The inorganic control had 10 g L^{-1} of glucose. Erlenmeyers were supplemented with an in-house inorganic heterotrophic culture medium as N source.

In addition, a fed-batch treatment started at 1 g L^{-1} glucose, was also maintained with 1 g L^{-1} of inverted molasse, which was added daily (Annex E).

2.11.2 Inversion of carbon source

Phosphate buffer solutions were prepared by mixing 0.2 mol L^{-1} of dipotassium phosphate, K_2HPO_4 with 0.2 mol L^{-1} of KH_2PO_4 in deionized water. Using the pHmeter (VWR pH 110) the pH of the solution was checked and adjusted with HCl (1 M) or NaOH (1 mmol L^{-1}) solution (if needed), until a pH of 6.5 was reached.

A solution of molasse was prepared with a 1:2 dilution from the source in the phosphate buffer with a final volume of 100 mL.

The conditions for the sugar inversion using enzyme MAXINVERT® 200000 MG, was 45 ° C and pH 5.8. The medium was continuously shaken and the enzyme suspension was added. Samples (1 mL) were taken every 30 min until the 8 h of reaction were complete. The experiment took place up to the maximum possible conversion with a maximum time of 17 h. Following sugar inversion, invertase activity in each sample collected was stopped by heat treatment (soaking in a bath for 5 min at 90-95°C).

2.11.3 Optimization of nitrogen source

Using the optimized autotrophic organic medium, the following N concentrations were tested, 1, 5 and 10 mmol L^{-1} in duplicate. The carbon source used was glucose 50 % (m/m) at 10 g L^{-1}.

Control had the standard inorganic in-house culture medium as source of N and glucose at 10 g L^{-1}.

2.11.4 Fed-batch using the optimized fermentation medium

The fermenter used was sterilized for 45 minutes (121 °C) and equipped with different sensors (dissolved oxygen, pH, temperature and foam level), temperature control, stirring rotor, baffles and a mass flow controller for aeration. The fermenter was connected to a control unit through a PID (proportional integral derivative controller) system, where these variables were monitored (Table3). Each variable had its own control cycle, with a set-point established by the operator.

Table 3 Reference value

Parameter	Set point	Unit
pH	6.5	
DO_2	50	%
Temperature	30	° C
Carbon feed standby	120	s
Carbon feed pulse	78	s
Speed variation	20	rpm
Speed time range	30	min
Carbon Flow rate	145	g min^{-1}

Peristaltic pumps, were used for the addition of acid or base in the control of the pH and to control the speed of agitation and/or aeration rate to control the dissolved oxygen concentration.

After all the elements were assembled in the fermenter (Figure 15), the pH probe was calibrated using standard solutions. The pH was corrected using a solution of sodium hydroxide (2 M).

Then, the peristaltic pumps and DO_2 sensor were calibrated. A sample was then taken before and after inoculation. Samples were collected twice a day for determination of growth parameters.

The optimized autotrophic medium (source of N, with final concentration of 7 mmol L^{-1}) was used in this assay, which was further supplemented with 550 μL of Fe. Hydrolysed molasse

was used as C source, with a final concentration of 3 g L^{-1} of glucose. This solution and organic culture medium were supplied to the fermenter, whenever the glucose reached values near zero (189 mL).

Figure 15 Bench 7L fermenter at the time of inoculation.

2.12 Growth assessment

Optical density (*OD*) was determined at a wavelength of 600 nm, used as an indirect measurement of the growth of the culture. Dry weight (*DW*) was determined by filtering a known volume of culture in glass microfibre filters (0.7 µm, VWR) that were later dried using Kern DBS moisture analyser. Calibration curves were established relating the *OD* and *DW* as shown below in equation 1:

$$OD_{600} = 4{,}9161 \times DW\,(g\,L^{-1}) - 0{,}1209; \quad (n = 29 \quad R^2 = 0{,}9254) \qquad (1)$$

The calibration curves generated are presented in Annex A.

The specific growth rate (μ) was measured using equation 2:

(2)

$$\mu\ (day^{-1}) = \frac{\ln(N_2/N_1)}{t_2 - t_1}$$

where N_2 and N_1 refers to biomass concentration and t_2 and t_1 to time (days).

The volumetric productivity in biomass (P_{max}) during the culture period was calculated through eq. 3, and is expressed in g L^{-1} day^{-1}, where X_f (g L^{-1}) is the *DW* of the biomass at the end of the exponential growth phase, while X_i (g L^{-1}) is the *DW* of the biomass in the beginning of the trial; $t_x - t_0$ represent the total time in days. From eq. 3 is possible to obtain the productivity of a specific component such as starch, lipids, proteins, $P_{component}$ as shown in eq 4, where $F_{component}$ as the mass fraction (w/w) of each component.

$$P_{max} = \frac{X_t - X_0}{t_x - t_0} \quad (3)$$

$$P_{component} = P_{max} * F_{component} \quad (4)$$

2.13 Nitrates and ammonia determination

Samples collected from the different culture systems were centrifuged for 15 min at 4500 G. Nitrates were determined according to Armstrong (1963), the collected supernatant was diluted and 300 µL of hydrochloric acid was added. Samples were measured using a spectrophotometer at 220 and 275 nm. The absorbance reading at 275 nm was subtracted two times from the reading at 220 nm.

Ammonia concentration was determined using an Ammonium-Ammonia Sera test (Sera, Heinsberg, Germany) by comparison to a calibration curve.

2.14 Determination of glucose and sucrose

For the determination of sugars, a YSI 2950D Biochemistry Analyzer was used. Briefly, samples were centrifuged in an eppendorf for 2 min in a microcentrifuge VWR (VWR MicroStar12). The supernatant was collected and filtered at 0.2 µm. Subsequently, a specific dilution, with the YSI buffer, was carried out to obtain a sugar reading between 0 and 3 g L^{-1}.

2.15 Analytical determinations

After cultivation, the microalgal biomass obtained during the growth phase was harvested by centrifugation at 4500 G for 15 min. The obtained biomass pellet was freeze-dried in a Telstar LyoAlfa 15 until a constant weight was achieved. The freeze-dried biomass was stored in a desiccator until further analysis.

2.15.1 Determination of total lipids

Total lipid content was determined following the Bligh & Dyer method (1959) with few modifications (Pereira *et al.*, 2011). Lyophilized biomass was weighed (w_i) in gass tubes, and 1 mL of chloroform, 2 mL of methanol and 0.8 mL of distilled water (1:2:0.8, v:v:v) were added to the biomass and homogenised with an IKA Ultra-Turrax disperser (IKA-Werke GmbH, Staufen, Germany) on ice for 60 s (Figure 16). Thereafter, 1 mL of chloroform was added and the mixture was again homogenised for 30 s. Finally, 1 mL of distilled water was added and homogenized for 30 s. Afterwards, the mixture was centrifuged at 2000 g for 10 min for phase separation. The organic phase (lower layer) was transferred to a clean tube with a Pasteur pipette, and later a known volume of chloroform (0.7 mL) was pipetted to a pre-weighed tube (w_1). The tubes were then placed in a dry bath at 60 °C to evaporate the chloroform.

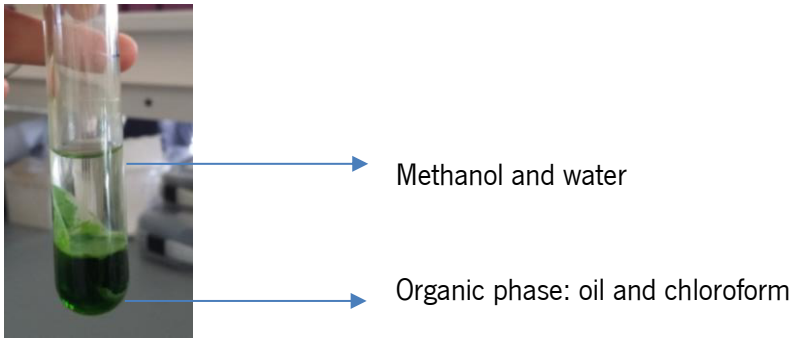

Figure 16 Biomass after centrifugation.

The resulting dried residue was weighed (w_2) in a precision balance and the percentage of lipids in each sample was calculated according to the following formula (5), where V_1 is the total volume of chloroform used (2 mL) and V_2 is the volume of chloroform evaporated (0.7 mL):

$$Total\ lipid\ (\%) = \frac{\left(\frac{(w_2 - w_1\ )x\ V_1}{V_2}\right)}{wi} \quad (5)$$

2.15.2 Elemental analysis (CHN)

Elemental analysis of C, H and N was achieved with a Vario el III (Vario EL, Elementar Analyser systeme (Figure 17), GmbH, Hanau, GermanyLiophylized biomass was weighed in specific aluminium caps (5-10 mg), according to the procedure provided by the manufacturer. Total protein was estimated by multiplying the N content by 6.25 (Nunez & Quigg, 2015).

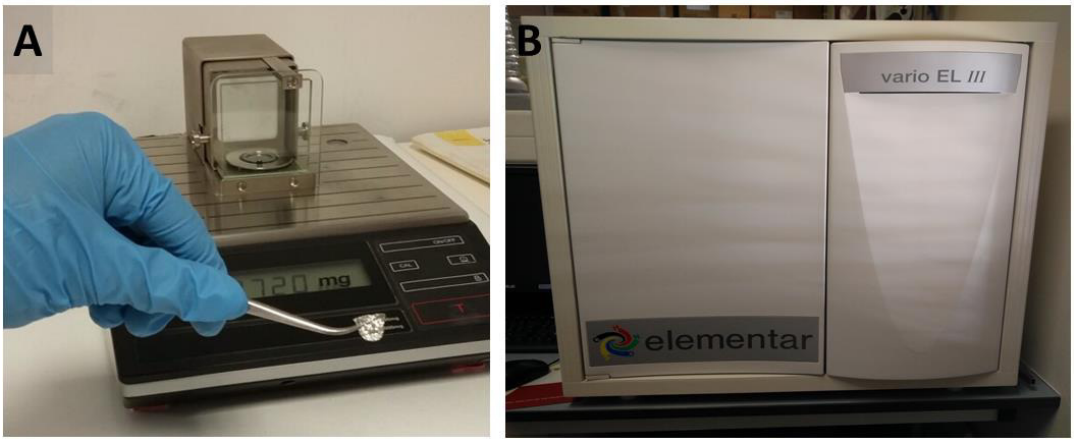

Figure 17 A: Precision balance to weigh the aluminum capsules. B: Elementar Analyser systeme.

2.15.3 Ash content

Total ash was determined by the weight difference before and after burning the produced biomass in a muffle. Biomass was weighed and placed in small ceramic cups and burned for 5 h at 550 °C using a furnace (J. P. Selecta, Sel horn R9-L).

2.15.4 Quantitative analysis of elements in each organic substrate

Determination of various elements was carried out using an Agilent Technologies 4200 microwave plasma-atomic emission spectroscopy (MP-AES) (Figure 18). The blank (nitric acid, 5%) was defined as a matrix solution, prepared with concentrated nitric acid (65%) and milli-Q water. A diluted solution of the target element (0.050 mg L^{-1}) near the inferior limit of detection by MP-AES analysis.

A calibration curve was prepared for each group of the following elements: Ca, Fe, K, Mg and Na (0-50 mg L^{-1}); Se, Sr, Zn, Cd, Sr, Ba, Cu, Ni, As, Co, Pb, Mo, Mn, Cr and Al (0-10 mg L^{-1}); V, Ag, Be, Th and Tl (0-10 mg L^{-1}); and P (0-50 mg L^{-1}). Each element was detected with a wavelength previously selected according to the manufactures procedure. If needed, organic substrates were diluted (1:10) before the instrumental analysis to fit within the calibration range.

The instrumental Limit of Detection (*LOD*) and Limit of Quantitation (*LOQ*) were determined based on sample replicates measurement. LOD and LOQ values were determined using the formulas below:

$$LOD = 3 \times s \qquad LOQ = 10 \times s \tag{6}$$

where s is the standard deviation of the replicates.

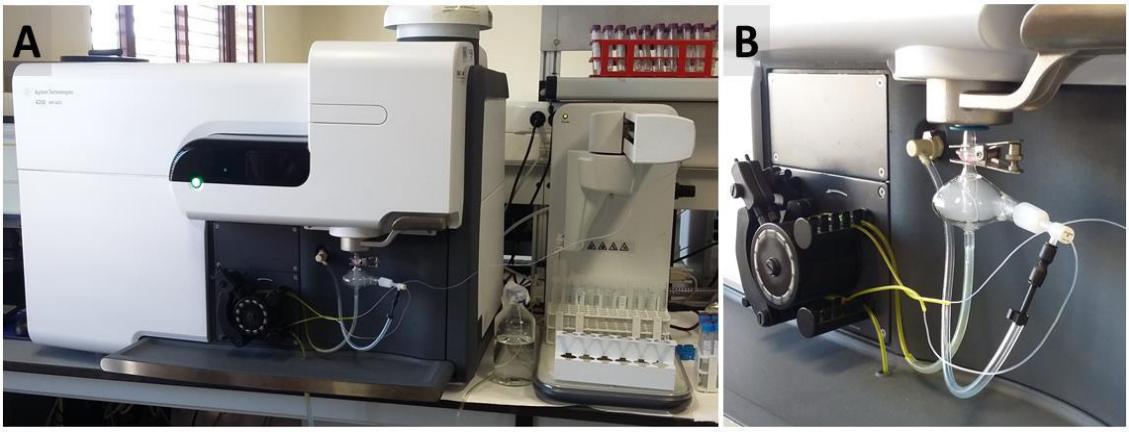

Figure 18 Agilent Technologies 4200 MP-AES.

2.16 Statistical analyses

Statistical analyses were performed using IBM SPSS 24 software. Experimental results are presented with 95 % confidence level. Shapiro-Wilk test allowed to verify if the data follow a population with normal distribution. If so, data were compared using *one-way* ANOVA, followed by a Tukey's multiple comparison tests. If not, the non-parametric Kruskall-Wallis test allows to compare populations of independent samples.

3. Results and Discussion

3.1 Organic substrates

3.1.1 Solubilization trials of organic substrates

Nitrogen is an important macronutrient constituent of biomass which can range from 7 % to more than 10 %, of biomass *DW* (Richmond, 2004). This element is commonly added to microalgal cultures in the form of nitrate or ammonia (Richmond, 2004). To assess the composition of N in four organic substrates (OS), solubility tests were performed at different dilutions (1, 5, 10, 50, 100 and 300 g L^{-1}).

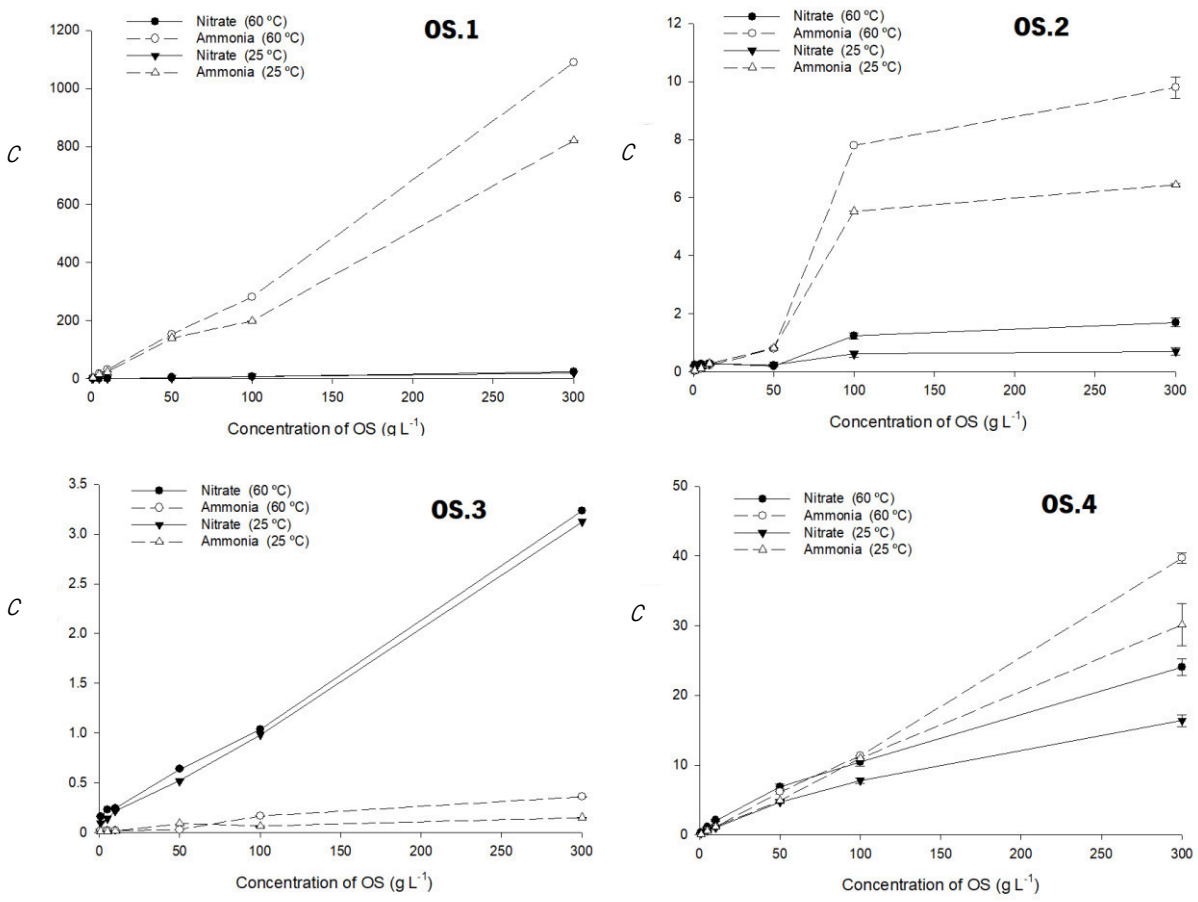

Figure 19 Solubilization at different concentrations of each product and the corresponding concentration (*C*) of nitrates (mmol L^{-1}) and ammonia (mmol L^{-1}) present.

The solubilization of N was more efficient at higher temperatures than room temperature, in most of the OS understudy (Figure 19). The only exception was OS.3, where temperature seems to display a lower influence ($p \geq 0.05$). The concentration of ammonia was higher than nitrates in almost all substrates, except in OS.3, where nitrates were present in higher concentrations. OS.1 was the medium that showed the higher concentration of N, with a total of 1113 mmol L^{-1} (at 60 °C), followed by OS.4 and OS.2, which presented significantly lower concentrations of ammonia (39.71 ± 0.71 and 9.80 ± 0.37 mmol L^{-1}) and nitrates (24.07 ± 0.42 and 1.7 ± 0.15 mmol L^{-1}). OS.3 had the lowest concentration of nitrates and ammonia, registering at 60 °C, only 0.36 ± 0.00 mmol L^{-1} and 0.15 ± 0.00 mmol L^{-1} respectively. This test was not finished in OS.5, since it formed a solution too viscous above 150 g L^{-1} that constrained the analysis. Nevertheless, the autotrophic growth assays at 100 g L^{-1} solution was prepared with 30.5 mmol L^{-1} ammonia. Due to the natural origin of OS, the concentration of N may vary among different lots.

3.1.2 Characterization of each organic culture medium

Since the OS studied are complex media, it is a determining factor to know the main elements that compose them, to correspond to the nutritional needs of microalgae. Table 4 shows the elements that can have the greatest impact on *Chlorella* growth.

Table 4 Elemental composition of OS at 300 g L^{-1}, OS.5 and MS at 100 g L^{-1}. AP correspond to the water used in this study. The control corresponds to the final concentration used in autotrophic growth

Element	Control (µM)	AP	OS.1	OS.2	OS.3	OS.4	OS.5	MS
P	180	<LOD	864.2	167.3	135.4	566.4	534.1	5.3
N:P	11.1	<LOD	1288.9	68.8	26.6	112.6	57.1	<LOD
Fe	20	15.4	101.4	59.1	34.6	66.8	34.5	13.3
K	0	114.3	15095.7	6581.5	3492.7	38320.2	4375.3	669.5
Mg	0.04	615.5	2950.1	349.8	231.0	282.1	695.0	638.3
Se	0	<LOD	10.3	5.7	2.7	16.9	3.7	<LOD
Zn	0.02	<LOD	1.0	0.8	0.3	0.7	3.2	<LOD
Cd	0	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Sr	0	23.2	4.1	1.9	0.2	1.4	0.7	27.5
Cu	0.002	<LOD	0.4	0.6	0.3	0.6	5.4	<LOD
Ni	0	0.1	1.1	0.2	0.1	0.4	0.7	0.1
Co	0	0.4	<LOD	<LOD	<LOD	<LOD	<LOD	0.3
Pb	0	0.1	2.0	0.8	0.3	2.9	0.7	0.1
Mo	0.02	0.4	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Mn	0.02	<LOD	28.6	0.9	0.5	0.1	1.2	<LOD
Cr	0	<LOD	1.4	0.1	<LOD	<LOD	<LOD	<LOD
V	0	2.0	4.5	0.3	1.6	<LOD	1.9	2.6

All OS present a wide variety of micronutrients in different concentrations. However, they do not present toxic heavy metals, such as, Hg, Pb, Ni, Cu, Zn, Cd and Cr in concentrations higher than those allowed by law (Regulation (EC) N.o 629/2008).

Phosphorus is another macronutrient key for effective growth of microalgae cultures, essentially used for energy transfer and DNA biosynthesis (Richmond, 2004). The optimum N:P ratio for freshwater algae growth is in the range of 6.8-10 (Martin, J. et al., 1985).

OS.1 registered the highest concentrations of micronutrients and a less balanced N:P ratio. In addition, this OS had the advantage of being rich in N, so it was possible to achieve about 2 mmol L^{-1} by adding 0.2 % (v/v) of a 300 g L^{-1} stock solution. The origin of this OS is vegetal, which allows the use for the production of biomass to supply the vegan market. Regarding the organoleptic characteristics of the OS understudy, OS.1 was the only that presented a favourable odour, a slight coffee aroma that does not affect the perception of the consumer in the final biomass product.

The N: P ratio is also considerably elevated in the remaining OS, mainly because of the low concentration of soluble P. The N:P (112.6) ratio determined in OS.4, showed once again the limitation of P in the medium. Only OS.3 (N:P, 16:1), presented a relation close to the optimum, however it had the lowest N content, resulting in a higher amount of solution (~32.7 %, v/v) to reach 2 mmol L^{-1} of N. During screening assay, the volume to be added to the final solution from each OS was 11.13 % v/v (OS.2), 5.93 % v/v (OS.5) and 3.2 % v/v (OS.4).

Fe is also a key element for microalgal growth, essential for the different metabolic pathways and for chlorophyll biosynthesis. Although the OS understudy did not reach ideal concentrations of Fe, the organic regulation allows the use of 2% Fe chelated with EDDHSA. Therefore, the major problem is the organic source of P, which will need further study to comply with the optimal N, C and P stoichiometry.

It is noteworthy, that the high concentration of suspended solids present in the OS after the solubilization can reduce the penetration of solar irradiance. In fact, the solids fraction present, create many difficulties in the preparation of the nutritive broth. Since these media were very dark, this factor negatively influenced spectrometric measurement and growth, so a better way to reduce turbidity of the medium was to centrifuge and/or filter all OS after solubilization. Nevertheless, even with the removal of all solids in suspension, all OS presented a medium brown colour to light brown depending on the final concentration of the OS, except OS.1 that showed a black coloration. MS does not show coloration and that can eliminate the limitation of the quantity to be added.

Therefore, it is important to concentrate as much as possible the stock solution of OS, to minimize the effect of colour in the absorption of light by the microalgae, and further reduce the process costs.

3.2 Autotrophic Growth

3.2.1 Screening of industrial organic substrates for autotrophic media

Five OS were submitted to a batch growth trial to compare the microalgal growth performance among them (Figure 20). Growth curves show that the microalgal growth with OS.1 and OS.4 were quite comparable to the control, whereas OS.3 did not promote the growth of *Chlorella*.

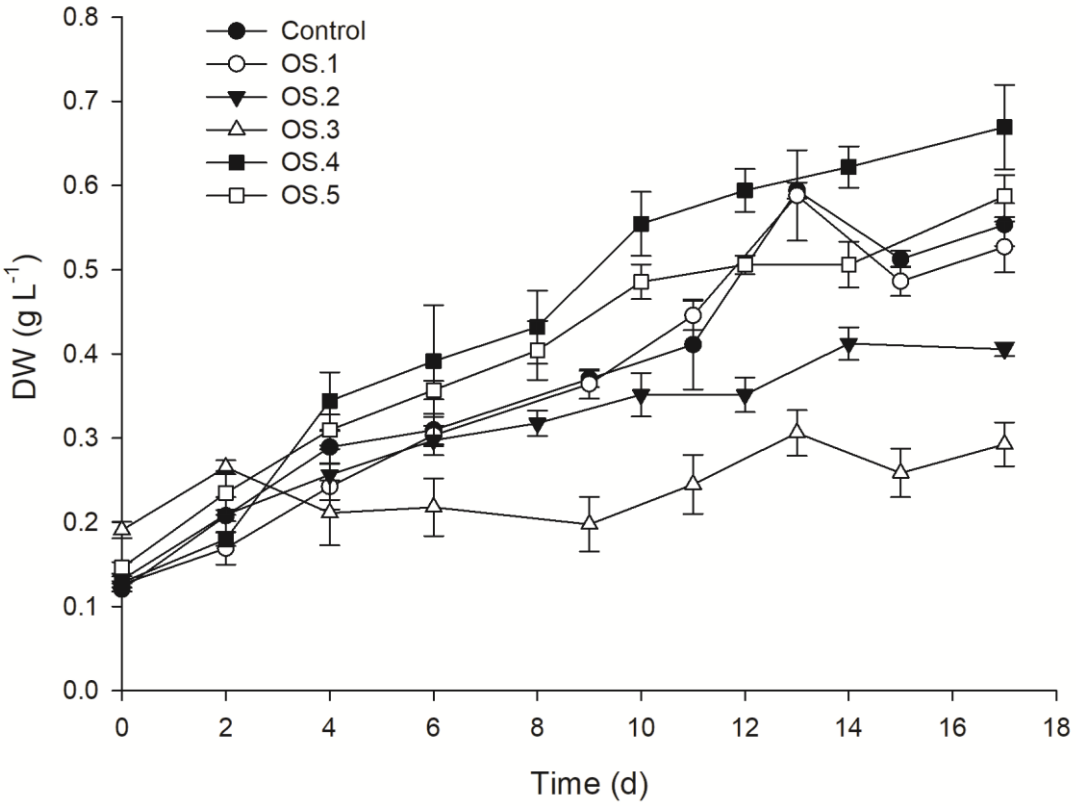

Figure 20 Screening of the growth of *Chlorella vulgaris* in 2 L reactors using different organic substrates.

The highest biomass concentration was reached by OS.4 (0.67 ± 0.05 g L^{-1}), since the medium presented a better balance of nutrients. In contrast, OS.3 that also had a promising N:P ratio, presented the lowest biomass concentration (0.31 ± 0.03 g L^{-1}), compromising the photosynthetic capacity due to the higher volume of added medium.

The specific growth rate (μ) was higher in OS.4 (0.057 ± 0.006 day^{-1}), followed by OS.5 (0.046 ± 0.003 day^{-1}) and OS.1 (0.045 ± 0.004 day^{-1}; Table 5). Higher biomass productivities were obtained according to the following order: OS.4 (0.033 ± 0.002 g L^{-1} d^{-1}), OS.1 (0.028 ± 0.002 g L^{-1} d^{-1}) and OS.5 (0.024 ± 0.001 g L^{-1} d^{-1}). There was no significant difference in biomass productivities between the control, OS.1 and OS.4 ($p \geq 0.05$), while OS.5, OS.2 and OS.3 displayed significant differences among them ($p < 0.05$).

Table 5 Biomass productivity and specific growth rate of batch cultures grown in 2 L reactors using different organic substrates. Different letters from the same colum represent significant differences ($p < 0.05$).

Treatment	Biomass productivity	Specific growth rate
	g L^{-1} d^{-1}	d^{-1}
Control	0.035 ± 0.002a	0.048 ± 0.003ab
OS.1	0.028 ± 0.002ab	0.045 ± 0.004b
OS.2	0.016 ± 0.001c	0.017 ± 0.002c
OS.3	0.005 ± 0.001d	0.000 ± 0.01d
OS.4	0.033 ± 0.002a	0.057 ± 0.006a
OS.5	0.024 ± 0.001b	0.046 ± 0.003b

Abeliovich and Azov (1976), reported that concentrations of ammonia over 2 mM and pH values over 8, might inhibit photosynthesis. Considering the initial and final nitrate and ammonia concentrations, the nutrient consumption was calculated during the culture period (Table 6).

Table 6 Inicial pH and composition of nitrate and ammonia present in diferent organic substrate and nutrient consumption from batch cultures grown in 2 L reactors

Growth condition	Inicial pH	Initial concentration		Nutrient consumption (%)	
		Nitrate (mM)	Ammonia (mM)	Nitrate	Ammonia
Control	6.79	2.31 ± 0.05	0.02 ± 0.00	89.6 ± 2.3	-
OS.1	6.69	0.2 ± 0.01	1.48 ± 0.06	14.3 ± 0.0	99.2 ± 0.2
OS.2	7.10	0.35 ± 0.11	1.51 ± 0.03	57.4 ± 16.3	99.1 ± 0.2
OS.3	6.92	1.43 ± 0.68	0.33 ± 0.01	17.1 ± 4	91 ± 0.2
OS.4	6.94	0.82 ± 0.00	1.57 ± 0.03	43.1 ± 4.1	98.8 ± 0.2
OS.5	6.62	0.48 ± 0.11	1.85 ± 0.03	12.6 ± 0.2	98.6 ± 0.6

Results show that all cultures grown using OS consumed preferentially ammonia over nitrates. In all organic treatments ammonia were almost fully consumed (except OS.3), while nitrates were consumed at much lower percentages. Perez-Garcia *et al.* (2011) reported that N is preferably assimilated in the following order: $NH_4^+ > NO_3^- > NO_2^- > CH_4N_2O$. The assimilation of nitrates requires the transport through the cell membrane and its reduction to ammonia, which demands energy, carbon and protons, resulting in the raising of the pH of the culture medium, the opposite of that is verified by the direct assimilation of ammonia.

3.2.2 Optimization of micronutrients

The growth of *Chlorella* was evaluated with different concentrations of MS (0.1, 1, 100 g L^{-1}), using the same solution and concentration of macronutrients and Fe in all cultures. There were no significant differences between MS treatments compared to the control cultures, with all cultures displaying a similar growth pattern (Figure 21).

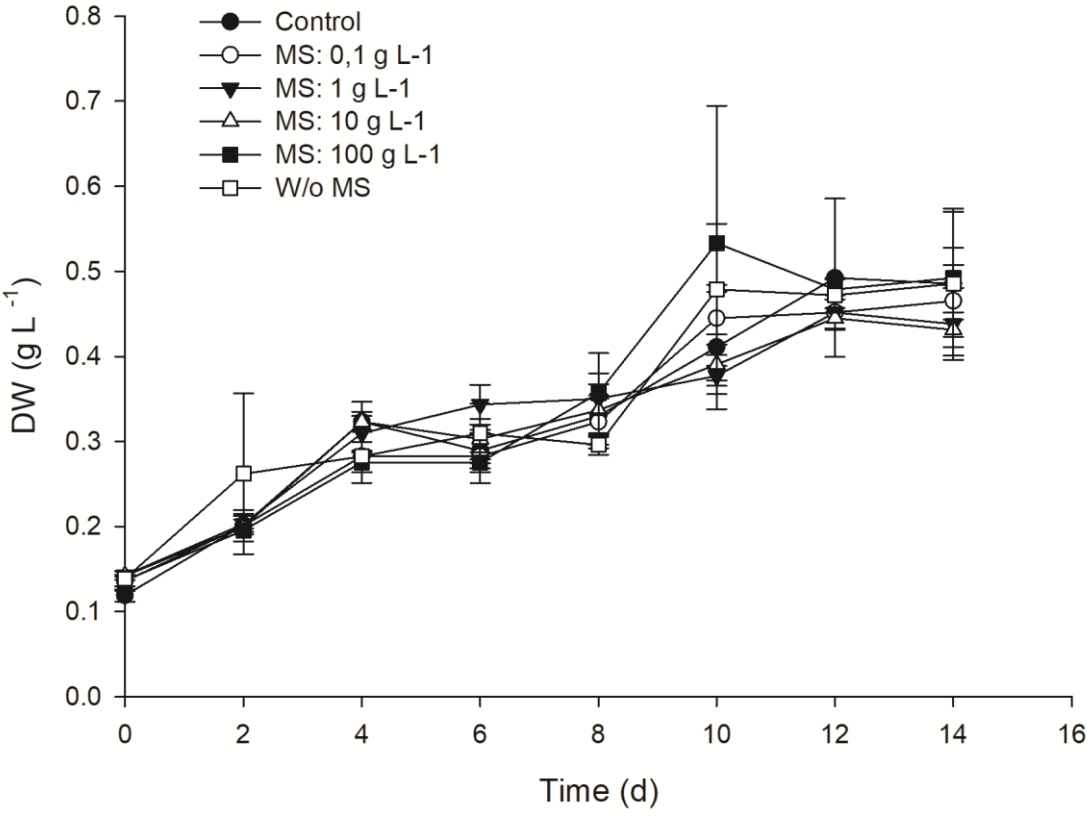

Figure 21 Batch growth of *Chlorella vulgaris* in 2 L reactors using different concentrations of MS.

Regarding the specific growth rate (0.026 ± 0.003 d^{-1}) and biomass productivities (0.031 ± 0.007 g L^{-1} d^{-1}), once again no significant differences were observed in all treatments ($p \geq 0.05$). However, the treatment with 100 g L^{-1} of MS, showed slightly higher *DW* values. A possible explanation for the lack of significant differences observed among the different concentrations of MS, might be related with the natural presence of microelements present in the water used (AP), which was probably sufficient to maintain a similar growth to the control conditions (Table 4).

3.2.3 Pilot scale and industrial production of organic *Chlorella vulgaris*

Using the data previously obtained with all OS, a composed medium for pilot-scale production was established using a mixture of OS.1, OS.4 and MS at 125, 150 and 20 g L^{-1}, respectively. After the establishment of the medium, a pilot-scale growth assay was performed in 125 L GW, using the composed organic medium and the inorganic medium commonly used in Algafarm as control. Figure 22 shows that both organic and inorganic culture media displayed the same growth pattern, without significant differences throughout the culture growth period ($p \geq 0.05$).

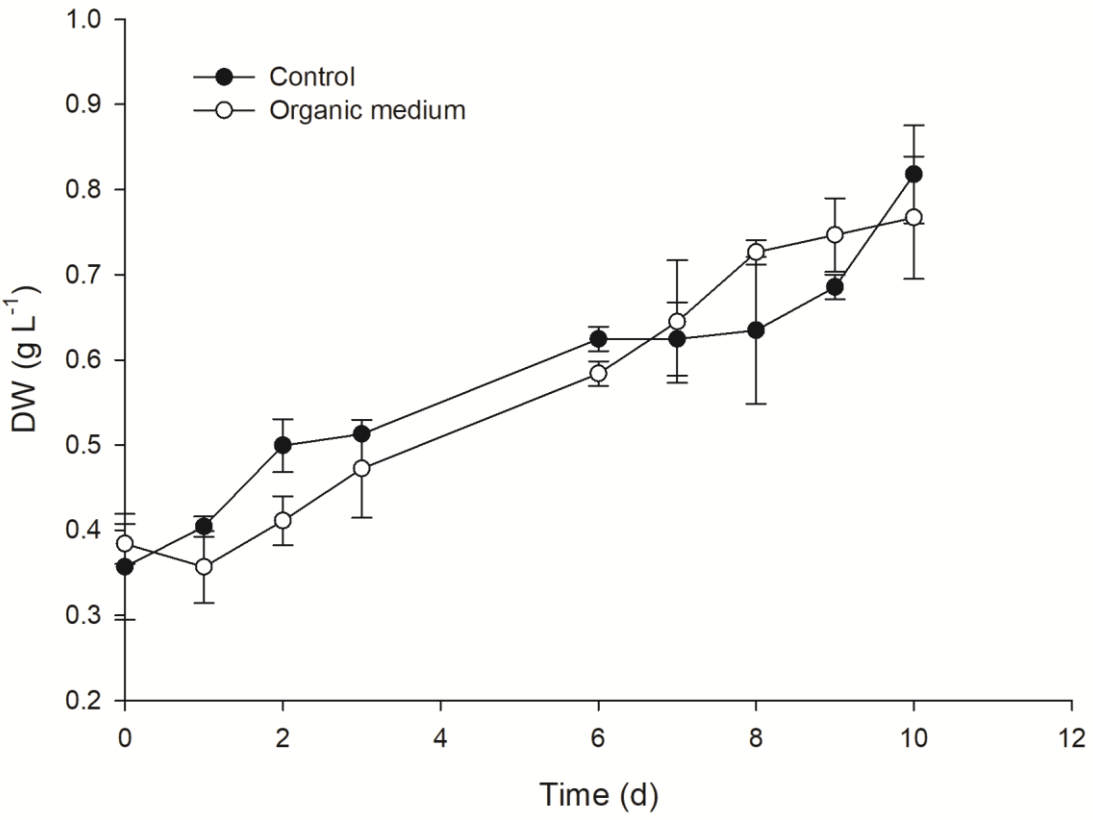

Figure 22 Growth curves of *Chlorella vulgaris* grown in 125 L reactors using the optimized medium.

In addition, Table 7 shows the volumetric productivity, which were again similar between the inorganic (control) and organic media. The growth performance and final *DW* obtained using GW were higher than those obtained in the 1.5 L assays, due to the higher sun exposure of the culture system that potentiate the growth of microalgae cultures. The pH generally ranged from 7.79 to 8.72 with tendency to rise over the culture period, but this was corrected with pulses controlled from CO_2.

Table 7 Comparison between volumetric productivity in organic and inorganic treatments and pH variations

GWP	Volumetric productivity g L^{-1} d^{-1}	pH range
Inorganic	0.06 ± 0.01	7.79 – 8.08
Organic	0.08 ± 0.02	7.87 – 8.72

Finally, a preliminary growth test was also performed using the established organic medium in an industrial 10 000 L tubular PBR (Fig 14). The aim of this assay was to assess the performance in a significantly higher culture volume, however, due to operational reasons, this test was performed using a single replicate. The growth of cultures was followed for 13 days and the results are shown in Figure 23. Overall, the developed culture medium was efficient and supported the growth of *Chlorella* cultures throughout the culture period, reaching a final *DW* of 1.36 g L^{-1}. Therefore, the results obtained in pilot and industrial scale production systems, show that the developed organic culture medium as the potential to be used for large-scale production of organic biomass.

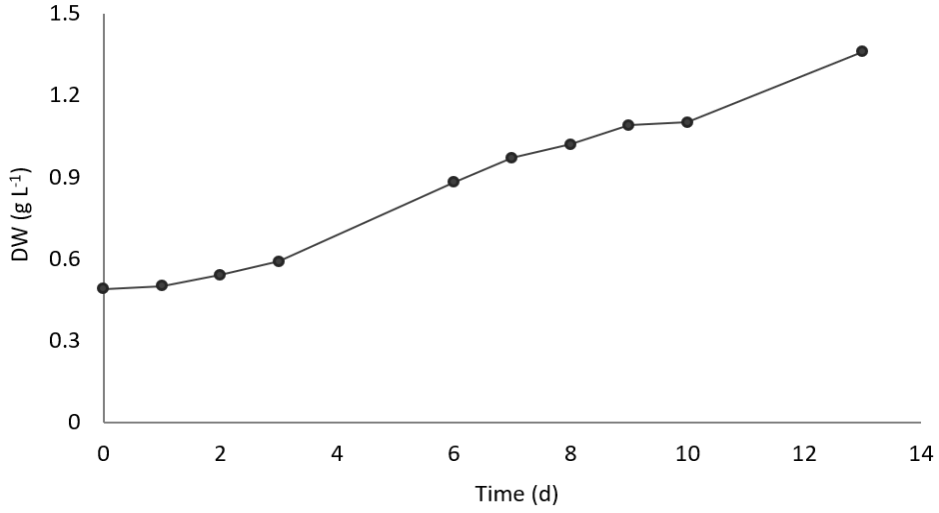

Figura 23 Growth curves of *Chlorella vulgaris* grown in 10 000 L reactors using the optimized medium.

3.2.4 Biochemical composition of biomass

The biochemical composition of biomass produced in pilot-scale GWP (Table 8) shown significant differences between both organic and inorganic culture media. Organically produced biomass presented a significantly higher content of proteins compared to the control inorganic medium, 48.1 and 36.5 % of *DW*, respectively. This increase of proteins in the organic biomass was achieved at the expenses of carbohydrates (36.2% of *DW*), resulting in a significantly higher content of carbohydrates in the biomass produced with the inorganic medium (45.1% of *DW*).

In some cases, P deficiency in the environment generates a physiological stress situation, which may affect the content and the lipid profile of the cell (Li *et al.*, 2014). Nevertheless, the lipid content of both biomasses was similar, presenting a mean lipid content of ~10.3 % of *DW*.

Regarding ashes, a lower content was detected in the biomass produced with organic medium when compared to the inorganic counterpart, 5.5 and 8.0% of *DW*, respectively.

Table 8 Elemental composition of *Chlorella* biomass produced in pilot-sclae GW with organic and inorganic culture media

Culture medium	Proteins (%)	Lipids (%)	Carbohydrates (%)	Ashes (%)
Organic	48.10 ± 1.13	10.27 ± 0.25	36.15 ± 1.22	5.49 ± 0.09
Inorganic	36.54 ± 0.34	10.33 ± 0.02	45.13 ± 0.24	8.00 ± 0.09

3.3 Heterotrophic growth

3.3.1 Enzymatic inversion of molasse

The inversion of sucrose was carried out in a concentrated solution of molasses (50 % m/m) with different concentrations of invertase (Table 9).

The purpose of this experiment was to convert complex sugars (e.g. sucrose) increasing the content of monosaccharides (e.g. glucose and fructose) to make molasses a substrate of excellence, as carbon source for fermentation. In addition, the optimum time and concentration of enzymatic inversion was studied to assess the most cost-effective procedure to be implemented in large-scale production facilities.

Table 9 Enzymatic hydrolysis of molasses (200 g L-1 sucrose) at 45 °C and pH = 4.6 with various concentration of invertase

Enzyme concentration (mg mL^{-1})	Time (h)	% conversion
1.5	0.5	100
0.15	1.5	100
0.015	17	74
0.0015	20.5	30

Although the activation temperature was not the optimum of the enzyme, at 65 °C (Najafpour & Shan, 2003), it was possible to achieve conversions of 100% at 1.5 and 0.15 mg mL^{-1} of enzyme. The fastest enzymatic inversion occurred at a concentration of 1.5 mg mL^{-1}. At the lowest concentration tested (0.0015 mg mL^{-1}), the conversion dropped 70 %. The result displaying the time to convert sucrose is inversely proportional to the amount of invertase required.

In a larger scale the conversion becomes too expensive at 65 °C, so setting the temperature at 45 °C with the adequate concentration of enzyme (0.015 mg mL^{-1}) enables the conversion of 80 % of substrate, even if the inversion is done over-night.

3.3.2 Optimization of carbon source

Following the optimization of sucrose inversion in molasse, the carbon source was optimized by using different concentrations of molasses and unhydrolyzed molasses (Figure 23).

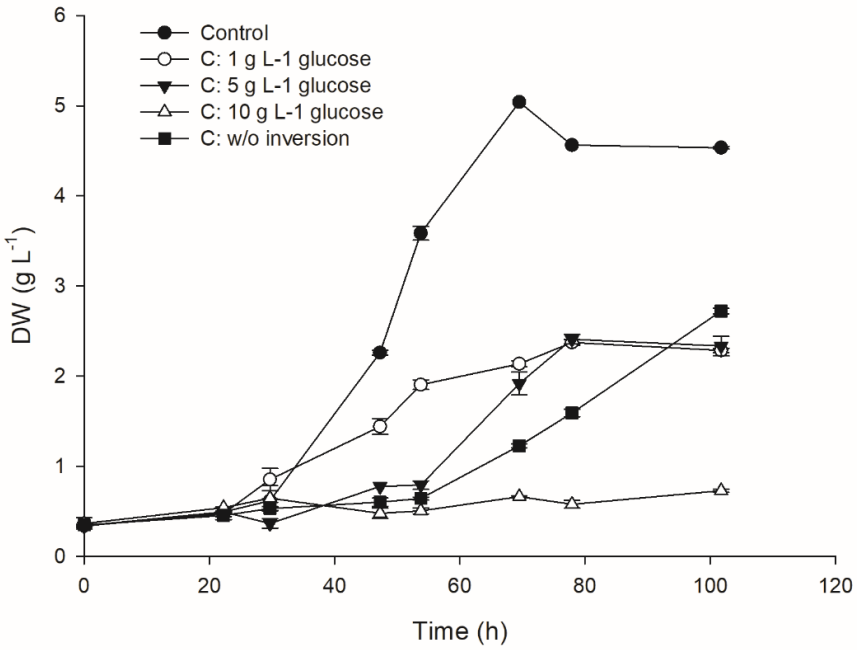

Figure 23 Growth curves of *Chlorella vulgaris* cultivated in different molasses concentrations.

The assay lasted approximately 4 days. The control and 1 g L^{-1} of glucose showed a similar lag phase of 30 and 22 hours, respectively. The 5 g L^{-1} of glucose and unconverted molasse treatments took longer to enter the exponential phase (54 hours). A higher lag phase represents a longer adaptation period of microalgae to the environment conditions (Monod, 1949). The treatment with 10 g L^{-1} of inverted molasse inhibited the growth of *Chlorella vulgaris*.

The highest *DW* values were 2.72 ± 0.04 g L^{-1}, 2.40 ± 0.05 g L^{-1} and 2.37 ± 0.03 g L^{-1}, with the initial glucose concentration of 4.9 g L^{-1} (not inverted molasse), 1 g L^{-1} and for 5 g L^{-1} of glucose, respectively. Whereas the highest glucose concentration had the lowest biomass concentrations (0.73 ± 0.02 g L^{-1}).

Glucose consumption (Table 10) was practically total in all treatments, except at 10 g L^{-1}. *Chlorella vulgaris* possess an inducible active hexose transport system responsible for uptake of glucose from the medium (Haass & Tanner, 1974).

Table 10 Consumption of glucose and sucrose by *C. vulgaris* cultivated with different molasse conditions

Growth condition	Carbohydrate consumption (%)		HMF
	Glucose	Sucrose	mg/L
	g L^{-1}	g L^{-1}	
Control	100 ± 0.00	-	2.3
C source 1 g L^{-1}	100 ± 0.00	-	5.8
5 g L^{-1}	100 ± 0.001	-	9.1
10 g L^{-1}	15 ± 0.8	-	17
C w/o conversion	99.7 ± 0.4	0	9.8

Thus, the fact that microalgae does not consume the total sugar at 10 g L^{-1} could be mainly due to the inhibition by combinations of factors, as presence of another type of sugars or high concentrations of 5-hydroxymethylfurfural (HMF). HMF is an organic compound, mainly formed as an intermediate in the Maillard reaction during heat treatment as sterilization. This chemical when absorbed by the cells can be converted into a series of pathways that rise the formation of others compounds, some of them reported to be mutagenic, such as acrylamide and sulfoxymethylfurfural (SMF) (Capuano & Fogliano, 2011).

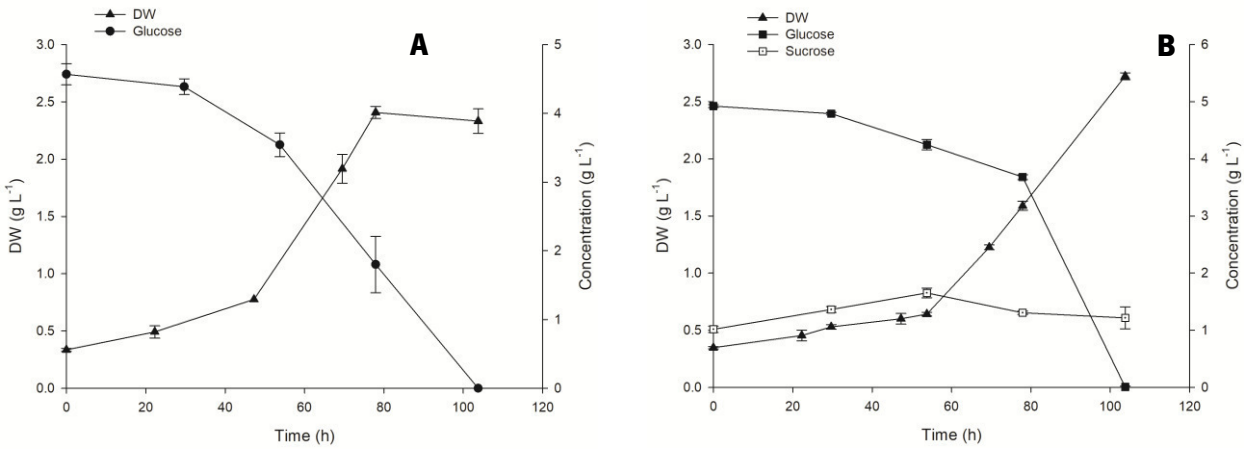

Figure 24 Comparison between growth curve and sugar consumption with hydrolysed (a) and non-hydrolysed (b) molasses with initial concentration of 5 g L of glucose.

The results presented above (Figure 24) demonstrate that cultures growing in molasses without the presence of sucrose begin the exponential phase when the medium reaches 3.5 ± 0.2 g L^{-1} glucose. The presence of sucrose does not seem to be inhibit growth in the treatment where molasses were not inverted growth, and glucose consumption was also more pronounced with 3.7

g L^{-1} glucose (t = 78 h). Since the high cost of the enzyme may represent a constraint on a large scale (Capuano & Fogliano, 2011), and the results show a final dry weight similar between the two treatments, the use of non-hydrolysed molasses should be considered as a solution for cost reduction.

The sucrose uptake does not occur, indicating that microalgae cannot assimilate sucrose. According to Komor *et al.*, (1985) disaccharides connected to carbon 1 or carbon 4 (sucrose and maltose) are not transported in *Chlorella*.

3.3.3 Optimization of nitrogen source

After the optimization of the organic carbon source, the autotrophic organic culture medium was tested as a source of N for heterotrophic growth, using different concentrations (1, 5 and 10 mmol L^{-1}; Figure 25). The highest concentration tested did not inhibit growth, registering the highest biomass concentration at the end of the trial (3.19 ± 0.08 g L^{-1}). Although, the 1 mmol L^{-1} treatment was supplemented with fresh culture medium every 2 days (1 mmol L^{-1} of N, see Annex F), lower biomass concentrations (1.67 ± 0.06 g L^{-1}) were obtained. These results show the significant influence of higher supplementation of nutrients in heterotrophic cultures due to the higher biomass concentrations obtained.

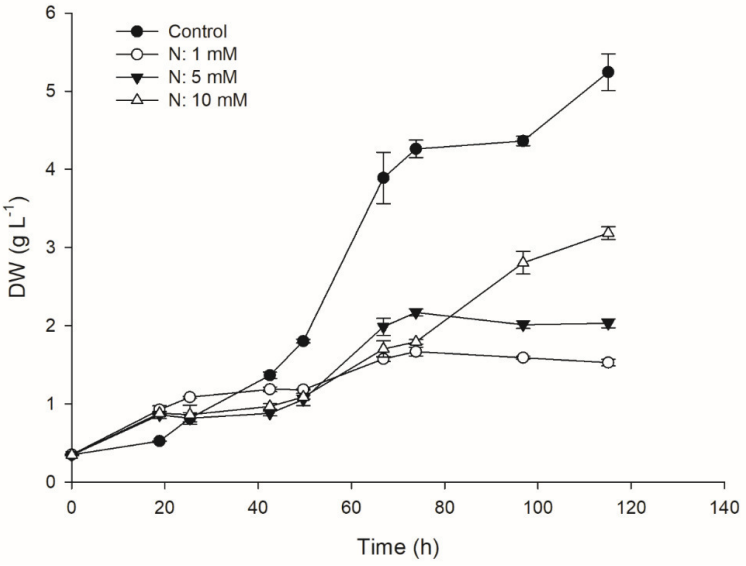

Figure 25 Growth curves of *Chlorella vulgaris* cultivated in different organic medium concentrations.

The broth pH was measured in all fermentations, showing significant variations among treatments. The conditions with higher amount of N added, presented a higher growth

performance, followed by a greater decrease in the pH value. Usually the pH values reach approximately pH = 4 (which can reduce growth) at the end of the exponential phase. With the depletion of the sugars in the first 25 h, the pH dropped from 7.53 to 3.96 and from 7.36 to 3.76, in the treatments of 5 and 10 mmol L^{-1} of N, respectively. However, every time a sample was collected the pH was restored to 6.5, by the addition of NaOH (0.5 M).

Table 11 Global overview on biomass and specific growth rate of heterotrophic cultures using different molasses concentrations (C source) and medium concentrations (N source). Different letters from the same colum represent significant differences (p<0.05).

Treatment		**Biomass productivity**	**Specific growth rate**
		g L^{-1} h^{-1}	h^{-1}
Control		0.049 ± 0.004a	0.038 ± 0.001a
N source	1 mM	0.016 ± 0.001c	0.014 ± 0.001b
	5 mM	0.023 ± 0.002b	0.018 ± 0.00c
	10 mM	0.025 ± 0.001b	0.016 ± 0.00d
Control		0.12 ± 0.001a	0.057 ± 0.00 a
C source	1 g L^{-1}	0.03 ± 0.00b	0.025 ± 0.00b
	5 g L^{-1}	0.07 ± 0.00c	0.051 ± 0.00c
	10 g L^{-1}	0.00 ± 0.00d	0.0 ± 0.00d
w/o conversion		0.04 ± 0.00e	0.030 ± 0.00e

The treatments that exhibited the highest volumetric productivities were the 5 and 10 mmol L^{-1} of N (0.023 ± 0.002 and 0.025 ± 0.001 g L^{-1} h^{-1} respectively), without significant differences between them (p≥0.05). The biomass productivity and specific growth rate of the 1 mmol L^{-1} of N treatment, were the lowest, 0.016 ± 0.001 g L^{-1} h^{-1} and 0.014 ± 0.001, respectively.

In relation to the carbon source, molasses concentration was the factor that most influenced the growth. From the study conditions, 5 g L^{-1} of glucose showed higher biomass productivity (0.07 ± 0.00 g L^{-1} h^{-1}), followed by non-inverted molasses (0.04 ± 0.00 g L^{-1} h^{-1}) and the treatment with 1g L^{-1} of glucose (0.03 ± 0.00 g L^{-1} h^{-1}). At 10 g L^{-1} glucose the growth was inhibited, which may be related to the HMF value or inhibiting concentrations of another sugar present in the system (e.g. fructose). Statistically these values (biomass productivity) are significantly different (p<0.05) between the different carbon source concentrations.

The only operational change between the two tests was a reduction in the rpm of the orbital shaker due to in-house operational reasons. Consequently, the growth of cultures was affected,

leading to lower biomass yield and specific growth rate than the control used for the optimization of the carbon source.

3.3.4 Fed-batch growth

Figure 26 shows the main control parameters that have more influence on the growth of *Chlorella* and the time at where medium (N + C) was added. There were difficulties in reading the absorbance due to the colour of the medium, since whenever a pulse was supplied the absorbance increased, which might not correspond exactly to an increase in biomass. Therefore, the *DW* of cultures was measured regularly, revealing that from an initial concentration of 0.9 g L^{-1}, the fermenter grew up to 13.1 g L^{-1} of biomass. Thus, heterotrophy growth showed to be the best method for production of microalgal biomass in a short period of time. In the first few hours the agitation (300 rpm) and aeration (4 L h^{-1}) had a manual adjustment since DO_2 was unstable.

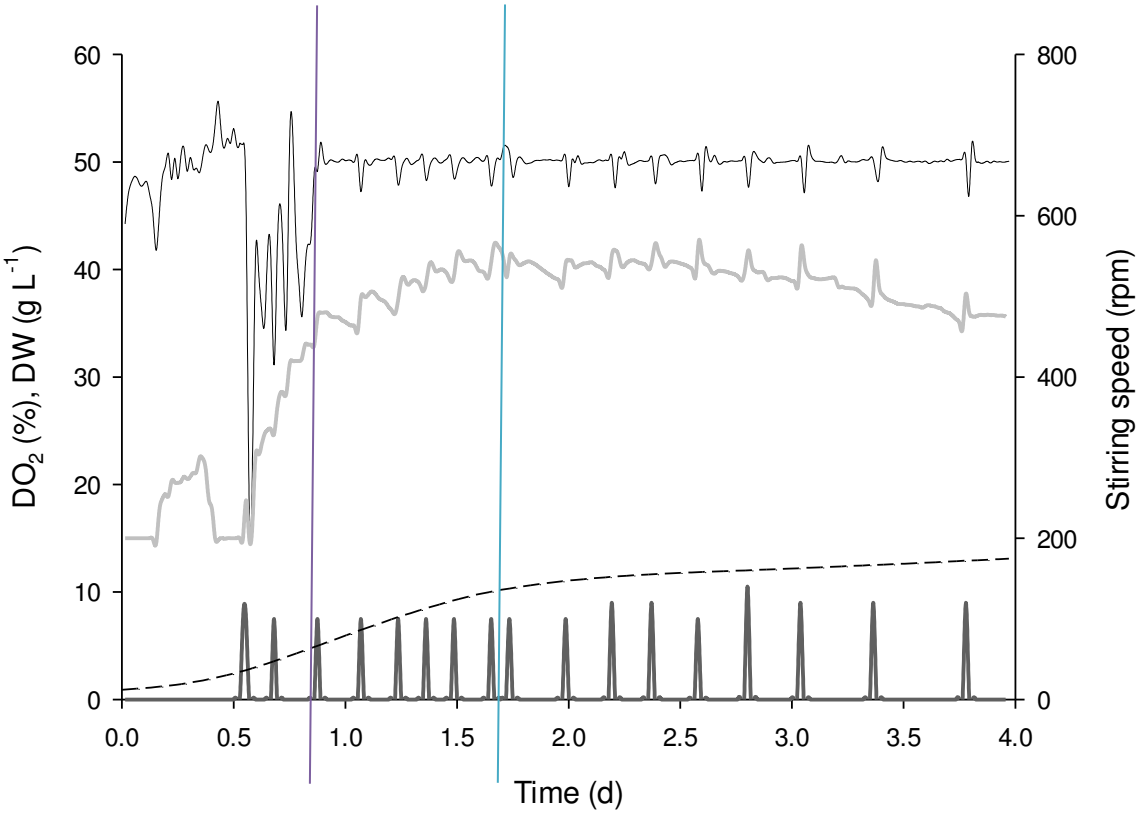

Figure 26 Growth curve in fed-batch and controlled parameters: DO_2 (thin blackline); Stirring speed (light grey line); *DW* (dash dark line), Carbon pulse (bold grey line). Stabilization of DO_2 (purple line), removal of volume (blue line).

Dissolved oxygen and stirring are parameters that inversely correlated. Whenever there is an increase in DO_2 there is an inverse response in the agitation, so that DO_2 returns to the steady state. The feeding method was established knowing that depletion of glucose was followed by a

rapid increase in the concentration of dissolved oxygen in the culture (Perez-Garcia *et. al.*, 2011). The fermenter was limited by these medium pulses more at the end of the assay. For example, it is possible to denote that at approximately t = 3.25 d, there is a clear increase of DO_2 without a corresponding carbon peak pulse. This period of starvation may be related to the established carbon feed standby being very large (t = 120 min), *i.e.* the minimum time required between pulses so that there is no excess glucose added may be too large for the time consumption of glucose. The best way to control would be hadding carbon manually and control the volume of the fermenter, to obtain a non-limited growth curve.

The pulses (medium and molasses) added, were approximately 200 mL, which limited the volume available to add more medium. After 2 days of fermentation, it was necessary to collect culture, in order to gain space in the fermenter. The N concentration used in the culture medium was around 7 mmol L^{-1} when the requirements for the fermentation process were at least about 100 mmol L^{-1}; this is the main reason, why the culture medium was added simultaneously with the inverted molasses. This carbon and medium source concentration is low for fed-batch high-cell density processes, leading to high final volumes of fermentation.

The consumption of fructose did not occur. Initially, 3.81 mg of fructose were present, while at t=1.6 d 10.81 mg were detected, reaching at the end of the growth period a final value of 27 mg fructose (with residual glucose levels). In the presence of substrates as glucose, the consumption of others “less preferred sugars” as fructose may be aborted because the enzymes that catalyse uptake of an alternate substrate cannot be synthesized in the presence of the “preferred” substrate (Lewin & Hellebust, 1978). Nevertheless, the consumption of all sugars present is important, to enable the transfer to autotrophy without a significant microbial growth. Thus, a fructose consumption strategy is needed, otherwise, molasses may not be at all feasible as organic carbon source for Algafarm.

4. Conclusion and Recommendations

The results in this study show that it is feasible to cultivate *Chlorella vulgaris* under an organic medium. All OSs present a wide variety of micronutrients in different concentrations, but neither of them have a balanced ratio between N and P macronutrients, due to the low concentration of soluble P.

The best compositions of the media under evaluation were reflected in growth. OS.1 and OS.4 showed no significant difference in biomass productivities between them and the control, while OS.3 presented the lowest biomass concentration (0.31± 0.03 g L^{-1}). Since the volume added of OS.3 to the final solution represent the highest amount (~32.7 %, v/v), it can be deduced from the results that autotrophic growth is strongly influenced by the light that the culture receives. So, it is important to concentrate as much as possible the stock solution of OS, to minimize the effect of colour in the absorption of light by the microalgae, and further reduce the process costs. Another observation to retain is that all cultures grown using OS consumed preferentially ammonia over nitrates.

It could be deduced from this result that the growth performance and final *DW* obtained using GWP were similar between the inorganic (control) and organic media, which can enhance the use of this medium on a large scale. The development and optimization of this type of culture medium for microalgae is still a promising area that needs further investigation.

In heterotrophic growth the sucrose uptake did not occur, therefore, the best compromise between the price of the invertase and the conversion time was 0.015 mg mL^{-1} of enzyme with 80 % of substrate converted in 17 hours.

With regard to the effect of molasses the maximum detected initial concentration of 5 g L^{-1} to avoid inhibition phenomena may be related to the HMF value in addition to the substrate inhibition phenomena of fructose and sucrose. On the other hand, the effects of different N supplementation towards the growth of *C. vulgaris* increased with the increase in their concentration to 5 and 7 mmol L^{-1}. However, this C and N source concentration is low for high cell density processes in fed-batch, leading to high final fermentation volumes. This way the fermenter volume had to be renewed in a short time.

The fed-batch growth in the 7 L fermenter had a final concentration of biomass of 13.1 g L^{-1}, showing that it is possible to grow heterotrophically to obtain higher, faster and better-quality cell concentrations.

In general the objectives were achieved, but there are a few recommendations for future work, in order to increase the concentrations of biomass obtained while reducing cost: 1) Extraction of MS can be enhanced by the addition of acid in certain amounts authorized by the regulation as citric acid; 2) Improvement of medium preparation with filtration or centrifugation at higher temperature at larger scale; 3) Screening of novel OS that can be a source of P; 4) Trial of other heterotrophic media richer in N (as OS.1) that do not require a significant addition of volume to the medium; 5) Use of hydrolyzed sucrose in a trial to liberate the HMF inhibition factor and verify the ability of *C. vulgaris* to use fructose.

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ANNEX A – CALIBRATION CURVES

The growth of *Chlorella* sp. was monitored by measuring the optical density of cultures on a UV / Vis spectrophotometer at 600 nm. The corresponding calibration curve is shown in Figure A1 and Figura A2.

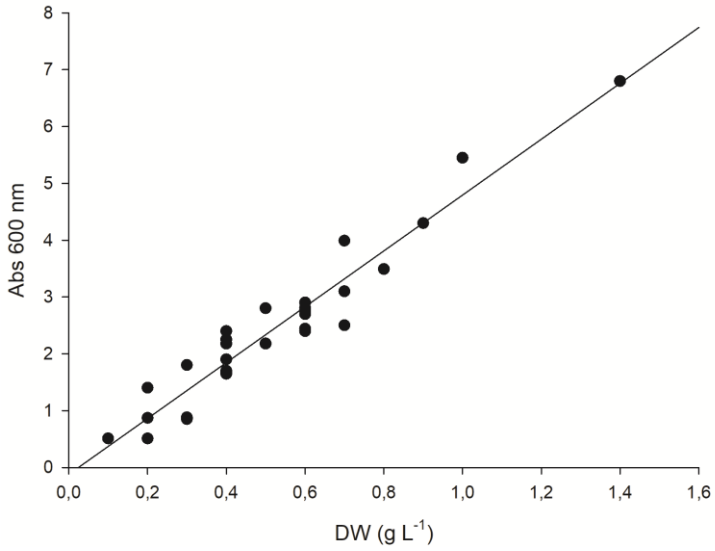

Figure A1 Absorbance of Chlorella sp. measured at λ = 600 nm vs dry biomass concentration for autotrophic growth.

R^2 =0.9254.

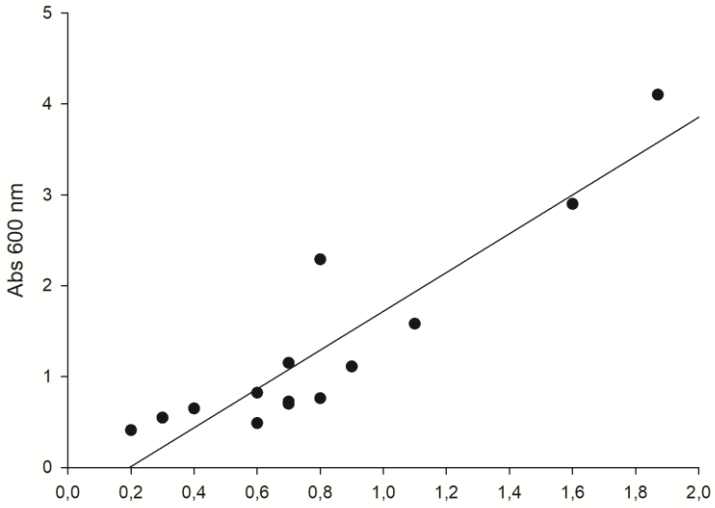

Figure A2 Absorbance of *Chlorella* sp. measured at λ = 600 nm vs dry biomass concentration for heterotrophic growth.

R^2 =0.8788

ANNEX B – OS. SELECTION

Prior to the OS screening test, a simpler assay, was performed (without replicated), only to identify whether the growth occurred or was absent. Thus, it was possible to select the 5 best OS. to the next phase.

Table B Pre-selection of OS

OS	Growth	Granular / Liquid (G/L)
1	++	G
2	+	G
3	+	G
4	++	G
5	++	G
6	Absent	L
7	Absent	L
8	Absent	L
9	Absent	G

ANNEX C –MPAES-MS DATA

Table C Limit of Detection (LOD) Limit of Quantification (LOQ), and its calibration curve for each element

	λ	LOD	LOQ	Calibration Curve (ppm)	R^2	Recovery (%)
Fe	**371.993 nm**	0,0060	0,0199	[0-50]	0,9998	120
K	**766.491 nm**	0,0132	0,0439	[0-50]	0,9953	119
Mg	**383.829 nm**	0,0074	0,0248	[0-50]	0,9995	93
Se	**196.026 nm**	0,1021	3402,0000	[0-10]	0,9995	101
Zn	**213.857 nm**	0,0043	0,0144	[0-10]	0,9992	109
Cd	**228.802 nm**	0,0004	0,0014	[0-10]	0,9996	120
Sr	**407.771 nm**	0,0004	0,0012	[0-10]	0,9998	80
Cu	**324.754 nm**	0,0026	0,0087	[0-10]	1,0000	113
Ni	**352.454 nm**	0,0012	0,0041	[0-10]	1,0000	103
Co	**340.512 nm**	0,0067	0,0225	[0-10]	1,0000	116
Pb	**405.781 nm**	0,0028	0,0092	[0-10]	0,9993	100
Mo	**379.825 nm**	0,0056	0,0188	[0-10]	0,9996	84
Mn	**403.076 nm**	0,0005	0,0017	[0-10]	1,0000	97
Cr	**425.433 nm**	0,0008	0,0027	[0-10]	1,0000	97
P	**213.618 nm**	0,04065	0,13551	[0-50]	0,9999	99
V	**309.311 nm**	0,00260	0,00870	[0-10]	1,0000	109

ANNEX D– DETERMINATION OF ENZYME CONCENTRATION

Data:

- MAXINVERT® 200.000 have Invertase Activity of 200 000 SU/g
- One SU is defined as the amount of enzyme required to form 1 mg of invert sugar, under standard conditions (pH 4.5, temperature 20°C)
- Molasse solution diluted 50 % m/m has 279 mg mL^{-1} of sucrose, in 100 mL, 20 000 SU
- It will be needed 0.14 g in 100 mL^{-1} ≈ 1.5 mg mL^{-1} of invertase

ANNEX E – CARBON AND NITROGEN OPTIMIZATION

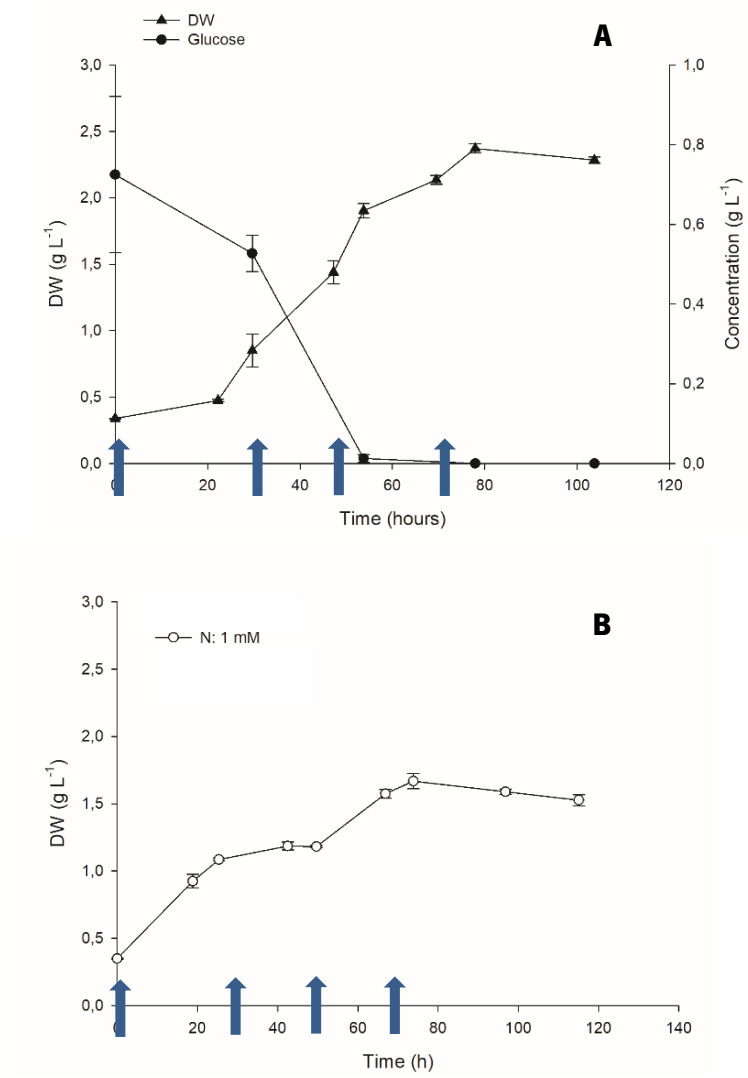

Figure D (A) Growth curve with initial concentration of 1 g L-1 glucose and glucose consumption of over time. (B) Growth curve with initial concentration of 1 mM of N. The moments of addition of more glucose (A) or medium (B) are represented in arrows on the the time axis.

ANNEX F – FED-BATCH DATA

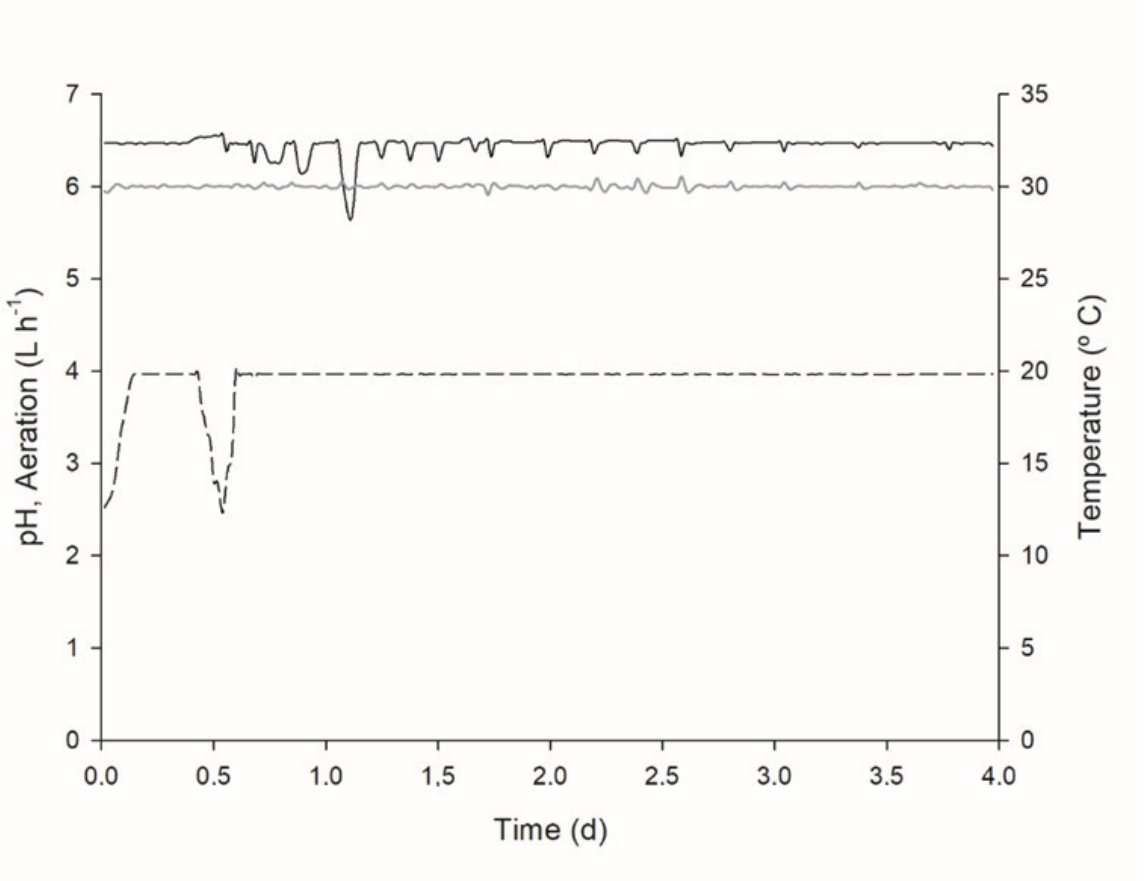

Figure F Operational condition of fed-batch: pH (blackline); Temperature (grey line); aeration (dash line).