Universidade do Minho
Escola de Engenharia

Francisco José Gonçalves de Veras Pereira

Organic heterotrophic medium for *Chlorella vulgaris*: formulation and validation

Tese de Mestrado

Mestrado Integrado em Engenharia Biológica

Ramo Tecnologias do Ambiente

Trabalho efetuado sob a orientação do(s)

Professor Doutor António Augusto Martins de Oliveira Soares Vicente

E da

Doutora Joana Gabriela Laranjeira da Silva

E supervisão de

Mestre Ana Isabel Faustino Barros

Outubro de 2019

AGRADECIMENTOS

Gostava de agradecer a todos que fizeram parte desta jornada, principalmente, aos que me ajudaram a concretizar, especificamente, os meus objetivos e que estiveram sempre ao meu lado, motivando-me a fazer mais e melhor de forma a abrir novas expectativas sobre a investigação científica das microalgas.

Individualmente, queria agradecer aos meus orientadores, Professor Doutor António Vicente e Doutora Joana Laranjeira da Silva, expressando a minha sincera gratidão pela oportunidade, confiança, incentivo, correções e apoio científico. Um especial agradecimento à minha co-orientadora Ana Barros, pois foi quem idealizou esta dissertação, dando-me sempre suporte e apoio em todas as minhas decisões e dúvidas. À Margarida, pois mesmo chegando à empresa depois de mim, contribuiu bastante para a concretização desta dissertação. Com o seu apoio e motivação tudo foi mais fácil. Agora em relação à equipa UI&D 2019, não existem palavras suficientes para explicar estes 6 meses. Foram 6 meses de muito suor (principalmente à volta do raceway do Pedro 😉), mas o que interessa é o sentimento de realização que ficou no interior de cada um dos estagiários. Assim, um muito obrigado, ao Pedro (o Grande Irmão e o ******* de sabão), à Inês (a Irmãzinha), à Joana (a pequenina Trabalhadora), à Susana (a Rapariga das dicas), à Rosângela (a Partner da fermentação) e à Sara (a Rapariga dos conselhos). Ao laboratório de fermentação, Ana, Maria, Bernardo, Adriana e Mafalda, por todos os ensinamentos dados ao longo destes 6 meses. Ao laboratório geral, pela disponibilidade em ajudar e ensinar as técnicas lá realizadas. Ao Quelhas e ao Nuno pelo carinho com que nos acolheram e pela ajuda na integração na empresa. Não esquecendo o Fábio, pois tornou-se um grande amigo e estava sempre lá para ajudar. Ao professor João Varela e à sua equipa de investigação do CCMAR pela disponibilidade e amabilidade em nos receber, com especial apreço ao Hugo e à Tamára. À minha família, especial aos meus pais e à minha namorada, pois foram eles que estiveram sempre lá a ouvir, a apoiar, a incentivar quando eu estava a ter os meus momentos de reflexão, principalmente, como controlar o pH de forma biológica, e a comemorar com as minhas vitórias. Aos meus amigos de longa data, Ricardo, Alex, Zais, Miguel, Hugo, Reis, Pedro e Catarina, pois sem o apoio deles não teria atingido os meus objetivos com tanta facilidade, seja no decorrer da dissertação, como na faculdade e no futuro que se avizinha. Por fim, agradeço à Spezialzucker – raffinerie e Biocal | LDM Minerais pelo fornecimento de amostras de produtos necessários para o desenvolvimento de todo este trabalho.

O meu muito obrigado a TODOS vocês.

STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

ORGANIC HETEROTROPHIC MEDIUM FOR *CHLORELLA VULGARIS*: FORMULATION AND VALIDATION

The industrial production of microalgae is a practice under development and has become very promising, having various applications, such as the food industry, cosmetics, biofuels and wastewater treatment. Microalgae are preferably photosynthetic organisms. However, there are some with the capacity of growing using of organic carbon sources, i.e. hetero and mixotrophically. The most commonly used carbon source is glucose, due to its simplicity and energy potential.

The objective of this thesis was to formulate an heterotrophic medium for *Chlorella vulgaris*, suitable for organic certification in accordance with European legislation. In relation to the biomass produced from this medium, it should meet Allmicroalgae requirements.

Initially, ammonia concentrations in the MNBIO growth medium was optimized. Yeast extract was also tested, however, cultures achieved growth values below those obtained with MNBIO medium. As carbon sources, a hydrolyzed sugar with organic certification, together with a sterilization by filtration or sterilization by autoclavating were tested. The organic sugar sterilized by filtration was selected once, there were no statistically significant differences between *C. vulgaris* growth. Potassium bicarbonate was selected against calcium bicarbonate and sodium bicarbonate as a buffer to use during fermentation process. With this optimized heterotrophic medium, it was possible to obtain an organic medium suitable for the *C. vulgaris* growth, taking into account the European legislation.

The optimized medium was tested in a 7 L bench-top fermenter, achieving dry weights of 39.2 ± 7.3 g L^{-1}. These fermentations had productivities and global growth rate of 15.5 ± 1.4 g L^{-1} d^{-1} and 1.68 ± 0.23 d^{-1}, respectively. In relation to biochemical analysis, the protein and chlorophyll content obtained were 22.9 ± 11.8 % and 9.76 ± 1.90 mg g^{-1}_{DW}, respectively. Comparing the results obtained with the inorganic production of Allmicroalgae for *C. vulgaris*, in terms of productivity and protein content are lower for the organic medium studied, but higher in the chlorophylls content.

KEYWORDS

Chlorella vulgaris; Heterotrophy; Industrial cultivation; Organic Certification EU

MEIO BIOLÓGICO HETEROTRÓFICO PARA *CHLORELLA VULGARIS*: FORMULAÇÃO E VALIDAÇÃO

A produção industrial de microalgas é uma prática em desenvolvimento e que se tem tornado bastante promissora, tendo várias aplicações, como a indústria alimentar, a cosmética, os biocombustíveis e o tratamento de águas residuais. Microalgas são organismos preferencialmente, fotossintéticos. No entanto, existem algumas microalgas com capacidades para crescerem usando fontes de carbono orgânicas, isto é, crescimento heterotrófico e mixotrófico. A fonte de carbono mais utilizada é a glucose, devido à sua simplicidade e ao seu potencial energético.

Esta tese teve como objetivo a formulação de um meio heterotrófico para a *Chlorella vulgaris*, apto para certificação biológica, de acordo com a legislação europeia, devendo a biomassa produzida com este meio satisfazer as necessidades da Allmicroalgae.

Inicialmente, a concentração de amónia foi otimizada no meio MNBIO. Foi igualmente testado extrato de levedura, contudo, as culturas alcançaram valores de crescimento inferiores aos obtidos no meio MNBIO. Testou-se um açúcar biológico hidrolisado como fonte de carbono, em conjunto com uma esterilização (por filtração ou na autoclave). O açúcar biológico esterilizado por filtração foi o selecionado, não havendo, diferenças estatisticamente significativas entre os crescimentos da *C. vulgaris*. Selecionou-se bicarbonato de potássio, em detrimento do bicarbonato de cálcio e de sódio, como tampão para usar durante o processo de fermentação. Com o meio biológico otimizado, foi possível obter um meio biológico adequado para o crescimento da *C. vulgaris*, tendo em atenção a legislação Europeia. O meio otimizado foi testado no fermentador de bancada de 7L, alcançando pesos secos 39.2 ± 7.3 g L^{-1}. Estas fermentações tiveram produtividades e taxas de crescimento global de 15.5 ± 1.4 g L^{-1} d^{-1} e 1.68 ± 0.23 d^{-1}, respetivamente. Em relação à análise bioquímica, o conteúdo proteico e de clorofilas obtido foi de 22.9 ± 11.8 % e 9.76 ± 1.90 mg g^{-1}_{DW}, respetivamente. Comparando os resultados obtidos com os da produção inorgânica da Allmicroalgae para a *C. vulgaris*, em termos de produtividades e conteúdo proteico foi inferior para o meio biológico estudado, mas superior em termos de clorofilas.

PALAVRAS-CHAVE

Chlorella vulgaris; Heterotrofia; Cultivo industrial; Certificação Biológica UE

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

List of General Nomenclature

A	Absorvance
ANOVA	Analysis of variance
R	Correlation Coefficient
DW	Dry weight
EU	European Union
FAME	Fatty acid methyl esters
GMO	Genetically modified organism
HMF	5 – hydroxymethylfurfural
LOD	Limit of detection
LOQ	Limit of quantification
MFA	Monounsaturated fatty acid
PBR	Photobioreactor
PUFA	Polyunsaturated fatty acid
P	Productivity (g L^{-1} d^{-1})
SFA	Saturated fatty acid
***p* – value**	Significance value
μ	Specific growth rate (d^{-1})
SD	Standard Deviation
t	Time
K_La	Volumetric mass transfer coefficient
YE	Yeast extract

1. CONTEXTUALIZATION

1.1. SECIL/ALLMICROALGAE

The work of this dissertation was done at Allmicroalgae, located in Pataias, Alcobaça. This is part of the SECIL group, the largest producer of Portuguese cement with operations in four continents always with an established sustainability policy that seeks new applications to factory wastes, including CO_2 and heat.

SECIL's microalgae project started at laboratory and pilot scale, with the development of the company's unique technology. Today, SECIL, through Allmicroalgae, has almost 10 years of experience in microalgae research, production and application. The company mission is to develop the various markets that use and intend to use microalgae as a natural source of nutrients and phytochemicals both nationally and internationally. This business is developed based on the properties of each microalgae, and its potential in various applications, such as, dietary supplements, food products for human consumption, feed and cosmetics. However, the initial and main objective would be to sequester part of the carbon dioxide (CO_2) emitted by neighboring SECIL cementer company, "Fábrica Cibra-Pataias", since this industry emits a lot of CO_2 in the manufacture of cement.

Figure 1 – Aerial view of Allmicroalgae.

The microalgae sold by Allmicroalgae comes from a production facility (Allmicroalgae) which is certified according to ISO 9001, ISO 22000, ISO 14001, OSHAS 18001 and *Hallal*. It is also noteworthy that Allmicroalgae already has the study and development of the industrial growth of several species such as *Chlorella vulgaris*, *Nannochloropsis oceanica*, *Phaeodactylm tricornutum*, *Tetraselmis chuii* and *Scenesmus* sp. The products may be supplied as powder or as paste. The biomass produced at Allmicroalgae results mainly from closed systems, either in photobioreactors or in fermenters.

1.2. Research aims

The main goal of this dissertation is the formulation of an heterotrophic organic medium for the production of *Chlorella vulgaris* and to verify that the characteristics of the resulting biomass meets the needs of Allmicroalgae. Thus, it was necessary to study several media to be applied bearing in mind that it should comply with the rules for organic production in the European Union. In addition, it was also important to analyze the differences obtained from inorganic (already existing) to the organic production developed, in terms of biochemical characteristics of the biomass.

1.3. Thesis outline

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

In Chapter 1, a brief description of the history and work developed by Secil/Allmicroalgae and the main objectives of this thesis are described.

In Chapter 2, a critical review of the literature is presented, addressing the main characteristics of microalgae and their applications. Information of the *Chlorella vulgaris* and microalgae growth technologies is also discussed. In the context of an organic certification approach, there is also a review of the European legislation on organic production.

In Chapter 3, the methodologies, materials, reagents and equipment are mentioned.

In Chapter 4, the work carried out is qualitatively evaluated, considering the initially imposed objectives. The results obtained are mentioned, together with a discussion about them.

In chapter 5, the final considerations are made, together with the limitations encountered during the execution of the experiments and suggestions for future work.

2. INTRODUCTION

2.1. Microalgae

Microalgae are unicellular organisms whose size can range from nanometers to millimeters, with a simple structure, which makes them organisms with high technological potential (Mata, T. M., Martins, A. A., Caetano, 2010; Drews- *et al.*, 2013; Jankowska, Sahu and Oleskowicz-Popiel, 2017). They are typically photosynthetic autotrophic organisms, using light energy and inorganic nutrients to synthesize components, such as lipids, proteins, carbohydrates, pigments, among others (Markou and Nerantzis, 2013). Nevertheless, some microalgae species have the ability to grow with organic carbon source supplementation through heterotrophic and mixotrophic metabolic pathways (Perez-Garcia *et al.*, 2011). In addition, they are versatile organisms as they tolerate a wide range of temperatures, salinities, pH values and light intensities (Khan, Shin and Kim, 2018).

Microalgae, due to their simple cellular structure and the diverse environment in which they live, are more efficient in nutrients, water and CO_2 exchanges than plants, and, consequently, they reach higher rates of conversion of cellular energy to biomass. It should also be noted that they are found in the marine environment, fresh water and soil, accounting for at least 60% of Earth's primary production (Benedetti *et al.*, 2018).

Due to microalgae's great adaptability to different physico-chemical conditions, they have developed a defense strategy producing new secondary metabolites, i.e. natural bio-products, including polysaccharides, proteins, amino acids (for example, glutamine and asparagine), fatty acids (such as linoleic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)), pigments (such as β - carotene, astaxanthin), vitamins and antioxidants, as shown in Figure 2 (Khan, Shin and Kim, 2018; Mobin, Chowdhury and Alam, 2019). Due to this high metabolic production, microalgae are presented as new model organisms for a wide range of biotechnological applications, such as biodiesel, health supplements, pharmaceuticals, and cosmetics (Demirbas and Fatih Demirbas, 2011; Enzing *et al.*, 2014; Jha *et al.*, 2017; Joshi, Kumari and Upasani, 2018). From an environmental point of view, some species have shown potential in wastewater treatment, with the removal of organic matter and assimilation of nutrients from effluents, and in atmospheric CO_2 mitigation (Benemann, 1997; Queiroz *et al.*, 2007; Pires *et al.*, 2012). Microalgae are microorganisms that represent a renewable and sustainable raw material, and

therefore, a new focus has been given to the biorefinery concept as being of potential interest (Khan, Shin and Kim, 2018).

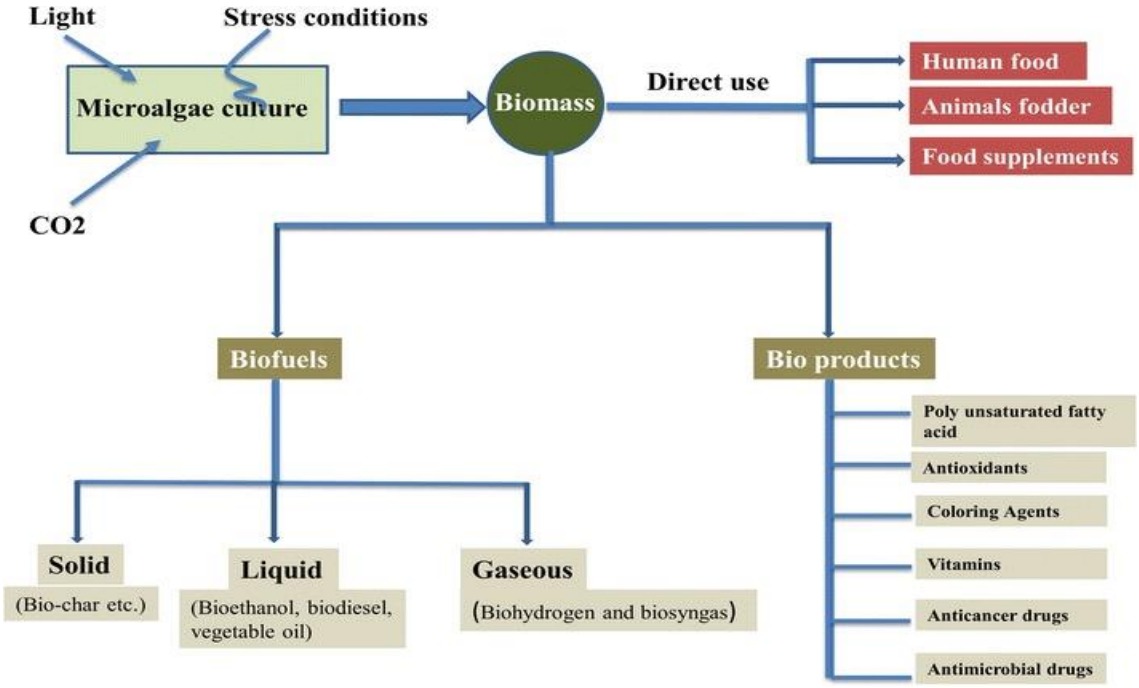

Figure 2 – Possible compounds resulting from microalgae biomass production under stress conditions, following the biorefinery concept. Adapted from Khan et al., 2018.

2.2. Technologies for microalgae cultivation

Microalgae growth characteristics are significantly dependent on the cultivation conditions applied. Metabolically, it is possible to use the photosynthetic route in photobioreactors or the respiratory route in heterotrophic bioreactors. In addition, these mechanisms can be combined in mixotrophy (Ahmad *et al.*, 2011; Amaro, Guedes and Malcata, 2011).

The cultivation of microalgae by a photoautotrophic mechanism can mitigate CO_2 emissions from human activities. It is estimated that 100 tons of microalgae biomass production result in around 183 tons of CO_2 fixation (Rodolfi *et al.*, 2009; Kumar *et al.*, 2010). On the other hand, heterotrophic cultivation also has a high potential for mitigating environmental impacts. These impacts are caused by effluent emissions from industrial processes and effluents can represent good nutrient medium for the microalgae growth (Khademi *et al.*, 2014).

2.2.1. Autotrophic Cultivation

In photosynthetic systems, biomass production is accomplished through the conversion of carbon dioxide, light energy and inorganic nutrients to oxygen and biomass (Davis, Aden and Pienkos, 2011). In terms of configuration, photosynthetic cultivation allows the use of open and closed systems. For microalgae growth there is a variety of open and closed reactor systems, where the main objective is the continuous maintenance of the microalgae conditions taking into consideration optimum conditions of productivity (Richmond, 2000).

In open systems, microalgae productions are directly exposed to the atmosphere. These are the cheapest option for large-scale biomass production since they have low cost of construction, installation, maintenance and are easy to operate (Jankowska, Sahu and Oleskowicz-Popiel, 2017). The most commonly seen open reactors are rectangular and circular tanks and raceway types (Figure 3).

Figure 3 – Open systems for microalgae production. ***A:*** *Circular pond located at the An-Nan campus, National Cheng Kung University, Taiwan (Chang et al., 2018);* ***B:*** *Raceways located in Qualitas farm, New Mexico (Nordrum, 2018);* ***C:*** *Raceway pilot scale installed in Allmicroalgae, Pataias, Portugal.*

Unlike the open systems, closed photobioreactors aim at the maintenance of an axenic culture of microalgae. This system, where contact with the atmosphere is significantly reduced or non-existent, is characterized by the regulation and control of most biotic and abiotic parameters (Suali and Sarbatly, 2012). Water, necessary nutrients and CO_2 are provided in a controlled way, while oxygen has to be removed (Barad, 2007). Closed photobioreactor design configurations include horizontal or tubular, bubble column, air-lift, flat panel and stirred tank, as can be seen in Figure 4 (Pereira, C.M.P.; Hobuss, C.B.; Maciel, J.V.; Ferreira, L.R.; Pin, 2012). Tubular photobioreactors are one of the most suitable types for outdoor production (Molina *et al.*, 2001). The design of PBRs for photoautotrophic cultivation maximizes the exposure to light irradiation to provide microalgae cells in order to ensure the optimum photosynthetic conditions. (Rodolfi *et al.*, 2009). However, this strategy has difficulties to achieve high biomass densities since light penetration is inversely proportional to cell concentration (Borowitzka, 1999; Grima, Acie and Chisti, 1999).

Figure 4 – Different closed system production of the microalgae biomass; ***A:*** *Industrial Tubular Photobioreactor;* ***B:*** *Bubble Column Reactors in laboratory scale;* ***C:*** *Flat Panel Photobioreactor;* ***D:*** *Tubular Photobioreactor in pilot scale. Pictures were kindly provided by Allmicroalgae, Pataias, Portugal.*

2.2.2. Heterotrophic Cultivation

While most algae grow photo-autotrophically, some have the ability to grow heterotrophically using organic substrates as a source of energy and carbon (Singh and Dhar, 2011). This type of metabolism mostly happens in the dark and so it is expected that the necessary carbon sources used to grow microalgae, substrate or intermediate energy metabolism pathways, might substitute for the carbohydrates produced during photosynthesis (Chen and Chen, 2006). Heterotrophic microalgae are grown in stirred tank bioreactors, commonly known as fermenters, and they provide a high degree of growth control in a sterile environment (Figure 5).

Figure 5 – Fermenters with a working volume of: ***(A):*** *7L;* ***(B):*** *200L;* ***(C)****: 5000L. Pictures were kindly provided by Allmicroalgae, Pataias, Portugal.*

Thus, the ability to grow heterotrophically is present in several microalgae genes and is mainly related to the following characteristics (Wen and Chen, 2001; Chen and Chen, 2006; Morales-Sánchez *et al.*, 2013):

- Ability to divide quickly in sterilized medium;
- Cellular permeability to organic carbon source;
- Active transport of organic carbon source:
- Resistance to induced hydrodynamic stresses in fermenters.

Heterotrophic systems operate in the absence of light, and for this reason, conventional industrial materials can be used in the construction of bioreactors, allowing for a simpler scale-up

as smaller surface/volume ratios can be used (Brennan and Owende, 2010). In addition, heterotrophic systems offer the possibility of greatly increase cell density and productivity (Riesenberg and Guthke, 1999; Miao and Wu, 2006; Shen *et al.*, 2010; Xiong *et al.*, 2010). Optimal growth and production can be easily maintained and axenic culture, through sterilization of the medium and reactor (Chen F, 1996).

A crucial factor for this type of cultivation is the oxygen supply because oxygen limitation lowers the growth rate and yields of microalgae. All heterotrophic microalgae are aerobic and the denser the culture, the greater the oxygen demand (Chojnacka, K.; Marquez-Rocha, 2004). In aerobic fermentation processes, volumetric oxygen transfer coefficient, k_La, is an important parameter, which is related to the agitation speed, aeration rate, geometrical characteristics the vessel, medium composition and the microalgae morphology. For example, k_La can be improved by increasing agitation and aeration, since microalgae are, traditionally, resistant due to their cell wall (Tobajas and García-Calvo, 2000; Zhou *et al.*, 2018).

Another crucial parameter is the pH of the culture medium, as it tends to decrease mainly in the exponential phase of growth. This happens due to heterotrophic growth using respiration route. Respiratory activity produces CO_2 which causes decreases in the pH medium (Yono, Budi ; Syaichurro, Iqbal; Sumardiono, Siswo; Sasongko, 2014). Growth rate, nutrient uptake and biochemistry of the cells are strongly affected by pH and an optimum pH range needs to be established (G *et al.*, 2018). A way to control pH in order not to limit the growth is with the application of buffers, according to the desired pH range. However, buffers can affect biological systems, specifically, cell growth, enzyme activity and form radical species (Nagira *et al.*, 1995; Grady, 1998). Traditional buffers such as phosphate, citrate, borate and succinate have some disadvantages when used in biological cultivations, for example, inhibition in enzymatic level and in biochemical reactions. Subsequently, many pH buffers were proposed to replace the traditional ones (Good, N.; Izawa, 1972; Ferguson, Wilfred; Braunschweiger, 1980). These buffers, such as, ADA, HEPES, MES, MOPS, PIPES, TES, Tris, etc, should cover pH between 6 and 8, have a maximum solubility, minimal interference with temperature, ionic strength and concentration and be stable, in order to not metabolized or act as inhibitory of enzymes (Ferreira *et al.*, 2015).

However, heterotrophic cultures also have drawbacks: only a small group of microalgae species can grow heterotrophically in conventional bioreactors; potential contamination by bacteria; higher energy and medium expenses; growth can be inhibited by high and/or low substrate

concentrations and some metabolites cannot be produced or are produced in very small amounts (Chen F, 1996; Chojnacka, K.; Marquez-rocha, 2004; Perez-Garcia *et al.*, 2011; Khan, Shin and Kim, 2018).

2.2.3. Mixotrophic Cultivation

Mixotrophic microorganisms can simultaneously perform photosynthesis and consume organic carbon (Wang, Yang and Wang, 2014). Although not as exploited in terms of microalgae production, when compared to autotrophy, this form of metabolism can result in competitive advantages and has been observed in the most varied environments, from oligotrophic to eutrophic. For example, mixotrophic growth can reduce problems related to light limitations resulting in better growth rates (Bohutskyi *et al.*, 2014). For a species to be able to grow under mixotrophic conditions, enzymes of the heterotrophic pathway cannot be inhibited by the presence of light (CHOJNACKA, K.; MARQUEZ-ROCHA, 2004).

2.3. Chlorella vulgaris

Chlorella vulgaris ("*Chloros*": green; "*ella*": diminutive) is an eukaryotic, unicellular, green microalgae with a fast growth rate when compared to related microalgae. It was discovered in 1890 by a German researcher as the first microalgae with a well-defined nucleus (Zeitum and Wortiriann, no date). The taxonomic position of Chlorella is depicted in Table 1. This microalgae grows both in fresh and marine environments, and due to this versatility, it is one of the most commercialized in the world (Coelho *et al.*, 2019).

Table 1 – Taxonomic Classification of Chlorella vulgaris

Phylum	Chlrophyta
Class	Trebouxiophyceae
Order	Chlorellales
Family	Chlorellaceae
Genus	*Chlorella*
Species	*Chlorella vulgaris*

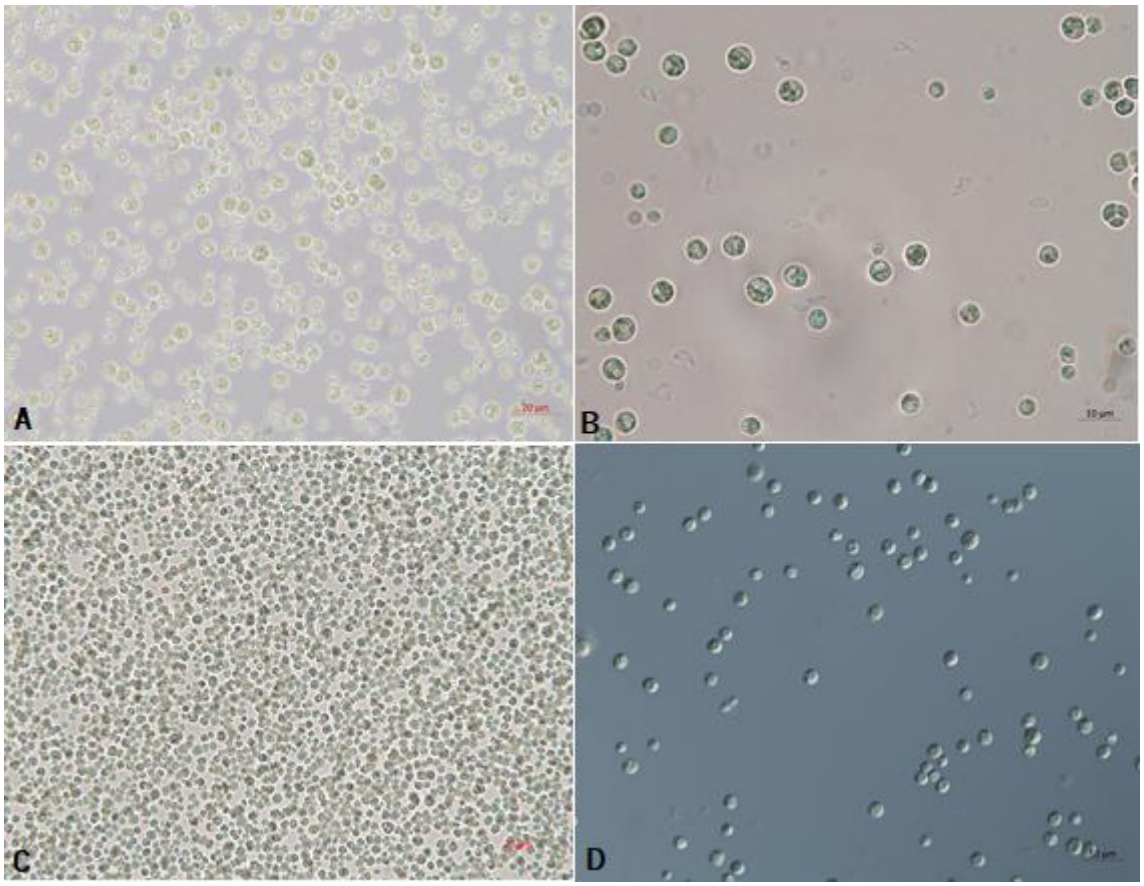

Figure 6 – Microscopic (Zeiss axio scope a1) view of Chlorella vulgaris *grown in Allmicroalgae R&D Department. A: Magnification 40x (Phase-contrast); B: Magnification 63X (Bright field); C: Magnification 40x (Bright field); D: Magnification 63x (DIC).*

C. vulgaris, one of the most studied species of the *Chlorella* gender, is sold as a "natural all-in-one supplement" in market due to its content in pigments, protein, amino acids, unsaturated fatty acids, vitamins, etc (Safafar *et al.*, 2016). *C. vulgaris* has no mobility and a single chloroplast, where it accumulates pigments such as chlorophyll a and b, β-carotene and xanthophyll. As there is a predominance of chlorophylls in relation to carotenoids, the cell presents a green color (Safafar *et al.*, 2016; Velichkova and Sirakov, 2018). The amino acid profile of *C. vulgaris* is complete concerning human nutrition due this microalgae contain all of the essential amino acids (humans are not able to synthesize) (Mason, 2001; Becker, 2007; Barkia, Saari and Manning, 2019). In addition, the amino acids profile of *C. vulgaris* is similar, in terms of quality, to that found in soy and egg white (Williams and Laurens, 2010). In relation to fatty acid profile, according to the conditions imposed by the growth, different profiles can be obtained, which are suitable for different applications. For example, for biodiesel production, saturated fatty acids, palmitic acid (C16:0), stearic acid (C18:0) and monounsaturated, palmitoleic acid (C16:1) and oleic acid (C18:1) are valorized (Mallick *et al.*, 2012; Yeh and Chang, 2012; Rushan *et al.*, 2019). However, under

favorable growth conditions FAME profile is more concentrated in polyunsaturated fatty acids, linoleic acid (18:2), linolenic acid (C18:3) and eicosapentaenoic acid (C20:5), which is more favorable for nutritional application (Freitas, 2017). Furthermore, this specie has a vitamin profile, constituted by vitamin A, B_1, B_2, B_3, C and E. Additionally, it is rich in essential elements for human nutrition, such as, potassium, magnesium and zinc (Tokuşoglu and Ünal, 2003; Panahi *et al.*, 2012; Yeh and Chang, 2012).

From a global perspective, *C. vulgaris* can be used in biofuels production, in human nutrition, as food supplement or additive, in animal feed, especially, in aquaculture, wastewater treatment, as it is capable of fixing large percentages of carbon dioxide and absorbing large quantities of nitrogen and phosphorus and, finally, in agrochemical applications (biofertilization using algae extract) (Benemann, 1997; Becker, 2007; Liang, Sarkany and Cui, 2009a; Fradique *et al.*, 2010; Singh and Dhar, 2011; Zheng *et al.*, 2012).

2.4. Nutritional requirements

Specific nutritional requirements of microorganisms used in industrial fermentation processes are complex. Not only it varies from microorganism to microorganism, but also within species and strains (Todaro, Celeste; Vogel, 2014). However, for any microalgae, some nutritional factors are of great importance for their development. Thus, essential macronutrients, micronutrients and vitamins, in lower concentrations, are needed. According to (Bruland, Donat and Hutchins, 1991; Markou and Nerantzis, 2013; Markou, Vandamme and Muylaert, 2014), the medium needs to contain C, N, P, K, Ca, Mg, Na, Cl (macronutrients) and Fe, Mn, Zn, Cu, B, Mo, Si, Se, V, Co, Ni (micronutrients), in small quantities (Bruland, Donat and Hutchins, 1991; Merchant *et al.*, 2006; Markou, Vandamme and Muylaert, 2014). Vitamins are organic compounds and function as coenzymes in the metabolism. Most frequently, thiamin and biotin, are the most used vitamins (Todaro, Celeste; Vogel, 2014).

The medium composition is obtained using compounds in its pure form and defined according to the yields of growth. However, in industrial fermentations, with the aim of reducing costs, completely unknown and complex medium, such as, molasses, steep liquor, meat extracts can be preferred (Leesing and Kookkhunthod, 2011; El-Sheekh *et al.*, 2014; Shakibaie, 2018).

According to the needs of microalgae, supplementation with other inorganic nutrients is necessary (Todaro, Celeste; Vogel, 2014).

An indication of carbon source importance is its percentage in cell dry weight: approximately 50% (w/w) (Markou, Vandamme and Muylaert, 2014). Respiration aims at producing energy and is linked to cell growth and division. In addition, this metabolism is related to the oxidation of organic substrates supplied to microalgae (Sun *et al.*, 2018). Thus, the main carbon and energy sources for microalgae heterotrophic growth are carbohydrates and organic acids, especially glucose, sucrose, acetic acid and glycerol (Liang, Sarkany and Cui, 2009b; Heredia-Arroyo, Wei and Hu, 2010; Kim *et al.*, 2013; Morales-Sánchez *et al.*, 2013; Shakibaie, 2018). The availability of the carbohydrate to the microorganism normally depends upon the complexity of the molecule.

Glucose is usually obtained from corn starch through hydrolysis and sucrose from sugar cane or sugar beets. Sucrose is most often purchased in the form of molasses (El-Sheekh *et al.*, 2014). Glucose is the most commonly used carbon source for heterotrophic growth (Perez-Garcia *et al.*, 2011). Therefore, respiration and growth rates tend to be better with glucose than with any other carbon source and this is because glucose has higher potential energy per mol than any other source (Boyle and Morgan, 2009).

Nitrogen is the second major nutrient and it accounts for 1 to 10% of the microalgae dry weight (major components in amino acids and nucleic acids). Microalgae can assimilate different nitrogen sources, from simple and complex sources. Simple nitrogen sources are ammonia (NH_4^+), nitrate (NO_3^-) and urea (Chen *et al.*, 2010; Wang and Lan, 2011). Ammonia is the preferred source of nitrogen for algae as it is the most energy-efficient, once less energy is required for its absorption (Wang and Lan, 2011). Nitrate is also an important nitrogen source and has a strong impact on microalgae metabolism and growth in general. In the case of ammonia consumption, there is a decrease in the pH due to the release of H^+ during assimilation. In the case of nitrate consumption leads to an increase in pH due to the release of OH^- during assimilation (Perez-Garcia *et al.*, 2011). There are also two more common sources, urea and complex nitrogen. On heterotrophy, normally, the order of preference for nitrogen source is:

Ammonia > nitrate > nitrite > urea (Perez-Garcia *et al.*, 2011)

Complex nitrogen sources include corn steep liquor, peptone, yeast extract and tryptone (Chen *et al.*, 2010). Yeast extract and tryptone are commonly used in microalgae growth to produce omega-3 polyunsaturated fatty acids (Wen and Chen, 2001). Besides nitrogen, these sources may serve as supplementation of amino acids, vitamins, carbon and trace nutrients.

Another important element is phosphorus (P). Phosphorus is element that influences cell development and the metabolism of microalgae. It is one of the basic components of RNA, DNA and ATP (Fan *et al.*, 2014). Supply of P also influences the biomass. In addition, when cultures have low amounts of P, they have a tendency to decrease protein and chlorophyll content (Wang *et al.*, 2008; Dean *et al.*, 2010).

Microalgae also need trace metals in smaller quantities: Cu, Se, Ni, Bo, Br, Zn, Mo, etc. However, supplementation with these elements depends on the microalgae being cultured and the composition of the medium. For example, if the nitrogen source was urea, an addition of Ni will be necessary once Ni is required for the functioning of the urease enzyme (Dupont, Barbeau and Palenik, 2008).

Microalgae adjust their nutrient uptake according to the availability of nutrients in the medium. These microorganisms are very versatile as they can grow under excess or limited nutrients (Lemesle and Mailleret, 2008). Thus, cultivation using nutrients limitation is often a strategy product valorization (Dragone *et al.*, 2011). This strategy leads to accumulate target molecules. For example, according to (Dragone *et al.*, 2011), nitrogen limitation leads to increase of carbohydrates content in *C. vulgaris*, according to (Panis and Carreon, 2016), nutrient deprivation induced an astaxanthin accumulation in *Haematococcus pluvialis* and according to (Forján *et al.*, 2007), phosphate limitation could be applied to accumulation of carotenoids, specially the xanthophyll, violaxanthin and zeaxanthin, in *Nannochloropsis gaditana*.

2.5. Organic Production

Nowadays, consumers are often looking for organically certified food, mainly, due to its better quality, in terms of taste, concern for the environment and animal welfare, and the fact that this it is the only production method that do not allow genetically modified organisms (GMO). Organic production obliges the respect for the principles, rules, and requirements of organic farming (*Aims of organic farming*, 2019).

The organic European market is the second in the world, in terms of turnover, after the organic North American market. Currently, there is an enormous development trend in the "organic world", since there are already 181 countries with organic activities and a market with, approximately, 90 billion euros (FIBL; IFOAM, 2018). However, as certified producers increase, it also increases the risk of non-compliance with the rules and loss of consumer confidence.

The European Union (EU) regulations on organic production have been created in order to ensure that all the rules are complied with. These regulations provide a clear, controlled and supervised idea of how to produce, transport and store organically (*Aims of organic farming*, 2019). 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 889/2008 complemented the previous laying down detailed rules for the implementation of previous regulation, concerning biological production, labelling and control (SATIVA, 2019).

The organic logo, seen in Figure 7, may only be used on products certified as organic by an authorized agency or inspection body and makes it easier for the consumer to identify that the product is certified.

*Figure 7 - **A:** EU organic logo; **B:** Example of a product with organic certification.*

According to the EU organic regulation, an organic identification mark must contain (Duteil, 2016):

- Name and signature or the producing/ manufacturing/ trading company
- Identification of the product/raw material
- Product-related organic reference (for example: “Organic Powder”)
- Code number of the control body (for example: PT – BIO – 03 where "PT" is the ISO code for the country, "BIO" is a term link with the organic production and "03" (SATIVA, Lda) is the reference number of the control authorities composed in 1 to 3 digits)

European legislation on the production of microalgae and algae is not yet fully developed. According to the legislation, there should be a restriction on nutritional inputs to the production system and a control of harvesting methods to assure long-term system productivity (Lembo and Menre, 2019). The content of the EU Organic Regulation comprises a general system for food production, including micro and macroalgae in the same lot, i.e. microalgae production must comply with the rules applicable to the production of any plant or seaweed. (Verzijden, 2016)

Reliable production of microalgae depends on the use of inorganic nutrients, nitrates, phosphates, silicates, etc. For the different algae species, these nutrients can be dosed at precisely the optimum for growth. In addition, synthetic ingredients are not allowed, except additives, such as vitamins and mineral supplements. However, they need to be really essential for microalgae growth and of natural origin (Lembo and Menre, 2019).

3. MATERIAL AND METHODS

Growth experiments were performed at Allmicroalgae within the period of February 11th and August 23th 2019. Biochemical analyses on the biomass produced at Allmicroalgae and were done at the University of Algarve, in cooperation with MarBiotech Group of the Centre of Marine Sciences, during the period of July 23th and August 9th of the same year.

3.1. Microalgae Strain and Medium culture

The freshwater microalgae *Chlorella vulgaris* used in all experiments was AGF002, the production strain used at Allmicroalgae. The seed inoculum used was obtained from a cryopreserved vial of the Allmicroalgae Working Cell Banks in standard heterotrophic medium (AGF_HM).

The culture medium used in Allmicroalgae for autotrophic organic production of *C. vulgaris*, MNBIO, composed by dilution of MNBBIO and MNMBIO, was tested. The composition of MNBIO is described in Table 2.

Table 2 – Elemental composition of MNBIO, the organic medium used by Allmicroalgae for the autotrophic cultivation of Chlorella vulgaris

		Element	*Concentration* $_{/\mu g\, g^{-1}}$
MNBIO	MNBBIO	N (NH_4^+ form)	2.88×10^4
		P	8.74×10^3
		K	3.32×10^4
	MNMBIO	Fe	2.02×10^4
		Mg	1.06×10^4
		Zn	1.01×10^4
		Mn	1.01×10^4
		Mo	1.63×10^2
		B	1.95×10^3
		Cu	9.76×10^2

pH variations in the medium during cultivation were controlled using PIPES (Sigma-Aldrich, Portugal) as a buffer. 300 g L^{-1} potassium bicarbonate (Proenol, VN Gaia, Portugal) and 70 g L^{-1} sodium bicarbonate (Maison-ecolo, Dardilly, France) were also tested for pH control tests.

Different ratios of MNBBIO were tested adjusting the ammonia concentration (N source) to 99, 83, 62 and 28 mmol L^{-1}. Yeast extract (Lallemand, Montreal, Canada) was also tested as a nitrogen source, at 20 g L^{-1} and 10 g L^{-1}.

Glucose 50% (w/w) (AGFERM_GLU) and organic sugar (Bio-Invertzuckersirup 73 %, Spezialzucker Raffinerie, Lage, Germany) were tested as carbon sources. Batched glucose concentration was set to 20 g L^{-1} based on the Allmicroalgae standard method of cultivation. Two sterilization procedures were tested for organic sugar, filtration (0.2µm, VWR, Lisboa, Portugal) and autoclaving at 121 °C for 40 min (Uniclave 88, Lisboa, Portugal).

A vitamin solution (AGFERM_VIT) composted of Thiamine-HCl (Sigma-Aldrich, Portugal), d-biotin (Sigma-Aldrich, Portugal), cyanocobalamin (Sigma-Aldrich, Portugal), calcium pantothenate (Sigma-Aldrich, Portugal) and p-aminobenzoic acid (Sigma-Aldrich, Portugal) was supplemented to the medium also based on the Allmicroalgae standard method of cultivation.

3.2. Setup of the experiments

3.2.1. Medium optimization

The assays were performed in 250 mL Erlenmeyer flask with 50 mL of culture in an orbital shaker incubator at 200 rpm and 30 °C.

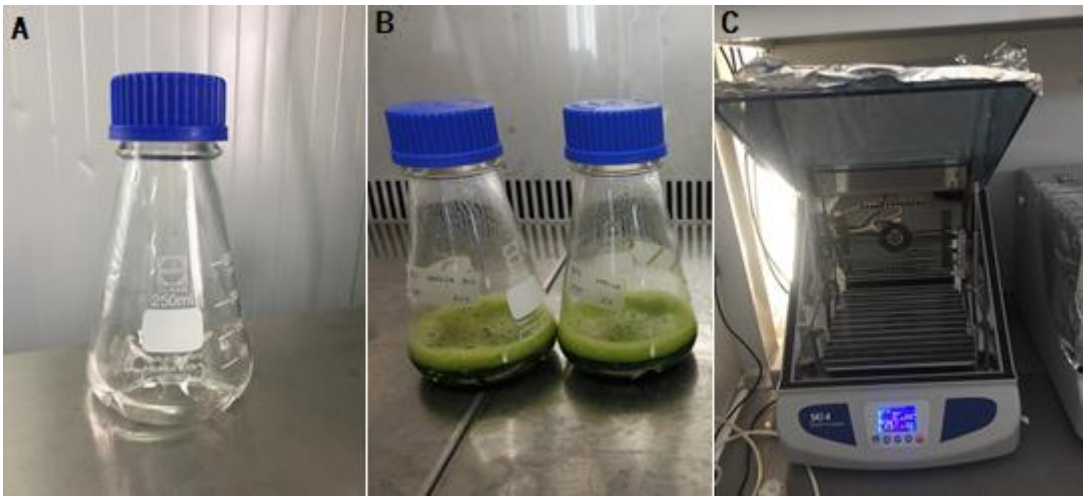

Figure 8 – Equipment used in the implementation of optimization. ***A:*** *250 mL Erlenmeyer flask;* ***B:*** *Erlenmeyer with 50 mL of culture;* ***C:*** *Shaker Incubator SKI-4.*

For all assays, the seed inoculum was grown in standard inorganic in-house culture medium and used to start trials at 0.4 % (v/v) of inoculum.

3.2.2. Fed-Batch cultivation using the optimized organic medium (Fermenter)

A 7 L bench-top fermenter (New Brunswick BioFlo®/CelliGen®115; Eppendorf AG, Hamburg, Germany) was inoculated with the optimized organic medium, $MNBIO_{optimized}$. The fermenter and the accessory flasks (with solutions of sugar, antifoam and base) were sterilized in autoclave for 45 min at 121 °C with 2.3 L of $MNBIO_{optimized}$ and equipped with different sensors (dissolved oxygen, pH and foam level), temperature control, stirring rotor, baffles. Peristaltic pumps were used for the addition of base for pH control, of carbon source and antifoam. The speed of agitation and the aeration rate were adjusted automatically in order to maintain 40% of dissolved oxygen. pH was maintained between 6 and 6.5.

The fermenter was operated in fed-batch mode, the solution of sugar was fed to the culture whenever the sugar was depleted in the medium. The $MNBIO_{optimized}$ containing 62 mM NH_4^+, organic sugar sterilized by filtration and 300 g L^{-1} Potassium Bicarbonate was used for pH control. Three fermenters were prepared, where 3 different reagents were tested to control the foam formed: organic extra virgin olive oil (Continente, Lisboa, Portugal), organic sunflower oil (Bratöl, Moers, Germany) and inorganic antifoam Foam Doctor (F112).

Figure 9 – 7 L bench-top fermenter (New Brunswick BioFlo®/CelliGen®115; Eppendorf AG, Hamburg, Germany) with organic medium.

3.2.3. Autotrophic growth assay

Biomass of heterotrophic *Chlorella vulgaris* culture produced in 7L bench-top fermenter to inoculate 70 L Flat Panel Photobioreactor, in triplicate. The air-lift system was cleaned and remained in the disinfection tanks with sodium hypochlorite. The compressed air introduced in the airlift system was filtered at 0.2 μm (VWR, Lisboa, Portugal). The water used in the process was disinfected with 100 ppm sodium hypochlorite and later neutralized. Organic medium was added every day up to a concentration of 2 mM of ammonia. The pH of the culture was controlled with the injection of CO_2 and the temperature was controlled with a sprinkler-like irrigation systems.

3.3. Growth Assessment

The growth of microalgae cultures was followed by optical density at 600 nm (OD) and used for dry weight estimation (DW) according to a calibration curve. Calibration curve for the heterotrophic *Chlorella vulgaris* given by Equation 1 and is represented in Appendix A*:*

$$DW = 0.5285 \times OD_{600nm} \text{ (n = 171} \quad R^2 = 0.945) \tag{1}$$

Calibration curve for autotrophic *Chlorella vulgaris* is given by Equation 2 and is represented in Appendix B:

$$OD_{600nm} = 2.2803 \times DW - 0.0929 \text{ (n = 640} \quad R^2 = 0.9372) \tag{2}$$

3.4. Ammonia concentration measurement

To determine ammonia concentration in the culture medium, Ammonia-Ammonia Sera Test (Sera, Heinsberg, Germany) was used. Briefly, samples were centrifuged to obtain the supernatant. Sera Test was added. After 5 minutes, absorbance was read in the Genesys 10S UV-Vis spectrophotometer (Thermo Scientific, Massachusetts, USA) at OD_{697nm}. Ammonia concentration was calculated according to the Allmicroalgae calibration curve.

3.5. Glucose/Sucrose concentration measurement

The sugars concentration was accessed by YSI 2950D Biochemestry Analyser (YSI Inc./Xylem Inc, Ohio, USA). Before analysis, samples were centrifuged (VWR MicroStar 12, Pennsylvania, USA) and 0.2 μm (VWR, Pennsylvania, USA) filtered.

3.6. Growth rate and biomass productivity

The specific growth rate (d^{-1}) was calculated based on Equation 3, the ratio between the difference of the final (t_2) and initial (t_1) biomass concentration, X_2 e X_1.

$$\mu = \frac{\ln\left(X_2/X_1\right)}{t_2 - t_1} \quad (3)$$

The productivity, P (g L^{-1} d^{-1}), can be expressed in overall productivity (initial and end point of the assay) and in maximum productivity (the highest productivity calculated based on two consecutive time points). It was calculated based on Equation 4, where X_f represents the biomass concentration at the final time, t_f, and X_0 the initial biomass concentration, at t_0.

$$P = \frac{X_f - X_0}{t_f - t_0} \quad (4)$$

3.7. Analytical Determinations

3.7.1. Pigments quantification

To determine the microalgae pigment content, a colorimetric method was applied. Biomass was placed in the Vortex for 15 min in tubes containing zirconia beads and 6 mL of acetone, in order to break the cell wall and extract the pigments. The procedure was repeated until complete loss of the pellet color. A scanning spectrum (between 380 and 700 nm) was obtained using a Genesys 10S UV-Vis spectrophotometer (Thermo Scientific, Massachusetts, USA) and the values were calculated according to the Allmicroalgae calibration curve.

3.7.2. Protein quantification

Protein content was determined by the Lowry method (Lowry, Randall and Lewis, 1951). 1 g L^{-1} fresh biomass was washed with distilled water. Biomass digestion was performed using 2 mol L^{-1} NaOH in 99 °C water bath for 10 min, so that all proteins are solubilized in the liquid phase. The residues were discarded by a new centrifugation (HERMLE Labortechnik GmbH, Wehingen, Germany) at 2547 g for 10 min. 1 mL of supernatant was removed to a new tube and 5 mL of reagent 1 (for 50 mL, a mixture of 48 mL sodium carbonate solution (5% w/v), 1 mL sodium-potassium tartrate solution (2% w/v) and 1 mL copper sulfate solution (1% w/v)) was added. Samples were kept for 10 min in the dark, and 1 mL of reagent 2 (1:2 dilution of Folin-Ciocalteau reagent with distilled water) was further added. Samples were kept in the dark for 30 more minutes. Absorbance was read at 750 nm on the Genesys 10S UV-Vis spectrophotometer (Thermo Scientific, Massachusetts, USA) and the results were calculated according to the Allmicroalgae calibration curve.

3.7.3. Ash Contents

Total ash was determined by the weight difference before and after the combustion of the biomass. In brief, biomass was weighed and placed in small ceramic cups and heated for 5 h at 550 °C using a furnace (*J. P. Selecta, Sel horn R9-L*). The resultant was weighed again.

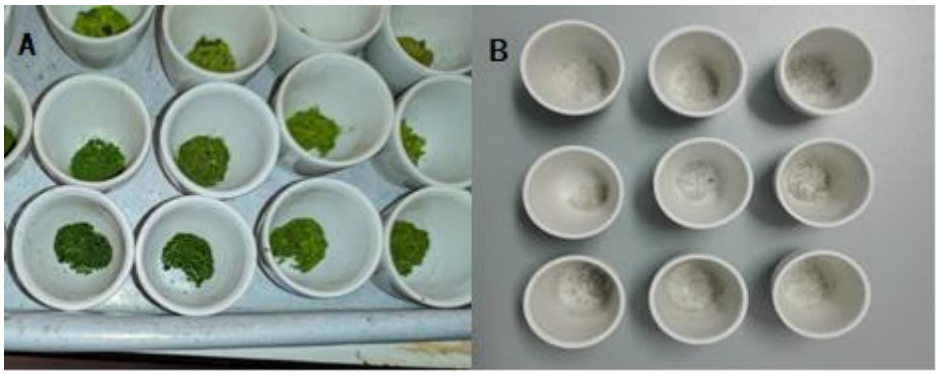

*Figure 10 – **A:** Moment before combustion of the biomass; **B:** moment after combustion of the biomass.*

3.7.4. Lipids quantification

Total lipid content was determined following the procedure described by (Bligh, E.G. and Dyer, 1959) with modifications described by (Pereira *et al.*, 2012). Lyophilized biomass was

weighed in glass tubes ($w_{biomass}$), where, 1 mL, 2 mL and 0.8 mL of chloroform, methanol and distilled water, respectively, were added. Sample were homogenized resorting to an IKA Ultra-Turrax disperser (IKA-Werke GmbH, Staufen, Germany) on ice for 60 s.

Further 1 mL chloroform was added, and the mixture was again homogenized for 30 s (on ice). Finally, 1 mL of distilled water was added, and a final homogenization performed for 30 seconds. The samples were centrifuged (Heraeus™ Multifufe™ X3, USA) at 2000 g for 10 min to allow phase separation. The organic phase (lower layer) was transferred into a clean tube using a *Pasteur* pipette. Then, a known volume of the organic phase was pipetted to a pre-weighed tube (w_i) and dried at 60 °C (Grant Instruments, England).

Figure 11 – Lipids Analyses Procedure A: Separation of lipids; B: Dry Bath at 60 .ºC.

The final weight (w_f) of the tube was measured and the lipids percentage was calculated according to Equation 5:

$$\%\, Total\ Lipid = \frac{\frac{(w_f - w_i) \times Volume_{Chloroform\ total}}{Volume_{Chloroform\ evaporated}}}{w_{biomass}} \quad (5)$$

3.7.5. Determination of FAME

Lipids and free fatty acids were converted to the corresponding FAME, according to adapted protocol of (Lepage and Roy, 1984). 5 to 10 mg of lyophilized biomass was homogenized by the Ultra Turrax (IKA T-25 ULTRA-TURRAX, Cole- Parmer, USA) with 1.5 mL of a methanol/acetyl chloride solution (20:1, v/v), for two cycles (60 and 30 s). 1 mL hexane was added to the samples and heated at 70 °C for 60 min. After cooling on ice, samples content was transferred to glass tubes and 1 mL of distilled water and 4 mL of hexane were added. Samples were vortexed (VV3, VWR, Portugal) at maximum speed in two cycles of 30 s and centrifuged for 5 min at 2000 g (Heraeus Multifuge X3, USA). The supernatant was then transferred to new tubes, extraction repeated twice, and supernatants combined. Anhydrous sodium sulfate was added, and the solution was filtered in 0.22 μm filters (EMD Millipore SLLGC13NL IC Spectra Syringe Filter, USA). A nitrogen gas flow was applied to evaporate the hexane and the dried fraction was immediately resuspended in 0.5 mL gas chromatography-grade hexane and transferred to vials for gas chromatography-mass spectrometry analysis (GC-MS) (SCION 456-GC, Bruker, USA).

A standard solution containing a mixture of 37 different FAMEs (Supelco® 37 Component FAME Mix, Sigma-Aldrich, Portugal) was used to establish calibration curves.

Figure 12 – Different steps on FAME determination. ***A:*** *Derivation vessels before homogenization with Ultra Turrax (lyophilized biomass + 1.5mL methanol/acetyl chloride);* ***B:*** *Biomass homogenization with Ultra Turrax;* ***C:*** *Hexane evaporation under nitrogen gas;* ***D:*** *Gas chromatography-mass spectrometry equipment with autosampler*

3.7.6. Determination of major and minor elements (MP-AES)

Quantitative analysis of major, minor, and some trace elements was performed in Agilent Technologies 4200 microwave plasma-atomic emission spectroscopy (MP-AES), shown in the Figure 13. A diluted solution of the target element (50 ppb) near the inferior limit of detection by MP-AES analysis was used as prepared in four groups as following:

(1) Ca, Fe, K, Mg and Na (0-50 ppm);
(2) Se, Sr, Zn, Cd, Sr, Ba, Cu, Ni, As, Co, Pb, Mo, Mn, Cr and Al (0-10 ppm);
(3) V, Ag, Be, Th and Tl (0-10 ppm);
(4) P (0-50 ppm)

Figure 13 – Agilent Technologies 4200 MP-AES.

Each element was detected with a wavelength previously selected according to the manufacture's procedure. 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:

$$LOD = 3 \times S \quad (6)$$

$$LOQ = 10 \times S \quad (7)$$

where s is the standard deviation of the replicates.

3.7.7. Statistical Analyses

Data are presented as mean values ± standard deviations (SD). The mean values were subjected to one-way ANOVA, followed by Tukey-Kramer multiple comparison method; p values ≤ 0.05 were considered statistically significant. All statistical analyses and graphs were performed using R software (version 3.6.1), through the RStudio IDE (version 1.2.5001). These statistical analyses were only applied to conditions with three biological replicates

4. Results And Discussion

The results are divided into five main sections, on which all details for all experiments are listed and discussed. Each condition was evaluated in terms of growth to choose the best condition to apply in the 7 L fermenter. Three fermenters were assessed in terms of growth and biochemical composition of biomass.

4.1. N concentration optimization

Heterotrophic growth requires a base medium for the microalgae to grow, along with a carbon source, a nitrogen source and a base to pH control. The medium available at Allmicroalgae for organic production of *C. vulgaris*, MNBBIO, is a medium with nitrogen in its constitution. In this sense, the concentration of this element was first optimized.

Nitrogen and phosphorus are macronutrients with a strong influence on microalgae growth. In MNB BIO, these two elements are present at approximate N:P ratio of 17.7:1, which is similar to the Redfield ratio 16:1 reported by previous studies (Leonardos and Geider, 2004; Chiu *et al.*, 2015; Moheimani *et al.*, 2015). However, this ratio is more suitable for marine phytoplankton and in the case of freshwater microalgae, according studies reported by (F. P. Healey, 1980; Hillebrand and Sommer, 1999) , the molar ratio N:P < 13 represented N limitation and N:P > 22 represented severe P limitation. Accordingly, a study elaborated by (Choi and Lee, 2015b), for *C. vulgaris* reported ratio N:P between 11:1 to 19:1 was the range with the highest productivity. Facing an organic production scenario, finding/formulating a growth medium needs to consider the correspondent European guidelines and certification. Allmicroalgae is already certified for organic production in the autotrophic cultivation of *C. vulgaris*. The medium used in autotrophy, consisting of a mixture of MNBBIO and MNMBIO, was also tested in heterotrophy. These two mediums are a complex fertilizer where the MNBBIO is composed of nitrogen (N), phosphorus (P), potassium (K) and organic matter, and MNMBIO has mostly iron (Fe), magnesium (Mg), zinc (Zn), manganese (Mn), molybdenum (Mo), boron (B), and copper (Cu) in its constitution.

The optimized elemental concentrations used for heterotrophic inorganic production were considered when formulating MNBIO from the aforementioned fertilizers. This medium has an acid pH, approximately 4.5, shown in Figure 14.

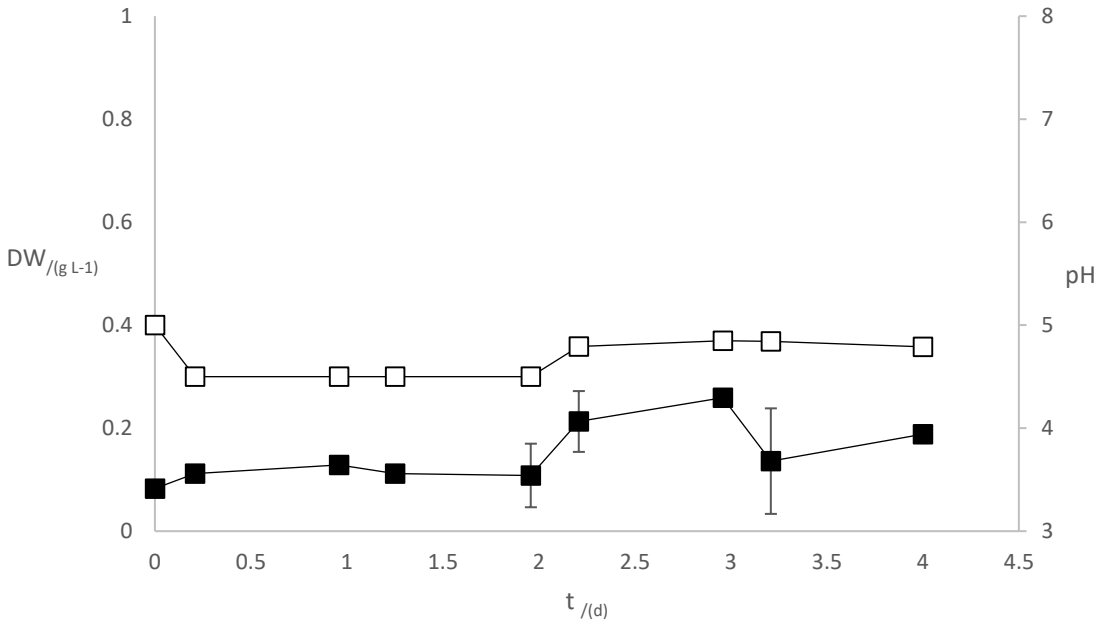

Figure 14 – Chlorella vulgaris *growth at 62 mmol L^{-1} ammonia without addition of buffer. Dry weight of culture (■) and pH variation (□).*

By the analysis of Figure 14, it was found that the pH has an inhibitory effect on *C. vulgaris* growth, i.e. with a too low pH in the medium, the microalgae does not have the capacity to grow. In relation to high pH values, study reported by (Shi, Jiang and Chen, 2002), showed that they have inhibitory effects on growth. Thus, the optimum pH for *C. vulgaris* growth is between pH 6 to 7 (Lustigman, Lee and Khalil, 1995; Sakarika and Kornaros, 2016).

Therefore, it was necessary to use a buffer, PIPES pH 7, to minimize pH interference. This was tested at 50 and 100 mmol L^{-1}, in parallel with the nitrogen concentration. Different concentrations of ammonia were tested:

1) 99 and 62 mmol L^{-1}, using 50 mmol L^{-1} PIPES (A99P50 and A62P50);
2) 83, 62 and 28 mmol L^{-1}, using 100 mmol L^{-1} PIPES (A83P100, A62P100 and A28P100).

The growth curves obtained are shown in Figure 15. The assays were compared with the standard *C. vulgaris* growth in the inorganic production medium (IP) and monitored until the beginning of the stationary phase, after depletion of the carbon and nitrogen source.

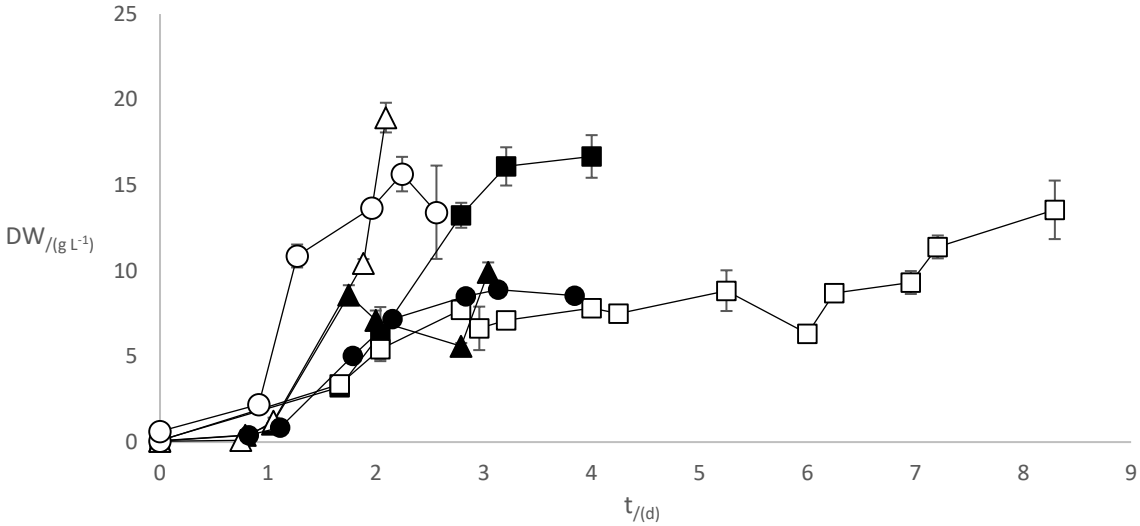

Figure 15 – Screening of Chlorella vulgaris *growth at different N concentration, in 250 mL Erlenmeyer flask. Dry weight of IP (control) (■); of A99P50 (□); of A62P50 (▲); of A62P100 (Δ); of A83P100 (●) and of A28P100 (○). All values refer to the average of data from three independent biological replicates, except in A83P100 which only value represent average from two biological replicates.*

The analysis of Figure 15 shows that a higher ammonia concentration results in low and slow growth. A83P100 resulted in the lowest dry weight at 8.90 g L^{-1} (min:8.67; max:9.14) indicating, possibly, an inhibitory effect. This can be explained based on ammonia toxicity, which affects microalgae growth, having an inhibitory effect at higher concentrations (Park *et al.*, 2010). The condition that resulted in a highest dry weigh was the adjustment to 62 mmol L^{-1} ammonia (100 mmol L^{-1} PIPES), 18.95 ± 0.56 g L^{-1}. The physico-chemical parameters, pH, Glucose in the medium and NH_4^+ consumption, controlled during the assay are shown in the Figures 16, 17 and 18, respectively.

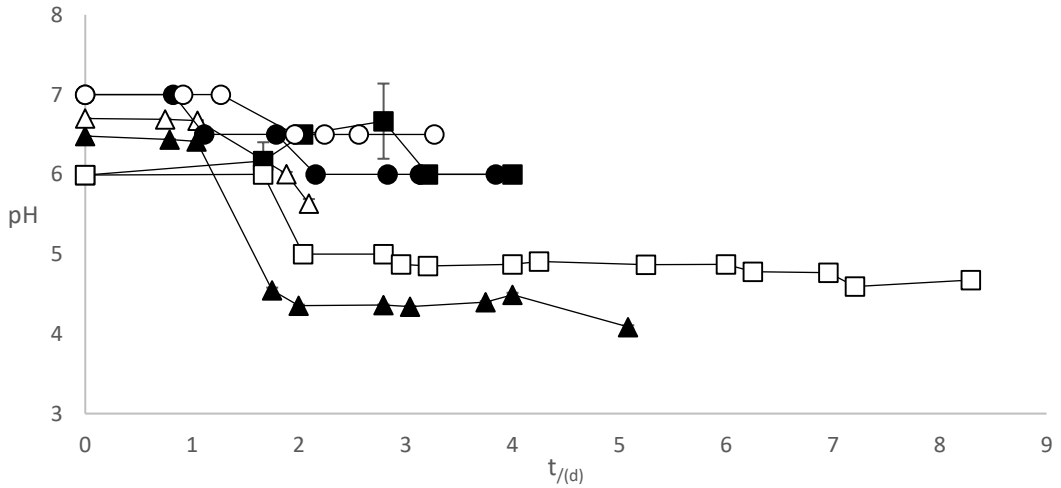

Figure 16 – pH variations of IP (control) (■); of A99P50 (□); of A62P50 (▲); of A62P100 (Δ); of A83P100 (●) and of A28P100 (○). All values refer to the average of data from three independent biological replicates, except in A83P100 which only value represent average from two biological replicates.

The analysis of Figure 16 shows the differences in *C. vulgaris* growth when the pH was controlled with the two buffer concentrations. When cultures were controlled with the 100 mmol L^{-1} PIPES, A62P100, A83P100 and A28P100, pH shows less variation and is maintained within the most suitable pH range. Therefore, in culture under these conditions, final dry weight was higher. In order to obtain greater consistency in the comparison with the other studies, 100 mmol L^{-1} PIPES was used as pH control for the remaining tests.

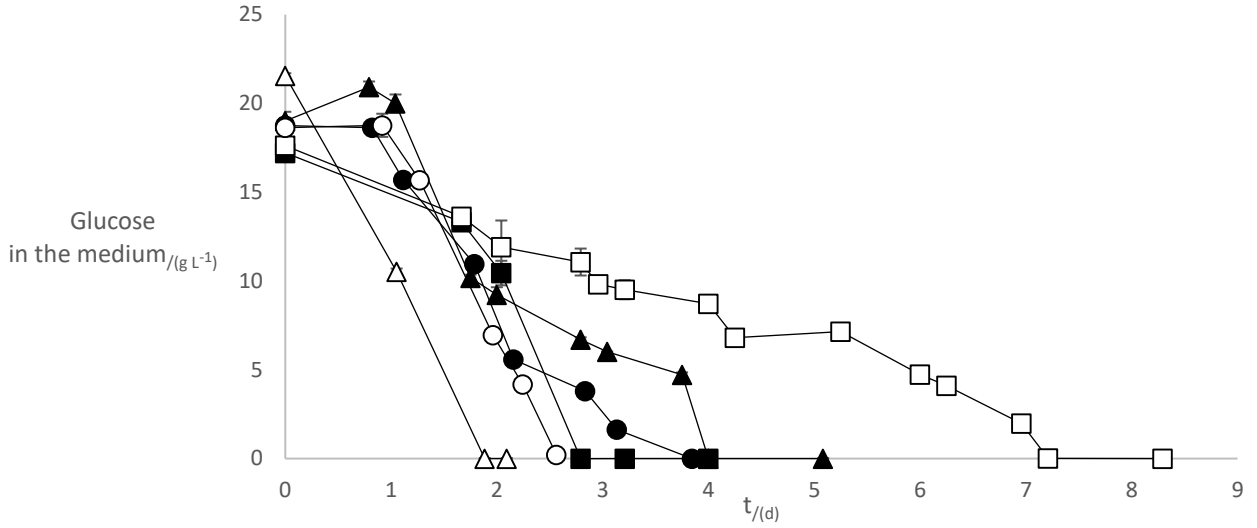

Figure 17 – Glucose concentration in the medium of IP (control) (■); of A99P50 (□); of A62P50 (▲); of A62P100 (Δ); of A83P100 (●) and of A28P100 (○). All values refer to the average of data from three independent biological replicates, except in A83P100 which only value represent average from two biological replicates.

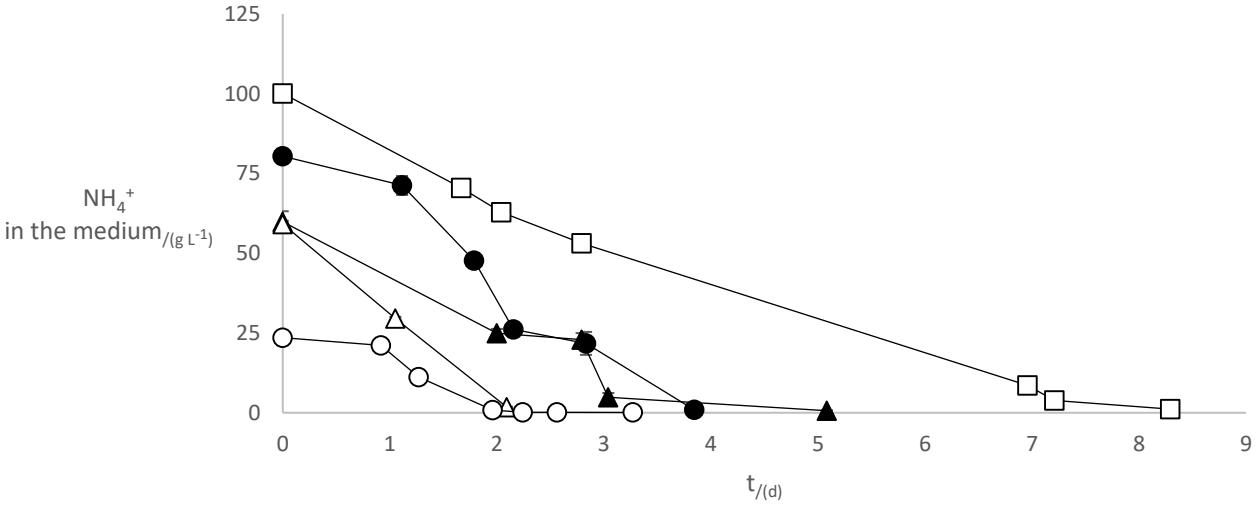

Figure 18 – NH_4^+ concentration in the medium of IP (control) (■); of A99P50 (□); of A62P50 (▲); of A62P100 (Δ); of A83P100 (●) and of A28P100 (○). All values refer to the average of data from three independent biological replicates, except in A83P100 which only value represent average from two biological replicates.

In relation to Figures 17 and 18, glucose and NH_4^+ in the medium, show that higher ammonia concentrations have a slower ammonia and glucose in the medium, A99P50 and A83P100. Consequently, as previously mentioned, slower growth.

The productivity and growth rate calculated are shown in Table 3. Accordingly, the biomass productivity was higher when using A62P100, 9.03 ± 0.42 g $L^{-1}d^{-1}$, and lower using A99P50, 1.63 ± 0.26 g $L^{-1}d^{-1}$. There was no statistically significant difference between A99P50 and A62P50, while the other conditions demonstrated significant differences between them ($p \leq 0.05$). In relation to the condition A83P100, ANOVA statistical analysis was not performed due to the fact that it had only two biological replicates.

The growth rate was also higher when cultures were grown in A62P100, 2.88 ± 0.12 d^{-1} and lower in A99P50, 0.61 ± 0.01 d^{-1}. There were no significant differences between IP and A62P50 ($p > 0.05$). The other conditions demonstrated significant differences between them ($p \leq 0.05$).

Table 3 – Biomass productivities and specific growth rates obtained in the different conditions. Different letters within each parameter indicate significant differences between values on the same column ($p \leq 0.05$). The numeric values represent the mean and standard deviation obtained from three replicates, except in A83P100, which the numeric represent average and (minimum value; maximum value), (min:; max:), due to only two replicates were performed

	$P_{overall}$ /(g $L^{-1}d^{-1}$)	μ /(d^{-1})
IP	4.14 ± 0.31 [a]	1.27 ± 0.02 [a]
A99P50	1.63 ± 0.26 [b]	0.61 ± 0.01 [b]
A62P50	2.86 ± 0.31 [c]	1.32 ± 0.04 [a]
A62P100	9.03 ± 0.42 [d]	2.88 ± 0.12 [c]
A83P100	2.82 (min:2.75; max:2.90)	1.59 (min:1.58; max:1.60)
A28P100	6.08 ± 0.02 [e]	2.19 ± 0.09 [d]

Study reported by (Kim *et al.*, 2013), showed that *Chlorella sorokiniana* when supplemented with ammonia as a nitrogen source in 1000 mL Erlenmeyer (25 °C and shaking speed of 140rpm), had a growth rate of 0.53 d^{-1}. Under the conditions studied, 62 mmol L^{-1} ammonia was the one with the best growth rate, 2.88 ± 0.12 d^{-1}.

As an alternative, another nitrogen source was considered. Organic Yeast Extract (YE) is often mentioned in the literature to grow *Chlorella protothecoides, Thraustochytrid, Aurantiochytrium sp.* and *Nitzschia laevis* (Wen and Chen, 2001; Chen *et al.*, 2010; Heredia-Arroyo, Wei and Hu, 2010; Anwar *et al.*, 2018). Different concentrations of YE were tested, considering as control the best result obtained in the medium MNBIO optimization. Thus, 20 g L^{-1} of YE (YE20), 20 g L^{-1} of YE supplemented with the same concentration used in MNBIO, 0.14 % MNMBIO (YE20+MNM), 10 g L^{-1} of YE without vitamins (YE10) and 10 g L^{-1} of YE with vitamins (YE10+Vit) were tested for the cultivation of *Chlorella vulgaris*. Growth curves are shown in Figure 19. The cultivation parameters are shown in Annex B. All microalgae need micronutrients to grow. The yeast extract is a nitrogen source and in order to optimize this source, it was supplemented with the micronutrient medium, MNMBIO (YE20+MNM). Micronutrients are also important because there are present in the elemental composition of the biomass, and thus need to be supplied.

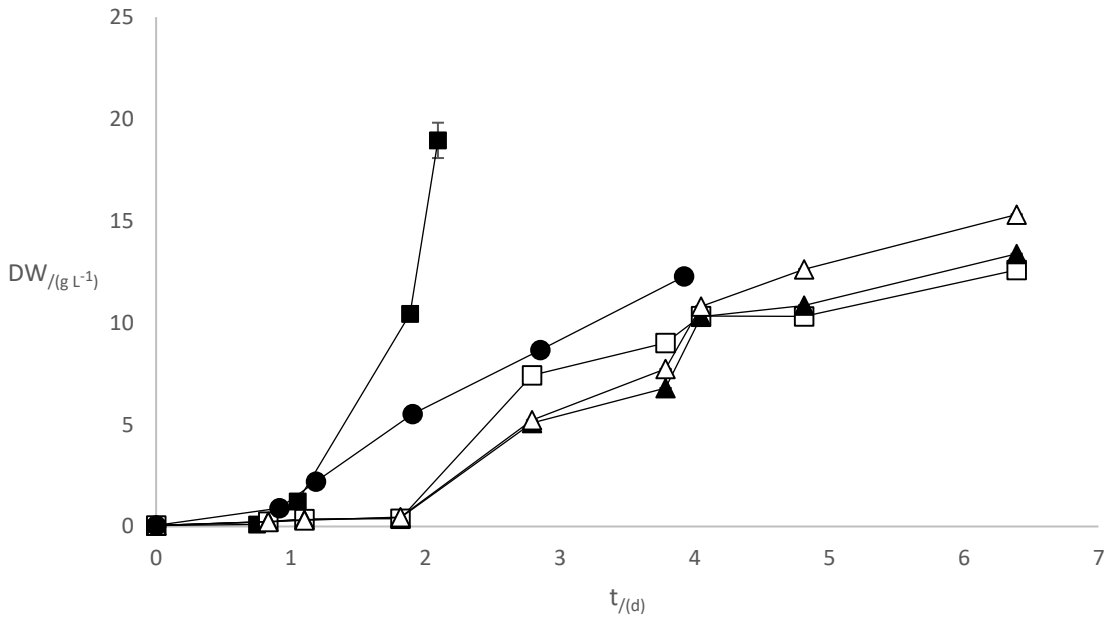

Figure 19 – Dry weight of control, with best condition of MNBIO optimization (■); dry weight of YE20 (□); of YE10 (▲); of YE10+Vit (Δ) and of YE20+MNM (●). Values represent averages of data from two biological replicates, except control, which value represent average from three independent biological replicates, and it is standard deviation.

The highest cellular concentration was obtained in the control (18.95 ± 0.56 g L^{-1}), followed by condition YE10+Vit, 15.31 (min:14.85; max:15.78) g L^{-1}. The remaining conditions, YE20, YE10 and YE20+MNM, dry weight did not show differences taking into account of the variance range. Besides few studies published optimizing heterotrophic medium of *C. vulgaris* with yeast extract, a study using *Chlorella protothecoides* and *Aurantiochytrium* sp. reports that YE is a

good complement to the medium, allowing to obtain a dry weight of 19.81 g L^{-1} with a concentration of 10 g L^{-1} yeast extract, and of approximately 30 g L^{-1} with a concentration of 20 g L^{-1} yeast extract. (Heredia-Arroyo, Wei and Hu, 2010; Gao *et al.*, 2013).

Growth parameters are presented in Table 4. All conditions showed lower productivity and growth rates when compared to the control, leading to the conclusion that *C. vulgaris* grows better in MNBIO. Nevertheless, within this N-source, the condition that, demonstrated higher productivity and growth rate was YE20+MNM, 4.28 (min:4.04; max:4.72) g $L^{-1}d^{-1}$ and 1.91 (min:1.84; max:1.97) d^{-1}, respectively. The remaining concentrations, with productivities of 1.96 (min:1.85; max:2.08) g $L^{-1}d^{-1}$ to YE20, 2.09 (min:2.01; max:2.16) g $L^{-1}d^{-1}$ to YE10 and 2.39 (min:2.31; max:2.49) g $L^{-1}d^{-1}$ to YE10+Vit had no differences between them, taking into account the variance range. The results are in accordance with the obtained for the specific growth rate.

Table 4 – Biomass productivities and specific growth rates obtained in the yeast extract study. In control, the numeric value represent the mean and standard deviation obtained from three replicates. In YE20, YE10, YE10+Vit and YE20+MNM, the numeric values represent the mean and (minimal value; maximum value)

	$P_{overall}$ /(g $L^{-1}d^{-1}$)	μ /(d^{-1})
Control	9.03 ± 0.42	2.88 ± 0.12
YE20	1.96 (min:1.85; max:2.08)	0.88 (min:0.87; max:0.90)
YE10	2.09 (min:2.01; max:2.16)	0.87 (min:0.85; max:0.88)
YE10+Vit	2.39 (min:2.31; max:2.49)	0.88 (min:0.88; max:0.89)
YE20+MNM	4.38 (min:4.04; max:4.72)	1.91 (min:1.84; max:1.97)

62 mmol L^{-1} ammonia in MNBIO was the condition that resulted in the higher *C. vulgaris* growth productivity and growth rate. Thus, it is applied hereafter.

4.2. Carbon Source Optimization

The carbon source is one of the constituents of the medium that most affects growth and the biochemical composition of the microalgae (Prathima Devi, Venkata Subhash and Venkata Mohan, 2012; Lin and Wu, 2015). Facing organic production, it was necessary to test medium components approved for organic production by the European certification. Therefore, Bio-

Invertzuckersirup 73 % was tested. In experiments with this organic sugar, 20 g L^{-1} glucose has been taken into account.

In order to verify the influence of this product on growth, two sterilization procedures were tested: filtration (SF+V) and autoclavation (SA+V), as represented in Figure 20. In this assay, these two sterilization procedures were evaluated since after autoclavation, the solution turned into a yellow-brown color, due to Maillard reaction. In this reaction, reducing sugars react with amino acids present in the medium leading to a series of different toxic compounds to microalgae, such as 5 – hydroxymethylfurfural (HMF) (Jandlova and Kucerova, 2016).

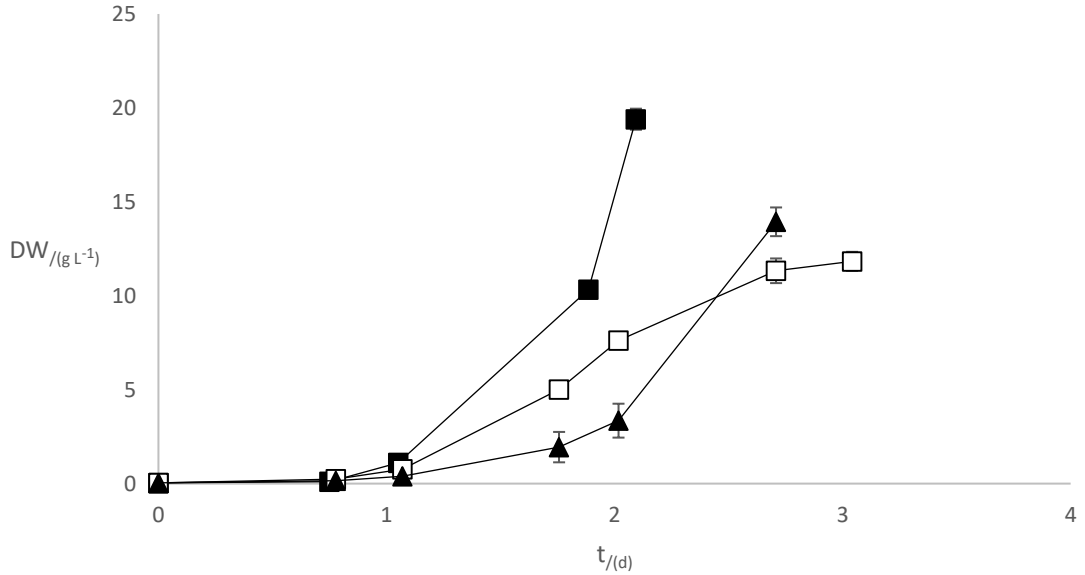

Figure 20 – Heterotrophic growth of Chlorella vulgaris *in 250mL Erlenmeyer flask using two procedures to sterilize organic sugar. Dry weight of control, growth with glucose (■); dry weight of SF+V (□) and SA+V (▲). All values represent averages of data from three independent biological replicates.*

Comparing the two sterilization procedures, *C. vulgaris* growth was higher in SA, 13.94 ± 0.76 g L^{-1}, however, this one presented a longer lag phase. This happened due to the presence of compounds formed in the Maillard reaction, HMF. HMF up to 1.13 g L^{-1} was reported to cause strong inhibition of *Spirulina maxima* growth (Yu *et al.*, 1990). This product is an organic certified sugar and it is 73 % hydrolyzed, which is an advantage because microalgae preferably consume simple molecules, monosaccharides (in this case, glucose and fructose), rather than more complex molecules, disaccharide (sucrose).

In an industrial fermentation and taking into account environmental sustainability, raw materials and waste (among others) must be minimized, maximizing yield and productivity. As the medium is composed by complex compounds, MNBBIO, MNMBIO and Bio-Invertzuckersirup 73 %, there may be components that equalize the effect of vitamins. In this sense, an evaluation was made for the influence of vitamins on *C. vulgaris* growth when organic carbon was used (Figure 21) to evaluate its need. Thus, *C. vulgaris* growth, where the carbon source was glucose, was studied with (Glu+Vit) and without (Glu) vitamins. The same method was applied for both sterilization procedures, by filtration with (SF+Vit) and without (SF) vitamins and by autoclavation with (SA+Vit) and without (SA) vitamins. The cultivation parameters are shown in Annex C.

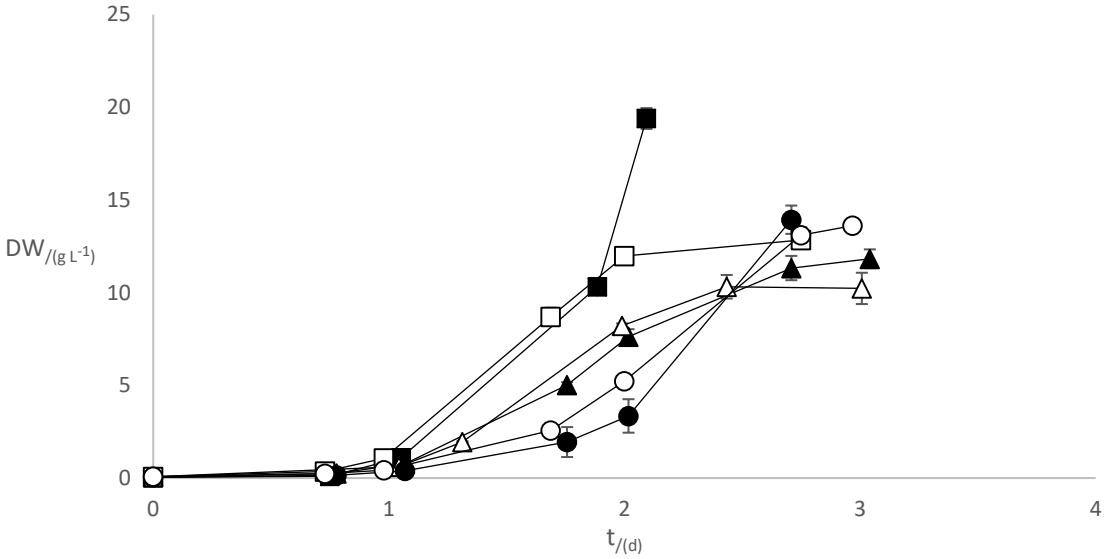

Figure 21 – Heterotrophic growth of Chlorella vulgaris *in 250mL Erlenmeyer flask verifying the vitamins effect. Dry weight of Glu+Vit (■); of Glu (□); of SF+Vit (▲); of SF (Δ); of SA+Vit (●) and of SA (○). Values represent averages of data from three independent biological replicates.*

The highest cellular concentration was in Glu+Vit, 19.40 ± 0.56 g L^{-1} and the lowest cellular concentration was in SF+Vit, 10.23 ± 0.84 g L^{-1} ($p \leq 0.05$). In relation to organic sugar and its sterilization procedures, statistically there are no significant differences between with and without vitamins conditions ($p > 0.05$). However, between the conditions with different sterilizations showed significant differences between them ($p \leq 0.05$), with higher cell concentration in SA+Vit, 13.94 ± 0.76 g L^{-1}.

The growth parameters about all conditions studied regarding the carbon source are presented in Table 5. In vitamin conditions, SA+Vit showed higher productivity, 4.98 ± 0.14 g $L^{-1}d^{-}$

^{1}, compared to SF+Vit, 3.97 ± 0.09 g $L^{-1}d^{-1}$ ($p \leq 0.05$). However, the same not happened with growth rate, with no significant differences between these two conditions ($p > 0.05$). When no vitamins were added to the medium, there were no statistically significant differences in productivity between the sterilization procedures, 4.27 ± 0.34 g $L^{-1}d^{-1}$ and 4.79 ± 0.24 g $L^{-1}d^{-1}$ to SF and to SA, respectively ($p > 0.05$).

Table 5 – Biomass productivities and specific growth rates obtained in the yeast extract study. Different letters within each parameter indicate significant differences between values on the same column (p ≤ 0.05). The numeric values represent the mean and standard deviation obtained from three replicates

		$P_{overall}$ /(g $L^{-1}d^{-1}$)	μ /(d^{-1})
With Vitamins	*Glu + Vit*	9.25 ± 0.26 ^{a}	2.94 ± 0.08 ^{a}
	SF + Vit	3.97 ± 0.09 ^{b}	1.90 ± 0.01 ^{b,c}
	SA + Vit	4.98 ± 0.14 ^{c}	2.18 ± 0.01 ^{b}
Without Vitamins	*Glu*	4.94 ± 0.01 ^{b,c}	1.95 ± 0.07 ^{b,c}
	SF	4.27 ± 0.34 ^{b,c}	2.07 ± 0.02 ^{b,c}
	SA	4.79 ± 0.24 ^{b,c}	1.76 ± 0.21 ^{c}

In this investigation, the growth parameters, dry weight, productivity, and growth rate, there were no significant differences between the conditions testing with and without vitamins. These results are not in accordance with (S. . Desouky, 2011; S. A. Desouky, 2011), who works with *Scenedesmus obliquus* and *C. vulgaris* that reported that growth, pigment and protein content were increased significatively under the presence of vitamins. This was, possibly, due to the medium being of inorganic origin. After the analysis of the carbon source study, it was found that the growth with and without vitamins did not have significant influence. Thus, in conditions without vitamins, the two sterilization procedures did not present significant differences between them and due to the formation of HMF, a possible inhibitory factor, it was chosen to use sugar sterilized by filtration.

4.3. pH control

The pH of the algal culture media plays a very important role in regulating the uptake of essential nutrients and microalgae (Zhang, Wang and Hong, 2014). Thus, heterotrophic reactions are run with pH control through addition on-demand of acid or alkaline solutions. According to

European legislation, three certified solutions were found: (1) calcium carbonate (CB), (2) potassium bicarbonate (PB) and (3) sodium bicarbonate (SB).

Calcium carbonate was tested but experiments were discontinued due to its poor solubility. As this reagent was poorly soluble in water, it did not dissolve. Therefore, it has not contradicted the acidity of the medium pH, not functioning as a pH controller. The other solutions were added at the initial moment in order to correct the pH of the medium (from, approximately, 4.5 to 6.5) and during the exponential phase, on which there is a tendency to rapidly decrease the pH. Pointing out that growth has only been controlled with these products, without the addition of PIPES (Figure 22 and 23). The cultivation parameters are shown in Annex D. *C. vulgaris* growing in a medium on which pH was controlled by sodium bicarbonate, had lower final DW, 8.72 (min:8.43; max:9.01) g L^{-1}, compared to 14.82 (min:14.18; max:15.46) g L^{-1} of potassium bicarbonate, taking into account the variance range.

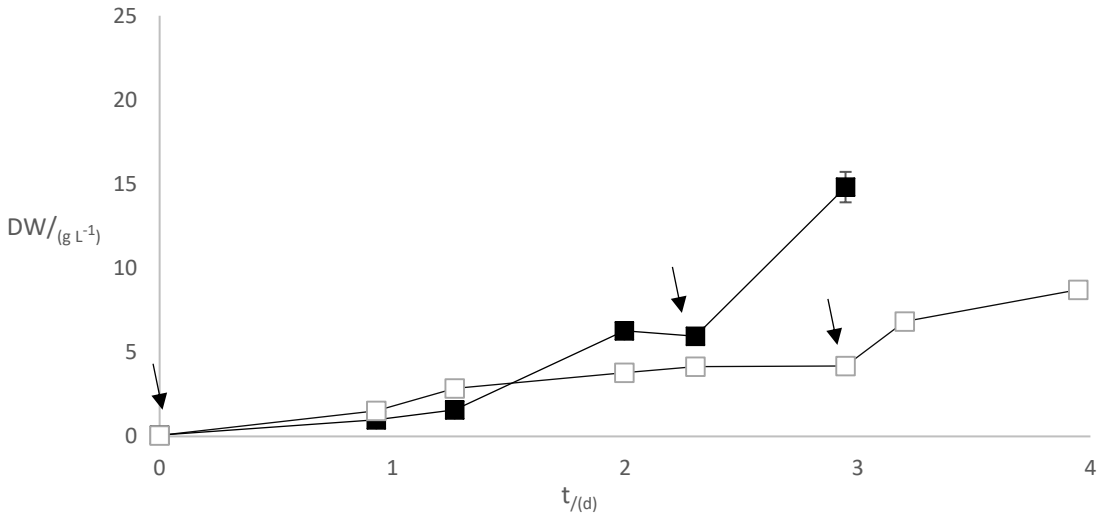

Figure 22 – Heterotrophic growth of Chlorella vulgaris *in 250mL Erlenmeyer flask with different solutions to pH control. Dry weight to culture with PB (■) and with SB (□). The symbol ↓ represents the time of the addition of solutions. First ↓ corresponds to both conditions. Values represent averages of data from two independent biological replicates.*

Biomass productivities and growth rates were obtained in both conditions and are resumed in Table 6. *C. vulgaris* growing in a medium on which pH is controlled by potassium bicarbonate showed, higher productivity and a higher growth rate, 5.00 (min:4.79; max:5.22) g $L^{-1}d^{-1}$ and 1.79 (min:1.80; max:1.78) d^{-1}.

The results obtained given that, in principle, the lower productivity and growth rate being obtained were in the condition where the pH of the medium is corrected with sodium bicarbonate.

In addition, the biomass produced is for human consumption. If the biomass contains sodium, this element is associated with problems of hypertension, cardiovascular disease, kidney malfunction and early aging. For these reasons, people are not very interested in food with a lot of sodium. Thus, the chosen base for controlling the pH in 7 L bench-top fermenter was the potassium solution.

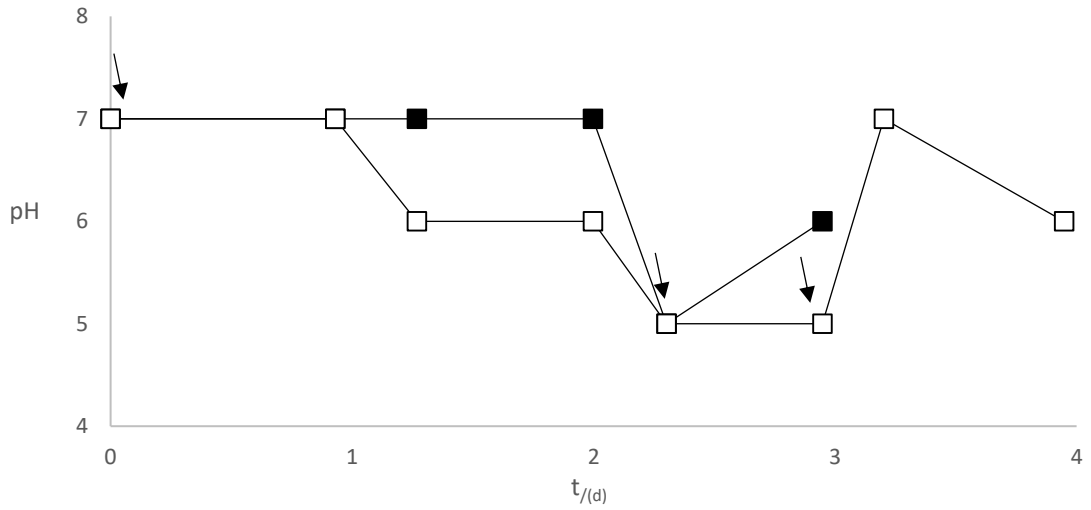

Figure 23 – pH variation to culture with PB (■) and with SB (□). The symbol ↓ represents the time of the addition of solutions. First ↓ corresponds to both conditions. Values represent averages of data from two independent biological replicates.

Table 6 – Biomass productivities and specific growth rates obtained in the choice of the best compound to pH control

	$P_{overall}$ /(g $L^{-1}d^{-1}$)	μ /(d^{-1})
Growth with PB as pH controller	5.00 (min:4.79; max:5.22)	1.79 (min:1.80; max:1.78)
Growth with SB as pH controller	2.19 (min:2.12; max:2.28)	1.26 (min:1.24; max:1.28)

4.4. Fed-Batch Growth in 7 L bench-top fermenter

A scale-up process was performed from a 250 mL Erlenmeyer flask to a 1000 mL Erlenmeyer flask and from there to the 7 L bench-top fermenter. To assess the viability of the organic medium optimized, $MNBIO_{optimized}$ in fed-batch mode, three assays were performed. The conditions implemented in each test are mentioned in Table 7. These were based on the results obtained in the previous tests. Basically, the requisites that change from fermenter to fermenter

were the carbon source, since the first was Glu and the rest with SF. In relation to antifoam, three different products were tested in order to verify their effectiveness and viability: (1) Organic extra virgin olive oil, (2) Organic sunflower oil and (3) inorganic antifoam Foam doctor. The latter antifoam was used to check the *C. vulgaris* growth potential in this medium, since it is used in the inorganic growth of the production department. These assays are shown in Figure 24, 25, 26, 27 and 28, being the Figure 25 to 28 referring to the cultivation.

Table 7 – Conditions implemented in three 7L bench-top Fermenter on the cultivation of organic Chlorella vulgaris

	Fermenter 1	*Fermenter 2*	*Fermenter 3*
C Source	Glucose	Organic Sugar	Organic Sugar
Antifoam	Organic extra virgin olive oil	Organic Sunflower Oil	Foam Doctor

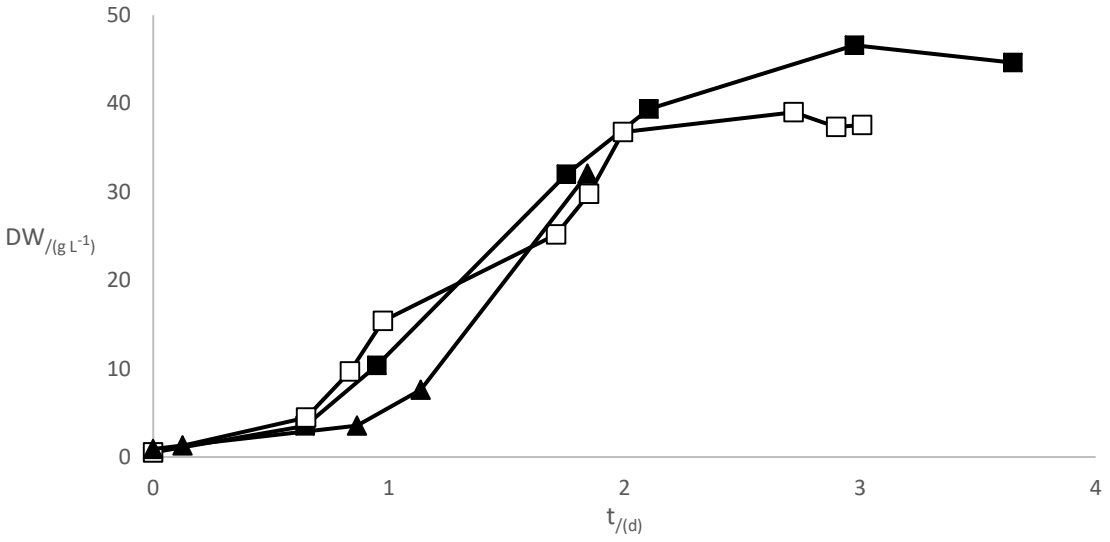

Figure 24 – Heterotrophic growth of Chlorella vulgaris *in 7L Bench Top fermenter. Dry weight of growth for fermenter 1 (■), for fermenter 2 (□) and for fermenter 3 (▲).*

Fermenter 1 reached a maximum concentration of 46.6 g L^{-1}. However, this biomass concentration may not be the real maximum. After two days of inoculation, the culture started to form foam and the anti-foam used, organic virgin olive oil, was not effective in a way that too much quantity was fed and the foam was not controlled. Due to this occurrence, the excessive amount of olive oil may have inhibited its growth. In relation to fermenter 2, the maximum dry weight was

39.0 g L^{-1}. Here, the growth may also have been inhibited by the previous reason. The antifoam used, organic sunflower oil, was also not effective in defoaming the culture. The fermenter 3 reached a maximum dry weight of 32.1 g L^{-1}. However, this fermenter was forced to finish during growth due to contamination. In addition, this had a higher lag phase, possibly due to the initial ammonia concentration batch being higher than expected (94 mmol L^{-1} ammonia), which could have been due to evaporation, as the fermenter had to be sterilized twice in this particular case.

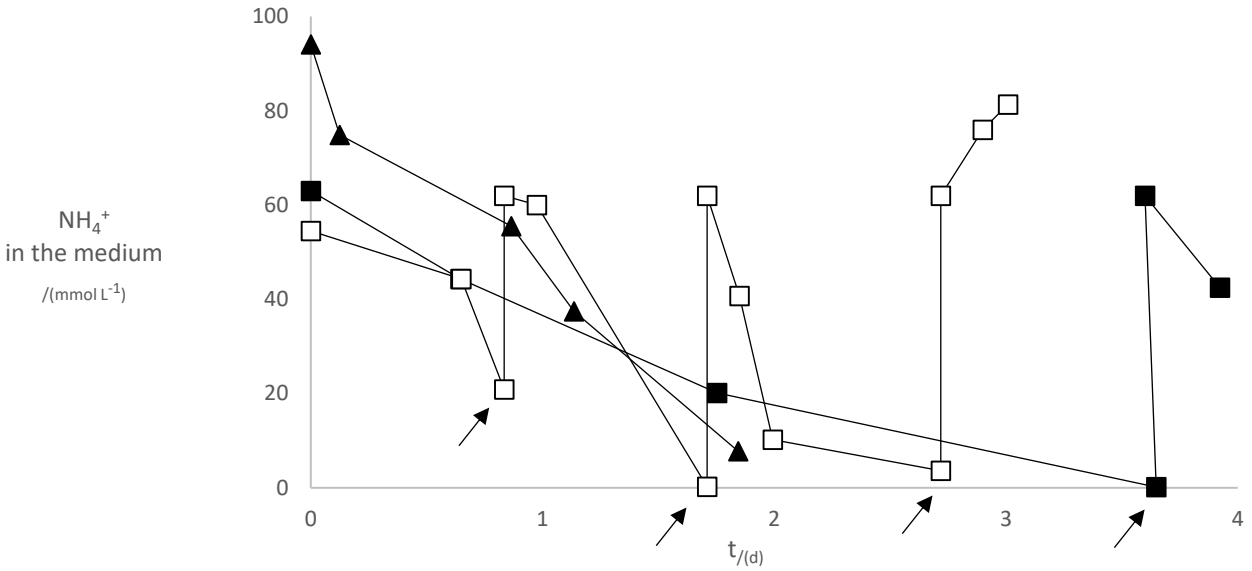

Figure 25 – NH_4^+ consumption of C. vulgaris *growth for fermenter 1 (■), for fermenter 2 (□) and for fermenter 3 (▲). The symbol ↓ represents the time of the addition of ammonia through MNBBIO.*

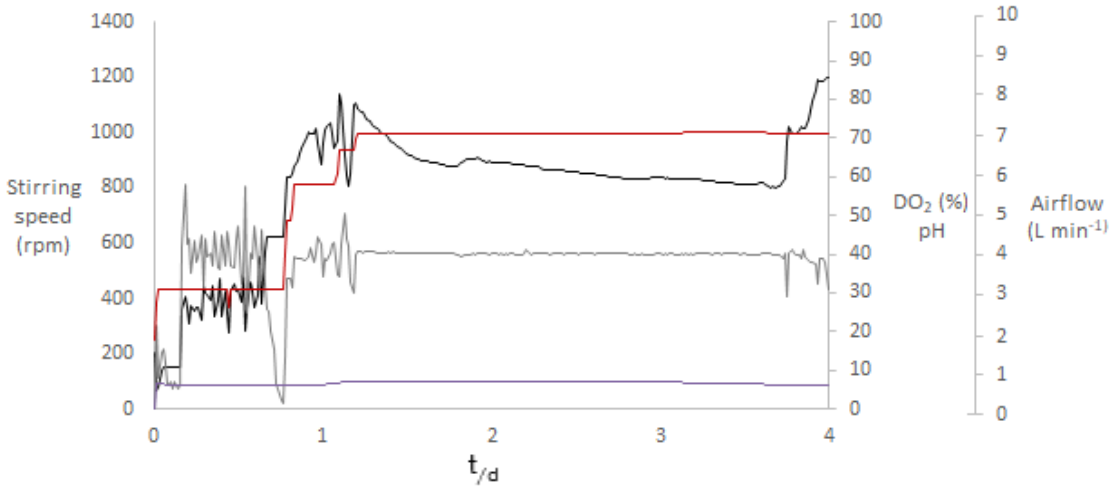

Figure 24 – Cultivation parameters of C. vulgaris *growth for fermenter 1, stirring speed (black line), DO_2 (grey line), pH (purple line) and airflow (red line).*

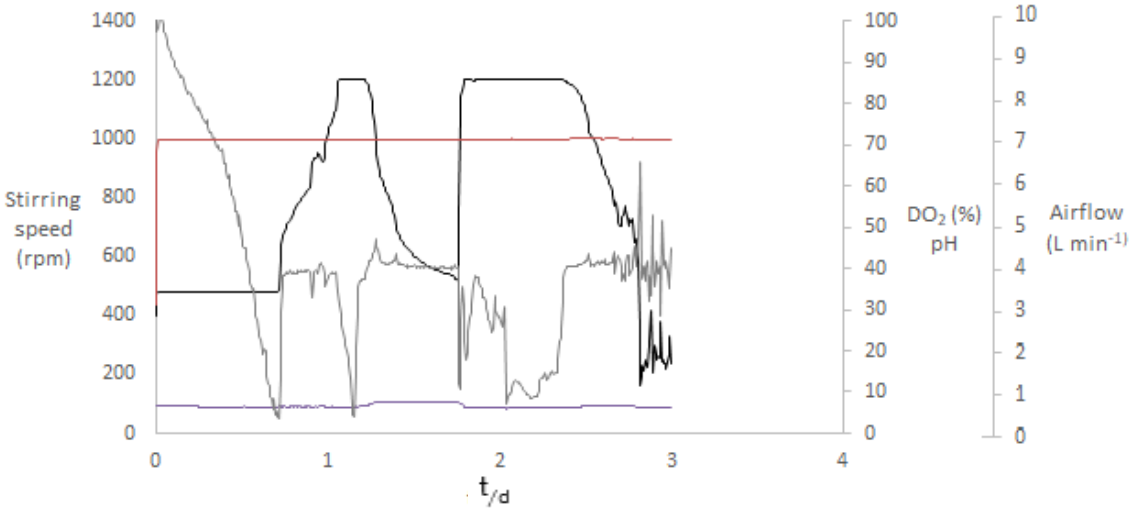

Figure 26 – Cultivation parameters of C. vulgaris *growth for fermenter 2, stirring speed (black line), DO_2 (grey line), pH (purple line) and air flow (red line).*

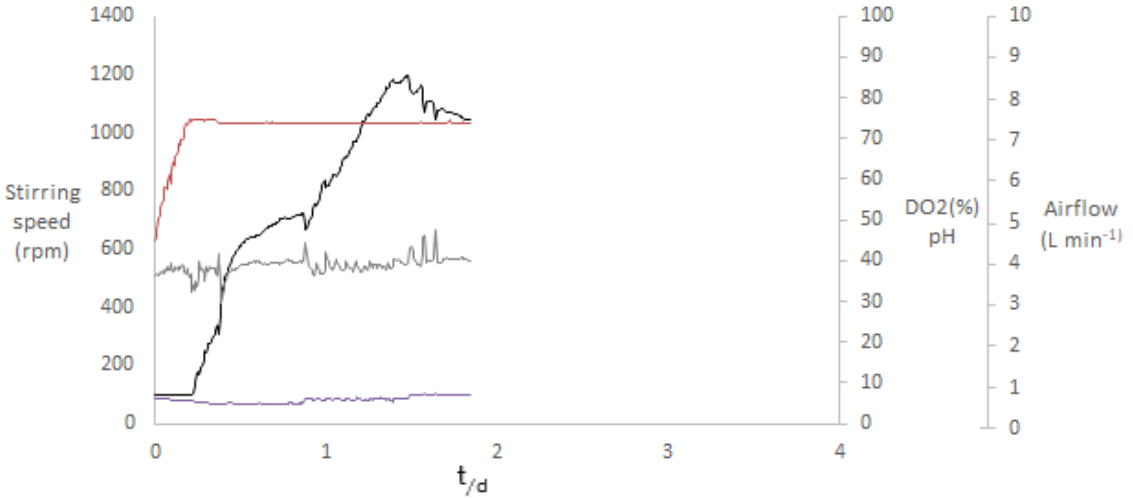

Figure 25 – Cultivation parameters of C. vulgaris *growth for fermenter 3, stirring speed (black line), DO_2 (grey line), pH (purple line) and airflow (red line).*

Observing Figure 27, it was noted that the cultivation parameters in fermenter 2 oscillated and were not stable compared to the other fermenters. This was due to the fact that in this fermenter was supplemented with ammonia several times, through MNBBIO. After each addition, the parameters were attempted to adjust (by automation) to that impact.

The values obtained for productivity and specific growth for fermenter 1, 2 and 3, are shown in Table 8.

Table 8 – Biomass productivities and specific growth rates obtained for the fermenter 1, 2 and 3. The numeric values represent the result obtained for a single test in each condition

	$P_{overall}$ /(g $L^{-1}d^{-1}$)	μ /(d^{-1})
Fermenter 1	15.5	1.51
Fermenter 2	14.2	1.59
Fermenter 3	16.9	1.93

According to the values obtained, it is believed that the full potential of the medium was not obtained. In relation to a study reported by (Barros *et al.*, 2019), it showed that the cultivation of *C. vulgaris* in the 7 L fermenter, using inorganic medium, achieved a biomass productivity of 27.3 ± 6.8 g $L^{-1}d^{-1}$, a higher value than that obtained in this thesis. This previous study practically works as a control since the same strain and conditions are studied, except the medium and carbon source. Besides few studies using organic certified medium of *C. vulgaris*, a study using *C. protothecoides* and inorganic medium, in 3.7 L fed-batch fermenter, reported biomass productivities of 4.8 g $L^{-1}d^{-1}$ and growth rate of 1.06 d^{-1} (Shi, Jiang and Chen, 2002). These values were lower than those obtained by the $MNBIO_{optimized}$.

4.5. Biochemical composition of biomass

One of the aims of this work was to understand if the growth of *C. vulgaris* using $MNBIO_{optimized}$ satisfied the requirements for a food product. Thus, by comparison of the biomass composition obtained in the first two fermentations, fermenter 1 and fermenter 2, (with organic virgin extra olive oil and organic sunflower oil as antifoam, respectively) with the standard composition of inorganic *C. vulgaris* heterotrophic biomass are mentioned in the Table 9 and a statistical analysis was made of the two fermenters tested. The biochemical analysis was only performed for two fermenters, since the third fermenter was only executed after the date of the biochemical analysis.

Table 9 – Elemental composition of biomass in inorganic and organic production with MN BIO developed for Chlorella vulgaris*. The numeric value represented were obtained for a single test in each condition*

Element	*Allmicroalgae Inorganic Production /(mg kg^{-1})*	*Organic Production Fermenter 1 /(mg kg^{-1})*	*Organic Production Fermenter 2 /(mg kg^{-1})*
Ag	0.05	< LOD	< LOD
Al	0.9	5.0	10.0
As	< LOD	< LOD	< LOD
Ba	1.2	< LOD	< LOD
Be	< LOD	< LOQ	< LOQ
Ca	3480.8	456.8	316.2
Cu	2.9	11.4	13.9
Fe	60.3	410.0	635.0
K	7325.0	18650.9	17728.8
Mg	701.7	841.0	1016.7
Mn	71.6	134.5	145.8
Mo	1.6	< LOD	< LOD
Na	2447.0	2316.0	1603.1
Ni	1.1	0.5	< LOD
P	480.7	941.3	929.0
Sb	3.6	< LOD	< LOD
Si	21.4	-	-
Sn	1.6	< LOD	< LOD
Sr	125.7	1.2	0.4
V	3.4	< LOD	< LOD
Zn	208.8	132.3	120.9

With the use of conventional fertilizers, an overall optimization of the elements is practically impossible since the concentrations are fixed. Thus, in an optimization with fertilizers based on a single but important element (in this case, nitrogen), the remaining elements, whether macro or micronutrients, may be in excessive or small quantities. When comparing inorganic with organic production on the cultivation of *C. vulgaris*, the differences obtained in elemental concentrations

are due to the initial balance made to the optimized elemental concentrations used in heterotrophic inorganic production. For this reason, it is necessary to pay attention to mass balances, as these differences may be limiting growth. Having made a mass balance, it was noted that the phosphorus was given in excess because the microalgae did not assimilate it large amount (approximately 28 % of the provided in the two fermenters). Some elements had lower concentrations (stand out, calcium, strontium and zinc) and others had higher concentrations (stand out, aluminum, copper, iron and phosphorus) in relation to the inorganic medium used in production department. However, of these elements mentioned above, for example, calcium, their percentage in the elementary description of the MNBIO is not quantified.

Regarding lipids, fatty acids and ashes, these were measured in the two tested fermenters (with olive oil and sunflower oil as antifoam). These values are listed in Table 10 and 11.

Lipid content in both fermenters was lower than the values reported by (Morowvat and Ghasemi, 2018), which, in 5 L fermenter with inorganic medium, obtained for concentrations of 3, 7 and 10 g L^{-1} glucose, 26, 39 and 44%, respectively. On the other hand, the value reported by (Liang, Sarkany and Cui, 2009b) (23.00 ± 2.00 %) was similar to the results obtained in fermenter 2. Fermenter 1 had a much lower lipid concentration than reported in the literature. In relation to carbohydrates content, the abrupt difference obtained between fermenter 1 and 2 was due to the biomass produced in fermenter 1 having little lipid content and protein content. Study reported by (Illman, Scragg and Shales, 2000), the maximum carbohydrate content obtained was 55.0 ± 3.2 %, while another reported by (Chia, Lombardi and Melão, 2013) was similar to that obtained in fermenter 2. Ash content is one of the critical parameters that determines the quality of algae biomass for various applications. The results obtained in this thesis were similar to the results obtained by (Roostaei *et al.*, 2018), of 9.1 ± 0.5 % ash content. Compared to Allmicroalgae's inorganic production, average value of 5.3 % (min:4.6 %; max:6.0 %), the content in organic cultivation was higher in both fermenters.

The FAME profile of biologically produced *Chlorella vulgaris* was mainly composed of polyunsaturated fatty acids (PUFAs) by, basically, linoleic acid with a maximum produced percentage value of 68.19 %. The quality of biomass produced by *C. vulgaris* is justified by the amount of PUFAs present in its constitution, since they are well know for their beneficial effects on human health and the nutritional value of microalgae (Guihéneuf and Stengel, 2013). The main fatty acids detected in the organic *C. vulgaris* here produced, arranged in descending order, were

linoleic (C18:2), oleic (C18:1), palmitic (C16:0), palmitoleic acid (C16:1). Other fatty acids were also contained in trace amounts. Results reported by (Lam and Lee, 2012; Choi and Lee, 2015a; Process *et al.*, 2015; Rohit and Venkata Mohan, 2018; Rushan *et al.*, 2019) lead to the conclusion that the FAME profile is a characteristic fingerprint for a specific microalgae. The percentages of majority FAME compound were the C18:1, 34.54 ± 2.06 %, following by C16:0, 15.54 ± 0.67 % and C18:2, 9.42 ± 1.31 %, to study elaborated by (Choi and Lee, 2015a). Results reported by (Rohit and Venkata Mohan, 2018), the majority compounds were the C16:0, 37.1 %, followed by C18:2, 20 %.

Table 10 – Biomass proximal composition. Values are presented in a dry weight basis. The numeric values were obtained for a single test in each condition

	Fermenter 1	*Fermenter 2*
Lipids (%)	6.13	17.75
Carbohydrates (%)	68.71	38.83
Ashes (%)	7.22	7.02

Table 11 – Fatty acid profile of the biomass produced. The numeric values were obtained for a single test in each condition

	Fermenter 1 (%)	*Fermenter 2 (%)*
C16:1	3.52	-
C16:0	18.87	12.30
C17:0	0.30	0.39
C18:2	49.83	68.19
C18:1	27.32	18.29
C18:0	0.16	0.83
TOTAL SFA	19.33	13.52
TOTAL MFA	30.84	18.29
TOTAL PUFA	49.83	68.19
ω – 6	49.83	68.19
PUFA/SFA	2.58	5.04

Protein and pigments were measured in the three fermenters. These values are listed in Table 12 to comparison with inorganic comparation. The biomass produced in fermenter 2 has more protein content than the inorganic production. However, the same does not happen in others, having less content. Protein content in fermenter 2, 36.41%, indicated an amount similar to that reported by (Liang, Sarkany and Cui, 2009b) (32 %), by (Xie *et al.*, 2017) (38.6 %) and by (El-Sheekh and Fathy, 2009) (36.5%), but superior to the other fermenter 1 and 3, 17.94 % and 14.36 %, respectively. In terms of pigments, the values obtained indicated that the maximum chlorophylls amount produced was 11.73 mg g^{-1} DW, higher than inorganic production, 6.58 ± 2.73 mg g^{-1} DW. This value was lower than that reported by (Doucha and Lívanský, 2012), where it obtained for *C. vulgaris* in 5 L fermenter, approximately, 1.6 % of total chlorophyll, i.e. 16 mg g^{-1} DW. However, study elaborated by (El-Sheekh and Fathy, 2009), the chlorophyll content of *C. vulgaris* biomass grown under heterotrophic conditions (250 mL Erlenmeyer flask, inorganic medium and 10 g L^{-1} glucose) was 0.37 %, i.e. 3.7 mg g^{-1} DW. This value was lower than obtained in this thesis.

Table 12 – Comparison of biochemicals parameters on cultivation of C. vulgaris using inorganic (standard) and optimized organic, in 7 L bench-top fermenter. The numeric value represented were obtained for a single test in each condition

	Inorganic Production	*Organic Production*		
		Fermenter 1	Fermenter 2	Fermenter 3
Proteins /(%)	24.52 ± 7.15 (n = 71)	17.94	36.41	14.36
Total Chlorophyll /(mg g-1)	6.58 ± 2.73 (n = 92)	9.59	11.73	7.95

Pointing out that, when cells grow under heterotrophic conditions using carbon sources in organic form, their photosynthetic system does not need to function and because of this, metabolites synthesis can be inhibited. This is probably why the chlorophyll and proteins content are lower in this growth regime (Taylor, 2014).

4.6. Hetero- to autotrophic sequential cultivation

The main objective of this assay was to verify the induced impact on biomass by the change from hetero- to autotrophic system. The growth of the culture was monitored for 6 days and the results are shown in Figure 29 and Table 13. The conditions implemented in this growth were similar to the current autotrophic conditions of large-scale production in the Allmicroalgae production department.

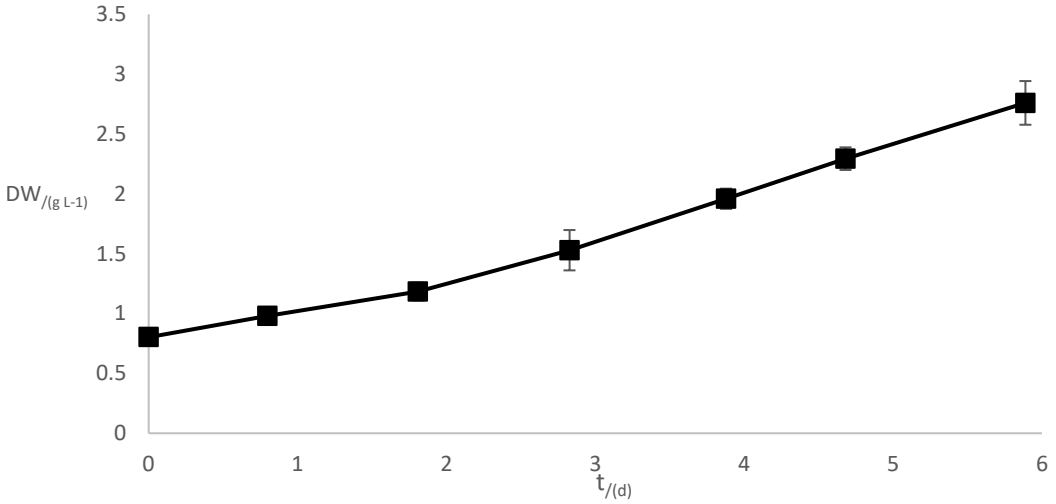

Figure 27 – Growth curves of C. vulgaris grown in flat panel photobioreactors using organic heterotrophic inoculum. Values represent averages of data from three biological replicates.

*Figure 28 – Two stage cultivation. **A:** Organic Heterotrophic Inoculum. **B:** Three flat panels with same conditions after inoculation. **C:** Flat panels after 4 days of inoculation.*

By the analysis of Figure 29, it was observed that with an organic heterotrophic inoculum, *C. vulgaris* grew and tripled its dry weight after six days, 0.80 ± 0.05 to 2.76 ± 0.18 g L^{-1}. Some microalgae present dual-trophic characteristics, i.e., they have the capacity to grow in photoautotrophic and heterotrophic conditions (Zheng *et al.*, 2012). For example, *C. vulgaris*, *C. sorokiana*, *Scenedesmus obliquus* and *Micractinium* sp. are microalgae with these characteristics (Paranjape, Leite and Hallenbeck, 2016; Santana *et al.*, 2017; Abomohra *et al.*, 2018). Their metabolism combines heterotrophic and photoautotrophic mechanisms, being able to switch between each other. In Allmicroalgae, the production of biologically certified *C. vulgaris* is achieved in two stages. The first is in heterotrophy starting in 7 L fermenter and is scaled-up to the 5000 L industrial fermenter. Subsequently, the biomass produced in the 5000 L fermenter is be used to inoculate the autotrophic industrial PBRs, on which cultures grow under autotrophic conditions. This dual procedure saves time and area required for the production of the inoculum (Barros *et al.*, 2019). Allmicroalgae's organic production system is optimized and applied for autotrophy. However, it starts from an heterotrophic inoculum produced under inorganic conditions. Only after inoculation of PBRs and after a few generations, does the cultivation of *C. vulgaris* complies with the requirements of European legislation for organic production. However, during this procedure, if the heterotrophic inoculum was already organically produced, it would save more time and there was the potential to create a new organic product. To simulate this process on a small scale, the biomass produced in fermenter was used to inoculate three flat panel photobioreactors with a volumetric capacity of 67 L (Figure 22).

One way to evaluate the impact was trough biochemical analysis of biomass in terms of proteins and pigments, which are presented in Table 13. The protein content of the inoculum obtained from heterotrophic growth was 17.9 %. Moving to photoautotrophic conditions, there was an increase in the amount of protein to 48.4 ± 5.5 % after six days. According to (Bertoldi, Sant'Anna and Oliveira, 2008), *C. vulgaris* cultivated in Bold Basal Medium (BBM) had a protein content of, approximately, 52.4%. Studies reported by (Yusof *et al.*, 2011) had a protein content of 42.55 ± 0.18 % with culture condition 12:12h photoperiod. The protein value obtained in this test is within the values found in the literature. Regarding the pigments, after 6 days of autotrophic growth there was an increase in the amount, of 9.59 to 23.32 ± 0.39 mg $_{\text{Total Chlorophyll}}$ g^{-1} DW. These results are similar to those obtained in the study reported by (Barros *et al.*, 2019), where the biomass produced in *C. vulgaris* originated from heterotrophy showed similar values of total chlorophyll.

Point out that the chlorophyll and proteins content is affected by the limitation of macronutrients (for example, N and P depletion) and light availability (Hu, 2004).

Table 13 - Comparison of biomass quality before and after the impact of the change in energy and carbon source. Evaluation in terms of proteins and pigments, more specifically, total chlorophyll. The numeric value in heterotrophic inoculum represented were obtained for a single test in each condition and in autotrophic growth, the numeric value represent average and standard deviation of three biological replicates

	Proteins (%)	*Pigments (mg g-1 DW)*
Heterotrophic inoculum	17.94	9.59
Autotrophic growth	48.44 ± 5.52	23.32 ± 0.39

5. Conclusions and Recommendations

Interest in microalgae biomass has been increasing in the global market due to their high nutritional value and the variety of technological applications. Therefore, many systems have been developed aiming to optimize biomass yields and added-value compounds production. In this context, new low-cost medium, different initial concentrations of microalgae and different processes in cultivation types have been studied.

Based on the results obtained in the optimization of the nitrogen source, it was concluded that the ammonia concentration has effects on cell growth, with concentrations higher than 62 mmol L^{-1} ammonia concentration induce a growth inhibition. pH value's outside the optimal range, between pH of 6 and 7, had also a significant effect on growth of *C. vulgaris*. Regarding the carbon source certified as organic it proved to be adequate for production, although with lower yields than those obtained with glucose. Thus, sterilize this sugar by filtration was the most suitable and most favorable method for this microalgae growth. Finally, potassium bicarbonate, the base selected to control pH, it was possible to maintain the pH within the range without causing stress in the *C. vulgaris* growth. With the optimized medium, $MNBIO_{optimized}$, applied for *C. vulgaris* growth proved to be very promising due it is a complex medium made up of the elements necessary for growth. However, the organically certified products used to control the foam were not effective, once they did not prevent the formation of foam. Simulating a sequential hetero to- autotrophic system in small scale, the organic heterotrophic inoculum has strong potential to switch to autotrophic conditions, since it had a much higher productivity. This cultivation strategy is promising to provide a more efficient way of microalgae biomass production due to increase in protein and chlorophyll content.

In relation to the biochemical analysis of biomass, in terms of fatty acid profile, the biomass produced was mainly composed by PUFAs, an important factor for the biomass quality. The chlorophyll and ashes content were higher in organic cultivation when compared to biomass of inorganic production. However, the protein content was lower in two fermenters performed faced with inorganic cultivation.

From a general perspective, a product with organic certification is an asset due it guarantees health benefits, taking into account the environment and food safety. Thus, MNBIO has become very interesting as a heterotrophic medium with organic certification. In addition, a carbon

source and a product to pH control found were certified for organic production and worked to make it possible to grow organic heterotrophic biomass according to guidelines of European legislation. Thus, in further approaches, the effect of temperature on sterilization of the carbon source, in this case, organic sugar, should be studied trying to understand the influence on the appearance of HMF. In addition to this, it would be interesting to use other antifoams with the approval of European legislation, such as certified silicone oil. It would also be very interesting to do a scale-up for the 200 L fermenter, checking the viability of this medium in this type of fermenter. In addition, as the medium used was a fertilizer mixture, filtering the medium before adding it to the fermenter could be a good approach to test.

References

Abomohra, A. E. F. *et al.* (2018) 'Effect of lipid-free microalgal biomass and waste glycerol on growth and lipid production of Scenedesmus obliquus: Innovative waste recycling for extraordinary lipid production', *Bioresource Technology*. Elsevier, 249(October 2017), pp. 992–999.

Ahmad, A. L. *et al.* (2011) 'Microalgae as a sustainable energy source for biodiesel production: A review', *Renewable and Sustainable Energy Reviews*, 15(1), pp. 584–593.

Aims of organic farming (2019). Available at: https://ec.europa.eu.

Amaro, H. M., Guedes, A. C. and Malcata, F. X. (2011) 'Advances and perspectives in using microalgae to produce biodiesel', *Applied Energy*. Elsevier Ltd, 88(10), pp. 3402–3410.

Anwar, S. H. *et al.* (2018) 'Utilization of different nitrogen sources for the growth of microalgae isolated from mangrove leaves in Banda Aceh - Indonesia', *IOP Conference Series: Earth and Environmental Science*, 207(1).

Barad, M. (2007) *Anatomical, Molecular, and Cellular Substrates of Fear Extinction.*, *Fear and learning: From basic processes to clinical implications.*

Barkia, I., Saari, N. and Manning, S. R. (2019) 'Microalgae for high-value products towards human health and nutrition', *Marine Drugs*, 17(5), pp. 1–29.

Barros, A. *et al.* (2019) 'Heterotrophy as a tool to overcome the long and costly autotrophic scale-up process for large scale production of microalgae', *Scientific Reports*. Springer US, (September), pp. 1–7.

Becker, E. W. (2007) 'Micro-algae as a source of protein', *Biotechnology Advances*, 25(2), pp. 207–210.

Benedetti, M. *et al.* (2018) 'Biomass from microalgae: The potential of domestication towards sustainable biofactories', *Microbial Cell Factories*. BioMed Central, 17(1), pp. 1–18.

Benemann, J. R. (1997) 'CO2mitigation with microalgae systems', *Energy Conversion and Management*, 38(SUPPL. 1), pp. 475–479.

Bertoldi, F. C., Sant'Anna, E. and Oliveira, J. L. B. (2008) 'Teor de clorofila e perfil de sais minerais de Chlorella vulgaris cultivada em solução hidropônica residual', *Ciencia Rural*, 38(1), pp. 54–58.

Bligh, E.G. and Dyer, W. J. (1959) 'Canadian Journal of Biochemistry and Physiology', *Canadian Journal of Biochemistry and Physiology*, 37(8).

Bohutskyi, P. *et al.* (2014) 'Mineral and non-carbon nutrient utilization and recovery during sequential phototrophic-heterotrophic growth of lipid-rich algae', *Applied Microbiology and Biotechnology*, 98(11), pp. 5261–5273.

Borowitzka, M. A. (1999) 'Commercial production of microalgae: ponds, tanks, and fermenters', *Progress in Industrial Microbiology*, 35(C), pp. 313–321.

Boyle, N. R. and Morgan, J. A. (2009) 'Flux balance analysis of primary metabolism in Chlamydomonas reinhardtii', *BMC Systems Biology*, 3(February 2009).

Brennan, L. and Owende, P. (2010) 'Biofuels from microalgae-A review of technologies for production, processing, and extractions of biofuels and co-products', *Renewable and Sustainable Energy Reviews*, 14(2), pp. 557–577.

Bruland, K. W., Donat, J. R. and Hutchins, D. A. (1991) 'Interactive influences of bioactive trace metals on biological production in oceanic waters', *Limnology and Oceanography*, 36(8), pp. 1555–1577.

Chen F (1996) 'High cell density culture of microalgae in heterotrophic growth', *Trends in Biotechnology*, 14(11), pp. 421–426.

Chen, G. *et al.* (2010) 'Optimization of nitrogen source for enhanced production of squalene from thraustochytrid Aurantiochytrium sp', *New Biotechnology*. Elsevier B.V., 27(4), pp. 382–389.

Chen, G. Q. and Chen, F. (2006) 'Growing phototrophic cells without light', *Biotechnology Letters*, 28(9), pp. 607–616.

Chia, M. A., Lombardi, A. T. and Melão, M. da G. G. (2013) 'Growth and biochemical composition of Chlorella vulgaris in different growth media', *Anais da Academia Brasileira de Ciencias*, 85(4), pp. 1427–1438.

Chiu, S. Y. *et al.* (2015) 'Cultivation of microalgal Chlorella for biomass and lipid production using wastewater as nutrient resource', *Bioresource Technology*. Elsevier Ltd, 184, pp. 179–189.

Choi, H. J. and Lee, S. M. (2015a) 'Biomass and oil content of microalgae under mixotrophic conditions', *Environmental Engineering Research*, 20(1), pp. 25–32. doi: 10.4491/eer.2014.043.

Choi, H. J. and Lee, S. M. (2015b) 'Effect of the N/P ratio on biomass productivity and nutrient removal from municipal wastewater', *Bioprocess and Biosystems Engineering*, 38(4), pp. 761–766.

CHOJNACKA, K.; MARQUEZ-ROCHA, F. J. (2004) 'Kinetic and stoichometric relationships of the energy and carbon metabolism in the culture of microalgae. Biotechnology'. Toronto, pp. 21–34.

Coelho, D. *et al.* (2019) 'Novel combination of feed enzymes to improve the degradation of Chlorella vulgaris recalcitrant cell wall', *Scientific Reports*, 9(1), pp. 1–11.

Davis, R., Aden, A. and Pienkos, P. T. (2011) 'Techno-economic analysis of autotrophic microalgae for fuel production', *Applied Energy*. Elsevier Ltd, 88(10), pp. 3524–3531.

Dean, A. P. *et al.* (2010) 'Using FTIR spectroscopy for rapid determination of lipid accumulation in response to nitrogen limitation in freshwater microalgae', *Bioresource Technology*. Elsevier Ltd, 101(12), pp. 4499–4507.

Demirbas, A. and Fatih Demirbas, M. (2011) 'Importance of algae oil as a source of biodiesel', in *Energy Conversion and Management*.

Desouky, S. . (2011) 'Response of Vitamins on the Growth Criteria and Some Mteabolic Activities of Stressed Scenedesums Obliquus Cultures .', *Australian Journal of Basic and Applied Sciences,* 5(6), pp. 89–99.

Desouky, S. A. (2011) 'Effect of some natural organic additives on the growth and photosynthesis of pollutant - Chlorella Vulgaris Beijer', *Journal of Applied Sciences Research*, 7(1), pp. 23–32.

Doucha, J. and Lívanský, K. (2012) 'Production of high-density Chlorella culture grown in fermenters', *Journal of Applied Phycology*, 24(1), pp. 35–43.

Dragone, G. *et al.* (2011) 'Nutrient limitation as a strategy for increasing starch accumulation in microalgae', *Applied Energy*. Elsevier Ltd, 88(10), pp. 3331–3335.

Drews-, P. *et al.* (2013) 'Microalgae classification using semi-supervised and active learning based on Gaussian mixture models', *Journal of the Brazilian Computer Society*, 19(4), pp. 411–422.

Dupont, C. L., Barbeau, K. and Palenik, B. (2008) 'Ni uptake and limitation in marine Synechococcus strains', *Applied and Environmental Microbiology*, 74(1), pp. 23–31.

Duteil, B. (2016) *EU Organic Label - Basics about the EU Sustainability standard*.

El-Sheekh, M. M. *et al.* (2014) 'Influence of Molasses on Growth, Biochemical Composition and Ethanol Production of the Green Algae Chlorella vulgaris and Scenedesmus obliquus', *Journal of Agricultural Engineering and Biotechnology*, (May), pp. 20–28.

El-Sheekh, M. M. and Fathy, A. A. (2009) 'Variation of some nutritional constituents and fatty acid profiles of chlorella vulgaris Beijerinck grown under auto and heterotrophic conditions', *International Journal of Botany*, 5(2), pp. 153–159.

Enzing, C. *et al.* (2014) *Micro-algal production systems*, *Microalgae-based products for the food and feed sector: an outlook for Europe*.

F. P. Healey, L. L. H. (1980) 'Physiological Indicators of Nutrient Deficiency in Lake Phytoplankton', *Canadian Journal of Fisheries and Aquatic Sciences*, 37, pp. 442–453.

Fan, J. *et al.* (2014) 'Lipid accumulation and biosynthesis genes response of the oleaginous Chlorella pyrenoidosa under three nutrition stressors', *Biotechnology for Biofuels*. Biotechnology for Biofuels, 7(1), pp. 1–14.

Ferguson, Wilfred; Braunschweiger, K. (1980) 'Hydrogen ion buffers for biological research', *Analytical Biochemistry*, 104(2), pp. 300–310.

Ferreira, C. M. H. *et al.* (2015) '(Un)suitability of the use of pH buffers in biological, biochemical and environmental studies and their interaction with metal ions-a review', *RSC Advances*. Royal Society of Chemistry, 5(39), pp. 30989–31003.

FIBL; IFOAM (2018) 'The World of Organic Agriculture. Statistics & Emerging Trends 2019', p. 353.

Forján, E. *et al.* (2007) 'Enhancement of carotenoid production in Nannochloropsis by phosphate and sulphur limitation', *Communicating Current Research and Educational Topics and Trends in Applied Microbiology*, 1(January), pp. 356–364.

Fradique, Ḿonica *et al.* (2010) 'Incorporation of Chlorella vulgaris and Spirulina maxima biomass in pasta products. Part 1: Preparation and evaluation', *Journal of the Science of Food and Agriculture*, 90(10), pp. 1656–1664.

Freitas, H. R. (2017) 'Chlorella vulgaris as a Source of Essential Fatty Acids and Micronutrients: A Brief Commentary', *The Open Plant Science Journal*, 10(1), pp. 92–99.

G, P. *et al.* (2018) 'Growing Chlorella vulgaris into bioreactors set in solar greenhouse, towards biofuel: Nutrient composition', *Journal of Applied Biotechnology & Bioengineering*, 5(2), pp. 102–106.

Gao, M. *et al.* (2013) 'Isolation and characterization of Aurantiochytrium species: High docosahexaenoic acid (DHA) production by the newly isolated microalga, Aurantiochytrium sp. SD116', *Journal of Oleo Science*, 62(3), pp. 143–151.

Good, N.; Izawa, S. (1972) 'Hydrogen ion buffers', *Methods in Enzymology*, 24, pp. 53–68.

Grady, J. (1998) 'Radicals from "Good's" buffers', *Analytical Biochemistry*, 173, pp. 111–115.

Grima, E. M., Acie, F. G. and Chisti, Y. (1999) 'Grima1999.Pdf', 70, pp. 231–247.

Guihéneuf, F. and Stengel, D. B. (2013) 'LC-PUFA-enriched oil production by microalgae: Accumulation of lipid and triacylglycerols containing n-3 LC-PUFA is triggered by nitrogen limitation and inorganic carbon availability in the marine haptophyte Pavlova lutheri', *Marine Drugs*, 11(11), pp. 4246–4266.

Heredia-Arroyo, T., Wei, W. and Hu, B. (2010) 'Oil accumulation via heterotrophic/mixotrophic chlorella protothecoides', *Applied Biochemistry and Biotechnology*, 162(7), pp. 1978–1995.

Hillebrand, H. and Sommer, U. (1999) 'The nutrient stoichiometry of benthic microalgal growth: Redfield proportions are optimal', *Limnology and Oceanography*, 44(2), pp. 440–446.

Hu, Q. (2004) 'Environmental Effects on Cell Composition', in *Handbook of Microalgal Culture: Applied Phycology and Biotechnology*, pp. 83–93.

Illman, A. M., Scragg, A. H. and Shales, S. W. (2000) 'Increase in Chlorella strains calorific values when grown in low nitrogen medium', *Enzyme and Microbial Technology*, 27(8), pp. 631–635.

Jandlova, M. and Kucerova, J. (2016) 'Hydroxymethylfurfural in syrups, doughs and in syrup´s biscuits', *MendelNet 2016*, (76), pp. 577–581.

Jankowska, E., Sahu, A. K. and Oleskowicz-Popiel, P. (2017) 'Biogas from microalgae: Review on microalgae's cultivation, harvesting and pretreatment for anaerobic digestion', *Renewable and Sustainable Energy Reviews*. Elsevier Ltd, 75(October 2015), pp. 692–709.

Jha, D. *et al.* (2017) 'Microalgae-based Pharmaceuticals and Nutraceuticals: An Emerging Field with Immense Market Potential', *ChemBioEng Reviews*, 4(4), pp. 257–272.

Joshi, S., Kumari, R. and Upasani, V. N. (2018) 'Applications of algae in cosmetics : an overview', *International Journal of Innovative Research in Science, Engineering and Technology*, 7(March), pp. 1269–1278.

Khademi, F. *et al.* (2014) 'An assessment of microalgae cultivation potential using liquid waste streams: opportunities and challenges', *13th International Conference on Clean Energy*, (January), pp. 2084–2097.

Khan, M. I., Shin, J. H. and Kim, J. D. (2018) 'The promising future of microalgae: Current status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products', *Microbial Cell Factories*. BioMed Central, 17(1), pp. 1–21.

Kim, S. *et al.* (2013) 'Growth rate, organic carbon and nutrient removal rates of Chlorella sorokiniana in autotrophic, heterotrophic and mixotrophic conditions', *Bioresource Technology*. Elsevier Ltd, 144, pp. 8–13.

Kumar, A. *et al.* (2010) 'Enhanced CO 2 fixation and biofuel production via microalgae: Recent developments and future directions', *Trends in Biotechnology*. Elsevier Ltd, 28(7), pp. 371–380.

Lam, M. K. and Lee, K. T. (2012) 'Immobilization as a feasible method to simplify the separation of microalgae from water for biodiesel production', *Chemical Engineering Journal*. Elsevier B.V., 191, pp. 263–268.

Leesing, R. and Kookkhunthod, S. (2011) 'Heterotrophic Growth of Chlorella sp . KKU-S2 for Lipid Production using Molasses as a Carbon Substrate', *Food Engineering*, 9, pp. 87–91.

Lembo, G. and Menre, E. (2019) *Organic Aquaculture. Impacts and future developments*.

Lemesle, V. and Mailleret, L. (2008) 'A mechanistic investigation of the algae growth "droop" model', *Acta Biotheoretica*, 56(1–2), pp. 87–102.

Leonardos, N. and Geider, R. J. (2004) 'Responses of elemental and biochemical composition of Chaetoceros muelleri to growth under varying light and nitrate:phosphate supply ratios and their influence on critical N:P', *Limnology and Oceanography*, 49(6), pp. 2105–2114.

Lepage, G. and Roy, C. C. (1984) 'Improved recovery of fatty acid through direct transesterification without prior extraction or purification', *Journal of Lipid Research*, 25(12), pp. 1391–1396.

Liang, Y., Sarkany, N. and Cui, Y. (2009a) 'Biomass and lipid productivities of Chlorella vulgaris

under autotrophic, heterotrophic and mixotrophic growth conditions', *Biotechnology Letters*, 31(7), pp. 1043–1049.

Liang, Y., Sarkany, N. and Cui, Y. (2009b) 'Biomass and lipid productivities of Chlorella vulgaris under autotrophic, heterotrophic and mixotrophic growth conditions', *Biotechnology Letters*, 31(7), pp. 1043–1049.

Lin, T. S. and Wu, J. Y. (2015) 'Effect of carbon sources on growth and lipid accumulation of newly isolated microalgae cultured under mixotrophic condition', *Bioresource Technology*. Elsevier Ltd, 184, pp. 100–107.

Lowry, Randall, R. J. and Lewis, A. (no date) 'Méthode de Lowry'.

Lustigman, B., Lee, L. H. and Khalil, A. (1995) 'Effects of nickel and pH on the growth of Chlorella vulgaris', *Bulletin of Environmental Contamination and Toxicology*, 55(1), pp. 73–80.

Mallick, N. *et al.* (2012) 'Green microalga Chlorella vulgaris as a potential feedstock for biodiesel', *Journal of Chemical Technology and Biotechnology*, 87(1), pp. 137–145.

Markou, G. and Nerantzis, E. (2013) 'Microalgae for high-value compounds and biofuels production: A review with focus on cultivation under stress conditions', *Biotechnology Advances*. Elsevier Inc., 31(8), pp. 1532–1542.

Markou, G., Vandamme, D. and Muylaert, K. (2014) 'Microalgal and cyanobacterial cultivation: The supply of nutrients', *Water Research*. Elsevier Ltd, 65, pp. 186–202.

Mason, R. (2001) 'Chlorella and spirulina: Green supplements for balancing the body', *Alternative and Complementary Therapies*, 7(3), pp. 161–165.

Mata, T. M., Martins, A. A., Caetano, N. S. (2010) 'Microalgae for biodiesel production and other applications', in *A review; Renewable and Sustainable Energy Reviews*, pp. 217–232.

Merchant, S. S. *et al.* (2006) 'Between a rock and a hard place: Trace element nutrition in Chlamydomonas', *Biochimica et Biophysica Acta - Molecular Cell Research*, 1763(7), pp. 578–594.

Miao, X. and Wu, Q. (2006) 'Biodiesel production from heterotrophic microalgal oil', *Bioresource Technology*, 97(6), pp. 841–846.

Mobin, S. M. A., Chowdhury, H. and Alam, F. (2019) 'Commercially important bioproducts from

microalgae and their current applications-A review', *Energy Procedia*. The Authors, 160(2018), pp. 752–760.

Moheimani, N. R. *et al.* (2015) *Past, Present and Future of Microalgae Cultivation Developments*.

Molina, E. *et al.* (2001) 'Tubular photobioreactor design for algal cultures', *Journal of Biotechnology*, 92(2), pp. 113–131.

Morales-Sánchez, D. *et al.* (2013) 'Heterotrophic growth of Neochloris oleoabundans using glucose as a carbon source', *Biotechnology for Biofuels*, 6(1).

Morowvat, M. H. and Ghasemi, Y. (2018) 'Maximizing Biomass and Lipid Production in Heterotrophic Culture of Chlorella vulgaris: Techno-Economic Assessment', *Recent Patents on Food, Nutrition & Agriculture*, 10(2), pp. 115–123.

Nagira, K. *et al.* (1995) 'Effects of organic pH buffers on a cell growth and an antibody production of human-human hybridoma HB4C5 cells in a serum-free culture', *Cytotechnology*, 17(2), pp. 117–125.

Panahi, Y. *et al.* (2012) 'Investigation of the effects of Chlorella vulgaris as an adjunctive therapy for dyslipidemia: Results of a randomised open-label clinical trial', *Nutrition and Dietetics*, 69(1), pp. 13–19.

Panis, G. and Carreon, J. R. (2016) 'Commercial astaxanthin production derived by green alga Haematococcus pluvialis: A microalgae process model and a techno-economic assessment all through production line', *Algal Research*. The Authors, 18, pp. 175–190.

Paranjape, K., Leite, G. B. and Hallenbeck, P. C. (2016) 'Effect of nitrogen regime on microalgal lipid production during mixotrophic growth with glycerol', *Bioresource Technology*. Elsevier Ltd, 214, pp. 778–786.

Park, J. *et al.* (2010) 'Ammonia removal from anaerobic digestion effluent of livestock waste using green alga Scenedesmus sp.', *Bioresource Technology*. Elsevier Ltd, 101(22), pp. 8649–8657.

Pereira, C.M.P.; Hobuss, C.B.; Maciel, J.V.; Ferreira, L.R.; Pin, F. B. (2012) 'Biodiesel renovável derivado de microalgas: avanços e perspetivas tecnológicas. Química Nova', 35, pp. 2013–2018.

Pereira, H. *et al.* (2012) 'Polyunsaturated fatty acids of marine macroalgae: Potential for nutritional and pharmaceutical applications', *Marine Drugs*, 10(9), pp. 1920–1935.

Perez-Garcia, O. *et al.* (2011) 'Heterotrophic cultures of microalgae: Metabolism and potential products', *Water Research*. Elsevier Ltd, 45(1), pp. 11–36.

Pires, J. C. M. *et al.* (2012) 'Carbon dioxide capture from flue gases using microalgae: Engineering aspects and biorefinery concept', *Renewable and Sustainable Energy Reviews*. Elsevier Ltd, 16(5), pp. 3043–3053.

Prathima Devi, M., Venkata Subhash, G. and Venkata Mohan, S. (2012) 'Heterotrophic cultivation of mixed microalgae for lipid accumulation and wastewater treatment during sequential growth and starvation phases: Effect of nutrient supplementation', *Renewable Energy*. Elsevier Ltd, 43, pp. 276–283.

Process, T. *et al.* (2015) 'Biotechnology & Biomaterials Assessment of Triacylglycerol Content in Chlorella vulgaris Cultivated in a', 5(4), pp. 2–5.

Queiroz, M. I. *et al.* (2007) 'The kinetics of the removal of nitrogen and organic matter from parboiled rice effluent by cyanobacteria in a stirred batch reactor', *Bioresource Technology*, 98(11), pp. 2163–2169.

Richmond, A. (2000) 'Microalgal biotechnology at the turn of the millennium: A personal view', *Journal of Applied Phycology*, 12(3–5), pp. 441–451.

Riesenberg, D. and Guthke, R. (1999) 'High-cell-density cultivation of microorganisms', *Applied Microbiology and Biotechnology*, 51(4), pp. 422–430.

Rodolfi, L. *et al.* (2009) 'Microalgae for oil: Strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor', *Biotechnology and Bioengineering*, 102(1), pp. 100–112.

Rohit, M. V. and Venkata Mohan, S. (2018) 'Quantum yield and fatty acid profile variations with nutritional mode during microalgae cultivation', *Frontiers in Bioengineering and Biotechnology*, 6(SEP), pp. 1–12.

Roostaei, J. *et al.* (2018) 'Mixotrophic Microalgae Biofilm: A Novel Algae Cultivation Strategy for Improved Productivity and Cost-efficiency of Biofuel Feedstock Production', *Scientific Reports*. Springer US, 8(1), pp. 1–10.

Rushan, N. H. *et al.* (2019) 'Effect of Immobilization Method on the Growth of *Chlorella vulgaris* and Fatty Acid Profile for Biodiesel Production', *Indonesian Journal of Chemistry*, 19(3), p. 767.

Safafar, H. *et al.* (2016) 'Enhancement of protein and pigment content in two Chlorella species cultivated on industrial process water', *Journal of Marine Science and Engineering*, 4(4).

Sakarika, M. and Kornaros, M. (2016) 'Effect of pH on growth and lipid accumulation kinetics of the microalga Chlorella vulgaris grown heterotrophically under sulfur limitation', *Bioresource Technology*. Elsevier Ltd, 219, pp. 694–701.

Santana, H. *et al.* (2017) 'Microalgae cultivation in sugarcane vinasse: Selection, growth and biochemical characterization', *Bioresource Technology*. Elsevier Ltd, 228, pp. 133–140.

SATIVA (2019) *Agricultura Biológica*. Available at: https://www.sativa.pt/agricultura-biologica/.

Shakibaie, M. R. (2018) 'Annals of Microbiology and Research', 2(1), pp. 45–50.

Shen, Y. *et al.* (2010) 'Heterotrophic culture of Chlorella protothecoides in various nitrogen sources for lipid production', *Applied Biochemistry and Biotechnology*, 160(6), pp. 1674–1684.

Shi, X. M., Jiang, Y. and Chen, F. (2002) 'High-yield production of lutein by the green microalga Chlorella protothecoides in heterotrophic fed-batch culture', *Biotechnology Progress*, 18(4), pp. 723–727.

Singh, N. K. and Dhar, D. W. (2011) 'Microalgae as second generation biofuel. A review', *Agronomy for Sustainable Development*, 31(4), pp. 605–629.

Suali, E. and Sarbatly, R. (2012) 'Conversion of microalgae to biofuel', *Renewable and Sustainable Energy Reviews*. Elsevier Ltd, 16(6), pp. 4316–4342.

Sun, H. *et al.* (2018) 'Biotechnology for Biofuels High - value biomass from microalgae production platforms : strategies and progress based on carbon metabolism and energy conversion', *Biotechnology for Biofuels*. BioMed Central, pp. 1–23.

Taylor, P. (2014) 'Growth Characteristics and Cellular Components of Chlorella regularis , Heterotrophic Fast Growing Strain Microalgae produce organic substances at high nutritional values with high efficiencies utilizing both light and carbon dioxide . culture of algae ba', 1369(June 2015), pp. 9–18.

Tobajas, M. and García-Calvo, E. (2000) 'Comparison of experimental methods for determination of the volumetric mass transfer coefficient in fermentation processes', *Warme- und Stoffubertragung Zeitschrift*, 36(3), pp. 201–207.

Todaro, Celeste; Vogel, H. (2014) *Fermentation and biochemical engineering handbook*, *The Chemical Engineering Journal*.

Tokuşoglu, Ö. and Ünal, M. K. (2003) 'Biomass nutrient profiles of three microalgae: Spirulina platensis, Chlorella vulgaris, and Isochrisis galbana', *Journal of Food Science*, 68(4), pp. 1144–1148.

Velichkova, K. and Sirakov, I. (2018) 'Growth parameters, protein and photosynthetic pigment content of Chlorella Vulgaris cultivated under photoautotrophic and mixotrophic conditions', *Bulgarian Journal of Agricultural Science*, 24(April), pp. 150–155.

Verzijden, K. (2016) 'Organic certification of food grade micro algae – wishful thinking or soon reality?', *Food Health Legal, legal and regulatory blog*.

Wang, B. *et al.* (2008) 'CO2 bio-mitigation using microalgae', *Applied Microbiology and Biotechnology*, 79(5), pp. 707–718.

Wang, B. and Lan, C. Q. (2011) 'Biomass production and nitrogen and phosphorus removal by the green alga Neochloris oleoabundans in simulated wastewater and secondary municipal wastewater effluent', *Bioresource Technology*. Elsevier Ltd, 102(10), pp. 5639–5644.

Wang, J., Yang, H. and Wang, F. (2014) 'Mixotrophic cultivation of microalgae for biodiesel production: Status and prospects', *Applied Biochemistry and Biotechnology*, 172(7), pp. 3307–3329.

Wen, Z. Y. and Chen, F. (2001) 'Optimization of nitrogen sources for heterotrophic production of eicosapentaenoic acid by the diatom Nitzschia laevis', *Enzyme and Microbial Technology*, 29(6–7), pp. 341–347.

Williams, P. J. L. B. and Laurens, L. M. L. (2010) 'Microalgae as biodiesel & biomass feedstocks: Review & analysis of the biochemistry, energetics & economics', *Energy and Environmental Science*, 3(5), pp. 554–590.

Xie, T. *et al.* (2017) 'Nitrate concentration-shift cultivation to enhance protein content of heterotrophic microalga Chlorella vulgaris: Over-compensation strategy', *Bioresource Technology*. Elsevier Ltd, 233, pp. 247–255.

Xiong, W. *et al.* (2010) 'Double CO2 fixation in photosynthesis-fermentation model enhances algal lipid synthesis for biodiesel production', *Bioresource Technology*. Elsevier Ltd, 101(7), pp. 2287–

2293.

Yeh, K. L. and Chang, J. S. (2012) 'Effects of cultivation conditions and media composition on cell growth and lipid productivity of indigenous microalga Chlorella vulgaris ESP-31', *Bioresource Technology*. Elsevier Ltd, 105, pp. 120–127.

Yono, Budi ; Syaichurro, Iqbal; Sumardiono, Siswo; Sasongko, S. (2014) 'Production of Spirulina platensis Biomass Using Digested Vinasse as Cultivation Medium', *Trends in Applied Sciences Research*.

Yu, S. *et al.* (1990) 'Furfural and hydroxymethylfurfural inhibition of growth and photosynthesisin spirulina', *British Phycological Journal*, 25(2), pp. 141–148.

Yusof, Y. A. M. *et al.* (2011) 'Fatty acids composition of microalgae chlorella vulgaris can be modulated by varying carbon dioxide concentration in outdoor culture', *African Journal of Biotechnology*, 10(62), pp. 13536–13542.

Zeitum, B. and Wortiriann, J. (no date) 'Beyerinck_Chlorella_1890'.

Zhang, Q., Wang, T. and Hong, Y. (2014) 'Investigation of initial pH effects on growth of an oleaginous microalgae Chlorella sp. HQ for lipid production and nutrient uptake', *Water Science and Technology*, 70(4), pp. 712–719.

Zheng, Y. *et al.* (2012) 'Two-stage heterotrophic and phototrophic culture strategy for algal biomass and lipid production', *Bioresource Technology*. Elsevier Ltd, 103(1), pp. 484–488.

Zhou, Y. *et al.* (2018) 'Effects of agitation, aeration and temperature on production of a novel glycoprotein gp-1 by streptomyces kanasenisi zx01 and scale-up based on volumetric oxygen transfer coefficient', *Molecules*, 23(1), pp. 1–14.

ANNEX A – CALIBRATION CURVES

The growth of *C. vulgaris* was monitored by measuring the optical density of cultures on a UV/Vis spectrophotometer at 600 nm. The growth was made heterotrophic and autotrophic pathways, where the respective calibration curves are shown in Figure A1 and A2.

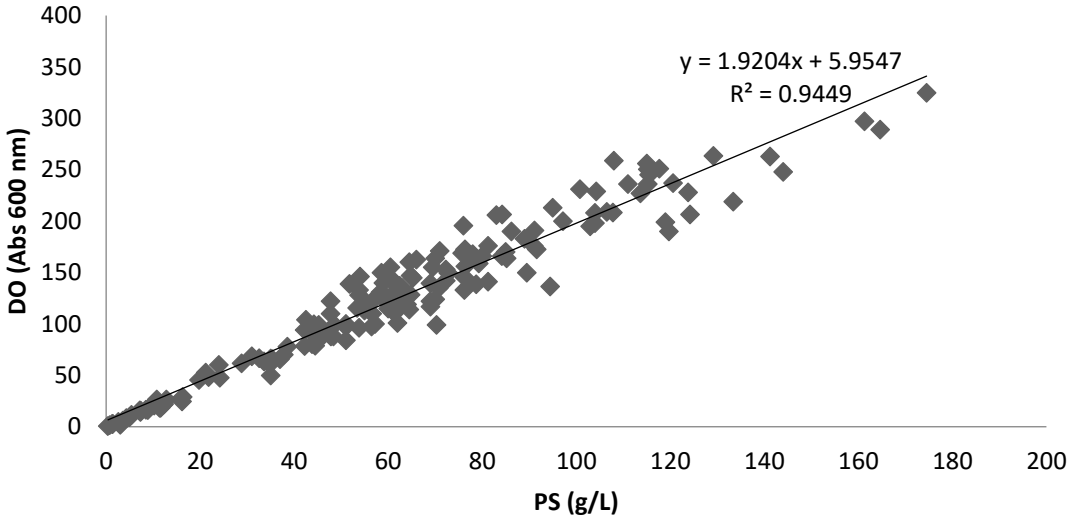

Figure A 2 – Absorbance of C. vulgaris measured at wavelength of 600 nm vs dry weigh for heterotrophic mode.

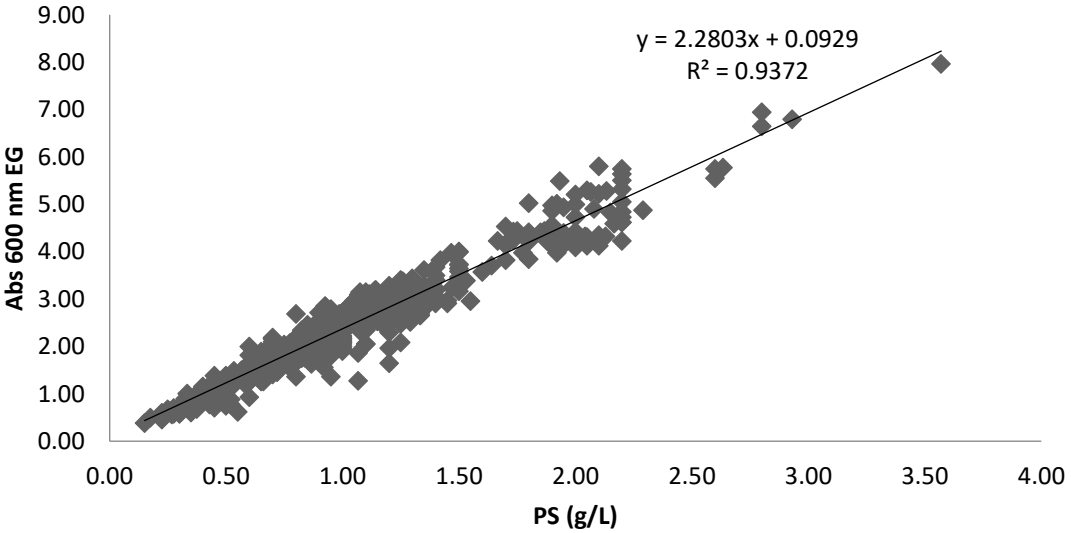

Figure A 1 – Absorbance of C. vulgaris measured at wavelength of 600 nm vs dry weigh for autotrophic mode.

ANNEX B – CULTIVATION PARAMETERS OF *C. VULGARIS* GROWTH WITH YEAST EXTRACT IN N OPTIMIZATION

C. vulgaris growth was monitored by analyzing the pH variation, glucose in the medium and ammonia in the medium over time.

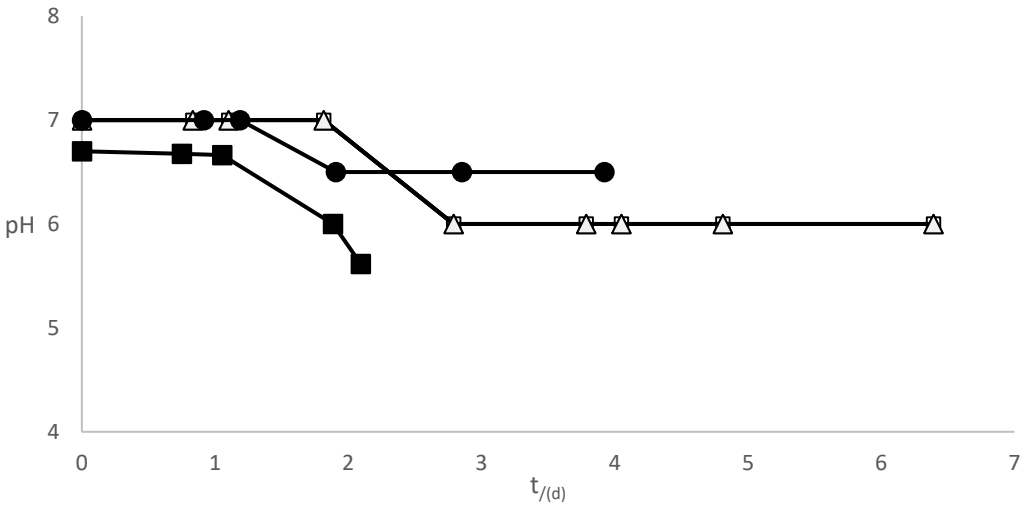

Figure B 1 – pH variation of control, with best condition of MNBIO optimization (■); pH variation of YE20 (□); of YE10 (▲); of YE10+Vit (Δ) and of YE20+MNM (●). Values represent averages of data from two biological replicates, except control, which value represent average from three independent biological replicates, and it is standard deviation.

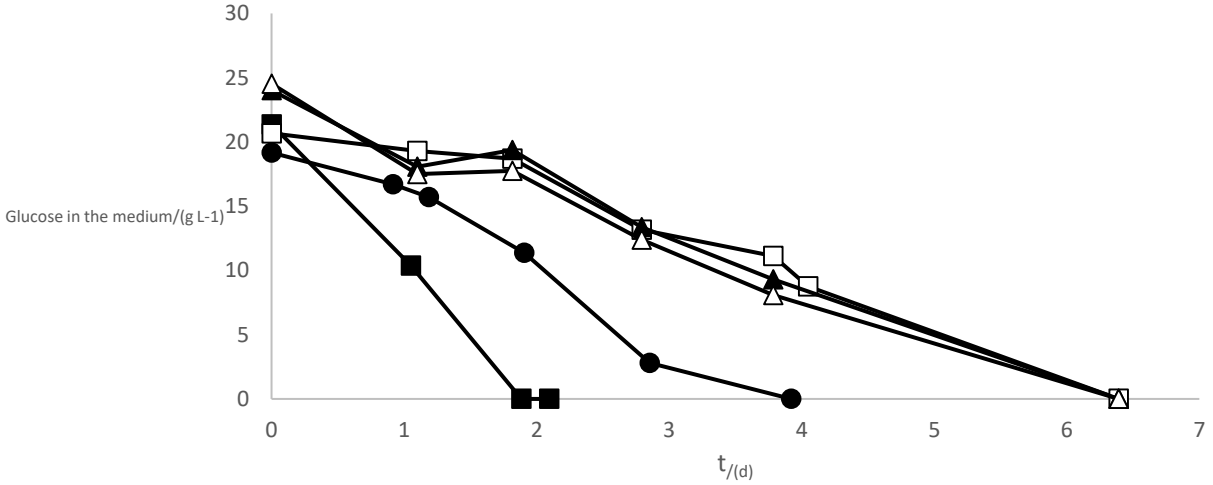

Figure B 2 – Glucose in the medium of control, with best condition of MNBIO optimization (■); Glucose in the medium of YE20 (□); of YE10 (▲); of YE10+Vit (Δ) and of YE20+MNM (●). Values represent averages of data from two biological replicates, except control, which value represent average from three independent biological replicates, and it is standard deviation.

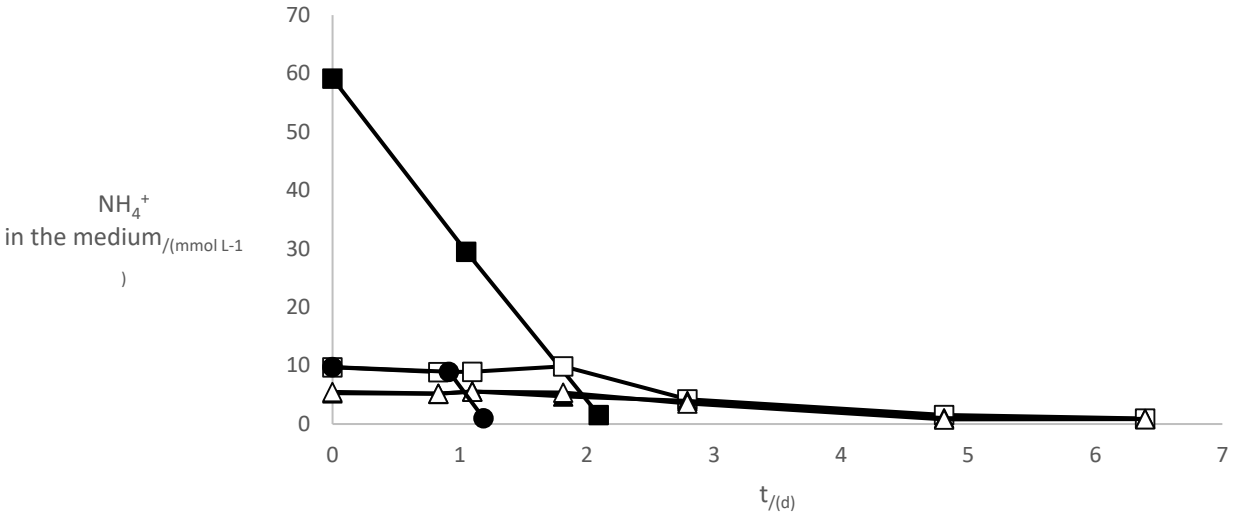

Figure B 3 – NH_4^+ in the medium of control, with best condition of MNBIO optimization (■); NH_4^+ in the medium of YE20 (□); of YE10 (▲); of YE10+Vit (Δ) and of YE20+MNM (●). Values represent averages of data from two biological replicates, except control, which value represent average from three independent biological replicates, and it is standard deviation.

ANNEX C – CULTIVATION PARAMETERS OF *C. VULGARIS* GROWTH IN C SOURCE OPTIMIZATION

C. vulgaris growth was monitored by analyzing the pH variation, glucose in the medium and ammonia in the medium over time.

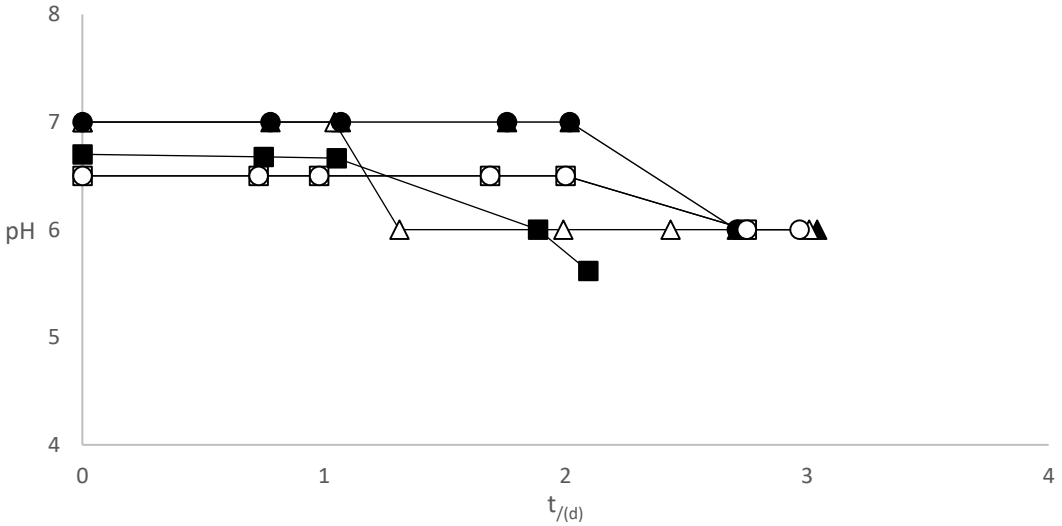

Figure C 1 – pH variation of Glu+Vit (■); of Glu (□); of SF+Vit (▲); of SF (Δ); of SA+Vit (●) and of SA (○). Values represent averages of data from three independent biological replicates.

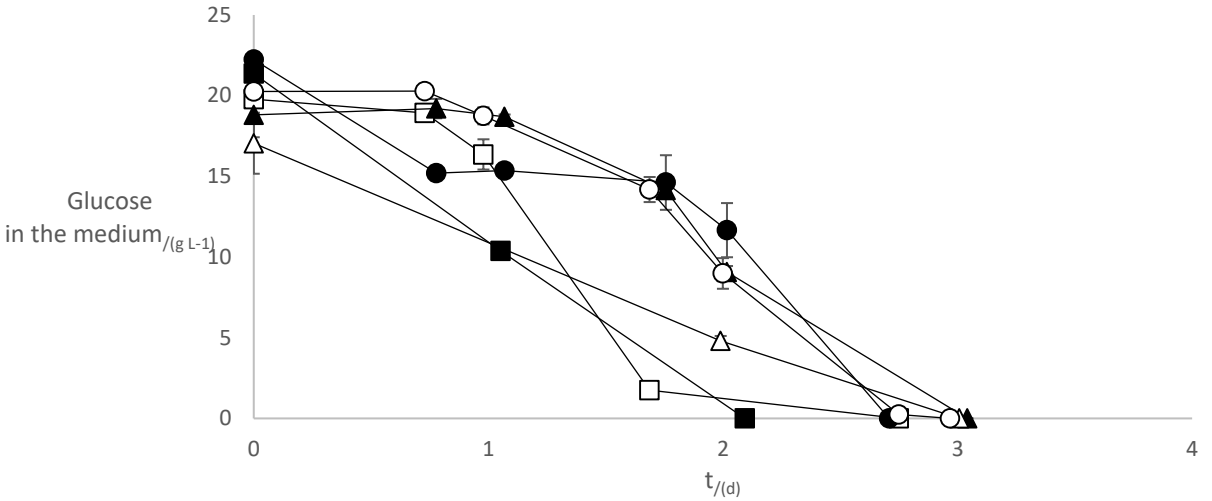

Figure C 2 – Glucose in the medium of Glu+Vit (■); of Glu (□); of SF+Vit (▲); of SF (Δ); of SA+Vit (●) and of SA (○). Values represent averages of data from three independent biological replicates.

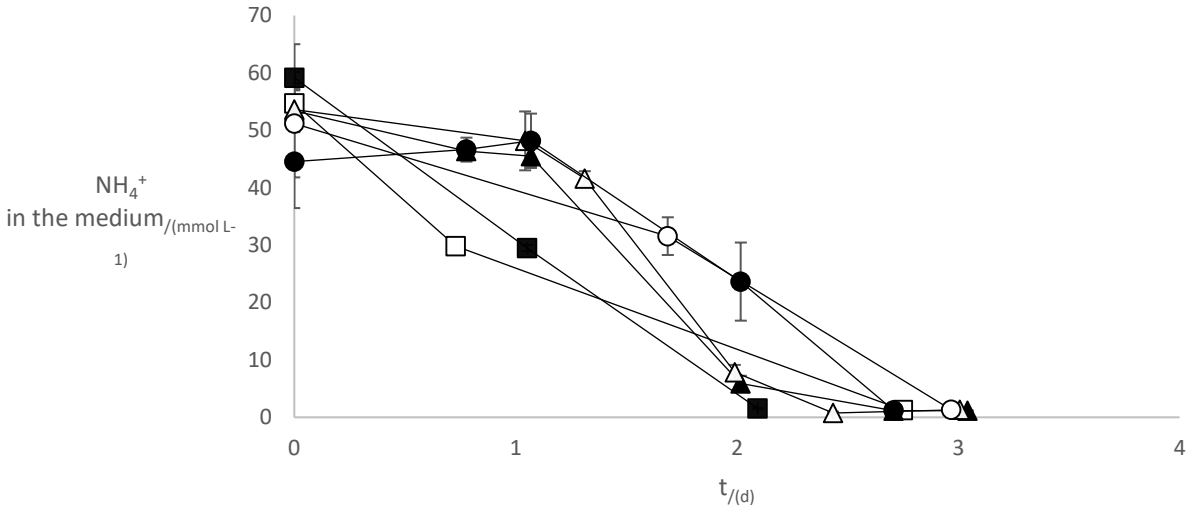

Figure C 3 – NH_4^+ in the medium of Glu+Vit (■); of Glu (□); of SF+Vit (▲); of SF (Δ); of SA+Vit (●) and of SA (○). Values represent averages of data from three independent biological replicates.

ANNEX D – CULTIVATION PARAMETERS OF *C. VULGARIS* GROWTH IN PH CONTROL

C. vulgaris growth was monitored by analyzing the pH variation, glucose in the medium and ammonia in the medium over time.

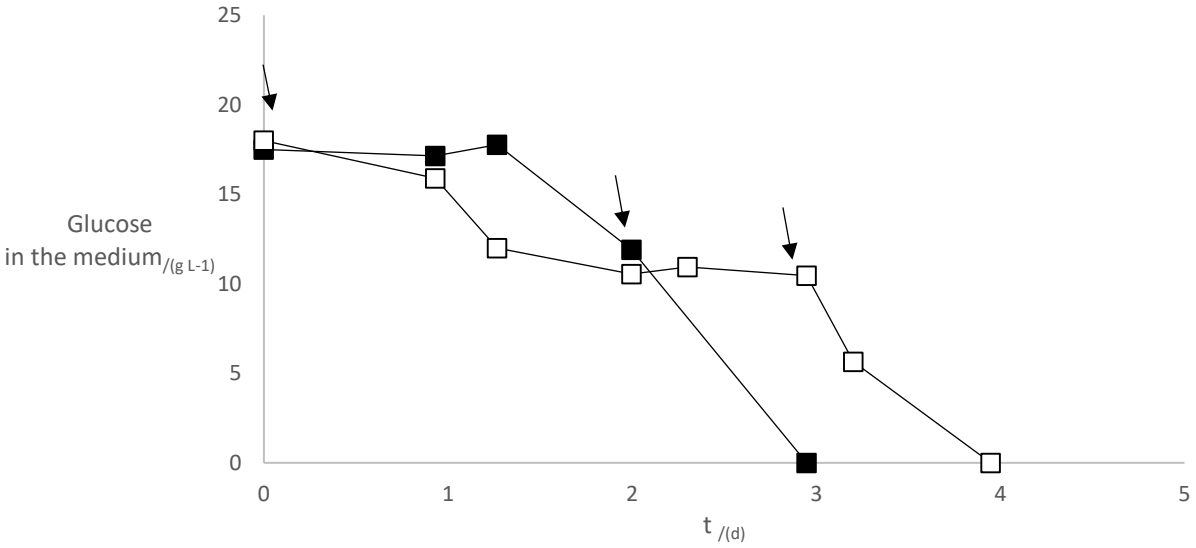

Figure D 1 – Glucose in the medium to culture with PB (■) and with SB (□). The symbol ↓ represents the time of the addition of solutions. First ↓ corresponds to both conditions. Values represent averages of data from two independent biological replicates.

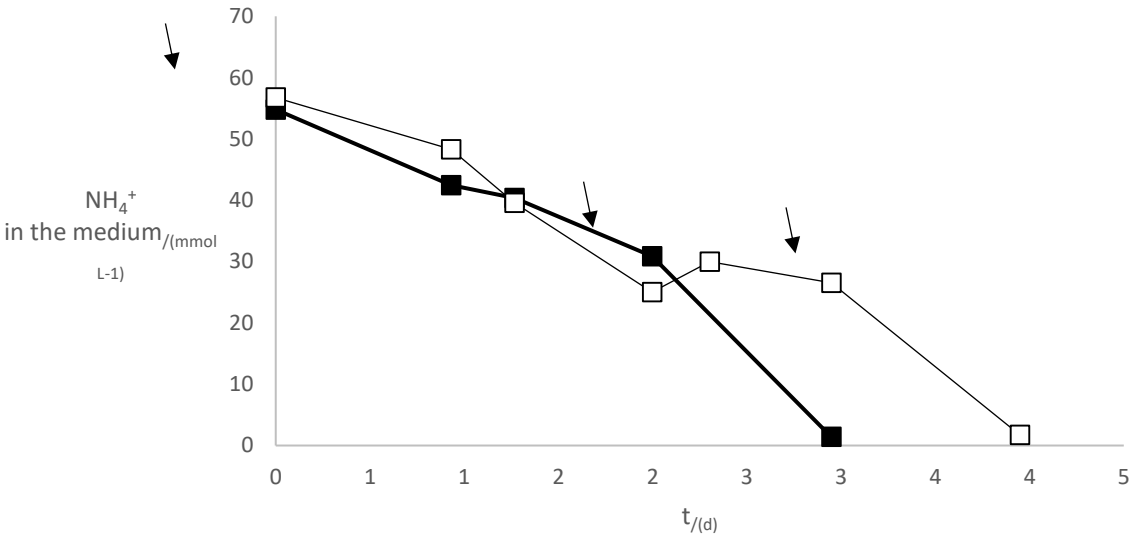

Figure D 2 – NH_4^+ in the medium to culture with PB (■) and with SB (□). The symbol ↓ represents the time of the addition of solutions. First ↓ corresponds to both conditions. Values represent averages of data from two independent biological replicates.