Scenedesmus rubescens production strategies for added value biomass

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Preface

The work presented in this thesis was performed at the Allmicroalgae facilities, during the period February-November 2020.

I declare that this document is an original work of my own authorship and I further declare that I have fully acknowledged of the Code of Conduct and Good Practices of the Universidade de Lisboa.

Gonçalo Moisés Simões do Espírito Santo

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Abstract

Nowadays, microalgae have attracted interest worldwide. One of the most high-value product from microalgae are carotenoids. They bring many health benefits due to their powerful antioxidant action and they have been claimed to reduce the risk of cardiovascular diseases and certain cancer types. For these reasons, carotenoids are a high-value industrial product.

In this way, the main goal of this work was the production of highly concentrated biomass of *Scenedesmus rubescens* (0037SA) in heterotrophic conditions to induce carotenoid production. Comparing the optimized medium for 0037SA strain and BBM medium, optimization was successful, improving the global productivity by 0.92-fold (from 1.94 to 2.79 g L^{-1} day^{-1}) and the specific growth rate by 1.26-fold (from 0.90 to 1.13 day^{-1}).

After medium optimization, scale-up was evaluated in 7L stirred-tank reactor. Two scale-up attempts were successful, achieving 72 g biomass L^{-1}. However, other species were described to reach higher biomass concentration, suggesting that *S. rubescens* may still reach higher cell densities.

The inoculum obtained from scale-up was used to induce carotenoid production in autotrophy. However, the conditions tested did not allow the carotenoid induction. In addition, 0037SA was also grown under heterotrophic conditions, in dark, testing different conditions. Different colours in the cell samples were observed and consequently different types of carotenoids were produced. In green cells, lutein and a small percentage of β-carotene were identified. In the orange sample, more carotenoids were found, such as astaxanthin. Thus, carotenoids induction in *S. rubescens* is feasible, enabling the use of this species for pharmaceutical applications.

Keywords: 0037SA strain, heterotrophic, fermenter, stirred-tank reactor, DoE, induction, carotenoids.

Resumo

Atualmente, as microalgas têm despertado interesse por todo o mundo. Um produto de alto valor são os carotenoides. Trazem muitos benefícios à saúde pela sua poderosa ação antioxidante e reduzem o risco de doenças cardiovasculares. Assim, os carotenoides apresentam-se como um produto de alto valor industrial.

O objetivo deste trabalho consistiu na produção de biomassa de *Scenedesmus rubescens* (0037SA) em condições heterotróficas, de forma a induzir a produção de carotenoides. Comparando o meio otimizado para a estirpe 0037SA com o BBM, a produtividade global foi 0.92 vezes maior (1.94 - 2.79 g L^{-1} dia^{-1}) e a taxa de crescimento específico aumentou 1.26 vezes (0.90 – 1.13 dia^{-1}).

Após a otimização do meio de cultura, as condições foram avaliadas em um reator de tanque agitado de 7L. Apenas dois ensaios de larga escala foram bem-sucedidos, atingindo as 72 g L^{-1} de concentração de biomassa. No entanto, outras espécies foram descritas que atingiram maior concentração de biomassa, sugerindo que *S. rubescens* ainda poderá atingir densidades celulares superiores.

O inóculo obtido do fermentador foi utilizado para produzir carotenoides em autotrofia. Porém, as condições testadas não permitiram esta indução. 0037SA também foi cultivado em condições heterotróficas, no escuro, testando diferentes condições. Foram observadas cores diferentes nas amostras e a produção de diferentes tipos de carotenoides. Nas células verdes foi identificado luteína e também uma pequena percentagem de β-carotenos. Na amostra laranja encontraram-se diferentes carotenoides, nomeadamente astaxantina. Assim, a indução de carotenoides por *S. rubescens* é viável, podendo esta espécie ser utilizada em aplicações farmacêuticas.

Palavras-chave: estirpe 0037SA, heterotrofia, fermentador, DoE, indução, carotenoides.

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Abbreviations list

AFDB - Ash free dry biomass

ANOVA - Analysis of variance

BBM - Bold's basal medium

CCD - Central composite design

DHA - Docosahexaenoic acid

DoE - Design of experiments

DW - Dry weight

EPA - Eicosapentaenoic acid

FP - Flat panels

HPLC - High-performance liquid chromatography

OD - Optical density

OVAT - One variable at time

PCB - Plate count broth

ROS - Reactive oxidative stress

rpm – Revolutions per minute

RSM - Response surface methodology

1. Context of work

1.1 Thesis outline

This present manuscript is divided into five chapters. The first chapter aims to present the a little of the company's history. Chapter two is a review of microalgae production – with special focus on *Scenedesmus* sp., industrial applications of microalgae, different type of growth conditions, and the design of experiments concept. The third chapter describes the main goal. The fourth chapter describes the materials and methods, where the experimental design of this work is presented. The chapter 5 lists all results and their analysis. The culture´s growth performance and their biochemical analysis are also discussed. Lastly, the chapter six is the conclusion of the work and the future perspectives towards improvement.

1.2 Allmicroalgae – company

The presented work was performed at Allmicroalgae – Natural Products, S.A. (facilities showed in Fig.1), located in Pataias, district of Leiria. This industrial-scale microalgae production company belonged entirely to Secil group until 2019. Secil is a cement producer company founded in 1930 in Portugal, internationalized in countries, like Brazil, Cape Verde, Angola, etc[1]. The microalgae production project appeared as an attempt to mitigate the CO_2 released during the cement production[2].

Figure 1 - Allmicroalgae S.A. facilities, Pataias[2]

Allmicroalgae is one of the biggest scale microalgae production plants in Europe with a total production volume of 1300 m^3 and an annual production capacity of 100 tons of dried biomass. At the production unit, microalgae are mostly cultivated in closed systems such as photobioreactors (PBRs) and fermenters. Biomass is often used for food and feed, pharmaceutical and cosmetic products[3]. There are several species regularly produced such as *Chlorella vulgaris*, *Nannochloropsis* sp., *Scenedesmus* sp., *Tetraselmis chui* and *Phaeodactylum tricornutum*. *C.vulgaris* is produced in a two-stage process. This process consists in heterotrophic growth of a concentrated inoculum that will be used to inoculate outdoor autotrophic PBRs. Allmicroalgae is certified by European Organic Production Certification, ISO 22000, Halal, Portugal Sou Eu and for the ISO 9001, ISO 14001 and GMP+ FSA[4].

2. Introduction

2.1 Microalgae – overview

Microalgae or microphytes are microscopic ancestral living organisms defined as oxygenic photosynthesizers that vary greatly in their morphology, physiology, and environmental range. These organisms comprehend over 300,000 species, of which about 30,000 are documented and they have been used over 50 years in domestic wastewater treatment and bioremediation of manure effluents[5]. Microalgae can be found in freshwater, seawater and hypersaline environments, but also in wet soils and rocks[6]. Most of these organisms are typically single-cell photosynthetic autotrophic microscopic organisms. In this way, microalgae use energy from light to convert inorganic carbon, like carbon dioxide (CO_2), to complex organic compounds. However, some microalgae also produce energy through organic carbon (glucose, acetate, glycerol, etc) [5, 6,7].

Microalgae are divided into four groups: cyanobacteria (blue-geen algae), chlorophytes (green algae), rhodophytes (red algae) and chromophytes (all other microalgae[5]). Apart from cyanobacteria (prokaryotes), all these microalgae are eukaryotes. The three most promising microalgae used to produce high-value products are the diatoms (*Bacillariophyceae*), the green algae (*Chlorophyceae*) and the golden algae (*Chrysophyceae*)[5].

Nowadays, microalgae are often consumed as dietary supplements as they have great nutritional value (table 1). However, it is also possible to extract a wide variety of bioproducts, such as proteins, lipids, carotenoids, astaxanthin, DHA (docosahexaenoic acid), EPA (eicosapentaenoic acid), etc[5]. These characteristics depend on the species and growing conditions, such as light, temperature and nutrients[8]. In the food industry, microalgae such as *Chlorella vulgaris*, *Arthrospira platensis*, *Dunaliella salina*, have been used as a protein source. However, the use of microalgae in the diet is still underdeveloped in Europe due to production costs, food safety, among other criteria[6].

Table 1- Nutritional values of different algae. Data from [9.]

Species	Proteins	Carbohydrates	lipids
Anabaena cylindrica	43-56	25-30	4-7
Aphanizomenon flos-aquae	62	23	3
Chlamydomonas rheinhardii	48	17	21
Chlorella pyrenoidosa	57	26	2
Chlorella vulgaris	51-57	12-17	14-22
Dunaliella salina	57	32	6
Euglena gracilis	39-61	14-18	14-20
Porphyridium cruentum	28-39	40-57	9-14
Scenedesmus obliquus	50-56	10-17	12-14
Spirogyra sp.	6-20	33-64	11-21
Spirulina platensis	46-63	8-14	4-9

Microalgae have a relatively complex and recalcitrant dynamic cell wall, and intracellular compounds are located mainly in globules or bound to complex membranes, making the extraction of a cellular content a major challenge[10]. The cell wall preserves the integrity of the cell, serving as a protective barrier against invaders and aggressive environments (e.g. extreme temperatures or pH)[11]. Thus, it exerts a biological and biomechanical control over cells, playing a fundamental role in their environmental interactions[11]. According to the species and its state of development, the composition of the cell wall differs, presenting distinct characteristics[12]. They could vary between extremely rigid like in *Haematococcus pluvialis* to extremely weak like *Porphyridium cruentum*[12]. The cell wall are usually three-layer structures composed of polysaccharides such as cellulose, pectin, mannan, xylan, of minerals (calcium, silicates) as well as of proteins and glycoproteins[10,11].

2.2 *Scenedesmus* sp.

Scenedesmus sp. is typically identified as green microalga (Chlorococcales; Scenedesmaceae) commonly found in fresh and various types of wastewater streams[13]. These algae are characterized by two-dimensional arrangement of two or more cells (Fig. 2) in regular aggregates called coenobia[14] and they are one of the first cultured algae *in vitro* due to rapid growth and easiness of handling[13]. The morphology between species of the genus varies according to nutrient concentration, pH or due to allochemicals released[13]. *Scenedesmus*' species, like specimens from other genus from coccoid green algae, present a highly resistant cell wall structure, exhibiting a characteristic trilaminar structure. This structure is resistant to treatment with several lytic enzymes such as celullases, hemicellulases, pronase, among others, well as to drastic non-oxidative chemical treatments[15].

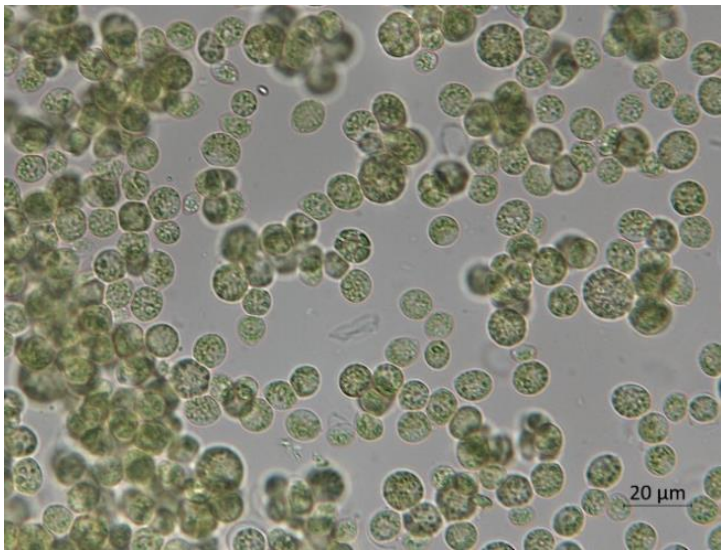


Figure 2 - Microscopic view of *Scenedesmus rubescens* in heterotrophic growth. Image obtained by Zeiss® Axio Scope.A1 coupled with ZEN Axiocam 503 color. Total magnification 400x.

Scenedesmus sp. reproduces mainly asexually by the formation of autospores[16]. In addition, these algae are known to be tolerant of extreme conditions as high salinity and alkalinity, unlike many other organisms. Therefore, these species can be grown in environments that are inhibitory to the growth of microorganisms.[17] This genus is one of the main feedstocks considered for biofuel productivity, given its high lipid content and capability to adapt to different environmental conditions (resistance to high concentrations of carbon dioxide and ammonia). In addition, the biomass has high protein (see table 1), but the pigments content which these algae can have is important too[18,19].

2.3 Microalgae cultivation

To create a culture medium is necessary to consider parameters as temperature, pH, light intensity and nutrients[14]. When considering these different parameters, models for different microorganisms growth can be created[14]. By modelling the growth of microalgae one can attempt to predict the optimization of growth conditions and also the quality of microalgae under different environmental conditions[20].

2.3.1 Autotrophic cultivation

Most microalgae are cultivated in photoautotrophic conditions, which means that they use carbon dioxide, sodium carbonate or sodium bicarbonate as inorganic carbon source during photosynthesis[21]. In this way, microalgae can grow under natural or artificial light[21,22]. Autotrophic growth does provide some advantages. One of these advantages is the use of inexpensive natural resources, such as CO_2[22]. Therefore, unlike other organisms due to the ability of algae metabolize CO_2 they can contribute to CO_2 reduction, mitigation. However, low biomass productivity makes autotrophic production a challenge[23].

There are two main types of reactors for microalgae production in autotrophic conditions (table 2): open pond systems and closed bioreactors[24] (Fig.3 A e B).

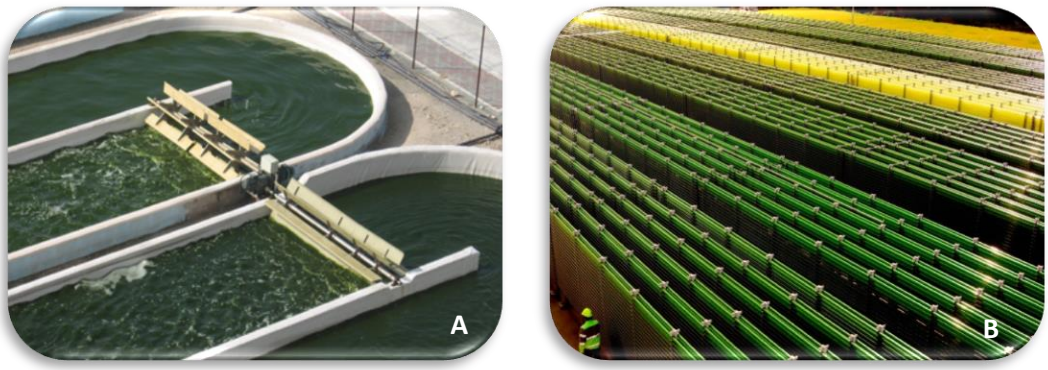


Figure 3 - Examples of bioreactor designs for A) open pond system[25] and B) closed bioreactors[26].

The open pond systems (table 2), are usually raceways. The artificial systems can be made of plastic, concrete, bricks or compacted earth in a variety of shapes and sizes. This type of system presents some advantages such as: ready access to sunlight, low construction and operation costs.

However, there are some limitations (table 2) like greater tendency to contaminations, evaporative losses and limitations of control conditions (temperature, pH, etc)[21,23,27]. To overcome the disadvantages of using open systems, numerous closed PBRs have been designed (table 2)[27]. Enclosed photobioreactor systems are reactors with higher degrees of sophistication when compared with open-pond systems[21]. The main goal of this system is to increase the biomass productivity. Since they permit to control different parameters such as pH, temperature, gas diffusion, among others, allowing to overcome the limitation of the growth rate. Thus, the use of PBRs has some advantages when comparing to open pond systems: better control of the culturing conditions, increased protection from environmental contamination, etc. However, enclosed photobioreactors systems are more complex and expensive (high investments) in comparison to open pond system[24,21].

Table 2 - Advantages and disadvantages of open pond System and closed photobioreactor.

	Open pond system		**Closed photobioreactor**	
	Advantages	**Disadvantages**	**Advantages**	**Disadvantages**
General features	Ready access to sunlight	Evaporative losses	Reduction in water loss	--
Operations	Simple operation	Limitations of control conditions (pH, temperature, light intensity)	Greater environmental control (pH, temperature, light intensity)	Complex operation
Investment	Lower investment	--	--	Higher investment
Contaminations	--	Prone to contamination	Less contaminations	--
Applications	--	Only a few species have a significant growth on a large scale	Greater effectiveness in the production of high-value products. Suitable for sensitive strains	--

2.3.2 Heterotrophic cultivation

Under heterotrophic conditions (table 3), microalgae use organic substrates both as energy and carbon sources, such as glycerol, acetate, glucose, etc[28]. Microalgae production is done in closed stirred

reactors named fermenters (Fig.4)[28]. In this way, contaminations from other microorganisms can be better prevented[23]. The heterotrophic growth allows faster biomass production, decreases the area needed for inoculum production[28,29] and does not require light, eliminating a variable that is limiting to the growth of microalgae in autotrophic PBRs[30]. Also, almost any fermenter can be used as a bioreactor to produce microalgae under heterotrophy.

Figure 4 - A) bench 7L reactor (fermenter) producing *Scenedesmus rubescens* 0073 SA B) industrial 5000 L reactor. From Allmicroalgae facilities.

However, this type of cultivation has also limitations. Only a few number of microalgae can grow heterotrophically [21,22]. In addition, the use of organic substrates can also promote the rise of the production costs. One of the disadvantages is the price of the fermentable substrates (e.g. acetate) and the competition with feedstocks for other uses such a food and biofuel productions[24]. Thus, in order to compensate the costs, high biomass productivity must be achieved[31]. Furthermore, the protein and pigment contents tend to be low, which can decrease the value of biomass[29,32].

To overcome limitations from solely autotrophic or heterotrophic cultivation, there are strategies that combine the two cultivation modes, taking the advantages of these two: the two-stage cultivation. In this strategy, the first stage in heterotrophy will increase the biomass production efficiency, obtaining highly concentrated inoculum for reactors operating under autotrophic conditions[29,33]. Thus, the heterotrophic process aims to reduce both costs and the time of autotrophic scale-up process increasing the overall biomass productivity[29]. Afterwards autotrophic cultivation aims to increase the protein and pigment contents of microalgae[29] resulting in higher quality biomass.

2.3.3 Mixotrophic cultivation

Mixotrophic conditions are the combination of autotrophic and heterotrophic metabolisms. In this way, the cells use organic carbon to produce energy. The carbon dioxide released from aerobic respiration will be used in photosynthesis. In turn, the oxygen released in photosynthesis will be reused in respiration[24]. Thus, this type of cultivation is advantageous when compared to other methods since it can achieve high biomass and lipid productivity are achieved when compared to autotrophic conditions[34]. The growth does not strictly depend on photosynthesis. Thus, the requirement of light is lower, not being an absolute limiting factor for microalgal growth[35]. Compared to the heterotrophy that depends only on a source of organic carbon, this type of cultivation can achieve higher yields. In addition, as the need for organic carbon sources contributes to increased costs and energy input, it is possible to reduce costs since this type of cultivation does not depend only on organic carbon sources[35,36]

Table 3 - Advantages and disadvantages for autotrophic and heterotrophic conditions.

	Autotrophic conditions		**Heterotrophic conditions**	
	Advantages	**Disadvantages**	**Advantages**	**Disadvantages**
Operations	Contribution to CO_2 reduction	Limitations of culture control (Temperature, pH)	Better culture control Simple daily management	
Costs	Use inexpensive natural resources (CO_2) .	.	The high productivity of the biomass balances the initial production costs	Expensive costs of aeration and agitation (oxygen control)[22] High operational costs[29]
Contaminations		Less control over contaminations.	More control over contamination.	
Applications	Production of specific metabolites.	Low biomass productivity and biomass concentrations	High biomass concentrations can be reached	Not suitable for most algae.

2.4 Microalgae biotechnological applications

Nowadays, microalgae have attracted interest worldwide due to their potential for several applications[37]. The production of high value bio-products, such as lipids, fatty acids, proteins,

carbohydrates, etc[38] extends significantly the array of uses. Thus, microalgae can be used for purposes such as biofuels, pharmaceuticals, animal feed, cosmetics, among others[24].

2.4.1 Biofuels

Fossil fuels contribute the most to greenhouse gases. Thus, mitigation strategies need to be found in order to neutralize carbon dioxide and alternative energies to this type of fuel must be widespread[39]. Third generation biofuels can be the produced from microalgae due to their high lipid productivity[24]. Also, when compared with cultures, such as palm oil (5950L ha^{-1}) or sunflower (952L ha^{-1}) the aerial lipid productivity (12 000L $^{-1}$) is superior[40]. Thus, microalgae require a smaller land area compared to other feedstocks for biodiesel from agricultural sources. Therefore, competition for arable soil with other crops, particularly for human consumption, can be greatly reduced[41] . Microalgae species that produce a large amount of carbohydrates, are excellent substrates for bioethanol production. Also, when compared to lignocellulosic materials, these carbohydrates are easier to convert to monosaccharides (due to absence of lignin)[42]. In addition, depending on the biomass application, microalgae could be used as a bio-sequester CO_2 from flue gases generated in power plants; this application can contribute to the reduction of greenhouse gases emissions.

2.4.2 Carotenoids

One of the most valuable compounds from microalgae to cosmetics and pharmaceuticals are pigments[43]. Pigments are common in nature and they can be melanin, chlorophyll, flavins, quinones, among others. One group of high-value products from microalgae is carotenoids (Figs. 5 and 6)[38]. This group of pigments has shown to have potential use in food, feed and cosmetic industries due to their characteristics: colour, aroma and remarkable nutrition[38,44]. Also, carotenoids bring many health benefits due to their powerful antioxidant action[,45,44]. For these reasons, carotenoids present high commercial values, whose market value is expected to reach 1.53 billions of dollars by 2021[38]. Also, these pigments have been associated with and claimed for reducing the risk of cardiovascular diseases, macular degeneration, certain cancer types, etc[43].

Chemically, carotenoids are lipophilic isoprenoid compounds, usually red, orange or yellow pigments. The presence of a conjugated double bond promotes this coloration, giving antioxidant properties to these compounds[46]. Carotenes, such as β-carotene (Fig.5A and Fig. 6C[47]- precursor of vitamin A) are hydrocarbons, while xanthophylls are the oxygenated carotenoids. These pigments are associated to light harvesting complex of photosynthesis and they protect the organism from reactive oxidative stress (ROS)[48,45,49]. In addition to carotene, there are other important pigments, such as lutein, astaxanthin, and others. Lutein (Figs. 5B and 6A[50]) is a primary xanthophyll pigment present in plants and green algae. It has antioxidant properties due to its long polyene structure with conjugated double bonds that has been implicated in protection against cardiovascular diseases[48]. Also, lutein is responsible for the bright yellow colour in flowers, fruits, etc. Nowadays, the current commercial supply of lutein is solely dependent of the genus Tagetus[48,51].

Astaxanthin (Figs. 5C and 6D[52]) is a secondary xanthophyll that is red/orange coloured. However, it can be commonly observed as pink colour in several marine microorganisms and the feather colour in birds (e.g. flamingo). In industry, astaxanthin is mainly used in aquaculture for fish and shrimp culture[48]. This pigment can be found in the cell membrane and lipoproteins, protecting microalgae from oxidative damage[48]. Like lutein, this pigment also possesses antioxidant and cardioprotective properties[51].

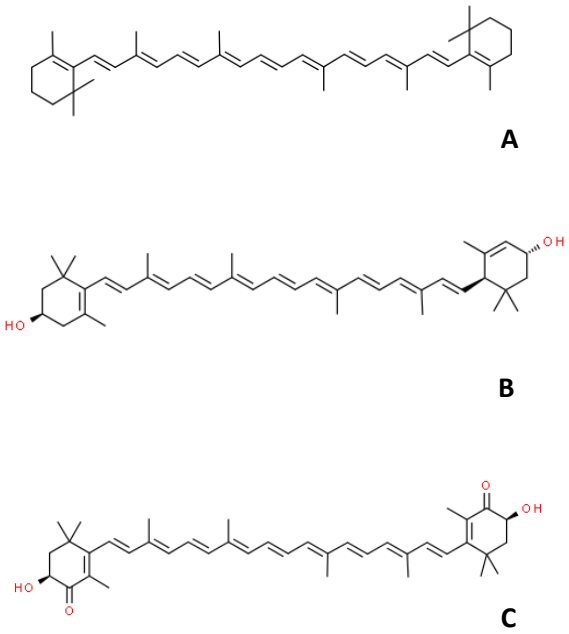


Figure 5 - Examples of microalgae carotenoids[53]. Chemical structures of: A) β-carotene. B) Lutein C) Astaxanthin.

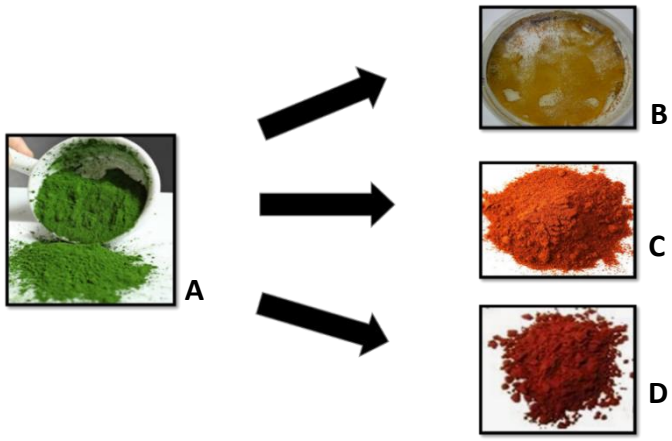


Figure 6 - Colour of microalgae pigments A) chlorophyll B) lutein C) β-carotene D) astaxanthin.

2.5 Cultivation parameters in heterotrophic growth

In order to culture microalgae and produce a given metabolite, a combination of parameters needs to be considered which are included in two categories: nutritional chemical factors, and environmental physical factors[54]. The first includes chemical elements in culture medium, such as carbon, nitrogen, phosphorus, calcium, sodium, silicon, metals such as iron and copper, vitamins, etc. Two of the most important factors (among limiting factors) are the nitrogen and carbon sources[55,56]. In addition, the interaction between carbon and nitrogen sources (C/N) must be considered[56]. The physical factors include pH, temperature, and intensity of aeration to the system[55].

2.5.1 Carbon source

Carbon is one of the most essential nutrients for microalgae growth since it is used as a nutrient and source of energy for microalgae[55,57] In heterotrophic conditions, the source of carbon is organic, such as glucose, starch, sucrose, acetate, etc[13].

2.5.2 Nitrogen source and phosphor

Like carbon, nitrogen (N) and phosphor (P) are important macronutrients in regulating cell growth and its metabolites[19,13], producing various biochemical compounds (which is predominantly used for synthesis of protein) and incorporation into ribosomal RNA. The change in the concentrations of nitrogen may influence the composition of biomass[58]. Thus, much research has been performed on nitrogen concentration and on its source. On *Scenedesmus* sp. cultures, the nitrogen sources usually used are ammonia, urea and nitrate[59]. However, it is not only the N source that is important but also the ratio C:N will impact on biomass concentration and productivity[31].

One of the conditions that is frequently studied is the limitation of nutrients, like P an N, to modulate biomass biochemical profile[59]. By limiting the availability of these nutrients, many metabolic responses in microalgae will result such as the degradation of protein (decrease the protein content) and then the accumulation of energy-rich compounds, such as lipids and carbohydrates[13,58]. Thus, limitation of nitrogen, as well as among other factors, will determinate cells' characteristics, both in composition and in morphology.

2.5.3 Temperature

Temperature is one of the physical factors that most affects an organism's growth rate, biomass composition, and metabolism (enzymes reactions, cell membrane system and other characteristics)[55]. Low and high temperatures promote alterations of cellular mechanisms and of fluidity of cell membranes[55]. To compensate a decrease of fluidity, the proportion of unsaturated fatty acids is increased[60]. However, low temperatures will limit the cell growth speed and consequently the biomass productivity. Too high temperatures will also promote decrease of cell growth rate [55,60].

2.5.4 pH

The pH is also one of the most critical parameters in microalgae cultivation, as it determines the solubility and bioavailability of nutrients and has a significant influence on microalgal metabolism[61]. This parameter also affects microalgal flocculation efficiency due to changes in the surface charge of the microalgal cells, the extent of coiling, and the degree of ionization of polymers[61,62].

2.5.5 Micronutrients

The elements needed in small quantities in the cell for its metabolism are mainly: iron (Fe), zinc (Zn), copper (Cu), nickel (Ni), molybdenum (Mo), manganese (Mn), boron (B) and cobalt (Co). Those are named micronutrients. They act mainly as enzymatic cofactors, which are important for the synthesis of many compounds[63]. Iron, for example, is needed for metabolic functions, such as in the electron transport chain, reduction of nitrates to nitrites, reduction of sulphates, detoxification of reactive oxygen species, among others[63] . Mo is also an important cofactor in the process of nitrogen reduction[63]. Co, Ni, Cu and Zn (transition metals) are important factors but at lower concentration than the other micronutrients. However, at high concentrations, they can be toxic heavy metals, interacting with proteins and change the enzymatic activities within the cell of an aquatic organism[64]. Then, in the search for adequate raw materials to compose algal culture medium, a large number of these micronutrients must be analysed to prevent growth inhibition. The right amount of each element in the culture medium will depend on the type/species of microalgae being studied[65].

2.6 Design of experiments

Traditionally, the optimization of a culture medium is done using the OVAT approach, which means "one variable at a time"[66]. This approach aims to adjust one reaction variable, while others are kept constant[66]. Although this process is quite simple, OVAT becomes a time-consuming and inefficient process since it does not determine possible interactions between different factors[67]. In addition, due to the time and labour-intensive approach, the final product will be obtained at higher development costs[68]. An alternative is the design of experiments (DoE), or factorial experimental design.

DoE is a statistical method of analysis of performance that allows the development of a model which, can predict some responses of a system to the change of its variables[66]. Furthermore, unlike OVAT, this procedure also allows the evaluation of any interaction among several important variables[66]. In the context of a microbial process, the responses could be biomass production, lipid productivity, etc, and the study variables could be the operation conditions[69]. In addition, DoE will determinate the importance of the factors (screening) and their interactions (optimization)[69].

Additionally, DoE determines the effect of each factor (variable in study) individually or by changing the level of other factors (interactions); which means that the level of one factor changes the effect of other factors on a specific response[70]. The variables can be numeric or categorical. In a two-level factorial (with k factors) each factor has two levels, a “high” and a “low” level that can be quantitative

or qualitative. An assay of such an analysis requires that all possible combinations to be made (2 x 2 x… x 2 = 2k). This method consists of adding centre points that decrease the error by providing protection against excessive curvature[70]. The factorial design is useful in the initial phases of a study to uncover the behaviour of a system, to establish the importance of the factors. Besides that, this method can be easily upgraded to perform further optimizations ((through graphical plots of the response, upon changes in the values attributed to the variables). DoE is used to approximate functions when the relationship between the dependent variable response and the parameters (independent variables, factors of the study) is unknown. The first-order model with 3 parameters and their interaction terms can be mathematically described; see regression equation bellow:

$$Y_i = b_0 + b_1 X_{1i} + b_2 X_{2i} + b_3 X_{3i} + b_{12} X_{1i} X_{2i} + b_{13} X_{1i} X_{3i} + b_{23} X_{2i} X_{3i} + b_{123} X_{1i} X_{2i} X_{3i} \quad (1)$$

The Y represents the predicted response, and X_{ij} values the input variables. The b_0 is a constant (average value of the result), b_1, b_2 and b_3 are the linear coefficients; and b_{12}, b_{13}, b_{23} and b_{123} are the interaction coefficients[71,72]. Adding interaction terms to main effects introduces curvature into the response function. If there is a limit curvature, the model is appropriate [73].

After obtaining the coefficients, the response surface methodology (RSM) could be used by the Box-Behnken method. RSM is an effective method for screening key factors from multiple factors. This design allows the assessment of any interaction between several parameters, ensures the development of a model to optimize the culture conditions. This method has been successfully utilized in many fields, as the chemical industry, biological engineering, etc [17,69]. Using DoE, the number of experimental trials for the optimization of a process can be reduced as both the parameters and their mutual interactions are studied in a statistically significant manner.

2.6.1 Screening design

Screening design is the first step to DoE in order to select the most important input factors and discard the non-significant ones. One of the used tools are Pareto charts, because they allow to visualise the factors and their interactions in order of significance (Fig. 7)[74]. However, this tool does not provide information on how the responses are affected by varying the factor level. Information provided by main effects and interaction plots are thereafter useful to identify synergism or antagonism between factors and responses[75].

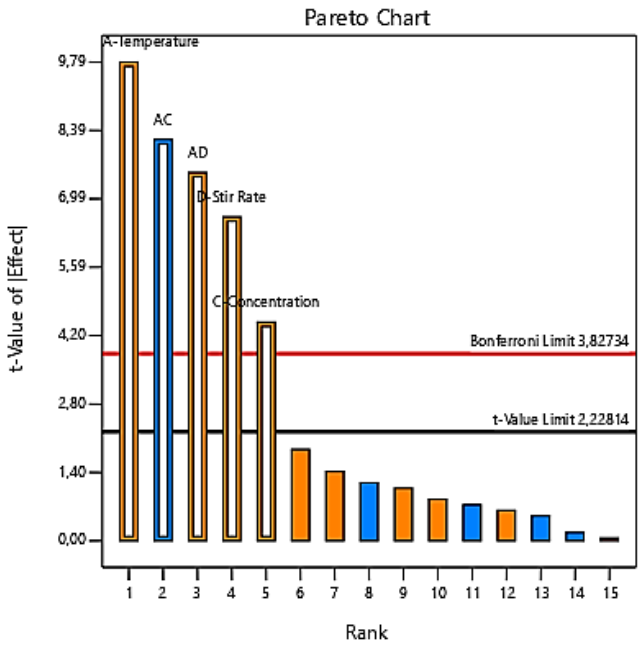


Figure 7 - Ordered bar chart (Pareto chart) from software "Design expert, 12 version". Below the t-value limit, the results are not significant. For the model, the most significant effects are temperature, AC and AD interaction, agitation rate and concentration, in order of importance. Blue columns are the positive effect. Orange columns are the negative effects. Based on Design expert tutorial.

2.6.2 Analysis of variance

ANOVA (Analysis of variance) is often used to analyse the obtained results. ANOVA is widely used as a statistical method to test differences between two or more variances. It can also identify the effects of individual variables and their interactions[75]. Based on ANOVA, it is possible to decide if a response model should include or to exclude the coefficients of linear, interaction and quadratic terms. ANOVA uses F-tests which will determinate if the specific regression terms and the equality of means. A good model is one that has the probability (*p*-value) lower than 0.05, which means the regression coefficient term under consideration is significantly different from zero. If the opposite happens, (*p*-value >0.05), then the factor from the model under consideration is not significant for the output response. Therefore, that particular regression coefficient term should be excluded from the model equation that predicts the response under study.

Multiple regression model adjustment should be assessed based in R-squared (R^2), which is also denominated as square of regression coefficient and used to measure whether the given model fits the observed data. The R^2 is the proportion of the variance in the output response that is predicted from factors. The value of R^2 varies between 0 and 1. The higher is the value, the better the model. If the predicted R^2 close to zero, the overall mean may be a better predictor of the response than the current model. In some cases, a higher order model may also predict better.

DoE is thus a powerful tool to optimize the growth of microalgae that helps to decrease the time in the process and increase work efficiency. In addition, this procedure was already used to improve the

medium composition of microalgae, using different methodologies, such as central composite design (CCD) and Placket-Burman [17,71].

3. Aim of the work

The main goal of this work was the production of highly concentrated biomass of *Scenedesmus rubescens* to induce carotenoid production. To achieve the main goal, the following strategy was considered:

- Optimization of heterotrophic culture medium and conditions for maximum biomass productivity;
- Heterotrophic culture scale-up to validate the previous goal and to assess this strain industrial production feasibility;
- Optimization of the strategy to maximize carotenoid production;
- Evaluate the potential biomass for food and pharmaceutical applications.

4. Materials and methods

All experimental trials were carried out at Allmicroalgae's research and development unit between February 17th and November 10th, 2020. However, due to the outbreak of the Covid 19 pandemic, between March and April it was not possible to develop any work at the facilities. The biochemical characterization of the produced biomass was performed at the MarBiotech group of the Centre of Marine Sciences (University of Algarve), between 23rd and 27th of November.

4.1 Strain and culture media

Scenedesmus rubescens (strain 0037SA) used in this work was axenic and obtained from Allmicroalgae's culture collection. This alga was stored in agar slant tubes and subsequently scaled to 250 mL Erlenmeyer flasks; cultures are constantly growing to maintain good conditions to start new culture assays in the exponential phase. Initially the culture media used was PCB (plate count broth). As optimization results were being obtained, cultures were subsequently grown in the most recent version of the optimized media. These tests were performed using one, two or three replicates, according to the type of tests. All culture media was sterilized by filtration in a 0.2 µm pore size PES Vacuum Filtration System (VWR, Pennsylvania, USA) and/or heat at 121 ºC for 40 min using an autoclave (Uniclave88 and uniclave77, A.J.Costa, Irmãos, Lda; Cacém, Portugal). Both the sterilization of the culture media by filtration and the inoculation phase and samples from the assays were handled in the laminar flow chamber Top Safe (LafTech, Melbourne, Australia).

4.2 Experimental Procedure

The experimental procedure consisted in mainly of 3 steps: (1) optimization of culture media; (2) heterotrophic scale-up and (3) increase in astaxanthin content (induction phase) in (3a) auto or (3b) heterotrophic conditions.

4.2.1 Experimental conditions

All experimental trials for medium optimization were conducted in 250 ml baffled Erlenmeyer flasks, ventilated with a 0.2 µm PTFE membrane (Duran™, Munich, Germany) with a working volume of 50 mL. Cultures were grown in an orbital shaker incubator (SKI 4, ARGOLAB, Carpi, Italy) at 28 ºC and 200 rpm (revolutions per minute). The heterotrophic assays were performed in axenic conditions using the same laminar flow chamber described in the point 3.1. For the optimization trials, the assays ended when cultures reached the stationary phase, or the carbon source was depleted. Erlenmeyer flasks were further scaled up to inoculate a 7L bench-top fermenter (New Brunswick BioFlo®/CelliGen®115; Eppendorf AG, Hamburg, Germany). Cultures were grown at 28ºC and pH was maintained at 6.5 by addition of ammonia (24% w/w).

For the induction tests, the culture grown in the 7 L reactors was used to inoculate 70L flat panels (FP), with low concentrations of culture medium (equivalent to 1 mM of nitrates) to induce carotenoid's production. The pH was kept in the range of 7-8 by the injection of CO_2.

4.2.2 Inoculation

The inoculation was done in order to guarantee an optical density (OD), at the wavelength of 600nm, between 0.150 and 0.250. The inoculum volume for the target initial optical density of the culture was calculated using the following equation:

$$\text{Vi (inoculum)} = \frac{\text{Vf (culture)} \times \text{Ci (OD culture)}}{\text{Cf (OD inoculum)}} \quad (2)$$

4.2.3 Growth assessment

The growth of *S. rubescens* was determined by OD and dry weight (DW). OD was measured at 540, 600 and 750nm, using a spectrophotometer (Genesis 10S UV-Vis - Thermo Scientific, Massachusetts, EUA). DW was determined by filtration of culture samples using pre-weighed 0.7 µm GF/C 698 filters (VWR, Pennsylvania, USA) and dried at 120 ºC on a DBS 60–30 electronic moisture analyser (KERN & SOHN GmbH, Balingen, Germany). In addition, absorbance values were converted into dry weight values using an in-house calibration curve (Appendix A1). These measurements were used to study cell growth, namely the specific growth rate and maximum and overall productivity were calculated.

The specific growth rate (µ) was calculated according to equation (3):

$$\mu\ (\text{day}^{-1}) = \frac{\ln(X2/X1)}{t2 - t1} \quad (3)$$

X_2 X_1 refer to biomass concentration (g L^{-1}) at time t_2 and t_1 (days) of cultivation within the exponential growth phase.

The volumetric biomass productivity (Pv) was calculated according to equation below (4):

$$Pv = \frac{Xf - Xi}{tf - ti} \quad (4)$$

Were Xf corresponding to final biomass concentration, Xi initial biomass concentration (g L^{-1}), tf, final time and ti the initial time (h) of cultivation within the exponential growth phase.

4.2.4 Microscopy

Culture was regularly microscopically observed to verify its axenicity and viability. For that, a Zeiss® Axio Scope A1 (Oberkochen, Germany coupled with ZEN Axicam 503 (Oberkochen, Germany) colour camera was used. To capture and edit the images, the Zen blue 2.5 lite software (ZEISS, Oberkochen, Germany) was used.

4.2.5 Nutrient quantification

The cultures sampled (50mL) were centrifuged for 10 min at 3500 rpm in VWR Mini Star microcentrifuge (VWR, Pennsylvania, USA). The supernatant was collected to quantify glucose, phosphate, ammonium, nitrate, magnesium and iron concentrations.

4.2.5.1 Nutrients (glucose, ammonium, phosphate, and nitrate, iron and magnesium)

The supernatant was diluted in saline solution (10% sodium chloride, 90% distilled water) when necessary. Freestyle precision Neo (Abbott, Witney, Oxon, UK) was used to determinate glucose concentration, in g/L.

Ammonia and phosphate Sera Tests (Sera, Heinsberg, Germany) were used to determine ammonium and phosphate concentrations, respectively. The supernatant was diluted with distilled water when necessary. The absorbance was measured at the wavelength of 697 nm for ammonium and 716 nm for phosphate. In case of nitrate concentration, the supernatant was diluted 1:80 in distilled water and hydrochloric acid (1M). The wavelengths 220 nm and 275 nm were used to measure the nitrate concentration. The three compounds were then determined based on in-house calibrations curves (Appendix 2) to mM (mmol/L) units. The absorbances were measured using Genesis 10S UV-Vis (Thermo Scientific, Massachusetts, EUA).

Iron and Magnesium Sera Tests were used to determine Iron and Magnesium concentrations respectively. The wavelength 561 nm was used to measure the iron. The absorbance was measured using Genesis 10S UV-Vis (Thermo Scientific, Massachusetts, EUA). The magnesium concentration is measured by the number of drops added.

4.2.6 Phase 1 – optimization of culture media

To optimize culture media, two type of tests were performed: OVAT tests and DoE tests. All assays were done with 20 g L^{-1} of glucose.

The following media were used for preliminary tests: TAP medium[76], Bold's basal medium; BBM[77] (5x concentrated) and MB16 Allmicroalgae fermentation medium (confidential composition). OVAT methodology was performed to test pH, phosphate concentration and vitamin effects. Every test was done in triplicate. Finally, DoE methodology was used for final media optimization, using Plackett-Burman methodology through the Mini Tab software (Mini tab, State College, Pennsylvania, EUA) and Box-Behnken methodology through the Design Expert software (version 12, Stat-Easy, Minnesota, USA).

4.2.7 Phase 2 – Scale-up process in heterotrophic route

In this stage, the optimized culture media previously obtained was used. Fifty millilitres of culture were used to inoculate an Erlenmeyer with 350 mL working volume. Then a 7 L bench-top fermenter (New Brunswick BioFlo®/CelliGen®115; Eppendorf AG, Hamburg, Germany) was inoculated with the initial working volume of 3L. This bioreactor was operated in fed-batch mode, supplemented with a 50% (w/w) glucose solution, 24% anti-foam solution and ammonia solution as nitrogen source. Ammonia (w/w) was also used to control the pH of the fermenter. Culture was maintained at 28ºC with a thermostatic bath, using a water jacked. The air flow intensity and the variation of rpm were automatically adjusted to assure favourable culture conditions.

4.2.8 Phase 3 - Induction of carotenoid production

Autotrophic and heterotrophic assays for carotenoid induction were performed. In the first case, when operational conditions existed, pilot-scale carotenoid production experiments were conducted in a 70L flat panel with limited MNS medium, a Guillard f/2-based medium[79] (1 mM of nitrate). CO_2 was injected to maintain pH in the range of 7 to 8.

For the heterotrophic assay, the medium was also limited. For that a DoE was performed, using a Box Behnken design from Design Expert (Stat-Easy, Minnesota, USA). The temperature was maintained at 28ºC, the pH ranged between 4 and 9 and the cells the cells grew in the dark. Fifty millilitres working volume was used to test these conditions in 350mL Erlenmeyer flasks.

4.3 Biochemical analyses

4.3.1 Protein content

Using a Vario EL III elemental analyser (Vario EL, GmbH, Hanau, Germany), total carbon, hydrogen and nitrogen (CNH analysis) of the freeze-dried biomass were analysed. About 1 mg of biomass was placed in tin capsules and then heated at 950 ºC. Total protein content was calculated by multiplying the nitrogen amount by the conversion factor of 6.25[80] after CHN analysis.

4.3.2 Lipid content

The lipid content was determined by a modified method of Bligh and Dyer (1959[81]), as reported by Pereira et al. (2011)[82]. From 20 mg of freeze-dried biomass, the lipids were extracted through a process of solvent addition and homogenization. The homogenization was carried out using an IKA T-25 Ultraturrax disperser (IKA-Werke GmbH, Staufen, Germany), proceeding to three steps. The first step was the addition of chloroform and methanol and 60s of homogenization. The second step was the addition of chloroform and 30s of homogenization. The last step was added only distilled water and 30 s homogenization. Then, the samples were centrifuged at 2500xg for 10 min in a Thermo Scientific Heraeus Megafuge (Thermo Scientific, Massachusetts, EUA). The chloroform phase, which remained in the bottom layer, was recovered with Pasteur pipette, put in a tube (pre-weighed), that evaporated overnight. Finally, in order to obtain the percentage of lipids, the dried residue was compared with dried mass of the culture. The percentage was calculated with the following equation:

$$\%\text{lipids} = \frac{\text{final weight} - \text{initial weight} \times \text{V olume total chloroform}}{\text{Volume evapoated cholorform} \times \text{weight of the dried sample}} \text{ x } 100 \qquad (7)$$

4.3.3 Ash content

Fifty milligrams of freeze-dried biomass were weighed in a crucible and taken to combustion at 550ºC for 8h, using a JP Selecta Sel horn R9-L furnace (JP Selecta, 22 Barcelona, Spain). After the combustion of the biomass, the weight difference resulted in the ash content.

4.3.4 Carotenoid content

The extraction of carotenoids was carried out on ice and under dim light to avoid oxidation. Twenty milligrams of freeze-dried biomass (pulverized material) were weight, put into a tube and methanol was added. With the addition of glass beads (500 -750µm), until filling 0.5 mL of Eppendorf tube, the cells were lysed using Retsch MM 400 mixer mill (Retsch Düsseldorf, Germany), at 30Hz, for 4 minutes. After disruption of cells, chloroform was added and the samples were vortexed (IKA vortex 3, China) and centrifuged using centrifuge z167 M (Hermle Laborator Technik - Germany)

for 5 min, at 3500 rpm. Then chloroform layer was removed from the bottom and replaced in a new 2mL Eppendorf tube. The extraction was repeated twice more. The chloroform collected was evaporated using a nitrogen flow.

Carotenoids analysis was performed by high-performance liquid chromatography (HPLC; Dionex Corporation, California, USA). Carotenoid separation was achieved using a mobile phase composed of solvent A chloroform and solvent B methanol (1:10) and filtered using 0.2 µm PTFE filter. The final volume was replaced on a glass via, adding 700µL of extract and the caps with pre-slit septa was placed. All carotenoids were detected at 450 nm and 280 nm and analysed with Chromeleon Chromatography Data System software (Version 6.3, ThermoFisher Scientific, Massachusetts, USA). The quantification was carried out using calibration curves for neoxanthin, lutein, zeaxanthin and β-carotene standards (Sigma-Aldrich, Portugal) astaxanthin was quantified with a calibration curve from Universidade do Algarve.

4.4 Statistical analysis

The statistical tests for OVAT were performed using R software (4.0.2 version), through RStudio 1.3.1073 version (R studio, Boston, USA). ANOVA analysis was followed by a post-hoc Tukey-HSD test when three or more conditions were compared. To compare groups of independent results a Student t-test was used with a confidence level ≥ 95 %. For each test, the mean and standard deviation of the triplicate were determined. Throughout the text, different letters were used to highlight the case significant differences. Statistically significant difference was considered at $p<0.05$. When the same letter is associated with different results, there was no significant differences among them as $p>0.05$.

The statistical tests for DoE methodology was performed using two software: Mini Tab (Mini tab, State College, Pennsylvania, EUA) based on a preliminary screening and Design Expert (version 12, Stat-Easy, Minnesota, USA) based on response surface methodology. Mini tab was used to do a preliminary screening through Placket-Burman design. Using Design Expert, the Box-Behnken design was conducted. ANOVA was also performed. Statistically significant difference was considered at $p<0.05$.

5. Results and discussion

5.1 Optimization of culture media

5.1.1 Influence of carbon source and culture media

As was previous mentioned, three different culture media were chosen to increase the growth of biomass: TAP (Fig.8), BBM 5-fold concentrated (Fig.9) and MB16 (Fig.10). This preliminary assay also aimed to infer about the most suitable source of carbon for *Scenedesmus rubescens* growth. Fig. 8 shows that the highest concentration of biomass occurred in the condition supplemented with glucose (0.801 of OD). These results suggested that the glucose was the carbon source that allowed the highest culture productivity and that with glycerol and acetate the microalgae do not grow.

Fig. 9 shows the results of heterotrophic cultivation of S. rubescens in Bold basal medium with or without supplementation of urea, where is clearly seen the positive impact of using this nitrogen source, since a higher biomass concentration is obtained (OD=10.6). However, the difference between cell growth outcomes may also be a result of the lack of nitrogen source before the culture had reached the stationary phase in the non-supplemented condition (only 5.5 mM of nitrates were used).

MB16 medium (ammonia as nitrogen source and glucose as carbon source) was tested at two different concentrations: 60 and 30 mM ammonia (Fig. 10). Besides the diluted medium had showed higher cell concentration until day 4, at this point the culture entered the stationary phase. In another hand, the concentrated medium still allowed the culture growth for one day more, suggesting that this medium may be inhibitory only at the initial growth phases.

In general, when comparing the growth among the culture media, in the 5-fold concentrated BBM, the culture achieved the highest cell density. However, it is not possible to conclude that this is the medium which better promotes the growth of *S. rubescens* since in the other two media cells suffered from a pH decrease, reaching values close to 4, which could had possibly inhibited the cell growth.

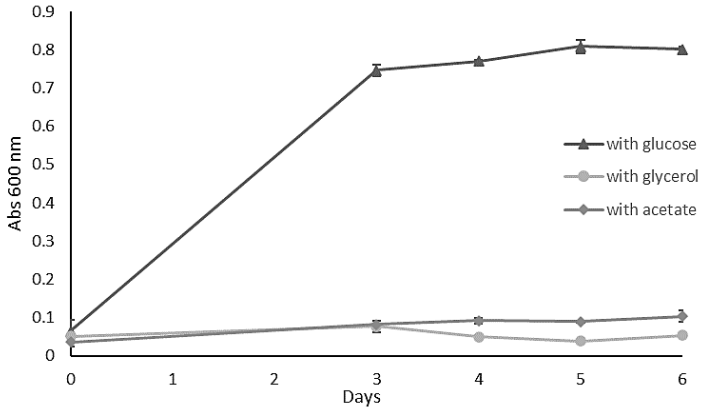


Figure 8 - *Scenedesmus rubescens* growth curves supplemented with carbon sources with the same concentration. The cultures grown under heterotrophic conditions in 250mL Erlenmeyer, using TAP medium. The experiment lasted 6 days. The values represent the average and respective standard deviation of 2 Individual experiments.

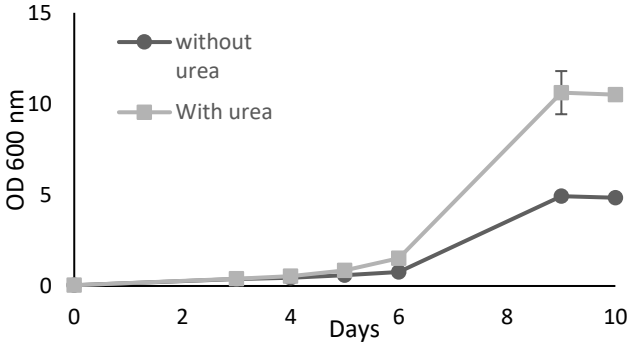


Figure 9 - *Scenedesmus rubescens* growth curves for supplementation with nitrates and nitrates + 30 mM urea as nitrogen sources. The cultures grown under heterotrophic conditions in 250mL Erlenmeyer, using of Bolds medium 5x concentrated. The experiment lasted 10 days. The values represent the average and respective standard deviation of 2 Individual experiments.

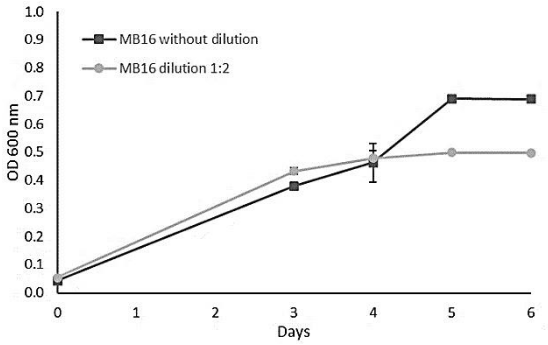


Figure 10 - *Scenedesmus rubescens* growth curves for supplementation with glucose as carbon source and ammonia as nitrogen source. The cultures grown under heterotrophic conditions in 250mL Erlenmeyer, using MB16 diluted 1:2 (30mM ammonia) and without dilution (60 mM of ammonia) The experiment lasted 6 days. The values represent the average and respective standard deviation of 2 Individual experiments.

5.1.2 Buffer and culture media validation

The media screening showed great variation on growth possibly due to pH decrease, with biomass increase. Hence, the application of a buffer to eliminate pH influence on growth was studied. Only BBM (pH 6.6) did not require the addition of any buffer. Furthermore, *S. rubences* growing in BBM achieved the highest biomass concentration, the pH tested was close to 7.

In Fig. 11, the highest biomass concentration was achieved by the culture supplemented with PIPES buffer, obtaining an OD value of 4.48. PIPES maintained the pH close to 7, while cultures growing with Tris buffer decreased to a value close to 6, which influenced the growth of the microalgae (Table 4). Thus, PIPES seemed to be a suitable buffer to control the pH, using PIPES for the next assays.

In comparison with BBM (supplemented with urea) and MB16 (buffered with PIPES), BBM-growing culture reached the highest biomass concentration (10.4 of OD). However, there was a better adaptation of *S. rubescens* at an early stage on TAP's medium. In this medium, cells reached the stationary phase earlier due to depletion of the nitrogen source (7.5 mM of ammonia).

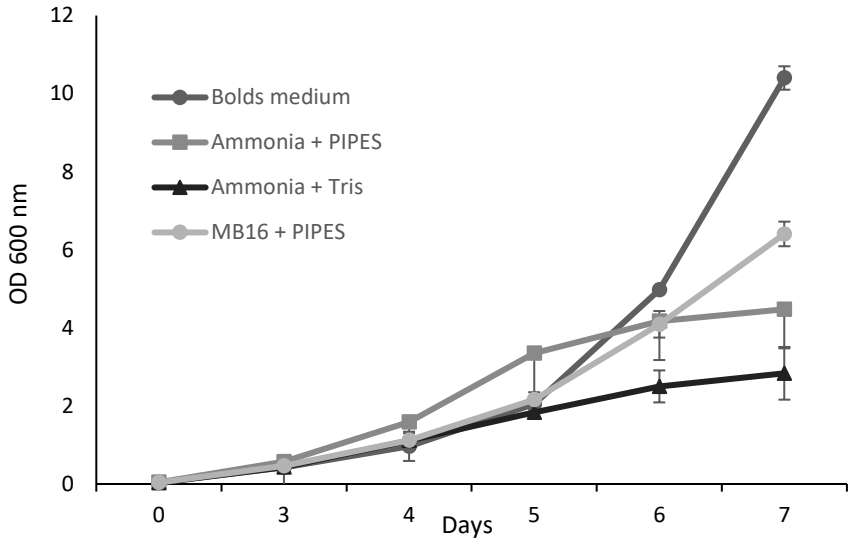


Figure 11 - *Scenedesmus rubescens* growth curves. The cultures grown under heterotrophic conditions in 250mL Erlenmeyer using Bolds medium 5-fold concentrated supplemented with urea and TAP medium supplemented with PIPES buffer and Tris buffer. MB16 medium was also tested buffered with PIPES. The experiment lasted 7 days. All the tests were supplemented glucose as carbon source. The values represent the average and respective standard deviation of 2 Individual experiments.

Table 4 - Variations of pH in different buffer conditions, using TAP and MB16 medium.

Conditions	Initial pH	Final pH
MB16 + PIPES	7	7
TAP + PIPES	7	7
TAP + Tris	7	6

5.1.3 DoE - screening

The development of the culture medium is a complex process due to the interactions between all the factors that influence cell growth, which promote its growth or even inhibit it. Thus, DoE was applied to better understand the interactions among factors.

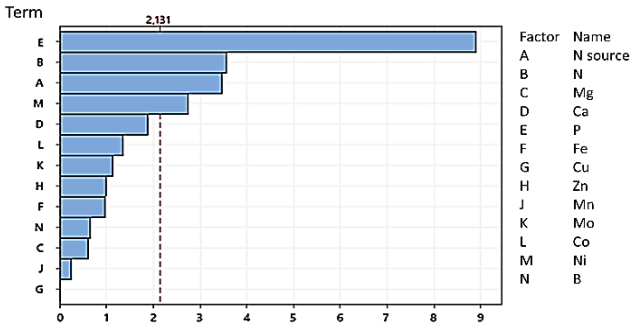
In order to optimize the culture medium, 12 nutrients were studied: N, Mg, Ca, P, Fe, Cu, Zn, Mn, Mo, Co, Ni and B. Screening was carried out to predict which nutrients influence the biomass productivity. Thus, using previously studied culture media, low and high concentrations were stipulated, obtaining a matrix through the Mini Tab software. The Placket-Burman design was used with two coded levels, using 13 variables, which are different nutrient concentration and the different source of nitrogen (nitrates and ammonia); starting with pH of 6.5. Thirty runs were employed (Appendix C1 and C2), with the chosen responses, which are biomass concentration, global productivity and maximum productivity. The factors that most influenced cell growth were the source of nitrogen, and P, Ni, and Ca concentrations. Nevertheless, this last nutrient influenced only on maximum productivity (p-value <0.05) (Fig. 12). This assay was important to analyse in more detail the elements that most influence cell

growth. At the early growth stages, cultures supplemented with ammonia presented higher biomass concentration than nitrates (Table 5). However, the multifactorial model indicated nitrates as the most suitable nitrogen source for *S. rubescens* growth. This is due the pH drops to 5, inhibiting the culture growth in the presence of ammonia and thus, compromising the growth. In the presence of nitrates, the pH increased between 7.5 and 8, not inhibiting cell growth, obtaining greater global productivity. Thus, the pH variation was a determinant factor to compromise the assay. Besides that, buffer concentration was not enough to keep the pH stable, being necessary to define a new concentration of PIPES. From this assay, the culture medium was modelled, except for the conditions that were consecutively tested in each assay (Appendix C4).

Table 5 - Biomass concentration and pH values at days 2 and 3 of assays from Placket Burman design. The chosen samples were the ones that reached the highest productivity.

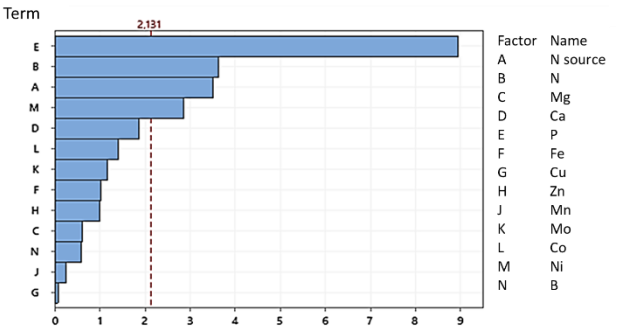
		Biomass concentration (g L^{-1})		**pH values**	
Sample	**Nitrogen source**	**Day 2**	**Day 3**	**Day 2**	**Day 3**
4	Nitrate	1.7	4.9	7	7.5
5	Ammonia	1.6	5.2	6	5.5
6	Nitrate	1.4	4.2	7	7
9	Ammonia	1.5	4.6	6	5
10	Ammonia	1.8	5.4	6	5
11	Ammonia	1.5	4.9	6	5
13	Ammonia	1.4	4.0	6	6
14	Nitrate	1.5	4.3	7	7
22	Ammonia	1.5	3.8	6	6
23	Ammonia	1.1	4.0	6	6
25	Ammonia	1.3	4.5	6	6
26	Ammonia	1.6	5.2	6	5
29	Nitrate	1.8	4.3	7	7.5
30	Nitrate	1.1	4.4	7	7.5

Term	Effect	Coef	P-value
Constant		10,275	0,000
N source	2,041	1,021	0,003
N	-2,575	-1,287	0,003
Mg	-0,439	-0,220	0,552
Ca	1,359	0,679	0,079
P	6,427	3,213	0,000
Fe	-0,701	-0,351	0,346
Cu	-0,015	-0,007	0,984
Zn	-0,711	-0,355	0,340
Mn	-0,185	-0,092	0,801
Mo	0,829	0,414	0,268
Co	0,983	0,491	0,193
Ni	1,989	0,994	0,015
B	-0,483	-0,242	0,513



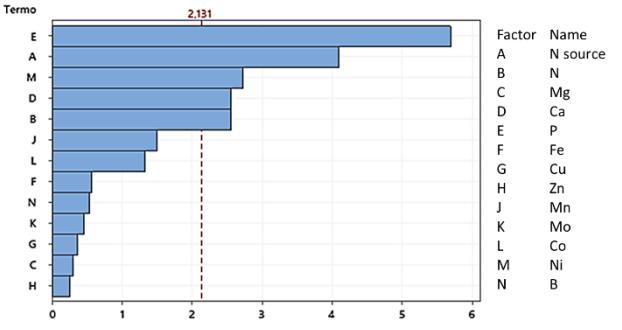
A

Term	Effect	Coef	P-value
Constant		0,09386	0,000
N source	0,01890	0,00945	0,003
N	-0,02383	-0,01191	0,002
Mg	-0,00412	-0,00206	0,540
Ca	0,01224	0,00612	0,082
P	0,05882	0,02941	0,000
Fe	-0,00681	-0,00340	0,316
Cu	-0,00051	-0,00026	0,939
Zn	-0,00665	-0,00333	0,327
Mn	-0,00175	-0,00087	0,794
Mo	0,00762	0,00381	0,264
Co	0,00926	0,00463	0,179
Ni	0,01873	0,00936	0,012
B	-0,00384	-0,00192	0,567



B

Term	Effect	Coef	P-value
Constant		0,4533	0,000
N source	0,3707	0,1853	0,001
N	-0,2824	-0,1412	0,022
Mg	0,0336	0,0168	0,765
Ca	0,2826	0,1413	0,022
P	0,6298	0,3149	0,000
Fe	-0,0628	-0,0314	0,579
Cu	-0,0404	-0,0202	0,720
Zn	0,0292	0,0146	0,795
Mn	-0,1654	-0,0827	0,156
Mo	-0,0506	-0,0253	0,654
Co	-0,1464	-0,0732	0,205
Ni	0,3014	0,1507	0,016
B	-0,0600	-0,0300	0,595

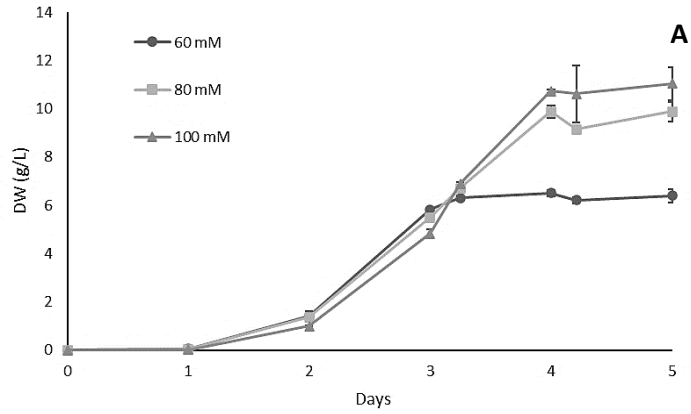


C

Figure 12 - Analysis of variance (ANOVA) and order bar chart (Pareto chart) obtained with the software *Minitab 19*, testing 13 factors for 3 responses A) Biomass concentration B) Global productivity C) Maximum productivity. The model was below to 0.05 of *p*-value (close to 0) which means that the model was significant.

5.1.4 Buffer concentration

Using ammonia as nitrogen source, different concentrations of buffer were tested: 60, 80 and 100 mM (Fig.13). 80 and 100 mM buffer concentrations resulted in an initial inhibition of growth and allowed keeping the pH at 6.5 for the longest time. In addition, 100 mM of PIPES allowed *S. rubescens* higher global productivity (0.112 g L^{-1} h^{-1}), followed by 80 mM. The condition 60 mM wasn´t effective maintaining pH=6.5 and so, the productivity (0.068 g L^{-1} h^{-1}) and the specific growth rate were lower (1.675 day^{-1}). Therefore, 100 mM was more effective in maintaining pH and allowed reaching the highest biomass productivity, which will be used in the next assays.



B

PIPES concentration (mM)	Initial pH	Final pH
60	6.5	5
80	6.5	6.5
100	6.5	6.5

Figure 13 - A) *Scenedesmus rubescens* growth curves under 3 concentrations of PIPES buffer: 60, 80 and 100 mM. The cultures grown under heterotrophic conditions in 250mL Erlenmeyer. B) Variations of pH when using the different PIPES concentrations. The experiment lasted 5 days. The values represent the average and respective standard deviation of 3 individual experiments. Base medium from section 5.1.3.

Table 6 – Global productivity and specific growth rate of S. rubescens under different PIPES concentrations. Base medium from section 5.1.3.

PIPES concentrations (mM)	Global productivity (g $L^{-1}h^{-1}$)	Specific growth rate (day^{-1})
60	$0.068^{a} \pm 0.001$	$1.68^{a} \pm 0.17$
80	$0.103^{b} \pm 0.003$	$2.05^{b} \pm 0.13$
100	$0.112^{c} \pm 0.001$	$2.58^{c} \pm 0.12$

5.1.5 Effects of different nitrogen sources

Once the previous results suggested that urea induces higher biomass concentration, an assay directly comparing the effects of supplementing different nitrogen sources was performed. Figure 14 shows the growth curves obtained, on which it is visible that cultures present the same behaviour when supplemented with different nitrogen sources. As was mentioned in section 3.2.7, ammonia is also used to maintain the pH in fermentation process (scale-up under heterotrophic conditions). Thus, to assure similarity in the process, ammonia was selected as the nitrogen source to be used further on.

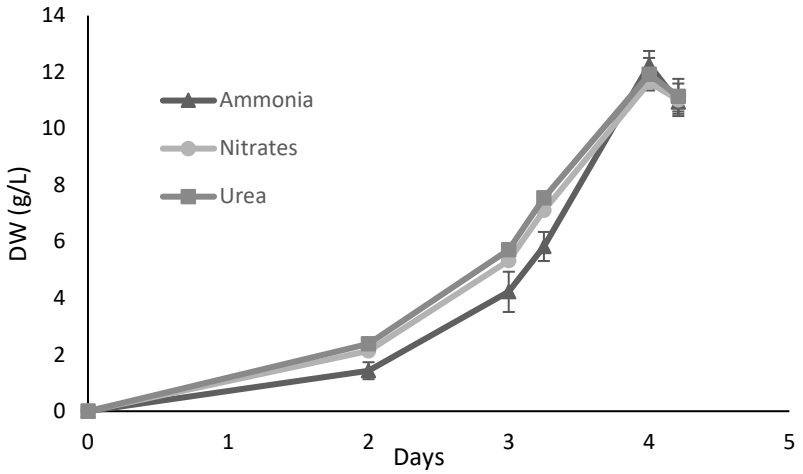


Figure 14 - *Scenedesmus rubescens* growth curves supplemented with different sources of nitrogen (with the same concentration). The cultures grown under heterotrophic conditions in 250mL Erlenmeyer. The values represent the average and respective standard deviation of 3 individual experiments. Base medium from section 5.1.3.

5.1.6 DoE – Optimization of medium culture using response of surface

"Design of Expert" Software was used to optimize the medium composition through "Box-Behnken design", a RSM method. As the effects of nitrogen sources had already been tested, 4 variables that most influenced the growth of *S. rubescens* were chosen (Table 7): N, P, Ni and Ca concentrations. The responses studied were biomass concentration, global productivity and maximum productivity. In this experimental design, a total of 26 sets of experiments was generated with 2 central points (Appendix C3). The central points determine the curvature and compensate the lack of fit values, indicating the significance of the model.

Table 7 – Levels of factors chosen for the experimental design.

Factors (mM)	Symbols	-1	0	+1
Ammonia	A	20	40	60
Phosphate	B	1	5.5	10
Nickel	C	0.3	1	1.7
Calcium	D	0	0.01	0.02

RSM allowed visualising a quadratic regression for biomass concentration and global productivity and a linear regression for maximum productivity. ANOVA (Fig. 15) indicated that the model was significant, F-value = 19.02 for biomass concentration, 14.23 for global productivity and 15.77 for maximum productivity. Also, the factor that most influenced cell growth was the concentration of phosphate (*p*-value = 0.0001). Only this factor in all responses is less than 0.05. These results suggest that the other factors and their interactions are not significant for culture growth.

Source	F-value	p-value	
Model	19,02	< 0.0001	significant
A-N	0,0050	0,9451	
B-P	185,18	< 0.0001	
C-Ca	0,5839	0,4609	
D-Ni	0,1742	0,6845	
AB	0,0034	0,9542	
AC	4,32	0,0619	
AD	0,3997	0,5401	
BC	0,0108	0,9191	
BD	0,8998	0,3632	
CD	1,72	0,2169	
A^2	0,7387	0,4084	
B^2	42,66	< 0.0001	
C^2	0,7549	0,4035	**A**
D^2	0,0017	0,9681	

Source	F-value	p-value	
Model	14,23	< 0.0001	significant
A-N	0,1740	0,6846	
B-P	136,06	< 0.0001	
C-Ca	0,0119	0,9152	
D-Ni	0,2383	0,6350	
AB	0,5704	0,4660	
AC	0,3791	0,5506	
AD	2,56	0,1377	
BC	1,19	0,2982	
BD	0,8606	0,3735	
CD	0,4869	0,4998	
A^2	0,0003	0,9866	
B^2	38,06	< 0.0001	
C^2	0,0026	0,9599	**B**
D^2	0,0097	0,9232	

Source	F-value	p-value	
Model	15,77	< 0.0001	significant
A-N	2,49	0,1298	
B-P	60,17	< 0.0001	
C-Ca	0,3015	0,5887	
D-Ni	0,1281	0,7240	**C**

Figure 15 - Analysis of variances (ANOVA) for 3 responses A) biomass concentration, B) Global productivity C) Maximum productivity. According to F-value and p-value, the models are significant in relation to phosphate concentration. The other variables are not significant. Tables from Design of expert software.

Using "Design of expert", RSM was performed (Fig.16). Figure 16A shows the interaction between variables P and N for the concentration of biomass. When using Ni at the medium level and Ca at the lowest, the concentration of P and N need to be increased (10mM and 60mM, respectively) in order to achieve higher biomass concentration. In the case of global productivity (Fig.16B), the graph shows the interaction between Ca and P. When using N and Ni are at the medium level, P needed to reach its highest level and Ca its lowest in order to obtain higher values of global productivity. Finally, P and Ni at the higher level, and N and Ca at the medium level, induce higher values of maximum productivity (Fig. 16C). From those models, it was possible to conclude that to optimize culture medium, values close to the highest level for factors N, P and Ni and lower values of Ca were necessary. Thus, 60, 10, 0.02 and 0.3 mM were used for N, P, Ni and Ca, respectively.

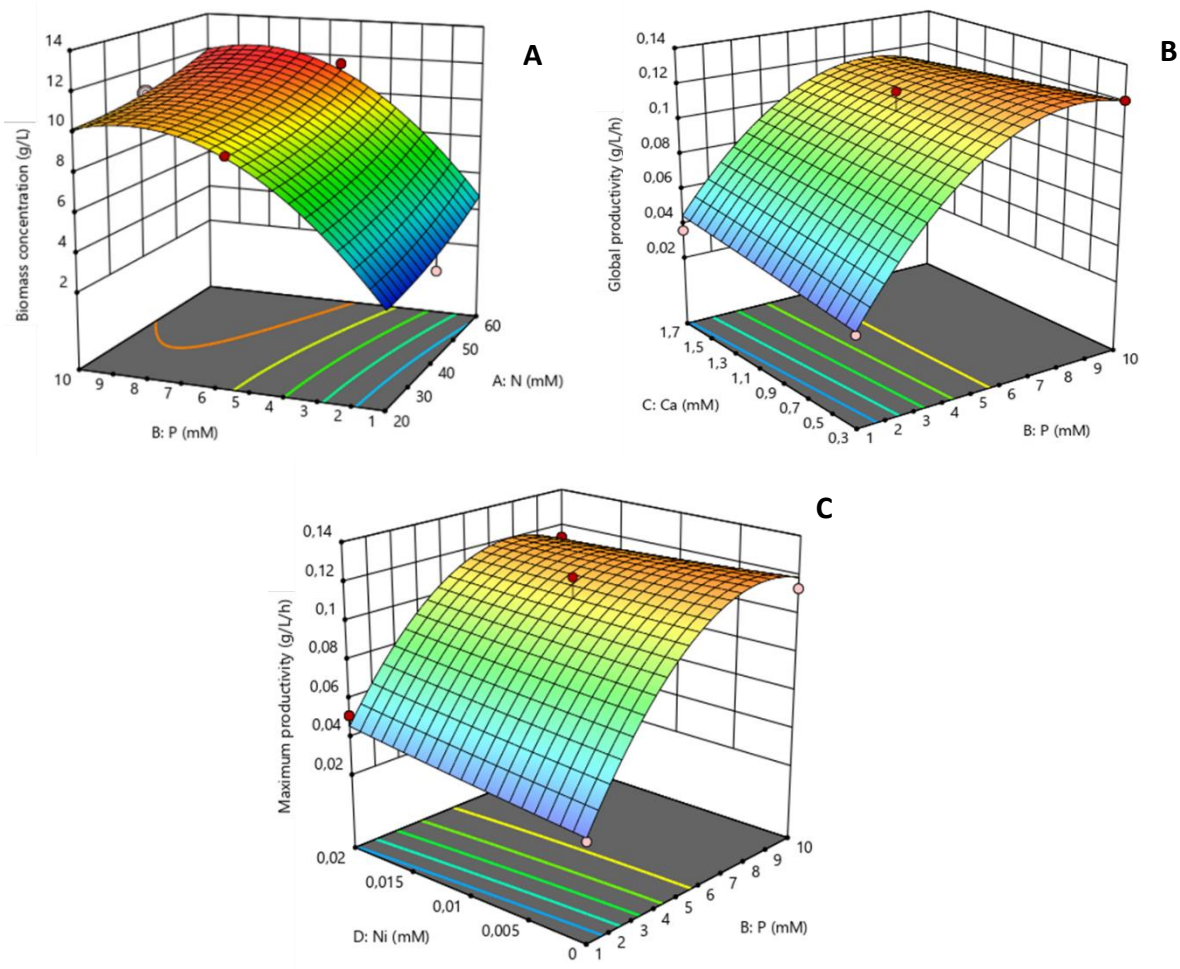


Figure 16 - Response surfaces showing the mutual effects of P and the other factors. A) Effects of the interaction between P and N factor for biomass concentration response. Ni was kept at maximum level and Ca was kept at low level. B) Effects of the interaction between P and Ca factor for global productivity response. N and Ni were kept at medium level. C) Effects of the interaction between P and Ni factor for Maximum productivity response. N and Ca were kept at medium level.

5.1.7 Effects of pH on *S. rubescens* growth

After performing the second experimental design (section 4.1.6) different pH values were tested: 6, 6.5, 7 and 8. The growth curves obtained are presented in Fig. 17. The results show a higher biomass concentration when culture is growing at pH 6.5 (11.2 g L^{-1}), followed by pH 7 (10.5 g L^{-1}), pH 6 (4.5 g L^{-1}) and, at last, pH 8 (2.3 g L^{-1}). However, the analysis of global productivity and the growth rate (table 8), showed no significant differences between pH 6.5 and 7 (*p*-value> 0.05). pH 6.5 was used for the following assays.

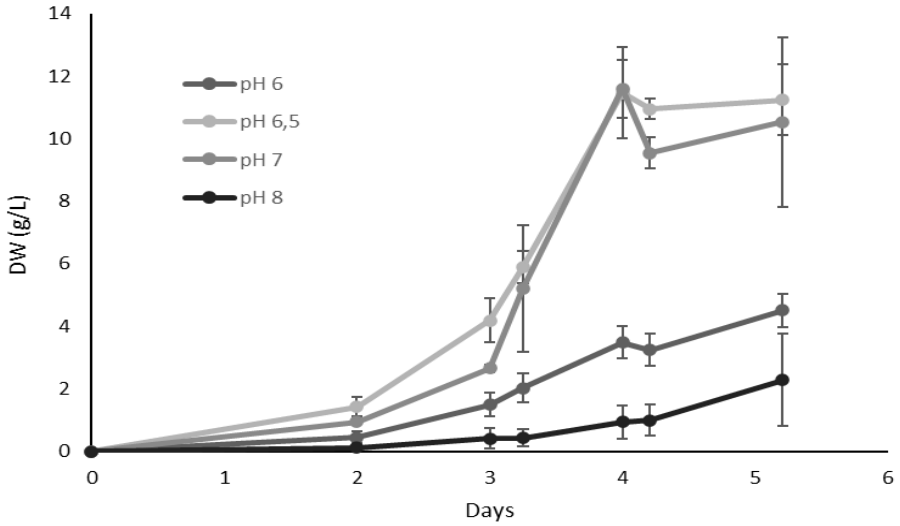


Figure 17 - *Scenedesmus rubescens* growth curves under different pH values. The cultures grown under heterotrophic conditions in 250mL Erlenmeyer. The values represent the average and respective standard deviation of 3 Individual experiments. Base medium from section 5.1.3.

Table 8 - Productivity and specific growth rate under different values of pH for *Scenedesmus rubescens*. Base medium from section 5.1.3.

pH	Global productivity (g L^{-1} h^{-1})	Specific growth rate (day^{-1})
6	$0.035^{a} \pm 0.004$	$1.77^{a} \pm 0.060$
6.5	$0.119^{b} \pm 0.012$	$2.37^{b} \pm 0.053$
7	$0.121^{b} \pm 0.008$	$2.38^{b} \pm 0.032$
8	$0.010^{c} \pm 0.005$	$1.07^{c} \pm 0.230$

5.1.8 Effects of vitamins

The effect of vitamins' presence was tested, and the growth curves are represented in Fig. 18. *S. rubescens* growing with vitamin supplementation have a higher growth when compared to the control, achieving a biomass concentration of 9.1 g L^{-1}. The same was observed for the global productivity and specific growth rate (Table 9).

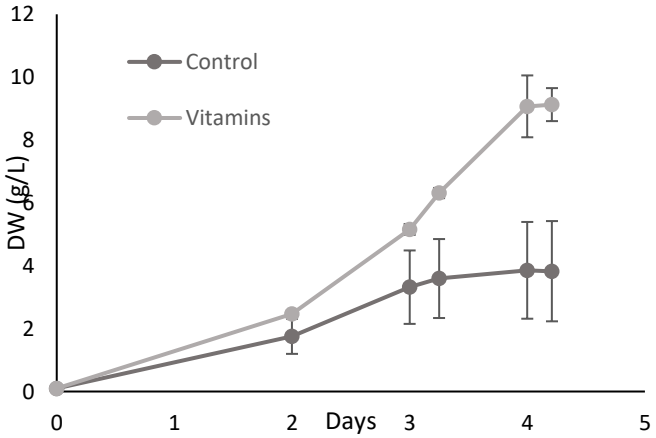


Figure 18 - *Scenedesmus rubescens* growth curves, testing the influence of vitamins. The cultures grown under heterotrophic conditions in 250mL Erlenmeyer. The values represent the average and respective standard deviation of 3 Individual experiments. Base medium from section 5.1.3.

Table 9 - Comparison of global productivity and specific growth rate of *S. rubescens* growing with vitamin supplementation. *B*ase medium from section 5.1.3.

Conditions	Global productivity (g L^{-1} h^{-1})	Specific growth rate (Day^{-1})
Control	0.037^{a}±0.015	0.90^{a}±0.092
Vitamins (0.075µL)	0.089^{b}±0.005	1.13^{ab}±0.027

5.1.9 Effects of different phosphate concentrations

In order to understand if nutrient concentration could be limiting to cell growth, phosphate was tested at 10 (control) 50 and 100 mM (Fig. 19). The growth curve showed that the use of 50 mM phosphate results in higher concentration of biomass (11.7 g L^{-1}), followed by the control (11.5 g L^{-1}), *p*-value >0.05). 100 mM phosphates induced an initial inhibition in the cell growth. However, at the third day, the culture reached the exponential phase and cell concentrations of 9.2 g L^{-1}, which is significant comparing to other conditions (*p*-value <0.05*)*. Comparing the global productivity and the specific growth rate (table) there was no significant difference between the use of 50 mM and 10 mM phosphates or between 50 and 100mM (*p*-value > 0.05), but there is significant difference between 10 and 100 mM.

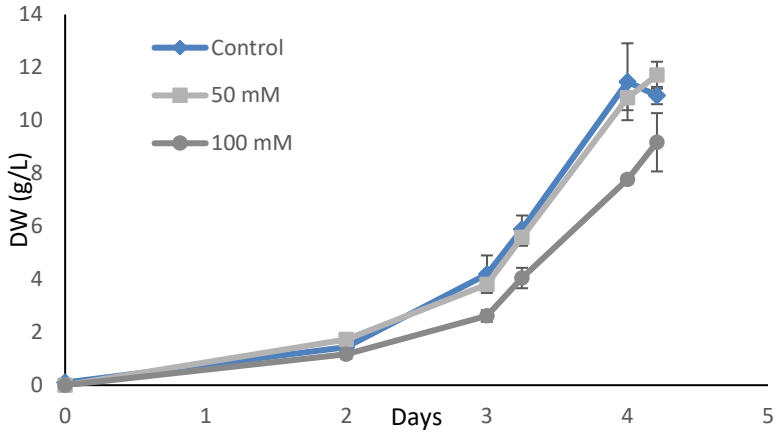


Figure 19 - *Scenedesmus rubescens* growth curves, testing different phosphate concentrations. The cultures grown under heterotrophic conditions in 250mL Erlenmeyer. The values represent the average and respective standard deviation of 3 Individual experiments.

Table 10 - Global productivity and specific growth rate under different concentrations of phosphate and under the presence of vitamins on optimised culture medium (0037SA medium).

Concentrations (mM)	Global productivity (g L^{-1} h^{-1})	Specific growth rate (Day^{-1})
control	0.119^{a}±0.012	1.18^{a}±0.027
50	0.115^{ab}±0.005	1.17^{ab}±0.011
100	0.090^{b}±0.011	1.09^{b}±0.005

5.1.10– Comparations of culture media

After the optimization tests, the optimized culture medium (0037SA medium) performance was compared to the initial growth medium (BBM 5-fold concentrated) in parallel cultures, as seen in Figure 20. BBM medium allowed to reach 8.14 g L^{-1}, while 0037SA allowed 11.5 g L^{-1}, an increase of about 40.8%. Concerning global productivity and specific growth rate (table 11), the global increased by 0.92-fold (from 1.94 to 2.79 g L-1 day-1) and the specific growth rate by 1.26-fold (from 0.90 to 1.13 day-1). In this way, it is possible to conclude that the growth was significantly optimized.

Table 11 - Comparison of *Scenedesmus rubescens* performance in cultures media: Bolds basal medium and 0037SA medium.

Concentrations (mM)	Global productivity (g L^{-1} day^{-1})	Specific growth rate (Day^{-1})
Bolds basal medium	2.79^{a}±0.232	0.90^{a}±0.026
0037SA medium	1.94^{b}±0.068	1.13^{ab}±0.025

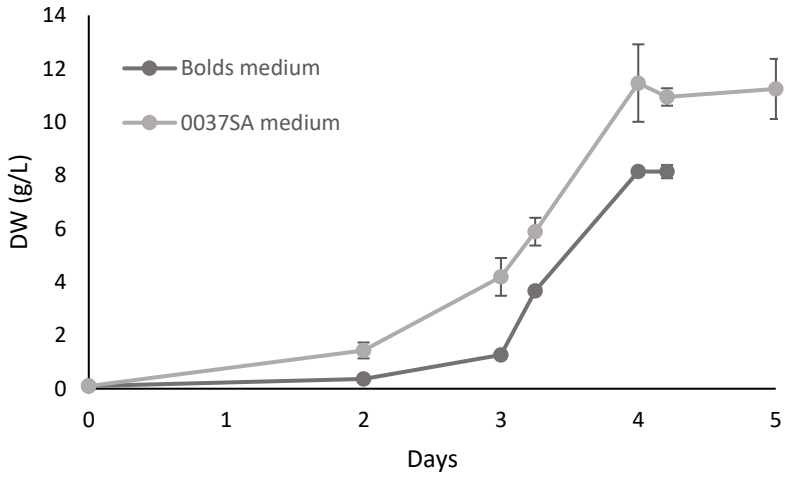


Figure 20 - *Scenedesmus rubescens* growth curves under Bold's basal medium and 0037SA medium obtained from this work. The cultures grown under heterotrophic conditions in 250mL Erlenmeyer.

Furthermore, 0037SA medium was compared with Jin *et al.* (2020), designed for *Scenedesmus acuminatus* (table 12)[32]. Jin et al. (2020) defined 6 as the optimum pH. For 0037SA medium, the pH 6.5-7 was defined as the optimum. In addition, when comparing both media (table 12), it is clear that 0037SA medium is formulation with higher nutrients concentration. Differences that expect to greater influence the cell growth are the nitrogen and phosphate, as previously discussed in section 5.1.3. In addition, in another study[60], the authors examined the effects of different nitrogen sources (ammonia, urea and nitrate) on ash free dry biomass (AFDB), cultivated in autotrophic cultivation. Among the nitrogen sources, the algae achieved the highest productivity AFDB under a mixture of urea and nitrates. Therefore, further studies on the nitrogen source and the different concentrations are needed.

Table 12 - Comparison between the medium developed in this work and the medium used for the species *Scenedesmus acuminatus* obtained from literature[49]. The main differences are the sources and concentration of nitrogen and phosphate. The optimum pH for S. acuminatus is 6 and for 0037 SA it is between 6.5 and 7.

Jin *et al.* (2020)		**0037 SA medium**	
Component	Concentration (mM)	Component	Concentration (mM)
KNO_3	29.6	$(NH_4)_2SO_4$	60
NaH_2PO_4	-	NaH_2PO_4	50 (total)
KH_2PO_4	8.82	K_2HPO_4	
$MgSO4.7H_2O$	4.87	$MgSO_4.7H_2O$	1.75
Trisodium citrate	0.77	Trisodium citrate	-
$FeSO_4.7H_2O$	0.06	$FeSO_4.7H_2O$	0.06
$CaCl_2.2H_2O$	0.14	$CaCl_2.2h_2O$	0.3
H_3BO_3	0.05	H_3BO_3	0.1
$ZnSO_4$	0.0014	$ZnSO_4$	0.003
$MnCl_2.4H_2O$	0.0091	$MnCl_2.4H_2O$	0.03
Na_2Mo_4	0.0001	$Na_2Mo_4.2H_2O$	0.03
$CuSO_4.2H_2O$	0.00036	$CuSO_4.2H_2O$	0.00325
$NiCl_2.6H_2O$	-	$NiCl_2.6H_2O$	0.02
$Co(NO_3)_2.6H_2O$	-	$Co(NO_3)_2.6H_2O$	0.01

5.2 Scale-up process under heterotrophic conditions

To find out if the developed culture medium is indeed an ideal medium for *S. rubescens*, a scale up process is essential to validate the results achieved. Thus, *S. rubescens* was grown on a bench-top bioreactor, in which parameter control is more effective.

Figure 21 shows the growth curves obtained during fermentation. The medium used (table 12) was adapted to the optimized conditions at the time of the assay. The first test (fermenter 1) started with 10mM of phosphate and this concentration was increased to 50mM in the second assay (fermenter 2). Vitamins were also used in the latter. In the first fermentation no initial inhibition was registered, however, during the second one cell sedimentation occurred, which prevented a better start of the culture. Besides, both the biomass concentration and the global productivity achieved were very similar (Table 13). In the first test, the maximum concentration of biomass was 69 g L^{-1} with a global productivity of 0.569 g L^{-1} h^{-1}. The second test, the maximum biomass concentration was 72 g L^{-1} with a global productivity of 0.600 g L^{-1} h^{-1}. However, differences in cell morphology were evident, as seen in Fig. 22. This is possibly due to the initial concentration of phosphate and its consumption throughout the test. The first fermentation consumed 95mM and, in this case, the cells presented a more elongated and smaller shape. The second fermentation consumed 140 mM and cells appeared similar to the ones in the Erlenmeyer assays. This points to the possibility that the availability of phosphate favours different morphologies in the cell but not interfere in the biomass productivity.

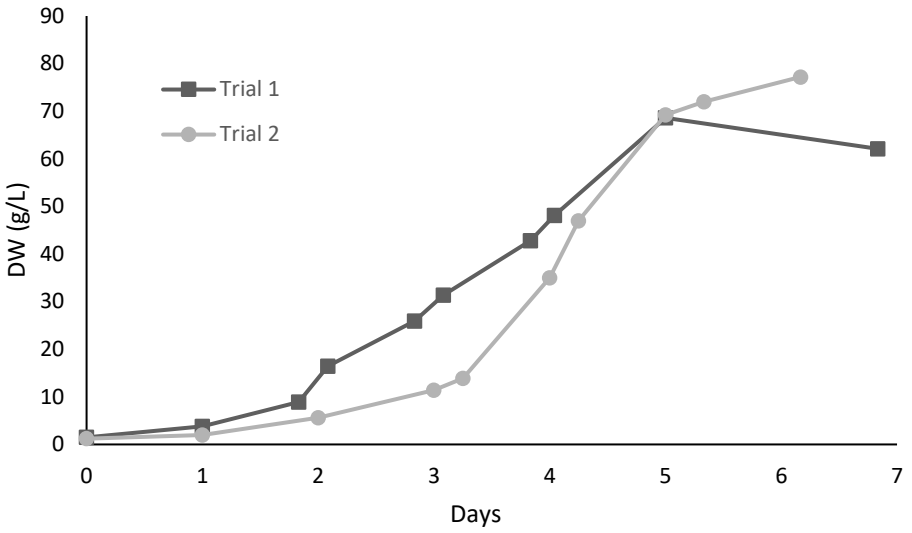


Figure 21 - *Scenedesmus rubescens* growth curves in a 7L working volume bench-top STR, under heterotrophic conditions (medium specificities indicated in text above). Detection of contamination on trial 2. At day 5, biomass was not validated.

Table 13 - Biomass concentration and global productivity for the two fermentation tests.

Fermentation	Biomass concentration (g L^{-1})	Global productivity (g L^{-1} h^{-1})
Trial 1	69	0.569
Trial 2	72	0.600

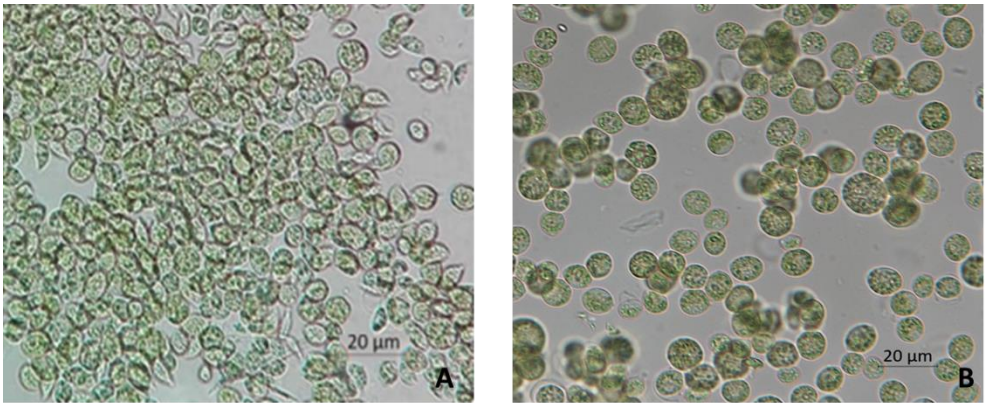


Figure 22 - Microscopic view of heterotrophic grown *Scenedesmus rubescens*. Different morphologies of 0037SA were observed. A- First fermentation. The cells presented an elongated form. B- Second fermentation. The cells have a more circular form and larger volume. Images obtained by Zeiss® Axio Scope.A1 coupled with ZEN Axiocam 503 color. Total magnification 400x

Only two scale-up were successfully performed. The main problem encountered was sedimentation, not allowing proper growth of this microalga, and thus more stirred bioreactor assays would be needed.

S. acuminatus is described to reach a maximum of 286 g L^{-1} in fed-batch, on a 7L fermenter, during 7 days which means that the biomass concentration reached was significantly higher than the biomass concentration achieved in this work[31]. In that case, the authors used 0.85 g L^{-1} of urea in fermenter batch medium, replacing KNO_3 changing the nitrogen source previously used, demonstrating once again the importance of the nitrogen source (section 5.1.8). Besides both not being the same species, they are genetically close, suggesting that *S. rubescens* may reach higher cell densities than the density achieved so far.

Comparing with *Chlorella vulgaris,* a biomass concentration of 174.5 g L^{-1} was obtained during this 5 L heterotrophic scale-up phase[29], which is also higher than 72 g L^{-1}. Thus in conclusion, it is still possible to optimize the culture medium to achieve greater growth for *Scenedesmus rubescens*.

5.3 Induction of carotenoid production

Carotenoid production is a response to different extreme conditions that are critical to microalgae growth. It is usually the combination of stress conditions, such as nutrient limitation (nitrogen), intense sunlight and high salinity[82]. This way, in order to verify the carotenoid induction in *Scenedesmus rubescens*, two strategies were adopted: (1) autotrophy with nitrogen depletion and (2) heterotrophy with nitrogen and phosphate depletion and pH variation.

5.3.1 Induction under autotrophic conditions

To induce carotenoid production, two autotrophic assays were conducted, as shown in Figs. 23 and 24. The inoculum obtained from the fermenters was used to inoculate flat panel reactors. Three flat panels were inoculated by each fermenter. In both assays, the inoculum no longer contained nitrogen, phosphate or glucose in the culture medium. The autotrophic company medium, MNS, containing of 1mM of nitrates was used. The pH was maintained between 7 and 8.

The conditions tested did not allow the carotenoid induction. In the first assay, the reason may have been difficulties stabilizing the pH that ranged between 6 and 9. In addition, the inoculum was already at the cell death phase, which may had compromised the viability of the cells.

During the second assay, there was cell growth, but the carotenoids induction was not achieved. Although the pH was more stable, the light intensity was lower (maximum of 640 W/m^2), which according to Nobutaka Hanagata and Zvy Dubinsky (1999), light intensity is an important factor combining with nutrient limitation to induce carotenoids[82]. In general, the results suggest a high complexity in the process, not being viable for the company's interests.

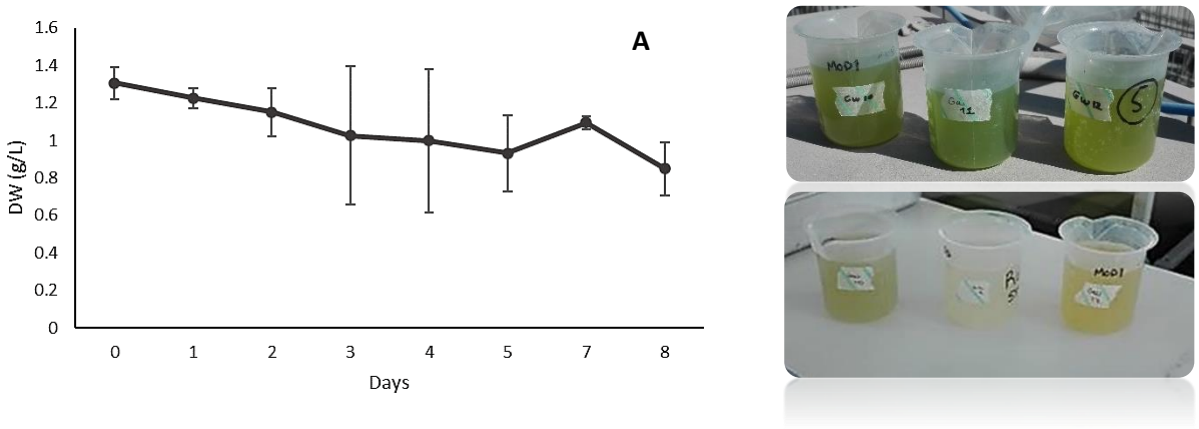


Figure 23 - A: *Scenedesmus rubescens* growth curve supplemented with 1mM of nitrates. The culture was operated under autotrophic conditions in 70L flat panels B: Samples of the three flat panels on day2 (top) and 12 (bottom). The experiment lasted 12 days.

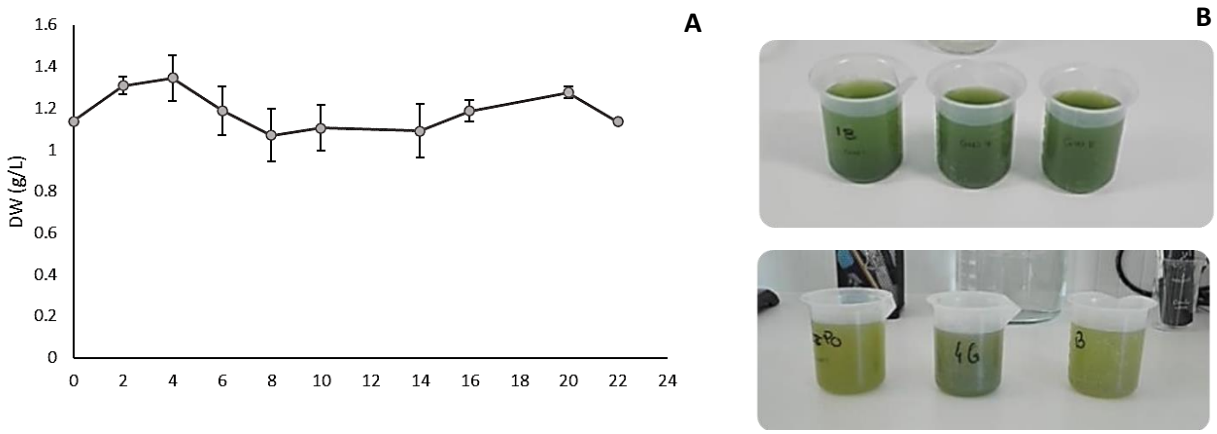


Figure 24 - A: A: *Scenedesmus rubescens* growth curve supplemented with 1mM of nitrates. The culture was operated in continuous mode under autotrophic conditions in 70L flat panels. B: Samples of the three flat panels on day 2 (top) and 24 (bottom). The experiment lasted 24 days.

5.3.2 Induction under heterotrophic conditions

As was mentioned, a DoE was performed. Through Box-Behnken design for RSM, 3 variables were tested: ammonia and phosphate concentrations and pH values (Table 14). The software predicted 13 runs (Table 15). In this test the responses were biomass concentration and carotenoid production. Due to the problems existing during the HPLC run, it was not possible to determine the pigments in all samples. In this way, a colour scale was carried out, from the greenest to the most orange, whose most orange sample was considered the sample with the highest concentration of carotenoids. RSM allowed visualising a quadratic regression for biomass concentration and a linear regression for carotenoid production. Through the analysis of the results (ANOVA), the models are significant (Fig. 25). In response 1, when culture is growing at pH 4, concentrations of biomass was lower. Algae grown at pH

6.5 and 9 reached similar concentrations, with no significant differences. Thus, different concentrations of ammonia and phosphate tested did not affect the growth of microalgae.

Table 14 - Levels of factors for design of experiment: ammonia, phosphate and pH.

Factors	Symbols	-1	0	+1
Ammonia (mM)	A	1	5.5	10
Phosphate (mM)	B	1	5.5	10
pH	C	4	6.5	9

Table 15 - Response surface method design in actual level of variables and observed responses functions for carotenoid induction in *Scenedesmus rubescens*.

	Factor 1	Factor 2	Factor 3	Response 1	Response 2
Run	N (mM)	P (mM)	pH	Biomass concentration (g L^{-1})	carotenoid production (colour scale*)
1	5.5	5.5	6.5	13	6
2	5.5	1	4	2.3	1
3	10	5.5	4	4.9	1
4	5.5	5.5	6.5	13.7	5
5	5.5	10	4	2.9	1
6	5.5	10	9	14.2	5
7	10	10	6.5	14.7	3
8	1	5.5	9	13.1	10
9	5.5	5.5	6.5	14.3	8
10	10	1	6.5	13.5	3
11	1	10	6.5	13.5	3
12	5.5	1	9	14.4	9
13	1	5.5	4	3.3	1

* 1- highest green colour; 10 - highest orange colour

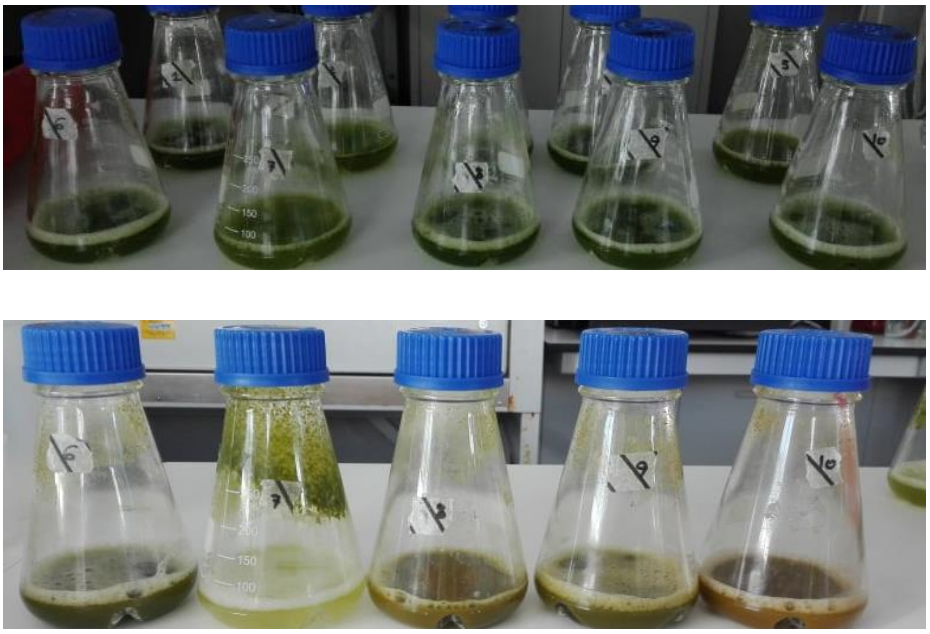
Significant differences were observed in case of carotenoid production (Fig. 26). Cultures grown at pH 4 remained green throughout the run. On the other hand, *S. rubescens'* colour varied between yellow, brown and orange, in the remaining condition. The condition that culture turned to orange colour was with 1 mM ammonia and 5.5 mM phosphate with pH 9. These results suggest nitrogen limitations promotes the production of carotenoids, as mentioned in other studies[82]. Also, low phosphate concentration is important to this induction.

	Source	F-value	p-value	
	Model	20,59	0,0151	significant
	A-Amonium	1,58	0,2971	
	B-phosphate	0,3405	0,6005	
	C-pH	94,37	0,0023	
	AB	1,35	0,3287	
	AC	1,13	0,3665	
	BC	0,1162	0,7557	
	A^2	0,0234	0,8882	
	B^2	0,0208	0,8946	**A**
	C^2	21,17	0,0193	

	Source	F-value	p-value	
	Model	10,07	0,0031	significant
	A-Amonium	1,04	0,3336	
	B-phosphate	1,83	0,2093	
	C-pH	25,55	0,0007	**B**

Figure 25 - Analysis of variances (ANOVA) for 2 responses A) biomass conce7ntration, B) carotenoid production. According to F-value and p-value, the models are significant. Tables from "Design expert" software.

However, studies mentioned of the production of carotenoids under conditions of high light intensity. In this case, the induction was performed in the dark, suggesting that the light intensity is not a fundamental factor in the carotenoid induction of Scenedesmus.



A

B

Figure 26 - *Scenedesmus.rubescens* growth under carotenoids induction during DoE. The cultures grown under heterotrophic conditions in 250mL Erlenmeyer. A: Samples on day 2 B: Samples on day 7. The experiment lasted 7 days

Although carotenoids quantification and detection were not performed in all conditions, comparison between two samples was performed: green sample (sample 5) and brown-orange sample (sample 12) were compared. Figures 27 and 28 consist of graphs plotted over data obtained by HPLC for samples 5 and 12, respectively. In sample 5, the most relevant carotenoids identified are lutein and

b-carotene. In sample 12, astaxanthin was still identified. These results confirm that in the most orange sample has the highest carotenoid production.

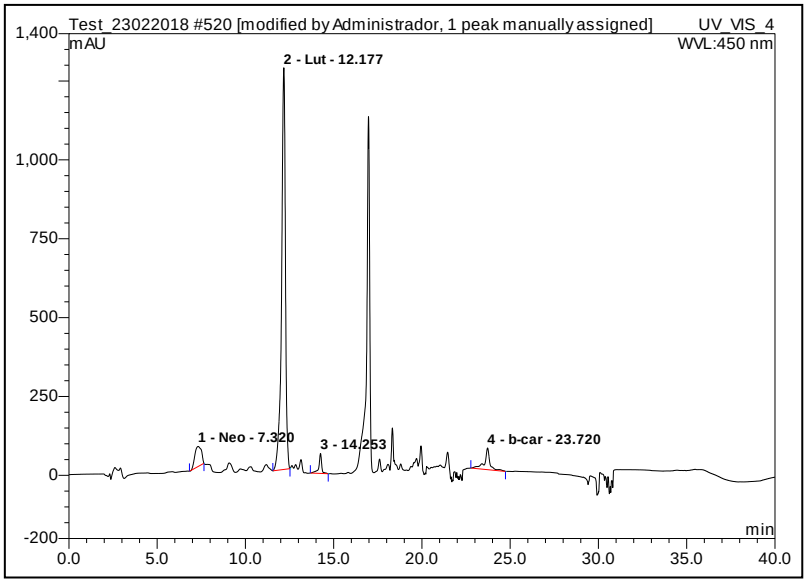


Figure 27 - HPLC chromatograms of photosynthetic pigments extracted from sample 5 at 7 days of heterotrophic culture. Peak identities: (1) neoxathin; (2) lutein; (3) unknown; (4) ß-carotene.

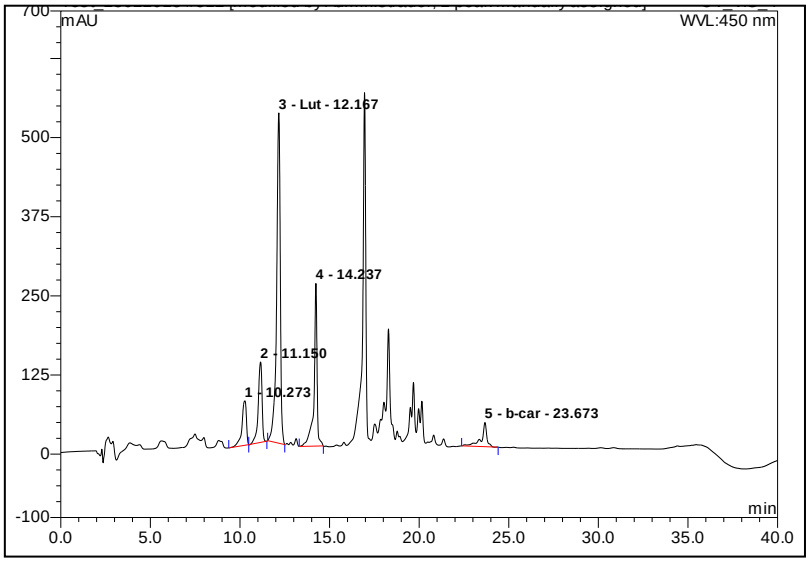


Figure 28 - HPLC chromatograms of photosynthetic pigments extracted from sample 12 at 7 days of heterotrophic culture. Peak identities: (1) astaxanthin; (2) adonixanthin; (3) lutein; (4) canthaxanthin (5) ß-carotene.

5.4 Biochemical analyses

The biochemical composition of the biomass obtained from the fermenters and from the last carotenoid induction assay was analysed. The content of proteins, lipids and ashes were accessed.

Table 16 shows the composition of the different samples. Regarding proteins, the biomass from trial two collected at 5 days of culture (see Fig. 21, Trial 2) achieved the highest concentration, 33.24% and 30.94%. These values are very close to the protein values of *Chlorella vulgaris* in heterotrophy[29] suggesting that *Scenedesmus* sp. has also a great potential to produce biomass for vegetal protein production purposes. Possibly, passing this species to autotrophy one could reach values between 50% and 56% as is the case with *S. obliquus* (Table 1)[9]. The stressed samples had a lower protein content, as already reported in the literature for species *Nannocloropsis* sp[82]., *Chlorella vulgaris*[83] and *Scenedesmus obliquuos*[84].

In addition, stress-induced cells have the highest lipid content (21.39% and 21.48%), followed by the cells collected from fermenter 2 (12.63% at initial and 12.35% at final growth phases) and, finally, the cells from fermenter 1 had the lowest lipid content (7.33%). These results agree with other studies[58] and suggest that when the cells are metabolically stressed, they tend to accumulate lipids, using other energetic components such as proteins and polysaccharides, as it is also reported in the species mentioned above[85,82 ,84].

Finally, the sample with the cells showing the highest ash content was collected from the fermenter 2 (3.23%). The samples from carotenoid-induction studies presented lower ash content (2.7 and 2.15%).

Table 16 - Proteins, lipids and ashes content of different samples, comparing the different contents of each sample. Samples referred as 1 and 10 mM were collected from carotenoid-induction assay.

Sample	Proteins (%)	Lipids (%)	Ashes (%)
10 mM of ammonia	15.20±0.87	21.39±2.10	2.7±0.0
1 mM of ammonia	13.14±3.74	21.48±1.88	2.15±0.15
Fermenter 1 (final phase)	24.73±1.09	7.33±0.44	-
Fermenter 2 (initial phase)	33.24±0.25	12.63±1.90	-
Fermenter 2 (final phase)	30.94±0.30	12.35±1.74	3.23±0.41

6. Conclusions and future perspectives

In this work, *Scenedesmus rubescens* (0037SA) was cultivated under heterotrophic conditions and optimization of culture medium was performed. This optimization resulted in a defined and proprietary culture media (0037SA medium). Then, the *S. rubescens* biomass reached in heterotrophic batch incubations, under BBM medium (5-fold concentrated) and cultivated under 0037SA medium, were compared. The results in this study show that the optimization was successful, improving the global productivity by 0.92-fold (from 1.94 to 2.79 g L^{-1} day^{-1}) and the specific growth rate by 1.26-fold (from 0.90 to 1.13 day^{-1}).

After the optimization tests, the optimized culture medium (0037SA medium) performance was compared to the initial growth medium (BBM 5-fold concentrated) in parallel cultures, as seen in Figure 20. BBM medium allowed to reach 8.14 g L^{-1}, while 0037SA allowed 11.5 g L^{-1}, an increase of about 40.8%. Concerning global productivity and specific growth rate (table 11), the global productivity by 0.92-fold (from 1.94 to 2.79 g L^{-1} day^{-1}) and the specific growth rate by 1.26-fold (from0.90 to 1.13day^{-1}). In this way, it is possible to conclude that the growth was significantly optimized.

After optimization, the potential of heterotrophic microalgae cultivation was verified, providing a better controlled environment (pH, temperature, aeration, etc). In this study, only two scale-up were successful, achieving 72 g L^{-1}. Other attempts were made, but due to the size of the cells, there was sedimentation, being necessary to improve the culture conditions (culture medium and other mechanical factors such as agitation and aeration). Furthermore, similar controlled cultures with other species were described to reach higher biomass concentrations, suggesting that *S. rubescens* may reach higher cell densities than the density achieved so far, and that further optimization of the culture medium is still needed.

The inoculum obtained from the fermenters was used to induce carotenoid production, in autotrophy. In this study, two assays were performed. However, the conditions tested did not allow the cells to produce carotenoids, suggesting that these conditions are not viable for the company's interests. In addition, 0037SA was also grown under heterotrophic conditions, in dark, testing different conditions such as depletion of nutrients and pH variations. When comparing the different conditions, different colours in the culture samples were observed and consequently the production of different types of carotenoids expected. After analysing two samples, a green and a brown-orange, different carotenoids were found. casein the green cells, large amounts of lutein have been identified and also a small percentage of b-carotenes. In the orange cells, more carotenoids were found, such as astaxanthin and its precursors. Thus, the results show that carotenoids induction in *S. rubescens* is feasible and that this species could be used in pharmaceutical applications.

Lastly, during the elaboration of this work, biochemical analyses were performed (proteins, lipids and ashes). Under fermentation mode, *S. rubescens* achieved about 31% of protein content, which was similar to the protein values of *Chlorella vulgaris* in heterotrophy[30]. This results suggest the great

potential to produce biomass for protein purposes. In addition, stress-induced samples have higher lipid content (21.39% and 21.48% of cell dry weight). These results suggest that there is accumulation of lipids upon nutrients depletion, as reported for other species[58,85,83,84].

In light of the conclusions that were reached, it would be interesting make other optimization assays. I would recommend to test nitrogen sources with different phosphate concentrations, due to the importance this factors to *S. rubescens* growth. In fed-batch procedure I would recommend different values of aerations and agitation in order to preventing cells sedimentation. Lastly, I recommend the validation of DoE for carotenoid production and also more assays in order to optimize production of carotenoids from *S. rubescens*.

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8. Appendixes

Appendix A – Calibration curves

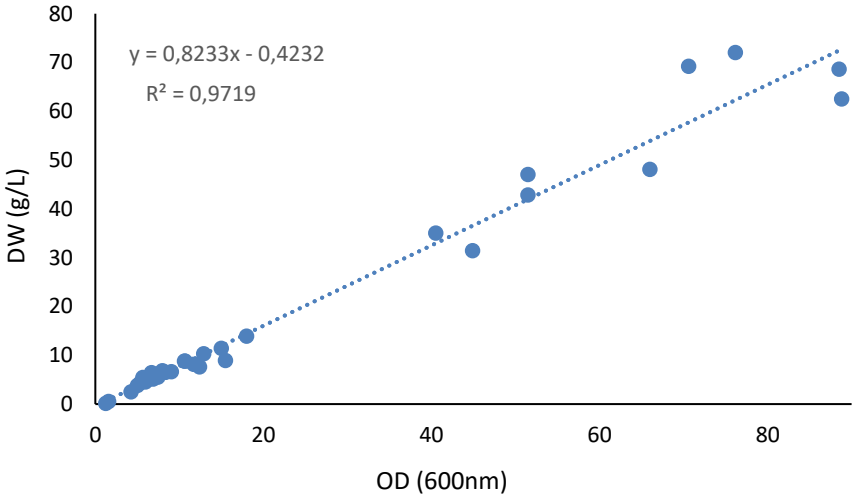

Figure A 1 - Dry biomass concentration (g L^{-1}) vs absorbance of *S. rubescens* measured at λ= 600 nm for heterotrophic growth.

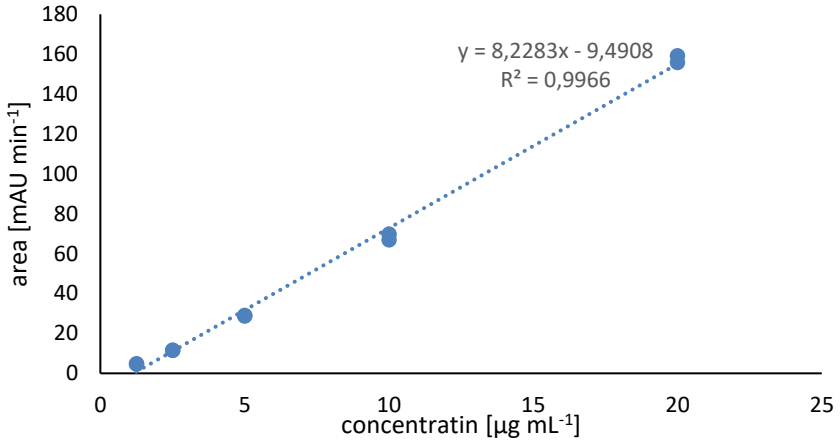

Figure A 2 - concentration (µ mL^{-1}) vs area (mAU min^{-1}) for astaxathin calibration curve.

Appendix B - Calibration curves equations for A) nitrates B) ammonia C) phosphate and D) Iron

A) - Y=3.596x + 0.002

B) - Y=13.927x - 0.092

C) - Y=12.706x + 0.051

D) - Y=0.463x - 0.019

Appendix C - DoE results

Table C 1 - Screening method design in actual level of variables through Mini Tab software for *Scenedesmus rubescens*.

Trial	Nitrogen source	N	Mg	Ca	P	Fe	Cu	Zn	Mn	Mo	Co	Ni	B
1	Ammonium	40	1.75	1	5.5	0.06	0.0325	0.0315	0.055	0.0105	0.0125	0.01	0.3
2	Ammonium	40	1.75	1	5.5	0.06	0.0325	0.0315	0.055	0.0105	0.0125	0.01	0.3
3	Nitrate	60	3	0.3	1	0.1	0.06	0.003	0.08	0.02	0.005	0	0.1
4	Nitrate	20	3	0.3	10	0.1	0.06	0.06	0.03	0.001	0.02	0.02	0.1
5	Ammonium	20	0.5	0.3	10	0.02	0.06	0.003	0.08	0.02	0.02	0.02	0.1
6	Nitrate	60	0.5	0.3	10	0.1	0.005	0.06	0.08	0.001	0.005	0	0.1
7	Nitrate	60	0.5	1.7	10	0.02	0.005	0.003	0.03	0.02	0.005	0.02	0.1
8	Ammonium	60	0.5	1.7	10	0.1	0.06	0.003	0.03	0.02	0.02	0	0.5
9	Ammonium	60	3	0.3	10	0.1	0.005	0.003	0.03	0.001	0.02	0	0.5
10	Ammonium	20	3	0.3	10	0.02	0.06	0.06	0.08	0.02	0.005	0	0.5
11	Ammonium	20	0.5	0.3	1	0.02	0.005	0.003	0.03	0.001	0.005	0	0.1
12	Nitrate	40	1.75	1	5.5	0.06	0.0325	0.0315	0.055	0.0105	0.0125	0.01	0.3
13	Ammonium	60	3	1.7	10	0.02	0.005	0.06	0.08	0.001	0.02	0.02	0.1
14	Nitrate	20	3	1.7	10	0.1	0.005	0.003	0.08	0.02	0.005	0.02	0.5
15	Nitrate	40	1.75	1	5.5	0.06	0.0325	0.0315	0.055	0.0105	0.0125	0.01	0.3
16	Nitrate	20	0.5	1.7	10	0.02	0.06	0.06	0.03	0.001	0.005	0	0.5
17	Nitrate	60	3	1.7	1	0.02	0.06	0.06	0.03	0.02	0.02	0	0.1
18	Nitrate	40	1.75	1	5.5	0.06	0.0325	0.0315	0.055	0.0105	0.0125	0.01	0.3
19	Ammonium	40	1.75	1	5.5	0.06	0.0325	0.0315	0.055	0.0105	0.0125	0.01	0.3
20	Nitrate	40	1.75	1	5.5	0.06	0.0325	0.0315	0.055	0.0105	0.0125	0.01	0.3
21	Ammonium	60	0.5	1.7	1	0.1	0.06	0.06	0.08	0.001	0.005	0.02	0.5
22	Ammonium	20	3	1.7	1	0.1	0.06	0.003	0.03	0.001	0.005	0.02	0.1
23	Ammonium	20	0.5	1.7	1	0.1	0.005	0.06	0.08	0.02	0.02	0	0.1
24	Ammonium	40	1.75	1	5.5	0.06	0.0325	0.0315	0.055	0.0105	0.0125	0.01	0.3
25	Ammonium	40	1.75	1	5.5	0.06	0.0325	0.0315	0.055	0.0105	0.0125	0.01	0.3
26	Ammonium	40	1.75	1	5.5	0.06	0.0325	0.0315	0.055	0.0105	0.0125	0.01	0.3
27	Nitrate	60	0.5	0.3	1	0.02	0.06	0.003	0.08	0.001	0.02	0.02	0.5
28	Ammonium	60	3	0.3	1	0.02	0.005	0.06	0.03	0.02	0.005	0.02	0.5
29	Nitrate	20	3	1.7	1	0.02	0.005	0.003	0.08	0.001	0.02	0	0.5
30	Nitrate	20	0.5	0.3	1	0.1	0.005	0.06	0.03	0.02	0.02	0.02	0.5

Table C 2 - Responses functions for optimization of media composition for heterotrophic cultivation of *Scenedesmus rubescens*. Minitab software was used.

Trial	Biomass concentration (g/L)	Global productivity (g/L/h)	Maximum productivity (g/L/h)
1	9.62	0.089	0.277
2	6.50	0.060	0.142
3	4.41	0.041	0.079
4	12.09	0.112	1.243
5	13.82	0.128	0.344
6	6.90	0.064	0.176
7	10.77	0.100	1.114
8	9.79	0.091	0.166
9	7.31	0.061	0.131
10	7.55	0.070	0.150
11	6.43	0.060	0.142
12	9.62	0.089	0.277
13	11.51	0.091	0.560
14	14.73	0.136	1.235
15	9.21	0.085	0.322
16	12.34	0.114	1.206
17	6.02	0.056	0.109
18	9.21	0.085	0.322
19	6.50	0.060	0.133
20	9.21	0.085	0.322
21	4.33	0.040	0.081
22	5.04	0.047	0.093
23	5.35	0.050	0.120
24	6.91	0.064	0.152
25	6.50	0.060	0.133
26	6.91	0.064	0.152
27	4.91	0.045	0.096
28	3.81	0.035	0.059
29	6.08	0.056	0.212
30	7.53	0.0697	0.150

Table C 3 - Culture medium obtained from the Placket-Burman experiment (0037SA medium)

0037 SA medium	
Component	Concentration (mM)
$MgSO_4.7H_2O$	1.75
Trisodium citrate	-
$FeSO_4.7H_2O$	0.06
$CaCl_2.2h_2O$	0.3
H_3BO_3	0.1
$ZnSO_4$	0.003
$MnCl_2.4H_2O$	0.03
$Na_2Mo_4.2H_2O$	0.03
$CuSO_4.2H_2O$	0.00325
$NiCl_2.6H_2O$	0.02
$Co(NO_3)_2.6H_2O$	0.01

Table C 4 - Response surface method design in actual level of variables and observed responses functions for optimization of media composition for heterotrophic cultivation of *Scenedesmus rubescens*.

	Factor 1	Factor 2	Factor 3	Factor 4	Biomass concentration (g/L)	Maximum productivity (g/L/h)	Global productivity (g/L/h)
Run	A:N	B:P	C:Ca	D:Ni	10.280	0.284	0.105
1	60	10	1	0.01	11.926	0.400	0.135
2	20	10	1	0.01	11.597	0.331	0.118
3	60	5.5	0.3	0.01	9.950	0.277	0.112
4	40	5.5	1.7	0.02	9.621	0.261	0.109
5	40	5.5	0.3	0	8.798	0.258	0.099
6	40	5.5	1	0.01	11.350	0.354	0.128
7	40	5.5	1.7	0	11.432	0.391	0.102
8	40	10	1.7	0.01	10.033	0.371	0.113
9	40	10	1	0.02	4.550	0.127	0.051
10	40	1	1	0.02	11.185	0.313	0.109
11	20	5.5	1.7	0.01	10.691	0.327	0.121
12	40	5.5	1	0.01	10.691	0.342	0.121
13	40	10	0.3	0.01	4.689	0.115	0.048
14	20	1	1	0.01	2.821	0.054	0.030
15	40	1	1	0	9.539	0.328	0.108
16	60	5.5	1.7	0.01	10.856	0.386	0.123
17	60	5.5	1	0.02	9.456	0.218	0.105
18	20	5.5	0.3	0.01	3.150	0.122	0.036
19	60	1	1	0.01	10.033	0.419	0.113
20	40	10	1	0	10.280	0.338	0.098
21	60	5.5	1	0	10.609	0.329	0.109
22	40	5.5	0.3	0.02	2.648	0.146	0.030
23	40	1	0.3	0.01	9.045	0.216	0.098
24	20	5.5	1	0.02	9.621	0.208	0.109
25	20	5.5	1	0	3.578	0.075	0.036
26	40	1	1.7	0.01			