Scenedesmus rubescens production strategies for added value biomass

Gonçalo E. Santo[1,2], Joana L. Silva[2], Marília Mateus[1]
1- iBB – Institute for Bioengineering and Biosciences, Department of Bioengineering, Instituto Superior Técnico, Universidade de Lisboa
2- ALLMICROALGAE, Natural Products S.A Avenida Engº Duarte Pacheco, 19 – 9º, 1070-100 Lisbon, Portugal

Nowadays, microalgae have attracted interest worldwide due to their potential to be used in several applications. One of the high-value products from microalgae is carotenoids. These group of pigments bring many health benefits due to their powerful antioxidant action, cardiovascular diseases and certain cancer types. For these reasons, carotenoids present high commercial values, being 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, (initial culture medium), 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}). After medium optimization, scale up was evaluated in 7L stirred-tank reactor. Only two scale-up were successful, achieving 72 g biomass L^{-1}. However, other species were described to reach higher biomass concentration. suggesting that *S. rubescens* may reach higher cell densities than the density achieved so far, being necessary to optimize the culture medium. The induction of carotenoid production was performed. Only in heterotrophic conditions the induction was succeeded. Different colours in the samples were observed, which was lutein, β-carotenes and astaxanthin. Thus, the results show that the carotenoids induction of *S. rubescens* is feasible, being able to use this species in pharmaceutical applications. Lastly, *S. rubescens* achieved about 33% of protein content, which was similar to the protein values of *Chlorella vulgaris* in heterotrophy. Stress-induced samples have higher lipid content (21.39% and 21.48% of dry cell dry weight).

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

1. Introduction

Microalgae or microphytes are microscopic ancestral living organisms defined as oxygenic photosynthesizers 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[1]. Nowadays, microalgae have attracted interest worldwide due to their potential for several applications[2].

To create a culture medium is necessary to consider parameters as temperature, pH, light intensity and nutrients.[5]. When considering these different parameters, models for different microorganisms growth can be created[5]. 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[6].

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[7]. In this way, microalgae can grow under natural or artificial light[7,8]. There are two main types of reactors for microalgae production in autotrophic conditions: open pond systems and closed bioreactors[4]

Under heterotrophic conditions, microalgae use organic substrates both as energy and carbon sources, such as glycerol, acetate, glucose, etc[9]. Microalgae production is done in

closed stirred reactors named fermenters. In this way, contaminations from other microorganisms can be better prevented[10]. This growth allows faster biomass production, decrease the area needed for inoculum production[9,11] and does not require light, eliminating a variable that is limiting to the growth of microalgae in autotrophic PBRs[12].

One of the high-value products from microalgae is carotenoids[3]. These group of pigments is shown to have potential use in food, feed and cosmetic industries due to their characteristics: colour, aroma and remarkable nutrition[3,14]. Also, carotenoids bring many health benefits due to their powerful antioxidant action[,15,14] and associated to reduce the risk of cardiovascular diseases, macular degeneration, certain cancer types, etc[16].

Carotenes, such as β-carotene -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)[17,15,18]. In addition to carotene, there are other important pigments, such as lutein, astaxanthin, and others. Lutein 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[17].

Astaxanthin is a secondary xanthophyll that is red/orange coloured. which is mainly used in aquaculture for fish and shrimp culture[17]. This pigment can be found in the cell membrane and lipoproteins, protecting microalgae from oxidative damage[17]. Like lutein, this pigment also possesses antioxidant and cardioprotective properties[19].

In this way, the main goal of this work was the production of highly concentrated biomass of *Scenedesmus rubescens* in heterotrophic conditions to induce carotenoid production.

2. Materials and methods

Algal strain and growth conditions

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 grown to maintain good conditions, using to start assays in the exponential phase.

To prepare the inoculants for fermentation, a single colony of *S. rubescens* 0037SA was inoculated into 50 mL of culture media in 250 mL baffled erlenmeyer. The cells grew in a shaking incubator at the at 28 ºC and 200 rpm.

Bench-scale fermentation were performed in a 7 L bench-top fermenter at 28ºC and pH value of 6.5. The fermenter 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 used as nitrogen source. Ammonia (w/w) was also used to control the pH of the fermenter.

Induction of carotenoid production

Autotrophic and heterotrophic assays for carotenoid induction were performed. In the first case, pilot-scale carotenoid production experiments were conducted in a 70L flat panel

with limited MNS medium, a Guillard f/2-based medium[20], (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 (Design of experiments) was performed, using a Box Behnken design from Design Expert. 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.

Biochemical analysis

Using a Vario EL III elemental analyser (Vario EL, GmbH, Hanau, Germany), total carbon, hydrogen and nitrogen (CNH analysis) of the freeze-dried biomass was analysed. 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[21] after CHN analysis.

The lipid content was determined by a modified method of Bligh and Dyer (1959)[22], as reported by Pereira et al. (2011)[23]. The lipids were extracted through a process of solvent addition and homogenization. The homogenization was carried out using an IKA T-25 Ultraturrax disperser 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. The chloroform phase, which remained in the bottom layer, was recovered with Pasteur pipette, putting 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 extraction of carotenoids was carried out on ice and under dim light to avoid oxidation. 20 mg of freeze-dried biomass were weight and a pulverized material into a tube and methanol was added. With the addition of glass beads (500 -750µm), the cells were lysed using Retsch MM 400 mixer mill at 30Hz, for 4 minutes. After disruption of cells, chloroform was added and the samples were vortexed and centrifuged using centrifuge z167 M for 5 min, at 3500rpm. 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 a high-performance liquid chromatography; HPLC. 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) The quantification was carried out using calibrations curves of lutein, zeaxanthin and standards (Sigma-Aldrich, Portugal). Astaxanthin was quantified with a calibration curve from Universiade do Algarve.

Statistical analyses

The statistical tests for OVAT (one variable ate time) were performed using R software (4.0.2 version), through RStudio 1.3.1073 version. ANOVA analysis was followed by a

post hoc Tukey-HSD test when three or more conditions were compared. Throughout the text. 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 based on a preliminary screening and Design Expert (version 12), based on response surface methodology. Mini tab was used through Placket-Burman. Using Design Expert Box-Behnken was conducted performed. ANOVA was also performed. Statistically significant difference was considered at $p<0.05$.

3. Results and discussion

Optimization of culture media

To optimize the culture medium, different elements for the growth of 0037SA species were studied. OVAT were performed for carbon and nitrogen source and pH. When comparing different sources of carbon (glucose, acetate and glycerol) glucose promoted the greatest cell growth (Fig. 1). In the case of the nitrogen source, nitrates, ammonia and urea were compared. (Fig. 2) No significant differences were found. Ammonia was chosen for the analysis of scale-up fermentation. In the case of pH, 4 values (6, 6.5, 7 and 8) were tested. (Fig. 3) The culture under 6.5 and 7 values reached higher values of cell productivity.

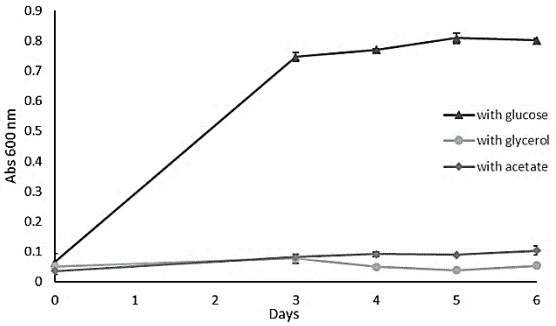

Figure 1 - *Scenedesmus rubescens* growth curves supplemented with different sources of carbon. The cultures grown under heterotrophic conditions in 250mL Erlenmeyer, using TAP medium. The experiment lasted 6 days.

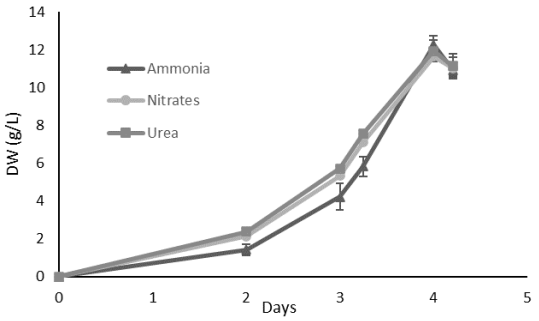

Figure 2 - *Scenedesmus rubescens* growth curves supplemented with different sources of nitrogen. The cultures grown under heterotrophic conditions in 250mL Erlenmeyer.

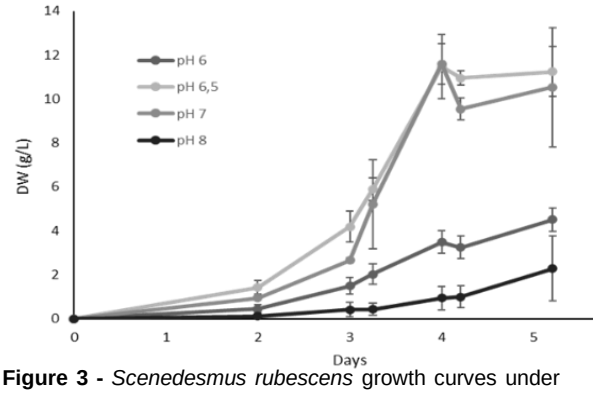

Figure 3 - *Scenedesmus rubescens* growth curves under different pH values. The cultures grown under heterotrophic conditions in 250mL Erlenmeyer.

Using DoE, 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, 30 runs were employed with the chosen responses, which are biomass concentration, global productivity and maximum productivity. The factors that most influenced cell growth was the source of nitrogen, P, Ni, and the Ca concentration. Nevertheless, this last nutrient influenced only on maximum productivity (*p*-value <0.05).

"Design of Expert" Software was used to optimize the medium composition through "Box-Behnken design", a Response surface method. 4 variables that most influenced the growth of *S. rubescens* were chosen: N, P, Ni and Ca concentrations. A total of 26 sets of experiments was generated with 2 central points.

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.

After the optimization tests, the optimized culture medium (0037SA medium) performance was compared to the previously tested growth medium, Bold's basal medium (BBM). 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, there was an increase 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.

Furthermore, 0037SA medium was compared with Jin *et al.* (2020), designed for *Scenedesmus acuminatus* (table 12)[24]. 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, it is clear that 0037SA medium is formulation with higher nutrients concentration. Differences that expects to be greater influence cell growth are the nitrogen and phosphate. In addition, in another study[25], 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.

Scale-up process under heterotrophic conditions

To find out if the culture medium is indeed an ideal medium for *S. rubescens*, it is essential to do a scale up process, in order to validate on the results achieved. Thus, *S. rubescens* was grown on a bench-top bioreactor, which parameter control is more effective. The medium used was adapted to the optimized conditions at the time of the assay. The first test started with 10mM of phosphate and this concentration was increased to 50mM in the second assay (Fig.4). Vitamins were also used in the last one. In the first trial (fermenter 1) did not registered initial inhibition, however, during the second trial (fermenter 2) there was sedimentation, which prevented a better start of the culture. Besides, both the biomass concentration and the global productivity achieved were very similar. In the first test, the maximum concentration of biomass was 69 g L^{-1} with a global productivity of 0.569 $gL^{-1}h^{-1}$. The second test, the maximum biomass concentration was 72 g L^{-1} with a global productivity of 0.600 g L^{-1} h^{-1}. Only two scale-

up were successfully performed. The main problem encountered was sedimentation, not allowing proper growth of this microalga and thus perform more assays.

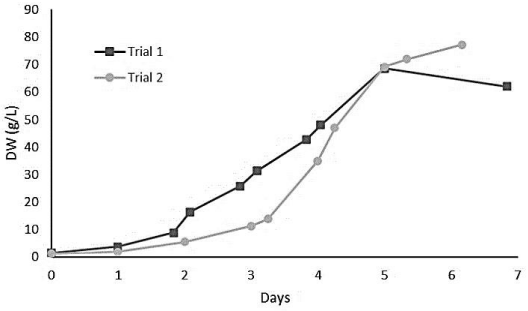

Figure 4 - *Scenedesmus rubescens* growth curves in a 7L working volume bench-top STR, under heterotrophic conditions. Detection of contamination on trial 2 at day 6.

S. acuminatus is described to reach a maximum of 286 g L^{-1} on a 7L fermenter, which means that the biomass concentration reached was significantly higher than the biomass concentration achieved in this work[24]. Besides both not being the same species, they are genetical closed, 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[11], which is 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*.

Induction of carotenoid production

To induce carotenoid production, two autotrophic assays were conducted, as shown in Figure 5A and 5B. 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.

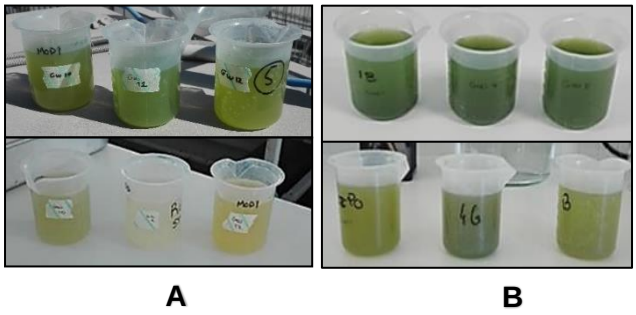

Figure 5 - A: Samples of the three flat panels on day 2 (top) and 12 (bottom). The culture grown under autotrophic conditions in a 70L flat panels. The experiment lasted 24 days. B: Samples of the three flat panels on day2 (top) and 24 (bottom). The experiment lasted 12 and 24 days, respectively.

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, which according to Nobutaka Hanagata and Zvy Dubinsky (1999), light intensity is an important factor combining with nutrient limitation to induce carotenoids[26]. In general, the results suggest a high complexity in the process, not being viable for the company's interests.

As was mentioned, a DoE was performed. Through Box-Behnken design" RSM, 3 variables were tested: ammonia and phosphate concentrations and pH values. The software

predicted 13 runs. 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.

Significant differences were observed in case of carotenoid production. 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[26]. Also, low phosphate concentration is important to this induction.

Although carotenoids quantification and detection were not performed in all conditions, comparation between two samples was performed: green sample (sample 5) and brown-orange sample (sample 12) were compared. Figure 6 and 7 consist of graphs made 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 are still identified. These results confirm that in the most orange sample has the highest carotenoid production.

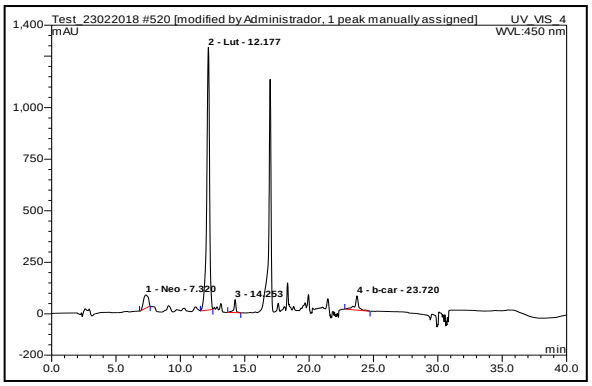

Figure 6 - HPLC chromatograms of photosynthetic pigments extracted from sample 5 at 7 days of heterotrophic culture. Peak identities: (1) neoxathin; (2) lutein; (3) unknown; (4) ß-carotene.

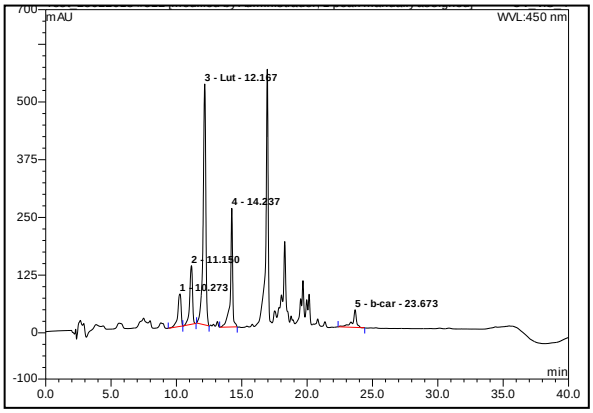

Figure 7 - HPLC chromatograms of photosynthetic pigments extracted from sample 12 at 7 days of heterotrophic culture. Peak identities: (1) astaxanthin; (2) adonixanthin; (3) lutein; (4) canthaxanthin (5) ß-carotene.

Biochemical analyses

The biochemical composition of the biomass obtained from the fermenters and the last carotenoid induction assay was analysed. The content of proteins, lipids and ashes were accessed. Regarding proteins, the biomass from fermenter 2 achieved the highest concentration, 33.24% and 30.94%. These values are very close to the protein values of *Chlorella vulgaris* in heterotrophy[11] suggesting that *Scenedesmus* sp. has also a great potential to produce biomass for protein purposes. Possibly passing this species to autotrophy could reach values between 50 and 56% as is the case with *S. obliquus*[27]. The stressed samples had a lower protein content, as it is already reported in the literature of species

Nannocloropsis sp.[28], *Chlorella vulgaris*[29] and *Scenedesmus obliquuos*[30].

In addition, stress-induced samples have higher lipid productivity (21.39% and 21.48%), followed by the second fermenter (12.63% of initial phase and 12.35% of final phase) and finally the sample from the first fermenter (7.33%). These results agree with other studies[31] 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 same species mentioned above[28,29,30].

4.Conclusions

In this work, *Scenedesmus rubescens* (0037SA) was cultivated under heterotrophic conditions. In this way, optimization of culture medium was performed, resulting (0037SA medium). Then, the biomasses reached cultivated BBM and 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 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 biomass L^{-1}. Other attempts were made, but there was sedimentation, being necessary to improve the culture conditions (culture medium and other mechanical factors such as agitation and aeration). Furthermore, other species was described to reach higher biomass concentration. suggesting that *S. rubescens* may reach higher cell densities than the density achieved so far, being necessary to optimize the culture medium.

The inoculum obtained from the fermenters was used to induce carotenoid production, in autotrophy. However, the conditions tested did not allow the carotenoid induction, suggesting not being 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 samples was observed and consequently the production of different types of carotenoids. When comparing two samples, a green sample and a brown-orange sample, different carotenoids were found. The first case, large amounts of lutein have been identified and also a small percentage of β-carotenes. The second sample, more carotenoids were found, such as astaxanthin and its precursors. Thus, the results show that the carotenoids induction of *S. rubescens* is feasible, being able to use this species 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 33% of protein content, which was similar to the protein values of *Chlorella vulgaris* in heterotrophy[11]. In addition, stress-induced samples have higher lipid productivity (21.39% and 21.48%), These results agree with other studies[31] suggesting that the accumulation of lipids upon nutrients depletion. as reported for other species[28,29,30].

5. References

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