Production of Chlorella protothecoides biomass, chlorophyll and carotenoids using the dairy industry by-product scotta as a substrate

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Abstract

Microalgae-based systems for the production of high value molecules are an emergent area, representing a great promise for industrial applications. The main challenge, however, is the development of high efficiency stra tegies for the large-scale production at low costs. The aim of this study was to evaluate the potential of ricotta cheese whey (scotta) to be used as a low-cost alternative substrate to grow the microalga Chlorella protothecoides. Furthermore, a salt and light stress condition was imposed in order to improve the carotenogenesis process. A significant reduction in lactose concentration was observed along the cultivation in the culture mediums containing scotta, indicating that the tested C. protothecoides shifted to mixotrophic growth, using the organic carbon source provided. Mixotrophic cultures presented a higher amount of biomass than the autotrophic one, however, the cellular accumulation of chlorophyll and carotenoids was higher in the latter culture. Despite this, the stress strategy that we applied enhanced carotenogenesis, allowing the cellular accumulation of well quoted car otenoids, namely astaxanthin and lutein/zeaxanthin. The results suggest that scotta has a great potential to be used as a culture medium to grow C. protothecoides. Moreover, through an adequate stress strategy it is possible to control carotenogenesis, allowing the production of high amounts of the desirable high value molecules.

1. Introduction

Microalgae may be exploited for synthesizing a range of products, including carbohydrates, proteins, essential amino acids, vitamins and pharmaceuticals, as well as bioactive molecules (Dufossé et al., 2005). In recent years, microalgae have emerged as attractive sources for many value-added molecules such as carotenoids (Dineshkumar et al., 2015; Rodrigues et al., 2014). However, the use of microalgae cultures to produce carotenoids has historically been limited, and the economic viability of algal biotechnology is hampered by processing costs and photosynthetic efficiency, as well as by productivity of algal cultures (Wichuk et al., 2014). The most common procedure for cultivation of microalgae is autotrophic growth. Under autotrophic cultivation, the cells harvest light energy and use CO2 as carbon source (Perez-Garcia et al., 2011). Despite several advantages, autotrophic growth leads to low biomass production. Hence, heterotrophic and mixotrophic mode of growth have been proposed as feasible alternatives to reach a higher biomass productivity (Perez-Garcia et al., 2011; Zhang et al., 2011). Under heterotrophic conditions organic molecules, such as sugars and organic acids, serve as carbon sources and the requirement for light is eliminated, whereas mixotrophic cultivation requires light and the simultaneous utilization of inorganic (CO2) and organic compounds as carbon sources. Mixotrophic growth, for some microalgae, can significantly increase the biomass and, furthermore, compounds characteristic of both photosynthetic and heterotrophic metabolisms are synthesized at high production rates (Abreu et al., 2012). However, to make this cultivation technique feasible, a cheap organic carbon source must be used (Girard et al., 2014; Rodrigues et al., 2014). Therefore, the ex ploitation of cheap and easily available agro-industrial byproducts as alternative culture media has been considered to grow microalgae. The ricotta cheese whey, also called scotta, is the main by-product of ricotta cheese manufacture process. The ricotta cheese is produced after the cheese making process, from raw residual cheese whey; fresh milk (up to 10%), milk fat and an acid solution of salts can also be added. The obtained mixture is maintained at high temperature (85–90 °C) to promote the precipitation of most of whey proteins that make ricotta cheese. The liquid solution remaining after ricotta cheese separation is the scotta, with different characteristics compared to cheese whey (Sansonetti et al., 2009). Scotta is widely produced in southern Europe and particularly in Italy where it represents a by-product that must be disposed of (Secchi et al., 2012). Most of scotta is used as supplement feed for livestock. However, this by-product is rich in lactose and contains organic nitrogen, hy drosoluble vitamins and a variety of minerals that could make it a good substrate in biotechnological processes for the production of commer cial high-value compounds. Although several studies have proved the viability of using scotta as a substrate for the production of high-value products, such as bio-ethanol and lactic acid (Sansonetti et al., 2009; Secchi et al., 2012), to the best of our knowledge, this by-product is still poorly used in biotechnological processes, despite its large availability and low cost. Microalgae biosynthesis of metabolites is species dependent and is influenced by the stress imposed by environmental conditions, such as deviations from normal values of salinity, temperature, heavy metal concentration and, above all, nitrogen availability and light intensity (Gouveia et al., 1996). C. protothecoides is a freshwater microalga which can provide appreciable amounts of proteins, chlorophyll and lipids as well as an interesting profile of well quoted carotenoids for nutraceutical and/or food and feed supplements (Campenni' et al., 2013). Moreover, since this freshwater microalga is known to exhibit endogenous β-galactosidase activity (Davies et al., 1994) it could be a potential candidate to be cultivated in a lactose-containing medium. Therefore, the aim of this study was to investigate the production of biomass, chlorophyll and carotenoids by C. protothecoides when cultured under mixotrophic condition in a medium containing scotta as organic carbon source.

Furthermore, the influence of salt and light stress upon the carotenogenesis process was also evaluated.

2. Materials and methods

2.1. Microorganism and inoculum

The freshwater microalga Chlorella protothecoides Krüger (ATCC® 30411™) was used in all the experiments. The strain was maintained in a standard inorganic medium at 25 °C (Araya et al., 2014). The mi croalgal inoculum was prepared in a BG-11 medium at 25 °C in 2 L glass photobioreactors under photoautotrophic conditions. The culture was aerated with CO2-enriched air (0.1 L min−1), under a photoperiod of 16:8 (light:dark).

2.2. Ricotta cheese whey (Scotta)

Two different kinds of ricotta cheese whey (scotta) were used as organic carbon sources: one obtained from ricotta cheese manufactured starting from a mixture of bovine and ovine cheese whey (90% and 10%, respectively) and called mixed scotta (MS) and the other obtained from ricotta cheese manufactured starting from 100% ovine cheese whey and called ovine scotta (OS). Both scotta used in this study were supplied by dairy industries located in Toscana region, central Italy. The physicochemical characterization of scotta was carried out for the following parameters: pH, acidity (g lactic acid 100 mL−1), reducing sugars (g lactose 100 mL−1), dry matter, fat, ash content, total nitrogen, calcium, magnesium, phosphorous, sodium, potassium, zinc, iron and manganese. All the analysis were carried out according to the methodologies proposed by AOAC (2005). To ensure that the reducing sugar present in scotta was in form of lactose an HPLC analysis was also carried out following the methodology proposed by Giovannelli et al. (2011).

2.3. Media and growth conditions

Six different cultivation conditions were carried out in triplicate (Table 1). For the autotrophic cultivation (run 1) the BG-11 medium was used, containing per liter: 1500 mg $NaNO_3$, 3.05 mg KH_2PO_4, 6 mg ferric ammonium citrate, 1.81 mg $MnCl_2{\cdot}4H_2O$, 75 mg $MgSO_4{\cdot}7H_2O$, 0.079 mg $CuSO_4{\cdot}5H_2O$, 36 mg $CaCl_2{\cdot}2H_2O$, 0.222 mg $ZnSO_4{\cdot}7H_2O$, 0.05 mg $CoCl_2{\cdot}6H_2O$, 2.86 mg H_3BO_3, 6 mg citric acid·1H2O and 20 mg Na_2CO_3 (Araya et al., 2014). For the mixotrophic cultivation (runs 2–3), the inorganic medium was partially replaced by the alternative substrate (30% v/v substitution). With the aim of evaluating the assimilation of organic carbon sources by C. protothecoides, under the cultivation conditions provided, pure lactose (12 g L−1), glucose (12 g L−1) and galactose (12 g L−1) were added to the BG-11 medium as controls (runs 4–6). The sugar concentration adopted was based on the lactose content in experiments containing 30% scotta. The batch cultivation of the microalga was performed by inoculating 10% (v/v) of starter culture (cell dry weight equal to 1.2 g L−1) into 1 L culture medium. Before inoculation, all culture mediums were sterilized at 121 °C for 20 min. The microalga was grown under aseptic conditions in 2 L photobioreactors.

2.4. Cultivation process

2.4.1. Green phase

Experiments were carried out at 25 °C in 2 L glass photobioreactors containing 1 L of medium, under a photoperiod of 16:8 (light:dark) for 7 days. The cultures were illuminated with a photon flux density of 400 µmol m−2 s −1 (photosynthetically active radiation) supplied by fluorescent lights. Agitation during cell growth was provided by bubbling CO2-enriched filtered air with 10% CO2 supplied at 0.1 L min−1 . Microscopic observation was done during growth to check the purity of the culture. During the cultivation, reducing sugar concentration, biomass concentration and cellular pigments were determined periodically (from day 3 up to day 7). The cultivations were performed in three replicates (n = 3).

2.4.2. Stress phase

In order to lead C. protothecoides metabolism to the accumulation of carotenoids, after the normal cultivation process (green phase) the culture was diluted (1:5) into a 20 g L−1 NaCl sterilized solution, resulting in salinity and light stresses, following a procedure similar to that described by Campenni' et al. (2013). Dilution of the culture enhance light penetration, leading the microalga to a higher exposure to light (Gouveia et al., 1996). The experiments were carried out in photobioreactors (2 L) for 5 days and the culture conditions such as tem perature, photoperiod, air supply and CO2 were the same as those used for the green phase. All experiments were performed in three replicates (n = 3).

2.5. Analytical methods

2.5.1. Biomass

Samples were aseptically collected regularly. Biomass concentration was estimated by cell dry weight (cdw) after centrifugation of the sample (3951×g for 10 min) and drying at 60 °C in a hot oven until constant weight.

2.5.2. Determination of carbohydrate concentration in the culture media

Reducing sugar concentration was determined regularly, according to the dinitrosalicylic acid (DNS) method proposed by Miller (1959). For the quantification a reducing sugar standard curve was adopted. Lactose, glucose and galactose were used as pure standards.

2.5.3. Chlorophyll and total carotenoids To extract pigments from the fresh biomass, 15 mL of the culture medium was collected in a falcon tube and centrifuged at 3951×g for 10 min, then the supernatant was discarded and the fresh biomass was mixed with 1.0 mm small glass beads (Sigma-Aldrich, Italy) and 5 mL of methanol. The mixture was vortexed for 2 min and the supernatant was collected after further centrifugation. The extraction process was repeated until both biomass and supernatant became colorless (three times). The amount of total chlorophyll and total carotenoids in the extract were determined spectrophotometrically according to the methodology described by Lichtenthaler and Buschmann (2001). Absorption spectra of extracts were measured in a spectrophotometer (SPECTROstar Nano, BMG Labtec, Ortenberg, Germany) reading between 380 and 700 nm. Total chlorophyll and total carotenoid contents were expressed on dry weight basis. C. protothecoides dry weight was measured by drying the fresh biomass at 60 °C until constant weight.

2.5.4. Astaxanthin, Lutein/Zeaxanthin and β-carotene

The analysis was conducted using a SpectraSystem HPLC instrument equipped with a UV–VIS detector (Thermo, Rodano, Italy). The column was a Kinetex C18, 250 × 4.6 mm ID, 5 µm particle size (Phenomenex, Torrance, CA, USA), eluted at 1 mL min−1 with solvent A (acetonitrile: methanol: Tris buffer 0.1 M pH 8 84:2:14) and B (methanol:ethyl acetate 68:32), according to the following program: 100% solvent A for 4 min, then a linear gradient from 0% to 100% B in 10 min, followed by 100% B for 15 min. The astaxanthin, lutein/zeaxanthin and β-carotene peaks were identified by comparison of the retention times of sample peaks with those of pure standards of β-carotene, lutein, zeaxanthin (Extransynthese, Lyon, France) and astaxanthin (Sigma-Aldrich, Italy). Astaxanthin, Lutein/Zeaxanthin and β-carotene quantifications were carried out by reference to calibration curves.

2.6. Statistical analysis

Statistical analysis was performed using one way analysis of var iance (ANOVA). Average values were compared using Tukey's test. Statistical analyses were performed by using software Statistica®, version 7.0 (Statsoft, USA). Differences were considered as significant when $p < 0.05$.

3. Results and discussion

3.1. Physicochemical characterization of mixed and ovine scotta The physicochemical characteristics of the two types of scotta used are shown in Table 2. As reported in the literature the main component present in both scotta is lactose (Sansonetti et al., 2009). Through the HPLC analysis (2.2) it was confirmed that the reducing sugar in scotta was only in form of lactose (Supplement Fig.). This disaccharide is a suitable carbon source that can support the growth of some freshwater microalgae strains (Girard et al., 2014). Furthermore, the organic nitrogen availability, the mild acidity and the variety of minerals found in both scotta support their application in biotechnological processes. Phosphorous, for example, is a macronutrient that plays a vital function in cellular metabolic processes by forming many structural and functional components required for normal growth and development of microalgae (Abreu et al., 2012). Both scotta were very similar in composition, although OS presented a higher amount of lactose, a milder acidity and a lower amount of sodium.

3.2. Biomass

Biomass concentration as cell dry weight (cdw) was estimated along the green phase (from day 3 up to day 7). Higher biomass productivity was achieved when the alternative substrates were used in the culture medium (runs 2 and 3). Substituting 30% (v/v) of BG-11 medium with both scotta led to important growth stimulation in C. protothecoides cultures under mixotrophic condition (Fig. 1). It can be observed that the final values of 3.60 g L−1 and 3.17 g L−1 achieved in the mixotrophic cultures using ovine scotta and mixed scotta, respectively, resulted in 2.8- and 2.5-fold increase when compared to the values obtained in the autotrophic culture. Mixotrophic cell cultivation, by using light and both organic and inorganic carbon sources, has been considered the most efficient process for the production of microalgal biomass (Lee et al., 1996). The stimulatory effect of both types of scotta on biomass production is probably related to the use of lactose as an organic carbon source (Fig. 2) and also to the presence of other nutrients, such as organic nitrogen and a variety of minerals (Table 1). Cheirsilp and Torpee (2012) also reported that the mixotrophic culture of freshwater Chlorella sp. provided a better growth than autotrophic culture. It is also important to mention that because of the faster growth of the mixotrophic cultures, no differences ($p > 0.05$) were found for the biomass concentration from day 3 up to day 7 for the medium containing mixed scotta and from day 4 up to day 7 for the medium containing ovine scotta. Therefore, in addition to the higher biomass concentration when compared with the autrotrophic mode, the use of both scotta as an organic carbon source could allow to shorten the time to harvest the cells, thereby increasing the biomass production for year. Regarding to the addition of pure sugars (runs 4–6) into BG-11 medium, no growth stimulation was observed as biomass concentration was similar or lower than that observed for the autotrophic culture (run 1). Along the cultivation process the biomass concentration in the medium containing pure lactose (run 4) was lower ($p < 0.05$) when compared to the medium without sugar addition (Fig. 1). These results were probably due to the lack of ability by C. protothecoides to use the organic carbon sources when pure sugars were added to the inorganic medium, under the cultivation conditions used as it is known that this microalga is capable of use glucose and galactose to support cell growth (Gao et al., 2014). Girard et al. (2014) also tested the addition of pure lactose into a synthetic medium to grow Chlorella spp. microalgae and observed that the pure sugar did not support the growth as results were similar for the medium without lactose supplementation. Regarding to the present study as the cultivation conditions were the same for all experiments it can be suggested that the nutrients in scotta may be presenting a biostimulant effect toward the use of the sugar by C. pro tothecoides to support its growth.

3.3. Consumption of the organic carbon sources by Chlorella protothecoides

It was observed that under the culture system provided, C. protothecoides strain used in this study, was unable to shift from autotrophic to mixotrophic mode of growth when pure sugars were added to the inorganic medium. Lactose, glucose and galactose concentrations did not present significant

($p > 0.05$) changes along the cultivation process which means that only the inorganic carbon source (CO_2) was used by the microalga (Fig. 2). These results can explain the low growth achieved when the BG-11 medium was supplemented with the pure sugars (runs 4–6), which was similar to the autotrophic cultivation (Fig. 1). According to some Authors the transition from autotrophic to mixotrophic mode in microalgae appears to be influenced by culture conditions such as temperature, light intensity, organic carbon source concentration and CO_2 supply (Chandra et al., 2014). Organic carbon concentration and light intensity in mixotrophic growth are for example critical factors that exhibit inhibitory effects at high doses (Li et al., 2014). On the other side, our results demonstrated that C. protothecoides was able to grow mixotrophically in the presence of lactose when scotta was used in the culture medium (runs 2–3). A significant decrease ($p < 0.05$) in lactose concentration was observed during the cultiva tion, leading to an almost complete depletion of the organic carbon source after 7 days of cultivation (Fig. 2). To assimilate lactose, living organisms have to synthesize a β-galactosidase enzyme to hydrolyze lactose into glucose and galactose and must absorb these molecules through the adequate transmembrane proteins (Girard et al., 2014). Endogenous β-galactosidase activity has already been demonstrated in some freshwater microalgae, including C. protothecoides (Davies et al., 1994). Girard and collaborators (2014) observed the ability of fresh water microalga Scenedesmus obliquus to use lactose as an organic carbon source. Along the cultivation the authors observed a decrease in lactose content followed by an increase in glucose and galactose con centrations, suggesting extracellular lactose hydrolysis by the micro algae. Previously, lactose had been shown to support Scenedesmus growth by Samejima and Myers (1958). However, to the best of our knowledge there are still no reports in the literature about the con sumption of lactose by C. protothecoides cultivated under mixotrophic condition in a lactose containing medium. It was previously reported that C. protothecoides cultured under heterotrophic conditions was unable to utilize lactose as an organic carbon source (Espinosa-gonzalez et al., 2014; Girard et al., 2014). However, it is well known that the culture conditions may be a limiting factor for the carbon source consumption by microalgae (Gao et al., 2014). From our results it can be suggested that the cultivation conditions used were decisive for the shift from autotrophic to mixotrophic mode of growth, allowing the consumption of lactose by C. protothecoides. Moreover it can also be suggested that the presence of some nutrients in scotta had a stimulatory effect on the growth and on the assimilation of the organic carbon source. Abreu and collaborators (2012) cultivated freshwater microalgae C. vulgaris under mixotrophic conditions using cheese whey as a substrate and observed that the biomass concentration at the end of the cultivation did not show sig nificant differences by comparing a medium containing non-hydrolyzed cheese whey (10 g L−1 of lactose) and an inorganic medium supple mented with glucose (5 g L−1) and galactose (5 g L−1). However, the authors did not report any data about lactose consumption.

3.4. Chlorophyll and carotenoids

Pigments production was evaluated in microalgal biomass cultured under mixotrophic (runs 2–3) and autotrophic (run 1) conditions. Total chlorophyll and total carotenoids were estimated along the green phase (Figs. 3 and 4) and after the stress treatment (Table 3). The maximum cellular accumulation of total chlorophyll (25.15 mg gcdw−1) and total carotenoids (6.38 mg gcdw−1) at the end of the green phase were ob tained when the microalga was cultured under the autotrophic mode of growth. The enhancement of chlorophyll biosynthesis by autotrophic Chlorella sp. strains compared with that resulting from mixotrophic cells has been previously reported (Cheirsilp and Torpee, 2012). It has been suggested that the formation of the photosynthetic apparatus in Chlorella may be disturbed by the presence of organic substrates, resulting in a decreased production of photosynthetic pigments when compared with that obtained in autotrophic mode (Yang et al., 2000). Moreover, high organic carbon concentration inhibits the photosynthetic CO_2 fixation and also modifies the pathways of photosynthetic pigment biosynthesis (Chandra et al., 2014). Among several

species of microalgae, Chlorella sp. is reported to have a high amount of chlorophyll (Harun et al., 2010). Chlorophyll is used in food industry as a natural pigment in processed foods. Because of its strong green color and consumers demand for natural foods, chlorophyll is gaining importance as a food additive (Humphrey, 2004). Regarding to the biosynthesis of carotenoids, the results obtained are in agreement with those of Abreu et al. (2012) who found a lower amount of carotenoids in mixotrophic cells of C. vulgaris when compared to cells grown on autotrophic culture. However, as shown previously, under the mixotrophic condition a higher amount of biomass was obtained (Fig. 1), therefore the differences in pigments production between autotrophic and mixotrophic mode of growth can be dampened by expressing the results as milligrams of pigments per unit volume of culture medium as in Figs. 3 and 4 (right y axis). In that way, the highest amount of chlorophyll (42.17 mg L−1) and carotenoids (11.98 mg L−1) were found in the medium containing ovine scotta after four days of cultivation. Moreover, under mixotrophic conditions there was no increase ($p > 0.05$) in cellular accumulation (mg gcdw−1) of total chlorophyll and total carotenoids from day 3 to day 7, therefore, for the production of pigments the cells grown mixotrophically could be harvested earlier, due to the faster growth, thereby increasing the annual productivity of pigments, making the process more viable and sustainable. After the salt and light stress a decrease in total chlorophyll content (mg gcdw−1) ($p < 0.05$) was observed for the autotrophic C. protothecoides (Table 3). Using a similar stress condition, also Campenni' et al. (2013) observed the degradation of chlorophyll in autotrophic C. protothecoides. This behavior is usually observed when stress conditions are applied on microalgae cultures and is generally followed by the revelation of carotenoids pigments (Gouveia et al., 1996; Ip and Chen, 2005). When compared with the green phase, no differences ($p > 0.05$) in total carotenoids content were found when C. protothecoides was exposed to the stress condition (Table 3). However, changes in the carotenoids profile were observed due to the stress applied. HPLC analysis revealed the presence of astaxanthin, lutein/zeaxanthin and β-carotene in the pigments extracts obtained from C. protothecoides samples. Astaxanthin content increased significantly ($p < 0.05$) for microalgae cultured under autotrophic mode and under mixotrophic mode when mixed scotta was used, whereas C. protothecoides cultured in the medium containing ovine scotta showed a significant increase ($p < 0.05$) only in Lutein/Zeaxanthin content, following the onset of the stress. Campenni' et al. (2013) observed that the dilution of the culture medium led to a higher exposure to light, therefore the enhancement of the carotenogenesis process was achieved through the dilution of the sample. The role of some carotenoid molecules in photoprotection is widely acknowledged (Müller et al., 2001). Furthermore, oxidative stress caused by high irradiance is effective for indu cing accumulation of carotenoids in algae (Park and Lee, 2001). Strong light can lead to the generation of reactive oxygen species (ROS), in the presence of which antioxidative carotenoids are produced in order to protect the cells against oxidative damage (Rise et al., 1994). Beside the increase in astaxanthin and lutein/zeaxanthin contents, after the stress, a lower amount of β-carotene was observed in all treatments, although the change was not significant ($p > 0.05$) for the medium containing mixed scotta. Known carotenogenic pathways show the possibilities of various oxidative transformations of β-carotene up to canthaxanthin, and of the hydroxylative pathways towards zeaxanthin/ lutein. Either of these compounds may be considered as precursors of the hydroxylated/oxidized astaxanthin (Gouveia et al., 1996). The ob served enhancement of the astaxanthin concentration through the stress period was an important feature as this carotenoid is a powerful antioxidant and has a high commercial value (Yuan et al., 2011), however, lutein/zeaxanthin were found as the main carotenoids present in C. protothecoides cells even after the stress, indicating that their oxidation to astaxanthin was low. Despite this, the relatively high amounts of astaxanthin, lutein/zeaxanthin and β-carotene present in this microalga, represent an important result as these pigments are well quoted in the market. There is a growing interest in the use of astaxanthin and lutein/ zeaxanthin as food-coloring agents, natural feed additive for the poultry industry and for aquaculture, especially as a feed supplement in the culture of salmon, trout, and

shrimp (Araya et al., 2014; Dufossé et al., 2005; Wichuk et al., 2014). Astaxanthin-rich extracts derived from Haematococcus pluvialis have been approved for several companies such as U.S. Nutra (USA), AstaReal AB (Sweden), Algatechnologies Ltd. (Israel), and Cyanotech (USA) (Campenni' et al., 2013). β-carotene serves as an essential nutrient (provitamin-A) and has high demand in the market as a food-coloring agent, as an additive to cosmetics and also as a health food ingredient (Raja et al., 2007). Furthermore, these carotenoids have been attributed with an extraordinary potential for promising applications in human health protection (Araya et al., 2014; Ip and Chen, 2005; Raja et al., 2007). These protective actions are likely to involve antioxidant mechanisms, including prevention of oxidative damage and cellular necrosis or apoptosis induced by oxidative stress (Yuan et al., 2011).

4. Conclusions

Chlorella protothecoides shifted to a mixotrophic growth when scotta was used in the medium as it was able to use lactose as a carbon source. Mixotrophic cultivation of C. protothecoides using scotta can be considered as a feasible strategy to reduce the costs of microalgal biomass production, while also contributing to solve the environmental problem caused by scotta disposal in dairy industries. High amounts of chlorophyll and carotenoids were found in both modes of growth, i.e. mixotrophic and autotrophic. Although the cellular accumulation of these pigments was higher in autotrophic C. protothecoides, their concentration per unit volume of liquid culture was greater under mixotrophic conditions and the peak value was achieved in a shorter time, thus raising interest toward this cultivation technique. Moreover, the imposition of a salt and light stress demonstrated that the carotenogenesis process can be controlled toward the accumulation of well quoted carotenoids, namely astaxanthin and lutein/zeaxanthin, thereby improving the efficiency of the pigments production by the mixotrophic C. protothecoides.

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Table 1. Different cultivation conditions of *C. protothecoides*.

Run	Culture medium	Carbon source
1	BG-11	CO_2
2	BG-11 + MS	CO_2 + Mixed *scotta*
3	BG-11 + OS	CO_2 + Ovine *scotta*
4	BG-11 + Lactose	CO_2 + Lactose
5	BG-11 + Glucose	CO_2 + Glucose
6	BG-11 + Galactose	CO_2 + Galactose

Table 2. Composition of *scotta* utilized in this work.

Parameter	Mixed *scotta*	Ovine *scotta*
pH	6.02 ± 0.03^{b}	6.20 ± 0.04^{a}
Acidity (g lactic acid 100 mL^{-1})	0.15 ± 0.01^{a}	0.12 ± 0.01^{b}
Reducing sugar (Lactose) (g 100 mL^{-1})	4.07 ± 0.21^{b}	4.69 ± 0.05^{a}
Dry material (g 100 g^{-1})	7.75 ± 0.13^{a}	8.24 ± 0.35^{a}
Total nitrogen (g 100 mL^{-1})	0.11 ± 0.2^{a}	0.11 ± 0.01^{a}
Fat (g 100 g^{-1})	0.18 ± 0.02^{a}	0.12 ± 0.04^{a}
Ash content (g 100 g^{-1})	0.94 ± 0.01^{a}	0.91 ± 0.02^{a}
Ca (g 100 g^{-1})	0.048 ± 0.004^{a}	0.037 ± 0.011^{a}
Mg (g 100 g^{-1})	0.030 ± 0.003^{a}	0.028 ± 0.006^{a}
P (g 100 g^{-1})	0.093 ± 0.002^{a}	0.114 ± 0.016^{a}
Na (g 100 g^{-1})	0.200 ± 0.004^{a}	0.186 ± 0.002^{b}
K (g 100 g^{-1})	0.119 ± 0.009^{a}	0.101 ± 0.002^{a}
Zn (mg 1000 g^{-1})	73.125 ± 0.884^{a}	72.500 ± 1.768^{a}

Parameter	Mixed *scotta*	Ovine *scotta*
Fe (mg 1000 g^{-1})	164.375 ± 0.884[a]	165.625 ± 13.258[a]
Mn (mg 1000 g^{-1})	3.125 ± 0.884[a]	4.375 ± 0.884[a]

Data are expressed as means of three replicates ± standard deviation.

[a-b] Means in the same row with different superscripts are significantly different ($p < 0.05$).

Table 3. Pigments production in green phase and stress phase as a function of the growth condition.

Green phase

Growth condition	Total chlorophyll (mg g_{cdw}^{-1})	Total carotenoids (mg g_{cdw}^{-1})	Astaxanthin (µg g_{cdw}^{-1})	Lutein/Zeaxanthin (µg g_{cdw}^{-1})	β-carotene (µg g_{cdw}^{-1})
$Autotrophic_{BG-11}$	25.17 ± 1.68a	6.38 ± 0.19a	96.54 ± 5.50b	1178.48 ± 101.93a	335.71 ± 37.24a
$Mixotrophic_{BG-11+MS}$	6.85 ± 1.44a	2.31 ± 0.32a	28.85 ± 4.42b	532.49 ± 67.40a	73.61 ± 12.73a
$Mixotrophic_{BG-11+OS}$	8.56 ± 2.03a	2.70 ± 0.22a	73.73 ± 9.77a	598.29 ± 21.06b	128.24 ± 8.47a

Stressed phase

Growth condition	Total chlorophyll (mg g_{cdw}^{-1})	Total carotenoids (mg g_{cdw}^{-1})	Astaxanthin (µg g_{cdw}^{-1})	Lutein/Zeaxanthin (µg g_{cdw}^{-1})	β-carotene (µg g_{cdw}^{-1})
$Autotrophic_{BG-11}$	14.17 ± 1.09b	5.64 ± 0.79a	131.02 ± 6.05a	1235.60 ± 152.59a	93.95 ± 27.60b
$Mixotrophic_{BG-11+MS}$	7.77 ± 0.99a	2.87 ± 0.30a	54.71 ± 6.32a	698.25 ± 41.85a	41.98 ± 3.63a
$Mixotrophic_{BG-11+OS}$	7.26 ± 2.19a	2.83 ± 0.52a	91.84 ± 2.69a	783.98 ± 10.26a	69.98 ± 10.02b

Data are expressed as means of three replicates ± standard deviation.

a-b: Different lowercase letters in the same column mean significant differences for the same growth condition by comparing the green phase and the stress phase at 5% level of probability.

Fig. 1. Biomass concentration along the cultivation process (green phase). Data are expressed as means of three replicates ± standard deviation.

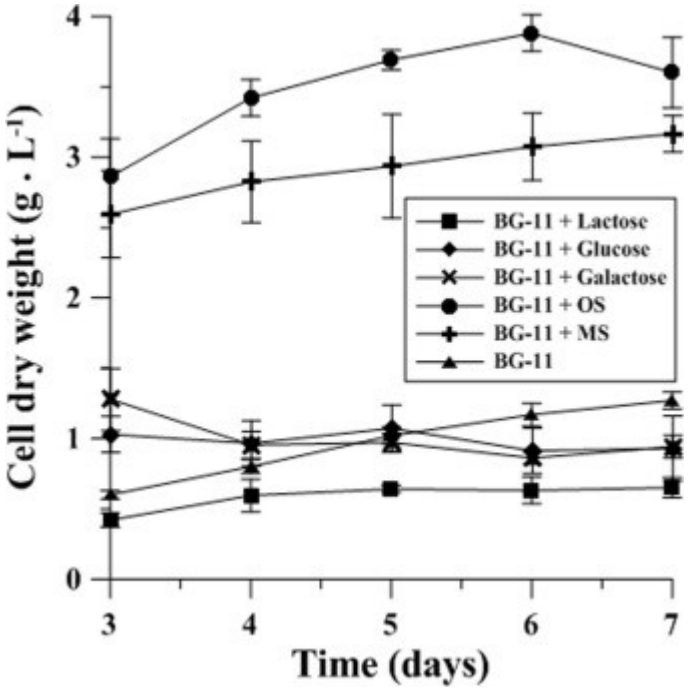

Fig. 2. Reducing sugar concentration along the cultivation process (green phase). Data are expressed as means of three replicates ± standard deviation.

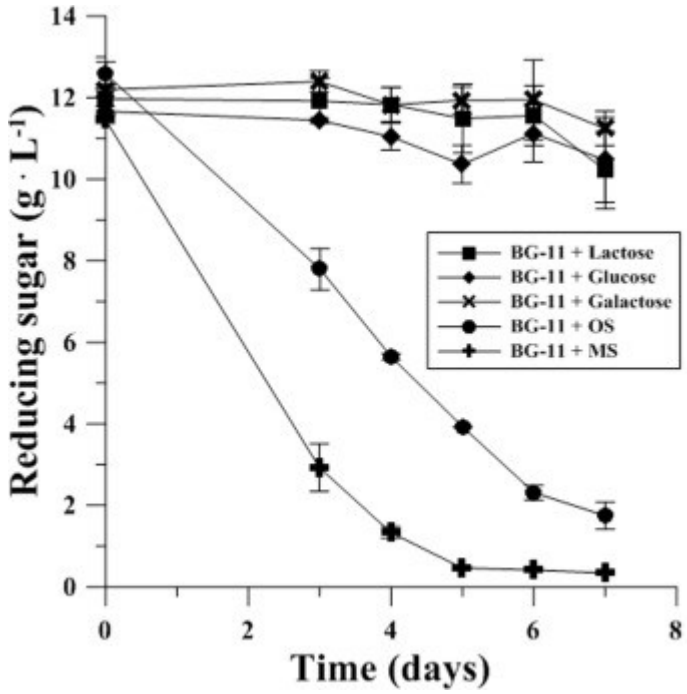

Fig. 3. Total chlorophyll in *C. protothecoides* along the cultivation process (green phase). Data are expressed as means of three replicates ± standard deviation.

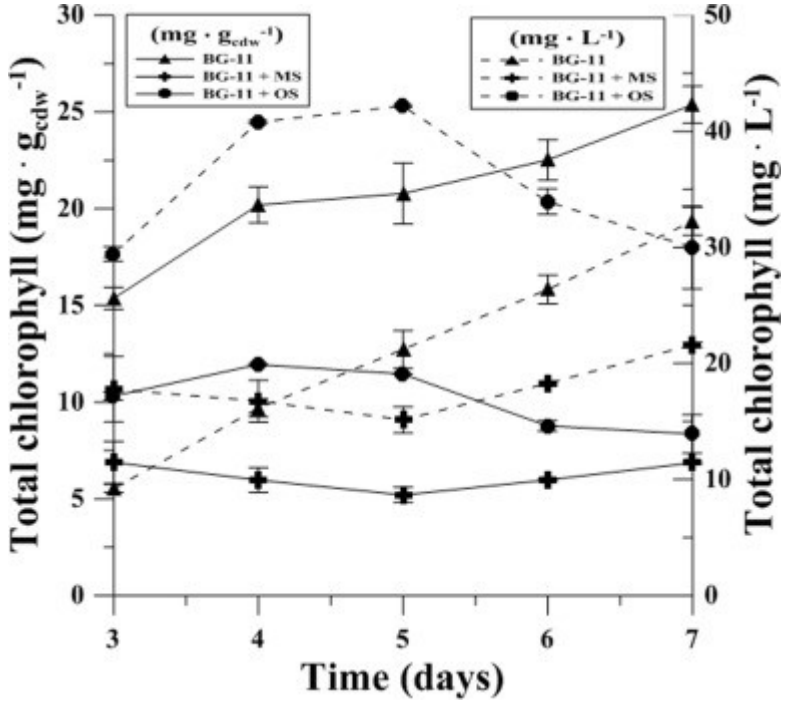

Fig. 4. Total carotenoids in *C. protothecoides* along the cultivation process (green phase). Data are expressed as means of three replicates ± standard deviation.