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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 strategies 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 carotenoids, 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 CO_2 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 (CO_2) 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 exploitation 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

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(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, hydrosoluble vitamins and a variety of minerals that could make it a good substrate in biotechnological processes for the production of commercial 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 microalgal inoculum was prepared in a BG-11 medium at 25 °C in 2 L glass photobioreactors under photoautotrophic conditions. The culture was aerated with CO_2-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).

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

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·$1H_2O$ 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 CO_2-enriched filtered air with 10% CO_2 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 temperature, photoperiod, air supply and CO_2 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