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Mixotrophic cultivation of *Chlorella vulgaris* using industrial dairy waste as organic carbon source

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HIGHLIGHTS

- ▶ Cheese whey was used as carbon source for *Chlorella vulgaris* growth.
- ▶ Mixotrophic microalgae grew faster than photoautotrophic cells.
- ▶ Maximum starch productivity was achieved under mixotrophic conditions.
- ▶ Highest pigment content (0.74%) was obtained in the photoautotrophic culture.

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ABSTRACT

Growth parameters and biochemical composition of the green microalga *Chlorella vulgaris* cultivated under different mixotrophic conditions were determined and compared to those obtained from a photoautotrophic control culture. Mixotrophic microalgae showed higher specific growth rate, final biomass concentration and productivities of lipids, starch and proteins than microalgae cultivated under photoautotrophic conditions. Moreover, supplementation of the inorganic culture medium with hydrolyzed cheese whey powder solution led to a significant improvement in microalgal biomass production and carbohydrate utilization when compared with the culture enriched with a mixture of pure glucose and galactose, due to the presence of growth promoting nutrients in cheese whey. Mixotrophic cultivation of *C. vulgaris* using the main dairy industry by-product could be considered a feasible alternative to reduce the costs of microalgal biomass production, since it does not require the addition of expensive carbohydrates to the culture medium.

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1. Introduction

Microalgae cultivation has been carried out throughout the world in order to produce animal feed or high-value added products, such as cosmetics, pharmaceuticals and health supplements (Das et al., 2011). More recently, microalgae have also been used for wastewater treatment, carbon dioxide (CO₂) mitigation, or as a feedstock for biofuel production (Brennan and Owende, 2010). These photosynthetic microorganisms can be cultivated either in open ponds or closed photobioreactors (PBR) using CO₂ and light as carbon and energy sources, respectively (Chen et al., 2011). Nonetheless, this culture mode, known as photoautotrophic, presents several disadvantages including low cell densities and long cultivation periods. Hence, heterotrophic and mixotrophic growth regimes have been proposed as feasible alternatives for the production of microalgal biomass (Yu et al., 2009).

Heterotrophic cultivation of microalgae involves the utilization of organic compounds as sole carbon source, while mixotrophic cultivation use simultaneously inorganic (for example CO₂) and organic compounds as carbon source (Dragone et al., 2010). Therefore, microorganisms cultivated under mixotrophic conditions synthesize compounds characteristic of both photosynthetic and heterotrophic metabolisms at high production rates. Additionally, lower energy costs have been associated with mixotrophic cultivation in comparison with photoautotrophic cultures, due to its relatively lower requirements for light intensities (Cerón García et al., 2005).

Despite mixotrophic cultivation of microalgae provides higher biomass and lipid productivities than cultivation under photoautotrophic conditions, the cost of the organic carbon substrate is estimated to be about 80% of the total cost of the cultivation medium (Bhatnagar et al., 2011). As a result, less costly organic sources have to be found in order to overcome the high carbon cost resulting from mixotrophic culture conditions (Liang et al., 2009). Cost reduction of growth media preparation with minimal undesired effects is crucial for a potential commercial application (Abad and

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Turon, in press). In this context, crude glycerol from biodiesel production, acetate from anaerobic digestion, and carbohydrates from agricultural and industrial wastes offer great promise as inexpensive organic substrates for the cultivation of microalgae on mixotrophic mode (Bhatnagar et al., 2011; Heredia-Arroyo et al., 2011).

Cheese whey (CW), the liquid by-product remaining from the cheese manufacturing process constitutes a serious environmental problem of dairy industries due to its high organic matter content (Dragone et al., 2009). Among the major components of whey, the disaccharide lactose, which on hydrolysis yields glucose and galactose, is greatly responsible for its high Biochemical Oxygen Demand (BOD = 30000–50000 mg/L) and Chemical Oxygen Demand (COD = 60000–80000 mg/L). In addition to this carbohydrate, CW also contains proteins, lipids, water-soluble vitamins and minerals (González Siso, 1996).

Exogenous sugars, such as glucose, galactose, mannose, fructose, sucrose and lactose have been commonly used for mixotrophic and heterotrophic cultivation of microalgae (Shi et al., 1999). However, these carbohydrates are transported and assimilated by microalgae with different efficiencies (Sun et al., 2008). A previous study revealed, for example, that some strains of *Chlorella* successfully utilize glucose and galactose during growth at different light intensities (Dvoráková-Hladká, 1966). Furthermore, recent reports indicate that *Chlorella vulgaris* grown on glucose medium may provide microalgal biomass for biofuel production and biorefinery (Kong et al., in press).

The objective of this work was to study the mixotrophic growth of *C. vulgaris*, using a hydrolyzed CW solution as an alternative approach to photoautotrophic microalgal cultivation. To our knowledge, no similar study has been previously carried out using this dairy by-product for cultivation of *C. vulgaris*.

2. Methods

2.1. Microalgal strain and inoculum preparation

The freshwater microalga *C. vulgaris* (strain P12) was used in all experiments. The microalgal inoculum was prepared according to previous studies (Fernandes et al., 2010) and conducted at 30 °C in 0.5-L glass photobioreactors under photoautotrophic conditions. The culture was aerated with CO₂-enriched air (2% v/v CO₂) at 0.4 vvm, and illuminated with continuous light (70 μmol/m² s) as reported by Dragone et al. (2011a).

2.2. Media and culture conditions

Four different cultivation conditions were carried out in duplicate (Table 1). The organic carbon sources used for mixotrophic cell growth were: a non-hydrolyzed CW powder solution, a mixture of pure glucose and galactose, and a hydrolyzed CW powder solution.

Sweet CW powder was supplied by Lactogal (Porto, Portugal). Its composition included (w/w): >73% lactose, 12% proteins, 1.5% lipids and <5% moisture. Non-hydrolyzed CW powder solution (nhCW) was prepared with distilled water and deproteinised by heat treatment as described elsewhere (Dragone et al., 2011b). Initial lactose concentration in nhCW was about 10 g/L. Hydrolyzed CW solution (hCW) containing glucose and galactose was obtained by hydrolyzing nhCW with β-galactosidase (>8.0 units/mg, Sigma-Aldrich) from *Aspergillus oryzae*. Enzymatic hydrolysis was performed at 30 °C and pH 4.5 with 0.02% of enzyme during 24 h. Glucose and galactose concentrations attained a maximum of 5 g/L at the end of the reaction and no lactose was detected in hCW.

All the experimental assays were carried out at 30 °C in 0.5-L glass photobioreactors containing 400 mL of medium, under a light intensity of approximately 70 μmol/m² s measured by a LI-250

Table 1

Different cultivation conditions of *C. vulgaris* and respective carbon sources.

Growth condition	Carbon Source
Photoautotrophic	CO ₂
Mixotrophic _{nhCW}	CO ₂ + Non-hydrolyzed CW solution (10 g/L lactose)
Mixotrophic _{hCW}	CO ₂ + Hydrolyzed CW solution (5 g/L glucose + 5 g/L galactose)
Mixotrophic _{G + G}	CO ₂ + Glucose (5 g/L) + Galactose (5 g/L)

Light Meter with a LI-190 quantum sensor (LI-COR, USA). Agitation during cell growth was provided by sparging CO₂-enriched air (2% v/v CO₂) from the base of the photobioreactors at 0.400 vvm. Initial cell concentration was about 0.5 g/L for all the cultivation conditions.

After reaching the stationary growth phase, cells were collected and centrifuged at 8750×g for 10 min, washed with distilled water and then freeze-dried for further biochemical characterization. The supernatant was also collected and frozen for subsequent sugar analyses.

2.3. Determination of microalgal cell concentration

Cell concentration in the photobioreactor cultures was measured regularly by using an improved Neubauer hemocytometer. Biomass concentration was estimated by cell dry weight after centrifugation of the sample (8750×g for 10 min), washing with distilled water and drying at 105 °C until constant weight.

2.4. Determination of carbohydrate concentration in culture media

Glucose, galactose and lactose concentrations in culture media were determined by High-Performance Liquid Chromatography (HPLC) in a Jasco chromatograph equipped with a refractive index (RI) detector (Jasco 830-RI, Japan) and a 300 × 6.5 mm Chrompack column (Chrompack, The Netherlands) at 60 °C, using 5 mM sulfuric acid as the eluent at a flow rate of 0.5 mL/min and a sample volume of 20 μL.

2.5. Determination of microalgal starch

The starch content of *C. vulgaris* was assayed by enzymatic hydrolysis of the microalgal starch to glucose with α-amylase and amyloglucosidase, according to Fernandes et al. (in press).

2.6. Measurement of lipid and protein contents in microalgae

Total lipids were determined by the classic Folch chloroform-based lipid extraction protocol. The protein content of microalgae was quantified according to the method of Lowry.

2.7. Measurement of chlorophylls and total carotenoids concentration

Chlorophylls and carotenoids in *C. vulgaris* were extracted with methanol and spectrophotometrically determined as described by Dere et al. (1998). Total pigment content was obtained by summing chlorophylls and carotenoids contents.

2.8. Determination of specific growth rate

The specific growth rate (μ, day⁻¹) was calculated from the Eq. (1), where N₁ and N₂ were the concentration of cells at the beginning (t₁) and at the end (t₂) of the exponential growth phase, respectively.

$$\mu = (\ln N_2 - \ln N_1)/(t_2 - t_1) \quad (1)$$

2.9. Determination of productivity of biomass, starch, lipids and proteins

Biomass productivity (P_{\max} , g/L d) during the culture period was calculated from the Eq. (2), where X_t was the biomass concentration (g/L) at the end of the exponential growth phase (t_x) and X_0 the initial biomass concentration (g/L) at t_0 (day):

$$P_{\max} = (X_t - X_0)/(t_x - t_0) \quad (2)$$

Productivity of starch, lipids and proteins at the end of cultivation were calculated from the Eq. (3), where $P_{\text{component}}$ was the productivity of starch, lipids or proteins, P_{\max} was the biomass productivity and $F_{\text{component}}$ was the mass fraction (w/w) of each component.

$$P_{\text{component}} = P_{\max} \times F_{\text{component}} \quad (3)$$

Data were compared using one-way ANOVA followed by a Tukey's multiple comparison tests with 95% confidence level.

3. Results and discussion

3.1. Growth parameters of microalgae cultivated under photoautotrophic and mixotrophic conditions

Specific growth rate, final biomass concentration and biomass productivity of *C. vulgaris* cultivated under photoautotrophic and mixotrophic conditions were compared and summarized in Table 2. The highest specific growth rates of *C. vulgaris* were 0.43 and 0.47 day⁻¹ when microalgae were cultivated under mixotrophic conditions using hydrolyzed CW powder solution, and a mixture of glucose and galactose as organic carbon sources, respectively. These values were almost 3.5 times higher than those obtained when cells were grown in inorganic medium supplemented with non-hydrolyzed CW powder solution, and under photoautotrophic mode of nutrition.

Biomass concentration at the end of cultivation and biomass productivity were also significantly influenced by the nutritional conditions. It can be observed in Table 2 that the highest values of X_{\max} (3.58 g/L) and P_{\max} (0.75 g/L d) achieved in the mixotrophic culture using hydrolyzed CW powder solution resulted in 2.9- and 7.5-fold increase respectively, when compared to the values obtained in the photoautotrophic culture. These results are in agreement with a previous study, which reported that mixotrophic *C. vulgaris* growth in glucose yielded higher biomass content and productivity than cells grown under photoautotrophic conditions (Kong et al., 2011). Mixotrophic cell cultivation utilizing both light and organic carbon source has been considered the most efficient

process for the production of microalgal biomass (Lee et al., 1996). When the light energy used for CO₂ fixation is decreased in mixotrophic cultures, most of the energy is used for carbon assimilation. Therefore, since the amount of energy dissipated is minimal, mixotrophy provides higher energetic efficiency than other cultivation modes (Lalucat et al., 1984). On the other hand, Shi et al. (1999) reported that glucose can be considered the best organic C-substrate for the growth of *Chlorella*.

It is worth mentioning that the organic substrate played an important role in promoting biomass accumulation of *C. vulgaris* during microalgae cultivation. As shown in Table 2, supplementation of the inorganic culture medium with hydrolyzed CW powder solution led to higher biomass concentration than supplementation with a mixture of glucose and galactose. The stimulatory effect of hydrolyzed CW powder solution on biomass production is probably related to the presence of some nutrients in CW powder composition, such as phosphorous and calcium. Ozmihi and Kargi (2007) reported that CW powder contains approximately 0.96% total phosphorous on dry weight basis. Phosphorous 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 (Richmond, 2004).

It should be stated that the mineral content in whey depends upon the processing techniques used for casein removal from liquid milk. Consequently, a higher microalgal biomass concentration than that found in our study could have been obtained by using acid CW powder due to the higher concentrations of calcium and phosphorous presented in that type of whey (Mavropoulou and Kosikowski, 1973).

The presence of nutrients might have also supported *C. vulgaris* growth when using non-hydrolyzed CW powder solution, which showed a specific growth rate similar to the photoautotrophic control but with a higher final biomass concentration (Table 2).

Due to the high content of nutrients, other valorization pathways for CW have recently been proposed. Viitanen et al. (2003) showed that CW can be applied as an alternative inducer in recombinant high-cell density fermentations. According to these authors, CW can be directly used without any pretreatment, not causing a dilution of the fermentation medium.

Therefore, a potential application of hCW could be related with its use as a fermentation additive for microbial cultivation.

3.2. Consumption of glucose and galactose by *C. vulgaris*

The above presented results demonstrated that the microalgal species used in this study was able to grow mixotrophically in the presence of glucose and galactose. Therefore, consumption of both carbohydrates by *C. vulgaris* cultivated under mixotrophic conditions is shown in Table 3.

It was found that glucose and galactose were consumed in larger quantities during microalgal growth in the presence of the hydrolyzed CW powder solution, in comparison to the culture supplemented with a mixture of pure sugars. In particular, glucose was completely consumed and only 4% of the initial galactose concentration remained in the growth medium at the end of cultivation when hydrolyzed CW powder solution was used as carbon source. On the other hand, after nearly 90 h of cultivation, initial contents of glucose and galactose dropped 80.5% and 49.5%, respectively, in the culture supplemented with both sugars. As discussed above, additional inorganic elements provided by hydrolyzed CW might have been responsible for the increased consumption of glucose and galactose derived from CW by *C. vulgaris*. These observations are in good agreement with a previous study where it was demonstrated that some components of hydrolyzed cheese whey enhanced carbohydrate utilization by microalgae (e.g. *Euglena gracilis*) (Freyssinet and Nigon, 1980).

Table 2

Growth parameters of *C. vulgaris* cultivated under photoautotrophic and mixotrophic conditions at 30 °C.

Growth condition	Growth parameters		
	μ_{\max}^* (day ⁻¹)	X_{\max}^{**} (g/L)	P_{\max}^{***} (g/L d)
Photoautotrophic	0.13 ± 0.01a	1.22 ± 0.12a	0.10 ± 0.01a
Mixotrophic _{hCW}	0.12 ± 0.00a	1.98 ± 0.43b	0.32 ± 0.13ab
Mixotrophic _{gCW}	0.43 ± 0.00b	3.58 ± 0.12c	0.75 ± 0.01c
Mixotrophic _{g + g}	0.47 ± 0.05b	2.24 ± 0.34b	0.46 ± 0.09bc

Data are expressed as mean ± standard error.

Means in the same column followed by different letters represent significant differences ($p < 0.05$).

* μ_{\max} = Specific growth rate during exponential growth phase.

** X_{\max} = Biomass concentration at the end of cultivation.

*** P_{\max} = Biomass productivity during the culture period.

Table 3
Consumption of glucose and galactose by *C. vulgaris* cultivated under mixotrophic conditions at 30 °C.

Growth condition	Carbohydrate consumption (%)	
	Glucose	Galactose
Mixotrophic _{nhCW}	–	–
Mixotrophic _{hCW}	100.0	96.0
Mixotrophic _{G+G}	80.5	49.5

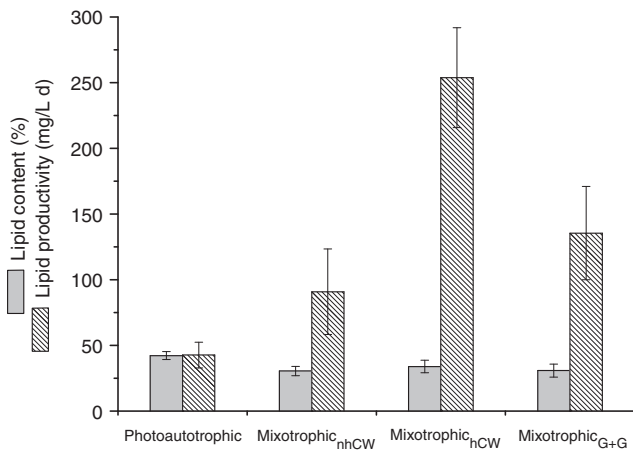


Fig. 1. Lipid content and lipid productivity of *C. vulgaris* under different nutritional conditions.

Regardless of the media used, glucose was more efficiently assimilated than galactose by *C. vulgaris* cells grown under mixotrophic conditions. Higher consumption of glucose compared to galactose for mixotrophic *C. pyrenoidosa* cultures was already described by Rodríguez-López (1966). A greater contribution to maintenance metabolism could explain the lesser assimilation of galactose when compared to glucose (Samejima and Myers, 1958).

3.3. Influence of nutritional modes on biochemical composition of *C. vulgaris*

The lipid content and lipid productivity of *C. vulgaris* under different growth conditions were compared and depicted in Fig. 1. When compared with mixotrophic cultures, higher lipid content (42%) was obtained in photoautotrophic mode at the beginning of the stationary growth phase (approximately 190 h). Other authors (Liang et al., 2009) have also shown that the amount of lipids accumulated in *C. vulgaris* under photoautotrophic growth conditions may surpass that from mixotrophic growth. On the other hand, the highest lipid productivity (253 mg/L d) was achieved when cells were grown in culture medium supplemented with hydrolyzed CW powder solution, due to the highest growth rate and cell density. Mixotrophic microalgal cultivation with hCW yielded six times higher lipid productivity than photoautotrophic culture (42 mg/L d). These results were remarkable in comparison with values presented in previous studies (Liang et al., 2009).

Different nutritional conditions had also different effects on starch content and starch productivity of *C. vulgaris* (Fig. 2). Although microalgal cells cultured photoautotrophically yielded the highest value of starch content (5.1%), maximum starch productivities were achieved mixotrophically using a mixture of pure glucose and galactose, and a hydrolyzed CW powder solution, as a consequence of the highest biomass productivity obtained under mixotrophic conditions. The lower content of nutrients in the medium containing pure carbohydrates as compared to that in hydrolyzed CW medium, promoted lower biomass growth and sugar

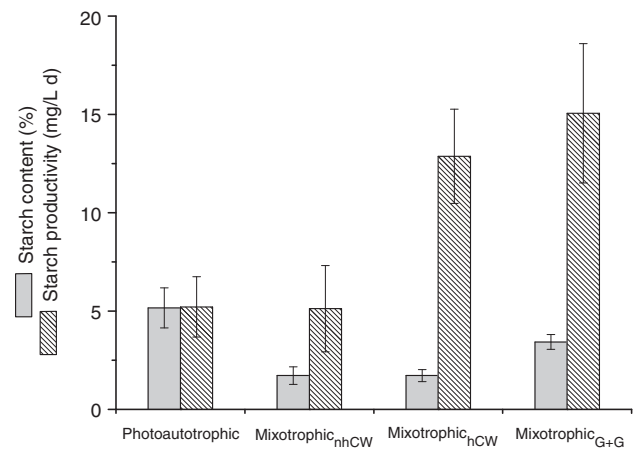


Fig. 2. Comparison of starch content and starch productivity of *C. vulgaris* grown under photoautotrophic and mixotrophic conditions.

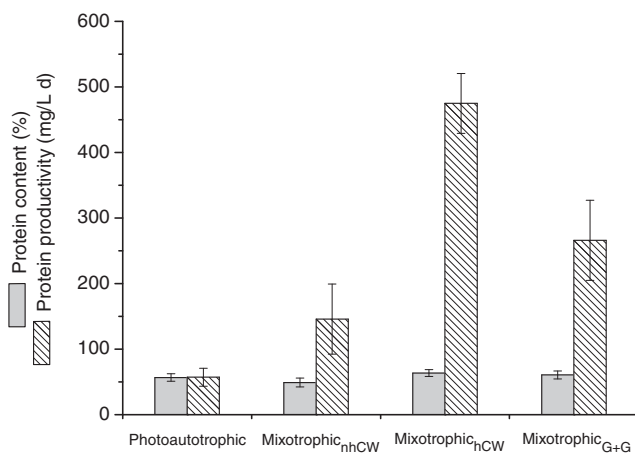


Fig. 3. Effect of nutritional mode on protein content and protein productivity of *C. vulgaris*.

consumption, and as a consequence of this stress condition, microalgal cells accumulated higher levels of starch. We have previously demonstrated (Dragone et al., 2011a) that higher starch accumulation in *C. vulgaris* P12 can be obtained under stressful growth conditions (e.g. by nutrient limitation). Therefore, since the starch productivity was calculated by multiplying the biomass productivity by the starch content (w/w) in microalgae, no differences on the values of this parameter were observed for the cells cultivated under mixotrophic conditions using hydrolyzed CW powder solution, and a mixture of glucose and galactose as organic carbon sources.

The protein content and protein productivity of photoautotrophic and mixotrophic microalgal cells were compared in Fig. 3. Cultivation of *C. vulgaris* P12 using hydrolyzed CW powder solution as organic carbon source led to the highest protein content (63.5%) and protein productivity (474 mg/L d). The highest protein content obtained in our study was significantly higher than that (26%) found in *C. vulgaris* (strain 31 #) cultivated in optimized mixotrophic medium with pure glucose as carbon source (Kong et al., in press).

The amount of total pigments in *C. vulgaris* cultured under photoautotrophic and mixotrophic conditions was also determined. As summarized in Table 4, the maximum pigment content (0.74%) was obtained in the photoautotrophic culture.

It has been suggested that the formation of photosynthetic apparatus in *Chlorella* may be disturbed by the presence of organic substrates (Yang et al., 2000), resulting in a decreased production

Table 4

Total pigment (chlorophylls + carotenoids) content of *C. vulgaris* cultivated under photoautotrophic and mixotrophic conditions at 30 °C.

Growth condition	Pigment content (%)		
	Chlorophylls (a + b)	Carotenoids	Total pigments
Photoautotrophic	0.51 ± 0.09c	0.23 ± 0.04b	0.74 ± 0.09c
Mixotrophic _{ChCW}	0.60 ± 0.17c	0.09 ± 0.03a	0.69 ± 0.17c
Mixotrophic _{ChCW}	0.22 ± 0.07a	0.04 ± 0.02a	0.26 ± 0.07a
Mixotrophic _{C + G}	0.37 ± 0.05b	0.08 ± 0.02a	0.46 ± 0.05b

Data are expressed as mean ± standard error.

Means in the same column followed by different letters represent significant differences ($p < 0.05$).

of photosynthetic pigments when compared with that obtained in photoautotrophic mode. The higher content of chlorophylls obtained in the photoautotrophic culture when compared to mixotrophic cultures confirms such observation. The enhancement of chlorophyll biosynthesis by photoautotrophic *Chlorella* strains compared with that resulting from mixotrophic cells have been previously reported by several authors (Ip et al., 2004; Kong et al., 2011). On the other hand, Yan et al. (in press) reported that low chlorophyll content in mixotrophic cells decreases the dependence on light. Therefore, reduced amount of chlorophylls in microalgae may relieve photoinhibition.

Among the different nutritional modes tested, the highest carotenoids content (0.23%) was also found in the photoautotrophic culture. This value dropped to 0.04% and 0.08% when cells were grown in inorganic medium supplemented with hydrolyzed CW powder solution, and with a mixture of pure glucose and galactose, respectively. These results are consistent with those of Liu et al. (2009) who found lower amount of carotenoids in mixotrophic cells when compared to cells grown on photoautotrophic culture.

4. Conclusions

When compared with the photoautotrophic control culture, mixotrophic microalgae grew faster, providing higher productivities of biomass, lipids, starch and proteins. Furthermore, microalgal biomass production and carbohydrate consumption were enhanced by supplementing the inorganic culture medium with hydrolyzed CW powder solution, than supplementing with a mixture of pure glucose and galactose, as a consequence of stimulatory effects arising from growth-promoting nutrients in CW. Mixotrophic cultivation of *C. vulgaris* using CW 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 CW disposal in dairy industries.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biortech.2012.05.055>.

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