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Research article

Valorization of second cheese whey through cultivation of extremophile microalga *Galdieria sulphuraria*

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Abstract: Second cheese whey (SCW) or "*scotta*" in Italian, is a side-stream from the manufacturing of "*Ricotta*" cheese, obtained after thermal coagulation of whey proteins residue in the cheese whey. *Galdieria sulphuraria* is a thermophilic red algae well known for its metabolic capabilities to grow on wastewater and other saline effluents. In this work, the valorisation of SCW as nutrient source for the growth of *G. sulphuraria* has been investigated using different concentrations of SCW. The biochemical and fatty acids composition of the biomass obtained has been evaluated too. Small differences have been observed in terms of biomass obtained after 12 days of cultivation between the SCW media and the relative control with the same amount of reducing sugars. The fatty acids composition of *G. sulphuraria* grown in SCW showed a higher content of polyunsaturated fatty acids compared to the control. The biomass productivity using SCW media has also been optimized through response surface methodologies with supplementation of nitrogen source obtaining a biomass dry weight higher than 10 g L^{-1}.

Keywords: sustainability, PUFA, food waste, microalgae, dairy wastewater, bioconversion

Abbreviations: SCW: Second cheese whey; PUFA: polyunsaturated fatty acids; CW: cheese whey; RSM: response surface methodology; SM: standard medium; DNS: 3,5-Dinitrosalicylic acid; FAN: free amino nitrogen; RS: Reducing sugars; CCD: central composite design; DW: dry weight; GC/FID: gas chromatography / flame ionization detector; FAME: fatty acids methyl esters; SM: standard medium

1. Introduction

The dairy industry is one of the most important food industries in Europe. Cheese manufacturing produces different effluents including the second cheese whey (SCW) which, in particular, comes from "ricotta" cheese production. The SCW is the result of whey proteins thermal coagulation, which are separated to make ricotta cheese, while the liquid residue is destined to be an effluent. The SCW is an interesting by-product due to the presence of important nutrients like lactose, nitrogen, free aminoacids, mineral salts, phosphorous etc. [1]. However, SCW production causes huge environmental and economic problems for its disposal by producers [2]. Only in Italy, where it is known as "*scotta*", more than 1 million tons per year are produced [1,3].

In the last years, many efforts have been carried out to evaluate the biotechnological utilization of dairy wastewater or cheese whey (CW) [4]. Actually, there is not a real utilization of this by-product and it is destined to disposal by the producers. Usually, dairy effluents are treated with physicochemical or biological processes. A biological process that has been evaluated in the last years involves the use of microalgae [5].

These aquatic protists are microrganisms widely known for their sustainable bioremediation capacity. In fact, they provide great opportunities to recycle nutrients present inside food waste or effluents such as sludge or saline wastewater [6,7]. The most used microalgae are *Chlorella* sp. and *Scenedesmus* sp., for which the growth on pretreated dairy effluents has been widely studied [8,9]. However, there is another group of microalgae that provides great opportunities as biorefinery platforms: the extremophile algae [10]. Extremophile algae are capable to grow in harsh conditions such as high salinity concentration or very low pH. Among them, *Galdieria sulphuraria* is a promising heterotrophic red algae that has been cultivated for the production of pigments, antioxidants and for the removal of nitrogen, sugars and phosphorus from wastewaters [5,11,12]. In fact, *G. sulhpuraria* is an important producer of C-Phycocyanin which exhibits ability to remain stable at high temperatures up to 60 ℃ [13]. This property makes this pigment very useful in various fields of forensic sciences including biotechnology, molecular biology, and recombinant technology.

Thanks to its great metabolic flexibility, this microalga is an interesting biomass for the bioconversion of food by-products and waste in molecules with high added value. Moreover, *G. sulphuraria* is a thermo-acidophilic microalga capable to grow at pH lower than 2 and at temperature higher than 50 ℃, which are important to prevent bacterial contamination that could affect the growth performance [12].

In scientific literature few works evaluated *G. sulphuraria* growth on food by-products. In particular, Massa et al., (2019) reported the biochemical composition for samples grown on spent cherry brine liquid [7]; while Zimermann et al., (2020) studied the growth kinetics of *G. sulphuraria* grown on whey permeate using, however, cell count as only growth parameter [5]. Scherhag and Ackermann (2020) instead, evaluated the sugar removal from fruit wastewater by *G. sulphuraria* (SAG 21.92) [11]. However, very few studies have been found on biomass optimization of these food wastes by using statistical methods such as response surface methodologies.

Therefore, for the first time, in this work SCW has been used as nutrient media for the cultivation of *G. sulphuraria*. The growth kinetics and the biochemical composition of the microalga have been determined. The biomass optimization of new SCW media has been established through response surface methodology (RSM).

2. Materials and methods

2.1. Galdieria sulphuraria standard growth conditions

G. sulphuraria (SAG 107.79) was obtained from Culture Collection of Algae at the University of Göttingen (Germany). Regular sub-culturing of photoautotrophic algae were made every 4 weeks on liquid and agar slants of Cyanidium Medium [7]. To obtain the transition from autotrophy to heterotrophy, the Allen medium [14] was used as standard media (SM), with an addiction of 30 g L^{-1} of glucose as organic carbon source and 1.32 g L^{-1} of $(NH_4)_2SO_4$ as nitrogen source. The cultures were placed in a dark room at 27 °C ± 1 and the mixing was provided through an air bubbling system equipped with a filter of 0.22 μm in order to prevent any contamination and to provide oxygenation to the culture. The pH of culture was set at 1.5 with the addition of H_2SO_4 5 N.

2.2. Characterization and treatment of Second Cheese Whey

The SCW was gently provided by a dairy industry in the area of Salerno (Italy) that produces mozzarella and other fresh cheeses. The samples were taken from the accumulation tanks of the company and immediately stored at −18 °C for the transport in the laboratory. In these tanks only the SCW was present and not any other type of dairy wastewater.

Prior any analysis, the SCW was filtered through a 1 μm filter to remove coarse solids. The chemical analyses involved the measurement of dry matter (g L^{-1}), volatile solids (g L^{-1}), ash (g L^{-1}), pH (using a Mettler-Toledo pH-meter), reducing sugars using 3,5-Dinitrosalicylic acid (DNS) method [15], protein content following Bradford method [16], nitrates (cadmium reduction method), ammonium (N-NH_4) (salicylate method), phosphate content (P total) (acid digestion method) [17] and free amino nitrogen (FAN). FAN content was estimated with ninhidrin reaction method described by Lie (1973) [18]. All the analyses were carried out in triplicate.

For the treatments, prior the cultivation, the SCW was heated up at 75 °C x 10 min to promote the precipitation of residual casein and then centrifuged at 4695 x g for 15 min. at 10 °C. The clean surnatant was then collected and used for the analyses and for the cultivation trials.

2.3. G. sulphuraria growth test and optimization using SCW

To evaluate the utilization of SCW as nutrient source, four different concentrations of this effluent have been investigated. The *scotta* was diluted to reach four different concentrations in reducing sugars (RS): 1.0%, 1.5%, 2.0% and 2.5%, corresponding to 22%, 34%, 45% and 57% of concentration (v/v) respectively. The dilution was made with distilled water. As control, *G. sulphuraria* was cultivated in Allen medium at 1.0%, 1.5%, 2.0% and 2.5% of glucose. For the samples with SCW, pH was adjusted to a final value of 1.5 using H_2SO_4 5 N as the SM.

For this test, *G. sulphuraria* cultivation was carried for 12 d in air-lift reactor of 3 L with a working volume of 2 L at 27 ± 1 °C in a dark room. The inoculum for the SCW test was previously acclimatized with the by-product to improve the growth performances.

After this, in order to enhance the biomass concentration using the new formulated media, a response surface method (RSM) was also used. A three level full factorial central composite design (CCD) was used to determine the effect of added nitrogen and glucose to the new media. The optimization consisted of 14 runs conducted in two blocks with 4 cubic points (or factorial points), 4 axial points (or star points) and 3 center points for each block. The independent factors used were glucose and $(NH_4)_2SO_4$. The three levels (−1, 0 and +1) set for glucose were 0, 5 and 10 g L^{-1} supplemented to the SCW media, while for NH_4SO_4 was 0, 0.4 and 0.8 g L^{-1}.

The mathematical relationship of the response (Y) to the significant independent variables X_1 and X_2 is given by the following quadratic polynomial Eq 1:

$$Y = \beta_0 + \Sigma i_{=1}\beta_i X_i + \Sigma_{i=1}\beta_{ii} X_i^2 + \Sigma_{i=1}\Sigma_{j=I+1}\beta_{ij} X_i X_j \quad (1)$$

Where Y is the predicted response; X_i and X_j are the coded values; β_0 the independent coefficient; $\beta_{i,,j}$ is the linear coefficient associated to each independent factor ($X_{i,j}$) and β_{ij} and β_{ii} are the coefficient for interaction and quadratic effects respectively [19]

The optimization test was conducted at 28 ± 1 ℃ using 1 L Erlenmeyer flask with a working volume of 600 mL and the mixing provided by an air bubbling system equipped with a filter (as described above).

2.4. Growth parameters

The monitoring of the growth performances for control and treated samples were obtained through growth curves using standard gravimetric methods on daily aliquots of cultures. The dry cell weight (DCW, g L^{-1}) was obtained after centrifugation at 4695 x g for 15 min and the pellet was rinsed twice to remove any residual salt. The pellet was dried at 70 ℃ until the constant weight was reached. The residual surnatant was filtered at 0.45 μm and immediately stored at −18 ℃ for further analysis. For the definition of the growth kinetics, the specific growth rate (μ), maximum specific growth rate (μ_{max} day^{-1}), maximum biomass obtained (X_{max}), and tX_{max} were calculated.

The maximum specific growth rate was calculated at the exponential stage following the Eq 2:

$$\mu max\ (\text{day}^{-1}) = \frac{(\ln DCW2 - \ln DCW1)}{t2 - t1} \quad (2)$$

Where DCW1,2 is the dry cell weight at time 1 and 2 respectively.

To evaluate the nutrient consumption by *G. sulphuraria*, the free amino nitrogen (FAN) in residual media was assessed. The FAN content was obtained using the ninhidrin assay and the residual reducing sugar by DNS method.

2.5. Biochemical composition and lipid analysis of biomass

After the period of cultivation (12 d), the biomass was harvested using a continuous centrifuge at 3005 x g and washed with distilled water. The wet biomass obtained was lyophilized for all the analysis. The carbohydrate determination was obtained with the Dubois method [20] using 1 gr of freeze-dried sample of *G.sulphuraria.* The ash content of the biomass was determined gravimetrically in a muffle furnace at 550 ℃ until achieving constant weight.

The lipid content of biomass was determined by the Bligh and Dyer method [21] using chloroform:methanol 2:1 (v/v). The lipids extracted were suspended in 1 mL of hexane and then converted to their relative methyl esters by adding 200 μL of KOH 2 N in methanol for 30 s at room temperature. The fatty acids profile was obtained in gas chromatography system (Shimadzu GC-17A) coupled with a flame ionization detector (GC/FID). The GC was equipped with a fused silica capillary column (SPTM-2560, 75m x 0,18 mm, i.d. 0,14 μm film thickness) and using helium as gas carrier. The identification of the fatty acids methyl esters (FAME) was obtained after the injection of pure standards FAME from Larodan (Malmoe, Sweden) and comparing the relatives retention time. Acquisition software used for identification of FAME was the Class-VP chromatography data system, vers. 4.6 (Shimadzu Italia, Milan).

The protein content of the biomass was obtained with Kjeldahl method [22]. The cellular protein value was calculated using the conversion factor of 6.25.

2.6. Statistical analysis

The data were analyzed using SPSS software, version 23 (IBM Corp., Armonk, NY, USA). All the analyses were carried out in triplicate, and average values with standard deviation were reported. One-way analysis of variance (ANOVA) was applied using raw data to test for significant differences among the samples. $P < 0.05$ was considered statistically significant. Tukey's test was used for post-hoc analysis when there were significant differences among the samples. The optimization process was evaluated with RSM analysis, performed in 'R' (RStudio with 'R' version 3.0.2, RCore Team, Vienna/Austria).

3. Results and discussion

3.1. Characterization of SCW

The chemical and physical composition of SCW is reported in Table 1. The characteristics of this by-product are not very different from cheese whey because it is slightly acid (pH 5.9) and characterized by a good content of reducing sugars, mainly lactose (up to 44 g L^{-1}). The sugars and nitrates content of SCW were higher than those reported in literature [23]. The high RS content is very interesting for heterotrophic cultivation of *G. sulphuraria*, which was reported to be able to grow on many different organic carbon sources [24,25]. The analysis showed a residual content of protein, probably due to an inefficient process of flocculation during the "Ricotta" cheese production. However, the residual organic nitrogen could be very interesting for cultivation of *G. sulphuraria* which is able to use both inorganic and organic forms of nitrogen for its growth (i.e. aminoacids) [26]. The C:N ratio of the SCW was approximately 30, while the ratio of the SM is 43. That could affect the biochemical and lipid composition of the biomass obtained, as reported by other authors [27]. Total phosphorus (P total) of SCW was 96 mg L^{-1}, that is lower respect to the standard medium (110 mg L^{-1}), and the resulting N:P ratio was 4.89 (higher than the SM). P is important for the synthesis of phospholipids and nucleic acids in microalgae [28].

Table 1. Chemical and physical characterization of second cheese whey (SCW) used for the growth of *G.sulphuraria*.

Parameters	Value
pH	5.9 ± 0.2
Ash (g L^{-1})	5.5 ± 0.5
Dry weight (g L^{-1})	58.4 ± 0.6
Volatile solids (g L^{-1})	53.3 ± 0.4
NH_4-N (mg L^{-1})	25 ± 1.3
NO_3-N (mg L^{-1})	80 ± 1.2
N total (g L^{-1})	0.59 ± 0.1
Free amino nitrogen (mg L^{-1})	231.14 ± 16.4
P total (mg L^{-1})	96.1 ± 0.4
Reducing sugars (g L^{-1})	43.4 ± 0.9
Protein content (g L^{-1})	3.1 ± 0.6

Note: Values expressed as mean ± SD (n=3).

The nutrient content of the SCW was not so far from the SM. Therefore, it was possible to test this dairy by-product for the cultivation of *G. sulphuraria*.

3.2. G. sulphuraria growth performances on SCW

The growth curves of *G. sulphuraria* grown using SCW are reported in Figure 1. The curves have been separated to better understand the growth performance on the various formulation of SCW medium with controls at the same amount of RS. No significant differences were observed for microalgae cultivated with *scotta* at 1% in RS respect to the relative control (Figure 1a). However the biomass obtained at 1% in RS is lower respect to the control.

For samples at 1.5% in RS, a difference can be observed (in terms of concentration) after 12 days of cultivation (5.1 g L^{-1} for the control and 4.2 g L^{-1} for the SCW samples), and the overall growth performance was lower respect to the control. However, these differences were not significant (Figure 1b). With SCW medium at 2% RS instead, *G. sulphuraria* showed a longer lag phase respect to the control, reaching only at 10^{th} day a concentration similar to the relative control. At SCW 2.5% RS, microalgae showed worst growth performance compared to the samples at 2.0% RS, with a longer lag phase respect to the control, and reaching the maximum concentration after 11 days of cultivation. The biomass obtained for SCW sample at 2.5% RS was significantly lower than the control (Figure 1d).

However, for SCW medium at 2.5% RS, the productivity is lower to the sample at 2.0%, this can be explained by an inhibition effect of higher concentration of "*scotta*". In fact, in the work of Zimermann et al., (2020) high concentrations of whey permeate resulted toxic for the growth of *G. sulphuraria*, and the author used concentration below the 40% (v/v).

To further define the growth kinetics of *G. sulphuraria* in SCW media, the growth rate, X_{max} and μ_{max} were calculated and reported in Table 2. After 4 days of cultivation, the growth rates of the controls are higher than the samples with SCW, except for the sample at SCW 1.5% RS. At 7^{th} day, significant differences between the controls and the SCW samples are reported for the samples at 2 and 2.5% RS, denoting the longer lag phase reported for the culture with higher concentration of *scotta* (Figure 1c,d). In particular for the samples at 2.5% RS the difference is more pronounced.

Table 2. Specific and maximum growth rate (μ_{max}), maximum concentration reached (X_{max}) and cultivation time for maximum concentration (tX_{max}) of *G.sulphuraria* growth on different concentrations of SCW diluted from 1.0 to 2.5% in reducing sugars (RS).

Sample	μ (4 d)	μ (7 d)	μ_{max}	X_{max} (g L^{-1})	tX_{max} (d)
Control 1.0% RS	0.305 ± 0.03	0.202 ± 0.01	0.174 ± 0.02	4.09 ± 0.21	8
SCW 1.0% RS	0.285 ± 0.01	0.191 ± 0.01	0.191 ± 0.01	3.87 ± 0.17	7
Control 1.5% RS	0.279 ± 0.02	0.219 ± 0.02	0.203 ± 0.03	5.15 ± 0.32	8
SCW 1.5% RS	0.287 ± 0.02	0.203 ± 0.01	0.186 ± 0.02	4.35 ± 0.19	9
Control 2.0% RS	0.325 ± 0.03	0.256 ± 0.02	0.199 ± 0.03	6.10 ± 0.29	8
SCW 2.0% RS	0.250 ± 0.02	0.212 ± 0.02	0.159 ± 0.03	6.02 ± 0.40	11
Control 2.5% RS	0.345 ± 0.02	0.259 ± 0.02	0.209 ± 0.01	6.89 ± 0.28	9
SCW 2.5% RS	0.259 ± 0.01	0.165 ± 0.01	0.147 ± 0.02	5.33 ± 0.21	11

Note: Values expressed as mean ± SD (n=3).

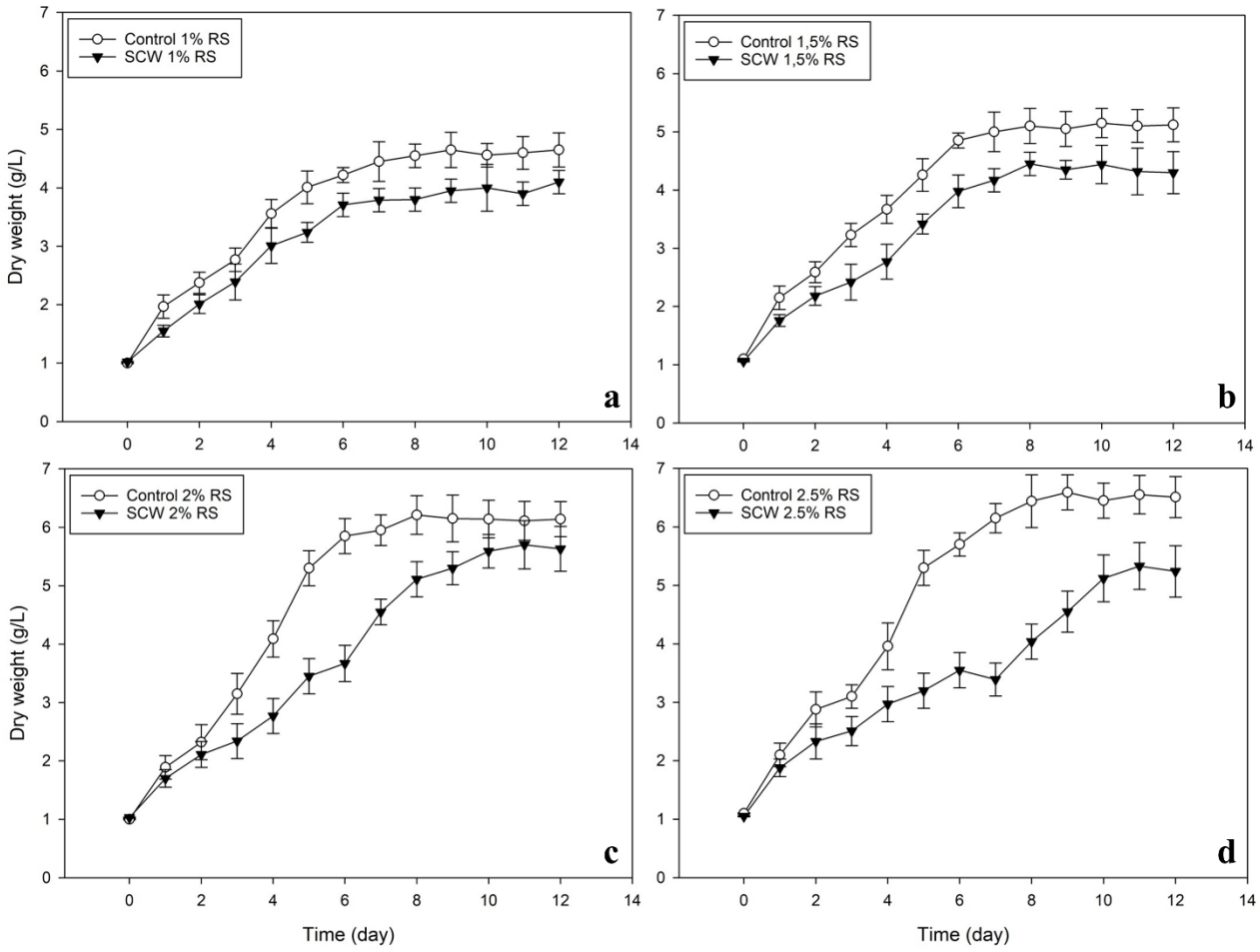

Figure 1. Growth curves (mean ± SD) of *Galdieria sulphuraria* growth with four formulation of second cheese whey medium diluted at 1.0% (a) 1.5% (b), 2.0% (c) and 2.5% (d) concentration in reducing sugars (RS). The controls refer to standard media with the same percentage of reducing sugars.

Both the samples at 2.0 and 2.5% RS reached the maximum biomass concentration after 11 days. This can be explained by a necessity of the microalga to adapt to the new media, which is something already known for this type of biomass [7]. About the uptake of lactose by *G. sulphuraria*, instead, some studies reported an utilization of this disaccharide by the alga [5,29], showing that lactose can be actually transported in to the cell by a low-affinity transport system. However it is actually not clear if lactose uptake is slower than the glucose uptake for *G. sulphuraria*.

The low free nitrogen present in SCW forced *G. sulphuraria* to assimilate organic nitrogen from peptides aminoacids (I.e. FAN) residual after pre-treatments. In Figure 2 is reported the FAN content on the supernatant collected after biomass harvesting. The FAN content is almost depleted in SCW at 1% RS after 12 days of cultivation. At 2.5% RS instead, the residual content of FAN is still high after cultivation. At 2.0% the residual FAN concentration is the same of the sample at 1.5%, proving a better uptake of nitrogen in that condition.

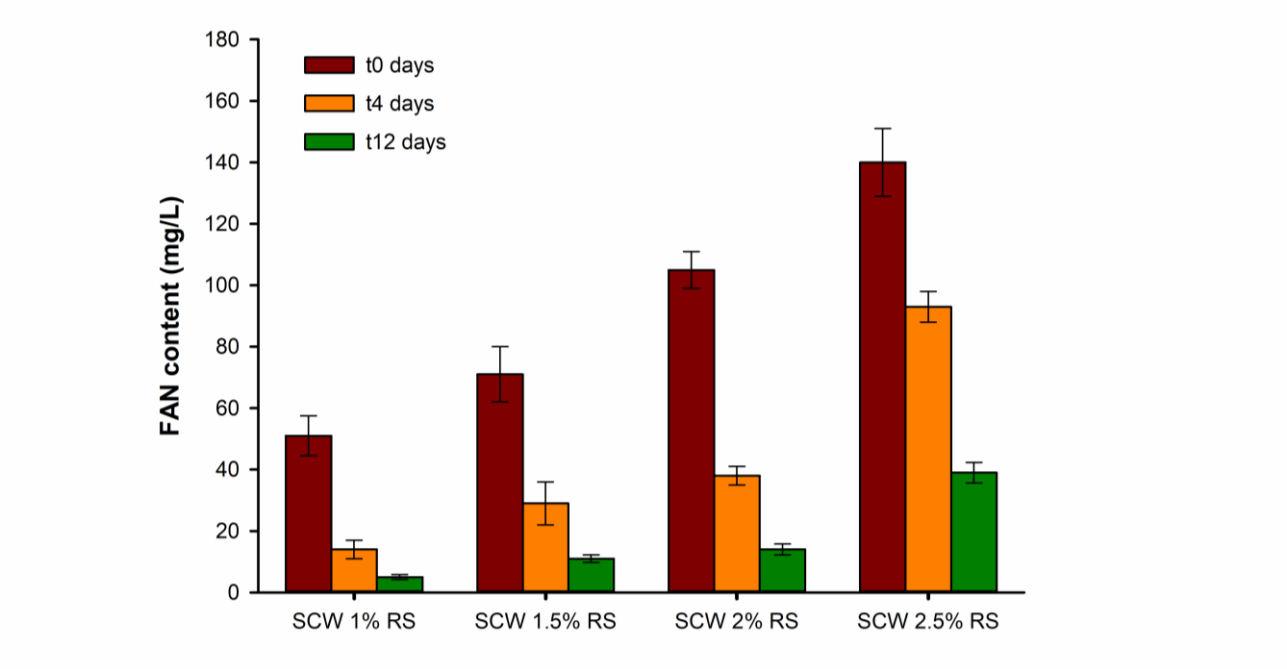

Figure 2. Free amino nitrogen content (mean ± SD) of residual water after centrifugation of biomass, sampled at beginning (t0), four days (t4) and twelve days (t12) of cultivation.

Other studies reported an assimilation of nitrogen from aminoacids and peptides present in growth medium by *Cyanidium caldarium* (formerly named *Galdieria sulphuraria*), but with a slower uptake [30]. This, combined to the presence of lactose as only carbon source, can explain the slower productivity compared to the controls. However, the SCW media formulation resulted interesting for the growth of *G. sulphuraria*, but an optimization of the medium is required to increase the biomass production.

3.3. Biochemical and fatty acids composition of biomass cultivated

The proximal composition of *G. sulphuraria* growth on various SCW formulations is reported in Figure 3. Carbohydrates results the principal biochemical component of the microalga, in agreement with other authors [7,31,32]. In general, the amount of carbohydrates increases with increasing concentration of SCW (in terms of RS); while the amount of proteins is lowered when the concentration of SCW increases. It is reported that heterotrophic cultures of *G. sulphuraria* accumulate carbohydrates (principally glycogen) when more glucose or reducing sugars are added to the culture media [33], which explains the higher content of carbohydrates in the samples with an higher percentage of RS. The α-glucan (glycogen) is the primary form of carbohydrates accumulation in *G.sulphuraria*. Moreover, a natural glycerol glycoside (named "floridoside") is an interesting molecule found in this red alga due to its therapeutic properties (bone formation promotion and modulation of immune system) [34].

The protein content showed no significant differences between the SCW samples and the relative controls, but the SCW 2.5% in RS showed a protein content lower than the control (26% vs. 32%). The protein content of SCW samples was in line with another study [32] that used standard culture conditions.

The lipids content was about 10% of DW, without significant differences between the samples. This value is higher than others found in literature [7,31]. However, on the fatty acids profile (Table 3) it can be observed that there are significant differences between the samples growth on SCW

media and the relative control in standard conditions. The control showed a greater concentration of oleic acid (up to 78% of Total fatty acids, TFA) respect to the SCW samples (30–60% of TFA) which showed instead a greater concentration in saturated fatty acids (SFA). The SFA content of SCW samples is significantly higher than the control, in agreement with another study on *G. sulphuraria* growth in heterotrophic conditions [33]. The amount of oleic acid obtained in the control is higher than another reported in literature for *G.sulphuraria* [31], while the amount of linoleic acid (C18:2) is lower.

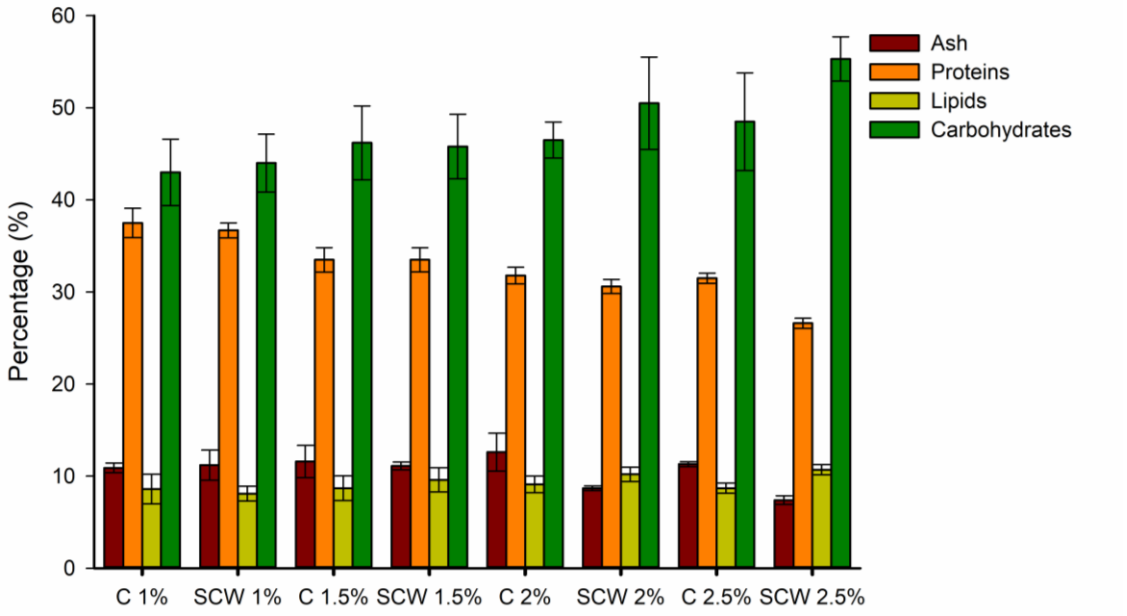

Figure 3. Chemical composition of *Galdieria sulphuraria* grown with SCW media diluted at four concentration of reducing sugars (1–2.5%) respect to the standard medium (C) with the same percentage of reducing sugars (error bars refers to SD). Values are expressed as percentage of dry weight.

Table 3. Fatty acids profile (g/100g) on the lipids extract from *G.sulphuraria* grown on SCW diluted at different reducing sugars (RS) concentrations (1–2.5%).

Fatty acids	Control	SCW 1.0%	SCW 1.5%	SCW 2.0%	SCW2.5%
C14:0	0.21 ± 0.07^{a}	4.54 ± 1.10^{d}	2.29 ± 0.37^{c}	1.6 ± 0.21^{b}	1.76 ± 0.42^{b}
C16:0	7.01 ± 1.02^{a}	26.26 ± 2.45^{b}	24.96 ± 1.24^{b}	18.89 ± 2.29^{c}	20.13 ± 1.93^{b,c}
C18:0	5.1 ± 1.13^{b}	10.8 ± 2.02^{a}	12.85 ± 1.41^{a}	5.96 ± 1.04^{b}	7.31 ± 0.56^{b}
Σ SFA	12.32	41.6	40.1	26.45	28.2
C16:1	0.11 ± 0.02^{b}	1.01 ± 0.24^{a}	0.53 ± 0.19^{a,b}	0.35 ± 0.11^{b}	1.14 ± 0.21^{a}
C18:1	77.84 ± 3.42^{c}	41.08 ± 5.45^{a,b}	34.9 ± 2.41^{a,b}	55.88 ± 3.57^{d}	49.63 ± 2.49^{b}
Σ MUFAs	77.95	42.09	35.43	56.23	50.77
C18:2	6.23 ± 1.13^{a}	5.72 ± 1.25^{a}	9.82 ± 1.44^{b}	6.05 ± 1.93^{a}	5.67 ± 1.26^{a}
C18:3	1.98 ± 0.26^{c}	4.24 ± 1.67^{a}	10.53 ± 2.18^{b}	8.43 ± 0.65^{a,b}	6.08 ± 0.41^{a}
Σ PUFAs	8.21	9.96	20.35	14.48	11.75

Values are expressed as means ± SD (n=3). SFA= saturated fatty acids; MUFA= monounsaturated fatty acids; PUFA= polyunsaturated fatty acids. Values followed by different letters on the same line are significantly different ($P < 0.05$).

Comparing the data of this study to the work of Graziani et al., (2013), the amount of α-linolenic acid (C18:3) of the samples growth in SCW is 2–3 times higher, and 5 times greater for SCW at 1.5% in RS. This can be explained by a difference in the culture conditions and growth medium of *G. sulphuraria*. In fact, in the work of Graziani et al. (2013) the microalga was cultivated at 36 ℃, while in this test at 27 ℃.

Moreover, an interesting result was obtained in terms of polyunsaturated fatty acids (PUFA) concentration, which was higher in the sample SCW 1.5% RS respect to the control (20% vs 8% of the control). The composition and characteristics of lipids and fatty acids of *G.sulphuraria* are regulated by the growth conditions [33]. In our case, the addiction of SCW in the growth media seems to affect the cells metabolism, stimulating the elongation and unsaturation of acyl chains in the algae cells, especially in the sample at 1.5% in RS. However, further studies are required to understand the regulation mechanisms of metabolic flow of fatty acids in *G. sulphuraria.*

3.4. Growth optimization of SCW media

The SCW formulation with higher biomass productivity is the one at 2.0% in RS, with 6.05 g L^{-1} of DW. For that reason, the optimization has been performed on this formulated medium.

Central Composite Design (CCD) was used to optimize the utilization of SCW media with the supplementation of organic carbon (glucose) and nitrogen (in forms of ($(NH_4)_2SO_4$). To the best of our knowledge, this is the first study to apply CCD for the biomass optimization of *G. sulphuraria* with a food waste.

The response surface design employed gave 14 combinations of selected nutrients (glucose and ammonium sulfate). In Table 4 is reported the design and the results with the responses. Biomass concentration (DW) was used as response, and was calculated at log phase (12 days). The significance of the model and its second-order Eq 2, derived from the multiple regression analysis of the data, was tested by analysis of variance (ANOVA) (Table 5) and *P*-value lower than 0.05 was considered significant in the analysis. The model fit is also expressed with coefficient of determination (R^2) which was 0.985, indicating that 98.5% of the variability in the Y (response) could be explained by the model. The *P*-value of the model was ($P < 0.005$) which implied that the model was significant, and also the lack of fit is non-significant ($P > 0.05$) proving the validity of the model. The regression equation obtained from the model has been shown in Eq 3.

$$Y = 6.393 + 0.2895\ X1 + 9.269\ X2 - 0.01887\ X1*X1 - 7.073\ X2*X2 + 0.0588\ X1*X2 \quad (3)$$

Where, X1 represent the amount of glucose added and X2 the $(NH_4)_2SO4$ added (in g L^{-1}) to the formulated SCW media.

Coded values; X1 glucose, X2 $(NH_4)_2SO_4$. The three levels (−1, 0 and +1) set for glucose were 0, 5 and 10 g L^{-1} supplemented to the SCW media, while for $(NH_4)_2SO_4$ was 0, 0.4 and 0.8 g L^{-1} respectively.

Based on ANOVA analysis, both the factors showed significant impact on the growth of *G. sulphuraria*. The most significant factor was ammonium sulfate ($P = 0.005$) followed by glucose ($P = 0.013$). In the run n. 12, without the addiction of glucose or nitrogen, the biomass obtained was 6.11 g L^{-1}, while the highest DW value was obtained in run 7 (10.65 g L^{-1}) with a combination of 0.8 g L^{-1} of $(NH_4)_2SO_4$ and 5 g L^{-1} of glucose added. The predicted values are also reported, which are very similar to the experimental values, proving the validity of the model.

Table 4. Growth optimization of SCW media with supplementation of glucose and $(NH_4)_2SO_4$ for *Galdieria sulphuraria* using Central Composite Design (CCD).

Run	Factor Assignment		Biomass (Y)	
	X_1 (Glucose)	X_2 ($(NH_4)_2SO_4$)	Experimental value (g L^{-1})	Predicted value (g L^{-1})
1	0	0	9.92	10.06
2	0	−1	7.45	7.27
3	0	0	10.24	10.06
4	1	0	10.18	10.21
5	−1	0	9.22	8.87
6	0	0	10.01	10.06
7	0	1	10.71	10.42
8	−1	1	9.10	9.28
9	1	−1	7.39	7.21
10	0	0	10.22	10.06
11	1	1	10.65	10.79
12	−1	−1	6.11	6.41
13	0	0	9.69	10.06
14	0	0	10.19	10.06

The supplementation of inorganic nitrogen source, lead to an increase of biomass yield by 58%. In that way, is possible to obtain a good productivity using SCW as principal nutrient source for *G. sulphuraria* cultivation.

Table 5. Analysis of variance for *G.sulphuraria* biomass optimization using coded values and regression equation.

Source	DF[a]	Adj SS[b]	Adj MS[c]	F-Value	*P*-Value
Model	6	24.2291	4.0382	97.73	0.001
Glucose (X1)	1	2.3188	2.3188	56.12	0.013
$(NH_4)_2SO_4$ (X2)	1	14.6328	14.6328	354.14	0.005
Linear	2	16.9516	8.4758	205.13	0.001
Square	2	5.9442	2.9721	71.93	0.000
X1*X1	1	0.6067	0.6067	14.68	0.006
X2*X2	1	3.4927	3.4927	84.53	0.000
X1*X2	1	0.0552	0.0552	1.34	0.286
Error	7	0.2892	0.0413		
Lack-of-Fit	3	0.0816	0.0272	0.52	0.689
Pure Error	4	0.2077	0.0519		
Total	13	24.5184			

Note: R^2 = 98.51 ([a]DF, degree of freedom; [b]SS, sum of squares; [c]MS, mean squares; F, probability of distribution; *P*, probability).

The biomass obtained in optimized condition is lower than other studies [7,32] where a concentration higher than 12 g L^{-1} was obtained. However different factors should be taken into account, such as the inoculum concentration and the culture conditions (I.e. temperature). Other studies reported a lower productivity than our work when cultivating *G. sulphuraria* on waste material [12]. Moreover, in a recent study where *G. sulphuraria* was grown on fruit-salad

wastewater, micronutrients and ammonia where added to promote the complete consumption of the nutrients present in the effluent [11]. This is a similar case as SCW media utilization. An interesting way to exploit the nutrients presents in this type of by-product would be the blend with other food waste (I.e. molasses as carbon source). In that way the supplementation with glucose or ammonium sulphate could be not necessary.

4. Conclusion

SCW can actually be used as alternative and sustainable medium for the cultivation of *Galdieria sulphuraria*. The suitability of this food waste has been tested at different concentrations and compared with SM. When diluted at 2.0% in RS the biomass obtained was higher than the other formulated media. Biochemical composition of biomass reported slightly difference between the algae growth in standard condition respect to the algae growth with SCW media. Fatty acids profile was affected by the new SCW media, obtaining a higher PUFA content respect to the SM. The biomass optimization with SCW media supplemented with glucose and nitrogen led to a good biomass production, proving that this dairy waste can be used as nutrient source for the cultivation of this extremophile red alga. With these results, it is possible to evaluate new economically and environmentally sustainable biotechnological process, using low cost food effluents for the cultivation of *G. sulphuraria*.

Conflict of interest

The authors have no conflict of interest to declare

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