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Assessment of water reuse potential for the cultivation of the red microalga *Galdieria sulphuraria*

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Abstract

The red microalga *Galdieria sulphuraria* has emerged as a promising biotechnological platform for large-scale cultivation and production of high-value compounds, such as the blue pigment phycocyanin. However, its extremophilic nature requires cultivation in an appropriately acidified culture medium, resulting in strongly acidic wastewater exceeding legal limits for industrial wastewater release. Additionally, the high freshwater usage, together with the supply of the necessary nutrients, is among the main costs associated with large-scale algae cultivation. This study investigates the possibility of recycling water and metals for multiple *G. sulphuraria*'s cultivation cycles to reduce costs and make microalgae cultivation more sustainable. Firstly, 25% water recycling showed no significant differences in consecutive cycles compared to the control, reaching the same final biomass concentration. Further, recycling the whole permeate derived from the harvesting process with the only re-integration of nitrogen and phosphate sources, did not affect *G. sulphuraria* growth in a first cycle of water recycling. Finally, the study evaluated the potential of *G. sulphuraria*'s phycocyanin (*C-PC*) accumulation achievable at high cells densities within the flat panel PBR, with values between the highest reported in the literature (10.8 % w/w). No negative effects of the recycled water on the *C-PC* accumulation were observed, suggesting that this approach could lead to a more sustainable and cost-effective strategy for large-scale *G. sulphuraria* cultivation and production of high-value compounds.

Keywords: ***Galdieria sulphuraria, Water Recycling, Phycocyanin, Closed Flat-panel Photobioreactor***

1. Introduction

Microalgae cultivation has become increasingly popular due to its potential for commercial applications across various industries [1]. As a result of CO_2 fixation, Microalgae are capable of accumulating significant amounts of carbohydrates, proteins, lipids, and other valuable compounds, such as pigments and vitamins, making them a promising energy feedstock with versatile applications in the production of dietary supplements, cosmetics, food, animal feed, and biofuels [2–5]. However, one of the main challenges associated with microalgae cultivation is, among the others, the large amount of water required for biomass production, as well as the continuous replacement of freshwater in photobioreactors to support the biological functions and growth of algae. The high cost of freshwater and its limited availability, especially in water-stressed regions, have made it difficult for the microalgae industry to grow [6,7]. Therefore, optimizing the harvesting process and investigating the feasibility of recycling water would consistently lead to the reduction of the environmental and management costs associated with microalgae cultivation [8], moving towards a more circular economy approach.

The polyextremophile red microalga *Galdieria sulphuraria* has gained extensive attention for its ability to survive in harsh conditions such as low pH (as low as 0.2 for some strains) [9], high temperatures (up to 57°C) and high osmotic pressure [10]. *G. sulphuraria* has been found to be a rich source of proteins, insoluble dietary fibers, and antioxidants [11,12], as well as it contains a high proportion of essential sulphur amino acids compared to other protein sources like *Chlorella*, *Spirulina*, and soybean protein [13]. Its blue-green color is attributed to the presence of blue phycobiliproteins C-phycocyanin (C-PC) and allophycocyanin, and chlorophyll a. Furthermore, C-phycocyanins extracted from *G. sulphuraria* are more stable at low pH and high temperatures than those extracted from *Arthrospira platensis*, nowadays the almost exclusively *C-PC* production platform, indicating their potential industrial applications. These characteristics position *G. sulphuraria* as a promising candidate for large-scale production as a food and feed source.

Since *G. sulphuraria* cultivation medium requires low pH conditions, sulphuric acid is commonly added, leading to highly acidic wastewater exceeding the permissible Italian limits for industrial wastewater discharge after biomass harvesting (Annex 5, Third Section, Legislative Decree n. 152/2006, [14]) Therefore, the aim of this work is to provide a preliminary assessment of the possibility of re-using the same cultivation medium, after biomass harvesting, for one or more cycles of *G. sulphuraria*

cultivation at pilot scale. Additionally, the potential *C-PC* content achievable within the employed PBR, in the control conditions (distilled water plus salts) as well as when grown in recycled water, is reported.

2. Materials and Methods

2.1. The Planar photobioreactor

The flat-panel PBR used in this work has been recently described in the literature [15], differing only from the implementation of a LEDs artificial light source instead of fluorescent tubes, allowing for less energy consumption and better regulation of specific wavelength requirements for the selected microalgae species. Briefly, the PBR is composed of two interconnected units: a photo-stage loop and a mixing tank. The photo-stage loop consists of two parallel alveolar flat panels illuminated by an interposed optical guide which re-directs the light, coming from two LEDs rods arranged on the top and bottom of the latter, perpendicularly to the panels. The alveolar flat panels are made of transparent polycarbonate with a light exposed surface area of 1.5 m^2 each and an internal path of 13 mm, for a total volume of 17L. The mixing tank is made up of a darkened HDPE (High-density polyethylene) material with a total useful volume of 50L. A hydraulic circulator is connected at the bottom of the mixing tank and upstream of the photo-stage loop, driving the liquid flow into both flat panels, from the bottom to the top. Temperature, pH, conductivity, dissolved oxygen, and carbon dioxide are constantly monitored by specific sensors (Mettler-Toledo®, USA) located at the output of the flat panels. The signals from the sensors are transmitted, through a multi-parameter transductor, to a Programmable Logical Controller (PLC, Unitronics, Israel) for data storage, online monitoring, and control.

2.2. Microalgae growth and cultivation conditions

Galdieria sulphuraria strain 074W was kindly donated by Prof. Antonino Pollio (University of Naples, Italy). All the experiments were conducted in batch mode and axenic conditions under constant artificial illumination with specific light spectra based on previous experiments (data not shown). The PBR was inoculated with Allen medium (control conditions) acidified at pH 2 with sulphuric acid [16], or with water permeate after centrifugation plus distilled water in a ratio 1:4 (with nutrients reintegration), or with water permeate only (with N and P reintegration), and microalgae cells for a total volume of 45 L. The initial biomass dry weight was about 0.25 g L^{-1} for all the tests. The injection of CO_2 was carried out with a flow rate of 0.06 NL min^{-1}, keeping constant the CO_2 concentration threshold in the PBR at 15 mg L^{-1} using the combination of solenoid valve and mass flow meter. The cultures were maintained at a constant temperature of 37 °C ± 2, and under constant 24/24 h artificial illumination (averaged 120 µmol m^{-2} s^{-1}). Microalgae growth was gravimetrically quantified as dry biomass concentration as previously reported [15]. The averaged biomass productivity (P_x, g L^{-1} d^{-1}) was calculated as:

$$P_x = \frac{X_t - X_0}{t - t_0}$$

X and X_0 are the final and initial concentrations (g L^{-1}), and t is the time passed between the two measurements.

2.3. Microalgae harvesting

Cells reaching the stationary phase were collected and centrifuged by using a CLARA-20 centrifuge (Alfa Laval, Sweden) model operated at a flow of 150 L hr^{-1} and a counter pressure of 1 bar. The concentrated biomass (approximatively 2 L) was freezed at -85°C and subsequently lyophilized (ScanVac CoolSafe Touch 55-4 Freeze Dryer, LaboGene, Denmark) to facilitate further extractions. The permeate water was collected and used as (as 25% or 98% of total working volume as previously described) medium for the assessment of water reuse potential.

2.4. C-PC extraction and quantification

The C-phycocyanin (*C-PC*) from G. sulphuraria was quantitatively extracted by bead beating (Mixer Mill MM 400, Retsch, Germany) ≈ 1 g of lyophilized biomass. Lyophilized cells were resuspended in 100 mM Na-phosphate buffer at pH 7 and exposed to 3 x 5 min beating cycles at a frequency of 30 Hz with 5 min breaks on ice between each cycle. Cell debris was removed through centrifugation at 16.000 rpm for 10 min and the supernatant was collected in fresh tubes. This extract is called crude extract. The *C-PC* contents were calculated measuring the absorbance at 620 nm and 652 and converting the measured absorbance to concentration using the Kursar and Alberte equation [17].

2.5. Macro- and micro-nutrients monitoring

With the aim of effectively reusing the permeate water as a medium for microalgae growth, macronutrients and micronutrients were quantified after centrifugation and eventually re-integrated in the permeate solution to achieve the same concentrations of the ideal Allen medium. Nitrogen (N) and phosphorous (P) were spectrophotometrically (Onda UV-31 Scan spectrophotometer, China) quantified by using standard reagent kits for highly sensitive photometric measurements (NANOCOLOR® test kit, Macherey-Nagel, Germany). All the other metals, namely Magnesium (Mg), Potassium (K), Manganese (Mn), Sodium (Na), Iron (Fe), Cobalt (Co) and Molybdenum (Mo), were quantified by Inductively Coupled Plasma Metal Analysis (OPTIMA 2000 ICP optical emission spectrometer, PerkinElmer, U.S.A.).

3. Results and Discussion

In recent years, *Galdieria sulphuraria* has emerged as a promising biotechnological platform for large-scale cultivation and production of a high-nutritional value biomass, for nutraceutical purposes, as well as to produce high-value molecules such as the blue pigment

phycocyanin. However, being an extremophilic species, it requires cultivation at high temperatures, and most importantly, in an appropriately acidified culture medium. The acidification of the medium is achieved using sulfuric acid, which results, at the end of the process and after biomass harvesting, in a strongly acidic wastewater outside the legal limits for industrial wastewater release. Moreover, since the high use of freshwater is among the main costs associated with large-scale algae cultivation, the possibility of recycling the unused water and metals for multiple cultivation cycles would significantly reduce the costs. In this study, the recycling of the 25% permeate water was at first experimented. This has been decided to firstly assess the feasibility of the process hardware components (PBR cultivation, harvesting setup), since operating at pilot scale, but also to initially avoid the potential effects of permeate that, being yellowish in color due to the likely presence of algae organic matter (*AOM*), without dilution could lead to a strong attenuation of light, but above all contribute to the formation of important biofouling. After an initial batch cultivation with standard medium, 3 consecutive cycles of harvesting and re-inoculation, using the permeate water plus dH_20 in a ratio 1:4, were carried out. For these tests, only N and P concentrations were quantified in the permeate and re-integrated to the starting concentrations. All the other salts were added to the final working volume as from the medium recipe. Despite the dilution, the pH remained between 3-3.5, therefore no pH adjustment was performed. Fig. 1 depicts the *G. sulphuraria* growth in control conditions and in each cycle of water recycling. After 16 days of cultivation, the biomass concentration reached 3.26 g L^{-1} ± 0.15 in control conditions, with an average biomass productivity (P_x) during exponential phase of 0.21 g L^{-1} d^{-1} ± 0.06. The *G. sulphuraria* growth in the consecutive cycles of 25 % water recycling showed no significant differences with respect to the control, reaching the same final concentrations at the end of the cultivation period, and an average P_x during the exponential phase of 0.22 ± 0.10, 0.20 ± 0.07, and 0.20 ± 0.05 g L^{-1} d^{-1}, respectively.

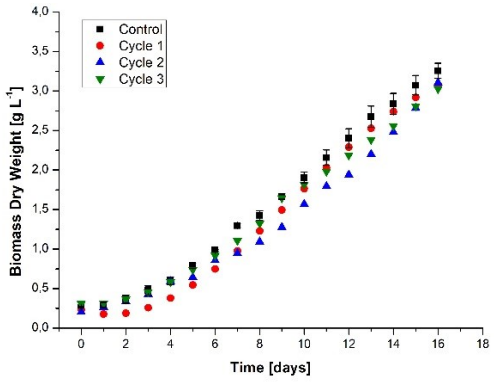

***Figure 1**:Biomass concentration over time. Black squares: Control (n =3). Red circles: 1st Cycle 25% water recycling. Blue triangles: 2nd Cycle 25% water recycling. Green triangles: 3rd Cycle 25% water recycling.*

Encouraged by these preliminary results, it has been decided to assess the possibility of recycling the whole permeate derived from the harvesting process. More precisely, the centrifugation allowed to retrieve the 99% of the total volume (44.55 L), but 44 L were used as new culture medium (98%) to which 1 L of fresh microalgae inoculum was added. For these tests, only one cycle of water recycling was assessed, in duplicate and on independent trials. Additionally, all the nutrients were quantified in the permeate water (Table 1). Since the concentration of all the monitored metals did not decrease significantly, indicating an excess of nutrients in the ideal medium, only N and P were re-integrated at the initial concentration for the growth with permeate water. As expected, no pH adjustment was required.

As can be seen from Fig. 2, *G. sulphuraria* growth in both the independent trials has not been affected using almost all the permeate water. The averaged P_x during exponential growth was 0.25 ± 0.08 and 0.24 ± 0.06 g L^{-1} d^{-1} for the control batches and the growth on permeate, respectively. Despite the strong yellowish colour of the permeate, indicating the presence of *AOM*, no differences in growth neither in terms of biofouling formation were observed with one cycle of 98% water recycling. Further experiments will be necessary to address the feasibility of recycling water for more consecutive cycles and a proper integration of nutrients according to the microalgae needs and to economic and sustainibility criteria.

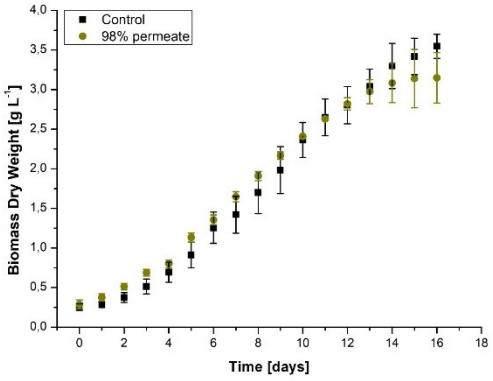

***Figure 2**: Biomass concentration over time. Black squares: Control (n =3). Yellow circles: 98 % water recycling (n = 2).*

***Table 1**:Concentration of metal and ions in the culture medium at the starting of batch controls and in the permeate water after centrifugation.*

Metal/Ion	***Starting concentration [mg L^{-1}]***	***Concentration in permeate water [mg L^{-1}]***
Mo	2.38 ± 0.02	1.126 ± 0.026
Co	0.028 ± 0.00	0.029 ± 0.00
Fe	2.223 ± 0.021	1.904 ± 0.027
Mn	2.285 ± 0.011	2.019 ± 0.047
Mg	39.35 ± 0.042	33.02 ± 0.684
Na	10.73 ± 0.24	11.088 ± 0.282
K	101.2 ± 0.707	67.793 ± 1.392
NH_4^+	470.7 ± 2.22	47.55 ± 0.84
PO_4^{3-}	250.10 ± 1.46	147.18 ± 4.25

Finally, it has been decided to evaluate the final *C-PC* accumulation in *G. sulphuraria* achievable within the flat panel PBR, and if the strategy of water recycling may somehow affect the production of this commercially relevant compound. At the end of the cultivation period, the *C-PC* accumulated was found to be the 10.80 ± 0.36 %

w/w in the control conditions (Fig. 3). This value is between the highest ever reported for several *G. sulphuraria* strains grown with different trophic modes [13,18,19]. Nevertheless, it is worth mentioning that the *C-PC* accumulation at the end of a batch cultivation process is maximized as cultures are very dense and light becomes the limiting factor. Continuous cultivation experiments should be done, appropriately selecting the most appropriate conditions (namely biomass concentration, dilution factor and light intensity), to evaluate and maximize the *C-PC* volumetric and areal productivities. As shown in Fig. 3, the final *C-PC* accumulation did not vary for all the experiments performed in this work. Especially concerning the 98% of water recycling, this indicates no major stress factor associated to possible presence of *AOM* over the *C-PC* accumulation.

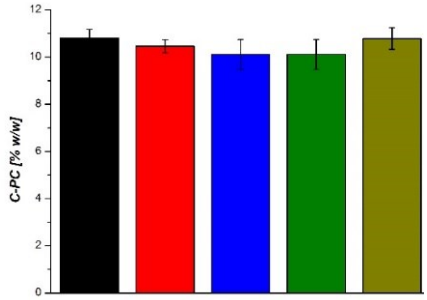

Figure 3*:G. sulphuraria C-PC accumulation as % weight/weight at the end of each cultivation cycle (n = 3). Black: Control. Red: 1st Cycle 25% water recycling. Blue: 2nd Cycle 25% water recycling. Green: 3rd Cycle 25% water recycling. Yellow: 98 % water recycling.*

4. Conclusion

The recycling of wastewater in *Galdieria sulphuraria* cultivation has been successfully demonstrated in this study. Results showed that 25% water recycling did not significantly affect the growth of G. sulphuraria, with no evidence of biofouling. Moreover, the possibility of recycling the whole permeate derived from the harvesting process did not affect the *G. sulphuraria* growth nor the final *C-PC* accumulation at least for one cycle of reusage. The assessment of more consecutive recycling operations is currently under investigation. Nevertheless, these findings suggest the feasibility of the recycling the unused water and metals for *G. sulphuraria* cultivation, which may represent a significant step forward in making microalgae cultivation more sustainable by reducing the amount of freshwater used and minimizing the release of acidic wastewater. The potential of this approach for further optimization and scale-up should be investigated in future studies, with the aim of achieving higher levels of water and metal recycling, and ultimately, a more efficient and environmentally friendly process for large-scale microalgae cultivation.

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