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RESEARCH ARTICLE

BIOTECHNOLOGY PROGRESS

Semicontinuous system for the production of recombinant mCherry protein in *Chlamydomonas reinhardtii*

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Abstract

Biotechnology advances have allowed bacteria, yeasts, plants, mammalian and insect cells to function as heterologous protein expression systems. Recently, microalgae have gained attention as an innovative platform for recombinant protein production, due to low culture media cost, compared to traditional systems, as well as the fact that microalgae such as *Chlamydomonas reinhardtii* are considered safe (GRAS) by the Food and Drug Administration (FDA). Previous studies showed that recombinant protein production in traditional platforms by semicontinuous process increased biomass and bio product productivity, when compared to batch process. As there is a lack of studies on semicontinuous process for recombinant protein production in microalgae, the production of recombinant *mCherry* fluorescent protein was evaluated by semicontinuous cultivation of *Chlamydomonas reinhardtii* in bubble column photobioreactor. This semicontinuous cultivation process was evaluated in the following conditions: 20%, 40%, and 60% culture portion withdrawal. The highest culture withdrawal percentage (60%) provided the best results, as an up to 161% increase in *mCherry* productivity (454.5 RFU h^{-1} – Relative Fluorescence Unit h^{-1}), in comparison to batch cultivation (174.0 RFU h^{-1}) of the same strain. All cultivations were carried out for 13 days, at pH 7, temperature 25°C and, by semicontinuous process, two culture withdrawals were taken during the cultivations. Throughout the production cycles, it was possible to obtain biomass concentration up to 1.36 g L^{-1}.

KEYWORDS

biotechnology, bubble column photobioreactor, heterologous protein, microalgae cultivation

1 | INTRODUCTION

Microbial processes have been used for providing biomass or numerous metabolic bio products commercially interesting, and microalgae stand out for being a sustainable platform for food, feed and fuels.[1] There are a number of microalgae species that can now be genetically modified, therefore, researchers and biotechnology companies are exploring microalgae as a platform for synthesizing heterologous proteins.[2]

Fluorescent proteins have been widely used as molecular reporters for studies on biological recombinant platforms. These proteins are easily detected, which enable much faster studies on protein accumulation, gene expression,[3] cell and tissue biology.[4,5] mCherry is one of the fluorescent protein successfully expressed by *Chlamydomonas reinhardtii*,[6] it is an efficient fluorescent reporter to evaluate protein expression and to monitor its localization inside or outside the cell.[7,8] This fluorescent protein presents maximum excitation at 587 nm, with respective emission at 610 nm and extinction coefficient of 72,000 M^{-1} cm^{-1},[9,10] and with

other well-known fluorescent protein, GFP (Green Fluorescent Protein) can be co-expressed with minimal spectral interference.[11] Another advantage of employing this protein is the generation of transgenic microalgae without any undesirable markers such as those based on bacterial antibiotic resistance genes.[12]

One of the necessary steps to be improved for an optimal heterologous protein production is to develop important cultivation parameters, for example, photobioreactor configuration and cultivation processes choices, as well as the scale up from flasks to photobioreactors. For this purpose, a few different cultivation processes can be applied. Batch[13] and semicontinuous processes are available and they may be selected according to the expression platform, substrate characteristics, the desired product, etc..[13] In the industry, one of the bigger challenges is to perform long period cultivation processes without contamination.[14] This issue is one of the technological challenges and is linked to the implementation, control and quality assurance within bio-industry.

In batch (or discontinuous) cultivation process (generally carried out for food and beverage production) all nutrients are supplied at the beginning of the cultivation and there is no supplementary nutrient addition or product removal until the process ends.[15] The semicontinuous cultivation process (or repeated batch) allows maintaining the culture at high specific growth rate, avoiding longstop periods between cultivations, and lag phase elimination in a subsequent cultivation cycle, since adapted growing cells are kept in the photobioreactor. These aspects result in higher productivities, in comparison to traditional batch process.[13,16] The main semicontinuous process particular feature is the culture portion withdrawal. When the first peak of maximum biomass concentration is achieved, the same withdrawal volume is fed with fresh culture medium to start the following cycle. Subsequent withdrawal portions can be an important variable, since it influences the amount of replenished medium and the biomass concentration that the following cycle will start.[16-18]

Regarding the photobioreactor configurations for microalgae cultivation, those employing pneumatic system for agitation are suitable for biomass production, since they feature ease of construction and lower cell shear stress, in comparison with mechanical agitation, which presents the advantage of a better homogeneous cell suspension.[13]

In the literature, it is possible to find semicontinuous process applied to wild type *C. reinhardtii* cultivation, for instance for hydrogen production.[19] However, there are no studies specifically related to *Chlamydomonas reinhardtii* semicontinuous process for producing heterologous protein by microalgae. With the goal of contributing to this field gap, the influence of different cell culture portions withdrawal was evaluated, in semicontinuous *C. reinhardtii* cultivation in a bubble column photobioreactor, for the production of heterologous *mCherry* protein.

2 | MATERIALS AND METHODS

2.1 | Microorganism, culture medium, and inoculum preparation

In this work, all cultivations were carried out employing the recombinant *Chlamydomonas reinhardtii* expressing the heterologous m*Cherry* protein. *C. reinhardtii* CC1690 nuclear genome was transformed by Molino et al[6] for heterologous production of fluorescent m*Cherry* protein fused to a signal peptide (pJP30), which promoted m*Cherry* secretion from the cell to the cultivation medium.

Modified TAP culture medium[20,21] was used for the production of heterologous m*Cherry* protein. It was prepared by adding (to final volume of 1 L): 10 ml of stock solution *A* (NH_4NO_3, 41.43 $g.L^{-1}$; $CaCl_2$ 5, $g.L^{-1}$; $MgSO_4$, 10 $g.L^{-1}$), 1 ml of buffer stock solution (K_2HPO_4, 205.2 $g.L^{-1}$; KH_2PO_4, 106.8 $g.L^{-1}$), 20 ml of Tris base stock solution (Tris hydroxymethyl-aminomethan, 1 M), 1.9 ml of glacial acetic acid, and 1 ml of Hutner's trace elements.[22] The modification in the traditional TAP medium refers to the replacement of NH_4Cl by NH_4NO_3 in accordance with Arias (2017).[23] After adjusting the pH to 7.0 ± 0.2 by adding KOH 1 M, the culture medium was autoclaved at 121°C for 15 minutes.

Recombinant *C. reinhardtii* inoculum was grown in 500 ml Erlenmeyer flasks with 200 ml working volume of the modified TAP medium for 5 days under continuous light intensity of 50 ± 10 μmol photons.$m^{-2}.s^{-1}$, at 25°C, on a rotative shaker at 250 rpm. When absorbance value (λ = 750 nm) achieved 0.800, 22 ml of the culture were used to inoculate the bubble column photobioreactor containing 198 ml of fresh modified TAP medium, resulting in initial biomass concentration of 100 $mg.L^{-1}$ (Abs =0.080)

2.2 | Bubble column photobioreactor

The column photobioreactor consists of borosilicate glass tube (25 cm × 5 cm) (Figure 1). This glass tube presents: pH electrode and silicon pipes for sampling, aeration, and gas release. The total working volume was set at 220 ml, and distilled water was daily added to reset the volume lost by evaporation. The temperature was adjusted to 25° ± 1°C, and the agitation was performed through a column of air bubble provided by an air pump (model S-600; Seven Star Electronics, Taiwan) and an airflow rate of 0.5 vvm. LED lamps provided continuous illumination at 65 ± 5 μmol photons m^{-2} s^{-1}.

The pH was kept at 7.0 ± 0.2 with the addition of pure CO_2. pH electrode was connected to a pH transmitter (model M300; Mettler Toledo, Switzerland), which controls a solenoid valve (ACL 20E; Cavenago di Brianza, Italy) for releasing CO_2 when the pH values increased in the cultivation medium.

2.3 | Semicontinuous cultivation for m*Cherry* protein production

The process started with an initial batch process step, carried out with initial biomass concentration of 100 mg L^{-1}. Once biomass concentration achieved its maximum value, the semicontinuous process was implemented by withdrawals of the exhausted medium and feeding with the same volume of fresh culture medium for starting the first cycle. When the biomass concentration achieved its peak again, another withdrawal was performed to begin the second cycle. Three

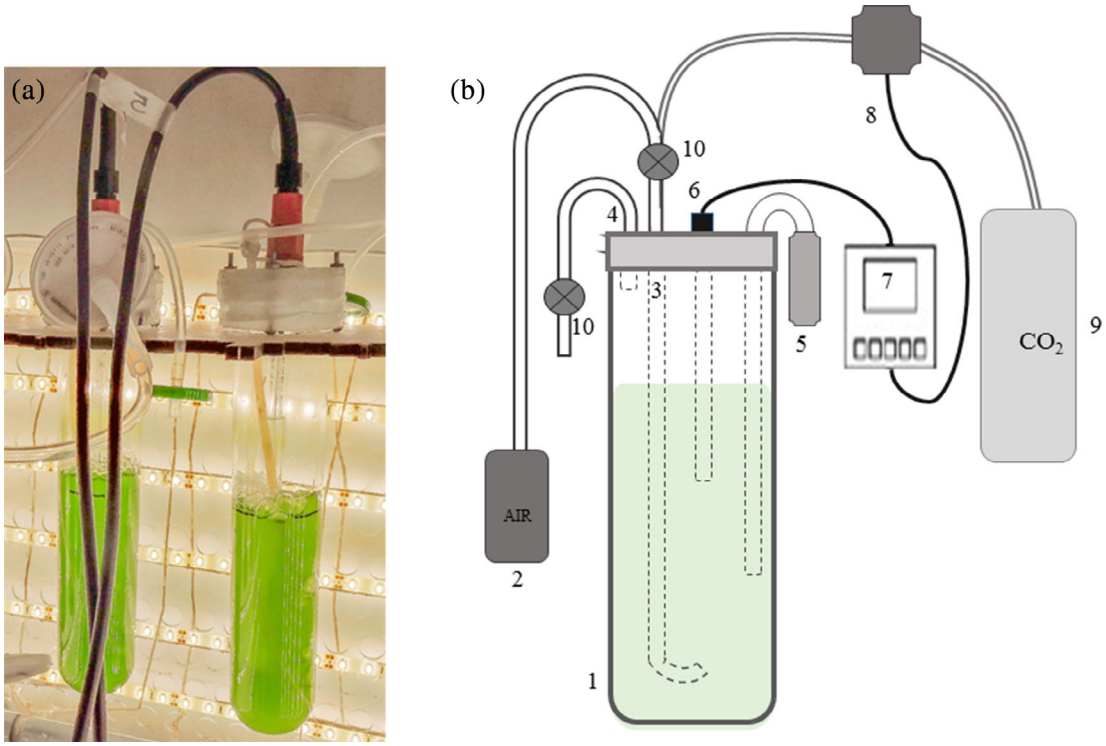

FIGURE 1 (a) Bench-scale bubble column photobioreactor illuminated by LED lamps. (b) Bubble column photobioreactor scheme: (1) glass column; (2) air pump; (3) air inlet; (4) air outlet; (5) sampling system; (6) pH probe; (7) pH transmitter; (8) solenoid valve; (9) CO_2 from cylinder; (10) 0.22 μm air filter

different culture portions withdrawal were evaluated: 20%, 40%, and 60%. All cultivations were performed in duplicates.

2 ml samples were taken daily, and centrifuged at 1,484 *g* for 10 min. m*Cherry* fluorescence was measured in the supernatant, and the pellet was resuspended in the same initial volume fresh medium for biomass concentration measurement.

2.4 | Analytical techniques

Biomass concentration was measured at 750 nm (spectrophotometer, model 700 Plus—Femto, São Paulo, Brazil). The absorbance was correlated to dry biomass per volume (mg L^{-1}) in a calibration curve. From an algal culture, culture dilution with TAP medium was performed, followed by their respective absorbance measurements. From the same algal culture, in triplicate, 25 ml was vacuum filtered (cellulose acetate membrane, 1.2 μm pore) and washed with the same volume of water for salts residue removal, following by drying at 60°C for 12 hr, and weighting after the room temperature is achieved. Absorbance was measured in black 96-well, clear bottom microplate (Corning Costar, USA).

mCherry protein was quantified by excitation at 575/9 nm and measuring fluorescence emission at 608/20 nm, with gain set to 200 in a sample of 100 μL. These measurements were performed in a black clear bottom 96-well microplate (Corning Costar) by the Plate reader Infinite® M200 PRO (Tecan; Switzerland)

2.5 | Parameter calculations and statistical analysis

Biomass productivity (P_x, g L^{-1} h^{-1}), was calculated as the ratio between the biomass amount produced per volume unit ($X_m - X_i$) and the cultivation time (t), according to Equation 1.

$$P_x = \frac{X_m - X_i}{t} \quad (1)$$

Maximum specific growth rate (μmax, d^{-1}) was calculated considering μ as maximum and constant throughout the exponential growth phase.

Results were compared by one-way analysis of variance (ANOVA) in Minitab 17 program, considering the results statistically significant when $p < .05$ (confidence level > 95%).

3 | RESULTS AND DISCUSSION

In this study, with the goal of increasing the recombinant mCherry expressed by a recombinant *Chlamydomonas reinhardtii*, semicontinuous cultivation process was performed in three different conditions: 20%, 40%, and 60% of culture portion withdrawal.

All cultivations were carried out as semicontinuous processes, but they started with a batch process. This initial batch process achieved the maximum biomass concentration average value of

1.30 ± 0.04 g L^{-1} in 5 days. Volumetric biomass productivity was calculated as 0.0100 ± 0.0004 g L^{-1} h^{-1}, and the specific growth rate average was 0.58 ± 0.08 d^{-1}. m*Cherry* protein production reached an average concentration of 23,422 ± 665.3 RFU (Relative Fluorescence Unit) and m*Cherry* productivity was 174 ± 5.8 RFU.h^{-1}.

In the fifth cultivation day, the first culture portion withdrawal was: 20% (Figure 2), 40% (Figure 3), or 60% (Figure 4). Table 1 shows all the results: maximum biomass concentration (Xm), biomass productivity (Px), maximum specific growth rate (μmax), and m*Cherry* protein productivity (Pp).

As it is possible to see in Table 1 and Figures 2–4, the maximum biomass concentration was satisfactory in all runs, which is demonstrated by microorganism growth even with the consecutive culture portions withdrawal. The maximum biomass concentration (Xm) ranged from 1.15 to 1.36 g.L^{-1}. As a result of different culture portions withdrawal, the initial biomass concentrations were different in the following cycle: the lower the culture portion withdrawal, the higher the initial biomass concentration. Although the time for achieving the stationary phase was different, ANOVA statistical analysis showed that there was no difference between Xm values (p = .084), neither among different portions withdrawal or among different cycles.

On the other hand, the independent variables of this study had statistically significant influence on Px (ANOVA, p < .001) and considering the Tukey test, it is possible to infer that the lower the withdrawal percentage, the lower the biomass productivity value. In fact, only for the variable 60% of culture withdrawal, Px results were similar to the initial batch cultivation (P_x = 0.0082, 0.0110, and 0.0100 g L^{-1} h^{-1}, for first cycle, second cycle and initial batch cultivation, respectively). In experiments carried out as 20% culture withdrawal, there was a reduction of approximately 80% in P_x (P_x = 0.0021 and 0.0017 g L^{-1} h^{-1}, for the first and second cycle, respectively).

For a specific withdrawal percentage, the fact that the X_m and P_x is maintained throughout the cycles is a very promising result. Boonma et al[24] observed a decrease in biomass productivity from the second cycle, when cultivating microalgae consortium by semicontinuous process and culture aeration with low CO_2 concentration 1% vol/vol CO_2 (ambient air). It shows the importance of coupling the CO_2 addition to the pH transmitter and a solenoid valve, in an automated gas/nutrient feeding.

Bresaola et al (2019)[16] emphasize that the advantage of employing semicontinuous process is the possibility of using adapted growing cells left in the bioreactor, which helps to reduce or eliminate lag phase in the following cycle of the culture, and avoiding "dead time" in the reactor, represented by the time required for cleaning, for instance.

Considering that specific growth rate (μ) is maximum and constant during the exponential growth phase, maximum specific growth rate (μ_{max}) was calculated as the average value of μ during this period. By the results in Table 1, it is possible to assume that μ_{max} values were influenced the same way as those observed for P_x, that is, there was a statistically significant influence of withdrawal percentage on this dependent variable (ANOVA, p < .001), and the reduction in withdrawal percentage resulted in lower μ_{max} values. In fact, only when applying 60% of culture withdrawal, it was possible to achieve μ_{max} values (μ_{max} = 0.3735 and 0.4740 d^{-1}, for first and second cycles, respectively) similar to those obtained in the initial batch cultivation (0.5800 d^{-1}). In experiments carried out as 20% culture withdrawal, μmax presented a reduction of approximately 90% (μ_{max} = 0.0550 and 0.0500 d^{-1}, for first and second cycles, respectively) in comparison with initial batch process step.

Despite the fact that there was no difference in Xm values, the reduced values of P_x and μ_{max} for experiments performed with lower withdrawal percentages shows the importance of culture renovation with fresh medium that grant good conditions for cell metabolism and growth. Another topic to be considered is the lower light availability for cells in the beginning of a new cycle with higher biomass concentration, as a result of self-shadowing effect.[18,25] Indeed, it is possible to observe in Figure 2 that a lag phase occurred in the beginning of first and second cycles of the semicontinuous culture with 20% of culture withdrawal. Therefore, considering the results so far observed, 60% of culture

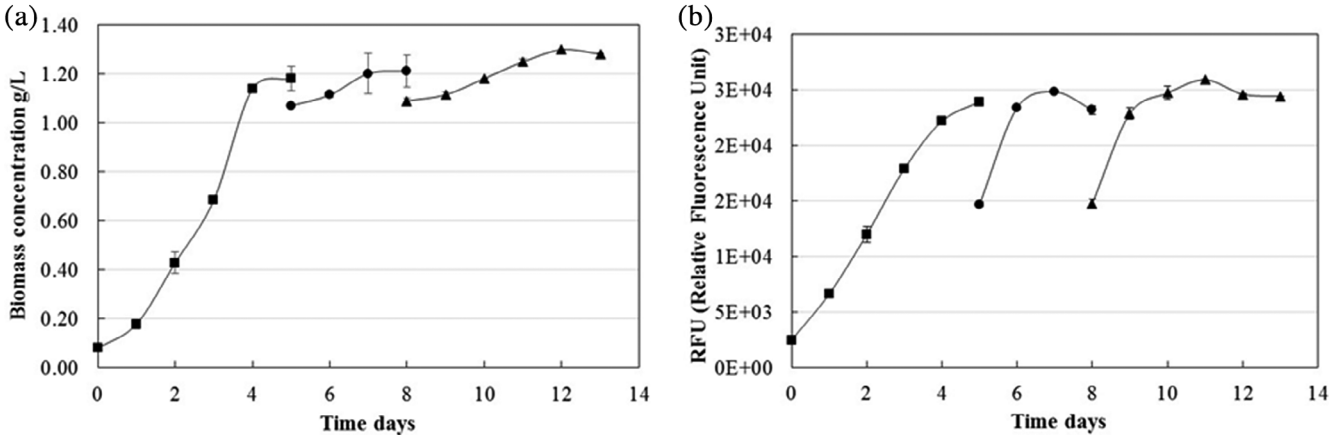

FIGURE 2 Biomass concentration and heterologous m*Cherry* protein production in semicontinuous cultivation of *Chlamydomonas reinhardtii* pJP30 with culture withdrawal of 20%. (a) Biomass concentration (g L^{-1}); (b) heterologous m*Cherry* production (RFU, relative fluorescence unit). (■): initial batch cultivation; (●): first cycle; (▲): second cycle

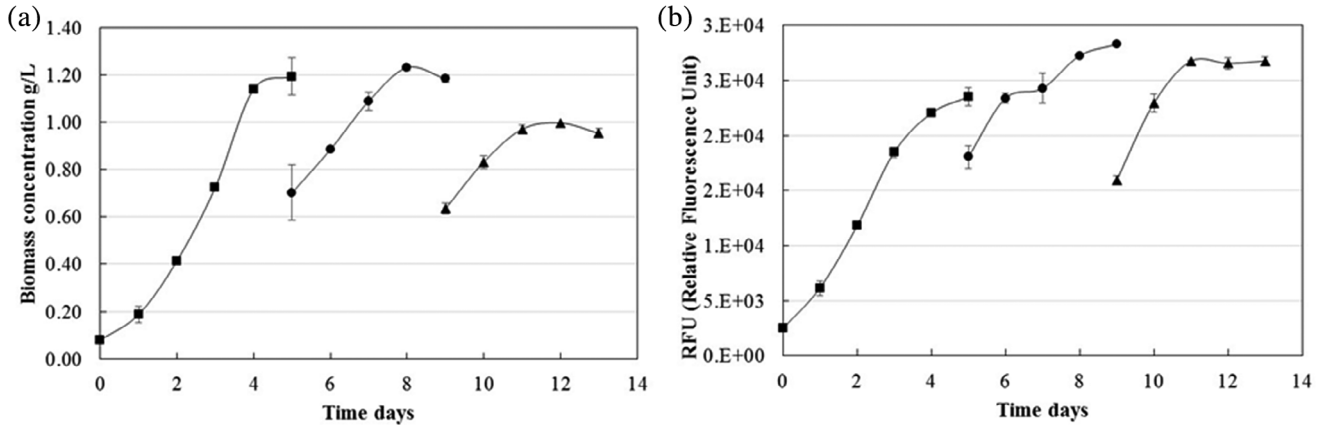

FIGURE 3 Biomass concentration and heterologous m*Cherry* protein production in semicontinuous cultivation of *Chlamydomonas reinhardtii* pJP30 with culture withdrawal of 40%. (a) Biomass concentration (g L^{-1}); (b) heterologous m*Cherry* production (RFU, relative fluorescence unit). (■): initial batch cultivation; (●): first cycle; (▲): second cycle

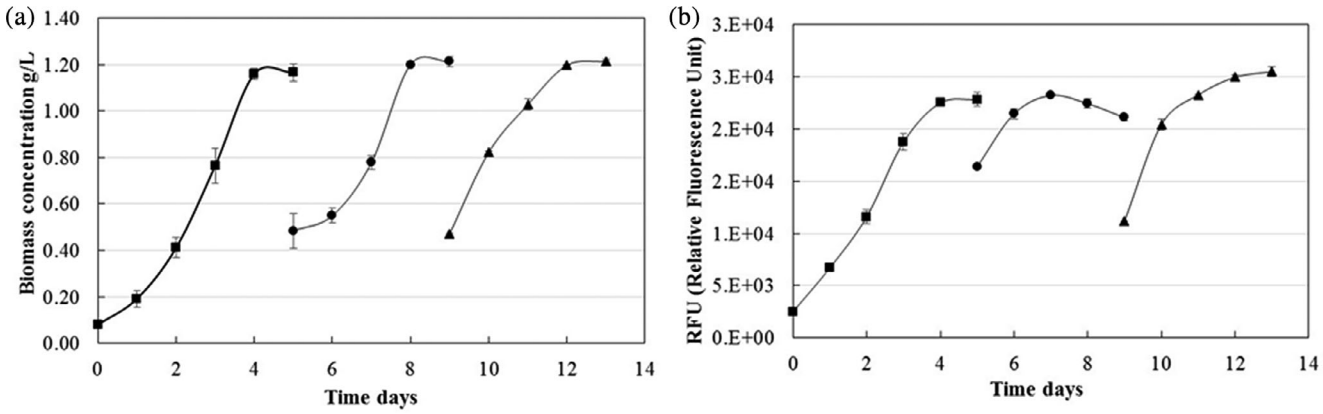

FIGURE 4 Biomass concentration and heterologous m*Cherry* protein production in semicontinuous cultivation of *Chlamydomonas reinhardtii* pJP30 with culture withdrawal of 60%. (a) Biomass concentration (g L^{-1}); (b) heterologous m*Cherry* production (RFU, relative fluorescence unit). (■): initial batch cultivation; (●): first cycle; (▲): second cycle

TABLE 1 Maximum biomass concentration (X_m), biomass productivity (P_x), maximum specific growth rate (μ_{max}) and relative m*Cherry* productivity (P_p, relative fluorescence productivity unit, RFU, per unit time)

Run	Cycle	X_m (g L^{-1})	P_x (g L^{-1} h^{-1})	μ_{max} (h^{-1})	P_p RFU h^{-1}
20%	1	1.33 ± 0.07[a]	0.0021 ± 0.0010[c]	0.055 ± 0.035[c]	118.5 ± 2.1[b]
	2	1.36 ± 0.08[a]	0.0017 ± 0.0001[c]	0.050 ± 0.002[c]	102.5 ± 5.0[b]
40%	1	1.33 ± 0.02[a]	0.0054 ± 0.0015[b, c]	0.213 ± 0.098[b, c]	106.6 ± 10.5[b]
	2	1.15 ± 0.07[a]	0.0051 ± 0.0021[b, c]	0.203 ± 0.025[b, c]	397.0 ± 41.0[a]
60%	1	1.33 ± 0.04[a]	0.0082 ± 0.0006[a, b]	0.374 ± 0.032[a, b]	141.5 ± 3.5[b]
	2	1.33 ± 0.04[a]	0.0110 ± 0.0000[a]	0.474 ± 0.072[a]	454.5 ± 50.2[a]
—	Batch	1.30 ± 0.04[a]	0.0100 ± 0.0004[a]	0.580 ± 0.078[a]	174.0 ± 5.8[b]

[a, b, c]means that do not share a letter are significantly different according to the Tukey test ($p > .05$).

withdrawal condition seems to be the most suitable for semicontinuous recombinant *Chlamydomonas reinhardtii* cultivation.

Reichert et al[17] cultivating the cyanobacterium *Spirulina platensis* by continuous process in closed photobioreactor, noticed that employing the lowest evaluated withdrawal percentage (25%) there was a reduction in the values of biomass productivity and maximum specific growth rate. Indeed, the lower the medium renovation rate, the lower the removal of residual material released by the cell metabolism, which may be toxic for cells or even increase the cultivation medium turbidity, consequently contributing to the shadowing effect. Matsudo et al[25] produced *Arthrospira platensis* biomass employing repeated fed-batch process, which differs from semicontinuous process by the feeding of nutrients during the initial batch process step, and also throughout the cycles. They observed the same result

concerning the influence of culture portion withdrawals on biomass productivity and maximum specific growth rate.

Still regarding cell growth, the bubble column photobioreactor employed in this work showed to be suitable for *Chlamydomonas reinhardtii* cultivation, since it was possible to obtain up to 1.36 g L^{-1} of biomass (dry weight), without any contamination. The device known as "Glass Green Wall Photobioreactor", made with stainless steel and with one side of glass, was employed by Zedler et al[26] that obtained 0.438 g L^{-1} of *C. reinhardtii* CW 15 biomass and up to 0.311 g L^{-1} of wall-deficient transgenic *Chlamydomonas reinhardtii* biomass. In their case, the high volume (100 L of medium) was probably one of the causes for this low maximum biomass concentration.

On the other hand, further studies could be performed aiming to optimize the physical–chemical conditions (light intensity, nutrients concentrations, etc.) for recombinant *Chlamydomonas reinhardtii* growth in the bubble column photobioreactor by semicontinuous process. For instance, Bresaola et al[16] was able to increase maximum biomass concentration from 1,099 to 2,355 mg L^{-1} by changing nitrogen ($NaNO_3$) concentration, when cultivating *Ankistrodesmus braunii* in bench-scale tubular photobioreactor by semicontinuous process.

Regarding fluorescent m*Cherry* protein, the fluorescence was detected in the supernatant from all the cultivations, which showed the secretion signal peptide working. Culture portion withdrawal and the cycles had statistically significant influence on the heterologous protein production (ANOVA, $p < .001$). There was no difference in most of the cycles, but in the second cycles of the runs with 60% and 40% of culture withdrawal, there was an increase of 161% (P_p = 454.5 RFU h^{-1}) and 128% (P_p = 397.0 RFU h^{-1}), respectively, of the m*Cherry* protein productivity, in comparison to initial batch process (174.0 RFU h^{-1}).

Considering these results, it is possible to state that the microalgae cells were perfectly adapted after the portion culture withdrawal conditions, mainly by the 60% of culture withdrawal, which could explain its influence on the higher values of biomass productivity, maximum specific growth rate, and recombinant protein productivity when compared to the other conditions. According to Weiner et al. (2018),[27] the main challenge, when scaling up the production of bioproducts by recombinant microalgae, is the poor overall expression of functional heterologous genes. In this context, the application of semicontinuous process seems to be very promising likely taking advantage of adapted growing cells left from the previous to the following cultivation cycle.

In effect, Lauersen et al[28] highlight that culture conditions must allow high-cell densities for the production of recombinant products. Notwithstanding, further studies with higher number of cycles should be performed for understanding the correlation between cell growth, cell physiology, protein biosynthesis, and protein secretion. Additional investigations should also be carried out concerning photobioreactor configuration, cultivation process and nutrients concentrations, mainly nitrogen and phosphorus, since these parameters may affect protein biosynthesis and accumulation.[29-31] Indeed, Michelon (2016)[30] found that depletion of nitrogen or phosphorus, in the cultivation of *Chlorella* spp., stimulated the accumulation of carbohydrate with the reduction in protein production.

Finally, Lauersen et al[32] also point out the advantage of harvesting secreted heterologous protein independently from the microalgae biomass, which must also be considered as a valuable coproduct. In this sense, 60% of culture withdrawal could so far be highlighted as the best condition for cell growth, biomass productivity and heterologous protein production.

4 | CONCLUSION

This study showed that semicontinuous cultivation with an adequate culture portion withdrawal may be an alternative process to efficiently increase the biosynthesis and secretion of heterologous fluorescent m*Cherry* protein by *Chlamydomonas reinhardtii* in bubble column photobioreactor. The highest culture withdrawal percentage (60%) provided the best results in terms of biomass productivity (P_x up to 0.0110 g L^{-1} h^{-1}) and maximum specific growth rate (μmax up to 0.474 d^{-1}), which were similar to those obtained in the initial batch cultivation (P_x = 0.0100 g L^{-1} h^{-1} and 0.580 d^{-1}, respectively). Still employing 60% of culture withdrawal, m*Cherry* productivity increased 161% (454.5 RFU h^{-1}) in comparison with the initial batch. Moreover, maximum cell concentration was maintained throughout the cycles with no contamination. This work demonstrates the importance of photobioreactor configuration and cultivation processes for improving cell productivity, growth rate and recombinant protein production. Despite the promising results here obtained, further investigations should be addressed for better understanding the correlation between cell growth, cell physiology, protein biosynthesis, and protein secretion.

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AUTHOR CONTRIBUTIONS

Cesar Arias: Data curation; formal analysis; investigation; methodology; validation; visualization; writing-original draft; writing-review and editing. **Marcelo Matsudo:** Formal analysis; visualization; writing-original draft; writing-review and editing. **Livia Ferreira-Camargo:** Funding acquisition; methodology; visualization; writing-review and editing. **João Vitor Molina:** Formal analysis; investigation; methodology; validation; visualization; writing-review and editing. **Stephen Mayfield:** Resources; visualization; writing-review and editing. **Joao Carlos Carvalho:** Conceptualization; formal analysis; funding acquisition; project administration; resources; supervision; visualization; writing-original draft; writing-review and editing.

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