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BIOLOGY

Harvesting of *Chlorella sorokiniana* BR001 cultivated in a low-nitrogen medium using different techniques

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ABSTRACT: *The harvesting process is a current challenge for the commercial production of microalgae because the biomass is diluted in the culture medium. Several methods have been proposed to harvest microalgae cells, but there is not a consensus about the optimum method for such application. Herein, the methods based on sedimentation, flocculation, and centrifugation were evaluated on the recovery of Chlorella sorokiniana BR001 cultivated in a low-nitrogen medium. C. sorokiniana BR001 was cultivated using a low-nitrogen medium to trigger the accumulation of neutral lipids and neutral carbohydrates. The biomass of C. sorokiniana BR001 cultivated in a low-nitrogen medium showed a total lipid content of 1.9 times higher (23.8 ± 4.5%) when compared to the biomass produced in a high-nitrogen medium (12.3 ± 1.2%). In addition, the biomass of the BR001 strain cultivated in a low-nitrogen medium showed a high content of neutral carbohydrates (52.1 ± 1.5%). The natural sedimentation-based process was evaluated using a sedimentation column, and it was concluded that C. sorokiniana BR001 is a non-flocculent strain. Therefore, it was evaluated the effect of different concentrations of ferric sulfate (0.005 to 1 g L^{-1}) or aluminum sulfate (0.025 to 0.83 g L^{-1}) on the flocculation process of C. sorokiniana BR001, but high doses of flocculant agents were required for an efficient harvest of biomass. It was evaluated the centrifugation at low speed (300 to 3,000 g) as well, and it was possible to conclude that this process was the most adequate to harvest the non-flocculent strain C. sorokiniana BR001.*
Key words: *microalgae, ferric sulfate, aluminum sulfate, sedimentation, centrifugation.*

Colheita de *Chlorella sorokiniana* BR001 cultivada em um meio com baixo teor de nitrogênio utilizando diferentes técnicas

RESUMO: *O processo de colheita é um desafio atual para a produção comercial de microalgas porque a biomassa é diluída no meio de cultivo. Diversos métodos têm sido propostos para coletar células de microalgas, porém não existe um consenso sobre um método ótimo para tal aplicação. Neste estudo, métodos baseados em sedimentação, floculação e centrifugação foram avaliados na recuperação de Chlorella sorokiniana BR001 cultivada em um meio com baixo teor de nitrogênio. C. sorokiniana BR001 foi cultivada em um meio com baixo teor de nitrogênio para induzir ao acúmulo de lipídeos e carboidratos neutros. A biomassa de C. sorokiniana BR001 cultivada em um meio com baixo teor de nitrogênio apresentou um teor de lipídeos 1,9 vezes superior (23,8 ± 4,5%), quando comparada à biomassa produzida em um meio com alto teor de nitrogênio (12,3 ± 1,2%). Adicionalmente, a biomassa da linhagem BR001 cultivada em um meio com baixo teor de nitrogênio apresentou alto teor de carboidratos neutros (52,1 ± 1,5%). O processo baseado em sedimentação natural foi avaliado utilizando uma coluna de sedimentação e concluiu-se que C. sorokiniana BR001 é uma linhagem não floculante. Portanto, o efeito de diferentes concentrações de sulfato férrico (0,005 a 1 g L^{-1}) ou sulfato de alumínio (0,025 a 0,83 g L^{-1}) foram avaliados no processo de floculação de C. sorokiniana BR001, mas altas doses de floculantes foram necessárias para uma colheita de biomassa eficiente. Também foi avaliada a centrifugação em baixa velocidade (300 a 3.000 g), e foi possível concluir que este processo constituiu o mais adequado para a colheita da linhagem não floculante C. sorokiniana BR001.*
Palavras-chave: *microalgas, sulfato de ferro, sulfato de alumínio, sedimentação, centrifugação.*

INTRODUCTION

Harvesting of microalgal biomass is considered a bottleneck in algae farms because the biomass is generally diluted in the medium (0.5 to 4 kg of dry weight per m^{-3}), and many microalgae with biotechnological potential are planktonic (*i.e.* free-floating) organisms that show density values similar to water (TIRON et al., 2017). Microalgal cultures are considered stable systems because the surface of microalgal cells presents negative charges that repel other cells, and microalgae are

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generally found in a dispersed state (SINGH & PATIDAR, 2018).

Harvesting of microalgae biomass requires costly and complex processes that can reach up to 30% of the total costs of production (FASAEI et al., 2018). Different methods to harvest microalgal cells have been proposed for a large number of species (TAPARIA et al., 2016). Sedimentation shows low operational costs when compared to other methods of biomass harvest (FASAEI et al., 2018), but the slowness of this process may be a problem for microalgae with a fast metabolism. Catabolism reactions may occur during the harvest process leading to undesired changes in the biochemical composition of the microalgae before their extraction.

To overcome the slow settling of non-flocculent microalgae strains, the use of flocculants have been proposed as a promising and cheap alternative to improve the harvest processes (WAN et al., 2015). Chemical flocculation is widely used in industries for water treatment (VANDAMME; FOUBERT; MUYLAERT, 2013), and different chemical flocculant agents have been successfully used in the harvest of several microalgal strains (WAN et al., 2015). Flocculation is also used as a secondary harvesting method to shorten the duration of the primary harvesting process (*e.g.* centrifugation) and increase the maximum cell recovery rate (KNUCKEY et al., 2006).

Centrifuges are a robust alternative to process large volumes of microalgae culture in a short time (SPOLAORE et al., 2006). Many types of centrifuges are commercially available (*e.g.* disc stacked centrifuge and scroll centrifuge), and the equipment can be readily incorporated in microalgal downstream processes. Centrifuges diminish or abolish the use of chemical flocculant agents which are not desired in some specific applications, like the use of microalgae as food and feed.

Despite the several harvest methods proposed in the literature, it is unlikely to determine an optimum harvesting method for all microalgae strains. Indeed, it is expected that the method and conditions of biomass harvesting should be specific for each microalgal strain. *Chlorella* is currently the second most commercially-produced microalga, and it has been considered a promising feedstock for advanced biofuels production (FALCONÍ et al., 2021; LIU & CHEN, 2014; ROCHA et al., 2017). Specifically, the strain *C. sorokiniana* BR001 shows a fast growth synthetic media in comparison to other Chlorophyta strains isolated from Brazilian freshwater reservoirs (ROCHA et al., 2017), and it is considered a promising strain for the treatment of wastewaters from sugarcane ethanol biorefinery which are largely produced in Brazil (FALCONÍ et al., 2020).

Although *Chlorella* has been used at a commercial scale and novel algae farming application have been proposed, the harvesting process requires investigation because the self-flocculation is a trait observed only in some strains of the genus *Chlorella* (ALAM et al., 2014; ESCAPA et al., 2015; RAS et al., 2011). Previous studies showed the efficiency of different methods on the harvest of *Chlorella* cultivated in rich-nitrogen media (AHMAD et al., 2014; NGUYEN et al., 2014). However, little is known about the harvesting of *Chlorella* cells cultivated in a low-nitrogen medium (ILLMAN; SCRAGG; SHALES, 2000). The main objective of this study was to determine the best method of biomass harvesting for a specific *Chlorella* strain cultivated in a low-nitrogen medium. The methods of sedimentation, centrifugation, and flocculation were evaluated on the harvesting of *C. sorokiniana* BR001 cultivated in a low-nitrogen medium. Algae farming using low-nitrogen media is largely adopted as a strategy to trigger the accumulation of neutral lipids and neutral carbohydrates (LIU & CHEN, 2014).

The strain *C. sorokiniana* BR001 was first cultivated in rich- and low-nitrogen media for evaluation of the accumulation of C-rich biochemical classes (*i.e.* total lipids and total neutral carbohydrates). Then, the different methods of biomass harvesting were evaluated on the harvesting of *C. sorokiniana* BR001 cultivated in a low-nitrogen medium. It was evaluated the natural sedimentation to evaluate if of *C. sorokiniana* BR001 presents the self-flocculation phenotype. A careful evaluation of flocculant agents was performed because their optimum dosage may vary one order of magnitude for different microalgae (DEMIR et al., 2020). Different centrifugation speeds and times were evaluated because centrifuges will be common equipment in algae farms and biorefineries as they are required in biorefining processes (AMORIM et al., 2020).

MATERIALS AND METHODS

Strain and growth conditions

C. sorokiniana BR001 was obtained from the Collection of Microalgae of the Department of Plant Biology, Universidade Federal de Viçosa (Minas Gerais, Brazil). The BR001 strain was maintained in a rich-nitrogen medium for *Chlorella ellipsoidea* (WATANABE, 1960).

Cultivation of C. sorokiniana BR001 in rich- and low-nitrogen media

The BR001 strain was cultivated in 2 L photobioreactors containing 1.6 L of the low-nitrogen medium proposed by ILLMAN et al. (2000) or the rich-nitrogen medium for *Chlorella ellipsoidea* (WATANABE, 1960). Photobioreactors were maintained in photoautotrophic growth conditions at 25 ± 2 ºC, 16/8 h photoperiod (light/dark cycle), and irradiance at bench height of 83 µmols photons m^{-2} s^{-1} obtained using 40-watt daylight fluorescent lamps. A diaphragm pump was used to provide mixing for cultivations in flasks. Cultures of *C. sorokiniana* BR001 were collected on day 14, and the biomass was harvested by centrifugation (20,000 *g* for 20 min), freeze-dried and stored at -20 ºC. Freeze-dried biomass was used for the determination of total neutral carbohydrates based on the phenol-sulfuric acid method (CRAIGIE & HELLEBUST, 1978), and the determination of total lipids was performed according to the Bligh and Dyer method (BLIGH & DYER, 1959; ZHU, 2002).

Cultivation of C. sorokiniana BR001 for evaluation of the harvesting methods

The BR001 strain was cultivated in 20 L photobioreactors containing 16 L of low-nitrogen medium proposed by ILLMAN et al. (2000). Photobioreactors were maintained in the aforementioned photoautotrophic growth conditions. Samples of *C. sorokiniana* BR001 culture were collected on day 17 for evaluation of the following methods of biomass recovery: sedimentation, flocculation, and centrifugation. An independent microalgae cultivation was carried out for each biomass harvesting method evaluated in this study. The harvesting methods were evaluated in quadruplicate.

Biomass harvest by sedimentation

Natural sedimentation of *C. sorokiniana* BR001 was evaluated using an acrylic sedimentation column with a diameter of 0.1 m and a height of 1 m. The sedimentation column was filled with a culture of *C. sorokiniana* BR001, and the cell suspension was homogenized using a rod for 1 min to ensure its uniform distribution along the column. Then, 10 mL of samples were collected from top to bottom of the column using the column scale as reference (0, 20, 40, 60 and 80 cm) in different times (0, 30, 180, 240, 300 and 360 min).

Optical density at 670 nm of the samples was determined using a UV-Vis spectrophotometer. Biomass dry weight was determined according to a standard curve correlating the optical density against different dry weights of the *C. sorokiniana* BR001. Recovery efficiency was calculated according to equation 1.

$$Recovery\ efficiency\ (\%) = \frac{mass\ of\ microalga\ recovered \times 100}{mass\ of\ microalga\ initial\ culture} \quad (1)$$

Biomass harvest by flocculation

Flocculation of *C. sorokiniana* BR001 biomass was performed using ferric sulfate or aluminum sulfate. The culture of *C. sorokiniana* BR001 was poured into a 500 mL beaker and the flocculant was added. The beaker was placed in the jar test apparatus and maintained for 10 seconds at 160 rpm and 25 ºC, then the rotation was reduced to 20 rpm and kept for more 5 min. The jar test apparatus is the equipment used for the uniform stirring of multiple samples for the evaluation of different types and doses of flocculant. The maximum speed achieved by the jar test apparatus was 160 rpm (velocity gradient of about 340 s^{-1}). Rotation of the jar test apparatus was turned off and samples were taken at different times (15, 30 and 60 min) during the flocculation process for determination of the recovery efficiency (Equation 1).

The following concentrations of ferric sulfate were used (g L^{-1}): 0; 0.005; 0.01; 0.025; 0.05; 0.1; 0.17; 0.25; 0.33; 0.5 and 1. The concentrations of aluminum sulfate used where (g L^{-1}): 0; 0.025; 0.05; 0.083; 0.17; 0.25; 0.33; 0.42; 0.5; 0.67 and 0.83. Those optimum concentrations of flocculants were previously determined in preliminary tests. pH of the flocculent solutions was adjusted to 6 using 0.1 M L^{-1} NaOH prior the test.

Biomass harvest by centrifugation

Centrifugation of the culture of *C. sorokiniana* BR001 was performed at room temperature using five different speeds (300; 600; 1,400; 2,200 and 3,000 *g*) and times (15, 30, 60, 120 and 180 min). After the centrifugation samples of the upper phase were taken for estimation of the recovery efficiency (Equation 1).

Statistical analysis

The experiment was performed in a completely randomized factorial delineation. The results of the cultivation of *C. sorokiniana* BR001 in rich- and low-nitrogen media were submitted to analysis of variance, and means were compared by Duncan's test at a 5% significance level. Results of biomass harvest by sedimentation were evaluated by response surface methodology, and the results of biomass harvest by flocculation and centrifugation were submitted to

non-linear regression analysis. The results of this study are presented as mean ± standard deviation.

RESULTS AND DISCUSSION

The strain *C. sorokiniana* BR001 cultivated in a low-nitrogen medium showed a content of total lipids 1.9 times higher (23.8 ± 4.5% in dry weight basis, DW) when compared to the cultivation with a rich-nitrogen medium (12.3 ± 1.2% DW). Cultivation using a low-nitrogen medium also allowed a significantly higher (P-value < 0.05) content of total neutral carbohydrates of (52.1 ± 1.5% DW) in comparison to the biomass produced using a rich-nitrogen medium (48.3 ± 3.3% DW). The high content of C-rich molecules suggests that the BR001 is a promising strain for advanced biofuels production (*e.g.* biodiesel and bioethanol). Thus, the evaluation of harvesting methods was evaluated using a culture of *C. sorokiniana* BR001 produced using a low-nitrogen medium.

Sedimentation is a low-cost process to harvest microalgae biomass. However, microalgae cell densities are generally similar to water density (MILLEDGE & HEAVEN, 2013). The microalgal cells separate from the medium during the sedimentation process due to gravitation forces, but the similar density of microalgae and medium results in a slow separation (MILLEDGE & HEAVEN, 2013). Media also show a density similar to the water since few grams of nutrients are added to them; for example, the low-nitrogen medium contains 99.4% (w w^{-1}) of water in its composition (ILLMAN; SCRAGG; SHALES, 2000). Therefore, the efficiency of the sedimentation process was evaluated in *C. sorokiniana* BR001 cultivated under nitrogen starvation condition because self-flocculation is a trait observed only in some strains of the genus *Chorella* (ALAM et al., 2014; ESCAPA et al., 2015; RAS et al., 2011). The sedimentation was evaluated using a response surface methodology, and the results showed that the recovery efficiency increased along the top to middle regions of the sedimentation column (Figure 1A). The highest recovery efficiencies were observed on the top of the column (*i.e.* 0 cm) and after 300 min of sedimentation (Figure 1A). However, the sedimentation process was slow and inefficient to harvest the *C. sorokiniana* BR001 biomass, and it was possible to recover only 30% of the biomass after 350 min (Figure 1A). These results clearly show that *C. sorokiniana* BR001 is not a self-flocculent strain.

A study showed that *C. vulgaris* JSC-7 is a self-flocculent strain and cell wall-associated polysaccharides containing a phosphodiester functional group might play an important role in the flocculent phenotype (ALAM et al., 2014). Self-flocculation of a *C. sorokiniana* strain was observed when this microalga was cultivated in swine manure wastewater and medium BG11 at the very high pH of 12 (ZHANG & CHEN, 2015). However, the self-flocculation of *C. sorokiniana* was not observed at pH 7 (ESCAPA et al., 2015; XU; PURTON; BAGANZ, 2013; ZHANG & CHEN, 2015). Those results suggest that some *C. vulgaris* strains but not *C. sorokiniana* are able to self-flocculate at different values of pH. Moreover, flocculation using pH 12 requires high consumption of alkali, especially if the microalgal biomass shows buffering capacity, which might limit the adoption of this strategy in commercial algae farms.

The development of a fast process to harvest microalgae biomass produced in open cultivation systems is necessary to avoid contamination by fast-growing heterotrophic microorganisms that are unavoidably present in cultures produced in open cultivation systems, and changes in the biomass composition like the catabolism of carbohydrates and lipids. For that reason, flocculating agents can be used to promote the aggregation of microalgae cells and increase sedimentation rates (MILLEDGE & HEAVEN, 2013). A detailed evaluation of flocculating agents is required because the type and dosage of the flocculation agent, medium composition, and microalgae species play an important role in the flocculation process (GRIMA et al., 2003).

Herein, the flocculating agents aluminum sulfate and iron sulfate were evaluated on the sedimentation process of the non-flocculent *C. sorokiniana* BR001 using non-linear regression models that showed high coefficients of determination ($R^2 \geq 0.92$) (Figures 1B and 1C). Aluminum sulfate and iron sulfate were considered some of the best flocculating agents to harvest *Chlorella* cells (PAPAZI et al., 2010), and they resulted in higher efficiency recoveries of *C. sorokiniana* BR001 in comparison to the sedimentation process (Figures 1A to 1C). It was possible to recover more than 80% of the biomass using 0.5 g L^{-1} of aluminum sulfate and iron sulfate (Figures 1B and 1C). Aluminum sulfate and iron sulfate also reduced the duration of the process of biomass harvest, and it was possible to achieve high biomass recovery efficiencies after 15 min (Figures 1B and 1C).

According to the non-linear regression models, the flocculant dosage was the most important parameter to achieve high recovery efficiencies (Figures 1B and 1C). Both flocculating agents showed little differences in the recovery rates of BR001 biomass (Figures 1B and 1C). Interestingly, the different harvesting times evaluated in this study

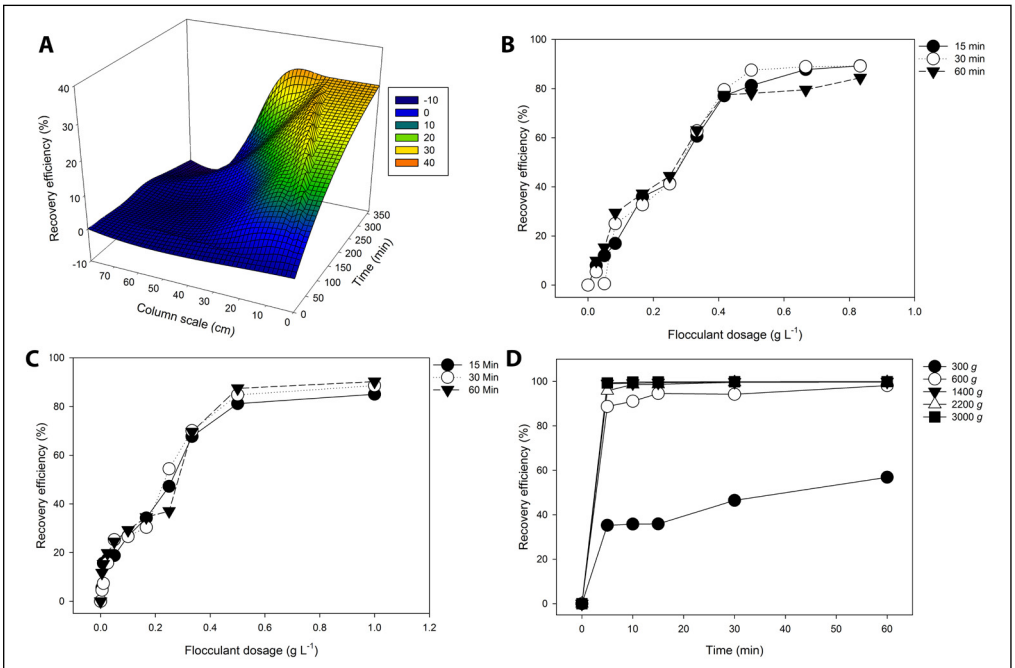

Figure 1 - Effect of different methods on the recovery efficiency of the biomass *Chlorella sorokiniana* BR001 cultivated under a nitrogen starvation condition. (A) Effect of the sedimentation process using different heights (top to bottom) and times in a sedimentation column. (B) Effect of different harvesting times and dosages of aluminum sulfate and (C) ferric sulfate. (D) Effect of the centrifugation process using different speeds and times.

also showed little effect on the maximum recovery of biomass (Figures 1B and 1C). However, the flocculating dosage of 0.5 g L^{-1} resulted in a satisfactory biomass recovery and higher dosages showed little effect on the biomass recovery (Figures 1B and 1C). For instance, iron sulfate dosages of 0.5 g L^{-1} and 1 g L^{-1} resulted in recovery efficiencies of 81.2% and 85.1% after 15 min, respectively (Figure 1C). These results are in agreement with a previous study that showed that increasing the dosage of aluminum chloride induced the flocculation of *C. sorokiniana* (ZHANG & CHEN, 2015), and the optimum doses observed herein are in agreement with a previous study that evaluated the harvest of *C. minutissima* (PAPAZI; MAKRIDIS; DIVANACH, 2010).

ZHANG & CHEN (2015) showed that the optimum dosage of the flocculant varies according to the composition of the medium and pH. Low levels of aluminum chloride (*e.g.* 10 mg L^{-1}) resulted in efficient flocculation of *C. sorokiniana* cultivated in medium BG11 (ZHANG & CHEN, 2015). In this current study, the use of 10 mg L^{-1} iron sulfate and 25 mg L^{-1} aluminum sulfate did not result in efficient flocculation of *C. sorokiniana* BR001 cultivated in a low-nitrogen medium (Figures 1C and 1D). Indeed, the low-nitrogen medium (ILLMAN; SCRAGG; SHALES, 2000) contains 3.7 times more nutrients (*i.e.* 6.3 g L^{-1}) than the medium BG11 (*i.e.* 1.7 g L^{-1}) (ANDERSEN, 2005). These different compositions of media are possibly related to the different efficiency recoveries observed in these studies.

Centrifugation is considered an efficient process to harvest microalgae biomass (BOROWITZKA & MOHEIMANI, 2013). Moreover, centrifugation can also be used in combination with other processes like flocculation, sedimentation, and filtration to develop a cheap and efficient process (BOROWITZKA & MOHEIMANI, 2013). However, previous studies did not evaluate the use of centrifuges to harvest the biomass of *C. sorokiniana* (XU; PURTON; BAGANZ, 2013; ZHANG & CHEN, 2015). High costs associated with the use of centrifuges can be reduced with a proper adjustment of the centrifugation process and the use of more efficient and low-cost centrifuge models (BOROWITZKA & MOHEIMANI, 2013). Thus, the effect of different centrifugation speeds and times harvest of *C. sorokiniana* BR001 cultivated in a low-nitrogen medium was evaluated in detail

using non-linear regression models that showed high coefficients of determination ($R^2 > 0.99$) (Figure 1D).

High recovery efficiencies were observed using centrifugation in comparison to the sedimentation and flocculation processes (Figure 1). It was possible to achieve high recovery efficiencies in this study using low centrifugal forces (*e.g.* 600 *g*) that are easily achieved by most of the industrial centrifuges. These results suggest that a robust and expensive centrifuge is not necessary to harvest the cells of *C. sorokiniana* BR001 cultivated in the low-nitrogen medium. High centrifuge speeds clearly improved the recovery efficiency using the different centrifugation times evaluated herein (Figure 1D). On the other hand, the centrifuge speed of 300 *g* was inefficient to harvest the biomass of *C. sorokiniana* BR001, and these recovery efficiencies were similar to those observed in the sedimentation process that resulted in a recovery efficiency lower than 40% (Figures 1A and 1D). However, centrifugation was much faster than the sedimentation process which increases the productivity of algae farms. Increasing the centrifuge speed to 600 *g* resulted in a significant increase in the biomass recovery, even using the shortest time of centrifugation evaluated in this study (Figure 1D). A remarkable advantage of centrifuges is the abolishing or reduction of the demand for flocculating agents that are potential contaminants for the biomass and water sources.

CONCLUSION

The free sedimentation-based process does not result in an efficient harvest of the biomass of the non-flocculent strain *Chlorella sorokiniana* BR001 cultivated in a low-nitrogen medium. Conversely, the inclusion of ferric sulfate or aluminum sulfate in the sedimentation-based process allows recovery efficiencies higher than 80% in less than one hour, but a high concentration of these flocculent agents is necessary to achieve adequate recovery efficiencies. Centrifugation presents high recovery efficiency, and the centrifugation speed at 600 *g* can harvest more than 90% of the *C. sorokiniana* BR001 biomass in 5 min. Therefore, centrifuge-based methods are the best alternative to harvest the biomass of the non-flocculent strain *Chlorella sorokiniana* BR001 cultivated in a low-nitrogen medium.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflicts of interest. The founding sponsors had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

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