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Biochemical Composition of Chlorella Sorokiniana Grown in a Novel Design of Hybrid Photobioreactor

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Abstract

The aim of this study was to assess the feasibility of a 180 L photobioreactor in which a submerged ultrafiltration system was used to maintain continuous nutrient inflow without biomass loss from the culture. After exponential growth, approximately 15% of the total volume was removed and replaced with a modified medium to induce algal physiological responses as a biochemical manipulation procedure. In this system, Chlorella sorokiniana was kept under healthy conditions, according to protein: carbohydrate ratio. *C. sorokiniana* was grown exponentially for 4 days up to $8.9x10^6$ cells mL^{-1}. The culture medium used for biochemical manipulation (72 h exposure) consisted of LC Oligo medium without nitrates or phosphates, and with $7x10^{-7}$ $molL^{-1}$ total copper. The results confirmed the effectiveness of the submerged membrane and showed that algae exposure to a stressing medium resulted in intracellular carbohydrate increase, thus protein: carbohydrate (P:C) ratios, and affected lipid class composition. This novel photobioreactor configuration has the potential to improve microalgal yields and/or specific intracellular constituents, inasmuch as biochemical manipulation of the biomass is facilitated and the continuous system is operated without biomass loss.

Keywords: Hybrid photobioreactor; Lipid classes; Carbohydrates; Proteins; Copper; Nutritional stress; Chlorella sorokiniana

Introduction

Microalgae have wide range of applications in pharmaceutical, cosmetic, and food industries [1,2] In addition, these organisms have high growth rates and the capacity to adapt themselves to changes in environmental conditions by producing a variety of biomolecules, which is known as physiological plasticity [3-5].

During microalgal growth in batch cultures, chemical and physical variations in cell surroundings can affect photosynthetic efficiency [6] and βbiochemical composition [3,7,8] argued that such effects can be expected because cell division decreases and carbon is allocated to lipids and carbohydrates, so these molecules are increased at the expense of proteins.

The quality of the biomolecules of interest is particularly important when the final product is subject to regulatory oversight [9]. However, batch culture does not offer such stability, which can result in undesirable variations from batch to batch in a production system. A photobioreactor that furnish constant growth conditions and reproducible conditions for manipulation of the biomass composition can be suitable for large scale production of microalgae. In theory, continuous culture would result in biomass of more constant composition than batch growth. According to [10], one of the impediments to the commercial use of high-density photoautotrophic algal cultures for the production of high-value products is the availability of suitable photobioreactors. Open ponds and closed photobioreactors share the problems of microalgae harvesting and growth medium enrichment that together contribute to the high cost of algal biomass production [11,12]. Open ponds are also predisposed to culture contamination. Continuous or semi-continuous systems applied to closed photobioreactors for algal culture is one of the best methods to maintain cultures close to their maximum growth rate, hence under healthy physiological conditions [6]. Continuous cultures have the advantage of producing algae of more predictable quality (biochemical composition) and keeping cells under healthy conditions for longer periods than batch or fed-batch systems. In addition, continuous systems are amenable to technological control and automation, increasing the reliability of the system and reducing labor cost [13]. However, continuous systems also have disadvantages, such as relatively high cost and complexity.

Considering the above mentioned difficulties and to make cultivation of microalgae in large scale economically feasible, membrane processes have long been tested in different stages of microalgae cultivation and processing with positive results [14-16]. Most existing large-scale microalgal plant systems still use energy-intensive centrifuges to harvest microalgae. Besides the often claimed lower energy consumption, especially for a submerged system [17], membrane filtration also offers the possibility of permeate recirculation without the accumulation of chemicals from flocculating agents, as encountered in the coagulation/flocculation technique [18]. According to Hwang et al. hydrophilic membranes are more resistant to fouling. However, it may depend strongly on the microalgal species too, since they excrete different amounts and types of organic materials that adhere onto surfaces are known to play important role in membrane fouling [14].

In this research we propose a novel system for microalgal growth whose important characteristic is the coupling of continuous cultures

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without biomass loss. The algae were cultured in semi-closed units, permitting evaporation as a means of heat dissipation, in continuous or semi-continuous cultivation mode, so that microalgae are kept under ideal growth conditions, and without biomass loss owing to a submerged polymeric membrane that is used to permeate used medium while retaining the biomass. Culture medium change for further biochemical manipulation of the biomass is conveniently done through membrane permeation. The polymeric membrane also permits biomass concentration prior to biomass harvesting. It is a reusable and commercially available membrane.

Material and Methods

Stock cultures

Chlorella sorokiniana (211-32) was kindly furnished by the Institute of Plant Biochemistry and Photosynthesis (IBVF), University of Sevilla, Spain. Stock batch cultures (200 mL) were kept in modified LC Oligo medium (AFNOR, 1980) under laboratory controlled conditions of light intensity (150 μE m^{-2} s^{-1}), light/dark cycle (12:12 hours), and temperature (24 ± 2°C), at pH 7 (Logen Scientific, LS 300-HH, Brazil).

Inocula preparation

Inocula for the 180 L photobioreactors were prepared in a scale-up system. C. sorokiniana was grown in 20 L low density polyethylene photobioreactors, in which culture medium was supplemented daily up to a final cell density of 106 cells mL^{-1}. This was further inoculated in the 180 L photobioreactor. Therefore, initial cell density of 180 L culture was 105 cells mL^{-1}. The choice of 106 cells mL^{-1} maximum inoculum density was based on the results of Lombardi and Maldonado [6] that showed that 106 cells mL^{-1} is the maximum density permitted for high photosynthetic efficiency, a measurement microalgae health status. Thus, healthy beginning of the 180 L cultures was guaranteed by carefully controlling the inoculum density and cells' physiological conditions

Photobioreactor and ultra filtration system

A photobioreactor, referred here as *hybrid photobioreactor* (HPBR), was used for the experiments. A schematic diagram of the unit is shown in Figure 1. It consisted of a glass tank, a mechanical mixing device, a CO_2 (3%)/air (97%) bubbling system installed below the membrane, a supply of fresh culture medium fed by a peristaltic pump and an ultrafiltration system. The mixing device was composed of a mechanical mixer (Fisatom, 713DS, Brazil) and

a propeller. Culture tank dimensions were 0.8 m length, 0.5 m width, 0.8 m depth with 180 L working volume; an air space of 0.08 m was maintained in the HPBR, so that the depth of culture medium was 0.72 m. For temperature regulation, water evaporated from the surface of culture medium, condensed on the lid and dropped back into the HPBR. In the conditions used, heat dissipation through this mechanism was enough for cells to keep on its maximum growth. Inside the HPBR there was a hydrophilic polyether sulfone membrane to process ultrafiltration with 0.6 m2 filtration area and 0.005 μm pore size and a hydraulic circuit to collect the permeate. To operate the HPBR as a continuous flow system, the polymeric membrane was employed to remove used growth medium free of microalgal cells (confirmed under light microscope), while a peristaltic pump (Masterflex, Model 77122-22, USA) introduced fresh culture medium. Culture pH was adjusted daily to pH 7.0 through the CO_2: air bubbling.

Photobioreactor cultures

The 180 L photobioreactor was placed in a greenhouse under semi-controlled environmental conditions. Natural illumination varied from 300 μE $m^{-2}s^{-1}$ to 1200 μE $m^{-2}s^{-1}$ and temperature control was promoted by exhausters and humid refrigeration systems. Culture temperatures varied within 20-28°C. The photobioreactor was operated as both continuous and batchculture modes at different moments in a unique culture module. First, a continuous system

maintained healthy growth conditions for microalgae through continuous flow of fresh culture media. For the continuous culture mode, the ultrafiltration system was operated continuously to permeate approximately 2 L day^{-1} in constant pressure without cell loss during 5 days. In this case, hydraulic pressure of the culture column above the membrane promoted the permeation. A ball valve was installed in the hydraulic circuit of the ultrafiltration system to control permeate output with no volume change of the culture during the 5 days. On the 6th day, the continuous input of fresh culture medium was ceased and 30 L of used medium were removed through the complete opening of the valve. This allowed a maximum initial flow of permeate of 40 L h^{-1} m-2 ± 10 and a steady-state flux at the end of the ultrafiltration process of 4 L h^{-1} m^{-2} ± 15.1. In this case, the pressure was not constant and flow rate decreased along permeation time. At every ten minutes the permeate flux was collected in order to evaluate its flux as function of time [19]. A summary of the fluid-dynamic conditions is given in Table 1.

After ultrafiltration, 30 L of fresh LC Oligo culture medium without nitrate and phosphate, plus $7x10^{-7}$ mol L^{-1} copper was fed into the photobioreactor to induce intracellular physiological transformation, such as biochemical synthesis of storage molecules. In this condition, the culture was operated in batch mode for 72 h. The experiment lasted

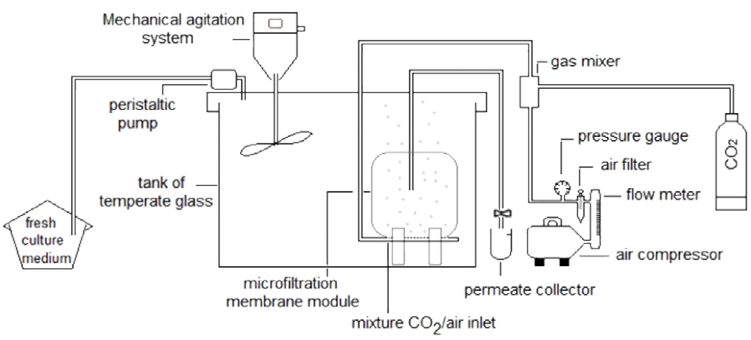

Figure 1: Schematic diagram of the hybrid photobioreactor (HPBR) used in this study.

Culture Phases	Pressure transmembrane	Filtration time (h)	Initial permeate flux (L $h^{-1}m^{-2}$)	Final permeate flux (L $h^{-1}m^{-2}$)
Continuous system (First 5days)	Constant	120	0.14	0.14
30 L medium removal	Variable	6	40	4.0

Table 1: Processing parameters of the ultrafiltration experiments.

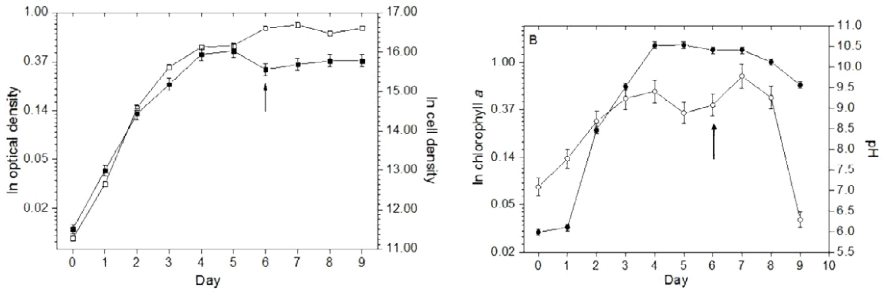

Figure 2: (A) Optical density (684 nm, natural logarithm; empty squares) and cell density (natural logarithm;full squares); (B) clorophyll*a* (natural logarithm, full circles) and pH (empty circles) during *Chlorellasorokiniana* growth in the 200 L hybrid photobioreactor. Continuous system was used up to day 5; arrowindicates exchange of 30 L used media with stressing medium (extra copper added).

9 days and cultures were sampled daily for optical and cell density, chlorophyll a concentration, pH, intracellular lipids, proteins and carbohydrates before and after culture medium exchange.

Cleaning procedure

The recovery of permeate flux is only possible after a chemical cleaning of the membrane [20]. In this case, at the end of the experiment, the system was rinsed with tap water with no chlorine to remove the polarized layer deposited on membrane surface. Subsequently, 0.5% alkaline detergent Extran® MA 01 (Merck KGaA, Darmstadt, Germany) at environmental temperature was circulated through the system during 60 minutes, after which it was rinsed with chlorine free tap water to completely remove the detergent. After this, a chlorine solution with 1% of sodium hypochlorite (NaClO) was circulated during 30 minutes, and finally the system was rinsed with chlorine free tap water. This cleaning procedure was performed between experiments and membrane performance checked through the mean time needed to remove 30 L of permeates (~ 6 h). Membrane performance was consistent throughout this study.

Physicochemical analysis

Chlorophyll a concentrations were determined by *in vivo* fluorescence, using a Turner Fluorometer (Promega Corporation, USA). Before measurements, culture samples were dark adapted for 20 minutes and chlorophyll a content determined considering a calibration curve, where chlorophyll a concentration (mg L^{-1}) was plotted against fluorescence intensity. Optical density was measured at 684 nm with a UV-VIS scanning spectrophotometer

(FEMTO 800 XI, Brazil). Cell density was determined by cells counting (samples in 5% acid Lugol solution) in a bright lined Fuchs-Rosenthal hemacytometer under optical microscope. Specific growth rates were obtained by plotting the natural logarithm of *in vivo* fluorescence intensity against experimental time and calculating the slope of the linear part of the curve by linear regression.

Total intracellular carbohydrates were quantified according to the modified phenol-sulfuric acid methodology described in [21], using glucose as standard. Total intracellular proteins were determined according to the procedure of [22] using bovine serum albumin (BSA) as standard and Coomassie Brilliant Blue G-250 as protein reagent. Protein extraction was performed as described by [23] using 0.5 N NaOH.

Lipid classes were quantified by Thin Layer Chromatography with Flame Ionization Detection using an Iatroscan TLC-FID (Iatron Laboratories Inc., Tokyo, Japan). Lipids were extracted twice in chloroform/methanol (2:1) and hexadecane-3-one was used as internal standard [24]. For lipid analysis, samples were concentrated in a rotary evaporator (RV05 S25, IKA, Germany). Concentrated lipid samples were spotted onto silica gel-coated rods (Chromarods-SIII), focused twice in acetone and scanned under the analytical conditions of 173 mL min^{-1} hydrogen flow, 2 L min^{-1} air flow and 4 mm s^{-1} scan speed. Lipid classes were identified by their retention times, compared to Sigma standards, and estimated from peak areas recorded and processed by Peak Simple software version 3.93 (SRI Instruments, Menlo Park, California, USA).

Results

Population density (cell mL^{-1}), optical density, chlorophyll a content and pH are plotted against experimental time in Figure 2. Optical density varied from 0.01 to 0.5 A.U, pH within 6.0 and 9.0 and chlorophyll a content from 0.02 mg L^{-1} to 1.44 mg L^{-1}. These results indicated that during continuous cultivation, exponential growth of *C. sorokiniana* was maintained up to the 4th culture day, during which cell density increased from 105 cells mL^{-1} to 9.3 x 106 cells mL^{-1} and the specific growth rate was 1.10 d-1 for *C. sorokiniana*.

The use of the submerged ultrafiltration membrane enabled culture medium exchange through which 15% of total volume was removed. This process led to - approximately 2.7 x 106 cells mL ^{1} becoming attached to the membrane surface, thereby decreasing the population density and polarizing the membrane. This was further confirmed by a decline in chlorophyll a content from 1.44 mg L^{-1} to 1.30 mg L^{-1}. Microscopic observations confirmed the absence of *C. sorokiniana* cells in the permeate. After 48 h of biochemical manipulation cell density rose, but 24 h later, cells began to aggregate and settle on the bottom of the photobioreactor. This event was followed by culture collapse, which was confirmed by sharp pH decrease (Figure 2). Protein: carbohydrate ratios confirmed the healthy condition of the controls (P:C 6.97), decreasing thereafter to 0.45 (24 h), 0.29 (48 h), 0.39 (72 h).

Figure 3 shows the relative lipid class composition. Exposure of

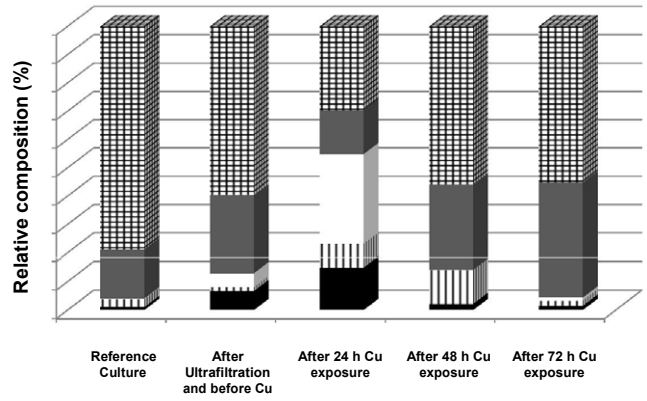

Figure 3: Bar graph showing the relative composition of lipid classesfor the several culture conditions.
Reference culture, after ultrafiltration and before biochemical manipulation, after 24 h biochemical manipulation, after 48 h biochemical manipulation and after 72 h biochemical manipulation. Bar patterns: Black=aliphatic hydrocarbons, stripe=free sterol, white=triacylglycerol, gray=acetone mobile polar lipids, grid=phospholipids.

culture to medium without N and P, and extra copper affected cell physiology and its biochemical composition. Under healthy growth conditions, the structural lipids acetone mobile polar lipids (AMPL) and phospholipids (PL) dominated, but after 24 h of biochemical manipulation, aliphatic hydrocarbons (HC~15%), free sterols (ST ~ 8%) and triglycerides (TG ~ 30%) increased at the same time that AMPL and PL decreased. Adaptation of the microalgae to the biochemical manipulation used to induce intracellular synthesis of biomolecules was confirmed by the return to almost normal values of the AMPL and PL lipid classes at 48 and 72 h. Free fatty acids (FFA), wax ester (WE) and free aliphatic alcohol (ALC) were present in trace amounts (0-1% of total lipids), so data are not shown.

Discussion

The pH variation (7.0 - 9.8) observed in the 180 L *C. sorokiniana* culture is in accordance with literature data for other *Chlorella* cultures [25,26]. This is explained by the consumption of nitrate and dissolved inorganic carbon species [27,9]. CO_2 bubbling that was used for pH control supported a hundred fold increase in biomass, from 105 to ~107 cells mL^{-1} in 4 days. Compared to literature data, the present results showed the greatest cell density increase, which can be related both to CO_2 bubbling and the nutrient influx provided by continuous feed of fresh medium during the exponential growth phase without biomass loss. [28] observed a 12.5 times increase in *Chlorella sp*. cell density (from 8×10^6 to 1×10^8 cells mL^{-1}) after 8 days of growth at 2% CO_2 bubbling [29] Studied *Chlorella vulgaris* growth in residual hydroponic solution and found that cell density increased 100 times in 8 days, from 105 to 107 cells mL^{-1}, after which pH reached 9.5, indicating that high CO_2 absorption accomplished. Note that the final cell density of 107 cells mL^{-1} obtained in this photobioreactor is very much similar to those obtained under controlled conditions in our laboratory, with LC Oligo culture medium.

Culture medium renewal using the ultrafiltration membrane presupposes removal of medium in the shortest time possible. In the present research, 15% of the medium was removed for the biochemical manipulation, with the membrane being used at its maximum permeation capacity. This led to algae fouling on membrane surface, which was detected as cell density decrease on the 6th culture day. According to [30], who used similar membranes for *Chlorella* filtration, the cake layer of *Chlorella* cells on membrane surface would not cause resistance higher than the membrane resistance itself if cells were not compressed. In this situation, membrane fouling may be attributed to the fact that initially, when cells are in low number, there is no compaction and, hence, no significant resistance. However, when the amount of algal cells being deposited on membrane surface increase, the extent of cell compaction increases at its maximum. This is controlled by the transmembrane pressure being applied as well as by algal cell shape and chemical surface interactions with the ultrafiltration membrane pores.

Babel and Takizawa [30] showed that, after maximum compaction, cake resistance increased linearly with the amount of cake. Therefore, air bubbling has been tested as a solution for membrane fouling, in which the coarse bubbles aeration generates direct shear on the membrane surface by inducing a secondary flow of liquid. This disrupts the mass transfer boundary layer, and promotes local mixing near the membrane surface [31,32].

In the present paper, during the continuous system, low permeate flux was maintained around 0.14 L $h^{-1}m^{-2}$ for five days. This flux was much lower than steady-state flux (4 L $h^{-1}m^{-2}$). In the continuous flow, low permeate flux, gas bubbling and culture mixing were essential factors to limit membrane fouling. Despite the bubbling aid to decrease membrane fouling, when the valve was fully opened to permit maximum permeation, a continued and accented decrease of the permeate flux was observed.

In the present research, during the 30 L medium removal, air bubbling was tested for *C. sorokiniana* removal from the membrane surface. These results showed that although there was less membrane fouling, the bubbling promoted algal stress and death. Therefore, during the 30 L medium removal, only gentle bubbling with CO_2/air mixture was used; the role of CO_2 /air bubbling in the culture system was exclusively for pH control, which was performed once a day throughout the experiment.

Chlorophyll a concentration varied from 280 pg cell-1 in the control to 87 pg cell-1 after 72 h exposure to the biochemical manipulation culture medium containing, representing a decrease of ~ 3 times. These results agree with those of Bajguz [33] that observed lower chlorophyll a content in stressed microalgae in comparison with the control.

The biochemical composition and P:C ratio of phytoplankton have been shown to be related to their nutritional status [34-36]. According to Healey and Hendzel, [37] an increase in P:C ratio is evidence of healthy cells, whereas its decrease an unhealthy or stressing condition. Thus, in the present results for *C. sorokiniana* at the beginning of exponential growth, when P:C ratio was 6.97, denotes healthy cells, whereas the P:C ratio decrease to 0.39 after 72 h exposure to the stressing culture medium denotes stressed cells. These results are in accordance with those of Ho [38], who observed a P:C ratio decrease from 4.4 to 0.37 for *Chlorella vulgaris* FSP-E during nitrogen starvation period in modified Basal medium. Ji [39] found a P: C ratio of 5.2 for *Chlorella vulgaris* cultured in 100-fold diluted monosodium glutamate wastewater (MSGW) and a P:C ratio of 1.79 with 800-time diluted MSGW. Similarly, in experiments of culture medium reuse, Rocha [40] obtained a ratio of 5.18 for *Scenedesmus quadricauda* control cultures, while in stressing conditions the authors obtained P:C ratios of ~ 1.0. Besides its variation with nutritional condition, it is also expected that the P: C ratio varies with different algal species.

We calculated P:C ratios from the biochemical composition data

presented in Illman [41], whose experiments were performed in batch cultures, and obtained approximate values of 0.57 for Chlorella vulgaris, 0.78 for *C. emersonii*, 0.73 for *C. protothecoids*, 1.18 for *C. sorokiniana* and 0.57 for *C. minutissima*. The higher level of protein in relation to carbohydrates observed in our P:C ratio in comparison to those calculated from the results of Illman[41] can be attributed to the different growth conditions furnished by continuous and batch systems, indicating a better physiological status in the first. The P:C ratios around 0.5, denoting that at least twice as much carbohydrates as proteins was present during culture manipulation with the modified culture medium in the present research, confirmed that cells were stressed. This is in agreement with several authors that showed reduced protein content in stressed cells, such as Lourenço [42] and Zhao [43] investigated the distribution of intracellular N in marine microalgae and showed that N-limited cells had lower protein content than the controls. Zhao [43] showed that nutrient stressed *Skeletonema costatum* had lower total proteins content than cells kept in high nutrient conditions. These authors showed that carbohydrates accumulated under N and P limitation, in which the highest carbohydrates content was obtained in cells grown with 64 μM N and 36 μM P.

It is well documented that lipids and carbohydrates are storage molecules, while proteins are structural biomolecules. Thus, an increase either in lipid or in carbohydrate content suggests phytoplankton cells can be in asituation unfavorable for growth. Several recent reports [44-46] have demonstrated that, under conditions of nitrogen-depletion, intracellular proteins can be transformed into lipids or carbohydrates in microalgae.

It is known that copper affects photosynthesis [6], growth rates [47] and chlorophyll *a* content in phytoplankton. In excess it disturbs important physiological functions and enzymes, interfering with cell biochemistry, including membrane integrity. The effects of copper on photosynthetic organisms include interference with fatty acids, membrane lipids, protein metabolism and inhibition of respiration and nitrogen fixation processes [48]. Similarly to the present results, the accumulation of carbohydrates in phytoplankton stressed by copper has been reported [49,50].

Contrary to what has been reported in several published data, the present results showed a reduction in lipid production/unit volume during and after culture manipulation. Several studies show lipid increase under nutrient stress [4,5,35,51,52]. Triacylglycerol (TG) accumulation was seen in *Stephanodiscus minutulus* when the cells were grown under Si, N or P limitation, while polar lipids decreased [53]. However, the present results agree with those of Xin [54], who did not obtain lipid accumulation in N or P limited *Scenedesmus sp.* cultures. Similarly, [55] demonstrated that combinations of stress factors can, under certain circumstances, inhibit neutral lipid synthesis. The authors investigated lipid synthesis in *Nannochloropsis sp*.

Regarding relative lipid composition, the present results showed that phospholipids (PL-membrane lipids) and a pigment class, acetone mobile polar lipids (AMPL), constituted most of the total lipids detected in *C. sorokiniana* under any growth conditions. This is in accordance to most literature data, which have shown that high concentrations of PL and AMPL, relative to other lipid classes, are expected in microalgae [56,57] and Parrish and Wangersky [58] investigated lipid class production in several marine phytoplankton and reported these lipid classes as major constituents (~ 70-80%). Similarly, Chia [4] reported high percent contribution of PL and AMPL to total lipids in *Chlorella sp*. However, TG increase denotes stressing conditions, and according to [57], any TG content higher than 15% of the total lipids can imply nutrient stress, which can justify the increase in TG after 24 h exposure to the biochemical manipulation medium. Similarly, [59] observed a rise in TG content at the expense of other lipid classes in nutrient-limited *Monodus subterraneus*.

The present results suggest that, immediately after the change in culture medium, a shift in the metabolism of *C. sorokiniana* was confirmed by a high percentage of TG, after which cells became acclimated and this storage lipid class returned to normal levels. According to [4], who obtained a negative correlation between TG and PL, such behavior may reflect a physiological pathway to structural lipid synthesis that is triggered under nutrient-replete conditions. In such situation, the precursors involved in both TG and PL synthesis are channeled towards the production of PL, needed in cell membranes [5,60,61].

Conclusions

The 180 L hybrid photobioreactor (HPBR), featuring a submerged polymeric membrane, supported continuous culture of *C. sorokiniana*, retaining its biomass inside the photobioreactor while used medium left the culture as permeate and was replaced by fresh medium. This system provided healthy growth conditions, according to the cell proteins: carbohydrates ratios, enabling the cells to accumulate proteins. The submerged polymeric membrane system permitted exchange of medium, thus facilitating biochemical manipulation process, which resulted in a change of relative lipid composition, favoring accumulation of the neutral lipid class, triacylglycerol short after exposure to stressing condition (24 h). However, 48 h and 72 h later, cells acclimated themselves, with accumulation of the polar classes PL and AMPL, and triacylglycerol returned to normal values.

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