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Research Article

Growing *Chlorella vulgaris* in Photobioreactor by Continuous Process Using Concentrated Desalination: Effect of Dilution Rate on Biochemical Composition

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Desalination wastewater, which contains large amount of salt waste, might lead to severely environmental pollution. This study evaluated the effect of dilution rate ($0.1 \leq D \leq 0.3$ day^{-1}) on microalgal biomass productivity, lipid content, and fatty acid profile under steady-state condition of *Chlorella vulgaris* supplemented with concentrated desalination. Continuous culture was conducted for 55 days. Results show that the biomass productivity (P_x) varied from 57 to 126 mg L^{-1} d^{-1} (dry mass) when the dilution rate ranged from 0.1 to 0.3 day^{-1}. At lowest dilution rate ($D = 0.1$ day^{-1}), the continuous culture regime ensured the highest values of maximum biomass concentration ($X_m = 570 \pm 20$ mL^{-1}) and protein content (52%). Biomass lipid content was an increasing function of D. The most abundant fatty acids were the palmitic (25.3 ± 0.6%) at $D = 0.1$ day^{-1} and the gamma-linolenic acid (23.5 ± 0.1%) at $D = 0.3$ day^{-1} ones. These fatty acids present 14 to 18 carbons in the carbon chain, being mainly saturated and polyunsaturated, respectively. Overall, the results show that continuous culture is a powerful tool to investigate the cell growth kinetics and physiological behaviors of the algae growing on desalination wastewater.

1. Introduction

The cultivation of microalgae in photobioreactors by continuous culture has been used worldwide to generate large amounts of biomass. This type of culture systems is well established and applied to the production of microbial biomass in fermenters (bacteria and fungi), for example, the alcohol industry [1].

Most of the photobioreactors in use have a cylindrical or tubular shape, with vertical or horizontal arrangement of the tubes. They can be installed indoors, receiving artificial light, or be prepared outdoors, getting solar power [2, 3]. Compared to tanks, photobioreactors are more expensive (especially the systems fed by artificial light). Nevertheless these relatively elevated costs can be compensated by generating components of high commercial value. For example, *Haematococcus pluvialis* is currently used for the production of astaxanthin pigment and *Chlorella* sp. has been used to enhance the nutrition value of food and animal feed [4, 5].

Many species of microalgae are able to grow efficiently in wastewater. In this case, it is important to find alternative culture media involving lower production costs than conventional ones, but which are sufficient to achieve suitable growth and nutrient composition [6]. An alternative culture medium

is the cultivation of microalgae integrated with wastewater from the desalination process of groundwater, which was suggested by Volkmann et al. [7].

The main method used for desalination of groundwater in the Brazilian Northeast is based on reverse osmosis. However, the effluent of desalination process has a potential for chemical (chlorides, sodium, carbonates, etc.). If not properly managed, it may cause environmental problems such as salination of agricultural land and subsequent soil infertility [8].

A great variety of photosynthetic microorganisms can be induced to overproduce specific fatty acids through relative simple manipulations of the physical and chemical properties of their culture medium, especially polyunsaturated fatty acids, such as linoleic acid (18:2 n-6) and alpha-linolenic acid (18:3 n-3), the latter being a widely recognized food supplement [9].

There are several studies about biomass composition or specific components of microalgae growing on wastewater [6, 7]. Nonetheless, the majority of the research is focused on batch and fed-batch cultivations. Despite the application of these processes on the evaluation of biomass composition, the microorganisms are submitted to many variables during cultivation, especially nutrient concentration, which makes it difficult to match a biomass composition variation to a certain cause. When cultivating by continuous process, the steady-state assures a better relation between operational conditions and biomass composition [10]. Sobczuk and Chisti [11] studied algal cultivation for biomass and biofuels using steady-state mode, in which the effect of dilution rate or temperature on the lipid contents and fatty acids profiles of the fresh water green microalga was evaluated. Moreover, steady-state studies are needed to be better understand and measure the specific growth parameters. Under steady-state conditions, cell density can be controlled and held constant by varying the reactor dilution rate, and constant irradiance levels can be readily maintained.

The continuous process has successfully been used to investigate the influence of the operating conditions on the kinetic parameters of *Chlorella* sp. growth using concentrated municipal wastewater [12]; however, there is no report in the literature about the kinetic parameters of *Chlorella vulgaris* cultivated in a culture medium based on desalination wastewater. On the basis of these issues, the aims of this study were to evaluate the influence of dilution rate on the continuous cultivation as well as to establish relationship between the desalination wastewater and protein/lipid contents along with fatty acid distribution in continuously cultivated *Chlorella vulgaris*.

2. Material and Methods

2.1. Desalination Wastewater Collection. Samples of brine discharges, titled as concentrated desalination (CD), were collected from brackish water reverse osmosis desalination plant, located in São João do Cariri, Paraíba, Brazil. Concentrated desalination samples were collected in a 100 L plastic container and stored at −20°C in the Laboratory of Food Biotechnology at Federal University of Santa Catarina (UFSC). The physicochemical characterization of the concentrated desalination (Table 1) was performed according to *standard methods for the examination of water and wastewater* [13].

Table 1: Composition and physic-chemical characteristics of concentrated desalination.

Parameters	Value
Cl (mg/L)	1,691.3
Ca (mg/L)	126.5
Fe (mg/L)	0.13
K (mg/L)	47.0
Mg (mg/L)	4.74
Na (mg/L)	987.5
NH_4^+ (mg/L)	1.35
Sulfate (mg/L)	138.0
Total phosphorus (mg PO_4^{3-} P/L)	0.70
Total hardness ($CaCO_3$, mg/L)	985.2
TDS (mg/L)	2,190.5
Total nitrogen (mg N/L)	30.0
Conductivity (μS/cm)	4,875.0
pH	8.11

Note: P is short for phosphorus, N is short for nitrogen, TDS is short for total dissolved solids, and NH_4^+ is ammonium.

2.2. Microorganism Maintenance. *C. vulgaris* was kindly supplied by the Chemical Engineering Laboratory, from Federal University of Campina Grande. Established cultures of the green microalgae *Chlorella vulgaris* were grown in 500 mL Erlenmeyer flasks containing 300 mL modified BBM medium [14] at pH 6.8 in a culture room at 25 ± 2°C, under a photoperiod of 12/12 h, at light intensity of ~72 μmol photons m^{-2} s^{-1}, with sparging saturated air-CO_2. The mineral salt medium composition, per liter of distilled water was 0.075 g K_2HPO_4, 0.014 g KH_2PO_4, 0.075 g $MgSO_47H_2O$, 0.09 g $NaNO_3$, 0.025 g $CaCl_32H_2O$, 0.025 g NaCl, 0.05 g EDTA-Na_4, 0.00498 g $FeSO_47H_2O$, 0.01142 g H_3BO_3, 0.232 mg $MnCl_2{\cdot}4H_2O$, 1.41 mg $ZnSO_4{\cdot}7H_2O$, 0.252 mg $CuSO_4{\cdot}5H_2O$, 0.192 mg $NaMoO_4{\cdot}5H_2O$, 0.080 mg $CoCl_2{\cdot}6H_2O$, and 10 g of yeast extract and supplemented with 250 mL L^{-1} of concentrated desalination. The conductivity of the medium was measured of about 1,250 μS/cm.

2.3. Continuous Culture Conditions and Equipment. Continuous culture was performed in a 6.0 L glass photobioreactor vessel (New Brunswick Bioflo 2000 fermenter) with working volume of 5.0 L at 30°C. The fermenter was previously sterilized by autoclaving 121°C for 15 minutes. Five liters of modified bold basal medium (BBM) was prepared. An initial biomass concentration of 15 mg L^{-1} was used at the start of batch culture preceding the continuous one. During the startup, *Chlorella vulgaris* was cultivated by the fed-batch process in the same reactor subsequently utilized for continuous cultivation. Continuous culture was started, once the batch culture had reached early stationary growth phase, by feeding the fresh medium at different dilution rates (D), specifically 0.1, 0.2, and 0.30 day^{-1}, corresponding to 10%, 20%, and 30%

of volume renewal per day. A peristaltic pump was used to feed the fresh medium. The composition of the feed medium was the same as that for the initial batch culture. It was assumed that steady-state condition was achieved when cell concentration, which was determined daily, was kept almost constant along a time period at least twice the residence time. The liquid volume was kept constant at 5.0 L during all the culture cultivation. The light intensity was about 108 μmol photons m^{-2} s^{-1} on the surface of the bioreactor exposed to six 20 W-fluorescent lamps (OSRAM Universal) during a photoperiod of 12/12 h light/dark cycle and under constant agitation of 100 rpm. The continuous culture was maintained for 55 days in total. Simultaneously, equal volumes of cell suspension were withdrawn from the fermenter. Samples were taken from the fermenter at every 12 hours on a daily basis for measuring the cell dry weight and pH.

2.4. Parameters Determination. Cell density (cells mL^{-1}) and biomass concentration (dry weight per liter) of cultures were measured according to the method reported previously [15]. The sample pH was directly determined using a SP990M pH meter (Sensoglass, São Paulo, Brazil). The pH meter was calibrated daily using standard solutions of pH 4 and 7. The biomass productivity in continuous culture (P_x; mg L^{-1} d^{-1}) was calculated, according to [16], as the product of the dilution rate (D; day^{-1}) and the biomass concentration under steady-state conditions (X_S; mg L^{-1}) $P_x = DX_S$.

2.5. Analytical Techniques. The exhausted broth was collected for analysis. One portion was used for determination of dry cell mass concentration by optical density (OD) measurements at 560 nm using calibration curve. Another fraction was filtered, and the resulting liquid phase was used for determination of pH.

After steady-state achievement, all the biomass contained in the bioreactor was centrifuged at 4000 rpm, at 10°C, for 30 min (Nova Técnica, Piracicaba, Brazil), washed twice with distilled water, dried in furnace at 55°C with air circulation for 12 h, and then stored in glass flasks at −20°C for cell mass composition analysis. Aliquots of dried biomass were analyzed for determination of total proteins by Kjeldahl method [17] using a conversion factor of 6.25. The total lipid content of dry biomass was determined in Soxhlet by reflux extraction with hexane for 4 h. Once the solvent had been removed, the residue was submitted to a new extraction with chloroform-methanol (2 : 1 v/v) for 4 h [17].

Fatty acids composition was determined after conversion of fatty acids with their corresponding methyl esters [18]. Analyses of fatty acids methyl esters (FAME) were characterized by gas chromatograph, model GC-2014 (Shimadzu, Kyoto, Japan), equipped with split-injection port, flame-ionization detector, Restek a 105 m-long capillary column (ID = 0.25 mm) filled with 0.25 μm of 10% cyanopropylphenyl, and 90% biscyanopropylsiloxane. Injector and detector temperatures were both 260°C. The oven temperature initially was set at 140°C for 5 min and then programmed at 2.5°C min^{-1}. Qualitative fatty acid composition was determined by comparing the retention times of the peaks with the respective standards of fatty acids. Quantitative composition was accomplished by area normalization and expressed as mass percent.

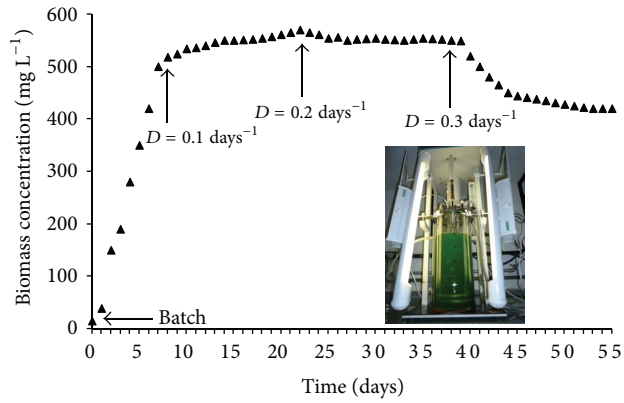

FIGURE 1: Cell concentration of *C. vulgaris* during batch and continuous culture carried out at different dilution rates (D) 0.1, 0.2, and 0.3 day^{-1}.

3. Results and Discussion

3.1. Effect of Dilution Rate on the Continuous Culture. Before starting continuous process, three different dilution rates (D) for *Chlorella vulgaris* cultivation in a culture medium based on concentrated desalination were studied. Our previous results showed that use of high concentration of concentrated desalination, above 25%, strongly affect the growth and biochemical composition of *Chlorella vulgaris*. Due to high salt waste presented in concentrated desalination and, in order to assess the applicability of growing *Chlorella vulgaris* by continuous process supplemented with desalination wastewater, the concentrated desalination was diluted as a proportion of 25%. Based on the literature data [19, 20] and preliminary studies, a value of dilution rate ($0.10 \leq D \leq 0.30\ d^{-1}$) was tested. In this way, fresh medium was added continuously until the end of cultivation with dilution rates of 0.1, 0.2, and 0.3 day^{-1}, respectively.

A gradual increase in pH was observed with the cell growth. On the 8th day of cultivation, the pH reached a value close to 10.0 ± 0.2, so the cell concentration stabilized. According to previous work [10], pH > 10.2 typically affects the growth of *A. platensis*. Thus, it was assumed that *Chlorella vulgaris* would behave similarly, so the continuing process initiated on the 8th day.

Figure 1 shows the results of *C. vulgaris* continuous cultivation carried out at different dilution rates using a culture medium based on concentrated desalination. The results showed that the *C. vulgaris* cultivated in concentrated desalination did not have negative growth effect until the 38th day, presenting a relative continuous growth on cell concentration. The continuous process started at $D = 0.1$ day^{-1} and reached steady-state conditions after about 6–8 days, at which cell concentration 518 mg L^{-1}; after 15 days, that is, a time equivalent to twice the residence time, the dilution rate was increased to 0.2 day^{-1}, and the cell concentration decreased

Table 2: Results of cell concentration and productivity obtained from continuous cultivations of *C. vulgaris* carried out at different dilution rates.

Continuous culture	Dilution rate (day^{-1})	X_m or X_S ($mg\,L^{-1}$)	pH	Biomass productivity (P_x, $mg\,L^{-1}\,d^{-1}$)
1	0.1	570 ± 20	10.0 ± 0.2	57.0 ± 1.2
2	0.2	550 ± 10	9.5 ± 0.1	110.0 ± 2.3
3	0.3	420 ± 16	9.3 ± 0.2	126.0 ± 0.8

until a new steady-state conditions was attained. After additional 16 days, D was further increased up to 0.3 day^{-1} following the same criterion. Steady-state conditions were observed at first two dilution rates, thus demonstrating an excellent ability of *C. vulgaris* to maintain stable conditions during at least two residence times. This result is quite promising taking into account that large production of these green algae is usually performed by continuous operation under steady-state conditions.

The values of X_S decreased with increasing D, while those of P_x increased (Table 2). The highest biomass concentration was obtained in the continuous culture carried out at $D = 0.1$ day^{-1} ($X_S = 570 \pm 10\,mg\,L^{-1}$) with biomass productivity ($P_x = 57.0 \pm 1.2\,mg\,L^{-1}\,d^{-1}$). This value was comparable to that ($473\,mg\,L^{-1}$) reported by Tang et al. [21] for continuous culture of *Chlorella minutissima* in glass photobioreactor vessel at dilution rate of 0.168 day^{-1} and higher than the one ($273\,mg\,L^{-1}$) reported by the same authors for continuous culture of *Dunaliella tertiolecta* at $D = 0.17$ day^{-1}. This comparison demonstrates the potential of *C. vulgaris* to grow quickly in glass photobioreactor vessel in continuous cultivation. On the other hand, Bezerra et al. [19] reported a value of ($5806 \pm 74\,mg\,L^{-1}$) higher than present study for *A. platensis* in helical tubular photobioreactor at $D = 0.1$ day^{-1}.

Changes in dilution rate of continuous culture had a significant impact on the steady-state cell density and biomass concentration [21]. At dilution rate $D = 0.2$ day^{-1} the biomass concentration decreased from 570 to 552 ± 20 $mg\,L^{-1}$. Moreover, the steady-state biomass concentration decreased with increasing D from 0.2 to 0.3 day^{-1} (Figure 2). As expected by the well-known mass balances in continuous bioprocess [22], the highest biomass concentration's was obtained at the lowest dilution rate ($D = 0.1$ day^{-1}). Nevertheless, under these conditions, lower value of productivity (P_x) was obtained (Table 2). It should be mentioned that in the beginning of the continuous culture the conductivity inside the vessel was 1,250 μS/cm and after 39 days of continuous culture, it was observed a decay of the cellular growth associated with 2-fold higher conductivity of about 2,570 μS/cm. Eventually, culture growth ceases due to depletion of the growth-limiting substrate or growth-salt stress, excessive excretion of toxic metabolites, accumulation of organic matter inside the bioreactor, and limiting light intensity due to the shadowing effect caused by high cell concentration [19, 21].

3.2. Effect of Dilution Rate on Chemical Composition of C. vulgaris. Biomass chemical composition of *C. vulgaris* varied in this study according to the imposed dilution rates

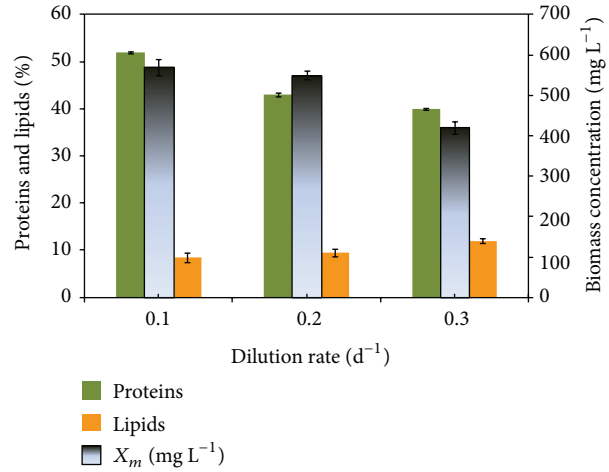

Figure 2: Proteins, lipids, and biomass concentration of *C. vulgaris* biomass grown on continuous cultivation as function of the dilution rate.

condition. The protein content decreased when the dilution rate increased from 0.1 to 0.3 day^{-1} (Figure 2). Its highest content (52.0%) was obtained at lowest dilution rate of 0.1 day^{-1} associated at first stationary phase where the biomass concentration is at the maximum level. Moreover, in this stationary growth phase, fresh culture medium is supplied to the homogeneously mixed culture with a high contribution of nitrogen supply. Such protein contents were much higher than those obtained by Zheng et al. [23] (34.1%) when cultivated *Chlorella vulgaris* in a closed photobioreactor and somehow higher (43.6%) than that observed by Sassano et al. [10] growing *A. platensis* by continuous process with dilution rate of 0.12 day^{-1}.

As far as the lipid content of biomass is concerned, the lipid content of cells varied from 8.1 to 12% at dilution rate in the range 0.1–0.3 day^{-1}. A progressive increase in D led to a clear increase in the lipid content, which achieved a maximum level of 12% at $D = 0.3$ day^{-1} (Figure 2). Eventually, in this lag phase, photosynthesis is still being performed and storage carbon products, such as starch and neutral lipid, are accumulated. Nonetheless, the lipid content in this present study was lower than that reported by Tang et al. [21] which observed a lipid content (38%) at $D = 0.328$ day^{-1} for *C. minutissima* continuous cultivation.

While the lipid content of the *C. vulgaris* biomass practically did not change with the change in dilution rate, there were differences noted in the fatty acids profiles of the total lipid fraction with dilution rate (Figure 3). The fatty acids found in the highest percentages were the palmitic acid

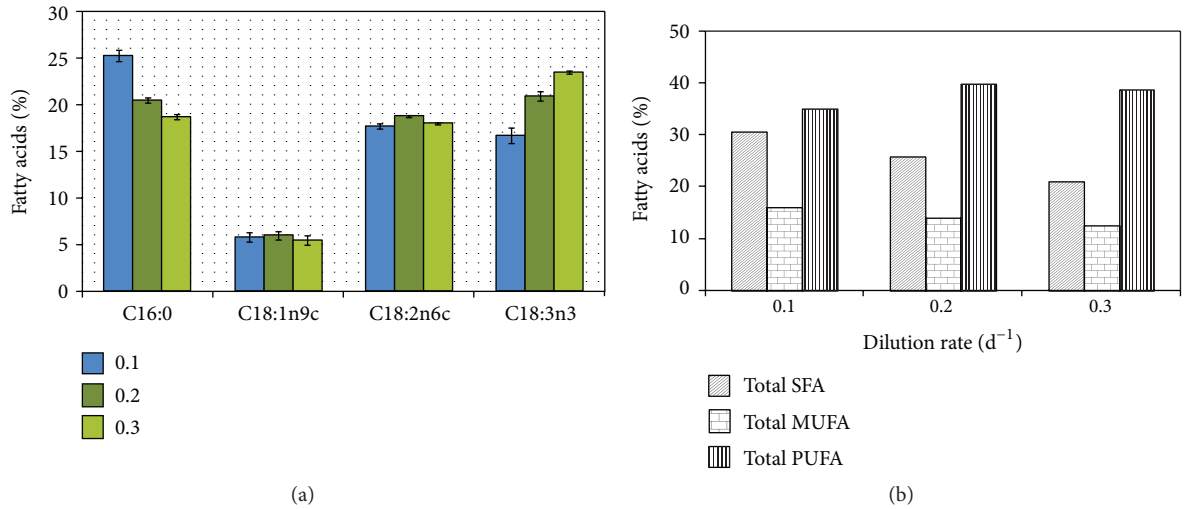

Figure 3: Effect of dilution rate on *Chlorella vulgaris* continuously cultivation in concentrated desalination as function of the fatty acids proportions. (a) The main fatty acids (FA) composition and (b) fatty acids proportions.

[C16:0] (25.3 ± 0.6%) at $D = 0.1$ day^{-1} and the gamma-linolenic acid [C18:3 n-3] (23.5 ± 0.1%) $D = 0.3$ day^{-1} (Figure 3(a)). What should also be noticed by these results is a clear predominance of fatty acids composed by 16 and 18 carbons in the carbon chain, as well as a certain equivalence between saturated and unsaturated ones (Figure 3(b)). The fraction of saturated FAME at lower dilution rate (from 0.1 to 0.2 day^{-1}) was more than that with the highest dilution rate of 0.3 day^{-1} (~30.5% versus ~21.0%). The γ-linolenic acid (C18:3 n-3c) composition with the highest dilution rate was significantly increased to 23.5% as compared with the one with the lowest dilution rate, while linoleic acid (C18:2 n6c) composition was the same ~18.0%. This observation is consistent with the results of Sobczuk and Chisti and is a consequence of dilution rate determining the average age of cells and thus affecting the fatty acid profile of algal cells [11].

The γ-linolenic acid (GLA 18:3 ω6) was the second most abundant fatty acid in *Chlorella vulgaris* biomass (23.5%), after the palmitic one. Polyunsaturated fatty acids (PUFAs) like linoleic (omega 6 fatty acid) and linolenic (omega 3 fatty acid), also known as essential fatty acids, were found to be high in *Chlorella vulgaris*. Considering that the sum of linoleic and γ-linolenic acids was approximately 41.5% at $D = 0.3$ day^{-1} of the total fatty acid content, an idea of the importance of these acids for the cell is evident. These essential fatty acids are an obligatory dietary requirements for humans and animals [24]. Most human diets are deficient in γ-linolenic acids. GLA has been associated with several beneficial health effects, such as LDL (low-density lipoproteins) reduction, precursor of C_{20} eicosanoids (prostaglandins, leukotrienes, and thromboxanes), anti-inflammatory effects, and among others [25]. It is noteworthy also the fact that the γ-linolenic acid is a widely recognized food supplement, in which fermentation of *Chlorella vulgaris* using photobioreactor by continuous process may be economically viable for such specialty product. On the other hand, due to its high capital and operating costs, however, fermentation of *Chlorella* may be economically viable only for high-value particularity products (γ-linolenic acid, pigments, and protein) but not for large-volume commodity products like biofuels [26]. The same authors suggest that the continuous process under steady-state conditions enables the cell to maintain constant levels of such essential and somewhat valuable metabolites, which could be advantageous for their production and recovery from *C. vulgaris*. Taking into account that *C. vulgaris* is presently commercialized as human food integrator and, besides, has the GRAS certificate (generally recognized as safe), more effort should be made in the future to characterize the chemical composition of its biomass when growing in a culture medium based on concentrated desalination.

4. Conclusions

The continuous mode of operation under steady-state conditions was shown to be feasible alternative way to establish the relation between a culture medium based on concentrated desalination and *Chlorella vulgaris* biomass composition. The highest biomass concentration (570 ± 20 mg L^{-1}) was obtained at dilution rate of 0.1 day^{-1} associated with high protein content (52%). The lipid content of cells varied from 8.1 to 12%. The fatty acid profile was slightly affected under different dilution rates. At highest dilution rate, polyunsaturated fatty acids were observed, especially the gamma-linolenic and linoleic acids representing around 41.5% of the total lipid fraction. With further optimization, the chemical composition of *Chlorella vulgaris* by continuous process has a great potential to benefit the high-value constituents from algal biomass based on desired applications.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

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