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Chlorella vulgaris biomass production using brewery wastewater with high chemical oxygen demand

Julieta Lois-Milevicich[1] · Nahuel Casá[1] · Paola Alvarez[1] · Ricardo Mateucci[1] · Víctor Busto[1,2] · Marina de Escalada Pla[1,3,4]

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Abstract

The aim of the work was to use an effluent with high chemical oxygen demand (COD) from small brewery as substrate for the production of *Chlorella vulgaris* biomass. A non-axenic strain from a Patagonian river was employed, and the effects of COD and pH value on the microalga growth were studied through a central composite design. A medium with COD 18300 mg O_2 mL^{-1} and initial pH: 6.5 optimized microalga growth. With the optimal condition, *C. vulgaris* adapted rapidly to medium and stationary phase was attained at 75 h. Moreover, lack of illumination did not affect μ_{max}, neither final biomass concentration, while supplementation with BG11 enhanced the biomass productivity at 0.47 ± 0.07 g L^{-1} h^{-1}, and pigment contents at least four times respect to the heterotrophic mode. For the first time, a brewery wastewater with high COD could be successfully used as substrate for *C. vulgaris* production, without presenting inhibitions, which represents a significant advance contributing toward more sustainable promising perspectives.

Keywords Microalgae · Valorization of waste · Optimal conditions · Growth kinetics · Biomass productivity

Introduction

Microalgae have been studied due to their promising role in the development of more sustainable processes. One of the genera with the greatest number of possible applications is *Chlorella*. The biomass obtained from its harvesting can have different uses, as nutritional supplement for animals, obtaining lipids for biodiesel, extracellular polymers, bio fertilizer, among others (Raposo et al. 2010; Mata et al. 2012; Song et al. 2013; Xiao and Zheng 2016). The main components of *Chlorella* species are proteins (≈ 42–58) %, lipids (12–55) %, and carbohydrates (5–40) %, which can vary depending on the conditions of cultivation. The genus *Chlorella* is the most promising as *Chlorella* species are suitable for heterotrophic cultivation and are highly productive and contain high concentrations of proteins with nutritional value that can be used in feed for both terrestrial and aquatic animals (Safi et al. 2014; Ende and Noke 2019). Lipids from *Chlorella vulgaris* find application in energy (biofuel) production and in nutrition due to the omega-3 and omega-6 families. In addition, biolipids as carotenoids offer great potential as antioxidants with the favorable effects on health benefits (Anthony et al. 2018). Although growth and biomass can be significantly higher under heterotrophic conditions than under photoautotrophic conditions, it is generally considered that the protein content is lower in microalgae grown heterotrophically compared with ones produced autotrophically or mixotrophically (El-Sheekh et al. 2014; Gami et al. 2014; Ende and Noke 2019). According to Kong et al. (2020), the biosynthesis of chlorophylls and carotenoids in *C. vulgaris* 31 cells was reduced under mixotrophic and heterotrophic cultivations; however, lipid production was promoted significantly. They stated

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✉ Marina de Escalada Pla
marina@di.fcen.uba.ar

[1] Centro de Tecnologías Químicas, Departamento de Ingeniería Química, Facultad Regional Buenos Aires (FRBA), Universidad Tecnológica Nacional (UTN), Buenos Aires, Argentina

[2] Instituto de Nanobiotecnología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, (NANOBIOTEC – UBA/CONICET), Buenos Aires, Argentina

[3] Instituto de Tecnología de Alimentos y Procesos Químicos, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, (ITAPROQ-UBA/CONICET), Buenos Aires, Argentina

[4] Departamento de Industrias, FCEN, UBA, Ciudad Universitaria, 1482 Ciudad Autónoma de Buenos Aires, Argentina

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that biomass, pigment, and lipid production of *C. vulgaris* 31 could be regulated by controlling the trophic mode and carbon source.

Nutrient and water supplies for microalgae cultivation are the major cost-contributory factors (Farooq et al. 2013). Wastewater has been proposed as an alternative to more expensive organic carbon sources (Abreu et al. 2012). Different microalga species have been grown in wastewaters from diverse sources, such as municipal (Mennaa et al. 2015; Ge and Champagne 2016); piggery (Wang et al. 2010), dairy (Abreu et al. 2012), dairy mixed with pulp, and paper wastewater (Gentili 2014); and distillery/brewery wastewater (Travieso et al. 2008; Mata et al. 2012). Nevertheless, the major concern for microalgae survivability has been to adjust the chemical oxygen demand (COD) of the effluents, since the high content of carbon in the wastewater inhibits microalgae growth (Wang et al. 2010, 2016). Moreover, in food waste digestates, high turbidity may inhibit cell growth while high ammonia levels can be toxic for microalgae (Nwoba et al. 2019). Gupta et al. (2017) assayed the tolerance of *Chlorella pyrenoidosa* to different COD concentrations at three dilutions of artificial wastewater (initial COD concentrations 5000, 3000, and 1000 mg L^{-1}). They reported optimal results for 1000 mg L^{-1} COD wastewater showing a steady increase in cell biomass, whereas in other samples (COD: 3000 and 5000 mg L^{-1}), the growth ceased no matter how much microalgal inoculum was initially added. They stated that the possible reason for that could be substrate inhibition, which is predominant in the wastewater at high concentration of COD and a high percentage of microalgae (COD 5000 mg L^{-1}; 0.3 g L^{-1} of microalgae).

According to the European Commission (EC) on the implementation of the Circular Economy Action Plan (2019), the circular economy is now an irreversible, global mega trend. Circularity means adapting industrial processes. Aspects as energy consumption, material use, waste prevention, recycling, and reduction of hazardous chemicals should be introduced in the adapted processes. Efficient waste management systems are an essential building block of a circular economy. One of the challenges has not been completely solved is to find out process to turn wastewater into new raw material saving the use of drinking water for dilution of COD. In this direction, Nwoba et al. (2019) proposed effluent recycling in microalgae-based treatment of high-level of ammonia. The EC also considered that small and medium-sized companies are the core of this transition of adapting industrial processes.

Annual sale of beer in Argentina is more than 20 million hectoliters, from which 2.2% is represented by micro and small breweries (Civitaresi et al. 2017; Ministerio de Agroindustria 2017). The beer industry generates wastewater from production, washing, and others, between 2 and 8 L per liter of beer produced, which have a chemical oxygen demand between 2000 and 6000 mg L^{-1} (Seluy and Isla 2014). The effluents of micro and small breweries might contain higher carbon loads. In this aspect, conversion of wastewater in new feedstock would ensure nutrients present in these streams to rejoin the economy.

The aim of the present work was to use the effluent from small brewery as substrate for the production of *C. vulgaris* biomass. A non-axenic strain from a Patagonian river was employed and the effects of the concentration of beer effluent and the pH value on growth were studied, detecting the conditions that optimized it. The growth curve of the microalga, biomass, lipid, protein, and pigments (chlorophyll and carotenoids) contents was also determined under optimal conditions.

Material and methods

Microalgae

A non-axenic *Chlorella vulgaris* strain LPMA39 was obtained from the Microalgae Laboratory of Universidad Nacional Patagónica San Juan Bosco from the Chubut River and is maintained by the Biotechnological Process Laboratory of UTN-FRBA in BG-11 medium (Stanier et al. 1971), which was prepared with analytical grade salts, analytical grade NaOH for pH adjustment, and membrane-deionized water.

Brewery wastewater

The brewery wastewater (BWW) was supplied by the brewery "Juguetes Perdidos," located in the department of Tres de Febrero, Buenos Aires. BWW was collected from piping purge of the wort boiling process. All wastewater batches presented a COD of 32,000 ± 3000 mg O_2 L^{-1}. They were pooled, characterized, and kept at − 18 °C until utilization. Physicochemical characteristics, along with the standard methods declared, are summarized in Table 1. The BWW was thawed in warm water at 30–40 °C prior to use.

Systems preparation

Culture media were prepared following the compositions and pH values determined by a central composed design (Table 2). COD values were adjusted by dilution with maintenance culture medium BG-11. Systems of 30 mL were placed in Erlenmeyer flaks of 125 mL capacity and sterilized for 20 min at 121 °C, in order to have a better control over the microbiological load after inoculation. After cooling, systems were inoculated with 3 mL of *C. vulgaris* suspension, with an inoculum in BG-11 medium of $OD_{680} \approx 2.0$. Erlenmeyer flaks were incubated at 26 ± 2 °C at an irradiance of 50 μmol photons m^{-2} s^{-1} PAR (measured with sensor PAR LP471

Table 1 Physicochemical characterization of the "Juguetes Perdidos" brewery wastewater

Physicochemical characteristics	Average value	Analytical method
pH	6.1	SM 4500-H^+ B
Conductivity (μS cm^{-1})	877	SM 2510 B
Turbidity–sedimentable solids in 10 min [mL L^{-1}]	8	SM 4500 F
Turbidity–sedimentable solids in 2 h (mL L^{-1})	17	SM 4500 F
Total organic carbon (mg-C L^{-1})	16,484	SM 22[a] ed. Met. 5310 B
Total nitrogen (mg-N L^{-1})	1238	Kjeldhal (catalyser Devarda)
Total phosphorus (mg-P L^{-1})	95.2	ICP-OES
Nitrate (mg-N L^{-1})	< 10	UNE-EN ISP 10304-1
Nitrite (mg-N L^{-1})	< 0.01	SM 4500-NO_2
Ammonia (mg-N L^{-1})	11.3	Ammonia test kit HACH. Salicylate Method

connected to a radiometer Delta Ohm HD2302.0), using cool white fluorescent tubes, and 100 rpm continuous orbital shaking (Cole Parmer, model OS-200, USA). Incubation was performed for 20 days, taking samples for measurement at 0, 6, 13, and 20 days.

Microalga and total mesophilic aerobic microorganism measurement

Microalga concentration was measured by means of optical density at 680 nm wavelength (OD_{680}) in a UV-visible spectrophotometer (Shimadzu, model UV-1700, Japan) according to Kuo et al. (2015) and Wang et al. (2010). Cell count was also performed with a Neubauer chamber as recommended by Raposo et al. (2010).

Total mesophilic aerobic microorganisms in the non-axenic *C. vulgaris* consortium were determined by direct streaking in Petri dishes containing Plate Count Agar (PCA), using serial dilutions when necessary. Petri dishes were incubated for 48 h at 36 ± 1 °C (APHA 1998).

Microalga growth curve

According to optimal conditions, different systems were prepared in order to characterize the growth curves of *C. vulgaris*. BWW was diluted with deionized water or BG-11 medium as detailed following.

- System 1: BWW diluted with deionized water and exposed to 12 h light cycle;

Table 2 Central composite design with two independent variables, COD and pH at five levels and central point in triplicate. Dependent variables: microalga growth measured through variation of ΔOD_{680} and changes in mesophilic aerobic microorganism (ΔCFU mL^{-1}) at 6, 13, and 20 days of incubation. Values are reported as mean ± SD (n = 2)

[1,2]COD Effluent	[2]Initial pH	$\Delta OD_{6d\text{-}0d}$	$\Delta OD_{13d\text{-}0d}$	$\Delta OD_{20d\text{-}0d}$	[3]$\Delta CFU_{6d\text{-}0d}$	[3]$\Delta CFU_{13d\text{-}0d}$	[3]$\Delta CFU_{20d\text{-}0d}$
9150 (0)	7.2 (0)	2.0 ± 0.1	3.5 ± 0.4	4.1 ± 0.2	1.8 ± 0.1	2.9 ± 0.1	1.1 ± 0.1
9150 (0)	7.2 (0)	1.9 ± 0.2	3.0 ± 0.3	5.9 ± 0.5	2.4 ± 0.1	2.8 ± 0.1	1.6 ± 0.1
9150 (0)	7.2 (0)	2.1 ± 0.1	2.4 ± 0.8	5.1 ± 0.4	2.4 ± 0.1	2.9 ± 0.1	1.6 ± 0.2
9150 (0)	6.5 (− 2)	2.6 ± 0.1	3.9 ± 0.5	6.5 ± 0.5	2.5 ± 0.1	2.9 ± 0.1	0.73 ± 0.09
9150 (0)	7.9 (+ 2)	2.6 ± 0.1	3.1 ± 0.6	5.2 ± 0.1	1.3 ± 0.2	1.3 ± 0.1	1.1 ± 0.2
4750 (− 1)	6.8 (− 1)	1.01 ± 0.03	2.8 ± 0.3	3.2 ± 0.1	2.72 ± 0.05	3.10 ± 0.02	3.09 ± 0.02
4750 (− 1)	7.5 (+ 1)	0.695 ± 0.002	2.6 ± 0.6	6.3 ± 0.5	2.53 ± 0.02	3.00 ± 0.01	3.03 ± 0.04
13,700 (+ 1)	6.8 (− 1)	2.8 ± 0.1	4.9 ± 0.4	8 ± 1	2.5 ± 0.6	2.76 ± 0.04	1.2 ± 0.3
13,700 (+ 1)	7.5 (+ 1)	2.7 ± 0.2	4.5 ± 0.5	6.1 ± 0.5	3.0 ± 0.2	1.6 ± 0.2	1.2 ± 0.1
180 (− 2)	7.2 (0)	0.9 ± 0.2	1.8 ± 0.2	2.6 ± 0.1	1.5 ± 0.1	1.3 ± 0.1	0.34 ± 0.07
18,300 (+ 2)	7.2 (0)	2.8 ± 0.2	5.049 ± 0.001	7.6 ± 0.6	1.5 ± 0.1	1.7 ± 0.1	0.51 ± 0.06

[1] *COD* chemical oxygen demand

[2] Coded values between brackets

[3] $\Delta CFU = (\log N - \log N_0)$. N, total mesophilic aerobic microorganisms, N_0, initial mesophilic aerobic microorganisms

- System 2: BWW diluted with deionized water and incubated in darkness;
- System 3: BWW diluted with BG-11 medium and exposed to 12 h light cycle.

Erlenmeyer of 250 mL, containing 60 mL of medium, was sterilized at 121 °C for 20 min. The 3 systems were incubated for 6 days at 26 ± 2 °C, with 100 rpm orbital shaking and an irradiance of 50 μmol photons m^{-2} s^{-1}, for 12 h photoperiod, using cool white fluorescent tubes, when applied.

Samples were taken during the incubation time for OD_{680} and cell count determinations.

The modified Gompertz equation was used in order to model the microalga growth through OD_{680} or cell count data (Zwietering et al. 1990):

$$\ln(N/N_0) = C.\exp\{-exp[(\mu_{max}/C).(lag-t)+1]\} \quad (1)$$

where N/N_0 represents biomass change determined by OD_{680} or cell count; C is the maximum change achieved, logarithmic value, μ_{max} is the maximal specific growth rate (h^{-1}), lag is strain lag phase time (h) and t is incubation time (h).

Every measurement was performed at least twice and growth curves were run two times each.

Study of biochemical composition of biomass

New batches of the three systems proposed were carried out. A fourth system composed by BG-11 medium without BWW and exposed to 12 h light cycle was analyzed as control. After 6 days of incubation at 26 ± 2 °C and 100 rpm orbital shaking, biomass was collected by centrifugation (Dragon Lab, mod D2012) at 6700 × g for 5 min. The pellet was washed with deionized water and centrifuged again. Dry weight was determined in accordance with Raposo et al. (2010) with a slight modification: a volume of 50 mL was centrifuged, washed, and dried at 90 °C until constant weight. Aliquots containing approximately 3 mg of dry biomass were centrifuged and the biomass obtained was used in the biochemical determinations. Lipid content was determined in centrifuged biomass by sulpho-phospho-vanillin method (SPV) described in (Mishra et al. 2014), using canola oil (Krol™, BA, Argentina) as standard. Protein content was determined as stated by Rearte et al. (2018). Briefly, pellet from centrifuged microalgae (≈ 3 mg) was hydrolyzed with NaOH 1 M at 95 °C for 1 h. Protein content was determined in the supernatant by the method described by Lowry et al. (1951), using BSA as standard. Chlorophyll and carotenoids were determined after extraction from centrifuged biomass with 90% acetone and ultrasound application (Qsonica, model Q55), running a spectral scanning of the extract between 400 and 700 nm wavelength and applying the following eqs. (2) and (3) (Wegmann and Metzner 1971). Results were expressed as a percentage of dry weight (dw) biomass.

$$Chl\ a\ (\mu g\ mL^{-1}) = 10.3.abs_{663nm} - 0.918.abs_{644nm} \quad (2)$$

$$\text{Carotenoids}\ (\mu g\ mL^{-1}) = 4.20.abs_{453nm} - 0.0264.[Chl\ a\ (\mu g\ mL^{-1})] \quad (3)$$

Experimental design and statistical analysis

As a means to evaluate the influence of COD and pH of substrate on microalgal growth, a central composite design (CCD) with two factors (independent variables) and at five levels (Table 2) was performed. The selection criterion for the lowest level for COD and the highest level for pH were determined based on preliminary results (not shown). The central point (0;0) was performed in triplicate. Table 2 shows all experimental runs. Dependent variables, ΔOD represents the OD_{680} differences and ΔCFU is referred to the differences in mesophilic aerobic microorganisms cell count, $\log(CFU\ mL^{-1})_t - \log(CFU\ mL^{-1})_0$. Both differences are respect to initial point, being the moment of inoculation, "zero time." The dependent variables were fitted to second degree polynomial eq. (4) with a multiple regression procedure:

$$\Psi = B_0 + B_1x_1 + B_2x_2 + B_{11}x_1^2 + B_{22}x_2^2 + B_{12}x_1x_2 \quad (4)$$

where Ψ is the dependent variable analyzed, x_1 and x_2 are independent (COD and pH) variables that affected Ψ value, B_0 is the value of the fitted response at the center point of the design ($x_1 = 0$ and $x_2 = 0$), B_1 and B_2 are the linear coefficients, B_{11} and B_{22} are the quadratic coefficients, and B_{12} is the cross coefficient between factors. This equation allowed to evaluate the effects of linear, quadratic, and interaction terms of independent variables on selected dependent variables (Montgomery 2008). The statistical significance ($p \leq 0.05$) of the coefficients in the regression equations was analyzed using analysis of variance (ANOVA), with a significance level of 95.0%. The adequacy of the model was evaluated through the coefficient of determination (R^2), adjusted R^2 and lack of fit test ($p \geq 0.05$) statistic. The desirability function was used to simultaneously optimize both responses (Nieto Calvache et al. 2016).

Statistical analysis of results was performed through ANOVA for a level of significance of 95% followed by a Tukey's multiple comparison post-test to identify significant differences between samples. The non-linear growth modeled curves were validated with (R^2_{adj}) greater than 0.95 and a Durbin Watson (DW) value greater than 1. The experimental design and the corresponding analysis as well as non-linear

regression were performed using the Statgraphics Centurion XV program (V 2.15.06, 2007, USA). For statistical analysis of results, Prism 5 V 5.01, 2007, (GraphPad Prism Software Inc., USA) was used.

Results

Experimental design

As it can be observed in Table 2, *C. vulgaris* could grow in all systems since a positive value for ΔOD was obtained in all cases. Moreover, generally, the highest ΔOD was observed at day 20, which represents that microalga growth did not cease, even though when high COD wastewater was used. On the other hand, the ΔCFU mL^{-1} of the total aerobic mesophilic bacteria belonging to the consortium that accompanies the microalga also grew until day 13, from that moment onwards the number of bacteria decayed, showing the lowest value at 20 days of cultivation in all systems.

A positive effect ($p < 0.05$) of COD on *C. vulgaris* growth can be observed over the cultivation period on Pareto's Charts (Fig. 1a, b, and c), while in the case of bacteria growth, only pH had a significant ($p < 0.05$) and negative effect at 13 days of cultivation (Fig. 1d). Significant ($p < 0.05$) quadratic effects were also observed for the independent variables on microalgal growth during the first 6 days (Fig. 1a). COD showed a negative quadratic effect, which means that there is a value within the evaluated range that maximizes the microalgal growth. Positive quadratic effect was observed for the initial pH ($p < 0.05$). In addition, a significant and antagonistic interaction between both independent variables

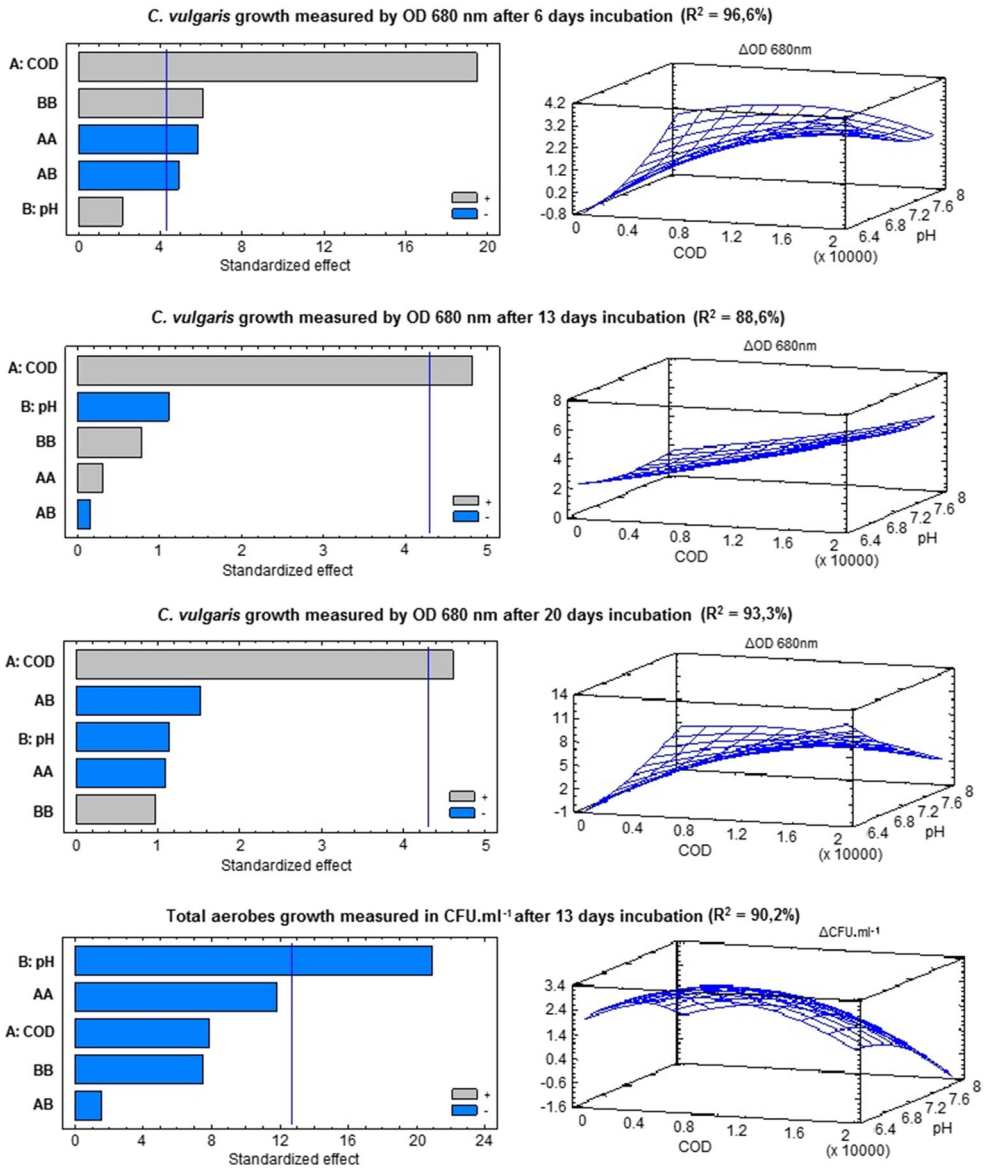

Fig. 1 Effect of COD and pH of BWW medium on *C.vulgaris* growth and on mesophilic aerobic microorganism belonging to the microalga consortium for different incubation time

was recorded. This indicates that the increase in the value of one of them partially compensates the main linear effect of the other. This behavior can be better observed on the response surface (Fig. 1a). The coefficients for the regression equations that represent the response surfaces, shown in Fig. 1, are also supplied as supplementary data (online resource).

From these analyzed results, the optimal, maximum values for microalgal growth, and minimum values for the growth of associated aerobic microorganisms were determined. The desirability function optimized several responses simultaneously. Two possible optimizations were tested and are shown in Table 3. The multiple optimization 1 considered initial wastewater concentration and pH values, to maximize growth of the microalga at 6, 13, and 20 days simultaneously, and the multiple optimization 2 considered initial wastewater concentration and pH values, to maximize growth of the microalga at 6, 13, and 20 days, and to minimize total aerobes growth at 13 days, simultaneously. In order to corroborate the statistical estimated optimal conditions, the new systems were tested and are also shown in Table 3. In general, the experimental responses were better than those statistically predicted (Table 3). However, in many cases, since the results obtained differed from those predicted, the initial design was fed back with the new systems. It should be also noted that in these new tested systems, no significant differences (one way ANOVA, $p > 0.05$, $n = 3$) were observed in the OD values at different incubation times. This leads to infer that under these optimal conditions, the microalga is able to grow more rapidly, reaching the stationary phase within 6 days. It must be highlighted that during the first 6 days, no significant effects of any independent variable on the growth of total aerobic microorganisms were observed. Then, with the feedback design, a new optimization was carried out in order to maximize the growth of *C. vulgaris* at 6 days of incubation. An optimal system with COD: 18300 mg O_2 mL^{-1} and initial pH: 6.5 was proposed, and statistical estimation response for ΔOD_{6d} was 7.8. These parameters are similar to those obtained experimentally for multiple optimization 2 (Table 3).

Growth kinetic characterization

Subsequent kinetic characterization was carried out taking into account these conditions. The microalgal growth curves determined with different diluents and also under darkness are shown in Fig. 2. The fitted parameters to the modified Gompertz model, C, μ_{max}, and lag period were also inserted in each curve. As it can be observed, the experimental values fitted adequately, R^2_{adj} (> 0.99) and DW (> 1.82), for the OD_{680} (Fig. 2a, b, and c) and R^2_{adj} (> 0.92) and DW (> 1.58) for the microscopic cell count in Neubauer chamber (Fig. 2d, e, and f). A similar profile was observed in all cases obtaining specific growth rates, μ_{max}, in the range of 0.15–0.17 h^{-1} and 3.6–4.1 day^{-1} and C values in the range of 2.7–3.2. It must be pointed out that lag phase could not be registered when cell count data was used to model the microalga growth. Nevertheless, a lag phase, of 7.0–9.2 h was registered when OD_{680} data was used instead of cell count. The curve registered in darkness tended to have a longer lag phase (Fig. 2b). Cell count rendered curves with a tendency to higher C and μ_{max} values, when medium was supplemented with BG-11 (Fig. 2f).

Biomass concentration, productivity, protein, lipid, and pigment contents

Figure 3a shows the results of the final biomass concentration and productivity, measured on the sixth day of cultivation. As it can be observed, the three systems assayed had higher biomass concentrations (1.68 ± 0.02–2.8 ± 0.4 g L^{-1}) and productivity (0.280 ± 0.003–0.47 ± 0.07 g L^{-1} day^{-1}) than the control system with photoautotrophic mode (0.46 ± 0.05 g L^{-1} and 0.08 ± 0.02 g L^{-1} day^{-1}). System 3 presented the highest

Table 3 Multiple optimizations using desirability function. Statistical estimated responses and experimental values reported as mean ± SD (n = 3)

Independent variables					Dependent variables			
Desirability			COD	pH	ΔOD_{6d-0d}	ΔOD_{13d-0d}	ΔOD_{20d-0d}	ΔCFU_{13d-0d}
Multiple optimization 1	Estimated optimal value	1.00	15,800	6.6	3.32	5.11	9.29	-
	Experimental value	-	-	-	5.5 ± 0.7	4.99 ± 0.03	5.5 ± 0.1	-
Multiple optimization 2	Estimated optimal value	0.95	18,300	7.0	2.55	5.03	7.00	1.34
	Experimental value	-	-	-	6.7 ± 0.4	7.1 ± 0.2	7.5 ± 0.8	0.70 ± 0.04

Multiple optimization 1, optimal system considers initial wastewater concentration and pH values, to maximize growth of the microalga at 6, 13, and 20 days simultaneously

Multiple optimization 2, optimal system considers initial wastewater concentration and pH values, to maximize growth of the microalga at 6, 13, and 20 days, and to minimize total aerobes growth at 13 days, simultaneously

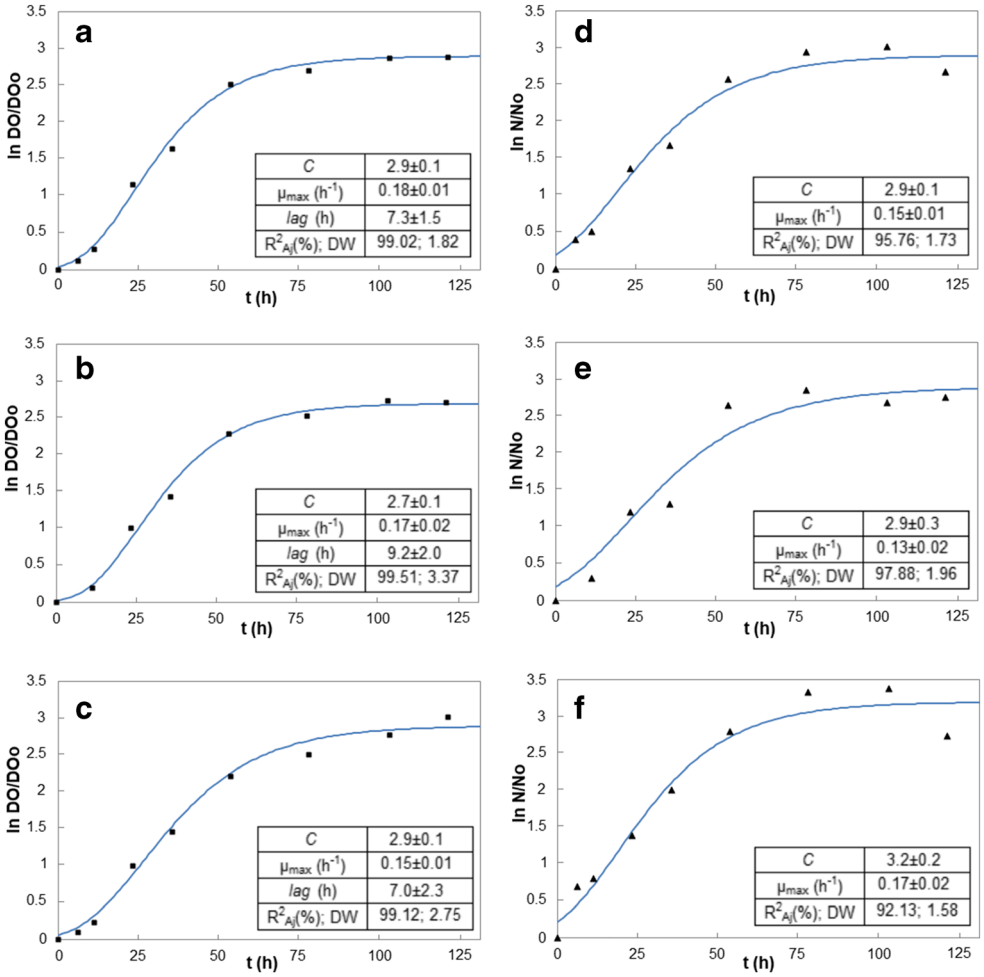

Fig. 2 Growth curves: **a**–**c** measured through OD_{680}; **d**–**f** measured through cell count. **a** and **d** System 1: effluent + water + light cycle; **b** and **e** system 2: effluent + water + darkness; **c** and **f** system 3: effluent + BG11 + light cycle. Square and triangle dots represent experimental values, while solid lines represent the fit to modified Gompertz model. $\ln(N/N_0) = C.\exp\{-exp[(\mu_{max}/C).(lag-t)+1]\}$. Fitted parameters are shown in inserted tables as estimated value ± $SE_{asymptotic}$, α: 95%

values (one way ANOVA, $F_{3,8} = 34$ for concentration and $F_{3,8} = 29.81$ for productivity, $p < 0.0001$, $n = 3$), while no differences between systems 1 and 2 were detected ($p > 0.05$). The biomass obtained was mainly composed by protein and lipids, being in the range of 30 ± 5–50 ± 8% dw for protein content while lipids content ranged within 7.5 ± 0.9–31 ± 4% dw (Fig. 3b). Regarding lipid content, systems 1 and 2 presented higher content than control (one way ANOVA, $F_{3,8} = 10.61$, $p = 0.004$, $n = 3$); without observing significant differences among systems formulated with BWW ($p > 0.05$). As regards protein content, no significant differences were detected except between system 1 and the control (one way ANOVA, $F_{3,8} = 5.17$, $p = 0.03$, $n = 3$). Regarding photosynthetic pigments, chlorophylls, and carotenoids, a significant difference was observed when comparing system 3 and the control to systems 1 and 2 (Fig. 3c) (one way ANOVA, $F_{3,8} = 213$ and $F_{3,8} = 300$ for chlorophylls and carotenoids respectively, $p < 0.001$, $n = 3$). Among systems formulated with BWW, system 3 presented the highest pigment content 1.91 ± 0.08 and 1.02 ± 0.03% m/m dw for chlorophyll and carotenoids respectively, being these values 4 times higher than those corresponding to the systems 1 and 2.

Discussion

Mixotrophy is a culture mode in which microalgae can drive both photoautotrophy and heterotrophy utilizing both inorganic and organic carbon sources (Kang et al. 2004). Heterotrophic production of *Chlorella* can be performed in a closed system with a better control of contamination from other microorganisms, as well as optimization of culture conditions to maximize the biomass yield (Ende and Noke 2019). Although systems prepared in this work were sterilized previously, the non-axenic strain is accompanied with aerobic microorganism. In the present work, *C. vulgaris* could continuously grow for days while aerobic microorganism count decayed. Therefore, it might be inferred that these bacteria could be controlled by the microalgal growth in the conditions herein assayed. It also has been reported previously that *Chlorella* strains, which were present in a consortium, were very competitive with other microalgae and presented biocidal activity against microorganisms (Hong et al. 2014; Marchão et al. 2018). Moreover, the optimal conditions to maximize the microalgal growth minimizing aerobic bacteria proliferation were found in the highest COD assayed, 18,300 mg O_2 mL^{-1}.

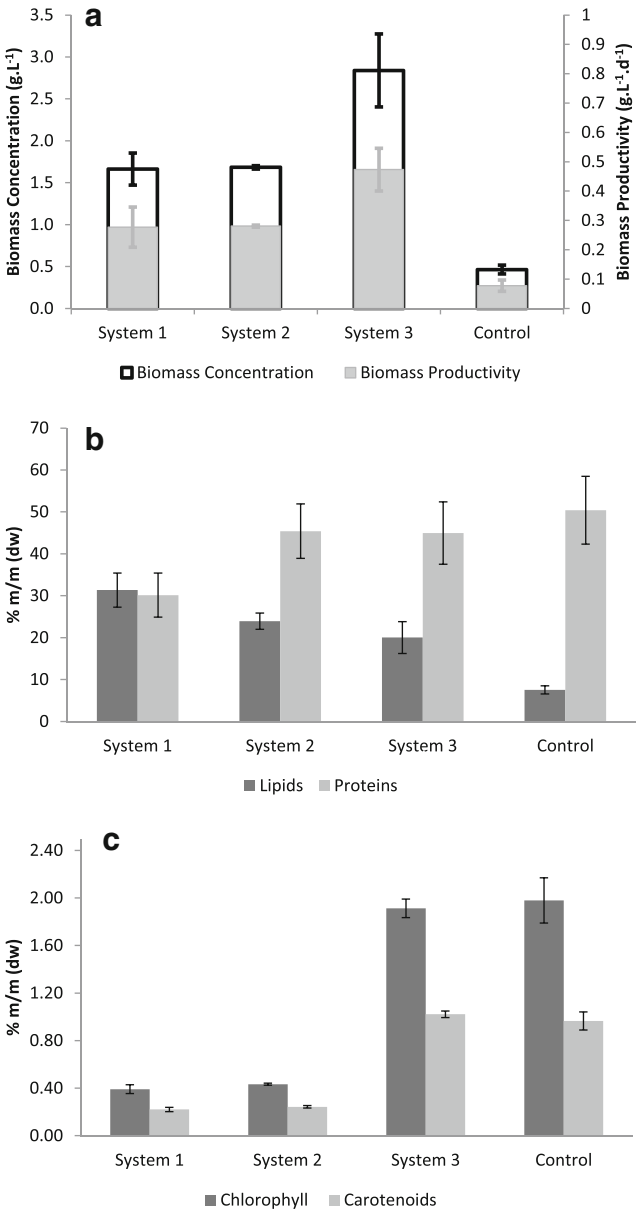

Fig. 3 **a** Biomass concentration and productivity; **b** lipid and protein; **c** chlorophyll and carotenoids content of *C. vulgaris* biomass obtained after 6 days cultivated in different systems. System 1: BWW + water + light cycle; system 2: BWW + water + darkness; system 3 BWW + BG11 + light cycle and control system: BG11 + light cycle. Bars reported means ± SD ($n = 3$)

This result is relevant since according to previous data, high COD might also inhibit microalgal growth. For instance, *C. pyrenoidosa* growth was inhibited by 5000 mg L^{-1} COD (Gupta et al. 2017). Some components of the effluents can be toxic, as ammonia (NH_3) considered inhibitory to *Chlorella* sp. in concentrations equivalent to 17 and 143 mg L^{-1} (He et al. 2013). The type of carbohydrates present in an effluent can also affect the growth of algal cells. Xylose did not stimulate *C. vulgaris* 31 growth nor lipid biosynthesis, while glucose and maltose promoted growth and lipid accumulation (Kong et al. 2020) in mixotrophic and heterotrophic mode. In the present work, effluent came from the piping purge of the wort boiling process. Therefore, it can be inferred that maltose and glucose might be the main carbon load (He et al. 2014). In addition, low ammonia content was registered in the BWW and the main nitrogen source was mainly from protein (Table 1). These facts may explain partially that *C. vulgaris* could grow without inhibition in this medium despite the high COD load. Furthermore, Ende and Noke (2019) stated that, if proteins are present in the substrate material, Maillard reaction could occur with sterilization, reducing bioavailability of amino acids and sugars. Nevertheless, the *C. vulgaris* strain adapted rapidly to the medium formulated with the effluent herein, as it can be observed with the absence of lag phase in the grow curve (Fig. 2d, e, and f), modeled with the count data from Neubauer's chamber. It is interesting to remark that the observation in the change in optical density was delayed approximately 7 h. This phenomenon could be explained because of the immediate beginning of cell division, which increases the number of cells; however, there is no appreciable change in optical density, which is measured in the chlorophyll absorption zone, until the biomass grows, and therefore, their total pigment content is enhanced (Fig. 2). The stationary phase was reached, in all cases, before 4 days (≈ 75 h) as it was inferred previously. It can also be observed that the values of C and μ_{max} for OD_{680} growth curves, generally, match those obtained from the results of the cell count in the Neubauer chamber. This would validate that the variation in OD_{680} was mainly associated with the growth of *C. vulgaris*.

It is interesting to note that the specific growth rate herein determined was ≈ 3 times higher than those reported by Farooq et al. (2013) of 0.97 day^{-1} for *Chlorella* sp. in BWW with COD lower than 3000 mg $O_2.L^{-1}$ and 0.839 day^{-1} for *Chlorella* sp. GD by Kuo et al. (2015) for a piggery wastewater (COD lower than 500 mg O_2 L^{-1}). Kong et al. (2020) reported that specific growth rate of *C. vulgaris* 31 increased in mixotrophic and heterotrophic mode when glucose (0.390–0.523 day^{-1}) and maltose (0.447–0.507 day^{-1}) were added as carbon sources. When comparing with other microalgae, Marchão et al. (2018) reported that the μ_{max} attained by *S. obliquus* in a BWW medium was 0.065 h^{-1} (1.5 day^{-1}) during the batch growth in the 6 L–photo bioreactor. These authors also reported a maximum cell concentration of 0.93 ± 0.01 g L^{-1} and the maximum biomass productivity of 156.80 ± 4.65 mg L^{-1} day^{-1}.

In the present work, higher biomass concentration and productivity were achieved in all systems prepared with BWW. In particular, in system 3, BWW was supplemented with BG-11 medium and cultured in mixotrophic mode. Taking into account that the stationary growth phase was attained before the 6 days (≈ 3 days), it can be inferred that the maximum productivity could be doubled if harvesting was to be

performed at the end of the exponential growth phase. Farooq et al. (2013) improved *C. vulgaris* (UTEX-265) productivity to 226.6 mg L^{-1} day^{-1} using BWW effluent in two stage photo-mixotrophic cultivation. Nwoba et al. (2019) reported a *Chlorella* sp. productivity of 0.2 g L^{-1} day^{-1} when cultivated in high-NH_3 food waste digestate, in batch, and fed-batch with and without recycling in a 5 L-photo bioreactor. Better biomass productivity (1.79 g.L^{-1} day^{-1}) was observed by Liu et al. (2013) for *Chlorella zofingiensis,* heterotrophically, in a cane molasses medium. Kong et al. (2020) stated that when *C. vulgaris* 31 was cultured in photoautotrophic control media, after 6 days, productivity was 0.061 g L^{-1} day^{-1} and similar to the value obtained for our control. They also observed that this value could be improved by supplementing the media with appropriate carbon source in mixotrophic and heterotrophic batch mode. The best results were obtained with glucose (0.226 and 0.191 g L^{-1} day^{-1}) followed by maltose (0.195 and 0.162 g L^{-1} day^{-1}) for mixotrophic and heterotrophic mode, respectively. These values are in concordance with data registered in the present work. It must be highlighted that BWW effluent came mainly from the wort boiling process. Standard brewery wort contains approximately 90% carbohydrates, among them sucrose, glucose, maltose, and maltotriose, with the two last the most abundant (He et al. 2014). When comparing systems 1 and 2, it was observed that exposure to light did not improve cell productivity. In addition, supplementation with BG-11 (system 3) raised the biomass productivity to 0.47 ± 0.05 g L^{-1} h^{-1}. It might indicate that under the tested conditions, using BWW as medium, *C. vulgaris* could adopt a fundamentally heterotrophic metabolism unless nutrients were supplemented with BG-11 addition.

In general, protein content of microalgae grown heterotrophically is lower when comparing with protein content in microalgae produced autotrophically or mixotrophically (Ende and Noke 2019). El-Sheekh et al. (2014) found around 600 mg g^{-1}dw of protein in *Chlorella* grown mixotrophically, whereas 400 mg g^{-1}dw of protein content was reported for heterotrophic mode, when using high concentrations of molasses. Despite the 12 h light cycle, system 1 had lower protein content than control (autotrophic) system, possibly due to lack of nutrients that, in the case of system 3, was compensated with BG-11 supplementation. System 2 tended to grow slower, as it can be observed through lag phase (Fig. 2b) or μ_{max} (Fig. 2e); therefore, starving conditions would not appear in the period tested. According to Ende and Noke (2019), the content of protein can be increased by harvesting at the right time. In *Chlorella* heterotrophically growth, the protein content approached a maximum value of ≈ 60% after glucose was consumed, and the cells entered the stationary phase. *Chlorella* can decrease the protein content and increase the weight percentage of lipids, when the medium is deficient in nitrogen (Ördög et al. 2012; Zhu et al. 2014a, 2014b). This trend can be observed when comparing systems 1, 2, and 3 with the control (Fig. 3b). Kong et al. (2020) stated that carbon sources affect lipid biosynthesis in *C. vulgaris* 31. For this strain, a lipid content of 7.76% was obtained with photoautotrophic culture, similar to the control system of the present work. They also reported that lipid biosynthesis was promoted by heterotrophic and mixotrophic cultivation with 2 g L^{-1} of maltose, achieving lipid contents of 25%. These data are in agreement with our results.

Photosynthetic pigments, chlorophylls, and carotenoids are important not only as bioactive molecules but also as indicators of the algal culture state. It has been suggested that high concentration of carbon source and the lack of light repress photosynthesis of the alga and make its trophic mode convert to heterotrophy (Kong et al. 2020). In addition, chlorophyll contents lower than 1% may indicate lack of nutrients in the culture (Veloso et al. 1991). In this work, supplementation with BG-11 medium generated percentages of pigments similar to those of the control with contents approximated to 2% m/m dw at 6 days of cultivation.

It can be concluded that BWW with high COD, 18300 mg O_2 mL^{-1}, could be successfully applied as substrate for *C. vulgaris* production, without presenting inhibitions. *Chlorella vulgaris* adapted rapidly to the medium and stationary phase was reached before 4 days. Moreover, lack of illumination did not affect μ_{max} or final biomass concentration, while supplementation with BG-11 enhanced biomass productivity and pigment contents. Further studies will be carried out in order to get deeper into the characterization of the biomass, aiming at assuring its right application for different purposes. The BWW could be converted to new feedstock, saving drinking water and ensuring that nutrients of these waste streams returned to the economy. It might improve resource efficiency and production processes, giving circularity especially to small and medium brewery producers.

E-supplementary data of this work can be found in online version of the paper.

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Authors' contributions JLM: conceptualization, formal analysis, investigation, and writing—original draft; **NC**: formal analysis and investigation; PA: methodology, investigation, and resources; RM: methodology, investigation, and resources; VB: project administration and funding acquisition; MEP: conceptualization, methodology, supervision, and writing—review and editing. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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