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Influence of Elevated CO_2 and Municipal Wastewater Feed on the Productivity, Morphology, and Chemical Composition of *Arthrospira (Spirulina) platensis*

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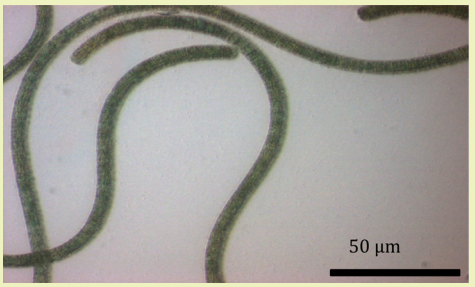
ABSTRACT: In this work, a marine strain of *Arthrospira (Spirulina) platensis* (CCMP1925) was submitted to high concentrations of municipal wastewater (WW) and carbon dioxide (CO_2). Under standard conditions (Zarrouk medium and optimal light), production reached 2.79 g/kg of culture after 8 days. Adding wastewater to the mixture led to an increased cell density reaching 3.29 g/kg below 40% wastewater, while above 40%, wastewater was shown to inhibit productivity. At an elevated CO_2 concentration fed to the mixture, production reached 3.55 g/kg, although increasing the concentration of CO_2 above 2% (v/v) was shown to inhibit the production of biomass. High concentrations of combined wastewater and CO_2 were generally shown to be detrimental for the production of algal biomass. However, some synergetic effects were observed for the optimal cell density that was obtained after 5 days for a mixture involving 40% wastewater and 8% CO_2, while after 8 days, the best results were obtained with a 40% WW mixture and 0% CO_2. A combination of elevated CO_2 and WW concentration (8% and 80%, respectively) led to poor cell growth, although the concentration in proteins, carbohydrates, and chlorophyll was generally higher when compared to the control cultures.



KEYWORDS: *Arthrospira platensis, CO_2, Wastewater, Productivity, Morphology, Chemical components*

INTRODUCTION

The increasing population density in major cities around the world has provided significant pressure on the water treatment facilities having to treat increasing amounts of wastewater (WW). Although many approaches are available to treat water, biological conversion remains a very efficient approach, and with the important growing demand of liquid fuel around the world, WW is getting increased attention as a cheap carbon-based feedstock to feed algae that could be used downstream as biomass for the production of biofuels. However, WW use may be a challenge for the production of algal biomass because of its high organic and mineral contents. Nevertheless, despite the complexity of the starting material, different works in the open literature have shown that the use of algal biomass could be very beneficial to remove contaminants from WW. As an example, Jiang et al. showed that *Chlorella vulgaris* could be used to remove traces of arsenate in water (up to 200 mg/L),[1] while Doke et al. reported that by using a *Spirulina* strain they managed to remove up to 95% of the BOD, 52% of nitrate, and 76% of phosphate, while reducing the bacterial count up to 75%.[2]

CO_2 is another readily available carbon-based feedstock that could be of interest as a feed to algae. Recent reports showed that the concentration of carbon dioxide reached a historical 400 ppm in the atmosphere in June 2013.[3] Thus, using it as a carbon source could transform a problem into an opportunity. Although it may not be the sole approach for the conversion of CO_2,[4] utilization of photosynthesis is a logical approach given the fact that the sun provides the energy required for the reduction of the very stable CO_2. A wide variety of plants could therefore act as CO_2 mediators, but algae have this net advantage of providing more biomass per unit of surface. Because CO_2 is one of their main feeds (depending on if they act in an autotrophic, mixotrophic, or heterotrophic fashion), it is usually considered as very efficient for biological conversion.[5] Numerous publications in the open literature tend to interact algae with high concentrations of CO_2. For example, Morais and Costa have shown that *Spirulina* sp. could achieve a daily biofixation of 53% of the carbon dioxide in a 6% mixture and 46% in a 12% mixture of CO_2 (v/v) in flue gas.[6] An even more favorable tendency was observed by Borkenstein et al. who fed *Chlorella emersonii* with CO_2 produced from the cement industry at a concentration in the magnitude of 15% in air.[7] The results showed that they were able to reach a density superior to 2 g/L with a growth rate reaching 0.13 g/L/d when industrial CO_2 was used directly in the culture, as compared to a reference culture feeding on pure CO_2 that showed a maximum growth rate of 0.10 g/L/d.

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Table 1. Components of Used Zarrouk and F1/2-si Medium

(A) modified Zarrouk medium		(B) F/2-si medium	
components	**quantity (g/L)**	**components**	**quantity (g/L)**
$NaHCO_3$	16.8	$NaNO_3$	0.075
K_2HPO_4	0.5	NaH_2PO_4 H_2O	0.005
$NaNO_3$	2.5	trace metal solution	1 mL
K_2SO_4	1	vitamin solution	0.5 mL
NaCl	1	**trace metal solution (g/L)**	
$MgSO_4{\cdot}7H_2O$	0.2	$FeCl_3$ $6H_2O$	3.15 g
$CaCl_2$	0.04	Na_2EDTA $2H_2O$	4.36 g
$FeSO_4{\cdot}7H_2O$	0.01	$CuSO_4$ $5H_2O$	0.0098
Na_2EDTA	0.08	Na_2MoO_4 $2H_2O$	0.0063
solution A5	1 mL	$ZnSO4$ $7H_2O$	0.022
solution A5 (g/L)		$CoCl_2$ $6H_2O$	0.01
H_3BO_3	2.86	$MnCl_2$ $4H_2O$	0.18
$MnCl_2{\cdot}4H_2O$	1.81	**vitamin/solution (g/L)**	
$ZnSO_4{\cdot}7H_2O$	0.222	thiamine HCl (vit. B_1)	0.0002
$CuSO_4{\cdot}5H_2O$	0.079	biotin (vit. H)	0.001
MoO_3	0.015	cyanocobalamin (vit. B_{12})	0.001

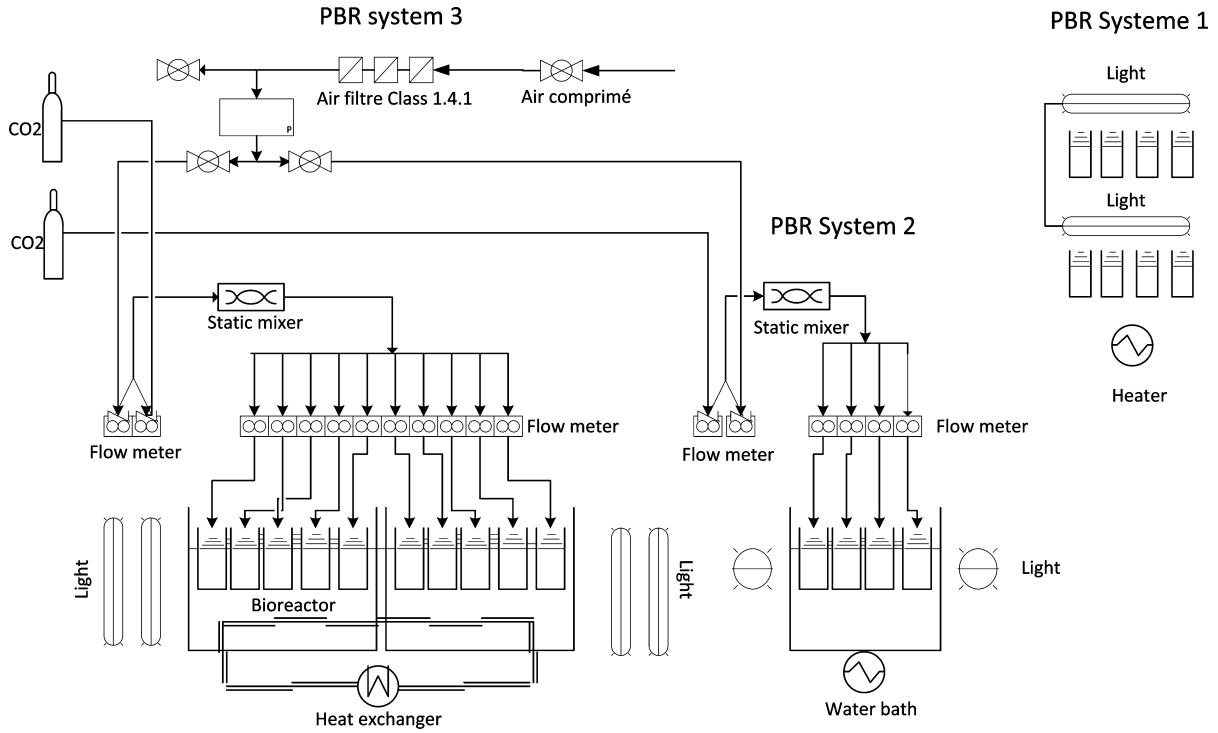

Figure 1. Process flow diagram of the three photobioreactor (PBR) systems used for cultivation of *Arthrospira platensis* in a CO_2−wastewater medium.

Another main interest of algal biomass is using it as feedstock for the production of biofuels. However, because fuel is a commodity with high demand and low value, it is crucial in order to put this industry forward that the price of the biomass be minimal because price is one of the most restrictive aspects of second and third generation biofuel production.[8] The utilization of both WW and CO_2 is a very interesting approach because it reduces cost of production while combining two problems into a valuable opportunity. Producing a low-value algal biomass may not lead to a suitable feedstock for the biodiesel industry, for example, because the lipid content may not be very high, but a low-priced carbon-based biomass could be a good candidate for thermal processes such as hydroliquefaction, pyrolysis, or gasification pending the use of an efficient dewatering process.

In this work, the effect of adding large concentrations of WW (0−80%) and CO_2 (0−8%) on the external and internal parameters of the species *A. platensis* was investigated. The algal biomass growth was monitored using dry weight and microscopy for each growth conditions, while chemical compositions (chlorophyll-a, total carotenoids, phycocyanin, proteins, and carbohydrates) were monitored using colorimetric techniques. In addition, the concentration of nitrate and phosphate in the solution was monitored using ICP-MS.

MATERIAL AND METHODS

Algal Strains and Growth Medium. A marine strain of *A. platensis* (CCMP1295) was purchased from the Provasoli-Guillard National Center for culture and marine phytoplankton. A fraction of the purchased sample (labeled CPF2 below) was adapted to the modified Zarrouk medium[9] (labeled CPZA below). Additionally, fresh-water strains of *A. platensis* (LB2340) and *A. maxima* (LB2342) were purchased from the culture collection of algae at the University of Texas in Austin (UTEX), which were labeled LB40 and LB42, respectively, in this research. The CPF2 strain was maintained in a F/2-si medium,[10] while all other strains were maintained in a modified Zarrouk medium that only differed from the original Zarrouk medium composition by the lack of the B6 microelement. However, the apparent difference for growth yield with and without the B6 solution could be considered as trivial because the difference from reported results was 0.03 unit of optical density according to Zarrouk, corresponding to an algal biomass density of 0.002 g/L. The detailed information on used mediums is provided in Table 1.

Wastewater Sampling and Composition. Municipal wastewater was sampled at the Sherbrooke municipal wastewater treatment plant (Sherbrooke, Canada). Samples were taken after decantation, before biofiltration on March 31, 2011, at 2:30 pm. According to the information provided by the plant, the water contained 31 mg/L of suspended solids. The BOD_5 value was of 22 mg/L, and total COD was 60 mg. The amount of nitrogen found as ammonium ions in the wastewater reached 11.1 mg/L. The samples were then transported to our laboratory at Université de Sherbrooke and decanted for a few hours. After isolation of the solid fraction, the supernatant was sterilized at 121 °C for 1 h in an autoclave, after which samples were filtered through a GF/G6 filter (1 μm) followed by a 0.45 μm micropore membrane filter before finally being autoclaved one more time at 121 °C for 1 h. Nitrogen and phosphate were quantified based on the U.S. Environment Protection Agency (EPA 2008) test methods. Results showed a relatively low nutrient content with 38.7 mg/L and <0.5 mg/L for phosphate and nitrate, respectively. The heavy metal content was analyzed using ICP-MS (Perkin-Elmer Sciex, Elan DRC II), and while most metal concentrations were found to be below 10 ppb, results also showed relatively high concentrations of aluminum Al(III), reaching 168 ppb.

Photobioreactors (PBR) and Growth System. Figure 1 presents the process flow diagram for photobioreactors (PBR) that were designed, assembled, and used in this study. The PBR system 1 was conceived for maintenance of the algal source. The latter was operated at atmospheric pressure using a light source provided from above at an intensity of 30 μEinstein m^{-2} s^{-1} from one cool-white fluorescent lamp (GE, Ecolux, 4100 K). The second system (PBR system 2) was used essentially for inoculum preparation and small-scale experiences such as strain selection. It can contain up to four 1 L erlenmeyers simultaneously, and lighting was provided from above as well at an intensity of 78 μEinstein m^{-2} s^{-1} from two cool-white fluorescent lamps (GE, Ecolux, 4100 K). Finally, the last system (PBR system 3) was designed specifically for the central part of this research. It was composed of 10 2 Lerlenmeyers installed in two glass tanks separated in two rows. Illumination was provided through both sidewalls of the tank with eight cool-white fluorescent lamps (GE, Ecolux, 6500 K) providing an average intensity of 225 μEinstein m^{-2} s^{-1}. In system 2 and 3, temperature was regulated at 32 °C using a heat exchanger. Contrary to PBR 1, PBR 2 and 3 were equipped with bubbling systems with air−CO_2 mixing units (static mixer and manifold) in which atmospheric air is filter-sterilized and mixed with pure CO_2 (Praxair, Canada) when necessary. The mixture is then distributed through a manifold, thus allowing bubbling at the bottom of each erlenmeyer for agitation and aeration of the culture. All gas output tubes were equipped with their own flow meters ensuring a steady flow.

In the actual work, inoculums were prepared during 3 or 4 s using PBR 2 to ensure that the algal mixture was at a comparable development stage, and 50 mg/L of inoculum was prepared for each culture for which only atmospheric air was bubbled at 1 L/min/L at 32 °C. The effect of WW was investigated through five different concentrations ranging from 0% to 80%, while the effects of CO_2 were investigated at three different concentrations ranging from 0 to 8% (v/v) with atmospheric air that was bubbled constantly at 1.5 L/min in the 1 L culture vessels.

Selection of Strain. Prior to the tests involving CO_2 and WW, the growth performance of the previously mentioned four strains was tested in the PBR 2 system under favorable growth conditions, which were inspired by the reported literature[9,11,12] at 32 °C with a constant flow (1 L/min) of bubbling (air) under a constant illumination of 78 μEinstein m^{-2} s^{-1} (about 3.3 klux). Among the studied strains, the CPF2 strain had the longest filament (1000 μm), while LB42 had the shortest (215 μm). After 14 days of culture (Figure 2), the biomass

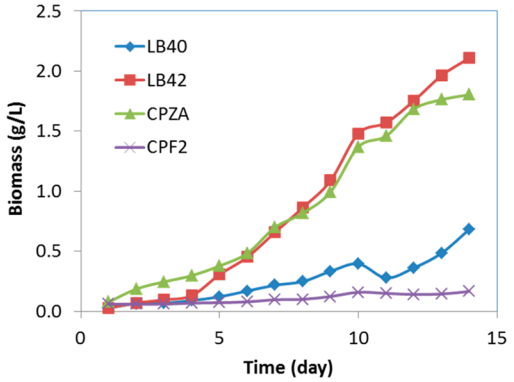

Figure 2. Biomass concentration for LB40, LB42, CPZA, and CPF2 estimated by optical density at 560 nm for a 14- growth period. A calibration curve between optical density and dry weight was established for each strain.

production of CPZA reached 1.8 g/L, a rate12 times higher than the original strain of CPF2 grown in a F/2-si solution, as well as being comparable to that of LB42. However, more than one year of observation in the laboratory revealed that the CPZA strain is very robust and needs little maintenance, while LB42 is very sensitive to environmental changes such as sudden temperature drops and repeated vessel transfer. In light of these observations, the CPZA strain was selected for the interaction with the high WW and CO_2 concentrations.

Growth Measurement. During strain selection, the optical density method used for estimation of the algal productivity was shown to be rather hard to adapt to the CPZA strain because it was very difficult to get a homogeneous algal distribution during the measure, a prerequisite for this kind of analysis. Such a phenomenon may be explained by the long filament-shape of these algae and/or their buoyancy. Therefore, because spectroscopic analytical tools could not efficiently measure the concentration, the biomass concentration was estimated simply by dry weight. Because of the abundance of the tests that had to be performed on the algal biomass on a daily basis, only a small quantity of sample was taken, and its volume could only be estimated if the concentration was to be expressed in terms of g biomass/L of culture solution. Of course, such an approach would have led to a significant error on the measures and justified the utilization of mass as reference instead of a volume because masses can be measured more accurately and objectively than volumes (with analytical balances). Therefore, concentration for the algal culture was expressed in terms of grams of algae per kilogram of culture solution. Such concentrations, although they may be different from the classical g/L, are easier to adapt to an industrial scale where masses can be more easily quantified than volume. Every three aliquots of samples were aseptically taken for the whole growth period. After being filtered with a Whatman GF/D filter under vacuum, the cells were washed with 100 mL of distilled water in order to eliminate excess mineral salts and other possible extracellular material. After drying at 105 °C up to a constant weight, the biomass concentration was reported in

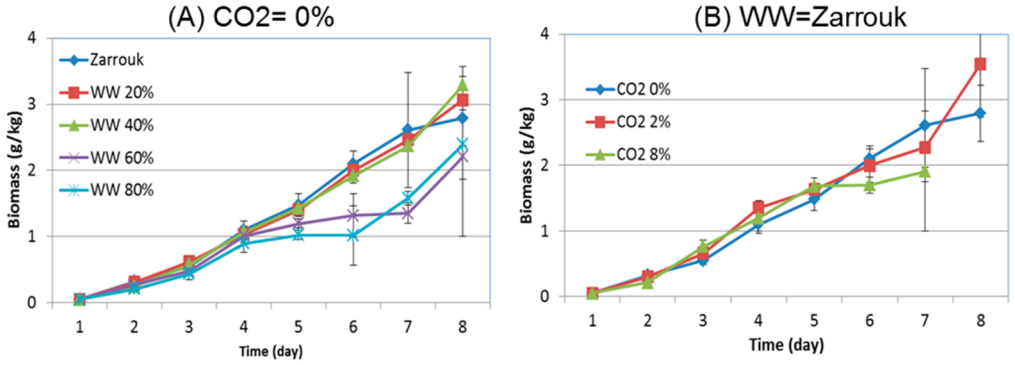

Figure 3. Biomass density under different growing conditions: (A) Various wastewater contents from a pure mixture of the Zarrouk medium up to 80% addition of wastewater at ambient CO_2 level (CO_2 = 0%) and (B) various CO_2 contents from ambient (CO_2 = 0%) to 8% CO_2 concentrations in pure Zarrouk medium. The mean values ± standard deviation (vertical bar) are presented (n = 6).

Table 2. Biomass Density, Growth Rate, and Doubling Time for *Arthrospira platensis* Grown in Different Mixtures of Zarrouk–Wastewater Medium at Different Intakes of CO_2

		Wastewater				
		Zarrouk	20%	40%	60%	80%
Biomass density (g/kg) at 5th day						
CO_2	0%	1.48	1.40	1.43	1.20	1.03
	2%	1.63	1.65	1.67	1.51	1.40
	8%	1.68	1.59	1.83	1.49	1.11
Biomass density (g/kg) at 8th day						
CO_2	0%	2.79	3.06	3.29	2.21	2.39
	2%	3.55	1.34	1.47	1.45	2.14
	8%[a]	1.91	1.14	0.72	0.81	0.64
Growth rate (g/kg/d) at 5th day						
CO_2	0%	0.36	0.34	0.35	0.29	0.24
	2%	0.40	0.40	0.40	0.36	0.34
	8%	0.41	0.38	0.45	0.36	0.27
Doubling time (d) at 5th day						
CO_2	0%	0.82	0.83	0.83	0.87	0.92
	2%	0.80	0.79	0.79	0.81	0.83
	8%	0.79	0.80	0.77	0.82	0.89

[a]The values were for 7th, 6th, 6th, 6th, and 7th day from Zarrouk to 80% wastewater due to excessive mortality of culture, respectively.

grams of biomass per kilogram of culture solution (g/kg) instead of (g/L).

The growth rate (p, g/kg/) was estimated as

$$p = \frac{X_i - X_0}{t_i - t_0} \quad (1)$$

where X_i represents the mass of biomass on the i^{th} day (t_i) as compared to X_0, the initial of biomass on initial day (t_0).

The double time (Td) was calculated using the equation

$$\mathrm{Td} = \frac{\ln(2)}{\mu} \quad (2)$$

where μ is the specific growth rate, which was calculated using the equation

$$\mu = \frac{\ln X_i - \ln X_0}{t_i - t_0}$$

Microscopical Observation. The color of each culture was monitored and microscopically examined on a daily basis, while on the sixth day, optical images were taken using a Leica system (Leica DMRX) equipped with a Sony 3CCD camera. The algal geometry was then analyzed using the image analysis software SigmaPro Scan (v.5, SPSS, Inc.).

Cell Composition Analysis. Concentration of all chemical components was determined with colorimetric measures using a Cary 100 Bio UV–visible spectrophotometer (Varian, Inc.). The soluble carbohydrates, proteins, and phycocyanin were extracted in a PBS (phosphate buffer saline) solution by sonification for 4 min with a 5-s pause in an ice bath under dimed light, after which the slurry was filtered. Soluble carbohydrates were measured as glucose equivalents using the method developed by Dubois.[13] The soluble protein concentration was estimated using an analytical technique developed by Lowry[14] using BSA (bovin serum albumin) as standard, and finally the phycocyanin concentration was estimated using the equation developed by Bennett and Bogorad[15]

$$\mathrm{PC} = \frac{\mathrm{OD}_{615} - 0.474 \times \mathrm{OD}_{652}}{5.34} \quad (4)$$

where PC is the C-phycocyanin concentration (mg/mL), OD_{615} is the optical density of the sample at 615 nm, and OD_{652} is the optical density of the sample at 652 nm.

For lipid–soluble pigments, chlorophyll-a, and total carotenoids, culture samples were extracted in an acetone/DMSO solution for 15 min in a covered vessel to avoid light and with occasional agitation. The chlorophyll-a concentration was estimated at 663 nm with an extinction coefficient of 88.67 l/g/cm, while total carotenoids were estimated at 480 nm with an extinction coefficient of 2500 l/100 mg/cm.[16]

RESULTS AND DISCUSSION

Cell Growth under Standard Conditions. In standard conditions (pure Zarrouk medium under ambient CO_2 level), the cell culture grew at a rate of 0.36 g/kg/d for the first 5 days and reached 2.79 g/kg on the eighth day (Figure 3, Table 2).

Such growth rate is relatively high as compared to the same species in a comparable medium.[12,17,18] Volkmann et al.[19] reported a much higher biomass density of 4.9 g/L in desalinator wastewater for the same species, although this concentration was reached after 23 days of growth. The color of the culture was a deep blue-green as expected, and the trichomes were about 1 mm long and 7 μ wide with about 8−10 coils (Figure 4).

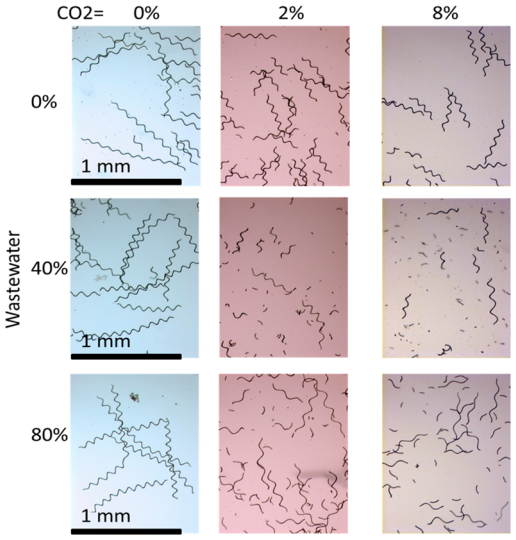

Figure 4. Optical microscopic images of *Arthrospira platensis* taken on the 6th day of growth in a medium composed of different concentrations of wastewater and CO_2.

The culture reached an alkaline pH (around 12) (Figure 5), while nitrate and phosphate concentrations in the algal solution were reduced by 37% and 30%, respectively, all of which observations were made on the sixth day (Figure 6). The chemical analysis indicated that the algal biomass contained about 34% of soluble protein, 6% of soluble carbohydrate, and about 9% of pigment (Figure 7).

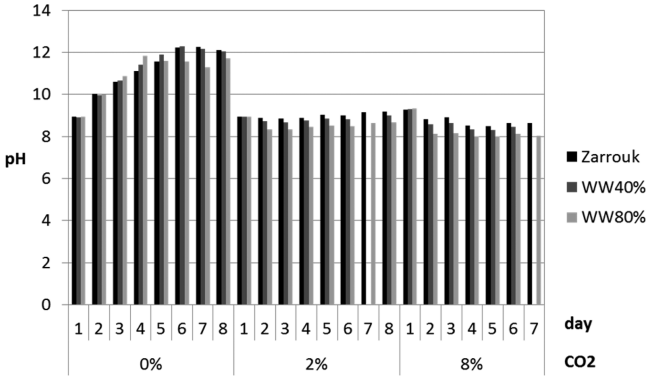

Figure 5. pH values in different wastewater concentrations (0−80%) measured from day 1 to day 8 in different CO_2 concentrations (0.04%, 2%, and 8%). For CO_2 = 8%, the measure was shown only up to day 7 due to culture destruction.

Effect of Wastewater. Adding wastewater was shown to have a distinctive effect on the production of biomass, which slightly increased by 18% with regard to standard conditions, reaching 3.29 g/kg solution on the eighth day in the 40% wastewater mixture (Table 2). Results also showed that the growth patterns and growth rates were similar up to this WW concentration (Figure 3 (A)). On the other hand, adding more than 40% WW to the mixture (as observed for the results obtained for the 60 and 80% WW mixture) has shown stagnation starting on the fifth day and for about 2 or 3 days after that. This resulted in a marked reduction of the growth rate as well as biomass production, reaching a density of 2.39 g/kg and 0.24 g/kg/d, respectively, for 80% wastewater mixture (Table 2).

Colla et al.[12] reported that sodium nitrate can be reduced from the Zarrouk medium up to 75% without any negative effects on biomass production when all other conditions are kept unchanged. As the WW sampled from the water treatment plant had a relatively low nutrient value (as mentioned previously), adding it to the Zarrouk medium could only act as a dilution effect on it (Figure 6), although same effect was not observed when only dilution the Zarrouk medium (unpublished data). The maximal amount of WW that could be added to the mixture without any detrimental effect could therefore be associated to the minimal nutrient content available for an efficient cell growth. Although such observations show that the addition of WW is somehow limited, the latter could still replace part of the expensive Zarrouk medium, which could lead to certain economical benefits if such cultures were produced at an industrial scale.

Even if close to 50% WW could be efficiently mixed with a standard medium (in this specific case), further dilution could cause growth stagnation starting on the sixth day. Interestingly, the onset time of the stagnation coincides with the maximum photosynthetic efficiency reported for this species.[20]

The color of the cultures was generally blue-green and was getting darker with increasing concentrations of biomass in the mixture. However, in 80% WW, the color of culture tended toward yellow-green after the stagnation (a symptom of chlorosis), after which the biomass concentration increased. Such phenomenon has also been observed by van Eykenlenburg,[21] who reported that chlorosis occurred after a few days in cultures with limited nitrogen source.

Although the phenomenon affected the color of the cultures, chlorosis did not seem to affect the morphological changes of cells. Optical images taken on the sixth day of experimentation showed that trichomes grown in all cultures were of similar dimension and with comparable helix geometry (Figure 4) with regard to the observations reported by van Eykenlenburg.[21]

As for the modification in the color of the cultures at high levels of WW, a chemical analysis revealed a marked difference between cells in the "pure" Zarrouk medium if compared to the 80% wastewater mixture (Figure 7). A very significant example of this difference is the soluble carbohydrates in the 80% wastewater mixture that were found to be 5 times superior with regard to the original Zarrouk medium. Such a phenomenon was observed by Gordillo et al.[22] as well as by Durangsri and Satirapipathkul[23] that reported an approximate 8-fold increase in carbohydrates in a limitative nitrogen environment.

In the actual research, the concentration of chlorophyll-a and phycocyanin decreased by more than 60%, while total carotenoids decreased by 34% for cultures grown with a mixture of 80% WW, as compared to the ones grown in pure

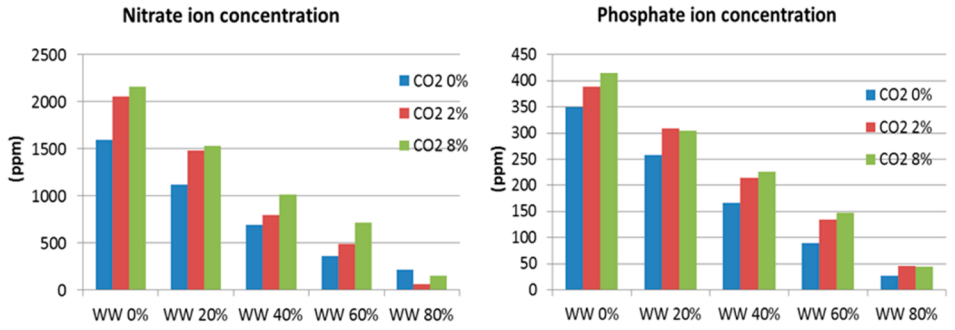

Figure 6. Concentration of nitrate and phosphate in the algal culture solution on the 6th day in a WW gradient from 0% to 80% at various CO_2 concentrations.

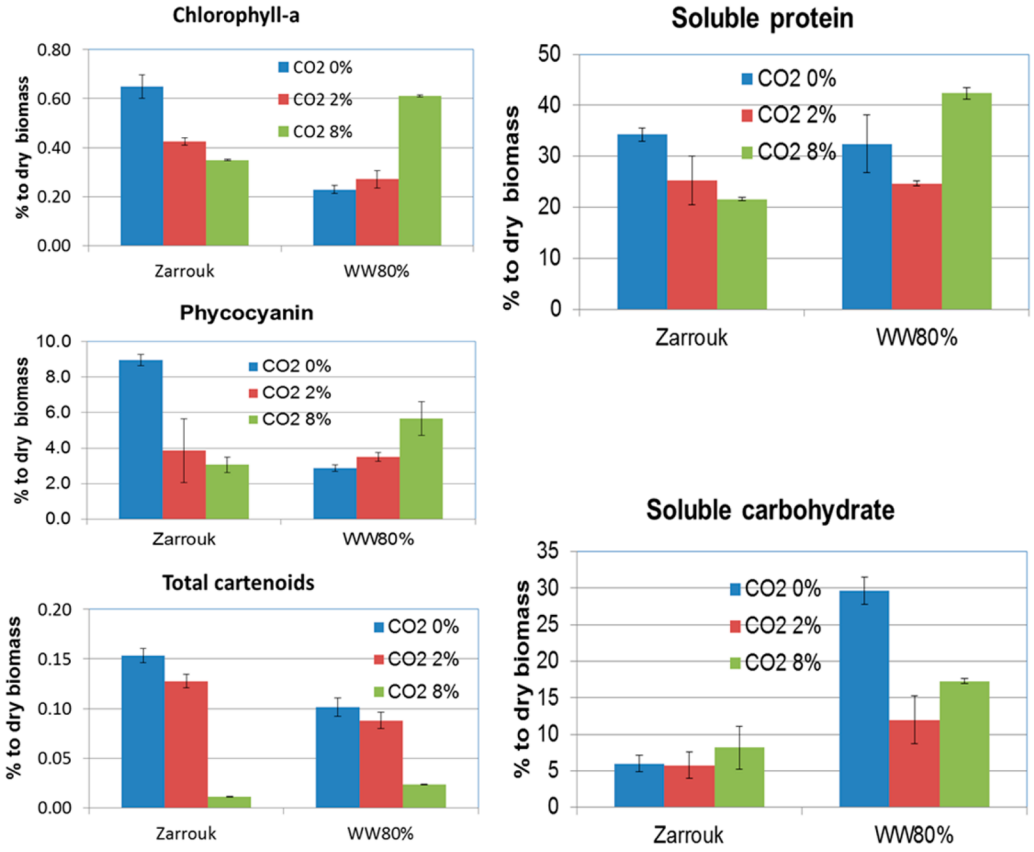

Figure 7. Concentration of chlorophyll-a, phycocyanin, total carotenoids, soluble proteins, and soluble carbohydrate for the CPZA strain grown in high concentrations of CO_2 and wastewater with regard to the same cultures in a Zarrouk medium. The mean values ± standard deviation (vertical bar) are presented (n = 6).

Zarrouk medium. The soluble protein content was shown to be very similar in both concentrations, which could be directly linked to the observations of Colla et al.[12] reporting that the nitrate in the Zarrouk medium can be reduced up to 75% without apparent reduction in protein content for the same species. However, this tendency was not observed by Durangsri and Satirapipathkul,[23] who reported a decrease of the protein content. An interpretation of the results obtained with high concentrations of WW when compared to the results reported in recent literature can suggest the possibility that this stagnation period may constitute an adaptation period for new extreme environments, confirmed by the changing color of the culture due to reduction in photopigment during this period.

Effect of CO_2. Adding CO_2 over atmospheric concentration had a clear impact on the pH that dropped to acidic (Figure 5). However, the impact of CO_2 is limited by the carbonate equilibrium, and therefore, despite the fact that effect is significant between 0% and 2% of CO_2, adding more of it does not generate another important decrease in pH. In pure Zarrouk medium, pH of the algal solution increased rapidly with time reaching about pH 12 on the sixth day when no CO_2 was added. In contrast, at the 8% CO_2 level, it was stabilized rapidly at around pH 8.5. Addition of wastewater also had an impact on the pH leading to more acidic medium proportionally to the fraction of WW in the mixture.

In pure Zarrouk medium (Figure 3 (B)), growth rate increased proportionally with increasing CO_2 during the first 5 days by 14% from 0.36 g/kg/d to 0.41 g/kg/d during the first 5

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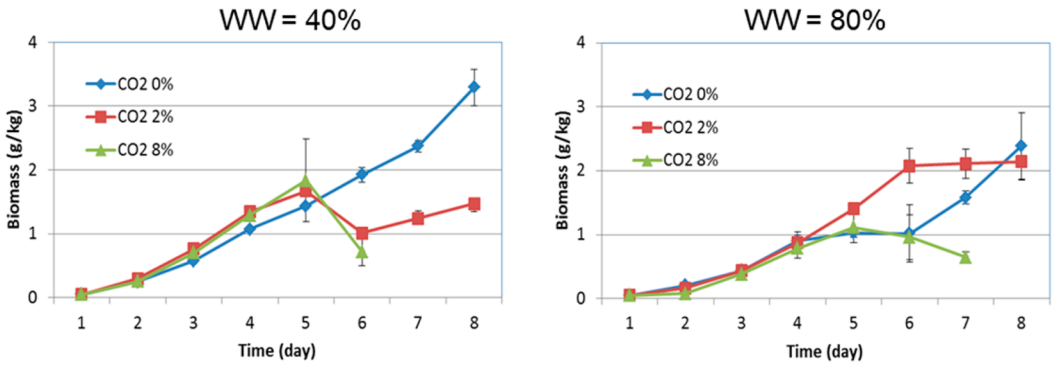

Figure 8. Biomass density with regard to growth period in 40% wastewater (WW = 40%) and 80% (WW = 80%) mixtures at various CO_2 concentrations. The mean values ± standard deviation (vertical bar) are presented (n = 6).

days, while a maximum biomass density of 3.55 g/kg on the eighth day was reported at 2% CO_2 (Table 2). At 8% CO_2 concentration, the cell growth stagnated rapidly after the fifth day, so that the final density of biomass was only of 68% of the ambient CO_2 concentrations (1.91 g/kg vs 2.79 g/kg). Gordillo et al.[22] also reported that the growth rate did not change at a 1% CO_2 concentration, but the maximum biomass density decreased. De Morais and Costa[6] observed a good growth pattern for the same genus under high CO_2 (up to 12%) with a rate of 0.2 g/L/d. Travieso et al.[24] also reported a rapid growth of 0.4 g/L/d with a 4% CO_2 intake in continuous mode, showing the possible wide variety of responses of this species under given growth conditions.

Such discrepancy has also been reported for other algal species. Chiu et al.[25] reported in their study on *Chlorella* sp. that adding up to 2% CO_2 promoted a higher biomass concentration, but higher concentrations tend to then inhibit growth, an effect that is generally more apparent for cultures inoculated at a low cell density. They suggested that such inhibition effects can be overcome by increasing inoculum density and applying suitable bioreactor design. In contrast, Chinnasamy et al.[26] scrutinized the CO_2 level from ambient CO_2 (0.037%) up to 20% and found that around 6% the CO_2 was optimum for *C. vulgaris*.

Contrary to adding wastewater, increasing the CO_2 intake in Zarrouk medium resulted in a shorter trichome length and slightly larger helix diameter (Figure 4). It is noteworthy that the concentration of nitrate and phosphate increased linearly with increasing CO_2 input (Figure 6). Biochemical analysis showed that all studied metabolites diminished rapidly, especially total carotenoids that diminished by almost 93% at 8% CO_2, while the soluble carbohydrate, in contrast, increased by 30% (Figure 7). Gordillo et al.[22] suggested that CO_2 may promote degradation of pigments that are not necessary for light harvesting while studying the same species. Ganesh et al.[27] observed that carotenoids are particularly sensitive to the presence of ROS (reactive oxygen species) for *S. maxima* even at a small concentration at which other pigments (chlorophyll or phycocyanin) are not sensitive. ROS are biomolecules believed to be produced by stressful situations such as high salinity. These metabolites have been linked to inhibition of the electron transport during photosynthetic activity, a phenomenon that can even lead to cell death. Although the role of CO_2 and ROS concentrations is not fully understood yet, it is possible that an 8% CO_2 increase could be so stressful for the CPZA strain that the carotenoid reduced dramatically. Our experimental data showed that total carotenoids diminished only by 16% from 0% to 2% CO_2, while a significant decrease (93%) was observed when CO_2 was increased from 0% to 8% (Figure 7).

Combined Interaction of Wastewater and CO_2. During the first 5 days of culture, all cell cultures involving mixtures with WW showed a higher growth rate of up to 40% WW. The highest increase (28%) was observed in a 40% WW mixture fed with 2% CO_2 with regard to the cultures grown in an exclusive Zarrouk medium and with atmospheric air (Table 2, Figure 8). However, those higher growth patterns disappeared after the sixth day for all cultures using higher CO_2 level. Binaghi et al.[20] reported that the photosynthetic efficiency of this species reached a maximum on the fifth day of culture. On the basis of this observation, it may be possible to imply that cell cultures at different wastewater and CO_2 levels can grow due to their high capacity to uptake carbon but are then overwhelmed by the inhibitory effects of the high CO_2 level.

The difference between CO_2 at 2% and 8% lies in that all cultures grown under a 2% CO_2 intake survived, while all cultures at a 8% CO_2 intake turned brown after the sixth day. It was also observed that massive foam was formed for these cultures starting from the fourth day. The microscopic observation revealed that cells in these cultures were rapidly disintegrated (Figure 4). The reason for such a sudden destruction remains unclear at this point, although a sudden so-called "mixotrophe lysis" was reported by Ciferri et al.,[28] which is believed to be due to insufficient inoculum quantity below 0.1 unit of optical density at 560 nm. In the current research, it corresponds to 49 mg/L, a quantity that is in the range of the inoculum that was used in the actual case. On the other hand, Belay[29] reported an auto-inhibition for the same species by excessive accumulation of organic matter generated from the fragmentation of trichomes during the harvesting and recycling of the medium.

It is noteworthy that the algae were able to grow in 80% wastewater at high CO_2 level (8%). In contrast to some of the other samples from this work as well as what was reported by van Eykenlenburg,[21] no chlorosis was observed in this extreme condition, but biomass production stopped after the fifth day (Figure 8). The cells were shorter with regard to those produced in pure Zarrouk medium, while the coils became very loose (Figure 4). Helix structures are known for being very adaptable to surrounding environmental conditions and culture state,[17] and loose helix were reported, for example, when the species was grown at lower temperature.[30,31] Recently, Durangsri and Satirapipathkul[23] reported a similar change with shorter trichome demonstrating loosen helix in *Spirulina*

grown in the wastewater obtained from a pickle factory, even though the wastewater was more saline that the Zarrouk medium. From this report and according to the fact that all cultures in this work were grown using the same temperature and light intensity, the morphological change may show in this specific case, for Durangsri and Satirapipathkul,[23] that the cells went through an adaptation mechanism related to the harsh growth conditions. Chemical analysis revealed that there may be a synergetic effect between elevated CO_2 and the 80% wastewater concentrations (Figure 7) because, as compared to lower CO_2 levels, the culture color was a deep blue-green with regard to what was observed in pure Zarrouk medium at ambient CO_2. Chemical analysis revealed that the chlorophyll-a content is almost the same for both cultures while other pigments, especially carotenoids, are still lower in severe conditions. Aakermann et al.[32] showed that carotenoid contents and the apparent color do not correlate for this species, which explained that the pigment composition was rather different although the color itself seemed comparable.

In an 80% WW medium, chlorophyll-a as well as phycocyanin contents increased more than 2-fold when CO_2 was increased from ambient up to 8%, and soluble proteins also tended to increase, which is an inverse tendency as compared to the pure Zarrouk medium, even though the total carotenoids contents still diminished when compared to the reference system. However, the reduction was much more attenuated (97% vs 77% reduction). Soluble carbohydrates were still more concentrated than in pure Zarrouk for each of the given CO_2 levels but tended to diminish by 58% when increasing CO_2 at high concentrations of WW. To our knowledge, such an interaction has never been reported in the open literature. It seems that there may be a synergetic overall positive effect (with regard to biomass density and cell composition) when growth conditions imply high contents of CO_2 and WW.

CONCLUSION

This work reported on the influence of municipal wastewater and elevated CO_2 on biomass production and morphology and on the cellular chemical composition of a marine strain of *A. platensis* (CCMP1295). The research made on this strain, originally adapted to grow in a Zarrouk medium, led to the conclusion that transferring a marine strain of *A. platensis* into a Zarrouk medium can accelerate the biomass production 12-fold, reaching 2.8 g/kg after 8 days at 32 °C under 78 μEinstein m^{-2} s^{-1}. At ambient CO_2 concentration, WW concentrations can reach 40% without any marked reduction in biomass production rate or quantity of soluble protein, soluble carbohydrate, chlorophyll-a, phycocyanin, and total carotenoids in cells. However, further addition of wastewater dramatically reduces all pigment components, while increasing the content of carbohydrates 6-fold by maintaining a similar protein content.

Higher concentrations of CO_2 can accelerate the growth rate in pure Zarrouk medium. Maximum production was 3.55 g/kg after 8 days at a 2% CO_2 intake. It lowers the concentration of pigments and soluble protein, while increasing the content of soluble carbohydrates.

A combination of elevated CO_2 and WW and accelerated growth with increasing CO_2 was observed only during early growth, although 8% of CO_2 was fatal for cell cultures after the sixth day.

The synergic effect of both studied factors is noteworthy because in an 80% mixture of WW, the increasing CO_2 had some beneficial effects on chlorophyll-a, phycocyanine, and soluble protein. Such results should be considered for algal culture development strategies under a phytoremediation approach.

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Notes

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