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Evaluation of Tolerant to CO_2 Excess Microalgae for the Production of Multiple Biochemicals in a 3G Biorefinery

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Abstract: To date, the positive environmental impact of microalgae-based technologies has been demonstrated in numerous studies. However, there is still a number of major technical and economic obstacles to overcome. Therefore, further research and innovation are needed for the development and commercial exploitation of large-scale integrated and sustainable processes, based on robust 'industrial' microalgal strains and novel photobioreactors (PBRs). Note that the advancement of intensified microalgal cultivation processes can facilitate the economically feasible co-production of microalgal biomass and value-added biochemicals. In this context, the goal of the present investigation was to compare several microalgal strains based on a set of productivity criteria, including the maximum biomass growth and the maximum concentration of total biochemicals (i.e., carbohydrates, proteins, and lipids) under CO_2 excess conditions (10% *v/v*). It was found that the wild type strain of *Stichococcus* sp. fully meets the above productivity criteria. In particular, a biomass concentration of 1.68 g·L^{-1} and a concentration of total biochemical products of 1.4 g·L^{-1} were measured in batch cultivation experiments in flasks using the selected strain. Further studies were performed in two different PBRs. Cultivation in a conventional stirred tank PBR showed successful scaling of the bioprocess, whereas cultivation in an innovative tubular recirculating PBR resulted in maximization of both biomass concentration (3.66 g·L^{-1}) and total biochemical products concentration (3.33 g·L^{-1}).

Keywords: microalgae; carbon dioxide; photobioreactor; biomass; carbohydrates; proteins; lipids

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1. Introduction

Today, the search for alternative resources and sustainable conversion processes that can be used in the production of renewable chemicals, fuels, and energy, and in addition mitigate the climate risk of greenhouse gas (GHGs) emissions, continues inexorably [1]. It is well-established that microalgae, among other biomass types, can largely address the above needs [2]. Microalgae are photosynthetic microorganisms with short doubling times that can be found both in marine and freshwater habitats. They are able to adopt different fixation pathways for CO_2, as well as to efficiently utilize nutrients (such as N and P) from wastes and wastewaters and convert them to valuable biomass [3,4]. Since the early 1950s, microalgal biomass has been utilized as feedstock for the production of biofuels (i.e., primarily biodiesel or 'green' diesel) due to its large content of lipids [5]. However, the economic feasibility of this single-product value chain has been questioned [6] and new applications of higher economic value have emerged; microalgal biomass is now used as a resource of valuable compounds for agricultural, nutraceutical, pharmaceutical, cosmeceutical, etc., products [7]. As a result, the simultaneous co-production of fuels and value-added biochemicals (e.g., proteins, carbohydrates, pigments, and bioactive compounds), commonly found in microalgal biomass, has been largely advanced in accordance with the general principles of a 3rd generation (3G) biorefinery [8,9].

The diversity and thus great potential of microalgae to produce numerous bioproducts has been recently reviewed in two excellent publications [10,11]. Indicatively, the protein content of *Arthrospira* biomass (also known as Spirulina, one of the most common commercial microalgal species) can be as high as 55–70% *w*/*w* per dry basis; it also contains polyunsaturated fatty acids (e.g., γ-linolenic acid), various phenolic compounds, and pigments (e.g., chlorophyll) of high nutritional value [12]. As a result, Spirulina is already considered one of the most known and effective dietary supplements [13–16]. On the other hand, carbohydrates and polysaccharides, which can be found both extracellularly and intracellularly in microalgal cell cultures, can be recovered and used directly in pharmaceutical applications, cosmetics, etc. Moreover, they can be depolymerized into simple sugars and serve as alternative raw materials for bioplastics [17–19]. Additionally, several other microalgal strains, such as *Chlorella*, are already exploited in the production of high-added value bioactive compounds, applied as antioxidants, thickeners, H_2O-binding agents, etc., especially in cosmetic applications [12,20]. For example, the Monaco-based company Exsymol has developed a protein-rich anti-aging product based on microalgal extracts, while other companies (e.g., LVMH and Daniel Jouvance, both in Paris, France) have set up their own microalgae production facilities focused on bioactive extracts [21].

In addition to the economic potential of 3G biorefineries, their environmental impact can be equal or even more significant, provided that CO_2, as the main carbon source for microalgae growth, is sequestrated from flue gases [22–24]. Compared to terrestrial plants, microalgae exhibit about 10 (up to 50) times larger CO_2 conversion capacity [25–27]. Note that the first research studies on the treatment of flue gases, generated from power and other industrial plants using various microalgae species, appeared in the open literature 25 years ago [28]. In recent years, a number of research groups also investigated the use of flue gases and synthetic effluents in microalgae processes [29,30]. Kumar et al. [31] explored the potential of *Chlorella sorokiniana* for CO_2 sequestration from flue gases for oil production in airlift and bubble column PBRs. Hosseini et al. [32] demonstrated the cultivation of *Scenedesmus* sp. in a top-lit gas lift open system, using an enhanced CO_2 air mixture (6% *v*/*v*). They showed that the process exhibited high productivity in lipids (i.e., three times larger than that obtained in traditional open raceways ponds). Yadav et al. [27] reported high biomass productivity for the cultivation of *Chlorella* sp. in a closed PBR, using in situ generated flue gases. Furthermore, da Rosa et al. [33] investigated the cultivation of *Spirulina* sp. LEB-18 in a vertical tubular PBR with simultaneous addition of monoethanolamine and CO_2. Their experimental results showed an increase in biomass productivity and CO_2 fixation rate. In all the above studies, the inhibition of microalgae growth and even microalgae death were observed and attributed to the 'toxic' compounds (e.g., H_2S, SO_x, NO_x, CO, etc.) contained in the gaseous effluents.

Commonly, the commercial cultivation of microalgae is carried out in open ponds and raceways because of their low construction and operation costs. However, this type of cultivation system exhibits several limitations including poor control of operational conditions, low biomass productivity, and high risk for contamination [34]. Typical examples of microalgae cultivation in commercial-scale open systems include Spirulina and *Dunaliella* strains [35,36]. On the other hand, closed microalgal cultivation systems (i.e., PBRs of numerous configurations) have been developed for efficient, safe, and high-yield production of multiple bioproducts [37]. In fact, there is a general consensus that closed PBRs are preferable for 3G biorefinery applications, because they have a number of distinct advantages over open cultivation systems, as highlighted in several publications [38,39]. However, the design of PBRs remains a challenge, especially for large-scale industrial applications. In addition to the large capital investment and operating cost (CAPEX and OPEX, respectively) of PBRs, there is a number of technological limitations that need to be addressed, including reduced light penetration at high-cell densities, auto-shading and photoinhibition phenomena, fouling of light transmission surfaces, ineffective mass transfer rates and fluid mixing patterns, gas hold up, huge shear rates, and major foaming, just to name a few [40]. Therefore, the optimal design and operation of PBRs are highly desirable

to enhance the biomass productivity and selectivity of value-added biochemicals from the cultivation of microalgal strains in novel PBRs and consequently improve the economic viability of a 3G biorefinery [41].

In the present study, a large number of microalgal species, originating from Greek strain collections, were cultivated in flasks and PBRs in order to assess their tolerance to CO_2 excess (up to 10% *v/v*). The main objective was to identify at least one robust strain that can grow unhindered, even under high CO_2 concentrations. An additional goal was to produce microalgal biomass at high concentration, exhibiting at the same time a high content and a total concentration of multiple biochemicals (i.e., carbohydrates, proteins, and lipids). Subsequently, the selected strain was cultivated in two different PBR configurations. Firstly, a conventional stirred tank PBR was used to demonstrate the scaling up of the bioprocess from flasks to the PBR. Subsequently, an innovative tubular recirculating PBR was employed to directly sequestrate CO_2 from a feedstock that resembles the composition (in terms of CO_2) of the gaseous effluents generated from the combustion of diesel oil and natural gas of the Public Power Company (PPC), Athens, Greece.

2. Materials and Methods

2.1. Microalgal Species and Cultivation in Flasks

In total, 13 microalgal strains (see Table 1), obtained from two proprietary collections (species #1–3, #6–9, and #11 from the TAU-MAC Culture Collection of the School of Biology, Aristotle University of Thessaloniki (AUTH), Greece; and species #4–5 from the ATHUL Culture Collection of the Department of Biology, National and Kapodistrian University of Athens (NKUA), Greece), and also isolated from the southern Greek seas near Crete (species #10 and #12–13, provided by the School of Chemical and Environmental Engineering, Technical University of Crete (TUC), Greece), were selected for cultivation and screening studies in flasks.

Table 1. Microalgal strains and respective cultivation media.

#	Microalgal Strain	Reference Code	Cultivation Medium
1	*Arthrospira* cf. *fusiformis*	TAU-MAC 0113	Zarrouk
2	*Arthrospira* cf. *maxima*	TAU-MAC 0213	Zarrouk
3	*Chlamydomonadales*	TAU-MAC 3510	3N-BBM + V_Mod
4	*Chlorella* sp.	ACA17	BG11
5	*Chlorella* sp.	ASP14	BG11
6	*Chlorella vulgaris*	TAU-MAC 1110	BG11
7	*Chlorella vulgaris*	TAU-MAC 3210	BG11
8	*Chlorophyta*	TAU-MAC 3917	BG11
9	*Komarekiella* sp.	TAU-MAC 0117	BG11
10	*Stichococcus* sp.	Wild-TUC	3N-BBM + V_Mod
11	*Stichococcus* sp.	TAU-MAC 0119	3N-BBM + V_Mod
12	*Stichococcus* sp.	EMS1-TUC	3N-BBM + V_Mod
13	*Stichococcus* sp.	EMS3-TUC	3N-BBM + V_Mod

The selected strains were initially cultivated in flask scale batch experiments to investigate their tolerance to large CO_2 concentrations (10% *v/v*) and biomass growth characteristics, as well as the production of specific biochemicals, namely, carbohydrates, proteins, and lipids. The cultures were hosted in 500 mL Erlenmeyer flasks. Initially, 185 mL of a fresh cultivation medium (see Table 1) was loaded and then inoculated with 15 mL of a mature preculture of a selected strain so that the initial biomass concentration was approximately equal to 0.2 g/L. The flasks were incubated for 15 days at 80 rpm in an orbital shaking incubator (3031, GFL, Burgwedel, Germany) at a controlled temperature of 25 ± 1 °C, with the aid of an air-circulating heating/cooling system. The microalgal culture was artificially illuminated using a custom-made lighting box, equipped with a series of cool white daylight LED lamps (type T8, 11 W, 950 lumens each, Osram Licht AG, Munich,

Germany) symmetrically placed at the internal box sides. A 16/8 h light/dark photoperiod, regulated by 8 lamps in total, was applied. The flasks were covered with an inlet–outlet airtight rubber stopper and the aeration of the culture was carried out via a suitable syringe nozzle, inserted through the top rubber stopper, and submerged to the culture, using a 50 mL·min^{-1} supply rate of a CO_2 air mixture (10% *v/v* CO_2 with synthetic air), regulated by a gas flow meter. The feeding gas stream was sterilized via a 0.2 μm PTFE filter (Whatman plc, Maidstone, UK) before entering the flasks.

Different cultivation media, such as BG11 for freshwater algae (e.g., *Chlorella* sp.), 3N-BBM + V_Mod for marine habitat strains (e.g., *Stichococcus* sp.), and Zarrouk, specifically for *Arthrospira* sp., were used for the cultivation of microalgae under N-sufficient conditions [42]. In Table 2, the exact chemical composition of each medium is presented. It should be noted that prior to sterilization, the pH of all media was adjusted to 7 ± 0.2 using a 2 N NaOH solution [43]. Then, the media were autoclaved for 20 min at 121 °C [44] using a Raypa (Barcelona, Spain) AES-75 steam sterilizer.

Table 2. Chemical composition of cultivation media.

Component	Concentration (g·L^{-1})		
	BG11	**3N-BBM + V_Mod**	**Zarrouk**
$NaNO_3$	1.5	0.75	2.5
$K_2HPO_4 \cdot 3H_2O$	0.04	0.075	0.5
K_2SO_4	-	-	1.0
KH_2PO_4	-	0.175	-
NaCl	-	0.25	1.0
$NaHCO_3$	-	-	16.8
$MgSO_4 \cdot 7H_2O$	0.075	0.075	0.2
$CaCl_2 \cdot 2H_2O$	0.036	0.025	0.04
Na_2CO_3	0.02	-	0.2
DiNa-EDTA	0.001	0.0045	0.08
Citric acid	0.006	-	-
Ammonium ferric citrate	0.006	-	-
TES + V *	1	1	1

* Trace elements solution (TES) and vitamin (V) contents according to a previous study [23]; TES did not contain the components that are already listed above in Table 2.

2.2. Cultivation in Photobioreactors

Batch cultures of the selected species were initially conducted in a 3-L stirred tank (diameter 22 cm and height 42 cm) glass photobioreactor (BioFlo 110 Bioreactor/Fermentor, New Brunswick Scientific, New Jersey, USA). The PBR was initially loaded with 1.4 L of the selected cultivation medium and then inoculated with 0.1 L of a mature preculture. The initial biomass concentration was 0.2 g/L. Based on a series of preliminary cultivation experiments and a previous optimization study [23], the temperature of the culture was controlled at 25 ± 1 °C and the pH of the medium was maintained at 7 ± 0.2 with the aid of two buffer solutions, namely HCl and NaOH (both 2 N), automatically supplied by suitable peristaltic pumps, while a CO_2 air mixture of 10/90 % *v/v* was supplied through a 0.2 μm PTFE filter and a ring sparger into the PBR at a rate of 200 mL·min^{-1}. The agitation rate was kept at 80 rpm, while the illumination profile was identical to the one used in the flask scale experiments, using the custom-made box with 8 LED lamps in operation and a 16/8 h photoperiod.

In a follow-up optimization experiment, the selected strain was also cultivated in a 5-L recirculating horizontal tubular glass PBR (xCubio phar, bbi-biotech GmbH, Berlin, Germany). The novel PBR consisted of two parts: one small vessel filled up to 1.5 L of culture, containing all the probes and measuring instruments, and one spiral tubular reactor of 9 m length, manufactured all-in-one without internal welded connections and with an extra clean surface for minimizing flow resistance and biofilm accumulation. The

photobioreactor was initially loaded with 4.125 L of the cultivation medium and then inoculated with 0.375 L of a mature preculture; the initial biomass concentration was again 0.2 g/L. The operational conditions were identical to the ones used for the strain cultivation in the stirred tank PBR (i.e., temperature 25 ± 1 °C, pH 7 ± 0.2, and supply of a CO_2 air mixture (10/90 % *v*/*v*) at a rate of 200 mL·min^{-1}). Illumination of the PBR was achieved via 660 nm (red color) and 450 nm (blue color) LED lamps, placed between the unit's small vessel and the spiral reactor tube. Once again, a 16/8 h light/dark photoperiod was applied while the light energy flux was equal to 1600 μE. The recirculation of the culture suspension in the two PBR compartments was driven by a magnetic (turbine) pump (Gather Industrie GmbH, Wülfrath, Germany) at a constant rate of 1.7 L·min^{-1}.

2.3. Analytical Measurements

In all the flask and PBR cultivation experiments, the microalgae growth was monitored by the periodic collection of 2 mL culture samples and measurement of their optical density (OD) at 600 nm, using a UV/Vis spectrophotometer (U-1800, Hitachi, Tokyo, Japan). For the measurement of biomass concentration (expressed as dry cell weight, DCW), a 5 mL sample collected from each individual culture was first filtered through a pre-weighted glass microfiber filter (934-AH, pore diameter 1.5 μm; Whatman plc, Maidstone, UK), and then dried overnight at 45 °C and finally weighed.

The biomass composition in the collected samples was determined by measuring its percentage content in carbohydrates, proteins, and lipids. Initially, a 6 mg freeze-dried collected sample was treated with a 2/1 *v*/*v* chloroform/methanol solution. The suspension was then subjected to sonication for 15 min (Vibra Cell VC-505 Sonicator, Sonics & Materials, Inc., Connecticut, USA; 40% amplitude, 45 sec on/15 sec off time intervals) to ease the disruption of microalgal cell walls and aid the extraction of lipids. Subsequently, the separation of lipids from the residual biomass was accomplished using a modified protocol of the original Folch method [45]. The quantification of extracted lipids was performed gravimetrically after overnight drying of collected lipids [46]. The measurement of carbohydrates was carried out by following the well-established phenol-sulfuric method, which is used to quantify the neutral monomeric sugars (as glucose-equivalent content) by measuring the UV/Vis absorbance of the unknown solution at 483 nm [47]. More specifically, 2 mg of freeze-dried collected sample was dispersed in an HCl solution (2.5 M) and incubated at 100 °C for 3 h. Standard solutions of D-glucose, prepared as a monosaccharide reference, were used for the construction of a glucose concentration–absorption calibration line.

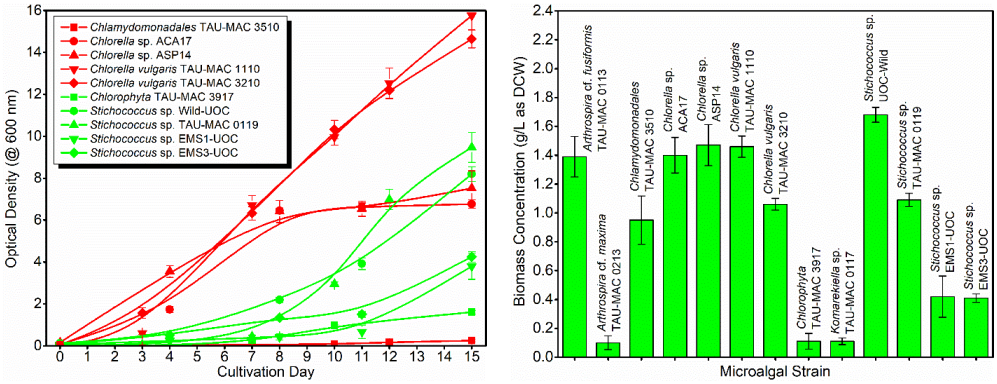
Accordingly, the intracellular proteins were measured by suspending a 2 mg freeze-dried sample in an aqueous solution of NaOH (0.5 N), containing 5% *v*/*v* methanol and a phosphate buffer (0.05 M). Subsequently, the suspension was heated at 100 °C for 30 min and then sonicated for 10 min (40% amplitude, 45 s on/15 s off time intervals) to ensure cell breakage and release of proteins. The protein's content in the resulting solution was quantified with the aid of a Micro-BCA kit (SERVA Electrophoresis GmbH, Heidelberg, Germany) and a microplate spectrophotometer (ELx808 Absorbance Microplate Reader, BioTek Instruments, Inc., Vermont, USA) [48]. Note that all the cultivation experiments and respective measurements were performed in triplicate and the reported values represent the mean values of the three runs.

3. Results and Discussion

3.1. Microalgal Species' Screening and Selection

The 13 microalgal species of Table 1 were initially cultivated in Erlenmeyer flasks to evaluate their growth, in terms of optical density (OD), final DCW, and tolerance to CO_2 excess (i.e., 10% *v*/*v*). The selected CO_2 composition represented the upper limit in all examined flue gas mixtures (pre- or post-treatment) generated from the combustion of diesel and natural gas, as specified by the Greek PPC.

Figure 1a shows the temporal evolution of the optical densities (ODs) of the selected microalgal cultures. It should be noted that the OD measurements of *Arthrospira* cf. *fusiformis*, *Arthrospira* cf. *maxima*, and *Komarekiella* sp. cultures were not possible due to the excessive heterogeneity of the collected samples. For these three cases, the biomass (as DCW) measured at the end of the culture is reported (see Figure 1b). Thus, OD measurements were used to monitor the tolerance of the investigated strains in CO_2 excess and not to determine the biomass growth rates. As can be seen in Figure 1a, the two *Chlorella vulgaris* strains (TAU-MAC 1110 and TAU-MAC 3210) exhibited the largest growth (biomass production), starting almost from the 1st cultivation day. The other two *Chlorella* sp. strains (ACA17 and ASP14) also exhibited high growth up to the 8th day of cultivation, followed by an almost constant value. On the other hand, the four *Stichococcus* strains displayed relatively high to medium growth after the 6th day of cultivation. Note that the two randomly mutated *Stichococcus* strains (EMS1-TUC and EMS3-TUC) had significantly lower growth than the respective rates of the wild type strains (Wild-TUC and TAU-MAC 0119). Finally, the *Chlamydomonadales* and *Chlorophyta strains*, especially the former one, showed the lowest growth.



(**a**) (**b**)

Figure 1. Comparison of growth of examined microalgal species: (**a**) temporal evolution of optical densities (ODs) of the selected microalgae cultures; (**b**) final values of the dry cell weight (DCW) of the microalgal strains of Table 1.

In Figure 1b, the biomass concentrations (expressed as DCW at the end of each culture) are depicted. As can be seen, the microalgal strains with the largest biomass concentrations are the *Stichococcus* sp. Wild-TUC, *Chlorella vulgaris* TAU-MAC 1110, and the two *Chlorella* sp. strains (ACA17 and ASP14). It should be noted that the identified high-growth species are already considered 'industrial' strains, capable of producing microalgal biomass at an economically feasible rate. In particular, *Arthrospira* cf. *fusiformis* TAU-MAC 0113 yielded a biomass concentration comparable to that reported in a similar study [49]. On the other hand, the DCW values measured for the *Arthrospira* cf. *maxima* TAU-MAC 0213, *Chlorophyta* TAU-MAC 3917, *Chlamydomonadales* TAU-MAC 3510, and *Komarekiella* sp. TAU-MAC 0117 cultures were significantly lower than those reported in the literature [50], an indication that these strains had a low tolerance to CO_2 excess. Thus, although it was expected that the *Arthrospira* cf. *maxima* TAU-MAC 0213 culture would yield a large DCW value because of the use of a sufficiently rich in nitrogen cultivation medium (Zarrouk), the measured DCW value was low owing to its low tolerance to CO_2 excess. Moreover, the mutated *Stichococcus* sp. strains failed to produce biomass concentrations at levels similar to those obtained by the wild type strains. This can be possibly attributed to the random mutations that these strains experienced in sequential cultures under C- and/or N-starvation conditions, as they were applied by the strain collection proprietor before they were made available for this study. As a result, the biomass growth with mutated *Stichococcus* sp. strains was low although the intracellular accumulation of lipids was significantly enhanced.

In summary, the present values on microalgal biomass yields for all the tested strains are not as high as those reported in the open literature, apparently due to the high CO_2 concentration and the non-optimized culture conditions, e.g., medium type and composition. However, some strains show increased tolerance to CO_2 while the final biomass yield is sufficiently high, supporting further optimization studies [23,51,52].

In addition to DCW measurements, all tested strains were evaluated with regard to the composition and concentration of intracellularly accumulated biochemicals. More specifically, the concentrations of carbohydrates, proteins, lipids, and total biochemicals were measured and shown in Table 3. It should be noted that the total biochemical product concentration was, along with biomass concentration, the criterion used for the evaluation of the individual cultures. In Figure 2, the measured weight percentages of intracellularly accumulated carbohydrates, proteins, and lipids are reported.

Table 3. Measured concentrations of intracellularly accumulated carbohydrates, proteins, lipids, and total biochemicals for all tested microalgal strains of Table 1.

Microalgal Strain	Carbohydrates ($g \cdot L^{-1}$)	Proteins ($g \cdot L^{-1}$)	Lipids ($g \cdot L^{-1}$)	Total Biochemicals Products ($g \cdot L^{-1}$)	Total Product Contents (% *w*/*w*)
Arthrospira cf. *fusiformis* TAU-MAC 0113	0.17	0.48	0.42	1.07	76.98
Arthrospira cf. *maxima* TAU-MAC 0213	0.02	0.01	0.03	0.06	60.00
Chlamydomonadales TAU-MAC 3510	0.03	0.03	0.03	0.09	9.47
Chlorella sp. ACA17	0.16	0.33	0.35	0.84	60.00
Chlorella sp. ASP14	0.27	0.63	0.33	1.23	83.67
Chlorella vulgaris TAU-MAC 1110	0.21	0.27	0.76	1.24	84.93
Chlorella vulgaris TAU-MAC 3210	0.17	0.21	0.56	0.94	88.68
Chlorophyta TAU-MAC 3917	0.03	0.02	0.02	0.07	63.64
Komarekiella sp. TAU-MAC 0117	0.04	0.02	0.02	0.08	72.73
Stichococcus sp. Wild-TUC	0.32	0.69	0.39	1.40	83.33
Stichococcus sp. TAU-MAC 0119	0.23	0.26	0.35	0.84	77.06
Stichococcus sp. EMS1-TUC	0.09	0.14	0.08	0.31	73.81
Stichococcus sp. EMS3-TUC	0.07	0.12	0.08	0.27	65.85

As can be seen in Table 3 and Figure 2a, the microalgal strains *Komarekiella* sp. TAU-MAC 0117, *Chlorophyta* TAU-MAC 3917, and *Chlamydomonadales* TAU-MAC 3510 had the highest content in carbohydrates, namely 40%, 34%, and 30% of DCW, respectively. Moreover, *Chlorella* sp. ASP14, *Stichococcus* sp. Wild-TUC, and *Arthrospira* cf. *fusiformis* TAU-MAC 0113 accumulated the largest amounts of proteins in their cells (43%, 40%, and 35% of DCW, respectively), as seen in Figure 2b. Finally, the two *Chlorella vulgaris* strains, TAU-MAC 1110 and TAU-MAC 3210, had the largest lipids percentages (52% *w*/*w*, lipids per DCW), as seen in Figure 2c. Regarding the total concentration of biochemicals in the dry biomass, all the *Chlorella* species (except ACA17 sp.) and all the *Stichococcus* species (except EMS3-TUC sp.) accumulated high amounts (i.e., larger than 70% of DCW). Note that almost half of the investigated strains had a total concentration of accumulated biochemicals larger than 0.8 $g \cdot L^{-1}$. From the above analysis of the intracellularly accumulated products, a number of relationships between the tested strains and measured biochemical products can be identified. Thus, *Chlorella* sp. ASP14 and *Stichococcus* sp. Wild-TUC species are very efficient in protein production, *Chlorella vulgaris* TAU-MAC 1110 and TAU-MAC 3210 strains are suitable for lipid production (although at a low overall efficiency), and *Chlorella* sp. ASP14 and *Stichococcus* sp. Wild-TUC are useful for carbohydrate production.

It should be noted that based on the measured biochemical contents, the cultivation of the examined strains does not appear to be significantly affected by the presence of CO_2 excess [49,53]. Thus, one of the main selection criteria for a strain regarding its industrial-scale cultivation in a PBR should be its ability to yield a maximum concentration in selected or total biochemical products, so that the notion of a modern 3G biorefinery can be satisfied. Indicatively, a biochemical content of >70–75% of DCW can largely improve the overall process economics associated with the extraction and recovery of the produced biochemical products. Moreover, a high microalgal biomass productivity that can be achieved by large

DCW and short cultivation times is required to reduce the operating costs in relation also to downstream biomass processing, including biomass dewatering, separation, and drying.

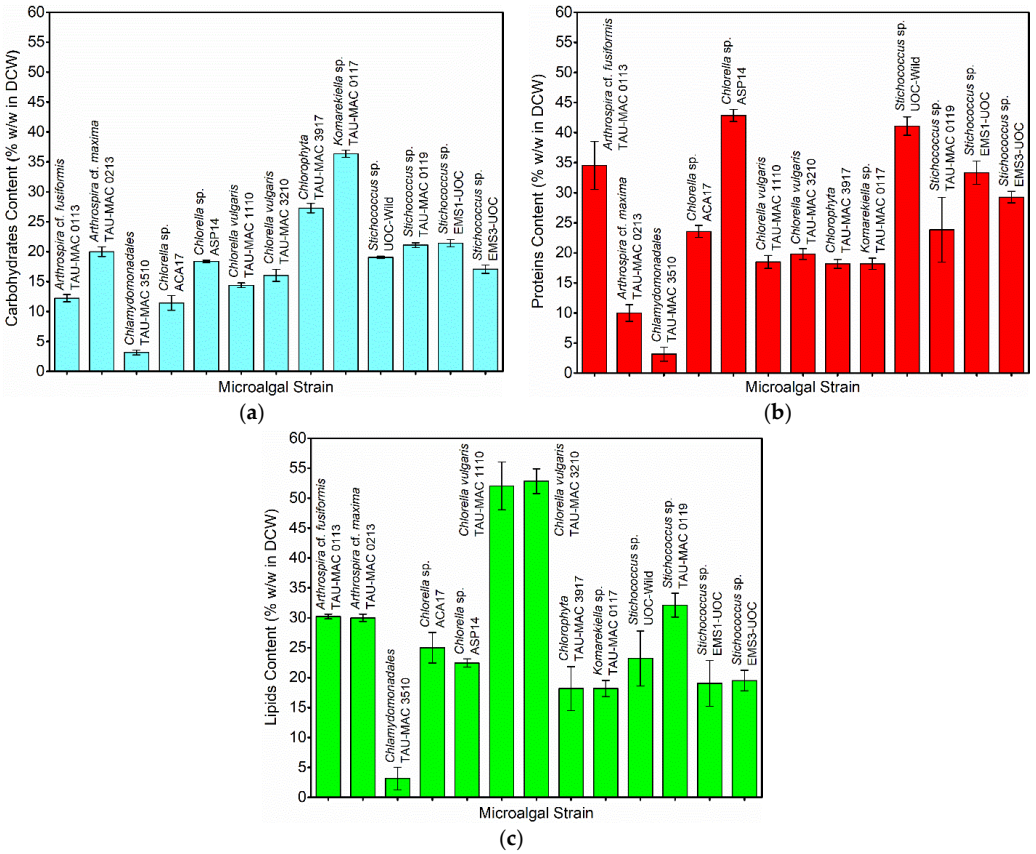


Figure 2. Intracellular contents of measured biochemicals: (**a**) carbohydrates, (**b**) proteins, and (**c**) lipids. Largest contents measured: (**a**) carbohydrates: 36.36% of DCW in *Komarekiella* sp. and 27.27% of DCW in *Chlorophyta* TAU-MAC 3917; (**b**) proteins: 42.86% of DCW in *Chlorella* sp. ASP14 and 41.07% of DCW in *Stichococcus* sp. Wild-TUC; and (**c**) lipids: 52.83% of DCW in *Chlorella vulgaris* TAU-MAC 3210 and 52.05% of DCW in *Chlorella vulgaris* TAU-MAC 1110.

Based on the above experimental results, *Stichococcus* sp. Wild-TUC, a marine microalgal species isolated from the sea region of Crete (southern Greece), was selected as the most promising strain due to its proven resilience to CO_2, general robustness as an 'industrial' strain and a high yield of 1.4 $g \cdot L^{-1}$ in total biochemicals, corresponding to 83.33% of DCW. In particular, the biochemical composition consisted of 0.32 $g \cdot L^{-1}$ (19.05% *w*/*w*) of carbohydrates, 0.69 $g \cdot L^{-1}$ (41.07% *w*/*w*) of proteins, and 0.39 $g \cdot L^{-1}$ (23.21% *w*/*w*) of lipids. It is worth noting that further to previous reports on the cultivation of *Stichococcus* sp. Wild-TUC regarding the maximization of biochemicals production in carbohydrates [23], lipids [54], and pigments [55], in the present investigation, a new cultivation policy was developed to maximize the accumulation of proteins in mid-cell-density cultures. This was achieved by simply feeding the *Stichococcus* sp. Wild-TUC cell cultures with a feedstock containing larger than 'normal' CO_2 concentrations. This new finding clearly underlines the great potential and versatility of the *Stichococcus* sp. Wild-TUC strain in producing different biochemicals by varying the environmental and culture conditions. As a result, it was selected for further investigation in PBRs.

3.2. Cultivation of Stichococcus sp. Wild-TUC in Photobioreactors

Following the selection of *Stichococcus* sp. Wild-TUC as the most promising strain, additional cultivation experiments were performed using two different PBR configurations, namely a conventional stirred tank photobioreactor (ST-PBR) and an innovative horizon-

tal tubular recirculating photobioreactor (HT-PBR). The objective was to investigate the cultivation characteristics of the selected strain under different mixing and intensification conditions. Note that all other operating and environmental conditions were identical to those applied to the flask scale cultivations. Figure 3a shows the temporal variation of biomass growth as indicated by the optical density (OD) and the measured dry cell weight (DCW) of the culture in the two PBRs. It is evident that both cultures show a similar variation of OD and DCW with time, as both process variables increase steadily to their respective maximum values, which are reached at day 14 (i.e., 1 day earlier than that found in the flask scale experiments). Thereafter, both cultures show a rapid decrease in OD and DCW values. It should be noted that the biomass growth rate in the HT-PBR is significantly higher than that measured in the ST-PBR, a clear indication that light penetration, mixing, and low cell shearing conditions in the HT-PBR are more favorable for the growth of the *Stichococcus* sp. Wild-TUC cells. As can be seen in Figure 3a, the maximum biomass concentrations (measured at day 14) in the HT-PBR and ST-PBR were 3.66 $g \cdot L^{-1}$ and 1.62 $g \cdot L^{-1}$, respectively. This large difference in the biomass growth in the two different PBRs can be attributed to the innovative design and operational characteristics of HT-PBR. More specifically, the small diameter (2.2 cm) of the spiral glass tube allows the artificial light to penetrate efficiently and uniformly the bulk culture even at high cell densities. As a result, cell auto-shading and inhibition phenomena are largely limited in the tubular PBR.

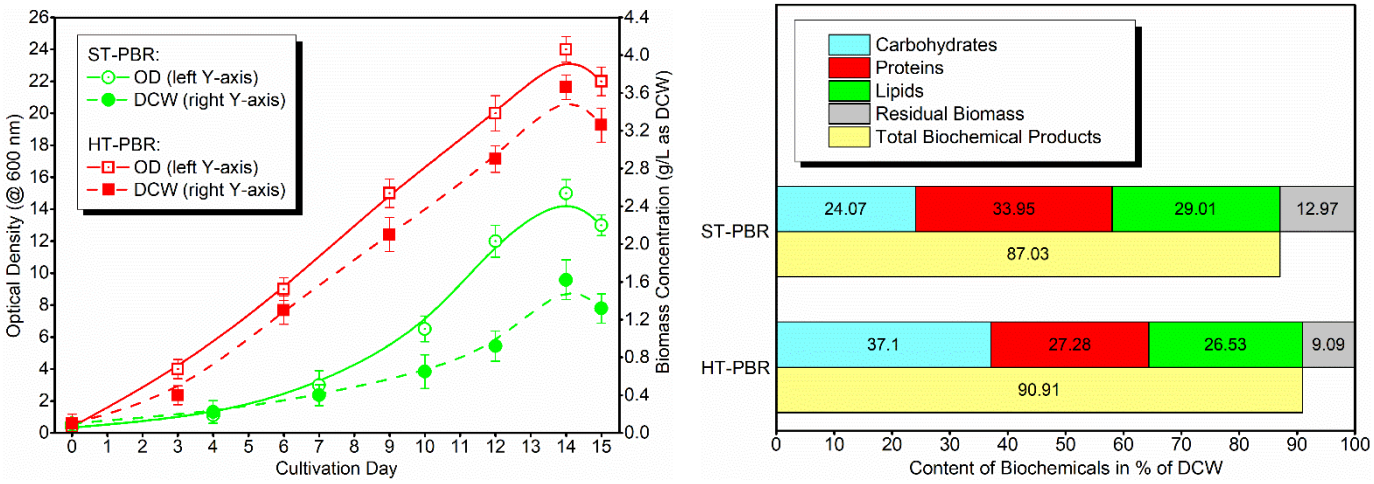


Figure 3. Comparison of *Stichococcus* sp. Wild-TUC growth in the two PBRs: (**a**) temporal evolution of OD and DCW; (**b**) biomass composition in carbohydrates, proteins, and lipids at day 14; ST: stirred tank, HT: horizontal tubular.

Moreover, the operation of the magnetic pump of the HT-PBR ensures the recirculation of the culture at a minimum shear rate but there is also a high volumetric rate, owing to the absence of mechanical mixing parts (i.e., agitator and baffles). As a consequence, the cells in the HT-PBR undergo minimum shearing, as was evident by the absence of foaming or/and cell adherence/sedimentation on the mechanical parts of the HT-PBR. Finally, the design, mixing, and operational characteristics affect the CO_2 and nutrient distribution in the two units and thus the respective mass transfer rates and, consequently, the microalgal biomass growth. It should be pointed out that the superior performance of the HT-PBR over that of the ST-PBR can be also attributed to the differences in the artificial illumination of the cultures in the two PBRs. The exposure of the cells in the HT-PBR to combine red and blue LEDs, instead of the typical white LEDs used in the ST-PBR case, increased biomass productivity and energy efficiency, as well as the accumulation of total biochemical products [55].

It is worth noting that the measured biomass concentration in the ST-PBR was very similar to the biomass concentration measured in the flask cultures (i.e., 1.62 $g \cdot L^{-1}$ in the

ST-PBR versus 1.68 g·L^{-1} in the flasks). However, despite the similarity in the biomass production in the ST-PBR and flask cultures, the composition of biochemicals (i.e., in carbohydrates, proteins, and lipids) was considerably different, as it is evident from the comparison of measured biochemical concentrations in the flask scale and ST-PBR cultures shown in Figures 2 and 3b. In fact, the cultivation of the *Stichococcus* sp. Wild-TUC strain in the ST-PBR, under CO_2 excess conditions (10% *v*/*v*) resulted in the production of 24.07% of DCW in carbohydrates, 33.95% of DCW in proteins, and 29.01% of DCW in lipids. On the other hand, the cultivation of the same strain in the flasks under identical conditions yielded a respective composition in the dry cell biomass of 19.05% in carbohydrates, 41.07% in proteins, and 23.21% in lipids. These comparative results clearly show that the biochemical composition in the cells is strongly influenced by the culture conditions in the flasks and the ST-PBR unit (i.e., light illumination, aeration, mixing and mass transfer conditions, etc.). Note that in these cultures, the metabolism of *Stichococcus* sp. Wild TUC cells is targeted primarily to the accumulation of proteins, and then lipids and carbohydrates.

Subsequently, a comparison of the biochemical composition of *Stichococcus* sp. Wild-TUC cultures obtained in the HT-PBR and ST-PBR was carried out. In the former case, due to the more favorable cell growth conditions (attributed to the intensified design of the HT-PBR), the *Stichococcus* sp. Wild-TUC strain cultures yielded a high biomass growth and a total concentration of biochemical products equal to 3.33 g·L^{-1}, which is very close to the reported 'normal' values in high cell density cultures [23]. Moreover, the biochemical composition of the collected dry cells was 37.1% *w*/*w* in carbohydrates, 27.28% *w*/*w* in proteins, and 26.53% *w*/*w* in lipids. In Table 4, the measured biochemical concentrations in the *Stichococcus* sp. Wild-TUC cultures in the two PBRs are shown. The higher total concentration in biochemical products (3.33 g·L^{-1}) obtained in the HT-PBR in comparison to the respective concentration (1.43 g·L^{-1}) obtained in the ST-PBR shows that the horizontal recirculating reactor is a more efficient cultivation system and should be preferred over the ST-PBR operation in an integrated 3G biorefinery. Note that in high cell density cultures, the *Stichococcus* sp. Wild-TUC strain approaches its 'normal' behavior as a growth-associated carbohydrate producer [23].

Table 4. Measured concentrations of the biochemical products, at day 14, in the *Stichococcus* sp. Wild-TUC cultures in the two PBRs.

Photobioreactor (PBR) Configuration	Carbohydrates Concentration (g·L^{-1})	Proteins Concentration (g·L^{-1})	Lipids Concentration (g·L^{-1})	Total Biochemical Products (g·L^{-1})
Stirred Tank: ST-PBR	0.39	0.56	0.48	1.43
Horizontal Tubular: HT-PBR	1.36	1.00	0.97	3.33

The experimental results in Figure 3 and Table 4 clearly demonstrate the ability of the selected strain to produce multiple biochemical products at high concentrations and a total biochemical mass fraction of 90.91% *w*/*w* in the HT-PBR and 87.03% *w*/*w* in the ST-PBR. These high yields in biochemical production can largely improve the overall process economics associated with the downstream extraction and recovery of the produced biochemical products.

4. Conclusions

The numerous advantages of microalgae need to be systematically exploited to alleviate the detrimental impact of CO_2 emissions on the global climate while, at the same time, they can deliver a renewable source of biomass and high added value biochemicals for the sustainable operation of 3G biorefineries. Toward this direction, several microalgal species were evaluated for their tolerance to high CO_2 concentrations. Among several candidates that demonstrated adequate tolerance to CO_2, the *Stichococcus* sp. Wild-TUC strain was the most promising, owing to its excellent cultivation characteristics in terms of

biomass yield and production of a series of biochemical products. It is envisioned that the selected species can be potentially utilized in closed PBRs for the treatment of flue gases generated from the combustion of diesel oil and natural gas in power plants in an effort to reduce GHGs emissions. Note that the exhaust gases from power plants could contain up to 10% CO_2 v/v, depending on the feedstock fuel. In the case of diesel or natural gas, the exhaust gases can be fed to the PBR with minimal or no treatment at all. Moreover, in other cases, CO_2-containing effluents produced in anaerobic digestion and composting units, bioethanol plants, pyrolysis, and hydrothermal liquefaction reactors, etc., can be directly used for the production of microalgal biomass and value-added biochemicals (i.e., carbohydrates, proteins, and lipids from CO_2 sequestration).

In the present study, it was shown that the *Stichococcus* sp. Wild-TUC strain is a robust and versatile 'industrial' microalgal strain that can be grown in various habitats since it is resilient to temperature, salinity, and pH variations. As an excellent carbohydrate and protein producer, it can be utilized as a 'cell factory' for the production of various value-added biochemicals even under non-optimized conditions, such as the medium composition. It is foreseen that the generated knowledge and results of this study can aid the development of a 3G biorefinery at a high technology readiness level (TRL). Beyond the positive environmental impact of microalgae technology for CO_2 sequestration, the efficient cultivation of the *Stichococcus* sp. Wild-TUC strain in intensified and scalable photobioreactors can also have a significant economic impact through the combined production of biomass and value-added biochemicals (i.e., carbohydrates and proteins).

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