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Effects of CO_2 and pH on *Stichococcus bacillaris* in Laboratory Scale Photobioreactors

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The effects of both the CO_2 concentration and the pH of the medium on biodiesel production by *Stichococcus bacillaris* (ACUF 158/11) were reported. Indoor cultures of *S. bacillaris* were carried out in both 0.6 L vertical bubble column photobioreactors and 1.7 L inclined bubble column photobioreactors. Batch, fed-batch and semi-continuous conditions were investigated as regards the biomass cultivation with Bold Basal Medium. pH was changed between 3 and 7. Tests were carried out at 23 °C, 200 μE/(m^2 s). The CO_2 concentration in the gas stream fed at the photobioreactors (0.5 vvm) was increased up to 15 %v to stimulate the growth process and to simulate the CO_2 level in exhaust gas.
Preliminary results showed that CO_2 concentration higher than the air (0.035 $\%_v$) improved the process performances in terms of productivity and concentration of lipids. No strong differences were observed for the selected CO_2 concentrations (5, and 15 $\%_v$). Tests at different pH carried out with 5 % CO_2 supplemented air showed that pH of about 7 maximize the biomass productivity. Tests in inclined bubble columns resulted in a significant higher biomass and oil productivity (256 and 80 mg/L d) than those assessed in vertical bubble column (124 and 42 mg/L d).

1. Introduction

Biofuels production coupled with carbon dioxide sequestration by means of photosynthetic microrganisms appeared a promising process since the end of the last century (Benemann et al., 1977). Microalgae biomass may be burned/gasified as crude dry matter in combustors/gasifiers or may be processed to produce liquid fuels (Sheehan et al., 1998; Chisti, 2007; Posten et al., 2009). In the latter case, bio-oil is extracted from microalgae and it may be either adopted as crude fuels or transesterified to biodiesel. Biodiesel production rate from microalgae cultures may be 1–3 orders of magnitude larger than that from oil crops (Chisti, 2007; Wijffels and Barbosa, 2010). As a matter of fact, oil yield per acre per year from microalgae is 10 times higher than that from palm oil. Microalgae grow extremely rapidly and many of them are exceedingly rich in oil (50 – 80 %). Moreover, the microalgal biomass fixes a large amount of carbon dioxide – 1.83 kg of CO_2 per kilogram of dry microalgae – and strongly contributes to the reduction of greenhouse gas emissions.
In spite of the documented environmental advantages of biodiesel production by microalgae cultures, this process does not yet compare with biodiesel from oil crops from an economic point of view. As of today microalgae are not yet economically competitive with other sources of energy. More than 100 such algae-to-fuel companies have popped up worldwide, but not a single commercial facility has been built (Waltz, 2009). Strong discussions associated with economic and technical evaluations are

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continuously proposed (Pienkos and Darzins, 2010; Stephens et al., 2010; Marzocchella et al., 2010). Key aspects of the industrialization of microalgae-to-biofuel processes are: i) selection and improvements of algal strains through genetic and metabolic engineering; ii) development of high-performance photobioreactors; iii) improvement of oil extraction and transesterification processes; iv) development of microalgae biorefineries. Yield and productivity of biomass and total lipid content are strongly affected by nutrient and light supply strategy.
Pollio et al. (1997) indicated *S. bacillaris* (ACUF 158/11) as a potential candidate for commercial-scale cultivations devoted to fuel production because of the high tolerance to large variations of temperature, salinity, and pH, and of its lipid content (33 %) and fatty acid distribution. In a previous work Olivieri et al. (2011) reported about tests carried with *S. bacillaris* cultivated in 0.6 L bubble columns with air as CO_2 source under a sequence of batch, fed-batch and semi-continuous conditions with respect to the liquid phase. They obtained values of biomass- and oil- productivity of 60 and 14 mg/(L d), respectively.
The present contribution moves one step further along the direction of characterizing the effects of both the CO_2 concentration in the gas phase and the pH of the medium on biodiesel production by *S. bacillaris*.

2. Experimental

Stichococcus bacillaris was from the algal collection available at the Department of Biological Science of University of Napoli (Algal Collection at University Federico II – ACUF (ACUF, 2012). Bold Basal Medium (BBM) supplemented with $NaNO_3$ as nitrogen source was adopted. BBM was autoclaved for 20 min. The final pH was about 6.8. Tests at pH = 3.0 were carried out adding glycylglycine as buffer to BBM. HCl and NaOH were adopted to adjust the pH.
Two typology of bench scale photobioreactor were adopted: vertical bubble column (VBC) and inclined bubble column (IBC) (Figure 1).The VBC was a 1 L cylinder (0.04 m ID, 0.80 m high) (Figure 1a). Gas stream was sparged at the bottom of the photobioreactor by means of a porous ceramic diffuser. The IBC was a 2 L parallelepiped (Figure 1b) with the longitudinal axis inclined with respect to the horizontal of 20°. Gas stream was sparged at the bottom of the ISBC by means of a manifold hole (1 mm ID) Teflon tube The head of photobioreactors was equipped with three ports for gas inlet, gas outlet and sampling operations.
The photobioreactors where hosed in climate chambers (M2M engineering) equipped with lamps either at the wall (for VBC photobioreactos) or at the ceiling (for IBC photobioreactos). Temperature and irradiance in climate chambers were set at 23±1 °C and 300 $\mu E/(m^2\ s)$, respectively. The volumetric flow rate of the gas stream - sterilized through a 0.22 µm filter - was set at 20 and 50 nL/h in the VBC and IBC, respectively. A gas mixing device (M2M engineering) provided the investigated level of CO_2 (0.035, 5, 10 and 15 %) in the gas stream by mixing air with pure CO_2 from a tank.
The contamination of the microalgae culture was checked by periodic observation of culture samples by microscope (Leitz Wetzler; 567146; Germany). A Nikon Eclipse 800 fluorescence microscope was adopted to visualize the content of *in vivo* chlorophyll-a in the algal cells. The concentration of algal biomass (X) was estimated as optical density at 600 nm with a spectrophotometer (Specord 50 – Analytic Jena). The biomass dry weight was measured by filtering the suspension on a Whatman filter and drying at 60 °C. A fluorometer (AquaFluorTM; Handheld Fluorometer/Turbidimeter; Turner Designs) was used to measure the content of in vivo Chlorophyll a in the untreated samples (Chl A). Photosynthesis (Φ) and respiration rate (OUR) were measured at the same irradiance of the climate chamber by means of an Oxygraph (Hansatech) connected to a PC. Total nitrogen (TN) and total inorganic carbon (IC) concentrations in the liquid phase were measured by means of a TOC-V CSH analyzer (SHIMADZU). The procedure for the analysis of the microalgae lipid content was: I) biomass harvesting by centrifugation for 30 min, 5000 rpm at 5 °C (Eppendorf - 5804 R); II) biomass freeze-drying at -50 °C (Labconco Freezon); III) lipid extraction with a 2:1 chloroform-methanol solvent mixture in a Soxhlet apparatus for 8 h; IV) lipid transesterification with methanol and 1.5 $\%_w$ NaOH at 65 °C for 3 min; V) methyl esters analysis through HPLC (Agilent 1100) (mobile phase: water and acetonitrile; column: Synergi 4u; detector: UV/Vis).

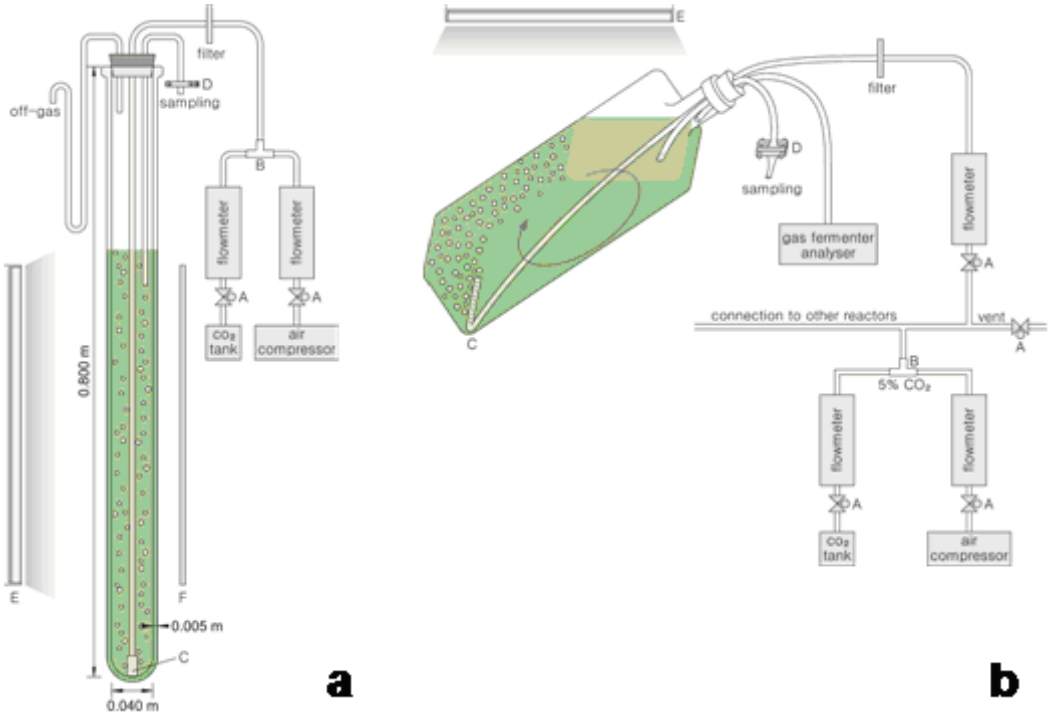


Figure 1: Sketch of the VBC (a) and of the IBC (b) photobioreactors. A) valve, C) gas diffuser, D) sterile clamp, E) light source

The pre-culture was carried out at 23 °C in continuously illuminated 100 mL Erlenmeyer flask. Periodic subculture were carried out each 4-5 days splitting the old culture into two flasks, filled up to the initial volume with BBM. The inoculum for photobioreactors was prepared in 3-4 weeks. Photobioreactors were inoculated with 1/10 of the final working volume. Tests were carried out in three different phase with respect to the liquid phase: batch, fed-batch and semi-continuous. The sampling operation took place two or three times a week.

3. Results and Discussion

Figure 2 reports data regarding two tests carried out in VBC feeding fresh air (Figure 2A) and 15 % CO_2 supplemented air (Figure 2B). The cultures were carried out under batchwise conditions until concentration of nutrients in the medium was sufficient, then the fed-batch mode started. The biomass concentration increased up to 0.26 g/L in the batch phase and then achieved a maximum value of about 4 g/L at the end of the fed-batch phase. At t = 42 d the semi-continuous mode was started with a weekly replacement of 35 % of the suspension corresponding to a dilution rate of 0.05 d^{-1}. Provided that a steady state condition was established, the reactors were cultivated under semi-continuous mode for about 6 weeks. The broth replaced with fresh medium was collected for the lipid analysis. Tests ended with microalgae cultivation under nitrogen starvation conditions for one week. The 12 week cultivations - from batch, fed-batch, and semi-continuous mode - were stopped and the residual biomass were collected for the lipid analysis.
Table 1 reports data of semi-continuous tests carried out with different CO_2 concentrations in VBC and different pH in IBC. *Stichococcus bacillaris* was able to grow in all investigated conditions. The biomass in the VBC photobioreactors reached steady state conditions within the culture time investigated provided that CO_2 was supplemented to the air stream. At steady-state conditions the biomass concentration and the biomass productivity (D·X) were about of 2.6 g/L and 130 mg/(L d).

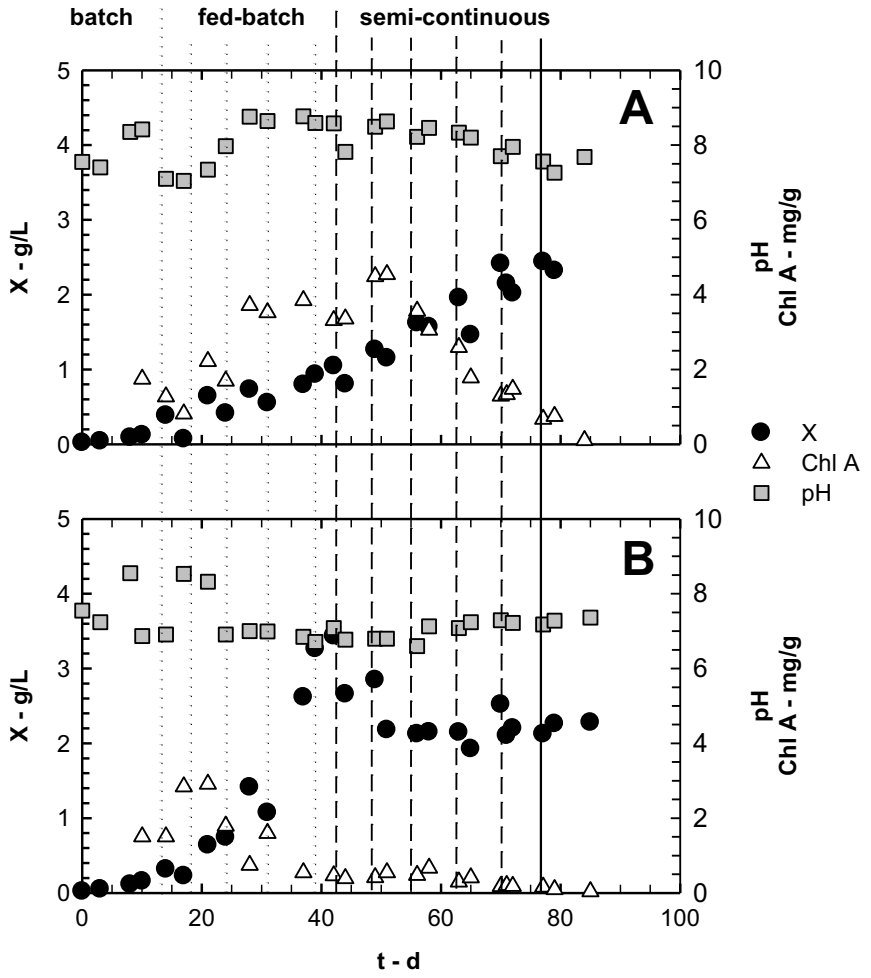


Figure 2: Semi-continuous cultures of S. bacillaris. Dotted lines mark BBM addition under fed-batch mode. Dashed lines mark culture replacement under semi-continuous mode. Continuous line marks the beginning of the nitrogen starvation conditions. A) fresh air. B) 15% CO_2 supplemented air

The Chlorophyll-a content was about 2.0 mg/g at the beginning of the tests and decreased with the time in the cultures sparged with CO_2 supplemented air. The gradual decrease of the Chlorophyll a content is in agreement with data reported by Huertas et al. (2000), Sergeenko et al. (2000) and Ge et al. (2011) regarding cultures of algal strains under high CO_2 concentrations. Due to the buffering action of carbonate and bicarbonate, the IC concentration was maximum in the reactors operated with the 15 % CO_2 supplemented air and minimum in the reactors operated with fresh air. As a consequence of the CO_2 addition, the average pH in the cultures decreased at about 7.

The total lipid fraction and the specific lipid productivity achieved their maximum in the cultures operated with 5 % CO_2 supplemented air: 34 $\%_W$ and 43 mg/(L d), respectively. Lower values were measured in the cultures operated at the highest value of CO_2.

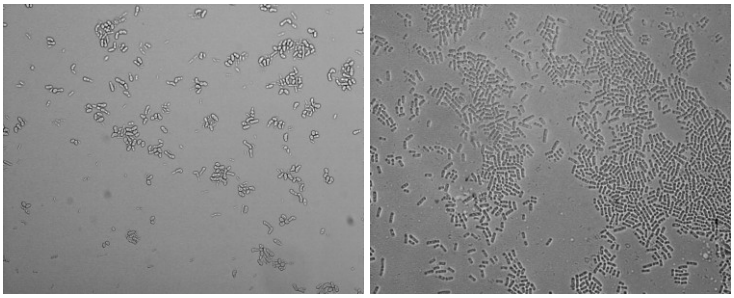
The CO_2 concentration in the air stream affected the fraction of the esterified lipids too, the fraction of lipids that can be used as biodiesel. The highest amount of total esterified lipids was achieved in cultures carried out at 15 % CO_2. As regard the lipid composition methyl-oleate was the highest identified chemical in the biodiesel.

Table 1: Steady state data of semi-continuous tests in VBC and IBC photobioreactors under N sufficient (+) and N starvation (-) conditions

Photobioreactor	**VBC**						**IBC**			
CO_2 - %	**0.035**		**5**		**15**		**5**			
initial pH			~7				3		7	
X – g/L	--		2.48		2.78		3.42		4.27	
X/t – mg/(L d)	--		124		139		205		256	
steady state pH	8.2		7.2		7.0		2.9		7.0	
IC - mg/L	68		128		146		5		64	
Chl-a –mg/g	2.56		0.30		0.27		0.79		0.57	
Φ - mg/(g h)	35.9		19.4		22.4		18.2		16.5	
N conditions	+	-	+	-	+	-	+	-	+	-
Lipid/X - %	29	28	34	27	27	27	28	24	32	40
Lipid/t – mg/(L d)	--	--	43	--	37	--	57	--	81	--
Esterif. lipid - %*	33	34	24	25	45	33	14	6.0	40	37
C16:0-palmitate**	--	--	--	--	--	--	--	--	5.6	--
C18:1cis – oleate**	26	27	18	18	33	24	11	3.9	23	22
C18:1 trans- elaidate**	--	--	--	--	--	--	--	--	3.8	5.3
C18:2 - inoleate**	3.2	4.0	2.7	3.4	4.1	4.4	1.7	0.9	4.4	4.3
C18:3-linolenate**	3.1	3.0	2.8	3.0	5.6	4.7	1.6	1.3	5.6	5.0

*data referred to the total esterified lipids. ** data refered to the total lipids

The pH effects on the microalgae cultures were investigated in the IBC photobioreactors operated with 5 % CO_2 supplemented air. Test carried out at pH=3.0 were characterized by much higher values of TN due to the nitrogen-rich component (glycilglycine) of the buffer system. The inhibiting effect of CO_2 on the Chl-a content was still observed. The best performances in terms of biomass concentration (4.27 g/L), productivity (256 mg/L d), lipid content (32 %), and productivity (81 mg/L d) were obtained at pH=7.0. In addition, the lipid content increased in the pH=7.0 cultures with the nitrogen starvation from 32 up to 40 %. Tests pointed out that *Stichococcus bacillaris* was able to grow under extreme conditions (pH about 3) with acceptable value of total lipid content (28 %) and productivity (57 mg/L d). However the total amount of esterified lipids was very low compared to the value achieved at pH=7.0.
Microscopic observations of the suspension sampled from cultures (Figure 3) pointed out that cells in cultures carried out at pH=7.0 appeared uniform, and characterized by high motility (Figure 3). At pH = 3.0 the agglomerated cells were: not motile, not uniform in size and shape, typically curved, in some cases very plump
Results of the tests carried in IBC were strongly different from those of tests carried out in VBC under similar operating conditions (pH = 7.0, CO_2 fraction in the air stream 5 %). The biomass productivity and the lipid productivity were higher in IBC (256 and 81 mg/(L d), respectively) than in VBC (124 and 43 mg/(L d), respectively). As a result, reactor shape affects the growth rate and the composition of microalgal cells.
The higher performances assessed fro the two reactors may be related to the hydrodynamic regime typical of the two units. It is possible to infer that both shear stress and light exposure pathway may affect the cell growth. As regards the light exposure it should be noted that in a concentrated culture of 2 g/L - optical density 5 - about 99.99 % of incident light is absorbed in the first millimeters of suspension. As a consequence, the biomass growth in the inner region of the photobioreactor is limited by the light supply to the whole culture. In IBC the culture re-circles continuously in the unit and cells renew in the upper region of the column with a frequency of about 2-3 Hz. In VBC operated under homogeneous bubbly flow conditions, the renewing frequency at the external irradiated-external region is of about tenths of Hz. The higher light efficiency of IBC vs VBC may be then explained.



pH = 3.0 pH = 7.0

Figure 3: Stichococcus bacillaris cells growth at different pH values in IBC photobioreactors

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