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# Biodiesel Production in Outdoor Cultures of *Scenedesmus Vacuolatus*

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The supply of renewable feedstocks for the production of convenience goods combined with the carbon capture and storage is considered a promising solution to both fossil resources depletion and global warming control. Photosynthetic microorganisms, e.g. microalgae, are good candidates for this challenging bet. The culture performances remarkably reduce when microalgal cultures move from laboratory to industrial scale. The basic requisite for the development of large-scale production of oil from microalgae is to make the process environmentally and economically sustainable/feasible. A key issue for the process based on outdoor photobioreactors (PBR) is the efficient utilization of the photosynthetically active radiation (PAR).
The present work reports the results of outdoor cultures of *Scenedesmus vacuolatus* strain *ACUF 053/95* in 1.7 L inclined bubble column (IBC) photobioreactors characterized by 250 $cm^2$ irradiated surface. Cultures were carried out in outdoor: i) during the May-July period under shadow conditions, irradiance maximum of 450 µE/($m^2$ s); ii) during the May-July period under direct sun light, irradiance maximum of 2100 µE/($m^2$ s); iii) during the September-November period under direct sun light, irradiance maximum of 2000 µE/($m^2$ s).
Harvested microalgae were processed to characterize the biodiesel composition thought direct alkaline transesterification. The biomass concentration at steady state conditions was kept within the range 3-4 g/L. Biomass volumetric productivity ranged in the interval 0.17-0.22 g/(L d) - area specific productivity of 11-15 g/($m^2$ d) - depending on the operation mode.

## 1. Introduction

The last century has been characterized by the explosive growth of the energy consumption and rapid increase of greenhouse effects (Etheridge et al., 1998). Moreover, the exploitation of renewable feedstocks for the production of convenience goods combined with bio-fuels production and carbon capture and storage is considered a promising solution to both fossil resources depletion and global warming control. Photosynthetic microorganisms, e.g. microalgae, are good candidates for this challenging bet. Indeed, autotrophic microalgae fix $CO_2$ and are feedstocks for several industries involved in human nutrition, animal nutrition, cosmetics, high-added value molecules, pharmaceuticals, biofuels and wastewater treatments (Chisti 2007; Wijffels and Barbosa, 2010; Li et al., 2015).
The concept of using microalgae to produce fuel was already being discussed 50 years ago. However, a concerted effort began with oil crisis and fuel cost increase in the 1970s (Hu et al., 2008). The advantages of microalgae as feedstock for biodiesel production with respect to terrestrial plants are: i) the microalgae can accumulate lipid at high concentration; ii) the microalgae do not require fertile land; iii) the microalgae can growth even on not freshwater; iv) the microalgae are characterized by high sensitivity to growth conditions and the different types of stress change the lipid composition; v) the microalgae can produce high value products.

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The success of biodiesel production from microalgae depends on the content of triglyceride (TAG) which are more than 70% of the lipid content (Eichenberger and Gribi, 1997; Spolaore et al., 2006; Dvoretsky et al. 2015; Tan et al., 2015). In general, TAGs are glycerol molecules esterified with three fatty acids. They may react with methyl alcohol to produce fatty acid methyl esters (FAMEs) and glycerol, as a by-product. The high concentration of unsaturated fatty acids in the extracted lipids is determinant for the fuel quality. Unsaturated FAMEs comprised over 82% of the total biodiesel content (Xu and Miao, 2006; Cheng et al., 2009). The FAMEs content is mainly composed of oleic, linoleic, linolenic, palmitic and stearic acids (Pratoomyot et al., 2005; Ruiz et al., 2009; Gao et al., 2010).

To reduce the biodiesel production costs is essential to use outdoor cultivation in open systems, but the main problem associated with this type of cultivation is the contamination of the culture by other algal species (Eustance et al., 2015). Moreover, during outdoor culture with solar energy as the light source, the biomass productivity is strongly affected by environmental factors such as irradiation and temperature. In an open pond without any temperature regulation, it has been shown that biomass growth was mainly limited by the temperature in winter and by irradiation in summer, since the culture temperature and irradiation likewise varied with the seasons (Vonshak et al. 1982). Lee and Low (1993) and Ho et al. (2014) reported the effect of weather and different operating condition on biomass growth, concluded that adaptation of the cell suspension to a high level of irradiation often increased the photosynthetic capacity and decreased the photosynthetic efficiency. The culture performances remarkably reduce when microalgal cultures move from laboratory to industrial scale (Rawat et al., 2013; Eustance et al., 2015). The basic requisite for the development of large-scale production of oil from microalgae is to make the process environmentally and economically sustainable/feasible (Rawat et al., 2013). A key issue for the process based on outdoor photobioreactors (PBR) is the efficient utilization of the photosynthetically active radiation (PAR) (Suali and Sarbatly, 2012). The efficient conversion of the sunlight – variable during the day and the year – requires the optimization of operating conditions and PBR design.

*Scenedesmus* sp. has been frequently proposed as possible candidate to produce biodiesel from microalgae (Griffiths et al., 2010; Rodolfi et al., 2009; Mata et al., 2010; Abd El Baky et al., 2012; Eustance et al., 2015) and the present work reports the results of outdoor cultures of *S. vacuolatus* strain *ACUF 053.* The cultures were carried out in 1.7 L closed inclined bubble column (IBC) photobioreactors characterized by 250 $cm^2$ irradiated surface. IBCs were operated according to Olivieri et al. (2013). The temperature was set at 23°C and pH was kept constant at 7. A 2% $CO_2$ gas stream (50 nL/h flow rate) was sparged at the bottom of the IBCs. Modified Bold Basal Medium (BBM) was adopted. Cultures were carried out in outdoor: i) during the May-July period under shadow conditions, irradiance maximum of 450 μE/($m^2$ s); ii) during the May-July period under direct sun light, irradiance maximum of 2100 μE/($m^2$ s); iii) during the September-November period under direct sun light, irradiance maximum of 2000 μE/($m^2$ s). Cultures were operated under fed-batch and semi-continuous modes at dilution rate set at 0.045 $d^{-1}$. The attention was also focused on the lipid production under different conditions of sunlight and nitrogen sufficient conditions.

## 2. Experimental

### 2.1 Organism and medium

*Scenedesmus vacuolatus* strain 053, belonging to the *Scenedesmus* genus ELIMINA, phylum Chlorophyta, was from the algal collection at the Department of Biological Science of the University of Naples “Federico II” (ACUF) (http://www.acuf.net ). Bold Basal Medium (BBM) supplemented with $NaNO_3$ – 40 mg/L – as nitrogen source was used. BBM was autoclaved for 20 min at 120°C. The pH of the autoclaved medium was close to 7.

### 2.2 Reactor setup and diagnostics

Microalgal pre-cultures were carried out in Erlenmeyer flasks housed in a climatic chamber (Gibertini) equipped with daylight fluorescent Philips lamps (TLD 30W/55). Microalgal cultures were carried out in Inclined Bubble Column (IBC) photobioreactors, volume of 2 L (Gargano et al., 2013; Olivieri et al., 2012). The working volume was set at 1.7 L, the longitudinal axis was at 20° with respect to the horizontal plane and the irradiated surface was 250 $cm^2$. Gas stream was sparged at the bottom of the IBC by means of multiple-orifice (1 mm ID). A hydrophobic filter (0.2 μm) sterilized the gas flow inlet. A gas mixing device (M2M engineering) provided the selected concentration of $CO_2$ in the gas stream fed to the photobioreactors by mixing air and pure $CO_2$. The temperature was set al 23°C and it was guaranteed by the climatic chamber. Cultures were carried out outdoor: i) a test campaign during the May-July period under shadow conditions characterized by a maximum irradiance level of 450 μE/($m^2$ s) and under direct sunlight with a maximum irradiance level of 2100 μE/($m^2$ s); ii) a test campaign during the September-November period under direct sun light where the irradiance can achieve a maximum of 2000 μE/($m^2$ s). Liquid phase was characterized in terms of pH,

measured with a pH-meter (Crison, Basic 20), and nitrate concentration, measured with nitrate probe (Matter). Biomass concentration (X) was measured with a spectrophotometer (Specord 50 – Analytic Jena) at 600 nm. Chlorophyll A content was detected in vivo with a fluorimeter (460 nm excited – 650 nm emitted) (AquaFluorTM; Handheld Fluorometer/Turbidimeter; Turner Designs). Bacterial and fungal contamination in the cultures was checked by microscope observations (Leitz Wetzler; 567146; Germany).

#### 2.2.1 Operation conditions and procedure

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 filling fresh medium. The inoculum for photobioreactors was prepared in 3 - 4 weeks. Photobioreactors were inoculated with 1/10 of the final working volume. The volumetric flow rate of the sparged gas stream was set at 0.5 vvm and the added $CO_2$ concentration was set at 2%. Tests were carried out in three different operating modes with respect to the liquid phase: (batch, fed-batch and semi-continuous) and in differential conditions with respect to gas phase. Under fed-batch conditions a concentrated BBM (10 times the concentration of medium) was supplemented to the photobioreactors when the nitrate concentration was lower than 10 mg/L. In this way nitrate concentration was never limiting for more than one day. Under semi-continuous operations 30% of microalgal suspension was weekly replaced with fresh medium. In this case the nitrate concentration depends by the growth and nutrient uptake rate determined by the average dilution rate (D) resulting to be 0.045 $d^{-1}$. The sampling operation took place two or three times a week. Data at steady state conditions were calculated as the average value: biomass concentration ($X_{ss}$), volumetric biomass productivity ($W_X = X_{ss} \bullet D$), chlorophyll A content (Chl A).
The biomass harvested during semi-continuous operations was used for lipid analysis. The procedure for the analysis of the microalgal lipid content was: I) biomass harvesting by centrifugation for 20 minutes, 5000 rpm at 5 °C (Eppendorf-5804 R); II) biomass freeze-drying at -50 °C (LabconcoFreezon); III) alkaline direct transesterification of biomass with methanol and 3.5% w/w NaOH at 70 °C for 10 min; IV) methyl esters analysis trough Agilent 7820A GC equipped with a Flame-ionization detector (FID) and Agilent DB-WAXTER column (30m x 0.320mm x 0.50 film thickness). The temperature was increased from 100 °C to 230 °C at 10 °C/min), hydrogen (1mL/min in constant flow) was used as carrier gas and the detector temperature was 300 °C. The identification and quantification of the fatty acid methyl esters (FAMEs) by chromatographic peaks was performed using commercial standards from Sigma-Aldrich. The identified FAME yield was reported as % FAME = total FAME mass/dry algal mass.

## 3. Results and discussion

Figures 1 reports data regarding three tests carried out in the Inclined Bubble Column photobioreactors: a) May/July under shadow conditions and direct sunlight; b) September/November under direct sunlight. The culture was operated under batch-wise conditions for about 10 days: the algal biomass concentration increased up to about 0.7 g/L for all conditions. The fed-batch mode started as the concentration of nutrients in the medium was not sufficient. Under fed-batch mode, microalgal growth continued and the biomass concentration achieved about 4.0 g/L in both conditions of direct sunlight, and 2.0 g/L under shadow conditions. After 28 day, the semi-continuous operation condition started. Provided that steady state conditions were established, the reactors were operated under semi-continuous mode for about 3-5 weeks. During the semi-continuous mode the harvested biomass was collected to assess produced lipids. At the end of semi-continuous mode, the cultures were stopped and the residual biomass was harvested. Results of the microalgal characterization under steady state conditions are reported in Table 2.

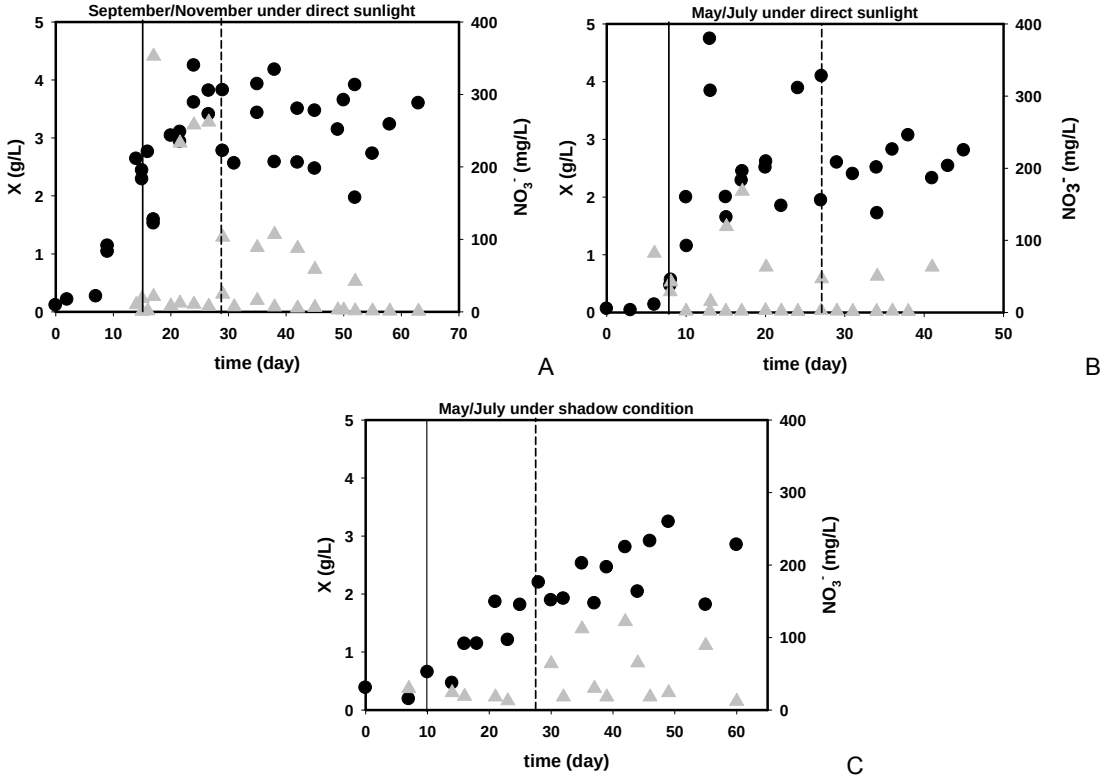


*Figure 1: S. vacuolatus cultures under a-b) shadow conditions; c) direct sun. Continuous vertical lines mark the beginning of the fed-batch, dashed vertical lines the semi-continuous mode. ● - biomass concentration (X); ▲ - nitrate concentration ($NO_3^-$).*

Table 2 reports the results of tests in terms of average biomass concentration ($X_{SS}$), chlorophyll A content (Chl A), volumetric biomass productivity ($W_X$), areal biomass productivity ($W_A$), and biomass/light conversion ($Y_{X/E}$) assessed under steady state conditions. The composition of identified FAMEs is also reported.

*Table 2: Steady state data of semi-continuous tests in inclined bubble column photobioreactors under different irradiance conditions.*

| | May/July | | September/November |
|---|---|---|---|
| | **Shadow condition** | **Direct sunlight** | **Direct sunlight** |
| % $CO_2$ | 2 | 2 | 2 |
| pH | 7 | 7 | 7 |
| I - µE/($m^2$ s) | 450 | 2100 | 2000 |
| $X_{ss}$ – g/L | 2.9 | 3.1 | 3.2 |
| $W_X$ – g/(L d) | 0.122 | 0.130 | 0.134 |
| $W_A$ – g/($m^2$ d) | 9.76 | 10.4 | 10.72 |
| $Y_{X/E}$ ($g_X$/E) | 0.17 | 0.06 | 0.06 |
| Chl A – mg/g | 4.94 | 5.11 | 5.12 |
| FAME yield - % | 4.80 | 4.20 | 6.42 |
| C18:1 – oleate/elaidate - % | 0.30 | 0.85 | 0.77 |
| C18:2 - linoleate - % | 0.42 | 1.04 | 1.20 |
| C18:3 – linolenate - % | 1.72 | 1.16 | 2.27 |
| C16:0 – palmitate - % | 1.08 | 0.74 | 1.27 |
| other - % | 1.28 | 0.41 | 1.11 |

The analysis of the results shows that *S. vacuolatus* was able to grow under all investigated conditions. The pH measured during tests was steady at 7 as a result of the equilibrium between acid action of the dissolved $CO_2$ and the buffer action of the carbonate/bicarbonate system. A very slight increase in biomass productivity from 0.12 to 0.13 g/(L d) and Chlorophyll A content from 4.9 to 5.1 mg/g may be observed under direct sunlight conditions. The invariance of the volumetric and areal biomass productivity rate may be interpreted considering that the basis of microalgal biomass production is directly proportional to the efficiency of photosynthesis. These results are confirmed considering the biomass/light conversion. The biomass/light conversion resulted three times higher on the biomass growth under shadow condition than under direct sun light. Under high light intensity the photosynthesis become less efficient (Camacho Rubio et al., 2003; Chisti, 2007). The considered irradiance under direct sun light resulted definitely very high and the photosynthesis was inhibited: the biomass/light conversion was low. Similar results were observed by Eustance et al.(2015). they did not observe variation on *Scenedesmus acutus* productivity passing from autumn to winter conditions. Those results, in association with the results reported by Eustace et al., 2015, can suggest that *Scenedesmus* strain can be a good candidate for outdoor culture of microalgae because it is able to adapt very easily to outdoor conditions.
For the characterization of the produced biodiesel, the determination of the FAMEs composition is an important step for. The conventional protocol consists of two phases: lipid extraction and transesterification. To reduce costs associated with lipids extraction, the attention was focused on the biodiesel production by a one-pot process. This approach is known as in situ or direct transesterification. The direct transesterification protocol optimized per *Stichococcus bacillaris* (Gargano et al., 2013) was used for the FAMEs characterization. The fraction of FAMEs – a relevant feature for biodiesel application – was characterized. No significant differences in terms of percentage of identified FAMEs were observed in all outdoor considered conditions. As regard the lipid composition methyl-oleate, linoleate, linolenate and elaidate were the identified chemicals present at the highest concentrations in FAME mixture.

## 4. Conclusions

The effects of the outdoor conditions on photobioreactor performances were investigated. Tests were carried out in inclined bubble columns at different irradiance values: under shadow condition (450 μE/($m^2$ s)) and under direct sun light (about 2000 μE/($m^2$ s)). The biomass productivity was between 0.122 and 0.130 g/(L d), the fraction of identify FAMEs using alkaline direct transesterification ranged in the interval 4.2 – 6.2 %, depending on the operation mode. *Scenedesmus vacuolatus* strain 053 was able to adapt easily to outdoor conditions.

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