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Photobioreactor-based procedures for reproducible small-scale production of microalgal biomasses

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Abstract

A simply designed photobioreactor (PBR) was developed with associated microalga production protocols for *Arthrospira platensis* and *Chlorella vulgaris*. 80-L culture medium batches of unstressed microalgal strains, still in their growing phase, were obtained. For the two microalgal strains used, biomass production cycles were found to be reproducible between replicate runs. On a dry weight basis (d.w.), productivity (P) and total biomass production (TBP) at the end of a production cycle (30 days) were respectively 30 mg d.w. L^{-1} day^{-1} and 0.9 g d.w.L^{-1} for *Spirulina*, and 20 mg d.w. L^{-1} day^{-1} and 0.6 g d.w. L^{-1} for *Chlorella*. Four sets of fluorescent light blocks (72 W) symmetrically set around the PBR tube provided the culture medium with permanent illumination of 336 µmol m^{-2} s^{-1} for *Spirulina*, and 168 µmol m^{-2} s^{-1} for *Chlorella*.

The unsophisticated design of this PBR unit makes it suitable for use in low-level technical environments, generally found in developing countries such as Côte d'Ivoire. The production protocols described enable even unskilled people to produce themselves these two microalgal biomasses that are locally used as food or feed supplements, or as partial substitutes for commercial fish feed used to raise tropical fish such as tilapia (*Sarotherodon melanotheron*), in small-scale recirculation breeding systems.

Keywords: Photobioreactor, PBR, microalgae, production, *Arthrospira platensis*, *Spirulina*, *Chlorella vulgaris*.

Running title: Reproducible small-scale PBR production of microalgae

Introduction

Microalgae are photosynthetic microorganisms capable of using light and carbon dioxide to produce biomass, with higher yields than photosynthetic plants. Japan started first large-scale cultivation of *Chlorella vulgaris* (*Chlorella*) (K. Yamaguchi 1997). Then extensive production of *Arthrospira platensis* (*Spirulina*) was first set in Mexico, and today, the major share of industrial microalga production is located in the Asia-Pacific rim, including China and India (Y.-M. Lu et al. 2011). The industrial-scale world production of the 3 major strains, including *Spirulina* spp., *Chlorella* spp. and *Dunaliella* spp., accounted for 8,000 t of dry weight/year, among them *Spirulina* was estimated to be over 3,000 t of dry weight/year (J. J. Milledge 2011). These biomasses are marketed as health food, nutraceutical products and for cosmetics (K. W. Gellenbeck 2012).

In developed countries, large-scale microalgal biomass productions use open-type production systems, such as artificial ponds, pools, or raceways, often placed under greenhouses, which involves extra building costs (A. Belay 2002). As an alternative, closed-type systems, namely photobioreactors (PBR), has been developed from the lab-scale to the industrial-scale to produce, as a continuous production flow or in batch mode, microalgal biomasses of several strains for niche markets. Well-controlled and well-managed cultivation conditions are required for successful production to fit with the objectives of the niche markets targeted (F. G. Acién Fernandez et al. 2013). Various PBR designs have been developed: plastic bags, stirred tanks, bubble columns, airlifts, large surface area flat glass panels or thin layer reactors, horizontal glass or light transparent plastic tubes connected together, vertically or horizontally, in a closed serpentine system. To allow continued growth of the biomass, the photoperiod has to be avoided during cultivation. Sophisticated internally illuminated photobioreactors (IIPBR), equipped with optical fibres to increase permanent light availability were also developed (A. K. Pegallapati et al. 2012; J. C. Ogbonna and H. Tanaka 2000). However, these PBRs also entail high construction costs (B. Wang et al. 2012; R. N. Singh and S. Sharma 2012; M. R. Tredici 2007).

In developing countries, microalgae are still considered as an interesting way to provide from aquatic plants, a relatively low-cost food supplement for low-income populations in poor health. Moreover, microalgal biomasses can be produced within a relatively short agricultural production chain, compared to the longer chain required to produce foods of animal origin. Only few practical books provide practical recipes for small-scale production of *Spirulina* in outdoor pounds (J. Jourdan 1996; R. D. Fox 1996; R. Henrikson 2013), but not for other microalgal strains, such as *Chlorella*, probably due to the lack of a

suitable small-scale harvesting technology. It was observed that many people from these countries (individual farmers or by village communities) have tried unsuccessfully to produce microalgae biomass because they did not well-monitor the numerous production parameters in the right way. Generally, both outdoor open or closed-type production systems, coupled with variable production methodologies, are not really accessible to these small producers. Many difficulties are encountered in gaining a real command of microalga cultivation, leading to low or irregular biomass productivity and quality, and even total loss of the entire production, leading to discouragement. Various types of biomass pollution from air-borne microorganisms with fast and competing growth rates have been encountered during microalgae outdoor productions (C. M. Monteiro et al. 2012; L. E. De-Bashan and Y. Bashan 2010). Generally, more expensive investments in closed-shading or green housing have to be made to minimize biomass contamination when using the less costly outdoor open-type production systems (tanks, pools, raceways). Otherwise, quality of the produced biomass can be poor, especially with strains of the *Chlorella* genus, which have a natural tendency to capture all type of environmental pollution (H. F. Olguin et al. 2000; M. M. El-Sheekh et al. 2000). In the case of *Spirulina*, such possible exogenous microorganism pollution is limited due to the relatively high pH level of the *Spirulina* culture medium. This is why, in African countries, microalga production is limited to open-type cultivation systems of *Spirulina sp*., that still remains difficult to well-manage for these people for many other reasons such as: lack of skilled manpower, relatively high-level of investments (land and construction costs) for operating raceways, air-borne bacterial pollutions, to master climate variations (successive dry and rainy seasons) and diurnal sunlight fluctuations that cause photoinhibition of the culture, preventing to achieve good biomass productivity (C. Sili et al. 2012).

Microalgae can also be used as an ingredient in the low-cost production of animal feed for fish farming (B. Raoof et al. 2006). In developing countries, the local small-scale aquaculture sector is also looking today for new low-cost feed sources to partially or totally replace the costly commercial fish feed, usually made with edible products from plant and animal origins (S. Hemaiswarya et al. 2011; J. Lu et al. 2006). Some of these fish nutrients are found in the composition of microalgal biomasses, produced from strains such as S*pirulina* or *Chlorella*. Small-scale production of such biomasses may provide local small-scale fish breeders with an alternative cheaper feed source for tropical fish breeding, e.g. tilapia (*Sarotherodon melanotheron*), grown in small-scale aquariums or small-volume ponds, than the industrial fish feed bought by the local fish breeders, entailing significant expense.

A small-scale and low-cost PBR was designed and biomass production protocols were drafted, at least for the two most popular microalgal strains: *Spirulina* and *Chlorella*. Cultivation protocols were set up to produce microalgae biomass, in a reproducible way, starting from the inoculum strains and ending with constant final biomass yields for each production cycle launched with the same inoculum. All production parameters were well-defined at the start of the production cycle and required from the operator no real monitoring until the end. Applying the described protocols, even unskilled people can produce, at their first attempt, several successive batches of microalgal culture medium showing reproducible biomass content and quality (dry weight yield, still growing and unstressed microalgae).

Materials and Methods

Microalga strains and inoculum cultivation

Two microalga strains *Arthrospira platensis* (*Spirulina*) and *Chorella vulgaris* (*Chlorella*) were collected from the inoculum collection maintained by an industrial microalga producer (Greensea, Mèze, France). The original strains were supplied to this producer by the Pasteur Institute in France from its own collection of pure microalga cultures. The inoculum samples were brought to the laboratory and cultivated under axenic conditions in 10-L glass bottles, previously cleaned by shaking the stoppered bottles containing 1L of distilled water and 100 ppm of NaOCl from time to time for 30 min. The bottles were rinsed with distilled water and stoppered. Aliquots (10 mL) of each collected *Spirulina* and *Chlorella* inoculum were added to different bottles already containing 5 L of the corresponding culture medium prepared for strain growth. The inoculum bottles were aligned on a glass-covered table, at room temperature (25°C), and continuously illuminated by a light block of two fluorescent daylight tubes placed parallel to the bottle line around 10 cm away. The bottles were placed against a sheet of white plastic to reflect part of the emitted light. The photon flux measured between the light block and the bottles, at the surface of each bottle, was found to be 84 µmol photon m^{-2} s^{-1}. Each bottle was stoppered with a cork perforated with 2 holes to allow air bubbling (200 mL min^{-1}) via a constantly filtered air flow manifold and an air outlet tube also equipped with an air-filter (PTFE 0.2 µm filter, Sartorius Stedim). For the *Chlorella* inoculum, air bubbling was enriched with 5% (v/v) CO_2. After five days' growth, the inoculums reached a biomass concentration of about 0.2 g L^{-1}, and were taken at this stage for inoculation of the PBR culture media.

PBR design and equipment

The open-type PBR consisted of a vertical plastic tube (methyl polymetacrylate, i. d. = 24.5 cm, height = 1.8 m, thickness = 3 mm, total vol. = 84 L) fixed at the centre of a parallelepiped-shaped cage (68 x 68 x 180 cm) formed by metal panels (5 x 5 cm) and positioned on a stainless steel platform with four legs equipped with wheels to allow movement of the PBR (**Fig. 1**). The tube was maintained securely in the cage between the upper cover and lower platform: the upper cover was made of plastic. Four light blocks were placed on each vertical panel of the cage and were centred at mid-height of the plastic

tube. They were 17 cm away from the outer surface of the tube. Each block was equipped with two daylight (6500K) fluorescent tubes (T8, F-36 W / 54-765, 2.6 cm wide, 1.2 m long). A large waste collection system was installed at the bottom of the tube, under the platform, to allow draining of the tube content at the end of each cultivation cycle or during the washing cycles. An air bubbling device was made with a ballasted circular porous tube (i. d. = 5 mm) placed at the bottom of the main tube and connected to a small plastic tube (e. d. = 3mm) conveying filtered air (0.2 µm PTFE filter, Sartorius Stedim) to the open top of the PBR. Air could be provided either by an aquarium pump placed on the upper cover or by an oil-free air-compressor continuously delivering a filtered air flow of 400 L/h controlled by an air flow meter (EFMP06VRG1, Metaworks Instruments, France). An aquarium-type temperature-controlled heater (18-34°C, TSRH 3614 type, 25-100, 100 W, Eheim, Germany), immersed at mid-height in the PBR tube, maintained the culture medium at a constant temperature (34°C) during production cycles, as the PBR was in a laboratory with the temperature set at around 25°C.

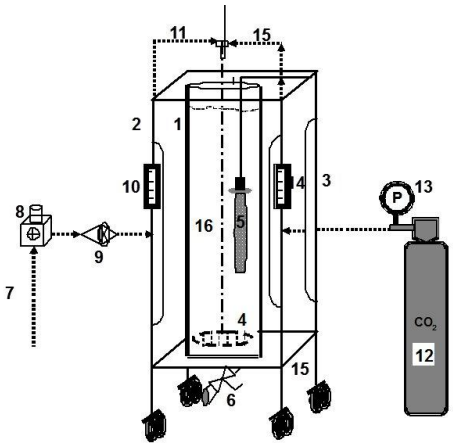

Fig 1 Photobioreactor

Light measurements

An illumination meter (Digital illumination meter, 1308SI, IHM, China) was used for light measurement (lux) at various spots around the PBR tube. The illumination level at the surface of the PBR tube was measured by placing the sensor on the surface of the PBR tube, exactly facing each light block, at mid-height. Illumination measurements were taken on each block position when all four blocks were switched on. They were found to be similar to each other (6 Klux). As the light was provided by daylight fluorescent tubes, it was possible to express the illumination level in photosynthetic photon flux density (PPFD) units, using the conversion factor of 0.014 (1 lux = 0.014 µmol photons s^{-1} m^{-2}) (Osram Sylvania 2000; S. Oncel and F. V. Sukan 2008). The total illumination level supplied to the culture medium was calculated by summing up the four illumination levels measured at each light block position. Total illumination levels were thus found to be 84, 168 and 336 µmol photons s^{-1} m^{-2} when one, two opposite and four light blocks were switched on respectively.

Preparation of the microalgal culture media

All the chemicals used for culture medium preparation were of technical grade (Panreac, Spain). The water used was of constant quality by using freshly pre-filtered (0.2µm) tap water to keep standardised conditions during comparative microalga production cycle runs. For each microalga, the same culture medium composition was used for inoculum growth and the PBR production cycle.

- *Stock solutions for Spirulina growth*

The composition of the culture medium used for *Spirulina* inoculum growth and for PBR production was basically similar to that suggested by Zarrouk (1966). Some slight modifications in certain nutrient concentrations were however made, as indicated by the medium compositions given hereafter. Four concentrated nutrient stock solutions were prepared separately as follows.

Solution A (10 L). 1280 g of sodium bicarbonate $NaHCO_3$ was added to 10 L of water under constant efficient stirring. The solution was over-saturated and remained whitish as its maximum solubility in water = 103g/L at 25°C (Properties of the Elements and Inorganic Compounds: Section 4 2005).

Solution B (2 L). 200 g of sodium nitrate $NaNO_3$ was dissolved in 2 L of water under magnetic stirring.

Solution C (2 L). The following salts were added and allowed to completely dissolve, in sequence, for 5 min under magnetic stirring, in 2 L of water: NaCl (80.0 g), K_2SO_4 (80.0 g), $KHPO_4$ (40.0 g), $MgSO_4 \cdot 7H_20$ (0.80 g), $CaCl_2 \cdot 7H_2O$ (3.2 g), $FeSO_4 \cdot 7H_2O$ (0.80 g), and EDTA (6.4 g).

Solution D (1 L). Minor salt components were added and allowed to completely dissolve, in sequence, under magnetic stirring, in1L of water: H_3BO_3 (2.6 g), $MnCl_2 \cdot 4H_2O$ (1.8 g), $ZnSO_4 \cdot 7H_2O$ (222 mg), and $CuSO_4 \cdot 5H_2O$ (79 mg).

As only small volume aliquots of this stock solution were used for each PBR culture medium preparation, stock solution D was stored in a cold room (5°C).

- *Stock solutions for Chlorella growth*

Two concentrated nutrient stock solutions were prepared separately as follows:
Solution E (2 L). The following salts were added and allowed to completely dissolve, in sequence, under magnetic stirring, in 2 L of water: $NaNO_3$ (16.0 g), $NaHCO_3$ (4.0 g), K_2HPO_4 (2.0 g), $MgSO_4 \cdot 7H_2O$ (2.0 g), $CaCl_2 \cdot 2H_2O$ (2.0 g), Na_2CO_3 (0.40 g), $FeSO_4 \cdot 7H_2O$ (0.10 g), EDTA (0.10 g), and $MnCl_2 \cdot 4H_2O$ (30mg).
Solution F (1 L). Minor salt components were added and allowed to completely dissolve, in sequence, under magnetic stirring, in1L of pre-heated (50°C) water: $ZnSO_4 \cdot 7H_2O$ (0.20 g); $CuCl_2 \cdot 2H_2O$ (0.20 g); $CoCl_2 \cdot 6H_2O$ (0.10 g); and H_3BO_3 (12 mg).
As only small volume aliquots of this stock solution were used for each PBR culture medium preparation, stock solution F was stored in a cold room (5°C)

- *Culture medium for PBR Spirulina production*

The PBR tube was first filled with 50 L of water and the following nutrient solutions were added in sequence: the whole volume of prepared sol. A (10 L), sol. B (2 L) and sol. C (2 L). Then 80 mL of sol. D was added (1 mL of sol. D per L of culture medium).
To provide sufficient mixing of the medium before the microalga production cycle began, air bubbling was started at 400 $L.h^{-1}$. The PBR was then filled with water up to a total volume of 80 L. The culture medium was allowed to homogenize until the immersed aquarium-type heater raised the temperature to 35 ± 1°C, which is the suggested optimum growth temperature for *Spirulina* (G. Torzillo et al. 1991).

- *Culture medium for PBR Chlorella production*

The culture medium was prepared by transferring the whole of sol. E (2 L) to the PBR. Then 8 ml of sol. F was added. The PBR was filled with water up to a total volume of 80 L. Air bubbling was started at 400 L h^{-1} to homogenize nutrients until the temperature of the culture medium stabilized at 26 ± 1°C, which was within the range of optimum growth temperatures for *Chlorella* (A. Converti et al. 2009).

Preparation of microalga inoculums

Each culture medium prepared for PBR production of *Spirulina* or *Chlorella* was inoculated using an aliquot of the corresponding microalga strain inoculum. Both microalga strains of the inoculum stock solutions were maintained in a growing phase by regularly sampling (every 5-8 days) 2.5 L of the inoculum stock solution, placed in a new cleaned 5-L bottle. Then, 2.5 L of a freshly prepared culture medium was added. The average microalga concentration of the inoculum C_i was generally within a range of 0.2 - 0.5 d.w. g L^{-1}. The initial biomass concentration at the start of a PBR production cycle was standardised at 50 ± 3 mg L^{-1} for *Spirulina* and at 20 ± 3 mg L^{-1} for *Chlorella*. A volume (mL) was taken from each inoculum stock solution (V_{Sp} for *Spirulina* and V_{Ch} for *Chlorella*) and added to the corresponding PBR culture medium, before the PBR production cycle started. These inoculum volumes were calculated as $V_{Sp} = (80 \times 50) / C_i$ and $V_{Ch} = (80 \times 20) / C_i$. Before adding the inoculum to the PBR, a volume of culture medium (V_{Sp} or V_{Ch} according to the strain produced) was removed. For each microalga production cycle, the total culture medium volume was 80 L and the initial microalga concentration was set at the standardised level stated above.

PBR microalga production cycles

As soon as the PBR culture media had been inoculated by the corresponding microalga strain, the PBR production cycle started by monitoring biomass growth and other characteristic parameters of the culture medium. All PBR production cycles were conducted in batch mode.
Generally a latency growth period of one day was observed at the start of the production cycle. This growth delay was the time needed for the microalga inoculum, diluted in the PBR culture medium, to adapt itself to its new environment. To avoid extra stress to the microalga culture, only two opposite light blocks were switched on for 24 h after the start of the cycle. The other two were then switched on and the 4 blocks were kept on for the whole production cycle (about 30 days).
For the *Spirulina* production cycle, air bubbling was set at 200 L h^{-1} at the start of the cycle and kept at that level for 2 weeks. It was then reduced to 100 L h^{-1} to avoid foaming at the surface of the culture medium. We noticed that reducing air bubbling at that time reduced *Spirulina* filament breakage, but had no significant impact on microalga growth.
As the PBR cover was not really a closed system (only a plastic dish covered the upper opening of the tube), some water evaporation occurred during the production cycle. The evaporated water had to be replaced every two days by an equivalent volume of culture medium, previously brought to the same temperature as that of the culture medium in the PBR. Depending on the air bubbling velocity during the production cycle, the volumes of culture medium added were between 0.3 and 0.7 L day^{-1}. Concentrations of inorganic carbons (HCO_3^- and CO_3^{2-}) were monitored to verify that their consumption by the

growing microalgae during the production cycle were at least compensated for by these periodically added volumes of culture medium.
For *Chlorella*, the same production protocol as for *Spirulina* was used, except that air bubbling was maintained throughout the cycle at its upper value of 200 L h^{-1}.

PBR post-cultivation cleaning and sterilizing

At the end of each production cycle, the culture medium containing the biomass produced was drained from the PBR tube using the waste pipe placed under the lower platform and connected to the PBR tube. The tube was carefully rinsed and washed out with tap water. The internal surface of the tube was carefully sprayed from the open top of the PBR with 3 L of an oxidant alkaline solution of NaOH (5 g L^{-1}) and NaOCl (0.5 g L^{-1}) to remove organic particles stuck to it. The alkaline solution was drained and the tube was washed with a minimum volume of tap water up to neutral pH of the wastewater. Another spray-washing session was carried out using 3 L of HNO_3 solution (2 g L^{-1}) to solubilise culture medium salt deposits. The tube was then rinsed up to neutral pH of the wastewater, washed with pre-filtered tap water and drained.

Chemical analysis

A hand conductimeter (Testo-240, no. 05602404, Testo, France) equipped with automatic temperature correction was used to measure the conductivity (C, mS cm^{-1}) of the culture medium. Standard solutions (C = 1.413 and 12.88 mS cm^{-1}) were used to calibrate the electrode responses. The total salinity content was expressed as NaCl equivalents (g L^{-1}).
A hand pHmeter (Testo-230, no. 05602304, Testo, France) equipped with automatic temperature correction was used to monitor pH variations during the microalga production cycles. Three calibration solutions (pH = 4, 7, and 9) were used.
The carbonate content was determined according to the described method (C. d. O. Rangel-Yagui et al. 2004). A sample of the culture medium was removed from the PBR by sinking a 500-mL plastic pot to the mid-height of the tube. An aliquot of 100 mL was taken from the stirred medium in the pot and immediately filtered at 0.6 µm (GF/A Whatman glass filter, Φ = 0.47 mm). Two aliquots of the filtrate (25 mL each) were consecutively titrated with an HCl (0.2 N) solution (V_1, mL), to pH = 8.3 and continued (V_2, mL) to pH = 4.6. The bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) contents were calculated according to the formulas:
HCO_3^- (mol L^{-1}) = 0.2 × (V_2 - 2V_1) / 25 and CO_3^{2-} (mol L^{-1}) = 0.2 × V_2 / 25
The dry matter (DM) of the microalga biomass was determined using a 100 mL aliquot of culture medium sampled by the pot technique above. The aliquot was first filtered (0.6 µm, Φ = 0.47 mm glass filter, GF/A, Whatman) and both the microalga biomass retained and the filter were weighed (m_1, g)). The biomass left on the filter surface was washed twice with 2 x 10 mL of slightly acidified (pH = 2-3) distilled water. The filter was dried at 105°C to constant weight (m_2, g) using an infrared heating balance (MRS 120-3, Kern-Sohn, Germany). Biomass DM was calculated as follows:
DM (g L^{-1}) = (m_2 - m_1) / 100

Kinetic parameters of microalga growth

As PBR production was conducted in batch mode, growth kinetics (biomass vs. time) were characterised by a well-known multi-phase shape curve modelled and described in the literature (J. M. Levert and J. L. Xia 2001): 1-lag phase, 2-exponential phase, 3-linear phase, 4-decelerating phase, 5-stationary phase and 6-death phase. After a few preliminary tests, we decided to stop the production cycles at the beginning of the decelerating phase, assuming that the nutritional requirements were still satisfied in the culture medium and that the growth temperature was set not far from the optimum.
The specific growth rate µ and the biomass volumetric productivity in batch mode P_b, were calculated as follows (B. Ketheesan and N. Nirmalakhandan 2012):
(C_{ef} / C_{ei}) = $e^{\mu/t}$ ➔ µ (day^{-1}) = (ln (C_{ef} / C_{ei})) / t
where $C_{ef,}$ and C_{iei} are the final and initial dry biomass concentrations (d.w., g L^{-1}), respectively, in the exponential growth phase and t is the duration of the exponential growth phase (days). The exponential growth period was decided by the localisation of the linear shape of the exponential scale curve drawn for dry biomass weight versus time.
Mean biomass batch productivity P_b was calculated as follows:
P_b (d.w., g L^{-1} day^{-1}) = (C_f − C_i)/ t
where C_f and C_i are the final and initial dry biomass concentrations (d.w., g L^{-1}), and t is the production time needed to reach the dry biomass concentration C_f, as long as biomass production was in its linear phase. Generally, linear production stopped just before entering the microalga decelerating growth phase and this time t was graphically determined on the biomass growth curves (about 25 days into this work).

Results and Discussion

Illumination settings during PBR production cycles

Light, carbon and mineral nutrients are the main production parameters that limit photosynthetic growth, assuming that the pH and temperature conditions are kept optimal during microalga growth. Of these parameters, only light accessibility by

photosynthetic microalgae is difficult to control or to optimize. It is widely recognized that PBR performance is governed by light energy transport inside the culture medium. This led to the development of PBRs with high illuminated surface-to-volume ratios. It has been shown that increasing light intensity first increases the growth rate up to a maximum level. Increasing the illumination again then leads to a dramatic decrease in biomass yield while the major fraction of the light provided cannot be used by the microalga culture. Excess light may also lead the biomass to be stressed and to produce undesirable metabolites (A. P. Carvalho et al. 2006). During biomass production, light penetration of the culture medium decreases as the cell density increases and photosynthesis is lower, even though the phenomenon of mutual cell shading can be minimized by adequate air bubbling of the culture medium in the PBR. The physical phenomenon of light transfer limitation in PBRs has been explained and modelled using the concept of a working illuminated volume and the notion of a dark volume taking place inside externally lightened PBRs which are not up-scalable and have to be kept on a small-scale, around 100 L per unit, for reasons of light efficiency limitation (J.-F. Cornet 2010; J. Pruvost et al. 2011).
Two illumination levels were tested during our PBR production cycles, run with the two microalga strains: full-power illumination with 4 light blocks switched on (set 1) and half-power illumination with only 2 light blocks (sets 2 and 3), as shown in **Fig. 2**..

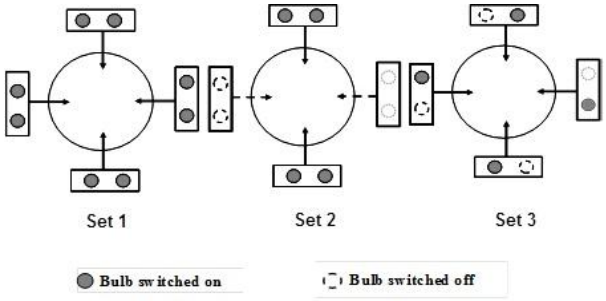

Fig. 2 PBR illumination using various fluorescent light block patterns

The illumination provided by each light block was not constant along its axis. The illumination profiles showed that the emitted and received light energies varied along the light block axis with similar shapes (**Fig. 3**). In this configuration, it was difficult to evaluate correctly the total light energy received by the PBR and more difficult to estimate the amount available for photosynthesis. To simplify evaluation of the light energy received by the PBR placed in the illumination situations of sets 1, 2 , and 3, it was decided to measure it at a spot placed against the PBR tube wall and at half-way from the switched on light block. To evaluate the total light energy supplied to the culture medium when several light blocks were used, these measurements were made for each illuminated light block and summed together. There was quite a large light energy loss (63% of the emitted light) due to the distance between the light blocks and the PBR tube surface, but the plastic wall of the tube did not absorb too much energy comparatively (17% variation for the inside and outside of the wall surface).

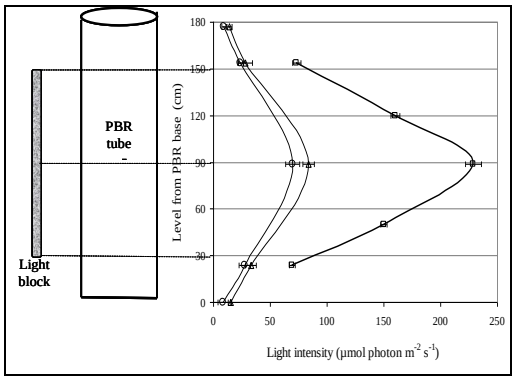

Fig. 3 Emitted light intensity profiles for a light block along the PBR tube axis

Measurements taken on the light block surface (□), on the external surface (Δ) and internal surface (O) of the PBR wall, with error bars

Although the distance between light bocks and tube surface could be easily reduced, using a specially installed device, it was found that a distance of 17 cm was appropriate for avoiding significant heating from light thermal radiation as the PBR was not equipped with a culture medium cooling system.

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Reproducibility of PBR production cycles for Spirulina and Chlorella

PBR production of *Spirulina* and *Chlorella* strains was launched in two identical PBR units. The kinetics for biomass growth were compared, and the reproducibility of microalga production was evaluated. For reproducibility purposes, three consecutive production cycles were run consecutively under the same operating conditions. Inoculation was carried out with the same inoculum stock solution. Each cycle was followed by a complete PBR cleaning run.

The illumination supplied to the PBR during the production cycles was that shown in set 1 for *Spirulina* and in sets 1 and 2 for *Chlorella* (**Fig. 2**). Microalga production was carried out at the respective optimum growth temperatures adopted for each strain: 34 ± 1°C for *Spirulina* and 25 ± 1°C for *Chlorella*.

The kinetic curves of biomass growth were traced for *Spirulina* (Sp1, Sp2 and Sp3) and for *Chlorella* (Ch1, Ch2 and Ch3), as shown in **Fig. 4**. The curves exhibited similar shapes with 3 phases: a short lag phase that lasted less than 1-2 days for both strains, the exponential growth phase from days 2-10 and the linear phase from days10-25.

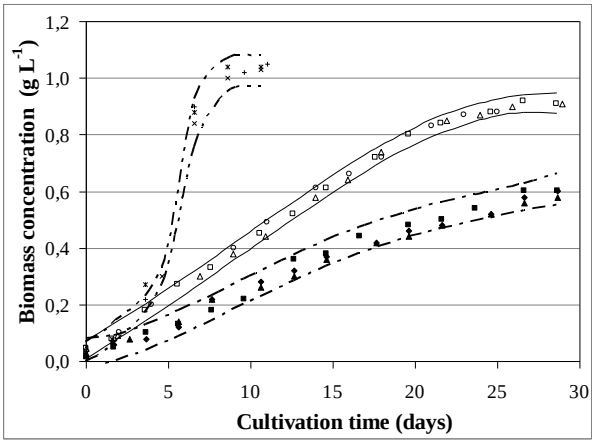

Fig. 4 Reproducibility of growth kinetics for Spirulina and Chlorella PBR production cycles

Spirulina cycles with permanent PBR illumination set1, □Sp1, Δ Sp2, and ◇Sp3; Chlorella cycles with permanent PBR illumination set 2, ■Ch1, ▲Ch2, and ◆Ch3; Chlorella cycles with permanent PBR illumination set 1, ×Ch4, ∗Ch5, and +Ch6

In order to establish the reproducibility of the PBR production cycles for the 2 strains, the Kruskal-Wallis non-parametric statistical test was applied to the data collected, using Statistica software (2008). The Kruskal-Wallis criteria obtained were 0.992 for the *Spirulina* and 0.986 for the *Chlorella* production cycles. At a confidence level of $p > 0.05$, the three cycles were considered as reproducible. Envelope curves were calculated from one item of production cycle data obtained for each microalga strain (Sp1 and Ch1) with a standard deviation of ± 5%. They were plotted using Tablecurve 2D software (Table Curve software 2009). For each microalga strain, the kinetic curves of the triplicate production cycles were positioned within the two limit-curves of the envelope curve, indicating that the reproducibility of the PBR productions was good for both microalga strains.

For *Spirulina*, the average specific growth rate was $\mu = 0.265$ day^{-1} and the productivity $P_b = 29.6$ mg L^{-1} day^{-1} (**Table 1**). At the end of the production cycles the average biomass concentration reached in the culture medium was 0.9 mg L^{-1} and the average total dry biomass harvested was 72.5 g.

Table 1 Characterisation of replicate PBR production cycles for *Spirulina* and *Chlorella*

Microalgal strains	*Spirulina*			*Chlorella*					
Permanent PBR illumination settings [a]	Set 1			Set 1			Set 2		
Replicate PBR production cycles	Sp1	Sp2	Sp3	Ch4	Ch5	Ch6	Ch1	Ch2	Ch3
Specific growth rate μ (day^{-1})	0.263	0.274	0.260	0.510	0.510	0.580	0.249	0.241	0.248
Batch Productivity P_b (d.w. [b], mg L^{-1} day^{-1})	30.3	29.9	28.8	94.3	95.3	93.6	20.3	19.6	20.3
Final biomass concentration [c] (mg d.w. L^{-1})	905	899	910	1020	1040	1050	600	580	600

[a] set 1 = 336 µmol photon m^{-2} s^{-1}, set 2 = 168 µmol photon m^{-2} s^{-1} ; [b] dry weight; [c] for 25-day production time, see Fig. 5a

Studies already published in the literature showed that there were infinite production techniques and operating culture conditions from which it was not possible to extract the results obtained under operating conditions close to ours. Nevertheless, we put together some data collected in the literature that may provide some insight into what can be obtained in the case of *Spirulina* production, either in raceways or in PBRs, and on various production scales. As can be seen from **Table 2**, it appears clearly that specific growth rates (μ) were higher in PBRs than in raceway systems.

Table 2 Comparison of Spirulina biomass cultivation in various production systems and operating conditions

Production system used	Production cycle conditions				Biomass produced at the end of a cycle		Specific growth rate (μ, day^{-1})	Photosynthetic efficiency	References
	Lighting mode [f]	Total cultivation volume (L)	Temp (°C)	Cycle duration (days)	Biomass concentration [h] (d.w. g L^{-1})	Total biomass (g)		Biomass produced per PPFD [g] unit (10^{-9} g μmol photon^{-1} m^2)	
Erlenmeyer [a]	A	0.2	30	18	1.5	0.3	0.37	980.4	(A. Çelekli and M. Yavuzatmaca 2009)
PBR	A	1.5	25	10	1.9	2.8	0.33	52.6	(S. Oncel and F. V. Sukan 2008)
PBR	A	2.5	30	12	2.3	5.7	0.53	5.0	(P. H. Ravelonandro et al. 2008)
PBR [a]	A - L/D	2.5	30	18	0.6	1.5		37.0	(B. Raoof et al. 2006)
PBR	M	3.5	32	11	1.3	4.7	0.49	32.2	(B. D. Rym et al. 2010)
Open tank	A	5	30	18	1.2	6.0		90.9	(C. d. O. Rangel-Yagui et al. 2004)
PBR [a, b, c]	A	5	32	17	3	15	0.42	1.4	(S. S. Oncel and O. Akpolat 2006)
PBR [a, b]	A - L/D	20	35	25	0.6	13.0	0.054	3.3	(L. M. Colla et al. 2007)
PBR [a, c, d]	A	21	30	12	2.6	55.2	0.03	7.7	(L. Travieso et al. 2001)
Open mini-tank [a]	A - L/D	5	30	8.5	0.4	2.0	0.13	76.9.0	(E. M. Radmann et al. 2007)
Open mini-tank [a, e]	A	5	31	18	1.1	5.6		50.0	(L. D. Sánchez-Luna et al. 2004)
Raceway	L/D	1350	12-28	15	0.5	715	0.18	-	(C. Jiménez et al. 2003)
Raceway	L/D	135,000	12-28	15	0.5	63,450	0.15	-	(C. Jiménez et al. 2003)
PBR	*A*	*80*	*34*	*25*	*0.9*	*72.0*	*0.26*	*83.3*	*Our study*

[a]: with pH adjustment; [b]: with additional CO_2 bubbling; [c]: with internal lighting; [d]: helical tubular PBR; [e]: with urea used as nitrogen feed source; [f]: A-photoautotrophic cultivation, M - mixotrophic cultivation, L/D - lighting in natural day and night sequences; [g]: PPFD - Photosynthetic Photon Flux Density; [h]: dry weight

Our results obtained in an 80-L PBR were of the same order of magnitude as the μ values reported for the different PBR cultivation conditions shown, except that higher figures were obtained when using small-volume PBRs (0.2-3.5 L total PBR volume). Although the total biomass produced under photoautotrophic conditions led to high biomass concentrations at the end of the production cycles (1.2-3.0 g L^{-1}), the total biomasses collected per production cycle were still low (2.8-15 g). They were far lower than those obtained in our work (72 g), assuming that up-scaling biomass production conditions from the literature data would probably not lead to a proportional up-scaling of the microalga yields per production cycle. Even the photometric efficiency was found to be higher in our 80-L PBR production cycle compared to that calculated from the literature data for smaller-sized PBRs (83 vs. 32-52 10^{-9} g μmol photon^{-1} m^2), as reported in **Table 2**.

For *Chlorella*, the PBR production cycles under illumination set 2 were characterised by an average specific growth rate of $\mu = 0.245$ day^{-1} and a productivity $P_b = 20.1$ mg L^{-1} day^{-1} (**Table 1**). At the end of the production cycles, the average biomass concentration reached in the culture medium was 0.59 mg L^{-1} and the average total dry biomass harvested was 47.5 g for the 3 consecutive cycles run.

PBR production cycles under illumination set 1 showed growth kinetics with higher growth rates, as shown in **Fig. 4**. The Ch4-6 curves were also reproducible but they reached a plateau (10 days after cycle start) far sooner than the Ch1-3 curves, obtained with the low illumination level (set 2), which was half of that used for set 1. The growth parameters were found to be in the same order of magnitude as the light levels supplied to the culture medium by set 1 and set 2: the specific growth rate μ was doubled and the productivity P_b was multiplied by 4.5 when using illumination set 1 (336 μmol photon m^{-2} s^{-1}), as shown in **Table 1**.

The three faster production cycles for *Chlorella* were stopped when microalga growth became very low. Biomass concentrations were about 1.04-1.05 d.w. g L^{-1}, a similar level to that reached with the *Spirulina* production cycles (Sp1-3) when the corresponding growth rates became low. At this biomass concentration level, PBR culture medium turbidity was so high that the light provided could not effectively penetrate the medium to allow good photosynthesis by the microalgae,

decreasing their growth rates. Because of the diameter of its tube, this PBR could not be used to reach higher biomass concentrations which are characterised by vey high turbidity.

PBR production cycles for Spirulina with various illumination levels

During the PBR production cycles, the temperature was maintained at its optimum value (35±1°C) by an immersed aquarium heater which will no longer be needed in tropical countries where the local temperature is often higher than that of our laboratory (25°C).

Five *Spirulina* production cycles (Sp1 to Sp5) were run comparatively with different illumination intensities and arrangements around the PBR tube (**Fig. 5**). The other production parameters (T, pH, nutrient supply, air bubbling) were maintained constant. Three different illumination patterns were used supplying two levels of light energy to the PBR surface (set 1, 336 µmol photon m^{-2} s^{-1} and sets 2-3, 168 µmol photon m^{-2} s^{-1}, see **Fig. 2**, **Table 1**).

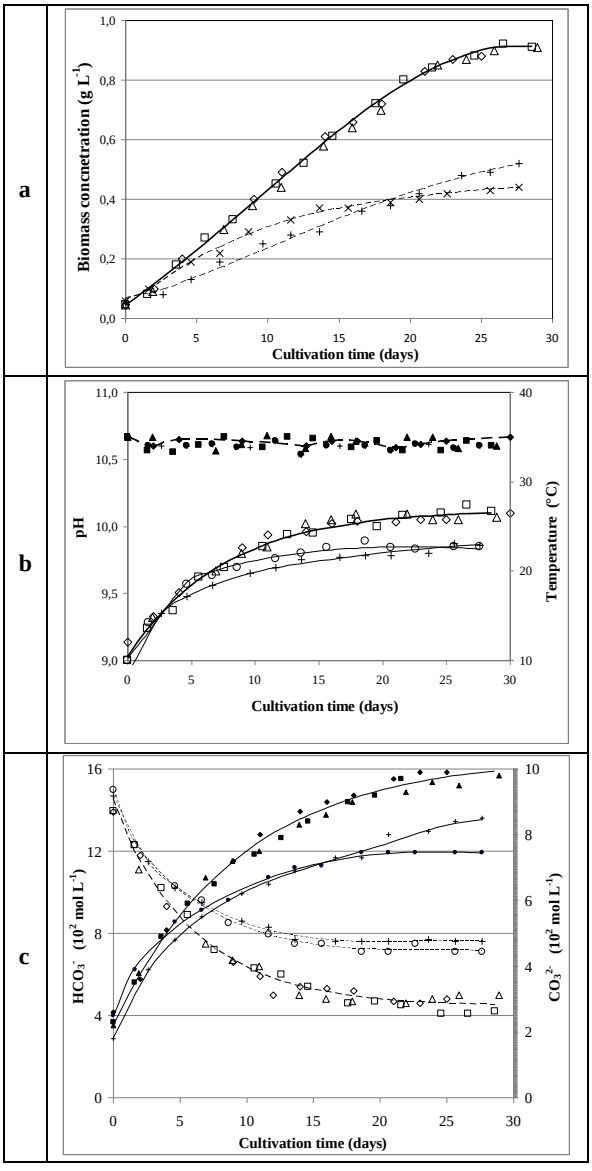

Fig. 5 Comparison of kinetics for *Spirulina* growth during different PBR production cycles

a: Growth kinetics - production cycles □ Sp1, Δ Sp2, ◇ Sp3, + Sp4, and O Sp5, PBR permanent illumination set1 for Sp1-3, set 2 for Sp4, set 3 for Sp5, see Table 3 and Fig. 2 for illumination settings; **b**: Temperature and pH variations of the culture media - medium pH for cycles □ Sp1, Δ Sp2, ◇ Sp3, + Sp4, and O Sp5, medium temperature for cycles ■ Sp1, ▲ Sp2, ◆ Sp3, + Sp4, and • Sp5; **c**: Content of major nutrients in the culture media - HCO_3- content for cycles □ Sp1, Δ Sp2, ◇ Sp3, + Sp4, and O Sp5, $CO_3$2- content for cycles ■ Sp1, ▲ Sp2, ◆ Sp3, + Sp4, and • Sp5

Growth kinetics for Spirulina PBR production under 2 illumination levels

Triplicate PBR production cycles (Sp1-3) were first run consecutively, using illumination set 1. Complementary cycles were run: Sp 4 using illumination set 2 and Sp 5 using set 3. Microalga growth kinetics were monitored for the different PBR

production conditions. Triplicate trials (Sp1-3) on the one hand, and production cycles Sp4 and Sp5 on the other, run respectively under the same operating conditions, showed very good reproducibility (**Fig. 5a**). Sp1-3 data points were found aligned on the same curve within a 5% s.d., but Sp4 and Sp5 showed distinct curves, but with similar shapes. The slopes of these last two curves were about half of those obtained for the triplicate trials Sp1-3. The productivity and growth obtained were higher. The total amounts of biomass per production cycle were also nearly doubled (**Table 3**). This result confirmed that, under these PBR production conditions, emitted light was the limiting factor for *Spirulina* growth.

Table 3 Composition of Spirulina culture media at the end of production cycles run with different illumination levels

PBR production cycles	Sp1-3 [c]	Sp4	Sp5
Light block sets [a]	Set 1	Set 2	Set 3
PBR Illumination (µmol photon m^{-2} s^{-1})	336	168	
Specific growth rate - µ (day^{-1})	0.26	0.14	0.15
Productivity - P_b (mg d.w. L^{-1} day^{-1})	30 ± 5	16	15
Final biomass concentration [b] (mg d.w. L^{-1})	905 ± 5	550	450
Photosynthetic efficiency [b] (mg d.w. µmol $photon^{-1}$)	1.7 10^{-6}	1.1 10^{-6}	1.7 10^{-6}
pH stabilisation level of culture media	10.1	9.85	9.8
Residual HCO_3^- concentration [b] (mol L^{-1} / g L^{-1} of $NaHCO_3$)	0.049 / 4.12	0.076 / 6.38	0.071 / 5.96
Residual CO_3^{2-} concentration [b] (mol L^{-1} / g L^{-1} of Na_2CO_3)	0.099 / 10.49	0.082 / 8.69	0.075 / 7.95

[a] see Fig. 2 for set design; [b] for 25-day production time, see Fig. 5a; [c] data from Table 1

Temperature and culture medium pH trends during PBR Spirulina production cycles

Apart from the light energy source (illumination), the most important parameters that might affect *Spirulina* growth were temperature, pH and nutrient concentration. As already seen, the PBR production cycles were reproducible when the same production conditions where used. The production cycles were run in batch mode with no monitoring of nutrient concentration in the culture medium during *Spirulina* growth, except for medium volume adjustments by a medium culture solution to compensate for water evaporation that occurred during the cycle. The way the production protocol was drawn up minimized technical intervention to adjust these parameters to their required levels for an acceptable biomass growth rate.

On the other hand, the medium pH was allowed to vary freely and no pH adjustment was made during the production cycles. Consequently, the pH was only monitored and its variations were the result of microalga photosynthetic activity towards nutrient concentrations that were modified during biomass growth. The initial pH value was pH = 9 ± 0.2, and it increased regularly to reach a steady-state at pH = 9.9-10.0, after 20 days of *Spirulina* growth.

Variations in pH over time in the triplicate production run (Sp1-3) were found to be particularly similar. Those for Sp4 and Sp5 showed similar kinetic curve shapes with a steady-state reached sooner, after 15 days, and at a lower pH = 9.7 than for the Sp1-3 runs (**Fig. 5b**).

The observed pH increase in the culture medium was the result of changes in the chemical balances of the different chemical entities solubilised in the cultivation medium: HCO_3^- , CO_3^{2-} , and OH^-. These species are involved in the following balances:

$2\ HCO_3^- \text{ <--> } CO_3^{2-} + H_2O + CO_2$ equation 1

$CO_3^{2-} + H_2O \text{ <--> } CO_2 + 2\ OH^-$ equation 2

$HCO_3^- \text{ <---> } CO_2 + OH^-$ equation 3

Spirulina, as a cyanobacteria sp., has the ability to use both CO_2 and HCO_3^- as inorganic carbon sources for its growth. The bicarbonate-carbonate buffer system may have provided the needed CO_2 for photosynthesis, as shown by eq. 1-3.

Experimentally, we observed a pH rise during *Spirulina* growth. Several possible pathways have been suggested to explain the pH rise during *Spirulina* growth.

According to Rangel-Yagui (2004), the pH rise would seem to be the effect of cell assimilation of HCO_3^- which is then converted into CO_2 and CO_3^{2-}, according to eq 1. CO_2 was used for photosynthesis while CO_3^{2-} was excreted in the extracellular environment (culture medium). It reacted with water to give OH^- according to eq 2. OH^- accumulation in the culture medium contributed to the observed pH rise during the microalga production cycles.

More recently, another possible pathway was proposed by Markou (2011). Bicarbonate ions (HCO_3^-) are the major carbonate entities at pH values between 6 and 10 according to the equilibrium expressions for the dissociation of bicarbonate and carbonic acid (C. B. Andersen 2002). Bicarbonate ions (HCO_3^-) are consumed during microalga growth. Within the intracellular medium of microalgae, the absorbed HCO_3^- accumulates in a specialised compartment, the carboxysome, where

it is converted into CO2 through the action of specific carboxysomal carbonic anhydrases, while its concentration in the culture medium decreases (V. J. Moroney and A. R. Ynalvez 2007). The CO2 dissolved in water generates a weak acid/base buffer system, namely the bicarbonate–carbonate buffer system. Although the dissolution of CO2 in water results in acidification due to the forming of carbonic acid, the photosynthetic process of CO2 fixation causes a gradual rise in pH due to the accumulation of OH^-

Evolution of carbonate nutrients during PBR Spirulina production cycles

The residual contents of inorganic carbon nutrients (HCO_3^- and CO_3^{--}) were found at similar levels (6.4-5.9 g L^{-1}) for PBR production runs under sets 2 and 3 and slightly higher (4.1 g L^{-1}) than those found for set 1 (**Fig. 5c**) These levels were probably not yet growth limiting factors at the end of the production cycle (25 days), as levels of about 10 g L^{-1} of NaHCO3 have been used to grow *Spirulina* in a small-volume laboratory photobioreactor (P. H. Ravelonandro et al. 2008). Growth curves kept the same rising shape levels around the 20th-25th day of production and the biomass concentration increased making the culture medium increasingly turbid, affecting the efficiency level of the emitted light settings.

Conclusion

The small-scale PBR designed associated with the production protocols provided could facilitate cultivations of *Spirulina platensis* and *Chlorella vulgaris* to produce reproducible culture media for microalgae in the growing stage with constant quality and yields.

On a research laboratory level, the reproducible culture media obtained could be used as standard and freshly made material for further scientific studies or technological developments, such as small-scale harvesting systems needed for microalga strains with a smaller cell size than *Spirulina* filaments. On a developing country level, this simple designed and low-cost PBR can be used for local microalga production in the low-level technical environments found in tropical countries. The coupled PBR/production protocol tool will allow village communities or individual small producers to safely cultivate and harvest by themselves their own microalga biomasses they can used as food supplement to improve their health and wellbeing. For fish farmers with modest means, this tool will be an affordable mean to produce low-cost and homemade feed ingredients for their fish breeding.

Temperature, light availability or illumination, and potent external pollution of the growing biomass, are critical microalgae production parameters to be mastered for optimising photosynthetic activity and productivity of microalgae cultures. Good management and fine-tune of these parameters remains the key for successful microalgae productions when using outdoor and open-type cultivation systems. In the case of our PBR cultivation methodologies, as soon as the cultivation started, no much skilled management of these parameters is required from the operator, except of cultivation medium volume adjustments, to obtain reproducible biomass productions.

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