SOPs for the dual chamber system available for the PROMICON consortium

Deliverable D4.2

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Álvaro Gómez Luengo[1], Fabián Moreno Avitia[1],Juan Nogales[1]

[1] Centro Nacional de Biotecnología, CSIC, Madrid

PROMICON

Harnessing the power of nature through PROductive MIcrobial CONsortia in biotechnology - measure, model, master

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Preface

Bioprocess parameters are highly relevant for the optimal operation of any biotechnological process. Cyanobacteria have been widely studied under different conditions, including standard photobioreactors. Due to the novel features of our dual chamber photobioreactor, it is mandatory to stablish a standard operational protocol which can be replicated keeping the bioprocess performance. Here, we describe the steps and considerations needed to operate the dual chamber photobioreactor.

Summary

The content of this document is structured as a SOP (Standard Operating Procedure) for cyanobacteria culture in a dual chamber photobioreactor (DC-PBR). We describe all the instructions for microorganism cultivation and bioreactor setup. A general description of the complete photobioreactor is included.

List of abbreviations

SOP	Standard Operational Procedure
DC-PBR	Dual-chamber photobioreactor
Scr	Sucrose

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Cyanobacteria are responsible for growth through photosynthetic processes, assimilating inorganic carbon (CO_2 and HCO_3^-) and consuming substrates (NH_4^+, NO_3^-, and PO_4^{-3}) while producing the necessary oxygen for bacteria to oxidise organic matter. Heterotrophic bacteria have the capability to thrive in both aerobic (oxygen-present) and anaerobic (where dissolved oxygen is depleted) conditions. These bacteria are responsible for hydrolysis processes and require the consumption of carbon. In this project, we take advantage of the potential interaction of phototrophic cyanobacteria and heterotrophic bacteria to produce desired compounds from renewable carbon sources such as CO_2. The coexistence of these kinds of organisms in the same space could promote light limitations in the cyanobacterium due shadowing effect thus decrising its performance; this is why we implemented a platform for cocultivation where the physical interactions are eliminated, but the metabolic exchanges remain possible.

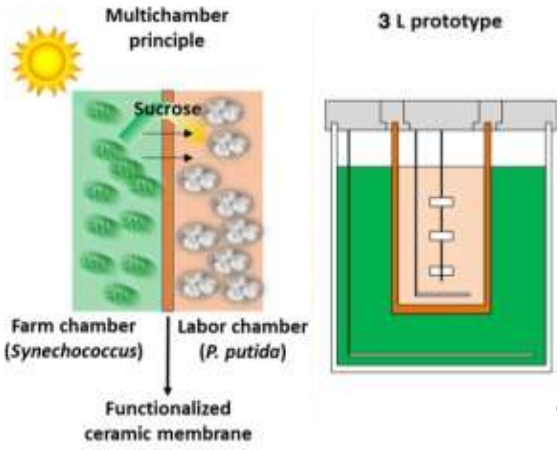

Figure 1. **Simplified scheme of the two-chamber bioreactor designed in this project**. Details can be found in D4.1

1.1 Culture media and inoculum preparation

The 2 L dual chamber photobioreactor (DC-PBR) is designed to have two functionally different spaces separated by a ceramic membrane. The inner chamber hosts the labourer strains, growing on sucrose produced by the farmer strain that is held on the outer chamber. The ceramic membrane keeps both strains physically separated, although allowing metabolites and molecules such as sucrose to pass through, see for details D4.1.

In this SOP we will focus on the establishment of the farmer strain to serve as feedstock for the labourer strain in the context of a working 2 L DC-PBR. In our case, we employ a recombinant strain of the model cyanobacterium *S. elongatus* PCC7942 able to produce sucrose upon IPTG induction and no need of saline stress, hereafter PromSucr strain. However, PromSucr strain as well as it parental strain PCC7942 are known to have very low growth rates compared to heterotrophic model organisms such as *E. coli* or *P. putida*. Moreover, we have seen in our hands that these microorganisms do not perform well when they are exposed to big dilutions, e.g. inoculating 1 L flasks from single colonies. In this regard, a few dilution steps had to be taken to increase biomass prior to inoculation of the 2 L DC-PBR. All cultivations were done in shake flask with continuous light conditions and 30ºC.

1. Starting from frozen stocks, we inoculated 10 mL flasks with BG11-HP media. Usually, it takes about 10 days to reach 1 OD_{720}. BG11-HP is a modified mineral medium described below.

Table 1. BG11-HP culture medium recipe

Compound	g/L
K_2HPO_4	$3.48 \cdot 10^{-1}$
$MgSO_4 \cdot 7H_2O$	$7.50 \cdot 10^{-2}$
$CaCl_2 \cdot 2H_2O$	$3.60 \cdot 10^{-2}$
Ácido cítrico · H_2O	$6.56 \cdot 10^{-3}$
Cit. férrico-amónico	$6.00 \cdot 10^{-3}$
$Na_2EDTA \cdot 2H_2O$	$1.04 \cdot 10^{-3}$
Na_2CO_3	$2.00 \cdot 10^{-2}$
H_3BO_3	$2.86 \cdot 10^{-3}$
$MnCl_2 \cdot 4H_2O$	$1.81 \cdot 10^{-3}$
$ZnSO_4 \cdot 7H_2O$	$2.22 \cdot 10^{-4}$
$Na_2MoO_4 \cdot 2H_2O$	$3.90 \cdot 10^{-4}$
$CuSO_4 \cdot 5H_2O$	$7.90 \cdot 10^{-5}$
$CoCl_2$	$2.20 \cdot 10^{-5}$
HEPES (pH ≈ 7.8)	2.38
$NaNO_3$	1.50

The concentration of the culture medium components is indicated in the table. The compounds are grouped by colour based on the stock solution recipe they come from. The partition of the compounds in different stock solutions is recommended to avoid potential precipitation of the culture medium components during storage or preparation.

2. At this point, we take the biomass by centrifuging 10 min at 4000 g, and then inoculating 100 mL of fresh BG11-HP media using the proper antibiotics. The sucrose secretion is induced in this stage by adding 1 mM IPTG.,

3. When the culture reach 1 OD_{720} (usually in 4 days), we perform a second dilution step following the same procedure to obtain 1L of freshly inoculated cyanobacteria in a 5 L flask. When cyanobacteril culture reach 0.4 OD_{720} we can start the DC-PBR operation.

1.2 Bioreactor setup and calibration

The particular design of the multi-chamber bioreactor implies a small set of special steps for its correct working. The main devices used to construct the DC-PBR were created by Bioprocess Technology company.

The adequate ceramic chamber has to be set in distilled water to absorb as much water as possible and avoid culture media absorption. It is important to have a humid ceramic in order to allow the diffusion of soluble compounds during the process.

The initial design of the ceramic holder was 3D printed in polypropylene for size testing. The same design was replaced with a steel structure, more suitable for routine sterilization procedures (figure 2).

a)  b) 

Figure 2. Bioreactor holders. a) Polypropylene 3D printed ceramic holder. b) Steel ceramic holder with ceramic chamber.

The ceramic chamber must be placed at the centre of the bioreactor jar, matching the circular lid indentation (figure 3).

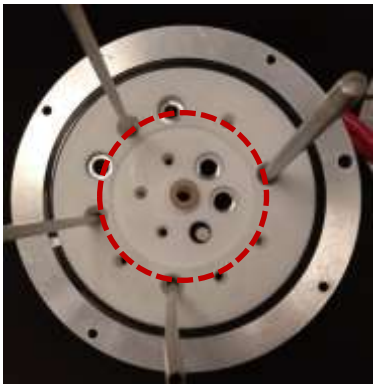

Figure 3. Biorector lid. In the figure we can observe the teflon made bioreactor lid with a circular indentation in the center. Such space is destinated to fit the cermic chamber, once the bioreactor is closed.

The temperature is controlled with the recirculation of water through the coated glass jar by using a water heater (figure 4). The temperature has to be set at 30 °C. This temperature is lower than the optimum temperature reported for *S. elongatus* (38 °C), but it is compatible with the labourer strain, which is *P. putida* KT2440.

Figure 4. Water heater recirculator

Setting up the bioreactor includes the sensors calibration and jar assembly. The DO2 sensor was calibrated with atmospheric air at 100 %, and the default electronic zero voltage was set to 0 %. The CO_2 sensor of the gas output is calibrated automatically with default settings. The pH potentiometer has to be calibrated with three points (4, 7, and 10). All the used sensors were acquired from Hamilton Company (figure 5).

a)  b)  c) 

Figure 5. Photobioreactor sensors. a) Dissolved oxygen sensor. b) pH sensor. c) CO2 sensor.

One starter litter is previously grown in a 5 L flask until reaching an optical density of 0.4 OD_{720}. The total operation volume in the bioreactor is 2 L, 1500 mL in the outer chamber and 500 mL in the inner chamber. The inner chamber is filled with fresh BH11-HP culture medium. The outer compartment is filled with 750 mL of *S. elongatus* culture at 0.4 optical densities and reconstituted with 750 mL of a fresh new BH11-HP medium (figure 6).

Figure 6. Operating DC-PBR

Light is the energy source for cyanobacteria growth, and this should be enough avoid any shadowing effect during cultivation. Two fluorescent lamps are set on two sides of the glass chamber (figure 7). The light intensity in each lamp is about 1000 luxes, which is about 750 photons µmol/m^2s. This could be considered high, but it is reported that glass and water can absorb up to 80 % of the light. This bioreactor is coated with a double glass wall filled with water. We consider that the light delivered inside the photobioreactor is about 500 photons µm/m^2s.

Figure 7. Fluorescent lamp used on the DC-PBR. The source of light are two fluorescent lamps on each side of the bioreactor. Each lamp carries two bulbs. The light shutter was closed to allow the visualization of the device.

The biomass and Scr production performance is a function of the CO_2 input and delivery. The dual chamber configuration is designed to be agitated by a magnetic stirrer in the outer compartment and a Rushton impeller in the ceramic chamber. The magnetic stirrer was set at 500 rpm in such a way that biomass mixing is visible.

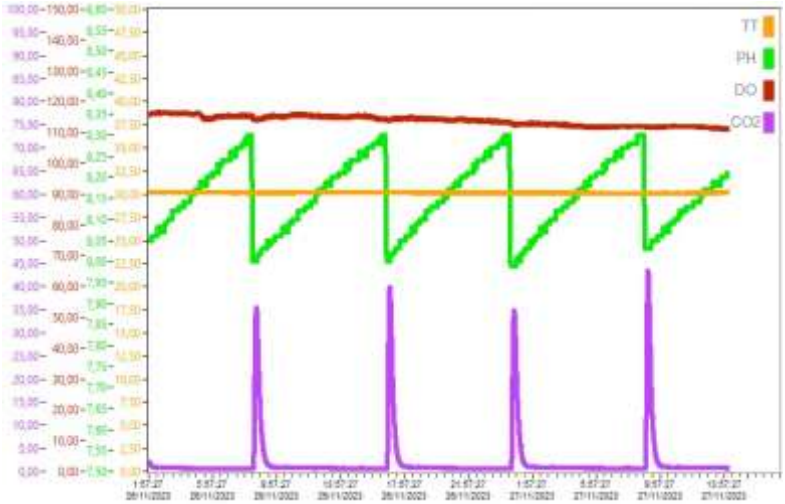

Figure 8. DC-PBR behavior under the described conditions. We can observe the changes or stability of the different process variables. The temperature (TT) and the disolved oxygen (DO) remain stable, while the pH and CO_2 emission change periodically

The CO_2 fixation metabolism removes protons from the culture medium, increasing the pH, but this change is counteracted by pure CO_2 injection at 0.2 VVM. The control action is triggered when the culture reaches a pH of 8.3, and the pH is decreased to 7.8, which is reported as optimal for *S. elongatus* and the labourer strain *P. putida* KT2440. Once the culture

is running, it is possible to observe a periodic pH increase in the range mentioned before. This periodicity is also observable in the CO_2 concentration in the air output. Once the pH is decreased by CO_2 injection, the concentration of this gas increase around 30% for a few seconds before decreasing again (figure 8).

Once the biomass is about to 0.8 OD_{720}, carbon source availability limits the growth rate. This forces the system to work with CO_2 enriched environment to provide a constant carbon source. This environment is generated by a constant airflow with CO_2 at 3 %. Air sparging takes a relevant place in non-tubular bioreactors for growing cyanobacteria. The delivery of CO_2. Once the CO_2 enriched air starts, the pH stays stable around 7.8. Neverless, the equipment can control the pH with NaOH 3 M in case this variable decreases below the setpoint threshold.

The Scr production occurs during the growing phase of *S. elongatus* and the diffusion of this carbohydrate should be followed during several days. The biomass production is followed by measuring the absorbance at 680 and 720 nm. Both wavelengths are helpful for monitoring cyanobacteria. The shortest wavelength is one of the absorbance peak of chlorophyll and the longest one allows the light dispersed by biomass. The ratio 680/720 nm is calculated to see the parallel increase of both measurements.

Once the inner chamber reaches the desired Scr concentration, the labour strains can be inoculated in the labour module. At this point, *P. putida,* the labourer strain, has to be inoculated at the desired biomass level. The Scr productivity is slower than this bacterium can consume it; thus, this strategy avoids the Scr utilization for exponential growth and endorses its transformation into PHA/PHACOS. The logic for PHA's production is implementing a low ratio nitrogen:carbon. This is a second reason to inoculate the labourer bacterium once the nitrogen availability in the culture media has decreased. The labour module is aerated at 1 vvm and stirred at 300 rpm. The internal growth is monitored by sample extraction and measuring the optical density at 600 nm.

1.3 Analytical methods

All the samples were taken and stored at -20 °C after optical density measurement. The culture samples are thawed and centrifuged at maximum speed. The supernatant is filtered for the measurement of carbohydrates in HPLC. For Scr detection, a Hi-plex H (300 x 7.7 mm) Waters column was used. This protocol uses an isocratic method with H2SO4 10 mM as the mobile phase. Carbohydrates are detected using the refraction index module.

1.4 Conclusion

All the procedures explained in this document are part of the standardization of *S. elongatus/P. putida* cocultivation. This document aims the generation of a first protocol for DC-PBR technology transference. The tasks performed in the WP4 are focused on constructing a lab-scale platform for PHACOS production, applying the concept of division of labour. Developing new technology is an iterative task that will be updated continuously until optimal performance is reached. This protocol allows the implementation of a DC-PBR, including bioreactor setup, microorganism cultivation, and Scr production.