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Biomass production in *Chlorella vulgaris* biofilm cultivated under mixotrophic growth conditions

Sandra Milena Rincon[a], Hernan Mauricio Romero[b], Wrya Moh Aframehr[a], Haluk Beyenal[a,*]

[a] The Gene and Linda Voiland School of Chemical Engineering and Bioengineering, Washington State University, Pullman, WA 99163, United States
[b] Department of Biology, Universidad Nacional de Colombia, Bogotá, Colombia

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ABSTRACT

The microalga *Chlorella vulgaris* can produce carbohydrates and lipids within short periods with high lipid productivity. Biofilm reactors can be used for microalga cultivation; however, the research on the use of biofilm reactors as a platform for algal biomass and oil production under mixotrophic conditions is limited. The goals of our work were 1) to develop a mixotrophic algal biofilm reactor using glycerol and urea as carbon and nitrogen sources; 2) to quantify biomass, total lipid, glycerol and urea consumption rates and estimate fatty acid profiles; and 3) to measure dissolved oxygen and pH depth profiles using microelectrodes under dark and light conditions. A membrane bioreactor in continuous mode with recycle under 50 μmol photons $m^{-2} s^{-1}$ light irradiance was used to grow the algae. No exogenous CO_2 was supplied. The productivities were 9.27 ± 0.47 g DW $m^{-2} d^{-1}$ and 12.64 ± 0.94 g DW $m^{-2} d^{-1}$ when 2 and 5 g L^{-1} initial glycerol concentrations were used. The total lipid contents were 13 ± 0.02% and 23.91 ± 0.03%, respectively. Depth profile measurements showed a maximum dissolved oxygen concentration of 3.5 mg L^{-1} near the surface and a minimum of 1.4 mg L^{-1} at the bottom of the biofilm. The pH in the biofilms did not vary with the depth, and it was around 6.8 in the dark and 7.5 in the light. The main fatty acids produced were C18:1, C18:2 and C16:0, and there was a low saturated fatty acid content of 23.37%. The designed biofilm reactor allowed *C. vulgaris* growth using very low light with a lower water demand. The composition of fatty acids detected in *Chlorella vulgaris* oil was significantly polyunsaturated. The biofilm growth mode affected *C. vulgaris* metabolism such that the cell met its CO_2 requirements internally and a high oil yield was obtained without nitrogen starvation.

1. Introduction

Algal biomass is used as feedstock for obtaining numerous products across a wide range of industries. Algal research focuses mainly on biofuel production, although many other biomass applications have emerged, such as food, feed, fiber, fertilizer, pigments, antioxidants and pharmaceuticals [1]. The prospects for integrating algae production into various industries is enormous since microalgae can amend greenhouse gas emissions and filter non-potable nutrient-rich water resources in the presence of light [2–7]. Many literature studies have shown that the production costs and the environmental footprint of whole manufacturing processes could be reduced using wastewater streams for microalgal biomass cultivation.

Microalgal biomass cultivation is commonly done using raceway ponds in planktonic growth mode. Biofilm reactors provide an alternative technology to the existing planktonic algae production because biofilms in these reactors can achieve high algal densities with low volumes of culture medium. The biomass production capacities reported so far for biofilm reactors are in the range of 2 to 6 g $m^{-2} d^{-1}$ [8,9]. The dry weight of the algal biofilms fluctuates between 100 and 200 g dry weight kg^{-1} wet biofilm [10]. Furthermore, microalgal biofilm production reduces harvesting costs with more efficient water utilization and a smaller areal footprint. However, to increase the general use of biofilm reactors for algae production, further biofilm reactor development and a better understanding of microalgal biofilm processes are needed.

In a biofilm reactor, microalgae grow on a surface while receiving nutrients and light [10–12]. The light is usually delivered from the top of the biofilm, whereas nutrients are delivered either from the top or from the bottom [11,13,14]. One type of biofilm reactor that has already been tested outdoors at a pilot scale is the rotating biofilm reactor, in which algal biofilms are intermittently exposed to the gas phase and the liquid medium. In two independent studies, *Chlorella* sp. was cultured in this type of reactor, with a light intensity of either 40 to 200 W m^{-2} (approximately 169 to 845 μmol $m^{-2} d^{-1}$ or 235 to 457 μmol $m^{-2} d^{-1}$) and medium supplied externally with atmospheric

* Corresponding author.
E-mail address: beyenal@wsu.edu (H. Beyenal).

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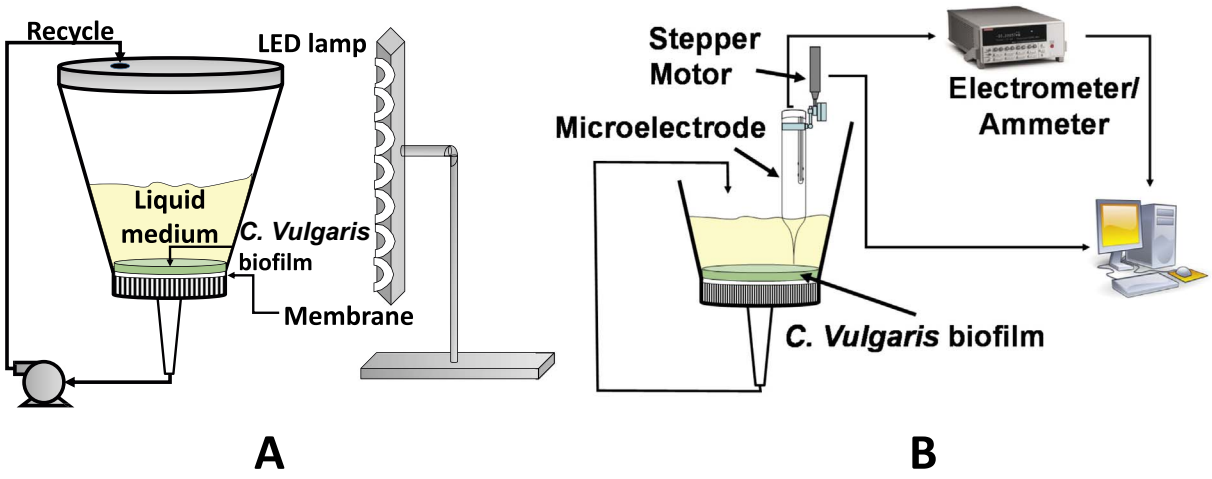

Fig. 1. A) Membrane biofilm reactor setup. A peristaltic pump was used to recycle. The illumination was provided by an external LED light source. B) Experimental setup used to measure depth profiles in biofilms using microelectrodes. Note that the depth profiles were measured while the solution was recycled (the same as in A). The figure is not drawn to scale.

CO_2 or flue gas from a biogas plant, respectively. The maximum surface biomass productivities obtained were 5.8 g m^{-2} d^{-1} [15] and 4.74 g m^{-2} d^{-1} [10]. However, when flue gas was used, the biofilm reactor was not efficient because of extreme pH fluctuations, temperature variations, and CO_2 limitations.

Even when the CO_2 limitations are overcome by technical adjustments to secure a constant CO_2 supply to the reactor, algal biofilm research conducted in autotrophic cultures has shown that beyond 5% CO_2 there are no further increments in photosynthesis under constant photon flux density [16,17]. For example, the growth rate and productivity of *Chlorella* sp. in planktonic cultures increased with CO_2 supply up to a CO_2 saturation point of 2% [18–21]. Furthermore, algal biofilms with and without bicarbonate amendment displayed similar growth rates, nutrient removal, and lipid accumulation [22]. Also, the algae photosynthetic productivity decreases along the thickness because of self-shading in the interior of the biofilm [17,22,23]. The reduction of the photosynthetic rate leads to an oxygen production decline in the biofilm [23], and there is a loss of biomass due to respiration and the synthesis of new biomass [17]. These literature studies indicate that additional CO_2 would not improve algal biofilm processes significantly and indicate the possibility of growing algal biofilms without external CO_2 supply under mixotrophic conditions, a process that has not been well studied.

Mixotrophic cultivation requires an external supply of organic carbon to avoid losses in productivity due to respiration and to overcome the photosynthesis limitations of the algal biofilm cultures. Several organic carbon sources have been shown to increase algal growth rate and lipid content [24–29]. Among them, glycerol is of interest because it is a by-product of biodiesel production and is inexpensive, which could improve the competitiveness of the biodiesel and reduce the algae production costs [28,30,31]. On the other hand, one of the main production costs associated with microalgal biomass production is the nitrogen supply. Urea has gained importance in large-scale algal cultivation because the cost of urea is low and the pH fluctuations over the growth cycle of the microalgae are smaller with urea than with other nitrogen sources. Urea is suggested as an excellent nitrogen source for culturing *Chlorella vulgaris* [32–34]. The hydrolysis of urea by microalgae is due to the activity of urease enzyme, or urea carboxylase, and allophanate lyase, or both where CO_2 can be produced [35,36]. Potentially glycerol and urea can be used in a biofilm reactor while a biofilm is generated mixotrophically. To the best of our knowledge, mixotrophic biofilms using glycerol and urea have not been studied.

The goals of our work were 1) to develop a mixotrophic algal biofilm reactor using glycerol and urea as carbon and nitrogen sources; 2) to quantify biomass, total lipid, glycerol and urea consumption rates and estimate fatty acid profiles; and 3) to measure dissolved oxygen and pH depth profiles under dark and light conditions. We chose *Chlorella vulgaris* because of its ability to grow rapidly. The *C. vulgaris* strains cultivated under mixotrophic growth conditions also have the highest lipid productivity [24,29]. The mixotrophic growth of *C. vulgaris* has shown that either 2 or 5 g L^{-1} glycerol is optimal; thus we used these two levels to observe the differences. Finally, the dissolved oxygen concentration profiles were measured to verify oxygen production in the absence of external CO_2 addition, and pH profiles were determined to demonstrate the existence of mixotrophic conditions in the biofilms.

2. Materials and methods

2.1. Microalgae

The green alga *Chlorella vulgaris Beijerinck* (UTEX 29-ATCC®30,581™) was grown for four days on Luria-Bertani (LB) agar plates to prepare it for inoculation. Then, a portion of the algae grown on an agar plate was transferred aseptically into an Eppendorf tube with 1 mL liquid medium. The resuspended microalgae were used to inoculate biofilm reactors aseptically as a resuspension of 0.0034 g wet biomass in 100 mL of the growth medium; this was equivalent to 2 g of algae per m^{-2} in the reactor. The volume transferred with this proportion of mass was taken from the Eppendorf tube with the suspension. The cell sizes were measured using a microscope (Nikon, Eclipse Ti-S). Five microliters of algae were poured onto a hemacytometer, and then microscope images were taken using Nikon NIS-Elements imaging software. The NIS-Elements radius tool was used to measure the radius of single cells. At least 10 single cells from each image were randomly selected to determine the average radius. Large-scale algal biofilms were imaged using a stereomicroscope (Leica stereo zoom from 10× to 70× magnification).

2.2. Membrane biofilm reactor and operation

The experimental setup is shown in Fig. 1. The experiments were carried out in a reactor that consisted of a polysulfone funnel vessel (14.5 cm × 8.5 cm) with 0.00152 m^2 of filtration area and an 85-mL working volume, and a lid with four ports and a filter holder support (base) 4.7 cm in diameter (Millipore). The water layer was approximately 2.5 cm in depth. The reactor was mounted on a filter holder and placed in a temperature-controlled incubator at 26 ± 2 °C for 192 h. The illumination was provided by an external light-emitting diode (LED) source (NeoPixel LEDs 8 × 8 - 64 RGB LED Pixel Matrix)