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Short Communication

Microfluidic perfusion bioreactor for optimization of microalgal lipid productivity

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HIGHLIGHTS

- Microfluidic perfusion bioreactor is designed for secondary metabolite production.
- Continuous supply of low concentration of nutrient permits microbes to grow at a minimum rate.
- Low nutrient allows same level of metabolite production compared to depleted condition.
- Stable feed of low level of nutrient is an effective way to enhance total TAG yield.

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ABSTRACT

Nutrient deprivation in a batch process induces microbes to produce secondary metabolites while drastically constraining cellular growth. A microfluidic continuous perfusion system was designed and tested to culture microalgae, *Chlamydomonas reinhardtii*, under constant nutrient concentration slightly lower than normal condition. When cultured in 7.5%/7.5% of NH_4^+/PO_4^{2-}, *C. reinhardtii* showed a 2.4-fold increase in TAG production with a 3.5-fold increase in biomass compared to level obtained under an only NH_4^+ depleted condition. The microfluidic continuous perfusion bioreactor with steady continuous nutrient flow can be used to optimize conditions for enhancing secondary metabolite production and increasing microbial biomass.

1. Introduction

Recently, demands for secondary metabolites such as antioxidants, antibiotics, and nutritional additives have markedly increased, and there is a need for technologies that can meet those demands (León-Bañares et al., 2004). Secondary metabolites, however, are produced in the cellular stationary phase, and in that phase, nutrients may become mostly depleted. Consequently, there is no cell growth, which results in a decrease in the total amount of metabolites produced. Although cell-strain development via genetic engineering could increase metabolite productivity per cell, genetically modified organism issues can arise and decreased growth could occur (León-Bañares et al., 2004; Radakovits et al., 2010; Wang et al., 2009).

A bioreactor process that provides a continuous supply of elementary nutrients is known to effectively increase biomass production and secondary bioproduct yield compared to that obtained from a traditional batch process. In addition, the steady state achieved in a continuous supply process contributes to a constant nutrient condition, under which the cells in the reactor proliferate with constant physiological phenotypes. This one-condition-to-one-phenotype feature can assist in the physiological analysis of cellular characteristics during cultivation (Dénervaud et al., 2013). In contrast, cells in a batch process system consume a finite amount of nutrients that are available in concentrations that decrease with time. Such time-dependent nutrient changes can make it difficult to analyze cellular properties due to the many-conditions-to-one-phenotype feature of batch processing (Arora et al., 2016; Gardner et al., 2013; Kamalanathan et al., 2016; Lee and Fiehn, 2008; Tevatia et al., 2012).

Development of microfluidic chips could help realize changes in continuous cultivation processes that can be applied at microscale

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to milliscale (Park et al., 2013). In order to produce a miniature, precise bioreactor that is capable of presenting the same physiological conditions as those on an industrial scale, the microfluidic chips should be capable not only of continuous processing but also capable of suspension cultivation, a second important factor in bioreactor processes. Suspension-based cultivation increases the cellular contact area available for nutrient uptake while maintaining the cells' physiological properties. It was demonstrated that trapping of *Corynebacterium glutamicum* could precipitate a physiologically undeniable deviation in the state of suspended cells; *i.e.*, 50% decreases in division rate and cell length (Dusny et al., 2015), and it was reported that immobilized *Chlamydomonas reinhardtii*, a microalgal model, exhibited 16% and 28% decreases in photosynthesis and respiratory activities, respectively (Garbayo et al., 2005). In addition, it was described a diffusible microfluidic chip that could be used to test the effects of a toxin on several marine microalgae and diatoms, including *Phaeodactylum tricornutum* (Feng et al., 2016; Zheng et al., 2013, 2014). Their device included diffusible chambers confined by semipermeable membranes above a continuous flow with different concentrations, and in which the cells were not immobilized but freely swimming and more sensitive to the toxin concentration.

In this study, a perfusion microfluidic chip is fabricated that meets both the continuous processing and suspension cultivation requirements. The microfluidic chip was applied in a continuous feed system for production of a secondary metabolite, triacylglyceride (TAG), from *C. reinhardtii*. TAG, a bio-derived neutral lipid, has been regarded as a precursor for biodiesel, an alternative to fossil fuel, and for ricinoleic acid (RA), which has healthcare and industrial uses (Kajikawa et al., 2016). Ammonium (NH_4^+) nutrient starvation is the most efficient way to obtain TAG from *C. reinhardtii*, but that approach limits biomass increases, thereby reducing the total lipid quantity produced. We assumed that conditions under which cells could sense being starved but still exert their primary metabolic activities, such as growth, would be the best cultivation conditions for achieving maximal TAG production.

2. Materials and methods

2.1. Fabrication of microfluidic perfusion bioreactor

The microfluidic continuous cultivating device was fabricated in PDMS (SylGard A, B) by applying soft lithography to produce Module I for nutrient gradient generation and Module II for microalgae growth. Module I had curved serpentine channels and embedded inlet and outlet holes. A glass coverslip was treated with air plasma (150 mTorr, 100 W) for 5 min and then attached to Module I. Subsequently, a polyethylene terephthalate (PET) transwell membrane (BD Biosciences, Cat. No.: 353091) and an upper PDMS piece were stacked in order, and the assembly was heated for 30 min in a 75 °C oven for irreversible bonding. The membranes were treated with PLL(20)-g-PEG(2) (0.1 mg/mL) in 10 mM Tris-HCl buffer (pH 6.8) for an hour at room temperature and washed twice with 10 mM Tris-HCl buffer (pH 6.8) to block microalgal cell adhesion (Lee and Spencer, 2008). Module II was prepared with two air-plasma-treated PDMS fragments between which other membranes were located. After loading the samples into the provided holes in Module I, Module II was placed on top of Module I to form a growth chamber that could be subjected to continuous perfusion mixed streams. Polyethylene tubing was inserted into the inlet and outlet holes to make the fluidic connections. The pieces of tubing were then connected to medium-containing syringes installed in a syringe pump (Fig. 1). Rhodamine solution (SigmaAldrich, Cat. No.: R6626, 0.7 mg/mL) was used to check the concentration gradient, and fluorescein isothiocyanate (FITC)-dextran solution (SigmaAldrich, Cat. No.: FD10S) 25 μg/mL in 10 mM Tris-HCl (pH 8.0) was used to check concentration stability.

2.2. Microalgae cultivation

CC-4349 (*cw15 mt-* strain) from the Chlamydomonas Center was cultivated in tris-acetate-phosphate (TAP) with NH_4^+ liquid medium for 5–7 d until the cells entered the stationary phase. A second inoculation of the suspension diluted the cells to 5×10^4 cells/mL in 30 mL of medium in order to maintain the cells in a healthy state for further cultivation. When cell abundance reached $3–4 \times 10^6$ cells/mL they underwent two deionized water (D.W.) washings, and the washed cells were loaded to Module I. Cell counting was performed by using a hemocytometer.

2.3. Triacylglycerol (TAG) staining

BODIPY 505/515 (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacen) (Thermo, Cat. No.: D3921) was dissolved in dimethyl sulfoxide (DMSO) to form a 5 mM stock solution (Govender et al., 2012). A microalgal suspension was mixed with 1/1000 volumes of the stock BODIPY solution and agitated for 30 s. After 10 min incubation in the dark, each sample was washed once with 5× lower volumes of 20 mM Tris-HCl buffer. Images of BODIPY-stained lipid droplets in cells were captured by using an epifluorescence Eclipse Ti inverted fluorescence microscope (Nikon) with a GFP_Quadruple filter. Images were processed with NIS-Elements AR v.4.20.00 for intensity measurement.

3. Results and discussion

3.1. Characteristics of microfluidic perfusion bioreactor

Laminar streams of two different solutions were sufficiently mixed by diffusion and a little inertial force in the curved serpentine channels (Fig. 2a and b). To assess the bioreactor's solution-mixing behavior, 0% rhodamine (i.e., D.W.) and 100% rhodamine solutions were introduced separately to the microfluidic chip through one or the other inlets. It was observed that concentrations at the five outlets were linearly generated, which meant that Module I could precisely produce proportionally linear gradients (Fig. 2c).

Module II included culture chambers sandwiched by top and bottom permeable membranes (Fig. 2d and e). After filling the microfluidic chip with D.W., FITC-Dextran solution was introduced at 10 μl/hr across the chambers. Fluorescence intensity graphs showed that the D.W. was rapidly exchanged (within a couple of minutes) with the FITC-Dextran solution; moreover, the FITC-Dextran content in the chambers remained constant over time (Fig. 2f).

3.2. Synergetic effect of phosphate depletion

This microfluidic chip was applied for assessing TAG production in order to determine whether, along with the effect of NH_4^+, there is a synergistic effect of phosphate PO_4^{2-}, which could be an important factor in the production of TAG. A NH_4^+-deplete medium with five different concentrations of PO_4^{2-} were continuously passed through the growth chamber where CC-4349 in the exponential state under normal conditions (7.5 mM NH_4^+, 1.0 mM PO_4^{2-}) had been loaded. After 120 h, starved cells in each chamber were collected and stained with BODIPY to detect TAG. A scatter plot graph (N = 313) showed that the slope of the sum fluorescent intensity of the cells was highest under 0% NH_4^+/0% PO_4^{2-} conditions (Supplementary Fig. S1). In addition, the percentile graph showed that the 50% median bar was 2.43 times higher under 0% NH_4^+/0% PO_4^{2-} than under the other conditions.