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Research paper

Determining the optimal cultivation strategy for microalgae for biodiesel production using flow cytometric monitoring and mathematical modeling

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ABSTRACT

Trade-off between growth and lipid accumulation is one of the main issues in cultivation of microalgae for biodiesel production. Advantages of two-phase cultivation systems, with growth and lipid accumulation occurring in separate tanks, have been proposed. However, because the two-phase cultivation system requires more cultivation space and complex operation procedures compared to a single tank system, its advantage for lipid production is still uncertain. Furthermore, previous studies have not explicitly determined an optimal cultivation schedule, defining timing of the transfer of cells to the second tank and harvesting in the second tank. We therefore developed a model for lipid accumulation in the microalgae *Chlorella sorokiniana* to compare theoretical maxima of lipid productivity in single- and two-tank cultivation systems. We first established a monitoring method of growth and lipid accumulation using a flow cytometer, and then modeled the data by a logistic equation to derive a model for lipid accumulation. This model was used to theoretically optimize cultivation methods and harvest times to maximize lipid productivity in four different cultivation systems: single- or two-tank setups, with batch or fed-batch cultures, respectively. Results theoretically demonstrated that a two-phase cultivation system is slightly more productive than a single-tank one. The mathematical model analysis indicates strategies for further improving the productivity of each cultivation system without any additional investments in cultivation systems (i.e., nutrient, light, CO_2). Our model analysis approach provides a theoretical basis for determining an optimal cultivation strategy of microalgae and identifies experimental data helpful for further improvement of cultivation systems.

1. 1Introduction

Microalgae capture carbon dioxide via photosynthesis and synthesize oil compounds. These microalgal oils can be used as feedstock for biodiesel [1–3]. They are renewable and would contribute to the reduction of greenhouse gas emissions, if we used them as an alternative to fossil fuels. To facilitate algal biodiesel, all production processes (e.g., selection of algal strains, cultivation, harvesting, oil extraction, and purification) would need to be refined to increase productivity and energy return on investment [4].

One of the central problems of microalgal cultivation for biodiesel production is the trade-off between growth and lipid accumulation. Microalgae accumulate neutral lipids (triacylglycerols; TAGs) as oil droplets in their cells, which can be used as biodiesel feedstock. However, the accumulation of neutral lipids is typically induced under adverse conditions for cell proliferation, such as nutrient depletion [5–9]. Consequently, lipid accumulation does not occur during biomass growth. Under favorable conditions for cell proliferation, the biomass increases rapidly, but lipid accumulation ceases. Our primary goal was to maximize the lipid production rate. To achieve this goal, we clearly need to increase both the cell proliferation rate and the lipid content in the cells.

As a solution to the problem of the trade-off between growth and lipid accumulation in microalgae, a “two-phase cultivation system”, with growth and lipid accumulation occurring in separate tanks, has been proposed. In the first tank, favorable conditions for growth are provided to maximize the cell proliferation rate. The algal cells are then transferred to a second tank, where lipid accumulation is induced under stress conditions, such as nutrient depletion. Optimization of this two-phase cultivation system has been studied in terms of optimal nutrient concentrations in the first tank, and optimal light intensity and CO_2 concentration in the second tank [10–16]. However, because the two-phase cultivation system requires more cultivation space and complex operation procedures compared to a single tank system, its advantage

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for lipid production is still uncertain. In fact, Stephenson et al. [11] and Griffiths et al. [13,14] reported that two-phase cultivation methods did not increase overall lipid productivity compared with single-tank ones. To the best of our knowledge, there is no theoretical basis for the superiority of two-phase cultivation systems. Furthermore, previous studies have not explicitly determined an optimal cultivation schedule, defining timing of the transfer of cells to the second tank and harvesting in the second tank. Adjustment of a cultivation schedule is a low-cost optimization method, because it does not require any additional investments in nutrients, enhancement of light intensity, or CO_2 bubbling.

We therefore developed a model to compare theoretical maxima of lipid productivity in single- and two-tank cultivation systems. First, we established a monitoring method of growth and lipid accumulation in microalgae using a flow cytometer. These monitoring data were modeled by a logistic equation to derive a model for lipid accumulation in microalgal cultures. This model was used to theoretically optimize cultivation methods and harvest times to maximize lipid productivity in various single- and two-tank systems.

2. Materials and methods

2.1. Study material

We selected *Chlorella sorokiniana* as our study material, because it is well known to accumulate neutral lipids under nitrogen depletion [17–21]. The strain *C. sorokiniana* NIES-2168 (hereafter *Chlorella*) was obtained from the Microbial Culture Collection, National Institute for Environmental Studies (NIES), Tsukuba, Japan.

2.2. Culture conditions

Bold's basal medium (BBM) was prepared, which contains $NaNO_3$ (250 mg), KH_2PO_4 (175 mg), K_2HPO_4 (100 mg), $MgSO_4{\cdot}7H_2O$ (75 mg), $CaCl_2{\cdot}2H_2O$ (25 mg), NaCl (25 mg), KOH (31 mg), $FeSO_4{\cdot}7H_2O$ (4.98 mg), H_3BO_3 (11.42 mg), $ZnSO_4{\cdot}7H_2O$ (8.82 mg), $MnCl_2{\cdot}7H_2O$ (1.44 mg), MoO_3 (0.71 mg), $CuSO_4{\cdot}5H_2O$ (1.57 mg), $Co(NO_3)_2{\cdot}6H_2O$ (0.49 mg), Na_2EDTA (50 mg) in a 1-L medium (The pH is 6.8). Precultured *Chlorella* cells were inoculated into a 1-L glass bottle with autoclaved BBM medium to obtain a cell density of 10^2 cells μL^{-1}. The bottle was illuminated for 12 h per day in front of fluorescent lamps (PPFD = 166 $\mu mol\ m^{-2}\ s^{-1}$), with air-bubbled (0.5 L min^{-1}) through under constant temperature (25 °C). Six replicate bottles were prepared; four of these bottles were harvested on the 11th, 18th, 25th, 32nd days of cultivation for lipid extraction and gravimetric measurement, respectively. Two bottles (Bottle-1, -2) were selected for daily monitoring of cell density and fluorescence intensity, until the end of the cultivation period (39th day), before being harvested (*See* Supplementary File 1 for illustration). Evaporative water loss of culture was replenished by distilled water daily.

2.3. Chemical changes in the culture medium

A 20 mL aliquot of the algal culture was extracted daily from two bottles (Bottle-1, -2) to monitor various chemical parameters. The pH was determined using a portable meter (HORIBA® B-212; Horiba Group, Kyoto, Japan). Nitrate and phosphorus concentrations were determined using ion chromatography (SHIMADZU® HIC-10AS; Shimadzu Corp., Kyoto, Japan), and the molybdenum-blue method coupled with a spectrophotometer (SHIMADZU® UV-1801; Shimadzu Corp.), respectively.

2.4. Flow cytometric monitoring of cell density

Three replicate samples of 200 μL were taken daily from Bottle-1 and Bottle-2 to determine cell density (*D*) using a flow cytometer equipped with 488-nm blue laser (Attune flow cytometer; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The laser excites chlorophyll autofluorescence, and the *Chlorella* cell population can be distinguished using fluorescence of the red emission channel (BL3-A, > 640 nm).

2.5. Flow cytometric monitoring of the lipid content per cell

Samples taken for monitoring cell density were diluted to 10^3 cells μL^{-1} and used for fluorescence measurement of their lipid content. Fluorescent beads (FluoSpheres®; Thermo Fisher Scientific, Inc.), comprising polystyrene microspheres of 1.0-μm size, with yellow-green fluorescence, were added to each sample (10^2 beads μL^{-1}) as a standard. Yellow fluorescence intensity (BL2-A, 563–587 nm) of the cells and beads was measured using the flow cytometer. The sample (1 mL) was then stained with a 10uL solution of Nile Red and acetone (0.1 mg mL^{-1}) for 10 min at 37 °C in darkness, and the BL2-A intensity was re-measured. A fluorescence index of the lipid content per cell (C_F) was calculated, as follows:

$$C_F = \left(\frac{B2_{s-cells}}{B2_{s-beads}}\right) - \left(\frac{B2_{us-cells}}{B2_{us-beads}}\right) \quad (1)$$

where $B2_s$ and $B2_{us}$ are the average yellow fluorescence intensities of particles in stained and unstained samples, respectively. Relative fluorescence intensity of cells versus beads was used to reduce instrument error during measurement.

2.6. Gravimetric determination of lipid content

A modified Bligh and Dyer [22] method was used to extract lipids from the algal cells. Culture bottles were harvested weekly from the 11th day of cultivation to determine the total lipid weight per bottle (mg L^{-1}). Algal cells were concentrated by centrifugation at 3000 rpm, washed twice with distilled water, and freeze-dried. The dried cells were divided into approximately 100 mg and disrupted in a 30 mL screw-capped tube containing stainless beads at 3000 rpm by a beads-beating instrument (Yasuikikai® Multi-beads shocker). Methanol (10 mL) and chloroform (5 mL) were added to the tube. The tube was voltexed and put into an ultrasonic bass (AS ONE® VS-100III) at a mixed frequency of 28 kHz, 45 kHz, and 100 kHz for 10 min, and placed on a shaker at 200 rpm for 20 h. Chloroform (5 mL) and distilled water (9 mL) was added to the tube, and the tube was voltexed and centrifuged at 3000 rpm for 5 min for separation. The upper layer was removed by micropipette. The chloroform layer and algal biomass was filtered through a disposable cartridge (Agilent® Bond Elut Reservoir, 60 mL) containing solid $NaSO_4$ to remove algal biomass and water. The tube was washed by 5 mL chloroform two times, and the chloroform was filtered through the cartridge. The all filtered chloroform was put in a pre-weighted aluminum cup and evaporated at 60 °C for 1 h, and lipid extract was weighted. Average lipid weight per cell (C_W) was calculated by dividing the total lipid weight by cell density, while lipid content per cell (%) was calculated by dividing by the total cell dry weight per bottle.

2.7. Fitting of the logistic equation model

A logistic equation model of the form shown below was used to fit daily cell density (*D*) and fluorescence lipid content index (C_F) values:

$$N(t) = \frac{K}{1 + e^{-r(t-\delta)}} \quad (2)$$

where *N* is *D* or C_F; *t* is time (day), *r* is the maximum rate of increase, δ is the inflection point (day), and *K* is the carrying capacity. Curve fitting was carried out using the software JMP®10.0 (JMP, Cary, NC, USA).

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