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Model based optimization of high cell density cultivation of nitrogen-fixing cyanobacteria

Swathi Alagesan, Sandeep B. Gaudana, S. Krishnakumar, Pramod P. Wangikar *

Department of Chemical Engineering, Indian Institute of Technology Bombay, Powai, Mumbai 400076, India

HIGHLIGHTS

- High cell density cultivation of unicellular cyanobacterium, *Cyanothece* 51142.
- Growth rate equation accounting for photolimitation due to self shading.
- Dynamic fed-batch model including variable biomass yield coefficient.
- Twelve fold higher biomass obtained compared to best reported in batch culture.
- Simulating flashing light effect by using turbulent regimes in photobioreactor.

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ABSTRACT

In the present study, fed-batch cultivation of *Cyanothece* sp. ATCC 51142, a known hydrogen producer, was optimized for maximizing biomass production. Decline in growth of this organism in dense cultures was attributed to increased substrate consumption for maintenance and respiration, and photolimitation due to self shading. A model incorporating these aspects was developed, and by using control vector parameterization (CVP), substrate feeding recipe was optimized to achieve 12-fold higher biomass concentration. The optimization results were verified experimentally on shake flask and bioreactor. The latter resulted in greater exponential growth rate possibly by overcoming photolimitation by simulating flashing light effect. Such a strategy can be readily applied for mixotrophic cultivation of cyanobacterial cultures in the first stage followed by photoautotrophic growth at the production stage.

1. Introduction

Eukaryotic microalgae and prokaryotic cyanobacteria have attracted significant attention recently due to their potential in the area of bioenergy (Kim et al., 2013; Prathima Devi et al., 2013). Cyanobacteria are photosynthetic microorganisms capable of converting solar energy into chemical energy; studied for their application in bioremediation, renewable energy production and as a source of commercially important products (Kaiwan-arporn et al., 2012; Rangel-Yagui et al., 2004). Nitrogen fixing cyanobacteria contribute to the nitrogen cycle and have been explored for nitrogenase linked hydrogen production. As nitrogenase is extremely sensitive to even trace amounts of oxygen, cyanobacterial cells separate photosynthesis and nitrogen fixation either spatially (e.g. multicellular species like *Anabaena*) or temporally (e.g. unicellular species like *Cyanothece*). However, ease of manipulation and cultivation, in addition to higher net hydrogen production per cell make unicellular cyanobacteria a favorable candidate for hydrogen production. *Cyanothece* sp. ATCC 51142 (henceforth *Cyanothece* 51142) is one such unicellular cyanobacterium, widely studied for its nitrogenase-linked hydrogen production (Bandyopadhyay et al., 2010; Sherman et al., 2010) among other applications. The availability of a fully sequenced genome, has favored in-depth study of *Cyanothece* 51142 for development as a "single cell factory" for hydrogen production. Commercial scale deployment of this organism, however, is limited by slow initial growth and low productivity. Therefore, for cost-effective industrial exploitation of *Cyanothece*, availability of dense starter cultures for production stages would be of tremendous value.

Numerous reports on high cell density cultivation of bacteria like *Escherichia coli* are available, with final biomass concentrations more than 100 g L^{-1} (Lee, 1996; Riesenberg and Guthke, 1999). These cultivation strategies mainly focus on optimizing the feeding recipe, cultivation method and reactor designs. Model based optimization of feeding recipe is commonly adopted in fed-batch cultivation to find the optimal feed-rates for maximizing biomass or product synthesis (Bapat et al., 2006; Maiti et al., 2011; Roubos et al., 1999). Here, the fed-batch process is represented by a non-linear dynamic model, which is solved analytically. However

* Corresponding author. Tel.: +91 22 2576 7232; fax: +91 22 2572 6895.
E-mail address: wangikar@iitb.ac.in (P.P. Wangikar).

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for complex processes, sequential methods like control vector parameterization (CVP) are used, where the problem is transformed into a number of non-linear programs (NLP) that are solved using a gradient method (Banga et al., 1997; Bapat et al., 2006; Biegler, 2007; Hirmajer et al., 2009).

Cyanobacteria, on the other hand, are slow growing. Cultures with biomass concentrations above 10 g L^{-1} are classified as ultra high cell density cultures (Gitelson et al., 1996; Hu et al., 1998). Ultrahigh densities have been reported for multicellular cyanobacteria like anabaena, while the maximal cell density observed for unicellular *Synechocystis* is 2.5 g L^{-1}(Wang et al., 2002; Yoon et al., 2008). The highest cell density obtained for *Cyanothece* 51142 in mixotrophic batch cultivation is 1.0 g L^{-1} (Feng et al., 2010). Cyanobacterial cultivation studies predominantly emphasize on augmenting growth by improving the photosynthetic efficiency, by enhancing the light availability. Photo-limitation due to self-shading is restricted by using different types of photobioreactor, altering the hydrodynamics or reducing the depth for light penetration (Grima et al., 1999; Grobbelaar, 2000). Another aspect dealt with in cultivation of photosynthetic organisms is the significance of mixotrophic growth (Chojnacka and Noworyta, 2004; Prathima Devi et al., 2013). *Cyanothece* 51142, shows enhanced growth under mixotrophic condition with glycerol (Feng et al., 2010). This ability can be usefully exploited in sustaining faster growth at low as well as high cell densities leading to dense cultures. Use of crude glycerol, a major by-product of detergent and biodiesel plants, as a low-cost additional carbon substrate will also help in its eco-friendly disposal.

Though light availability and mixotrophic growth have been dealt with independently in cultivation studies, implication of a model based fed-batch cultivation of cyanobacteria for maximal biomass production, combining these two aspects has not been described. In the present study, a mathematical model incorporating aspects like photolimitation, substrate inhibition and maintenance energy requirement was devised and used to optimize the fed-batch cultivation of *Cyanothece* 51142 in shake flask. Use of turbulent regimes with strategic feeding was also demonstrated to obtain higher growth in a lab scale photo-bioreactor. The model adequately predicted the feeding time, and when experimentally adopted resulted in a 12-fold higher biomass concentration. Such a strategy will be tremendously beneficial for large-scale production of dense seed cultures for production stages.

2. Methods

2.1. Strain and growth medium

Axenic culture of *Cyanothece* 51142 was grown and maintained in ASP2 medium (with nitrate) in Lab-Therm LT-XC shaker incubator (Kuhner AG, Switzerland) at 30 °C, 125 rpm under constant illumination (100 μmol photons m^{-2} s^{-1}) (Gaudana et al., 2013). Mid-exponential phase culture was used as inoculum by harvesting the cells, washing with 0.9% NaCl and resuspending in fresh medium such that the initial optical density was 0.2. For heterotrophic and mixotrophic studies, ASP2 medium was supplemented with glycerol of required concentrations.

2.2. Fed-batch cultivation

Fed-batch cultivation was carried out in 250 ml Erlenmeyer flasks with 100 ml ASP2 medium supplemented with 18 mM sodium nitrate and 30 mM glycerol. The growth conditions were same as that used for the seed culture. Samples were drawn every 12 h for monitoring the growth and substrate utilization. One ml each from 3 M glycerol stock and 1.8 M sodium nitrate stock, each prepared in 1X ASP2 medium (without nitrate) was fed based on the model predicted feed rates. When the biomass concentration crossed 7 g L^{-1}, 5 ml of 4X medium (without nitrate, and NaCl concentration equivalent to 1X medium) was added to replenish the medium salts.

Reactor studies were performed in a 2 L Biostat B plus bioreactor (Sartorius, Goettingen, Germany) with 1 L working volume. The culture was grown in ASP2 medium supplemented with nitrate and glycerol, as described earlier. Higher agitation of 600 rpm was used (Krishnakumar et al., 2013). Continuous aeration, through a sparger at the bottom of the vessel, was maintained at the rate of 0.1 volume/volume/min (vvm) using a rotameter. Cool white fluorescent lamps (40 W) (Oreva, Ahmedabad, India) were used as light source. Initially the intensity of illumination was set to 150 μmol m^{-2} s^{-1} which was stepped up to 300 μmol m^{-2} s^{-1} after 60 h. The pH and dissolved oxygen (DO) of the culture was recorded real-time using pH and DO probes (Hamilton, Bonaduz, Switzerland) respectively. The exit gas from the reactor was analyzed for volume percentage of CO_2 and O_2, using BlueInOne Cell exit gas analyzer (BlueSens, Herten, Germany). Samples were drawn aseptically through sampling port every 12 h to monitor growth and substrate concentration in the medium. Ten ml of 3 M glycerol and 1.8 M sodium nitrate (each prepared separately in 1X medium) was fed based on the model predictions. Medium salts were replenished when the biomass concentration crossed 7 g L^{-1}. Silicone oil (0.1% in 1X medium) was used as the antifoaming agent.

2.3. Dynamic optimization

The model for fed-batch cultivation was developed based on mass balances for biomass and substrates (Bapat et al., 2006; Maiti et al., 2011). The structure of the model used was as follows:

$$\frac{dX}{dt} = \mu * X - \frac{(u(1)+u(2)) * X}{V} \quad (1)$$

$$\frac{dS_g}{dt} = -(\mu/Y_{(x/g)} + m) * X + \frac{u(1) * F1}{V} - \frac{(u(1)+u(2)) * S_g}{V} \quad (2)$$

$$\frac{dS_n}{dt} = -\frac{\mu * X}{Y_{(x/n)}} + \frac{u(2) * F2}{V} - \frac{(u(1)+u(2)) * S_n}{V} \quad (3)$$

$$\frac{dV}{dt} = (u(1)+u(2)). \quad (4)$$

Where X, S_g, S_n – biomass, glycerol and nitrate concentrations (g L^{-1}), respectively; V – total culture volume (L); μ – monod equation for the growth rate (h^{-1}); $u(1)$, $u(2)$ – feed rates for glycerol and nitrate (L h^{-1}), respectively. $Y_{x/g}$, $Y_{x/n}$ – biomass yield coefficients for glycerol and nitrate (g g^{-1}), respectively; m – maintenance coefficient (g g^{-1} h^{-1}); $F1$, $F2$ – concentration of glycerol and nitrate in glycerol and nitrate feeds (g L^{-1}), respectively.

The light limitation caused by self shading was accounted in the growth equation by using a logistic function, which indirectly incorporates the effect of reduced light penetration with increasing culture density (Eq. (5)) (Shuler, 1992).

$$\mu = \mu_m * (1 - X/X_m)^n * \frac{S_g}{S_g + K_g + S_g^2/K_{Ig}} * \frac{S_n}{S_n + K_n + S_n^2/K_{In}}. \quad (5)$$

Where μ_m – maximum growth rate (h^{-1}); X_m – maximum biomass concentration (g L^{-1}); K_g, K_n – half saturation constant for glycerol and nitrate, respectively; K_{Ig}, K_{In} – substrate inhibition constant for glycerol and nitrate, respectively. The parameters for the model were obtained from preliminary experiments (Supplementary Table 1).

Furthermore, the glycerol taken up was not only utilized for growth but was also diverted towards other pathways like maintenance requirement and respiration. In addition, the rate of respira-