Biotechnology Reports 4 (2014) 151–155

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

Micro-compartmentalized cultivation of cyanobacteria for mutant screening using glass slides with highly water-repellent mark

Sayuri Arai [a], Mina Okochi [a], Taizo Hanai [b], Hiroyuki Honda [a,*]

[a] Department of Biotechnology, Graduate School of Engineering, Nagoya University, Furo-cho, Chikusa-ku, Nagoya,Aichi 464-8603, Japan
[b] Laboratory for Bioinformatics, Graduate School of Systems Life Sciences, Kyusyu University, 804 Westwing, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

ARTICLE INFO

Article history:
Received 13 July 2014
Received in revised form 8 October 2014
Accepted 9 October 2014
Available online 16 October 2014

Keywords:
Cyanobacteria
Droplet culture
Dodecane

ABSTRACT

Photosynthetic microorganisms such as cyanobacteria have attracted attention for their potential to produce biofuels and biochemicals directly from CO_2. Cell isolation by colony has conventionally been used for selecting target cells. Colony isolation methods require a significant amount of time for cultivation, and the colony-forming ratio is potentially low for cyanobacteria. Here, we overcome such limitations by encapsulating and culturing cells in droplets with an overlay of dodecane using glass slides printed with highly water-repellent mark. In the compartmentalized culture, the oil phase protects the small volume of culture medium from drying and increases the CO_2 supply. Since a difference in cell growth was observed with and without the addition of antibiotics, this compartmentalized culture method could be a powerful tool for mutant selection.

1. Introduction

Photosynthetic microorganisms, including cyanobacteria and microalgae, have attracted a growing interest in biofuel production. These organisms are efficient at converting solar energy and recycling CO_2, and thus, biofuel production does not compete with agriculture for water, fertilizer, and arable land. Estimates suggest that nearly 50% of the global net primary fixation of carbon by photosynthesis occurs in ocean waters dominated by phytoplankton. For these reasons, there is an increasing interest in utilizing photosynthetic microorganisms to fix CO_2 and produce biofuels. Photosynthesis-driven conversion of carbon dioxide to biofuels and biochemicals using genetically modified cyanobacteria has previously been investigated [1–5]. For example, ethanol, 1-butanol, and isobutyraldehyde (a precursor to isobutyl alcohol) have been produced directly from CO_2 [3–5]. Cyanobacteria are attractive candidates for biofuel production, since genome characterization has facilitated genetic engineering of host cells [6].

To improve biofuel productivity, it is important to develop an effective screening method for the selection of useful mutants. The general approach for mutant screening involves cell isolation following colony formation in agar nutrient media, followed by the identification of target mutants by evaluating their activity after culturing in liquid media. For a long time, “toothpicks and logic” were considered sufficient for screening [7]. However, cell isolation on agar plates cannot be carried out efficiently for organisms with low growth rates and/or low colony-forming ratios. In cyanobacteria, the doubling time for *Synechococcus elongatus* PCC7942 is more than 10 h (with 5% CO_2 bubbling), and the number of colonies formed in a solid medium is less than 10% of the number of cells before plating. A significant amount of time is required for culturing single cells into colonies that are large enough to visualize and select from agar plates. This inherently limits the throughput of mutant screening. To address this problem, some have proposed methods for encapsulating single cells in aqueous droplets [8–10] and agarose microparticles [11].

In this study, encapsulation of cyanobacteria in a droplet culture was investigated for cell screening without colony formation on agar plates. Using glass slides printed with highly water-repellent mark, we conducted micro-compartmentalized cultivation from single cyanobacteria cells by covering micro-droplets in an oil phase. This oil phase can protect small volumes of culture medium from drying and increase the transfer of CO_2 from the air to cells, since, it has a higher absorption constant than water. This micro-compartmentalized culture method offers promise for the screening of useful cyanobacteria mutants, such as high growth strains and strains resistant to specific metabolic products, and for single colony isolation for many kinds of microalgae that can fix CO_2.

* Corresponding author. Tel.: +81 52 789 3215; fax: +81 52 789 3214.
E-mail address: honda@nubio.nagoya-u.ac.jp (H. Honda).

http://dx.doi.org/10.1016/j.btre.2014.10.003

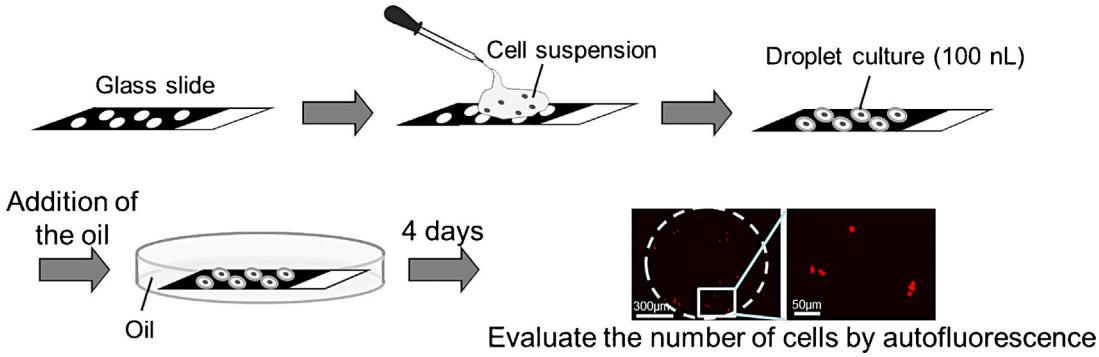

Fig. 1. Overview of the droplet culture method.
Droplets were prepared by laying the cell suspension on a glass slide printed with highly water-repellent mark. The volume of one droplet is approximately 100 nL. Cyanobacteria were cultured for four days after the glass slide was covered with oil. After culture, growth was evaluated using cell autofluorescence.

2. Materials and methods

2.1. Culture conditions

S. elongatus PCC7942 was cultured at 30 °C under a light irradiance of 50 μmol photons m^{-2} s^{-1}. The strain was grown on BG11 medium (1.5 g/L KNO_3, 0.4955 g/L $(NH_4)_3SO_4$, 0.006 g/L citric acid anhydrate, 0.006 g/L ferric citrate, 0.001 g/L Na_2EDTA, 1.03 g/L NaCl, 0.039 g/L K_2HPO_4, 0.0739 g/L $MgSO_4$, 0.038 g/L $CaCl_2{\cdot}2H_2O$, 0.020 g/L Na_2CO_3, 1000× trace minerals [2.86 g/L H_3BO_3, 1.81 g/L $MnCl_2{\cdot}4H_2O$, 0.222 g/L $ZnSO_4{\cdot}7H_2O$, 0.39 g/L $Na_2MoO_4{\cdot}2H_2O$, 0.079 g/L $CuSO_4{\cdot}5H_2O$, 0.0404 g/L $CoCl_2{\cdot}6H_2O$]) [12].

2.2. Selection of oil

The stationary phase culture fluid (5 mL) was mixed with 5 mL of oil (dodecane, mineral oil, oleyl alcohol, and oleic acid) in a test tube and cultured under light. After three days, cells were stained with YO-PRO®-1 iodide (Abs, 491 nm; Em, 509 nm; Y3603, Invitrogen, Life Technologies, Carlsbad, CA, USA) and the number of live and dead cells were counted by tallying red and green colors, respectively, using fluorescence microscopy (Model IX70, Olympus Co., Ltd., Tokyo, Japan) [13].

To confirm cell growth with overlaid oil, cyanobacteria were cultured with oil in 5% CO_2 for four days and the growth was monitored by measuring absorbance at 730 nm (OD_{730}) using a digital colorimeter (miniphoto518R, Taitec, Saitama, Japan) and an ultraviolet and visible spectrophotometer (V-630 BIO, JASCO Corporation, Tokyo, Japan).

2.3. Micro-compartmentalized cultivation

S. elongatus was cultured in test tubes under 5% CO_2 until OD_{730} = 0.8. To make the 5% CO_2 environment, Anaero Pack·CO_2 (Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan) was used. The culture was diluted in BG11 at 1 cell per 100 nL (10^4 cells/mL). Droplets were prepared by laying 1 mL cell suspension on a glass slide printed with highly water-repellent mark (high-density amino group introduction coat, 570 holes of 1 mm in diameter, 480 μm spaces between holes; Matsunami Glass, Osaka, Japan). Due to the patterning of the hydrophobic area (spacing between holes) and hydrophilic area (holes), droplets were formed. Based on the number of cells in a droplet and the cell concentration of the suspension, the volume of one droplet was approximately 100 nL. After the glass slide was covered with oil, the cells were cultured in micro-compartmentalized droplets for four days (Fig. 1). The oil phase was equilibrated with BG11 medium beforehand by mixing dodecane and BG-11 medium at a ratio of 1:1 by volume, followed by three periods of centrifugation at 5000 × *g*. Cell growth in each micro-compartmentalized droplet was evaluated by detecting cell autofluorescence (chlorophyll a and phycocyanin) using fluorescence microscopy. To detect autofluorescence, an excitation filter (520–550 nm), a dichroic mirror (565 nm) and an emission filter (580 nm) were used. The analysis of acquired images was performed using an EMCCD camera (Luca 658 × 496 pix, Andor Technology Ltd., Belfast, U.K.) and image analysis software (Andor IQ, Andor Technology Ltd.). The fluorescence images were taken under the condition that no signal was detected in a droplet lacking cells. We assessed the red points, which were supposed to indicate cells in the fluorescence images. After that, the cells in phase difference images were counted. The specific growth rate of droplet cultures was compared with that of normal liquid cultures without dodecane in 18 mm test tubes.

3. Results and discussion

3.1. Selection of oil

For the selection of an oil phase for micro-compartmentalized cultivation, *S. elongatus* in stationary phase were incubated for three days with an overlay of oil. The cell death rate of *S. elongatus* increased consecutively according to treatments with dodecane, mineral oil, oleyl alcohol, and oleic acid; in the case of oleic acid, dead cells comprised 28% of the sample (Fig. 2). Little toxicity was observed with an overlay of dodecane, with only 2% dead cells. Although both dodecane and mineral oil had low toxicity,

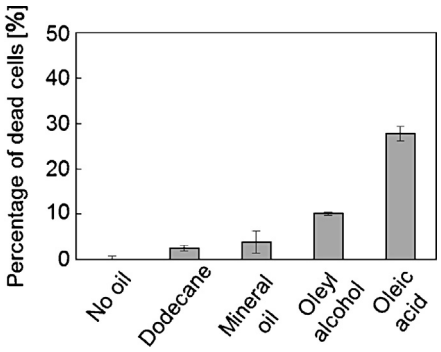

Fig. 2. The percentage of dead cells in the presence of oil.
Cyanobacteria in the stationary phase were incubated with an overlay of dodecane, mineral oil, oleyl alcohol, or oleic acid in the presence of light (50 μmol photons m^{-2} s^{-1}). After three days, cells were stained with YO-PRO and the numbers of live and dead cells were estimated by tallying counts of red and green cells, respectively, using fluorescence microscopy.