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A flexible microbial co-culture platform for simultaneous utilization of methane and carbon dioxide from gas feedstocks

Eric A. Hill [a], William B. Chrisler [a], Alex S. Beliaev [a], Hans C. Bernstein [a,b,*]

[a] Biological Sciences Division, Pacific Northwest National Laboratory, Richland, WA, USA
[b] The Gene and Linda Voiland School of Chemical Engineering and Bioengineering, Washington State University, Pullman, WA, USA

HIGHLIGHTS

- A co-cultivation technology that converts, CH_4 and CO_2, into microbial biomass.
- Robust bacterial growth on biogas and natural gas feedstocks.
- Continuous co-cultivation without air or O_2 feed to support CH_4 oxidation.
- A flexible co-culture technology constructed from genetically tractable bacteria.

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ABSTRACT

A new co-cultivation technology is presented that converts greenhouse gasses, CH_4 and CO_2, into microbial biomass. The methanotrophic bacterium, *Methylomicrobium alcaliphilum* 20z, was coupled to a cyanobacterium, *Synechococcus* PCC 7002 via oxygenic photosynthesis. The system exhibited robust growth on diverse gas mixtures ranging from biogas to those representative of a natural gas feedstock. A continuous processes was developed on a synthetic natural gas feed that achieved steady-state by imposing coupled light and O_2 limitations on the cyanobacterium and methanotroph, respectively. Continuous co-cultivation resulted in an O_2 depleted reactor and does not require CH_4/O_2 mixtures to be fed into the system, thereby enhancing process safety considerations over traditional methanotroph mono-culture platforms. This co-culture technology is scalable with respect to its ability to utilize different gas streams and its biological components constructed from model bacteria that can be metabolically customized to produce a range of biofuels and bioproducts.

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1. Background

Microbial biomass is a clean, renewable energy source that can significantly diversify and sustain future energy and transportation fuel requirements (Elliott et al., 2015; Tian et al., 2014). Production of microbial biomass and targeted bioproducts often depends upon costly substrates, such as glucose or other sugars, that limit the economic viability of the process (Kumar et al., 2012; Rodriguez et al., 2014). In contrast, various trends in algal cultivation have promised to develop renewable bioprocesses that convert inorganic carbon (CO_2/HCO_3^-) into microbial biomass using solar energy; however, process viability is often constrained by low biomass productivities and the inherent limitations of photosynthetic efficiencies (Grobbelaar, 2010; Quinn and Davis, 2015). Natural gas or biogas derived CH_4 represents an alternative, energy rich carbon source for generating microbial biomass and bioproducts (Kalyuzhnaya et al., 2015; Sheets et al., 2016) yet O_2 demands and mass transfer limitations remain challenging aspects for developing viable and safe bioprocesses (Rishell et al., 2004). Co-cultivation platforms of photoautotrophic and methanotrophic microbes represent a unique and promising option for concurrent capture of CO_2 and CH_4 within an integrated system (van der Ha et al., 2012)

CH_4 and CO_2 have the largest contributions to atmospheric radiative forcing caused by anthropogenic greenhouse gasses (GHG) (Robertson et al., 2000). Natural gas is an abundant resource that has played a central role in global energy production. Much of the natural gas that is co-produced with oil recovery (5 quadrillion BTU, ~5% of annual production) is unused, flared or vented representing a major GHG contribution that could be redirected and consumed as microbial feedstock (Fei et al., 2014). Biogas represents another major source of CH_4 and is an important renewable

* Corresponding author at: 902 Battelle Boulevard, P.O. Box 999, MSIN J4-18, Richland, WA 99352, USA.
E-mail address: Hans.Bernstein@pnnl.gov (H.C. Bernstein).

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energy source that can be upgraded to a gaseous transportation fuel or combusted to generate electricity. However, biogas utilization is constrained because of its high upgrade costs and presence of contaminants such as H_2S and organosilicon (i.e., siloxanes). A large fraction of biogas produced is also being flared or vented into the atmosphere, representing yet another wasted product that could be redirected into microbial biomass and targeted bioproducts.

Many state-of-the-art biotechnologies are seeking to capitalize on the productivity and stability advantages gained though co-cultivation of microbial consortia (Bernstein and Carlson, 2012; Bernstein et al., 2012; Gilmore and O'Malley, 2016; Lindemann et al., 2016). This study presents new bacterial co-culture platform for concurrent conversion of CH_4 and CO_2 into biomass by employing a cyanobacteria-methanotroph binary culture. The co-culture technology is based upon robust metabolic coupling between oxygenic photosynthesis and methane oxidation. Continuous steady-state operation can be achieved based on light and O_2 limitation of the complimentary trophic partners, resulting in balanced and, ultimately, self-regulated growth. This co-culture has been demonstrated using various mixtures of CH_4, CO_2, O_2 as well as raw biogas to test performance on different GHG feedstocks. Importantly, the bacteria used to build this co-culture are amenable to metabolic engineering which renders the platform flexible and scalable for the production of different bioproducts on cost-effective, renewable CH_4 feedstocks.

2. Materials and methods

2.1. Bacterial strains and media

Synechococcus sp. PCC 7002 and *Methylomicrobium alcaliphilum* 20z were grown under co-culture and axenic conditions. All cultures were grown in one of two previously described minimal salts media, P-medium (Khmelenina et al., 1999) or A-plus medium (Stevens and Porter, 1980).

2.2. CH_4 feedstocks

Raw biogas was collected from an anaerobic digester (located in Outlook WA, USA) that was operated on dairy farm waste. The biogas composition was 58% CH_4, 42% CO_2, 0% O_2, and 0.3% H_2S as measured by a Landtec biogas 5000 m (Landtech, Dexter, MI). A synthetic natural gas feed stream of 80% CH_4, 17% N_2, and 3% CO_2 was made by mixing pure gas flows through calibrated rotameters.

2.3. Batch cultivation

The maximum specific growth rates on various biogas, CH_4, CO_2 and O_2 mixtures were obtained using sealed 30 ml Balch tubes incubated under constant 250 μmol photons m^{-2} sec^{-1} (fluorescent light). Each tube was charged with 8 ml A-plus medium (pH 8.0), sparged with an appropriate gas mixture and sealed with the respective gas in the head-space maintained at 1 ATM. The optical density (OD_{730nm}) was measured over a 96 h period using a Spectronic 20D+ spectrophotometer (Thermo Spectronic, Thermo Fisher Scientific, Waltham, MA). Each culture Balch tube was inoculated to a starting $OD_{730nm} = 0.028 \pm 0.005$. Co-cultures were inoculated with a 1:1 ratio of *M. alcaliphilum* and *Synechococcus* 7002, respectively.

2.4. Photobioreactor cultivation

Continuous cultivation was performed under chemostat mode by previously described methods (Beliaev et al., 2014; Bernstein et al., 2015). Briefly, di-chromatic (680 and 630 nm LEDs) photobioreactors were operated as light and O_2 limited chemostats using the New Brunswick BioFlo 3000 fermenter charged with a 5.5 L working volume diluted at a 0.03 h^{-1}, 30 °C, pH 8.0 and controlled for constant incident and transmitted irradiance (250 and 10 μmol photons m^{-2} s^{-1}, respectively). Cells were never exposed to dark conditions during these experiments. The control volume was sparged at 0.25 L min^{-1} with a synthetic natural gas mixture, 80% CH_4, 17% N_2, 3% CO_2. Steady-state biomass concentrations were measured directly as ash-free cell dry weight (g_{CDW} L^{-1}) as previously reported (Pinchuk et al., 2010). The volumetric gas mass transfer coefficient, $k_La = 5.32\ h^{-1}$, was directly measured in the reactor under abiotic conditions by the unsteady re-aeration technique. Dissolved O_2 concentration in the reactor was measured with a Clark O_2 electrode (InPro® 6800Series, Mettler Toledo International Inc., Columbus, OH). The *in situ* net rate of O_2 production was calculated from the steady-state mass balance through the bioreactor control volume (Eq. (1)).

$$q_{O_2}x = D\left([O_2^{in}] - [O_2]\right) + k_la\left(k_H pO_2^{in} - [O_2]\right) \quad (1)$$

The specific rate of O_2 production q_{O_2} multiplied by the biomass concentration (x) is interpreted here as the net rate of O_2 production during photosynthesis (Bernstein et al., 2014) and is a function of the dilution rate (D), k_La, dissolved O_2 concentration ($[O_2]$) and Henry's law partitioning coefficient ($k_H = 1.08$ mM atm^{-1}). The specific rate of biomass production (q_x; Cmmol biomass h^{-1} g_{AFDW}^{-1}) was calculated by assuming the molecular weight of ash free dry biomass (AFDW) to be 24.59 g_{AFDW} $Cmol^{-1}$ (Roels, 1980). The net rate of photosynthesis was also determined as a function of incident irradiance (P-I curve) by collecting axenic *Synechococcus* 7002 cells from the bioreactor and measuring the volumetric O_2 production rates as a function of 'white light' (tungsten incandescent) inside an oxygraph chamber (Hansatech, Norfolk, UK) coupled to a 2π quantum sensor (LI-210SA Photometric Sensor, LI-COR Biosciences, Lincoln, NE). The P-I curve was fit to the Jassby-Platt (Eq. (2)) using a parametric nonlinear regression (Jassby and Platt, 1976).

$$P = P_{max} \cdot \tanh\left(\frac{I_i}{I_k}\right) \quad (2)$$

The net volumetric rate of oxygenic photosynthesis (P) was estimated as a function of incident irradiance (I_i) by fitting to the single response variable (I_k), which represents the theoretical saturating irradiance of photosynthesis. The maximum net volumetric rate of oxygenic photosynthesis (P_{max}) is a constant that was directly measured from the P-I curve.

2.5. Flow cytometry and imaging

The relative abundances of *Synechococcus* 7002 and *M. alcaliphilum* cells were determined by flow cytometry using a BD Influx Fluorescence Activated Cell Sorter (FACS, BD Biosciences, San Jose, CA). Upon harvesting, the cells were immediately treated with 50 mM Na_2EDTA (Sigma-Aldrich) and gently pipetted to disrupt large aggregates and then fixed with 2% paraformaldehyde. Using the 488-nm excitation from a Sapphire LP laser (Coherent Inc., Santa Clara, CA) at 100 mW, samples were analyzed using a 70-μm nozzle. Optimization and calibration of the FACS was performed before each analysis using 3 μm Ultra Rainbow Fluorescent Particles (Spherotech, Lake Forest, IL). The ratio of the two distinct populations of cells within a mixed microbial community were identified from 50,000 recorded cells via size and complexity gates using FloJo (FloJo, LLC Ashland, OR) flow cytometry software. Microscopic images were acquired on a Zeiss LSM 710 Scanning Confocal Laser Microscope (Carl Zeiss MicroImaging GmbH, Jena,

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