Bioresource Technology 169 (2014) 462–467

Contents lists available at ScienceDirect

Bioresource Technology

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

Boosting D-lactate production in engineered cyanobacteria using sterilized anaerobic digestion effluents

Whitney D. Hollinshead [a,1], Arul M. Varman [a,b,1], Le You [a], Zachary Hembree [a], Yinjie J. Tang [a,*]

[a] Department of Energy, Environmental and Chemical Engineering, Washington University, St. Louis, MO 63130, USA
[b] Biological and Materials Science Center, Sandia National Laboratories, Livermore, CA 94550, USA

HIGHLIGHTS

- Anaerobic digestion effluents provide N/P nutrients for cyanobacterial cultivation.
- Acetate-rich effluents enhance D-lactate synthesis from engineered cyanobacteria.
- Alkaline pH culture condition is important for cyanobacterial D-lactate secretion.

ARTICLE INFO

Article history:
Received 19 April 2014
Received in revised form 30 June 2014
Accepted 1 July 2014
Available online 10 July 2014

Keywords:
D-lactate dehydrogenase
Municipal waste
Photomixotrophic
Synechocystis 6803

ABSTRACT

Anaerobic digestion (AD) is an environmentally friendly approach to waste treatment, which can generate N and P-rich effluents that can be used as nutrient sources for microalgal cultivations. Modifications of AD processes to inhibit methanogenesis leads to the accumulation of acetic acid, a carbon source that can promote microalgal biosynthesis. This study tested different AD effluents from municipal wastes on their effect on D-lactate production by an engineered *Synechocystis* sp. PCC 6803 (carrying a novel lactate dehydrogenase). The results indicate that: (1) AD effluents can be supplemented into the modified BG-11 culture medium (up to 1:4 volume ratio) to reduce N and P cost; (2) acetate-rich AD effluents enhance D-lactate synthesis by ~40% (1.2 g/L of D-lactate in 20 days); and (3) neutral or acidic medium had a deleterious effect on lactate secretion and biomass growth by the engineered strain. This study demonstrates the advantages and guidelines in employing wastewater for photomixotrophic biosynthesis using engineered microalgae.

1. Introduction

High feedstock costs and environmental burdens remain major obstacles in the development of industrial-scale biorefineries. To overcome these problems, microalgae-based biorefineries have been proposed as an economical and environmentally friendly process that can be potentially integrated into CO_2 sequestration and wastewater treatment processes. Numerous wastewaters (livestock waste, poultry waste, and municipal slurries) contain significant levels of nitrate (N), phosphate (P), and other nutrients that can be used to support algal cultivations (Patil et al., 2010; Olguín, 2012; Cho et al., 2013). In addition, P and N stripping from waste water are often necessary to avoid eutrophication and environmental damage to local ecological systems. Therefore, it is ideal if the N and P in wastewater are consumed by photo-biorefineries, serving both bio-production and bioremediation (Cho et al., 2011; Cho et al., 2013).

Anaerobic digestion (AD) is an effective method for waste treatment, which involves four major conversions: organic wastes → simple sugars → organic acids → acetic acid → methane (Chen et al., 2008). The effluents from anaerobic digestion of municipal and agricultural wastes may provide cheap N and P sources for microalgal cultivations. In addition, AD can accumulate acetic acid at high concentrations by blocking methanogenesis using either acidic conditions or chemical inhibitors (Wilkes, 2008). The resulting effluents have higher N & P levels as well as abundant amounts of acetic acid and other organic acids. These acetate-rich effluents have previously been successfully used for biodiesel fermentation (Liu et al., 2013).

Previous wastewater studies on facilitating algal bioprocesses mainly focus on the search for new algal species, optimization of algal cultivations, and pre-treatment methods to avoid contamination (Cho et al., 2011; Huang et al., 2012; Ho et al., 2013). Recently,

* Corresponding author. Address: Campus Box 1180, One Brookings Drive, St. Louis, MO 63130, USA. Tel.: +1 314 935 3441.
E-mail address: yinjie.tang@seas.wustl.edu (Y.J. Tang).
[1] W.D.H. and A.M.V. have equal contributions to this work.

http://dx.doi.org/10.1016/j.biortech.2014.07.003
0960-8524/

metabolic engineering and synthetic/systems biology tools have been employed to create novel microalgal strains to produce biofuels and other commodity chemicals (Angermayr et al., 2012; Lan and Liao, 2012; Berla et al., 2013). Among these studies, cyanobacteria are a promising chassis because of their fast growth and effective photosynthetic production (Wang et al., 2012). Therefore, it is of great interest to develop economical bioprocesses by integrating waste treatment with cyanobacterial biorefinery. In this study, we have investigated the feasibility of using different anaerobic digestion effluents for cultivation of wild-type *Synechocystis* sp. PCC 6803 and its engineered variant (AV10 strain with a novel D-lactate dehydrogenase) (Wang et al., 2011; Varman et al., 2013). We monitor both cyanobacterial growth and D-lactate biosynthesis (a chemical important in food, pharmaceutical, and plastic industries) under the influence of wastewater supplementation. By studying this model cyanobacterial system, we can obtain knowledge on applying waste streams to promote engineered microalgal bioprocesses.

2. Methods

2.1. Anaerobic digestion

Effluents from anaerobic digestion processes were generated and provided to us by Professor Yan Liu's group at Michigan State University. The municipal sludge was obtained from the East Lansing Wastewater Treatment Plant (East Lansing, MI, USA). The sludge was subjected to anaerobic digestion under three conditions (Rughoonundun et al., 2010): (1) AD1 – Under normal condition; (2) AD2 – Under acidic condition (pH = ~5) to promote acetogens and inhibit pH-sensitive methanogens; (3) AD3 – Treatment with iodoform solution to inhibit methanogenesis (Liu et al., 2013). The compositions of the different AD effluents are shown in Table 1. The AD effluents were filtered then autoclaved before each experiment (stored at −20 °C).

2.2. Strains and growth conditions

The wild type *Synechocystis* 6803 cells were transferred from BG-11 agar plates into shaking flasks containing BG-11 medium and grown at 30 °C (Wang et al., 2011; Varman et al., 2013). During the mid-log growth phase, aliquots of culture were withdrawn and then resuspended in their respective media (combinations of BG-11 medium and AD effluents) to a starting biomass equivalent to OD_{730} of 0.1. The cultures were cultivated in 50 mL shake flasks (10~15 mL working volume) under 80–100 μmol of photons m^{-2} s^{-1}. The engineered strain of *Synechocystis* 6803 (AV10) employs a novel D-lactate dehydrogenase GlyDH* (mutated from glycerol dehydrogenase) and a soluble transhydrogenase to balance the cofactors (Wang et al., 2011; Varman et al., 2013). The seed culture for AV10 was grown in BG-11 media with 20 μg/mL of kanamycin. During the mid-log growth phase, aliquots of culture were withdrawn and then resuspended in their respective media (with 1 mM IPTG) to a starting biomass equivalent to OD_{730} of 0.4.

Cell optical density OD_{730} was used to monitor biomass growth in the BG11 medium culture. For culture media with AD effluents, *Chlorophyll a* was used as an indicator for cyanobacterial biomass to avoid the interference of light absorption by wastewater. In brief, the culture samples were centrifuged, and then *Chlorophyll a* was extracted from the biomass with 1 mL of methanol by vortexing for 5 min. After centrifugation to remove solids, *Chlorophyll a* was measured for its absorbance at 663 nm via a UV–Vis spectrophotometer (Agilent Cary 60, USA) as adapted from (Meeks and Castenholz, 1978). Biomass correlation between OD730 and OD663 (Chlorophyll a) can be estimated using equation: OD730 = 0.38 × OD663 (R^2 = 0.99).

2.3. D-Lactate quantification

D-Lactate concentrations in the supernatant were measured using an enzyme assay (D-Lactic acid/L-Lactic acid Enzyme Kit, R-Biopharm, Germany) on a 96 well plate reader (Infinite 200 PRO microplate photometer, Tecan, Switzerland). The intracellular D-lactate concentration in the engineered strain was estimated using a isotopomer-based approach as described in a standard protocol (Bennett et al., 2008). In brief, the engineered strain was grown in BG-11 medium with 4 g/L of fully labeled ^{13}C-sodium bicarbonate (Sigma–Aldrich, St. Louis) in a closed glass bottle for three days. The labeled cultures (OD_{730} = 1.9, 20 mL) were filtered at 4 °C and washed with fresh BG-11 medium (pH = 7) to remove the residual extracellular lactate. The biomass-containing filter paper was then placed in a mixture of methanol and chloroform (1:2 ratio, with 0.01 μg/mL unlabeled lactate in the extraction solution as the internal standard) to extract the intracellular lactate. The samples were lyophilized (FreeZone Freeze Dry System, Labconco, MO) and derivatized using methoxyamine hydrochloride and N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA). The samples were analyzed via a Gas Chromatography-Mass Spectrometry (GC–MS) (Hewlett Packard 7890A and 5975C, Agilent Technologies, USA). The MS peak [M-117] of the trimethylsilyl-derivatized lactic acid was used for analysis of D-lactate labeling, and the presence of natural isotopes were corrected (You et al., 2012). The intracellular concentration of lactate was approximately estimated based on the ratio of the ^{13}C lactate abundance to the ^{12}C lactate abundance.

2.4. Validation of organic carbon utilization by the engineered strain

The seed culture for AV10 was prepared in BG-11 media with 20 μg/mL of kanamycin. During the mid-log growth phase, aliquots of culture were withdrawn and resuspended in BG-11 media with 4 g/L of $NaH^{13}CO_3$ (initial OD_{730} < 0.01) and 20 μg/mL of kanamycin. Grown for four days, this labeled seed culture was then used to inoculate a control culture (BG-11 media with 2 g/L of $NaH^{13}CO_3$) and a culture with a mixture of AD2 and BG-11 media ($_{1:4}$ volume ratio, 2 g/L $NaH^{13}CO_3$). All labeled cultures started at an

Table 1
Composition of anaerobic digestion sludges.

AD conditions	AD 1 Normal	AD 2 Low pH	AD 3 Chemical Inhibition	BG11 Medium
Total phosphorus (mg/L)	183	100	185	30
Total nitrogen (mg/L)[a]	280	420	590	1495
Chemical oxygen demand (g/L)	4.5	15.3	24.9	ND
Butaric acid (g/L)	0.02	1.28	2.69	
Propionic acid (g/L)	0.49	1.29	1.41	
Acetic acid (g/L)	0.41	3.85	7.30	
D-Lactate (g/L)	<~0.025	~0.025	~0.025	

[a] Most nitrogen in AD sludge is in the form of ammonia (Sheets et al., 2014).