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# Photosynthetic biohydrogen production in a wastewater environment and its potential as renewable energy

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ABSTRACT

Oxygen ($O_2$) is a strong inhibitor of hydrogenase (HydA) activity and expression and altering the sulfur (S) oxidizing transitions in photosystem II (PSII) often allows algal photohydrogen production; however, this may not be practical in a wastewater environment. To counteract natural mechanisms of oxygen evolution in PSII, we utilized acetic acid and butyric acid, which are main volatile fatty acids (VFAs) found in anaerobic bacterial digestion in wastewater treatment, as oxygen regulators for photosynthetic biohydrogen production using *Chlorella vulgaris*. It was found that a VFA-containing synthetic wastewater promotes oxygen depletion in a photobioreactor (PBR), producing maximum hydrogen yield of $65.4 \pm 0.3$ μmoL $H_2$ $L^{-1}$ $mM^{-1}$ acetate without artificial sulfur or chloride deprivation. Butyric acids showed no significant effect on oxygen depletion and biohydrogen production in the PBR. The measurements of both relative expression level of mRNA and specific activities of reactivate HydA revealed that repetitive algal $H_2$ photo-evolution was possible by HydA synthesis in *C. vulgaris* followed by complete oxygen depletion controlled by acetic acid levels in the PBR. This emerging understanding of the role of VFAs on oxygen regulation in PSII in natural environments is expected to lead algal-driven bioenergy production technologies to the next level.



## 1. Introduction

For decades, the use of microalgae has been primarily focused on generation of feedstocks such as carbohydrates [1], lipids [2], and biogas via dark-fermentation processes [3]. Recently, direct biohydrogen production using microalgae has attracted public attention as an alternative to traditional biogas practices [4]. Several microalgae species (e.g. *Chlorella pyrenoidosa and Scenedesmus sp.*) showed their capability of producing molecular hydrogen ($H_2$) through the utilization of algal fermentative metabolism [5]. However, as the enzyme (i.e. HydA) responsible for biohydrogen production is extremely sensitive to oxygen and is deactivated at a partial pressure of 2% oxygen [6], the capability to induce hydrogen production in algal metabolism appears only after anaerobic adaptation and is lost in the presence of small quantities of oxygen [7].

Photosystem II (PSII) is a large multisubunit membrane protein complex, responsible for light-driven water oxidation as a key step in photosynthesis to produce oxygen in higher plants, algae, and cyanobacteria [8]. Particularly, water is oxidized at the oxygen evolving complex (OEC) in PSII which goes through five sequential oxidation states ($S_0$ to $S_4$) before $O_2$ is evolved [9]. Under anaerobic conditions, microalgae synthesize [FeFe] HydA, which preferentially limits the synthesis of D1 and D2 heterodimer proteins in PSII and thus inhibits oxygen evolution from microalgae, producing molecular hydrogen [10]. Sulfur and chloride ions ($Cl^-$), necessary for the S oxidizing transitions ($S_2$ to $S_3$ and $S_3$ to $S_0$) to produce oxygen in PSII, have been often depleted to achieve anaerobic conditions [11]. For example, chloride ions ($Cl^-$) are exchanged with other anions (e.g. $F^-$, amine and acetate) which can occupy the $Cl^-$ binding site of the PSII OEC, inhibiting oxygen evolution [11]. However, the adoption of sulfur or chloride ions deprivation for regulating oxygen in the PSII of microalgae may be impractical in a wastewater environment, considering the inherent abundance of these compounds and elements in wastewater [12].

The concept of fermentative metabolism of hydrogen evolution was discovered decades ago using *Chlamydomonas moewusii* [13]; however, engineering challenges limit the adoption of algal hydrogen production in a wastewater environment in terms of

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renewable energy production and performance characteristics. The roles of acetic acid and butyric acid, which are common volatile fatty acids (VFAs) from anaerobic bacterial digestion in wastewater treatment, on oxygen depletion and subsequent biohydrogen evolution, and its repeatability for continuous algal hydrogen production have not been fully explored in wastewater environments [14]. Here we show a novel biomimetic strategy for quantitatively designing oxygen depleting photosynthetic reaction using *Chlorella vulgaris* in an acetate-rich synthetic wastewater, based on the concept of acetate binding at the PSII OEC, replacing chloride with acetate (Fig. 1) [15]. Quantitative analysis of the use of VFAs required for renewable photosynthetic hydrogen production has important implications for sustainable design for biohydrogen production from wastewater.

## 2. Materials and methods

### 2.1. Strain isolation and growth conditions

*C. vulgaris* (UTEX 2714, UTEX Algae Culture Collection, USA) was selected as model microalgae which can be found in wastewater environments [16]. A solution of 20 mL of *C. vulgaris* UTEX 2714 suspended culture was inoculated in 500 mL of autoclaved Bold's Basal Medium (BBM) [17] in 1 L Pyrex glass bottle (KIMAX, Germany) flasks. Then, 0.03% $CO_2$ from air (filtered through a 1 μm pore-size hydrophobic glass laminate filter, Pall Corporation, USA) was supplied by sparging through a tube inserted into the bottle, and continuous illumination was provided with fluorescent lamps (110 μE $m^{-2}$ $s^{-1}$) for 3 weeks at room temperature (~26 °C).

### 2.2. Experimental set up

To demonstrate acetate-driven anaerobic and photosynthetic hydrogen production without controlling sulfur or other anions (e.g. $Cl^-$), batch tests were performed in duplicate using a 500 mL photobioreactor (PBR) with an effective volume of 300 mL. Each series of batch experiments was initiated by adding different sodium acetate concentrations (25–95 mM) and 20% BBM (v/v) under continuous illumination (110 μE $m^{-2}$ $s^{-1}$) under a stirred condition at 100 rpm and at room temperature (~26 °C). With the addition of acetate and BBM, 20% of the PBR volume was discharged once stirring was completely stopped for partial biomass sinking after oxygen production of each phase. A control experiment for oxygen depletion was also conducted without the addition of acetate. The chemical characteristics of the acetate-rich synthetic wastewater based on fermentation effluent were as follow: pH 7.2 ± 0.3, 26 ± 1 °C temp. 1320 ± 20 mg $L^{-1}$ chemical oxygen demand (COD), 28.4 ± 0.3 mg $L^{-1}$ total nitrogen (TN), 3.5 ± 0.2 mg $L^{-1}$ total phosphorous (TP), 6.12 mg $L^{-1}$ $Cl^-$. Acetate and butyrate of the synthetic wastewater were 1320 ± 20 mg $L^{-1}$ and 2640 ± 40 mg $L^{-1}$, respectively. The one-way ANOVA test function in the GraphPad Prism6 software was used to generate all P-values.

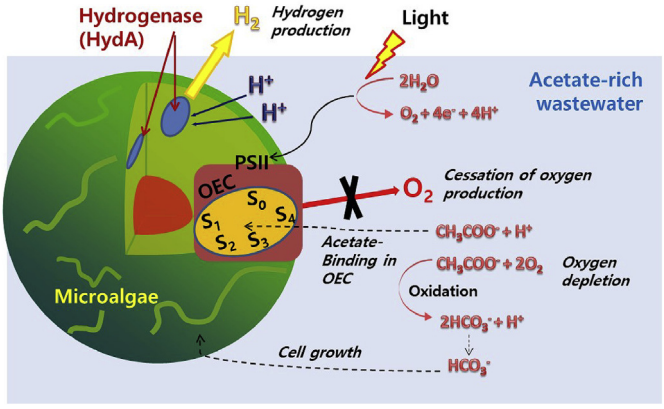


**Fig. 1.** A schematic diagram of renewable photobiological hydrogen production using acetate-rich wastewater.

### 2.3. Hydrogen ($H_2$), oxygen ($O_2$) and acetate measurements

$H_2$ and $O_2$ contents (μmol $L^{-1}$) accumulated in the headspace of the PBR were continuously monitored using microsensors ($H_2$-NP and OX10, Unisense A/S, Denmark). Pre- and post-calibration for sensor performance evaluation was conducted with different $H_2$ and $O_2$ concentrations. Acetate and butyrate were periodically analyzed by gas chromatograph (Shimadzu GC-14 A, Japan) equipped with a flame ionization detector (FID). The temperatures of injector port, detector and column were 220, 220, and 190 °C, respectively. We used calibration curves to convert measured acetic acid and butyric acid concentration to each acetate and butyrate concentration (Fig. S1).

### 2.4. Preparation of crude cell extract and measurement of HydA activity

The crude cell extract preparation and the measurement of HydA activity were performed as described by Ueno et al. [18]. In brief, microalgae cultivated under different oxygen concentrations controlled by acetate for 0–4 days were harvested by centrifugation (12,000 g, 4 °C, and 15 min) and washed twice in 20 mM phosphate buffered (pH 7.5) 1% NaCl solution. After 5 min of anaerobic adaptation with oxygen-free nitrogen gas, the cells were resuspended in a basal buffer containing 50 mM Tris (hydroxymethyl) aminomethane-HCl (pH 8.0), 2 mM $MgCl_2$ and 1 mM Dithiothreitol (DTT) followed by the addition of powdered sodium dithionite (50 mM). Protein concentrations in crude extracts were determined by the Bradford method [19] using Bovine Serum Albumin (BSA) as a standard. The samples were analyzed using NanoDrop 2000 UV–Vis Spectrophotometer (Thermo Fisher, USA). The HydA activities were quantified by the amount of hydrogen evolved from methyl viologen which had been reduced by sodium dithionite. Hydrogen production was determined using a microsensor ($H_2$-NP, Unisense A/S, Denmark). One unit of activity is defined as the amount of HydA evolving 1 μmoL $H_2$ per minute.

### 2.5. Total RNA isolation and HydA mRNA expression

Total RNA was isolated from the cells using the QIAGEN RNeasy kit (Qiagen, USA), according to the manufacturer's instruction [20]. The RNA concentration of each sample was determined using a spectrophotometer at 260 nm. The integrity of each RNA sample was evaluated using an Agilent 2100 BioAnalyzer (Agilent Technologies, USA). cDNA synthesis was performed with 1 μg of total RNA in 20 μL using random primers (Invitrogen, USA) and Superscript II reverse transcriptase (Invitrogen, USA). Real-time (RT)-PCR analyses were performed using a 7500 Real-Time PCR System (Applied Biosystems Inc, USA). PCR reactions were performed in a 25 μL vial containing 12.5 μL of 2× SYBR Green reaction buffer, 1 μL of cDNA (corresponding to 25 ng of reverse transcribed total RNA) and 5 pmol of each HydA specific primer obtained from *Chlorella fusca* (GenBank accession no. AJ298228). Data analyses were performed on 7500 system SDS software version 1.3.1 (Applied Biosystems Inc, USA). All analyzed samples were normalized by the corresponding expression of 18 S ribosomal RNA (18 S rRNA). The same primers used by Hwang et al. [21] were also used in this study.