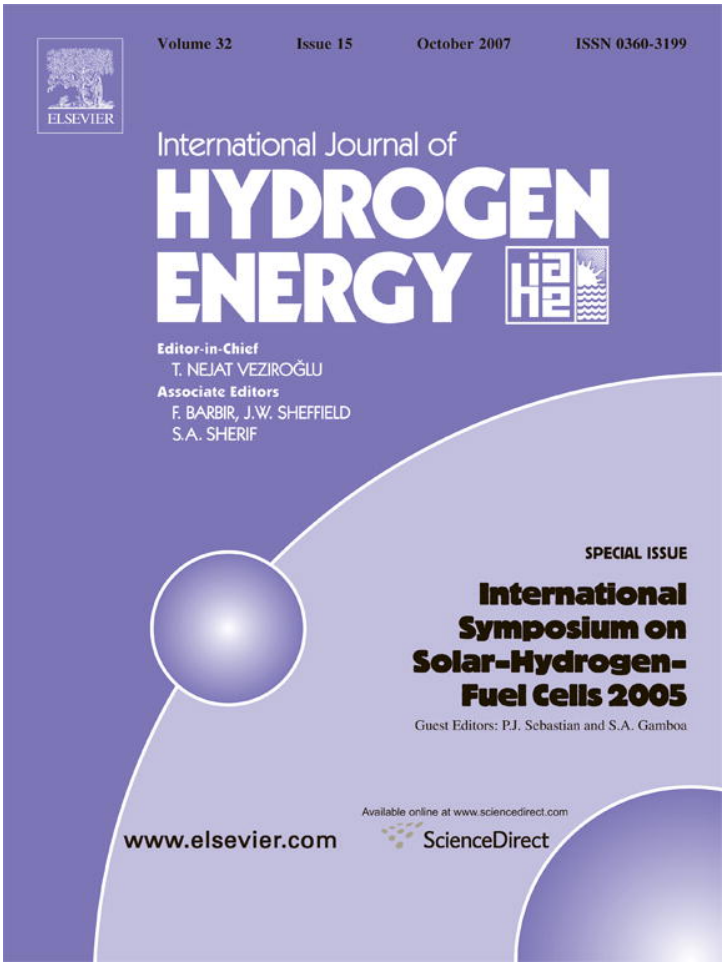

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The effect of butyrate concentration on hydrogen production via photofermentation for use in a Martian habitat resource recovery process

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Abstract

Biological hydrogen production from waste biomass has both terrestrial and Martian advanced life support applications. Several forms of this process exist, but one process, indirect biophotolysis, is suitable for a potential Mars mission. This process is two-stage, combining a dark fermentation of starch or sugars with photofermentation of the remaining waste organic acids to produce hydrogen gas. Since butyrate is expected as one of the major inputs into photofermentation from the first stage, *Rhodobacter sphaeroides* SCJ, a photoheterotrophic purple non-sulfur photosynthetic bacterium was examined for its potential in hydrogen production at 10–100 mM butyrate concentrations. As butyrate levels increased, hydrogen production increased up to 25 mM butyrate, and then decreased and ceased by 100 mM. Additionally, lag phase increased with butyrate concentration, possibly indicating substrate inhibition. Maximal substrate conversion efficiency was 8.0%; maximal light efficiency was 0.89%; and maximal hydrogen production rate was 7.7 μmol/(mg cdw h) (173 μL/(mg cdw h)). These are generally lower than values reported in the literature.

Keywords: Photofermentation; Biophotolysis; Advanced life support; Spaceflight; Butyrate utilization; Rhodobacter sphaeroides; Purple non-sulfur bacteria

1. Introduction

The main objectives of using a controlled ecological life support system (CELSS) for a Martian surface habitat are to reduce launch mass and decrease dependency on re-supply from Earth in terms of water, oxygen, and food, the latter two being integral to closure of the carbon cycle. Essentially, the carbon processing system has two components: a plant growth and preparation component and a waste-processing component. Carbon wastes can come in several forms, typically as inedible biomass, exhaled CO_2 and CO_2 reserves, human wastes, and uneaten food and related preparation wastes. When studying mass flows in a CELSS, a large fraction of waste predicted in models that involve plant growth tends to be inedible biomass from plants for all mission scenarios. Thus, there is an emphasis on biomass degradation and recovery of useful materials [1].

Plants oxidize water and reduce CO_2 into carbohydrates (CH_2O) during oxygenic photosynthesis according to the following equation [2]:

$$CO_2 + H_2O \xrightarrow{h\nu} (CH_2O) + O_2. \quad (1)$$

Therefore, waste carbon material contains a significant fraction of hydrogen taken in from spacecraft water reserves. Over time, waste biomass can also become a drain on hydrogen (and therefore water) resources.

For example, in a wheat consumption model from the Advanced Life Support (ALS) Baseline Values and Assumptions Document (BVAD) maintained by Johnson Space Center (JSC) [3], for an extended mission to Mars where all food crops are grown, the predicted total inedible biomass per crewmember-day (CM-d) is 1.271 kg/CM-d. Ash analysis of wheat tops grown at the JSC in an enclosed environment indicates that hydrogen accounts for about 5% of the total weight [4]. Therefore, roughly 0.0633 kg/CM-d of hydrogen or the equivalent

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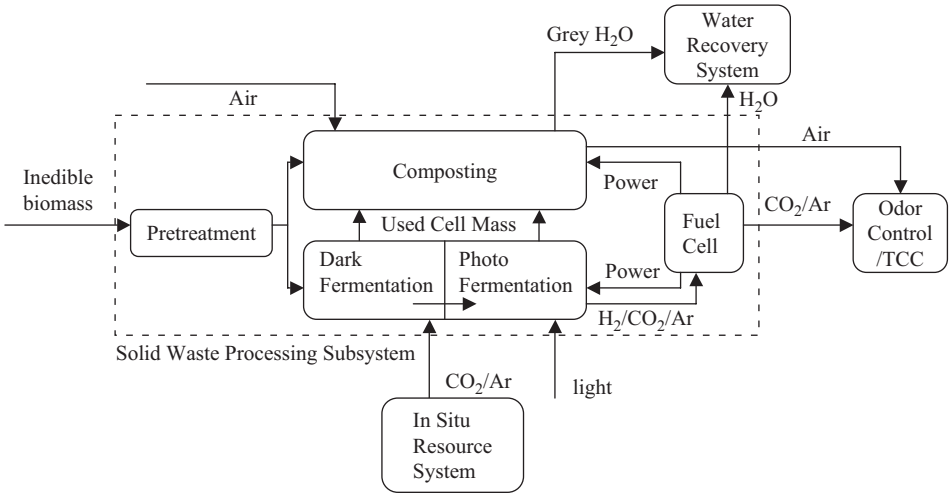

Fig. 1. Composting scheme with indirect biophotolysis co-generation of power.

of 0.572 kg/CM-d worth of water is lost in dry inedible wheat biomass.

For the Mars Design Reference Mission (DRM), a model of expected mission requirements for a short-term Mars mission, the expected surface stay for six crewmembers is 619 days [5]. Taking this as a baseline for an extended surface stay mission, a calculated loss of about 236 kg of hydrogen being sequestered in unprocessed inedible biomass occurs if continuous wheat growth is conducted. This is the equivalent loss of 2124 kg of water from water supplies for wheat growth alone. Since water will make up the largest supply resource by mass for a Mars mission [6], recovering this hydrogen from waste biomass will greatly reduce the overall launch mass.

Various methods exist for enabling hydrogen recovery. In indirect biophotolysis, purple non-sulfur photosynthetic bacteria have been shown to use waste carbon, typically in the form of organic acids, to produce hydrogen in a two-stage process [7]. In the first stage, acidogenic bacteria naturally present in the environment derive energy and produce some hydrogen by degrading waste carbohydrate matter into simple organic acids and alcohols. In the second stage, these organic acids are harvested and fed as a substrate to photoheterotrophic bacteria for additional hydrogen production. Photoheterotrophic bacteria of the genus *Rhodobacter* have been thoroughly studied under a variety of growth conditions. An anoxic inert gas environment best suits these bacteria. It has been shown that the best conversion efficiencies exist in low light (6.5–20 klux) and low nitrogen (2–10 mM) environments with a pH of 6.7–7.5. Ammonium and glutamate were found to be the best nitrogen nutrients, and acetate was shown to have the highest substrate conversion efficiency [8].

While these conditions require creation of an artificial environment on Earth, they are actually advantageous for Mars-based applications. Since carbon dioxide or argon (and not oxygen) are principal chemicals of the Martian atmosphere they can be potentially captured to constitute the plant growth chamber air without having to worry about oxygen contamination [9]. On Earth, careful measures must be taken to ensure that no oxygen enters the system. Also, the ambient insolation on Mars is often less than a half to a tenth of that on Earth, especially during a dust storm [10]. As it so happens, this is near the intensities reported in the literature for maximal light conversion efficiency. Indeed, both terrestrial- and space-based microgravity studies have shown that bacteria can potentially thrive in a reduced gravity environment and even produce useful bio-based products such as medicines or bioplastics [11,12]. A schematic of how indirect biophotolysis would fit in such an ALS configuration is outlined in Fig. 1. A co-generation scheme is depicted where hydrogen produced by indirect biophotolysis is sent to a fuel cell. Machinery used in composting is powered by electricity generated from the fuel cell. Water and CO_2 are the main byproducts of the fuel cell, which are sent to respective water and air revitalization subsystems for treatment. Spent cell mass can be used as compost, depending on composting conditions.

While substrate conversion efficiencies have been widely reported in the literature [8], recent work has centered on understanding the effects of using effluent of anaerobic digestion from *Clostridia* or mixed consortia as a feed source for photofermentation. Often, butyrate is found at a 2:1 or higher ratio with acetate in the effluent from dark fermentation in batch mode with mixed consortia [13]. It has been reported that *R. sphaeroides* can metabolize these substrates; however, tolerance ranges and inhibition effects by any of these substrates are not well documented. Previous researchers have noted that butyrate is converted to hydrogen with less efficiency than other substrates; while much published research exists detailing instances of butyrate utilization, sensitivity of *R. sphaeroides* SCJ (itself a new strain to hydrogen research) to a range of butyrate concentrations has not been studied (see Table 1). Therefore, this work focused on characterizing the metabolism of butyrate over a wide range of concentrations. Several specific issues were investigated:

1. The effect on hydrogen production by butyrate was systematically characterized and correlated with previous literature.

Table 1
Various butyrate results reported

Strain	Concentration	Rate hydrogen produced	Total hydrogen	Conversion efficiency	Conditions	Reference
Rhodopseudomonas sp. *TN3*	Butyrate 30 mM	30 μL/h/mg cdw	n.r.	n.r.	5 mM L-glutamate, 700 mL at 10 000 lux	[18]/1981
Rhodopseudomonas sp. *TN3*	Butyrate 30 mM + bicarbonate 10 mM	60 μL/h/mg cdw	n.r.	n.r.	5 mM L-glutamate, 700 mL at 10 000 lux	[18]/1981
Rhodopseudomonas sp.	Butyrate 24 mM	157–255 μL/h/cm^2	n.r.	54–67	10 mM ammonium 0.2% bicarbonate, 0.1% yeast extract, immobilized cells in 12 mL at 1 klux	[19]/1986
Rhodobacter sp.	Butyrate 24 mM	185–321 μL/h/cm^2	n.r.	44–67	10 mM ammonium 0.2% bicarbonate, 0.1% yeast extract, immobilized cells in 12 mL at 1 klux	[19]/1986
R. rubrum G-9 BM	0.2% Butyrate	14.31 μL/h/mg cdw	n.r.	n.r.	10 mM ammonium immobilized cells in 5 mL at 2 klux	[20]/1986
Rhodopseudomonas sp. *RV*	Butyrate 50 mM	57 μL/h/cm^2	n.r.	n.r.	10 mM Ammonium, 0.1% yeast extract, at 1 klux	[21]/1984
Rhodopseudomonas sp. *RV*	Butyrate 46.2 mM	205 μL/h/cm^2	604 mL	75.1	10 mM Ammonium, 0.1% yeast extract, immobilized cells at 1 klux	[21]/1984
Rhodopseudomonas sp.	Butyrate 27 mM	7.2 mL/L/h	100 mL	8.4	0.8 mM Na Glutamate, 480–680 μmol/m^2/s	[22]/2001
R. palustris	Butyrate 27 mM	0 mL/L/h	0 mL	0	0.8 mM Na Glutamate, 480–680 μmol/m^2/s	[22]/2001
"Microbiology Strain"	Butyrate 27 mM	0.2 mL/L/h	4 mL	0.3	0.8 mM Na Glutamate, 480–680 μmol/m^2/s	[22]/2001
Rhodopseudomonas capsulate ATCC 237B2	Butyrate 7 mM	32.9 mL/h/L reactor	23.44 mM	50.41	0.1% L-Glutamic acid, 350 mL at 100 W, Ar with 5% CO_2	[17]/1983 [15]/1975
Rhodopseudomonas capsulate ATCC 17013	Butyrate 7 mM	30.8 mL/h/L reactor	25.57 mM	37.6	0.1% L-Glutamic acid, 350 mL at 100 W, Ar with 5% CO_2	[17]/1983 [15]/1975
Rhodopseudomonas capsulata ATCC 17020	Butyrate 7 mM	21.5 mL/h/L reactor	8.34 mM	46.59	0.1% L-Glutamic acid, 350 mL at 100 W, Ar with 5% CO_2	[17]/1983 [15]/1975
Rhodopseudomonas capsulata DSM 152	Butyrate 7 mM	30.0 mL/h/L reactor	20.33 mM	58.09	0.1% L-Glutamic acid, 350 mL at 100 W, Ar with 5% CO_2	[17]/1983 [15]/1975
Rhodopseudomonas capsulata DSM 156	Butyrate 7 mM	25.4 mL/h/L reactor	24.53 mM	45.85	0.1% L-Glutamic acid, 350 mL at 100 W, Ar with 5% CO_2	[17]/1983 [15]/1975
Rhodopseudomonas capsulata DSM 157t2	Butyrate 7 mM	25.8 mL/h/L reactor	22.25 mM	55.76	0.1% L-Glutamic acid, 350 mL at 100 W, Ar with 5% CO_2	[17]/1983 [15]/1975
Rhodopseudomonas capsulata NCIB 8254	Butyrate 7 mM	27.1 mL/h/L reactor	23.01 mM	51.36	0.1% L-Glutamic acid, 350 mL at 100 W, Ar with 5% CO_2	[17]/1983 [15]/1975
Rhodopseudomonas sp. *RV*	Acetate 16.3 mM	37 μL/h/cm^2	117 mL	40.2	10 mM Ammonium, 0.1% yeast extract, immobilized cells at 1 klux	[21]/1984
Rhodopseudomonas sp.	Acetate 22 mM	25.2 mL/L/h	269 mL	72.8	0.8 mM Na Glutamate, 480–680 μmol/m^2/s	[22]/2001
R. palustris	Acetate 22 mM	2.2 mL/L/h	56 mL	14.8	0.8 mM Na Glutamate, 480–680 μmol/m^2/s	[22]/2001
"*Microbiology Strain"*	Acetate 22 mM	2.6 mL/L/h	134 mL	35.3	0.8 mM Na Glutamate, 480–680 μmol/m^2/s	[22]/2001

2. Substrate inhibition may exist at the butyrate concentrations expected in the inlet streams from a two-stage system. This would become evident with an increasing lag phase as butyrate concentration increases and was monitored during this study.
3. An optimal butyrate concentration for energy conversion efficiency could exist. This was assessed by comparing the total hydrogen produced while varying substrate levels.

2. Methods

2.1. Microorganism, media, and growth conditions

R. sphaeroides SCJ used in this study was originally isolated by Weaver et al. [14] from Jamaican soil. The culture was maintained in RCVBN Medium, according to Weaver et al. [14], with the exception that sodium acetate (50 mM) and NH_4Cl (10 mM) served as the carbon and nitrogen nutrients, respectively, along with $NaHCO_3$ (1%, w/v). Cells were cultivated in 15 mL of anaerobic sterile test tubes under light for 2 days at 30 °C.

2.2. Hydrogen production media

Experiments were conducted using either sodium acetate or sodium butyrate as the carbon substrate, 8 mM sodium glutamate as the nitrogen source along with 0.5% (m/v) $NaHCO_3$. Also, 28.8 mM KH_2PO_4 and 34 mM K_2HPO_4 buffer was used to stabilize pH at 6.8.

2.3. Experimental setup

Three hundred mL samples were incubated in 2 L capacity water-jacketed reactors held at 32 °C. Illumination for each reactor was provided with 65 W incandescent light bulbs (held at 34 cm) separated by black partitions. Reactors contained two ports, a septum port and a gas displacement line. The gas displacement line was evacuated through submerged and inverted graduated cylinders. Glassware was cleaned with deionized water and ethanol, and then autoclaved. Media and bacterial inoculum was added to the reactor first, followed by purging with argon before sealing. Data were collected in triplicate for sodium butyrate with concentrations ranging from 10 to 100 mM. Additionally, one trial of 25 mM sodium acetate was run for comparison. All activities were conducted in Colorado, USA at an altitude of 1630 m and a nominal pressure of 84.1 kPa.

2.4. Hydrogen analysis

Hydrogen was determined by injecting 1.0 mL of headspace gas from both the reactor and graduated cylinder to a HP 5710A Gas Chromatograph equipped with a Carboxen 1000 column (60/80 Mesh $15' \times 1/8''$ SS) at 80 °C for 4 min, incremented at 32 °C/ min to 200 °C for 4 min. Barometric pressure was read from a 429 Nova Princo Mercury Barometer with each data point. Hydrogen was quantified by calculating moles based on the pressure and volume using the ideal gas law. This was then converted to STP volume for comparison with the literature.

2.5. Cell dry weight

Twenty mL of grown cells were withdrawn, diluted, and washed once with deionized water. After drying at 100 °C, the cell dry mass was measured. A Genesys 10 Series Spectrophotometer was used to measure optical density at 660 nm. Optical density was linearly correlated with dried cell mass and used to measure cell mass density.

2.6. Organic acids

Two mL bacterial samples were withdrawn from the reactor through the septa port using a stainless steel cannula. The cells were spun and the supernatant filtered and run on a HP 1050 Series HPLC (Agilent Technologies) equipped with an Aminex HPX-87H Ion exchange column (300 mm × 7.8 mm) at 45 °C. The mobile phase is 4 mM H_2SO_4 with a flow rate of 0.6 mL/min. A sample of 20 μL was injected and detected via a UV detector at 210 nm.

2.7. Light efficiency

Light efficiency was calculated based on the following equation [8]:

$$\%\ \text{light conversion efficiency} = 100 \times H_{2\ \text{enth}}/E_{\text{light}}, \quad (2)$$

where $H_{2\ \text{enth}}$ is the energy of hydrogen produced, and E_{light} is the total emitted energy of the light source. Incandescent light bulbs were characterized at 34 cm from the light source with a LicorLi-1800 spectroradiometer that reported the light spectrum profile in $\mu mol/m^2/s/nm$ per nm interval that were integrated over 300–900 nm. The light spectrum profile was taken for each bulb before and after all the experiments and before and after integral values averaged (see Fig. 2). This was performed since incandescent light sources degrade a noticeable amount over the experiment duration. Hence, using only the original values can lead to under-reporting light efficiency. To determine the overall light energy emitted, this value was then multiplied by the tangent exposure area and used as E_{light}. The maximum hydrogen produced in each run was taken and multiplied by the average value of 203 kJ/mol to determine $H_{2\ \text{enth}}$.

2.8. Hydrogen efficiency and maximal hydrogen

Substrate efficiency was calculated using [8]:

$$\%\ \text{substrate conversion efficiency} = 100 \times \text{observed } H_2/\text{theoretical } H_2. \quad (3)$$

Theoretical H_2 yield from butyrate is according to the equation:

$$NaC_4H_7O_2 + 6H_2O + H^+ \rightarrow 10H_2 + 4CO_2 + Na^+. \quad (4)$$

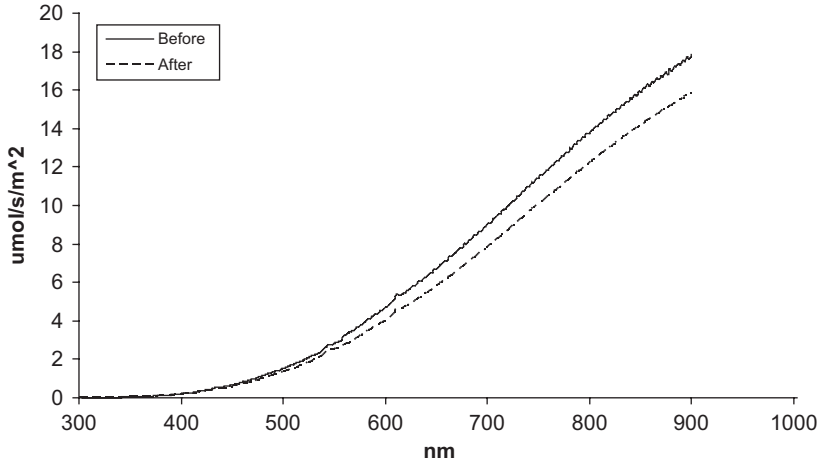

Fig. 2. Light spectrum of incandescent lighting before experiment and after 477 h.

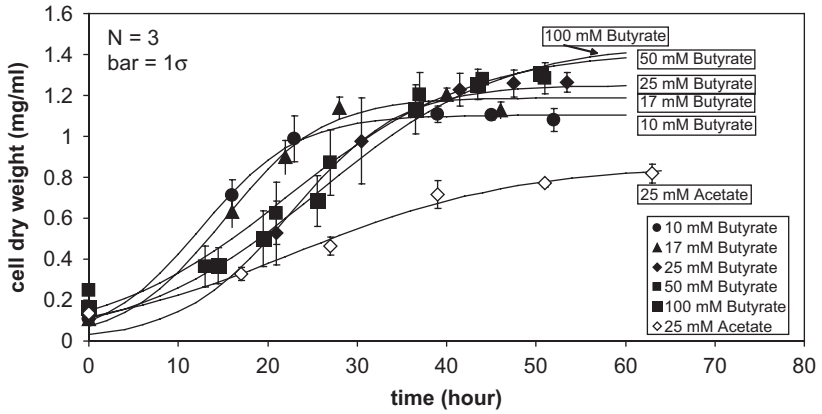

Fig. 3. Cell growth over time at butyrate substrate concentrations from 10 to 100 mM.

And for acetate:

$$NaC_2H_3O_2 + 2H_2O + H^+ \rightarrow 4H_2 + 2CO_2 + Na^+. \quad (5)$$

Maximum hydrogen produced and maximum hydrogen production rate were defined as the maximal total hydrogen produced and the maximal rate observed for every run.

2.9. Lag phase determination

Lag phase was calculated by fitting a logistic growth model to cell dry weight data [15]:

$$y(t) = \frac{A}{1 + \exp[(4\mu_m/A)(\lambda - t) + 2]}, \quad (6)$$

where $y(t)$ is the cell dry weight, A is the maximum population limit, μ_m is the maximal growth rate, and λ is the lag phase. However, lag phase was calculated indirectly from curve fitting cell dry weight data. An exponential growth tangent was drawn and lag phase found by intersecting that line with a line representing the initial concentration.

3. Results and discussion

3.1. Cell growth and lag phase at various substrate concentrations

Fig. 3 shows data for cell dry weight accumulation during cell growth at various butyrate concentrations. Data points were collected in triplicate and the values averaged. For cell dry weight, as butyrate concentration increases, the maximum growth increased until reaching 1.3 mg/mL density at butyrate concentrations of 50 mM or more. Acetate at 25 mM was completely utilized and cell mass did not reach similar levels as butyrate, indicating that at that concentration, it is still the limiting substrate. Data from Fig. 3 also indicated that although increasing butyrate concentration resulted in higher final cell mass, the lag phase was also increased. A Student's 1-tail unpaired t-test was performed on butyrate curves from 10 to 100 mM. Additionally, a Student's t-test was used to compare 25 mM acetate with 25 mM butyrate and was significant (data not shown). The data indicate the possibility for substrate inhibition even though the variance in the data was quite high.

Table 2
Rate and substrate conversion efficiency at various butyrate concentrations

Butyrate (mM)	Total hydrogen (mM)	Rate of production (μmol H_2/mg cell dry wt/h)	Carbon conversion efficiency (%)[a]
10	10.5	3.3	10.5
17	8.34	2.7	5.0
25	30.3	7.7	12.9
50	9.16	4.0	3.9
100	0	0	0

[a]Calculated based on amount of butyrate consumed.

Table 3
Maximum hydrogen production and efficiencies

	Butyrate	Acetate
Max total H_2, mmol	30 (25)[a]	20.7 (25)
Max H_2 rate, μmol/(mg cdw h)	7.72 (25)	4.70 (25)
Max average H_2 Rate, STP mL/(L culture h)	31.8 (25)	26.9 (25)
Max substrate efficiency	8.0 ± 3.7(10)	19.5 ± 6.0(25)
Max light efficiency	0.89 ± 0.23(25)	0.7 ± 0.23(25)

[a]Substrate concentration (mM) at max.

3.2. Rate of hydrogen production and conversion efficiency at various substrate concentrations

Rates of hydrogen production were determined by measuring hydrogen accumulation in the culture headspace during growth in various substrate concentrations. Data shown in Table 2 indicated that 25 mM butyrate yielded both the highest total amount of hydrogen and the highest specific rate of production. Hydrogen was not detected when the butyrate concentration is 100 mM even though growth did occur (Fig. 3). Using Eq. (4) we calculated substrate conversion efficiency based on the amount of butyrate consumed, not the amount added initially, by the microbe during hydrogen production. Calculated data shown in Table 3 again indicate that the highest substrate conversion efficiency occurred at 25 mM butyrate. It is likely that the carbon conversion efficiency (Table 2) is an underestimation, since an undetermined amount of the butyrate is being assimilated into new cell mass during growth instead of toward hydrogen production. Using 25 mM acetate as a control, a substrate conversion efficiency of 20.8% was determined, calculated based on the amount of acetate consumed according to Eq. (5). Light conversion efficiency was calculated using 25 mM butyrate and 25 mM acetate according to Eq. (2), the former yielded 0.89% while the latter near 0.7%, respectively.

3.3. Comparison of conversion efficiencies with the literature

Carbon conversion efficiencies reported in the literature range from 0% to 75% [8] for butyrate and 45% to 57% [16] for acetate(Table 1). Carbon conversion efficiencies determined in our study are lower than the literature values. Both light efficiencies observed and reported show that light efficiencies can range from 0.1% to 11.3% in literature [17]. The huge variations could be due to the different microbial strains used, or experimental conditions not recorded in literature. Typical maximal hydrogen reported from literature is 30–60 μL/mg/h [18]. We reported 0–7.7 μmol/mg/h (0–173 μL/mg/h) in this study, which is comparable with those documented in the literature. A more standardized methodology should be adopted in the scientific community to compare values more accurately.

4. Summary and conclusions

Indirect biophotolysis has the potential to allow hydrogen energy co-generation on Mars while at the same time degrading and recycling waste biomass to carbon dioxide. While many of the growth parameters of purple non-sulfur bacteria have been well studied, much work is currently being done to fully characterize the two-stage system described previously. In this study, hydrogen production response to butyrate was characterized. Based on these data, we show that optimal hydrogen production was found at 25 mM butyrate, falling off and ceasing by 100 mM. Yet, cell growth still occurred at higher concentrations of butyrate. Hydrogen produced from butyrate was greater than hydrogen produced by acetate in our study, with the latter likely being limited at the 25 mM level. Both the carbon conversion and light conversion efficiencies are lower than those reported in the literature, although similar production rates were observed. Furthermore, a correlation between lag phase and butyrate is suggested, possibly indicating substrate inhibition. The toxicity of butyrate could be overcome in a scale-up process either by adapting cells gradually to higher concentrations of butyrate or by continuously feeding a low dose of butyrate. The results suggest that hydrogen could be sufficiently captured for use in fuel cells from what would otherwise be wasted in a CELSS for a Martian surface stay.

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