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Investigation of the Photoheterotrophic Hydrogen Production of *Rhodobacter sphaeroides* KCTC 1434 using Volatile Fatty Acids under Argon and Nitrogen Headspace

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Abstract: In this study, the photo fermentative H_2 production of *Rhodobacter sphaeroides* KCTC 1434 was investigated using acetate, propionate and butyrate under argon and nitrogen headspace gases. The highest H_2 yield and Substrate Conversion Efficiency (SCE) were observed from butyrate (8.84 mol H_2/mol butyrate consumed, 88.42% SCE) and propionate (6.10 mol H_2/mol propionate consumed, 87.16% SCE) under Ar headspace. Utilization of acetate was associated with low H_2 evolution, high biomass yield and high final pH, which suggest that acetate uptake by the strain involves a biosynthetic pathway that competes with H_2 production. The use of N_2 in sparging resulted to a decreased H_2 productivity in propionate (0.49 mol H_2/mol propionate consumed, 7.01% SCE) and butyrate (1.22 mol H_2/mol butyrate consumed, 1.04% SCE) and was accompanied with high biomass yield and radical pH increase in all acids. High H_2 generation had shown to improve acid consumption rate. The use of the three acids in a mixed substrate resulted to a drastic pH rise and lower H_2 generation. This suggests that a more refined culture condition such as additional control of pH during fermentation must be kept to enhance the H_2 productivity. Overall, the study provided a background on the H_2 production using *R. sphaeroides* KCTC 1434 which might be a good co-culture candidate because of its high SCE on butyrate and propionate.

Keywords: Biohydrogen Production, Headspace Gas, N_2-Fixation, Photofermentation, *Rhodobacter sphaeroides*

Introduction

In the recent years, the issue of energy security and environmental pollution has gained considerable attention. Various scientific endeavors have been directed at generating petroleum-independent and low-carbon technologies as the global industry hopes to transition to a sustainable bio-based economy (Golden and Handfield, 2014; Lee, 2016). H_2 has been known as a clean and renewable energy carrier, a promising alternative to fossil fuels. It does not evolve CO_2 during combustion and has an energy yield (122 kJ/g) that is 2.75 times greater than any hydrocarbon fuels (Kapdan and Kargi, 2006). A more cost-effective, energy efficient and ecologically benign means of H_2 production is through biological method (Das and Veziroglu, 2001). With a levelized cost of energy at USD 2-3/kg from 2008 to 2042, biohydrogen can compete with the cost of conventional fossil fuel in the global market (Lee, 2016).

Among the different ways of producing H_2 from biomass, the use of Purple Non-Sulfur Bacteria (PNSB) such as *Rhodobacter sphaeroides* has been widely known due to their metabolic versatility, wide range of substrate utilization (Das and Veziroglu, 2001) and high product purity (Kars and Gunduz, 2010; Wu *et al*., 2012). The ability of these bacteria to photoheterotrophically convert organic acids into H_2 and CO_2 has made PNSB popular in wastewater remediation and alternative energy generation (Fang *et al*., 2005;

Argun *et al*., 2008; Uyar *et al*., 2009; Amrouche *et al*., 2011; Han *et al*., 2012). Organic acids such as Volatile Fatty Acids (VFAs) produced during the acidogenic phase of anaerobic digestion of organic wastes can be further converted to CO_2 and H_2 (Kapdan and Kargi, 2006). The efficiency of light energy used for the production of H_2 by photosynthetic bacteria is theoretically higher compared to cyanobacteria (Nath and Das, 2004).

Since the photoheterotrophic production of H_2 requires anaerobic condition, purging of reactor headspace is done using an inert gas. Unlike other fermentation studies where a less costly N_2 gas is employed, photo-fermentation commonly uses argon because N_2 is the natural substrate for nitrogen fixation (Tao *et al*., 2008; Uyar *et al*., 2009; Kim *et al*., 2011; 2012a; Han *et al*., 2012; Pandey *et al*., 2012). When dimolecular nitrogen (N_2) is present, fixation dominates (Koku *et al*., 2002) and H_2 production becomes minimal (Sasikala *et al*., 1990; Liu *et al*., 2009). Although N_2 has been known to inhibit the H_2 producing activity of pre-formed nitrogenase (Sweet and Burris, 1981; Liu *et al*., 2009) less has been written about the mechanism H_2 generation under a nitrogen-fixing environment. When members of the *Rhodospirillaceae* family were grown on N_2, *R. sphaeroides* and *R. capsulatus* showed the most rapid growth and highest *in vivo* nitrogenase activity among other species of the genus *Rhodopseudomonas* (Madigan *et al*., 1984). Hence, a further investigation in this line may lead to an opportunity of enhancing nitrogenase action for H_2 production without the need of rigorous changes in the overall physiology and metabolism of the bacteria (Koku *et al*., 2002).

In this study, three VFAs (acetate, propionate and butyrate) that are identified to be the main fermentation products in dark fermentation liquor (Uyar *et al*., 2009) were chosen and H_2 production under blue Light Emitting Diode (LED) and two different headspace gases, N_2 and Ar, was investigated. The blue LED was selected because it was observed that *R. sphaeroides* KCTC 1434 illuminated with blue light exhibited the highest growth rate and cell concentration (Lee *et al*., 2011). Specifically, the objectives of the study were aimed at determining the effect of two different headspace gases, N_2 and Ar, on the H_2 productivity of *R. sphaeroides* KCTC 1434 using individual and mixed acids such as acetate, propionate and butyrate.

Materials and Methods

Microorganism and Growth Medium

Rhodobacter sphaeroides KCTC 1434 was purchased from the Korean Collection for Type Culture (KCTC). The strain was initially grown on Van Niel's (VN) agar medium containing K_2HPO_4 (1 g L^{-1}), $MgSO_4$ (0.5 g L^{-1}), yeast extract (10 g L^{-1}) and 20 g L^{-1} agar. The strain was cultivated anaerobically on VN agar plates at 30±1°C under white LED lamp (4 k lux) (STK, Korea) inside an anaerobic chamber (COY Laboratory Products Inc., Grass Lake, MI) maintained using a gas mixture of 5% H_2, 5% CO_2 and 90% N_2. After ensuring the purity of the grown colonies, cells were scraped and transferred to serum bottles containing 60 mL of VN liquid medium (without agar) and incubated at 30±1°C, 120±2 rpm, in blue LED lamp for 24 h. Inoculums were then harvested by centrifugation at 4720 *x g* (Mega 17R, Hanil Science Industrial Co., S. Korea) for 10 min at 4°C. The pellets were washed with Modified Pfennig and Bieble (MPB) basal media prior to transfer in preactivation media. The MPB media used for preactivation and H_2 production experiment had the following basal composition: $MgSO_4 \cdot 7H_2O$ (0.2 g L^{-1}), NaCl (0.4 g L^{-1}), yeast extract (0.4 g L^{-1}), KH_2PO_4 (0.5 g L^{-1}), $CaCl_2 \cdot 2H_2O$ (0.05 g L^{-1}), ferric citrate (5×10^{-3} g L^{-1}), $ZnCl_2$ (0.07 mg L^{-1}), H_3BO_3 (0.06 mg L^{-1}), $MnCl_2 \cdot 4H_2O$ (0.1 mg L^{-1}), $CoCl_2 \cdot 2H_2O$ (0.2 mg L^{-1}), $CuCl_2 \cdot 2H_2O$ (0.02 mg L^{-1}), $NiCl_2 \cdot 6H_2O$ (0.02 mg L^{-1}), $(NH_4)_2MoO_4 \cdot 2H_2O$ (0.04 mg L^{-1}) and HCl (0.025%v/v).

Preactivation

Preactivation in MPB minimal media was done following growth on VN liquid. In both growth and activation stage, N_2 gas was used in purging the reactor headspace for 10 min to ascertain anaerobic condition. Preactivation of inoculums was also done at 30±1°C, 120±2 rpm, in blue LED lamp for 24 h. Preactivated cells were harvested by centrifugation as mentioned previously. The pellets were washed twice with MPB basal solution and resuspended in MPB H_2 production media to serve as the inoculum for H_2 production.

During preactivation stage, 7.5 mM malic acid and 10 mM sodium glutamate were used as carbon and nitrogen sources, respectively. For H_2 production experiment, the malic acid is replaced with other carbon sources while sodium glutamate is reduced to 2 mM as optimized elsewhere (Eroglu *et al*., 1999). As growth factors, a vitamin solution was added with the following composition: Nicotinic acid (0.2 mg L^{-1}), nicotinamide (0.2 mg L^{-1}), thiamine HCl (0.4 mg L^{-1}) and biotin (8.0 mg L^{-1}). The pH of the media for preactivation and H_2 production set-up was adjusted to 6.8 using 5 M NaOH. All reagents were purchased from Sigma-Aldrich Co (St. Louis, MO).

H_2 Production Experiment

Three sets of H_2-production batch experiments were conducted in duplicate using 160 mL serum bottles (Wheaton) containing 60 mL of inoculated medium. In the first set, H_2 production from individual acids (30 mM acetate, 20 mM propionate

and 15 mM butyrate) was determined under two different headspace gases (Ar and N_2). For the effect of headspace gases N_2 and Ar on H_2 production from mixed VFAs, a mixture of 15 mM acetate, 10 mM propionate and 7.5 mM butyrate was used as substrate and purging was carried using Ar in one set of reactors and N_2 in the other.

In all experiments, a 10 min purging of the reactor headspaces was done to ensure anaerobic condition, which was confirmed by Gas Chromatographic (GC) analysis. Sparging with either Ar or N_2 was carried only at the initial preparation of the batch cultures and the pressure build up in each subsequent sampling was released through a tube connected to an acidified brine solution. Incubation was carried at 30±1°C, 120±2 rpm, in blue LED lamp.

Analytical Methods

Gas samples were collected using a gas-tight glass syringe (Hamilton Co., Rem, Nevada) and biogas composition analysis was carried using GC (HP 6890 Series, Agilent Technologies, USA) equipped with ShinCarbon ST 100/120 micropacked (cat. #19808) column and a thermal conductivity detector. The pressure (P) in the bioreactors was measured using a pressure sensor (SIKA D-53695, Germany) and was converted into volume using the ideal gas law corrected to standard conditions (Pan *et al*., 2008; Kim *et al*., 2012b). Initial (P at time 0 and P measurement following pressure release) and final (P measurement at the start of each sampling) volumes were multiplied with the % gas composition from GC analysis and the difference gave the volume of H_2 gas generated. The pH of the culture media was measured using pH meter (Orion 290, CA, USA). Bacterial growth was monitored by measuring optical density at 660 nm using UV-Vis spectrophotometer (UV-1601PC, Shimadzu, Japan). Cell dry weight (DCW) was obtained using a standard curve where one unit of optical density corresponded to 0.27±0.06 g DCW/L medium. The concentration of the VFAs were analyzed after adding formic acid and 2-ethylbutyrate using GC (HP 6890 Series, Agilent Technologies, USA) with a flame ionization detector and a capillary column (PB-FFAP, 0.25 µm, J&W Scientific, Folsom, CA). All values were reported as standard deviation (±) between each replicate.

Kinetic Model Analysis and Other Indicators

The cumulative H_2 production in the batch experiments followed the modified Gompertz Equation (Han *et al*., 2012):

$$H = P\exp\left\{-\exp\left[\frac{R_m e}{P}(\lambda - t) + 1\right]\right\} \quad (1)$$

where, H is the cumulative H_2 production (mL), P is the H_2 production potential (mL), R_m is the maximum H_2 production rate (mL/h), e is 2.71828, λ is the lag phase time (h) and t is the incubation time (h). The values were determined by best fitting the H_2 production experimental data for Equation 1 using the solver function in Microsoft Excel 2010 version (Newtonian algorithm). The kinetic parameters obtained where then used to describe the H_2 production from each batch run.

The specific growth rate was calculated over the time interval where cell density increased linearly with time, using the following equation:

$$\mu_{max} = \frac{\ln\frac{x_2}{x_1}}{t_2 - t_1} \quad (2)$$

where, μ_{max} is the specific growth rate and x_1 and x_2 are cell densities at two different time points in the exponential phase, t_1 and t_2. The biomass yield is the total amount of cell produced during the entire fermentation over the total substrate consumed. The H_2 yield is computed as the cumulative H_2 production per substrate consumed (in moles). The substrate consumption is the final amount of substrate consumed when the highest H_2 production was achieved over the initial amount of substrate. The Maximum Substrate Consumption Rate (MSCR) is the amount of consumed substrate over the total time of fermentation when H_2 production was at maximum. The Substrate Conversion Efficiency (SCE) is the actual moles of H_2 produced over the amount of H_2 calculated from the amount of substrate consumed (in moles). The specific H_2 production rate (SHPR) is the cumulative H_2 production per dry cell weight over the total time of fermentation.

Results

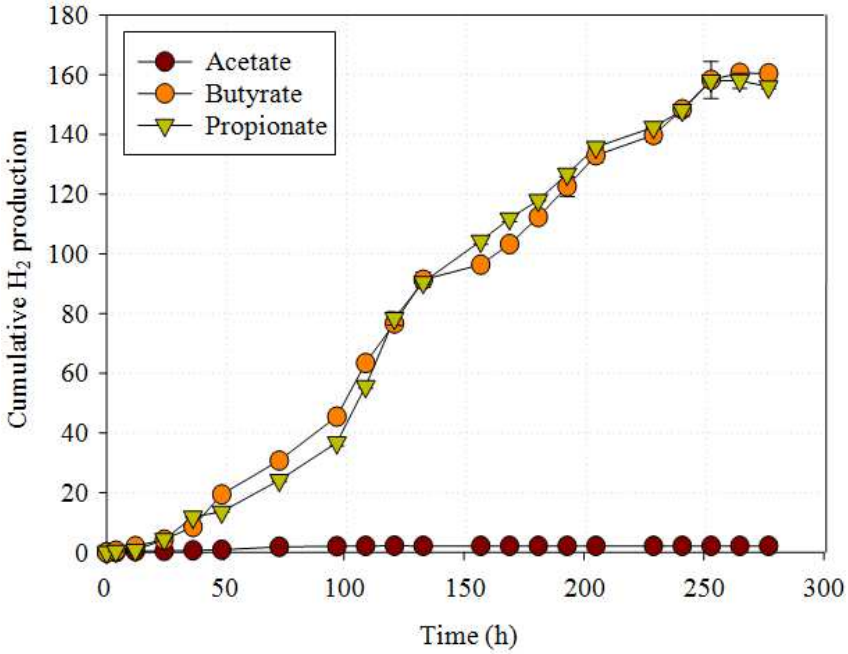
H_2 Production and Kinetic Profile from Individual Acids using Ar and N_2 Headspace Gases

H_2 productivity of *R. sphaeroides* KCTC 1434 was investigated using 30 mM acetate, 20 mM propionate and 15 mM butyrate. Similar to the method of Uyar *et al*. (2009), the VFA concentrations chosen were proportional to the number of carbon in each organic acid.

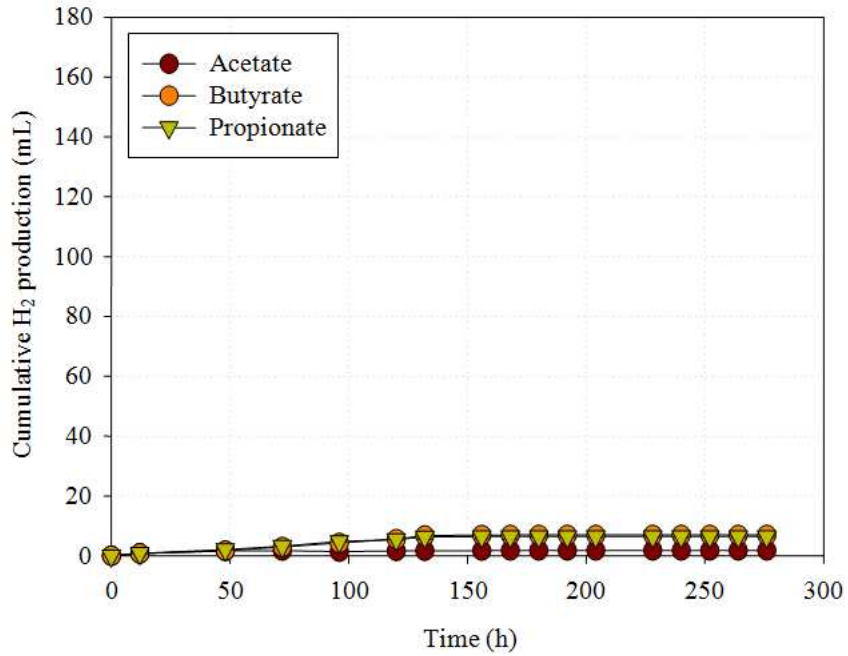
Propionate and butyrate exhibited a continuous H_2 buildup under Ar headspace while a minimal amount of H_2 was produced from acetate as illustrated in Fig 1a. Within a 276 h experimental duration, the amount of H_2 accumulated from acetate, propionate and butyrate in argon-purged reactorswere 2.20, 157.95 and 160.58 mL (Fig. 1a and Table 1), respectively. In the reactors with N_2-purged headspace, the H_2 generation time lasted for

165 h with a cumulative production of 1.66 mL in acetate, 6.41 mL in propionate and 6.93 mL in butyrate (Fig. 1b, Table 1). While acetate evolved a minimal amount of H_2 regardless of the headspace gas used, the Ar-sparged propionate and butyrate reactors gave H_2 volumes that were 24.64 and 23.17 times higher than their N_2- sparged counterparts.

The N_2-purged reactors exhibited a growth associated H_2 evolution. The volume of H_2 produced rose at the exponential phase of cell growth and leveled off at the onset of stationary phase. This trend was not seen in Ar-purged reactors wherein a sustained increase of H_2 volume was obtained along the stationary phase of cell growth.



(a)



(b)

Fig. 1. Cumulative H_2 production from acetate, propionate and butyrate under (a) Ar and (b) N_2 headspace

Table 1. Kinetic parameters from modified Gompertz analysis of H_2 production from acetate, propionate, butyrate under Ar and N_2 headspace

Gompertz Parameters	Acetate		Propionate		Butyrate	
	Ar	N_2	Ar	N_2	Ar	N_2
P (mL H_2)	2.200	1.660	157.950	6.410	160.580	6.930
R_m(mL/h)	0.020	0.050	1.200	0.070	0.900	0.060
λ(h)	7.000	0.000	63.000	0.000	40.000	0.000
R^2	0.989	0.974	0.997	0.995	0.996	0.995

Table 2. H_2 productivity and substrate consumption of *R. sphaeroides* KCTC 1434 from individual VFAs in Arand N_2 headspace

Carbon sources	Head-space gas	Amount of H_2 Produced (mL)	SC (%)	MSCR (mM/h)	H_2 Yield (mol H_2/mol substrate consumed)	%SCE	SHPR (mL H_2/g DCW·h)
Acetate	Ar	2.20±0.087	75.40±3.5	0.34±0.05	0.07±0.01	1.79±0.05	0.32±0.07
Acetate	N_2	1.66±0.15	22.53±1.2	0.16±0.04	0.06±0.02	1.50±0.03	0.15±0.01
Propionate	Ar	157.95±0.41	96.11±0.9	0.10±0.01	6.10±0.15	87.16±1.40	12.54±0.44
Propionate	N_2	6.41±0.24	41.45±0.7	0.06±0.02	0.49±0.06	7.01±0.95	0.94±0.03
Butyrate	Ar	160.58±1.24	91.71±1.3	0.05±0.05	8.84±0.08	88.42±1.52	11.68±0.88
Butyrate	N_2	6.93±0.13	29.29±1.1	0.03±0.03	1.22±0.07	12.16±0.86	1.04±0.09

Table 3. Growth kinetic parameters of *R. sphaeroides* KCTC 1434 from individual VFAs in Ar-and N_2-purged reactors

Carbon sources	Headspace gas	Biomass yield (g DCW/g substrate)	Final DCW (g DCW/L medium)	μ_{max} (h^{-1})	Final pH
Acetate	Ar	0.39±0.02	1.11±0.05	0.014±0.003	10.3±0.1
Acetate	N_2	0.32±0.03	1.00±0.04	0.028±0.005	10.3±0.2
Propionate	Ar	0.25±0.05	0.83±0.04	0.009±0.002	7.5±0.1
Propionate	N_2	0.33±0.07	0.87±0.03	0.003±0.004	8.9±0.0
Butyrate	Ar	0.33±0.03	0.87±0.06	0.005±0.002	7.2±0.1
Butyrate	N_2	0.47±0.06	0.95±0.01	0.003±0.001	9.1±0.2

Modified Gompertz fitting was done using the H_2 production data to determine parameters that can aid in interpreting the results (Table 1). The lowest H_2 production rates (R_m) were derived from acetate (0.02 mL h^{-1} in Ar- purged and 0.05 mL h^{-1} in N_2-purged reactors), while the highest rate was obtained from propionate (1.20 mL h^{-1}) followed by butyrate (0.90 mL h^{-1}) in Ar-purged reactors. Propionate (0.07 mL h^{-1}) and butyrate (0.06 mL h^{-1}) had lower H_2 production rates under N_2 headspace. The Gompertz-derived λ values showed no H_2 production lag times in N_2-purged reactors while 7, 63 and 40 h were determined as H_2 production lag times for acetate, propionate and butyrate containing Ar-purged reactors, respectively. The Gompertz-fitted data had correlation coefficient values ranging from 0.974 to 0.997.

The consumption rate of each substrate (SCR) was not substantially affected by the difference in headspace gas. As reflected from the VFA analyses (Table 2), there was only a slight variation in the acetate consumption rate under Ar (SCR = 0.17) and N_2 (SCR = 0.15).The same was true with propionate (SCR under Ar = 0.07, SCR under N_2 = 0.06) and butyrate (SCR under Ar = 0.05, SCR under N_2 = 0.03). Despite the low H_2 evolution, the highest SCR and cell growth were observed from acetate with specific growth rates (μ_{max}) of 0.028 and 0.014 for N_2- and Ar-purged reactors, respectively (Table 3). Furthermore, the final pH was also greater in N_2- than in Ar-purged reactors, with the exemption of acetate (pH = 10.3, under N_2 and Ar headspace). From an initial value of 6.8, the pH rose to 8.9 and 8.1 in propionate and butyrate containing N_2-purged reactors, respectively (Table 3). These final pH values were higher compared to those of Ar-purged reactors whose values were pH 7.5 (propionate) and pH 7.2 (butyrate) (Table 3).

H_2 yield, Substrate Conversion Efficiency (SCE) and Specific H_2 Production Rate (SHPR) are useful parameters in characterizing microbial H_2 production. H_2 yield is the amount of H_2 produced per amount of substrate consumed. As shown in Table 2, almost similar H_2 yields (0.07 mol H_2/mol substrate in Ar- and 0.06 mol H_2/mol substrate in N_2) were obtained from acetate under the two headspace gases, while yields from propionate and butyrate were higher in Ar- (6.10 and 8.84 mol H_2/mol substrate, respectively) than in N_2- (0.49 and 1.22 mol H_2/mol substrate, respectively) sparged reactors.

The theoretical production of H_2 from acetate, propionate and butyrate is described by the following equation:

$$Acetate: C_2H_4O_2 + 2H_2O \rightarrow 4H_2 + 2CO_2 \quad (3)$$

$$Propionate: C_3H_6O_2 + 4H_2O \rightarrow 7H_2 + 3CO_2 \quad (4)$$

$$Butyrate: C_4H_8O_2 + 6H_2O \rightarrow 10H_2 + 4CO_2 \quad (5)$$

SCE is equal to the ratio of moles of H_2 actually obtained divided by the moles of H_2 expected through stoichiometric conversion of the substrate consumed according to the chemical reactions (3), (4) and (5). This parameter reflects how much of the substrate has been utilized for H_2 production other than growth or alternative biosynthesis (Yiilmaz *et al*., 2010; Kim *et al*., 2011). From the experiment, there was no remarkable difference between the SCE of acetate in Ar- (1.79%) and N_2- purged reactors (1.50%), while argon-purged propionate and butyrate reactors have SCEs that were 12.4 and 7.3 times larger than their N_2-purged counterparts. Under Ar headspace, the SCE of butyrate and propionate were 88.42 and 87.16, respectively. SHPR values from acetate, propionate and butyrate were likewise 3.6, 12.4 and 11.2 times greater in Ar- than in N_2-sparged reactors.

The H_2 Production of R. Sphaeroides KCTC 1434 from Mixed VFAs using Ar and N_2 Headspace Gas

Using the conditions previously stated in the methodology section, H_2 production in mixed VFAs (15 mM acetate, 10 mM propionate and 7.5 mM butyrate) was investigated under Ar and N_2 headspace gases. As depicted in Fig. 2a, a low H_2 production was obtained with N_2 sparging while no remarkable difference was observed in the cell density under Ar and N_2 headspaces. The volumes of H_2 evolved were 31.44 mL in N_2 and 47.12 mL in Ar-sparged reactors. H_2 was produced until 168 h in Ar headspace, a duration that was twice longer than in N_2, where a leveling off of H_2 evolution was observed after 72 h.

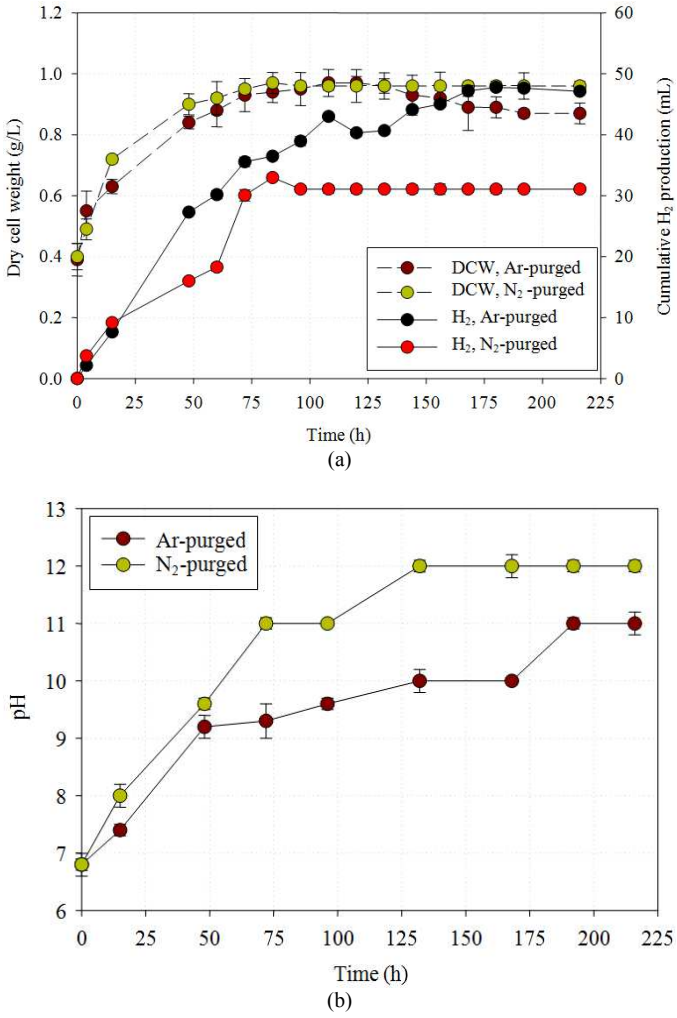


Fig. 2. (a) Cumulative H_2 production and cell density and (b) pH change during H_2 production of *R. sphaeroides* KCTC 1434 from mixed VFAs under Ar and N_2 headspace

Table 4. Kinetic parameters and substrate conversion during H_2 production of *R. sphaeroides* KCTC 1434 in Ar-and N_2-purged reactors using mixed VFAs

Headspace Gas	Kinetic parameters				Substrate consumption rates (mM/h)			
	P (mL H_2 produced)	R_m (slope)	λ (h)	R^2	Acetate	Propionate	Butyrate	SCE (%)
Ar	47.20	0.58	7	0.991	0.04	0.02	0.01	52.14
N_2	32.97	0.61	0	0.969	0.12	0.06	0.03	19.45

The pH quickly increased to 9.6 in Ar-purged reactors during the first 48 h, rising to a final value of 11 in 216 h (Fig. 2b). The rise of pH was more drastic in N_2 reactors where a linear increase to a value of 11 was obtained in 72 h and a final pH value of 12 in 130 h.

Using modified Gompertz analysis, λ was determined to be 7 and 0 h in Ar- and N_2-purged reactors respectively (Table 4). This observation conferred with the previous experiments in which the lag time values were lower in N_2 than in Ar headspace. Gompertz-predicted R_m values were 0.61 mL H_2/h in Ar-purged reactors and 0.58 mL H_2/h in N_2-purged reactors.

Under Ar headspace, consumption rates were 0.04, 0.02 and 0.01 mM h^{-1} in acetate, propionate and butyrate, respectively (Table 4). Relatively higher consumption rates were observed under N_2 headspace at 0.12 mM h^{-1} for acetate, 0.06 mM h^{-1} for propionate and 0.03 mM h^{-1} for butyrate. Hence, among the three acids, acetate was consumed the fastest regardless of the headspace gas. The SCEs were observed to be 30.70% under Ar headspace and 19.45% under N_2 headspace.

Discussion

The H_2 Production of R. sphaeroides KCTC 1434 from Individual Acids

Acetic, propionic and butyric acid generally accounts for 70-80% of the VFAs in acidogenic reactors of anaerobic digestion (Fang and Yu, 2002; Shi and Yu, 2006). Hence, these substrates were chosen for this investigation. As shown in the results, *R. sphaeroides* KCTC 1434 grew well in the three acids however high H_2 production was observed only in propionate (87.16% SCE) and butyrate (88.42% SCE) under Ar headspace. Although a wide range of studies on carbon utilization for H_2 production in PNSB is available, discrepancies exist even for different strains of *R. sphaeroides*. For instance, most studies reported highest H_2 production in acetate, followed by propionate, then butyrate (Uyar *et al*., 2009; Lo *et al*., 2011; Han *et al*., 2012). Using individual acid substrate, Uyar *et al*. (2009) obtained SCEs of 33, 31 and 14% in acetate, propionate and butyrate, respectively. Han *et al*. (2012) also had similar findings in *R. sphaeroides* RV when they investigated the effect of different carbon sources in H_2 productivity. In their report, the use of different concentrations of acids gave corresponding SCE values between 97.4-99.9% in acetate, 70.7-86.4% in propionate and 56.2-81.6% in butyrate. Other studies however reported dissimilar trends (Tao *et al*., 2008; Lo *et al*., 2011). In the investigation of Tao *et al*. (2008), isolated strains identified as *R. sphaeroides* ZX-2, ZX-3, ZX-4 and ZX-5 exhibited variable preference for acetate and butyrate. Strain ZX-2 was able to utilize butyrate at 6.45% SCE while acetate was not used for both growth and H_2 production; ZX-3 gave 63.20 and 4.61% SCE in acetate and butyrate, respectively; ZX-4 was not able to utilize both acids; while ZX-5 gave the highest SCE at 69.00 and 71.50% for acetate and butyrate, respectively. The variations in reported data from literatures confirm the complexity and flexibility of PNSB metabolism and therefore require investigation of each type of strain (Koku *et al*., 2002; Kim *et al*., 2012a).

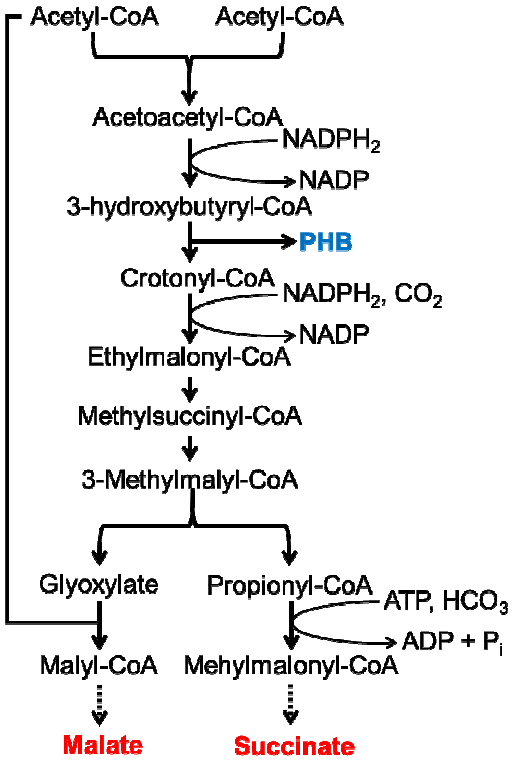


Fig. 3. Ethylmalonyl-CoA pathway operating in *R. sphaeroides* sp. (lifted from Kars and Gunduz, 2010)

In our study, the use of acetate was associated with low H_2 production (Fig. 1), high cell density increase and high final pH (Table 3). There was no substantial difference between the SCE of acetate in Ar and N_2-purged reactors (1.79 and 1.50, respectively). This may be indicative of a metabolic route that competes with nitrogen-fixation and is inherent only to acetate assimilation. Due to the missing isocitrate lyase of *R. sphaeroides*, the uptake of acetate may occur through the ethylmalonyl-CoA pathway (Fig. 3) with the following net stoichiometry (Erb *et al*., 2007; Balat *et al*., 2009):

$$3\ Acetyl-CoA + 2CO_2 + 2NADPH + 2NAD + < pmf > \rightarrow 2\ malate + 2NADH + 2NADP + 3CoA \quad (6)$$

In the aspect of biohydrogen production, this pathway holds two disadvantages. First, it requires Adenosine Triphosphate (ATP) and reducing power in the form of $NADPH_2$ which diminishes energy and electron output; and second, it shares common elements with Polyhydroxybutyrate (PHB) biosynthetic route (Kars and Gunduz, 2010). The production of polyhydroxyalkanoates (*e.g*., PHB) as an intracellular storage material is another route that competes with the H_2 production in disposing excess reducing equivalents in cells (Hustede *et al*., 1993). PHB production is often seen as one of the critical factors associated with low H_2 productivity during utilization of organic substrate in photo fermentation (Khatipov *et al*., 1998; Kobayashi *et al*., 2011; Koku *et al*., 2002; Han *et al*., 2012; Pandey *et al*., 2012). In the study of Pandey *et al*. (2012) using *R. sphaeroides* NMBL-01, lactate and PHB were end metabolites formed during photo fermentation of acetate and butyrate. Han *et al.* (2012) also found that increasing concentrations of acetate and butyrate gave lower H_2 production and higher PHB accumulation, while no PHB was detected during H_2 production from different concentrations of propionate. While they reported SCEs of 98.0% at 2.4 g $^{-1}$L acetate, 78.3% at 2.0 g L^{-1} propionate and 81.6% at 1.8 g L^{-1} butyrate, our study (using 2.5 g L^{-1} sodium acetate, 1.92 g L^{-1} sodium propionate, 1.65 g L^{-1} sodium butyrate) gave higher SCEs except for acetate. It is not however clear whether *R. sphaeroides* KCTC 1434 have the same assimilation pathway for acetate and butyrate, or whether it has assimilation pathway distinct from the strains reported in these literatures. Nevertheless, taking into account the ethylmalonyl pathway of acetate assimilation in *R. sphaeroides* and the fact that acetate is the most advantageous substrate for PHB production, our findings stand theoretically sound. Furthermore, H_2 production using acetate and PHB-producing systems in photoheterotrophic H_2 generation have been reported to exhibit elevated pH (Hustede *et al*., 1993; Khatipov *et al*., 1998; Uyar *et al*., 2009; Kim *et al*., 2011; 2012a). Hustede *et al*. (1993) showed that *R. sphaeroides* ATCC 17023 grown in un buffered mineral medium containing 10 mM acetate had complete consumption of the acid and had a pH increase of 10.0 within 1 day, while Khatipov *et al.* (1998) reported that *R. sphaeroides* RV grown in glutamate as N source had significantly higher pH and PHB content but very low H_2 production in acetate than in lactate and pyruvate. As the amount of PHB increases with increased pH, the use of acetate as substrate therefore facilitates PHB accumulation (Khatipov *et al*., 1998; Kim *et al*., 2012a). Although the determination of PHB was not within the scope of our study, the low pH and high biomass yield among acetate-fed reactors may signify the occurrence of an H_2-competing pathway such as the ethylmalonyl-CoA during acetate assimilation (Fig. 3).

It can be observed from Table 2 that high H_2 generation was associated with high substrate consumption (%SC) but low MSCR. MSCR is the amount of substrate consumed over the total fermentation time when H_2 production was at maximum. Propionate and butyrate had 96.11 and 91.71% SC under Ar headspace but with an MSCR of 0.10 and 0.05 mM h^{-1}, respectively. This indicates that majority of the propionate and butyrate consumed were converted to H_2 and only a minute portion were used for growth or alternative biosynthesis. SHPR is the biomass weight-normalized H_2 production rate. Acetate under Ar headspace had a 75.40% SC, 0.34 mM/h MSCR and an SHPR of 0.32 mL H_2/g DCW·h, which denote that *R. sphaeroides* preferred acetate for biosynthesis instead of H_2 production.

It is worth mentioning that H_2 generation improves organic acid utilization (Table 2). Ar sparged reactors exhibited 96.11% propionate consumption and 91.71% butyrate consumption with SHPRs of 12.54 and 11.68 mL H_2/g DCW·h, respectively. Higher acetate consumption was observed under Ar (75.40%) than N_2 headspace (22.53%) with SHPR values of 0.32 and 0.15 mL H_2/g DCW·h, respectively. Since the use of sequential dark fermentation and photo fermentation in a combined two-stage process has been seen as an attractive technology for the enhancement of H_2 production from waste material substrates (Su *et al.*, 2009; Laurinavichene *et al*., 2010; Chookaew *et al*., 2015), the strain may be further evaluated for its potential use in co-culture dual-stage fermentation system.

The Effect of Headspace Gas on H_2 Production

The presence of N_2 gas in reactor headspaces provided a nitrogen-fixing environment to the *R. sphaeroides* strain. An obvious inhibition was exhibited by all N_2-sparged reactors. SHPRs of 0.32 (acetate), 12.54 (propionate) and 11.68 (butyrate) mL H_2/g DCW·h under Ar sparging were reduced to 0.15 (acetate), 0.94 (propionate) and 1.04 mL (butyrate) H_2/g DCW·h in the presence of N_2. The lower H_2 evolution in N_2-sparged

reactors was accompanied with high biomass yield and high final pH (Table 3).

While lower production rates (R_m) were derived (Table 1), all N_2- sparged reactors had 0 h production lag times (λ). This indicates a growth-associated H_2 production in the presence of N_2. Under N_2 headspace, H_2 evolution from propionate and butyrate occurred along the exponential phase of cell growth and was diminished with the onset of stationary phase. In the study of Sasikala *et al*. (1990), resting cells of *R. sphaeroides* O.U. 001 did not produce any H_2 under a gas phase of pure N_2. Our study using growing cells of *R. sphaeroides* KCTC 1434 therefore supports the fact that H_2 is evolved as a side reaction during N_2 fixation since during active cell growth, nitrogenase functions to provide precursors needed for protein biosynthesis. On the contrary, a continued accumulation of H_2 was observed along the stationary phase of cell growth in Ar-purged reactors. In many studies H_2 evolution in *R. sphaeroides* starts in the mid- to late exponential growth phase (Koku *et al*., 2003: Eroglu *et al.*, 1999; Tao *et al*., 2008; Golomysova *et al*., 2010). Using cells in stationary phase, Melnicki *et al.* (2008) reported H_2 production in *R. rubrum* to be not strictly coupled with growth (Melnicki *et al.*, 2008). Under active cell growth, organic substrates enter the tricarboxylic acid pathway cycle to provide necessary biosynthetic precursors and Nicotinamide Adenine Dinucleotide phosphate (NADH) for biomass synthesis. During this time, nitrogenase functions as a "safety valve" (Gest, 1972) that disposes excess electrons in cells, provided N_2 fixation is absent. In the stationary phase where biosynthesis is minimal, most of the organic carbon is oxidized into CO_2 and most of the extracted electrons are directed toward H_2 production (Ormerod and Gest, 1962; Koku *et al*., 2002; Golomysova *et al*., 2010).

Despite the lower acid consumption rates, all N_2-sparged reactors exhibited high final pH values (Table 3). The radical increase in pH could be explained by the nitrogenase-mediated H_2 production via the reaction (Liang and Burris, 1988; Kars and Gunduz, 2010):

$$N_2 + 8e^- + 8H^+ + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi \quad (3)$$

From this metabolic reaction, 2 moles of ammonia is produced per mole of N_2 present. The dissolution of ammonia produces ammonium ion which could lead to the increase of pH in the medium. Additionally, NH_3 has a substantial solubility in water (Sander, 2015), hence the rapid climb of pH once N_2 fixation initiates. A similar account could be found in the study of Kim *et al.* (2012a) wherein an increase in NH_4^+ concentration during photofermentative H_2 production by *R. sphaeroides* KD131 resulted to an increased pH and PHB production. Nitrogenase operates at neutral pH with the highest activity at pH range of 6.5-7.5 (Peng *et al*., 1987). In our study, butyrate and propionate under Ar headspace had final pH values of 7.2 and 7.5. The presence of ammonia in nitrogen fixing environment renders a reversible inhibition on nitrogenase via the switch off effect. H_2 production in *R. sphaeroides* M-19 was reported to be completely inhibited at ammonia concentrations higher than 2 mM (Yokoi *et al*., 1998).

The H_2 Production of R. sphaeroides KCTC 1434 from Mixed VFAs using Ar and N_2 Headspace Gas

In their investigation of H_2 production using *R. sphaeroides* OU 001 in mixed and individual acids, Uyar *et al*. (2009) also reported highest biomass accumulation in acetate. Using VFA mixtures, they made an acetate-rich bioreactor (40 mM acetate, 10 mM butyrate and 5 mM propionate) and a butyrate-rich bioreactor (30 mM butyrate, 10 mm acetate and 5 mM acetate). They found out that among the three VFAs, acetate was consumed the fastest in the two bioreactors. They concluded that their strain preferred smaller substrates (2C in acetate versus 4C in butyrate). In this study, acetate was also consumed at a higher rate than propionate and butyrate. This consumption was however not attributed to H_2 production since the use of acetate in individual acid experiments gave low H_2 yields. In contrast to the results of Uyar *et al*. (2009) where SCE was improved with the use of mixed substrate, the results in this study showed lower SCE in mixed substrates. This decrease may have been due to acetate and the drastic pH evolution. Uyar *et al*. (2009) pointed out that they had to add high amount of buffer in acetate-containing medium since this substrate had the tendency to increase the pH during the run. Several studies reported the negative effect of alkaline pH on nitrogenase activity and phototrophic H_2 production. Peng *et al*. (1987) reported that the nitrogenase activity of *R. sulfidophilus* was highest at the pH range of 6.5-7.0 and decreased to zero at pH level of 8.0. Alkaline pH contributes more to the activity of hydrogenase while being detrimental to nitrogenase activity (Kim *et al*., 2012a). No pH adjustment was carried in this study and the buffer concentration used in MPB basal media (in the form of KH_2PO_4 and K_2HPO_4) was kept unchanged throughout the experiments.

Comparison to other Literatures based on SCE

The SCE is an indication of the specific H_2 conversion yield of photofermentative bacteria per mole of substrate consumed. This is computed based on the actual H_2 yield over the theoretical amount of H_2 produced per mole of substrate. Comparing the results of the study with the existing literatures (Table 5), *R. sphaeroides* KCTC 1434 has the highest H_2 conversion from propionate and butyrate based on SCE.

Table 5. Substrate Conversion Efficiencies (SCE) in this study as compared with other literatures*

	SCE (%)			
R. sphaeroides sp.	Acetate	Propionate	Butyrate	Reference
R. sphaeroides ZX-5	69.0	61.9	71.5	Tao *et al*. (2008)
R. sphaeroides OU 001	33.0	31	14.0	Uyar *et al*. (2009)
R. sphaeroides 131	17.5	---	24.7	Kim *et al*. (2011)
R. sphaeroides KD 131	10.9	---	12.3	Kim *et al*. (2012a)
R. sphaeroides NMBL-01	23.2	---	14.1	Pandey *et al*. (2012)
R. sphaeroides RV	98.0	78.3	81.6	Han *et al*. (2012)
R. sphaeroides CNT 2A	52.5	---	29.0	Subudhi *et al*. (2016)
R. sphaeroides KCTC 1434 (Ar-headspace)	1.8	87.2	88.4	This study
R. sphaeroides KCTC 1434 (N_2-headspace)	1.8	7.1	12.6	This study

*All studies from literature were kept under Ar-headspace

While the study of Han *et al.* (2012) reported the highest SCE on acetate and Tao *et al*. (2008) the highest on butyrate, only *R. sphaeroides* KCTC 1434 showed very low SCE for acetate but high SCE for propionate and butyrate. It can therefore be deduced that the strain used in the study prefer acetate for biomass formation while propionate and butyrate are directly converted to H_2. Once measures to control the negative effect of acetate in the culture media are taken, the strain can be a good co-culture candidate for an enhanced H_2 production using mixed substrate such as VFAs. Based on SCE, it has been observed that most of the H_2-producing microorganism prefer acetate in mixed acid fermentation (Uyar *et al*., 2009; Lo *et al*., 2011; Han *et al*., 2012).

Conclusion

H_2 production of *R. sphaeroides* KCTC 1434 was investigated under Ar and N_2 headspace. Our results indicated that unlike most PNSB, our strain utilizes propionate and butyrate better than acetate for H_2 production under Ar headspace. The use of acetate for H_2 generation is accompanied with high biomass yield and high pH increase in both headspace gases. N_2 inhibited H_2 productivity and the minimal amount of H_2 evolved was associated with cell growth. N_2-fixation during H_2 production leads to an increased biomass yield and radical pH rise. H_2 generation increased acid consumption rate. The utilization of mixed acid substrate in photofermentative H_2 production by *R. sphaeroides* KCTC 1434 necessitates refinement of culture parameters such as pH control, illumination and other operating conditions.

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