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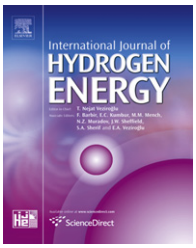

Combination of dark- and photo-fermentation to enhance hydrogen production and energy conversion efficiency

Huibo Su, Jun Cheng, Junhu Zhou, Wenlu Song, Kefa Cen*

State Key Laboratory of Clean Energy Utilization, Zhejiang University, Hangzhou 310027, China

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ABSTRACT

In this study, we investigated a two-phase process of combining the dark- and photo-fermentation methods to reutilize the residual solution derived from dark fermentation and increase the hydrogen yield (HY) from glucose. In dark fermentation, an orthogonal experimental design was used to optimize the culture medium for *Clostridium butyricum* (*C. butyricum*). The optimal culture medium composition was determined as glucose 20 g/l, NaCl 3 g/l, $MgCl_2$ 0.1 g/l, $FeCl_2$ 0.1 g/l, K_2HPO_4 2.5 g/l, L-cysteine 0.5 g/l, vitamin solution 10 ml/l, and trace element solution 10 ml/l. In this method, the maximum HY increased from 1.59 to 1.72 mol H_2/mol glucose and hydrogen production rate (HPR) from 86.8 to 100 ml H_2/l/h. The metabolite byproducts from dark fermentation, mostly containing acetate and butyrate, were inoculated with *Rhodopseudomonas palustris* (*R. palustris*) and reutilized to produce hydrogen in photo-fermentation. In photo-fermentation, the maximum HY was 4.16 mol H_2/mol glucose, and the maximum removal ratios of acetate and butyrate were 92.3% and 99.8%, respectively. Combining dark fermentation and photo-fermentation caused a dramatic increase of HY from 1.59 to 5.48 mol H_2/mol glucose. The conversion efficiency of heat value in dark fermentation surged from 13.3% to 46.0% in the two-phase system.

1. Introduction

Hydrogen is considered the ideal substitute for fossil fuels because of its high energy density and clean oxidation products [1–3]. Among the various hydrogen-producing methods, biological fermentation to convert organic wastes into hydrogen is a promising technology, because not only does it reduce waste pollution but also recovers renewable energy [4,5]. Starch and cellulose are used as feedstock to produce hydrogen by the following biological processes: complex organic polymers are first hydrolyzed into glucose and then fermented to alcohols and low-molecular-weight organic acids, which are further oxidized to acetic acid and hydrogen by hydrogen-producing acetogenic bacteria through an acetogenic process [6]. Therefore, glucose is an appropriate substrate for studying hydrogen-producing characteristics.

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2 \quad (1a)$$

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2H_2 + 2CO_2 \quad (1b)$$

$$C_6H_{12}O_6 + 6H_2O \rightarrow 12H_2 + 6CO_2 \quad (1c)$$

The Embden–Meyerhof–Parnas (i.e. glycolysis) or the Entner–Doudoroff pathways can metabolize glucose to pyruvate. Through these two pathways, 1 mol glucose can produce 2 mol pyruvate and 2 mol NADH. NADH possesses a strong reducing capacity and can supply electrons to reduce the H^+ to H_2 in the glucose-metabolizing pathways [7]. Many

* *Corresponding author.*
E-mail address: juncheng@zju.edu.cn (J. Cheng).
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researchers have found that a low NADH concentration results in a low hydrogen yield (HY) [8–10]. The theoretical conversion efficiency of heat value in hydrogen production by dark fermentation is low at 33.5%, which is the heat value ratio of 4 mol H_2 product to 1 mol glucose feedstock (Eq. (1a)) [6]. This low value greatly restricts the industrial application and commercialization of biological hydrogen production methods. Raising the HY and energy conversion efficiency in the hydrogen-producing process is a crucial but difficult challenge. In our previous studies, acidogenic bacteria production of hydrogen by dark fermentation of cassava starch yielded volatile fatty acids (VFAs) and ethanol byproducts, which the photosynthetic bacteria could reutilize to produce more hydrogen in the presence of light [11,12]. This two-phase process combining dark- and photo-fermentation could produce a theoretical maximum HY of 12 mol H_2/mol glucose (Eq. (1c)) and improve the theoretical conversion efficiency of heat value to 100.6% (without considering the input light energy). In this study, the combination of dark- and photo-fermentation was optimized to enhance hydrogen production from glucose and the energy conversion efficiency. In dark fermentation, an orthogonal experimental design was used to optimize the culture medium, HYs, and hydrogen production rates (HPRs). The soluble metabolite products (SMPs) from dark fermentation were diluted to an optimal concentration and reutilized to produce hydrogen by photo-fermentation.

2. Materials and methods

2.1. Bacterial strains and medium

Clostridium butyricum obtained from the Zhejiang Institute of Microbiology in China was acclimated for 72 h and inoculated with glucose to produce hydrogen by dark fermentation. The hydrogen production medium [11] contained (g/l): glucose, 10–40; peptone, 4.0; L-cysteine, 0.5; NaCl, 3.0–6.0; $MgCl_2$, 0.05–0.2; $FeCl_2$, 0.05–0.2; K_2HPO_4, 1.0–2.5; vitamin liquid, 10 ml; and trace element liquid, 10 ml. The vitamin liquid contained (g/l): glutamic acid, 0.01; ascorbic acid, 0.025; riboflavin, 0.025; citric acid monohydrate, 0.02; folic acid, 0.01; p-aminobenzoic acid, 0.01; and creatine, 0.025. The liquid trace element contained (g/l): $MnCl_2$, 0.01; $ZnCl_2$, 0.05; H_3BO_3, 0.01; $CaCl_2$, 0.01; Na_2MoO_4, 0.01; $CoCl_2{\cdot}6H_2O$, 0.2; ($AlK(SO_4)_2$), 0.01; and $NiCl_2{\cdot}6H_2O$, 0.01.

Rhodopseudomonas palustris obtained also from Zhejiang Institute of Microbiology was domesticated for 72 h and inoculated with the SMP from dark fermentation to further produce hydrogen by photo-fermentation. The basic medium for photo-fermentation (BMPF) [11] contained (g/l): KH_2PO_4, 0.5; K_2HPO_4, 0.6; NaCl, 0.2; $MgSO_4$, 0.2; $CaCl_2{\cdot}2H_2O$, 0.05; $NaHCO_3$, 2.0; sodium glutamate, 1.87; vitamin liquid, 1.0 ml; and trace element liquid, 1.0 ml. The BMPF and additional carbon resources were used as the medium for photosynthetic hydrogen production. The vitamin liquid contained (g/l): biotin, 0.1; nicotinic acid, 0.35; thiamine hydrochloride, 0.3; p-aminobenzoic acid, 0.2; pyridoxamine hydrochloride, 0.1; calcium pantothenate, 0.1; and vitamin B_{12}, 0.05. The trace element liquid contained (g/l): EDTA–2Na, 2.0; $FeSO_4{\cdot}7H_2O$, 2.0; H_3BO_3, 0.1; $CoCl_2{\cdot}6H_2O$, 0.1; $ZnCl_2$, 0.1; $Cu(NO_3)_2{\cdot}5H_2O$, 0.05; $MnCl_2{\cdot}4H_2O$, 0.1; Na_2MoO_4, 0.75; $NiCl_2{\cdot}6H_2O$, 0.02; and Na_2SeO_3, 0.001.

2.2. Experimental design for medium optimization in dark fermentation

The dark fermentation was performed in 300-ml transfusion bottles for batch tests. A quantity of 25 ml domesticated *C. butyricum* was inoculated with 225 ml culture medium, which always contained 4 g/l of peptone, 0.5 g/l of L-cysteine, 10 ml of vitamin liquid, and 10 ml of trace element liquid. The five compositions in the media which needed to be optimized were glucose (10–40 g/l), NaCl (3.0–6.0 g/l), $MgCl_2$ (0.05–0.2 g/l), $FeCl_2$ (0.05–0.2 g/l), and K_2HPO_4 (1.0–2.5 g/l). Four concentrations (levels) were prepared of each medium composition (factor), as shown in Table 1. The 16 orthogonal experimental conditions were designed to analyze the effects on hydrogen production of the five factors at four levels, as shown in Table 2. The experimental results noted were the averages (± standard deviation) of the values obtained in independent experiments conducted in triplicate.

The fermentation reactors were fitted with rubber stoppers and were purged with N_2 gas for 20 min to ensure an anaerobic environment. The initial pH of the fermentation medium was adjusted to 7.0 ± 0.02 and the temperature of fermentation was controlled at 35 °C by a constant temperature water bath. Biogas rich in hydrogen was discharged from the fermenter headspace and collected in a 1-l graduated container. Optimal medium compositions were determined according to HY, which is defined in Eq. (2):

$$H_2 \text{ yield (HY)} = \frac{\text{Amount of } H_2 \text{ produced (mol)}}{\text{Amount of substrate (glucose or VFAs) (mol)}} \quad (2)$$

2.3. Experimental method in photo-fermentation

To efficiently reutilize the VFA's byproducts from dark fermentation and raise the HY in photo-fermentation, the optimal concentrations of acetate and butyrate, respectively, were investigated. The residual liquid from dark fermentation, which mostly contained acetate and butyrate, was collected by centrifugation for 30 min at 4800 rpm followed by filtering with a vacuum pump. The residual liquid was diluted to an optimal concentration of butyrate (15 mM) and mixed with 25 ml 10 × BMPF for photo-fermentation.

Table 1 – Factors and levels in orthogonal experimental design.

Factor	Factors and symbols				
	A Glucose (g/l)	B $MgCl_2$ (g/l)	C $FeCl_2$ (g/l)	D K_2HPO_4 (g/l)	E NaCl (g/l)
Level 1	10	0.05	0.05	1.0	3.0
Level 2	20	0.10	0.10	1.5	4.0
Level 3	30	0.15	0.15	2.0	5.0
Level 4	40	0.20	0.20	2.5	6.0

Table 2 – Hydrogen production in dark fermentation under orthogonal experimental conditions.

Experimental conditions	A Glucose	B $MgCl_2$	C $FeCl_2$	D K_2HPO_4	E NaCl	H2 production[a] (ml)	H_2 yield (mol/mol glucose)
1	1[b]	1	1	1	1	427 ± 6	1.26
2	1	2[b]	2	2	2	495 ± 8	1.46
3	1	3	3[b]	3	3	488 ± 5	1.44
4	1	4	4	4[b]	4	448 ± 6	1.32
5	2	1	2	3	4	1078 ± 10	1.59
6	2	2	1	4	3	1044 ± 12	1.54
7	2	3	4	1	2	827 ± 8	1.22
8	2	4	3	2	1	882 ± 8	1.30
9	3	1	3	4	2	1312 ± 14	1.29
10	3	2	4	3	1	1241 ± 15	1.22
11	3	3	1	2	4	936 ± 14	0.92
12	3	4	2	1	3	1150 ± 12	1.13
13	4	1	4	2	3	949 ± 12	0.70
14	4	2	3	1	4	1031 ± 10	0.76
15	4	3	2	4	1	1519 ± 11	1.12
16	4	4	1	3	2	1248 ± 13	0.92
K_1[c]	1.370	1.210	1.160	1.093	1.225		
K_2	1.412	1.245	1.325	1.095	1.222		
K_3	1.140	1.175	1.198	1.292	1.202		
K_4	0.875	1.167	1.115	1.318	1.147		
R[d]	0.537	0.078	0.210	0.225	0.078		

a Data are the averages (±standard deviation) of the values obtained in independent experiments conducted in triplicate ($n = 3$).
b Designed levels1–4 for different factors (shown in Table 1).
c K: the average of HYs of four experiments at one level (for one factor).
d R: the range of K_1–K_4 for one factor.

A quantity of 25 ml domesticated *R. palustris* was inoculated with 225 ml culture medium in a 300-ml transfusion bottle. The initial pH of fermentation medium was adjusted to 7.0 ± 0.02. The fermenter with a rubber stopper was purged with N_2 gas for 20 min to ensure an anaerobic environment. The fermenter was placed in an illuminated incubator equipped with a microcomputer (Shanghai Boxun SPX-300I-G, China) at 30 ± 0.5 °C under a light intensity of 200 W/m^2 (about 6000 lux). Incandescent lamps (100 W) were used as input light resources and a light meter (TES-1334A, Taiwan) was used to measure illumination intensity.

2.4. Analytical methods

A graduated glass syringe was used to measure biogas volume in dark- and photo-fermentation every 12 h. The compositions of biogas sampled by the syringe were analyzed on a gas chromatograph (GC, sp 3400, China) equipped with a thermal conductivity detector (TCD). The temperatures of the injection port, oven, and TCD detector were 50, 50, and 100 °C, respectively. Helium was used as the carrier gas at a flow rate of 30 ml/min. The VFAs (mainly comprising acetate, propionate, butyrate, valerate and caproate) and ethanol, which were sampled before and after photo-fermentation and adjusted with HCl to pH 2.0, were analyzed on a second GC using a flame ionization detector (FID) and a 2-m glass column packed with Unisole F-200 (30/60 mesh). The temperatures of injection port, oven, and FID detector were 200, 240, and 280 °C, respectively. Here too helium was used as the carrier gas at a flow rate of 5 ml/min. All the experimental data were the averages (± standard deviation) of the values obtained in independent experiments conducted in triplicate.

2.5. Kinetic model and data analysis

Cumulative hydrogen production was simulated by a modified Gompertz equation (Eq. (3)) and the kinetic parameters (H_m, R_m and λ) were estimated via Matlab 7.5 [11,13].

$$H = H_m \exp\left\{ -\exp\left[\frac{R_m e}{H_m}(\lambda - t) + 1\right]\right\} \quad (3)$$

Here, H is the cumulative volume of hydrogen production (ml), H_m is the maximum hydrogen production potential (ml), R_m is the maximum hydrogen production rate (ml/h), e is 2.718, λ is the lag-phase time (h), and t is the incubation time (h). The overall volumetric hydrogen production rate ($R_{overall}$) defined in Eq. (4) is a performance indicator that takes into account both the rate of and time delay in hydrogen production [11,14].

$$R_{overall} = \frac{H_m}{(H_m/R_m) + \lambda} \bullet \frac{1}{V} \quad (4)$$

Here, V denotes the working volume of the culture.

The volume of hydrogen produced in a batch fermentation experiment with 250 ml medium was calculated according to the biogas volume, modified to standard temperature and air pressure, and the hydrogen concentration in the biogas. The specific hydrogen yield (mol/mol) was defined as the ratio of hydrogen produced (mol) to the initial volume of glucose (mol) or VFA (mol).

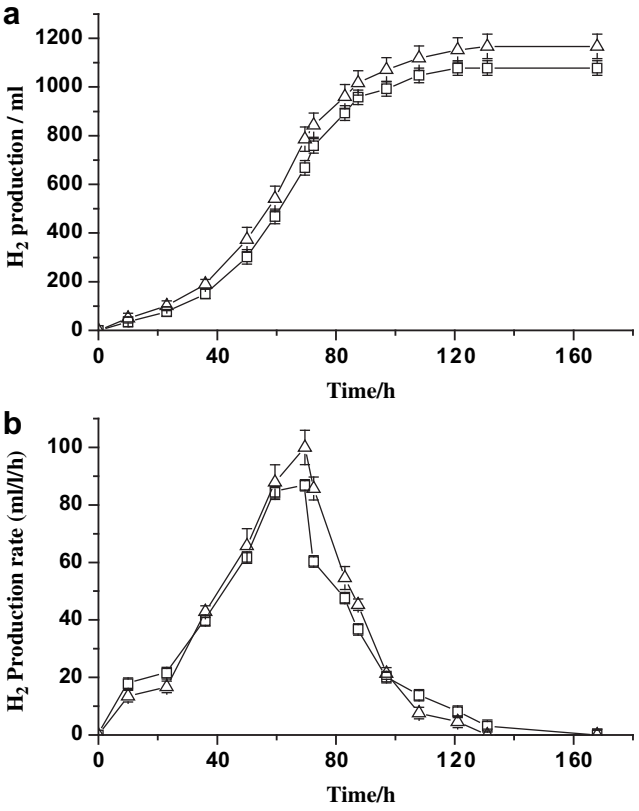

Fig. 1 – Hydrogen production from glucose with the theoretical optimal medium and experimental optimal medium (a) H_2 volume produced; (b) H_2 production rate. Δ- theoretical optimal medium (g/l): glucose 20, K_2HPO_4 2.5, $FeCl_2$ 0.1, $MgCl_2$ 0.1, NaCl 3.0. □- experimental optimal medium (g/l): glucose 20, K_2HPO_4 2.0, $FeCl_2$ 0.1, $MgCl_2$ 0.05, NaCl 6.0. Data are the averages (± standard deviation) of triplicate independent experiments ($n = 3$).

3. Results and discussion

3.1. *Medium optimization in dark fermentation*

The 16 orthogonal experimental conditions were designed to analyze the effects on hydrogen production of five factors (medium compositions: glucose, $MgCl_2$, $FeCl_2$, K_2HPO_4, and NaCl) at four levels (concentrations), as shown in Table 2. The parameter K was the statistical average of HYs at one level (for one factor). For example, the $K_1 = 1.370$ in the column A was the mean value of four HYs of 1.26, 1.46, 1.44, and 1.32, which were obtained in four experiments with the factor A (glucose) at level 1 (10 g/l). Parameters K_2, K_3, and K_4 in the column A (glucose) were the mean values of four HYs obtained at level 2 (20 g/l), level 3 (30 g/l), and level 4 (40 g/l), respectively. The parameter R was the statistical range of K_1–K_4 for one factor. For example, the $R = 0.537$ in the column A (glucose) was the difference between the maximum $K_2 = 1.412$ and the minimum $K_4 = 0.875$ in column A. The different values of R revealed the effects of the five factors on hydrogen production, while the different values of K showed the effects of the four levels on hydrogen production. It was found that glucose ($R = 0.537$) asserted the most important influence on hydrogen production, while NaCl ($R = 0.078$) and $MgCl_2$ ($R = 0.078$) the least. Because $K_2 = 1.412$ was the maximum value in the column A (glucose), the level two of factor A (marked as "A_2") was selected as a component of the theoretical optimal medium. The B_2, C_2, D_4, and E_1 were similarly selected as the other four components of the theoretical optimal medium. Accordingly, the theoretical optimal medium ($A_2B_2C_2D_4E_1$) compositions were (g/l): glucose 20, K_2HPO_4 2.5, $FeCl_2$ 0.1, $MgCl_2$ 0.1, and NaCl 3.0.

The experimental optimal medium $A_2B_1C_2D_3E_4$ (condition No. 5), which produced the experimental maximum HY of 1.59 mol H_2/mol glucose, was directly selected from the 16 practical experimental conditions (Table 2). The experimental optimal medium ($A_2B_1C_2D_3E_4$) compositions (g/l) were glucose 20, K_2HPO_4 2.0, $FeCl_2$ 0.1, $MgCl_2$ 0.05, and NaCl 6.0.

A new experiment with the theoretical optimal medium ($A_2B_2C_2D_4E_1$) was carried out to verify the practical hydrogen production, which result was compared with that of the experimental optimal medium ($A_2B_1C_2D_3E_4$) in Fig. 1. The theoretical optimal medium of $A_2B_2C_2D_4E_1$ derived from the orthogonal analysis yielded a specific HY of 1.72 mol H_2/mol glucose, which was slightly higher (by 8.2%) than 1.59 mol H_2/mol glucose produced by the experimental optimal medium $A_2B_1C_2D_3E_4$ (condition No. 5) selected from the 16 experimental conditions. The peak HPR of 100 ml H_2/l/h produced by the theoretical optimal medium was also higher (by 15.2%) than the 86.8 ml H_2/l/h produced by the experimental optimal medium. Therefore, the performance of the theoretical optimal medium was superior to that of the experimental optimal medium. The complete optimal medium was finally determined as glucose 20 g/l, NaCl 3 g/l,

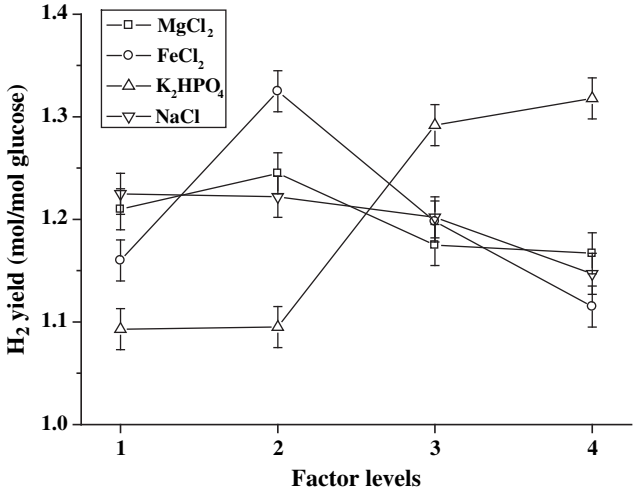

Fig. 2 – Effects of inorganic components on hydrogen production in dark fermentation. $MgCl_2$ levels: (1) 0.05 g/l, (2) 0.10 g/l, (3) 0.15 g/l, (4) 0.20 g/l. $FeCl_2$ levels: (1) 0.05 g/l, (2) 0.10 g/l, (3) 0.15 g/l, (4) 0.20 g/l. K_2HPO_4 levels: (1) 1.0 g/l, (2) 1.5 g/l, (3) 2.0 g/l, (4) 2.5 g/l. NaCl levels: (1) 3.0 g/l, (2) 4.0 g/l, (3) 5.0 g/l, (4) 6.0 g/l. Data are the averages (± standard deviation) of values of four experiments at one level (for one factor).

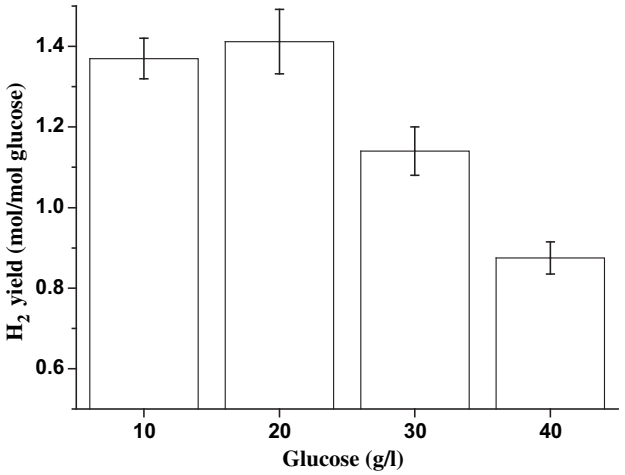

Fig. 3 – Effects of glucose concentrations on hydrogen production in dark fermentation. Data are the averages (± standard deviation) of values of four experiments at the same glucose concentration.

$MgCl_2$ 0.1 g/l, $FeCl_2$ 0.1 g/l, K_2HPO_4 2.5 g/l, L-cysteine 0.5 g/l, vitamin solution 10 ml/l, and trace element solution 10 ml/l.

3.2. *Effects of inorganic components on dark hydrogen production*

The effects on hydrogen production of K_2HPO_4, NaCl, $MgCl_2$, and $FeCl_2$ concentrations are shown in Fig. 2, in which the HYs correspond to *K* values at the four levels for each factor (Table 2). It is seen that the HY increased from 1.09 to 1.31 mol H_2/mol glucose when K_2HPO_4 concentration increased from 1.0 to 2.5 g/l. This is because a high concentration of phosphate buffer can prevent the pH value from dropping rapidly, which is favourable for hydrogen production in dark fermentation [15]. The HY rose from 1.16 to the peak of 1.33 mol H_2/mol glucose when $FeCl_2$ increased from 0.05 to 0.1 g/l, because Fe^{2+} participates in the ferredoxin synthesis in *C. butyricum*, which mediates electron transfer of reducing power en route to hydrogenase-catalyzed hydrogen production [16]. However, the HY declined to 1.12 mol H_2/mol glucose when $FeCl_2$ steadily increased to 0.2 g/l, because excessive Fe^{2+} inhibits hydrogen production [16,17]. The HY rose from 1.21 to 1.25 mol H_2/mol glucose when $MgCl_2$ increased from 0.05 to 0.1 g/l, because phosphorylase and enolase need Mg^{2+} for the growth of bacteria. Nevertheless, the HY declined to 1.17 mol H_2/mol glucose when $MgCl_2$ steadily increased to 0.2 g/l, because excessive Mg^{2+} inhibits hydrogen production. The HY fell from 1.23 to 1.15 mol H_2/mol glucose when NaCl rose from 3.0 to 6.0 g/l, implying that excessive Na^+ restricts hydrogen production.

3.3. *Effects of glucose concentrations on dark hydrogen production*

The effects of glucose concentrations on hydrogen production in dark fermentation are shown in Fig. 3. The peak HY of 1.412 mol H_2/mol glucose was obtained with 20 g/l glucose. Increasing glucose concentration to 40 g/l resulted in a reduced HY of 0.875 mol H_2/mol glucose because of the inhibition by fermentation products, which is similar to the result reported by Chen [17]. The kinetic parameters of hydrogen production derived from Eqs. (3) and (4) and SMP from dark fermentation at different glucose concentrations are shown in Table 3. The Roverall increased from 35.6 to 56.8 ml/l/h when glucose rose from 10 to 40 g/l.

The SMP from dark fermentation was mainly composed of butyrate (68.5–90.6 wt%), acetate (7.5–28.5 wt%), propionate, valerate, caproate, and ethanol. With increase in glucose concentration, there was increase in butyrate concentration and a considerable reduction in acetate concentration. The total concentration of butyrate and acetate was high at 93.5–98.6 wt%, implying that hydrogen was produced by *C. butyricum* through a butyrate-type fermentation [17–19]. Ethanol as a competitive electron-consuming product [20] had a very small concentration range of 0.2–2.1 wt%. There was a positive relationship between the acetate/butyrate ratio in SMP and the specific HY in dark fermentation. The HY increased from 1.12 to 1.59 mol H_2/mol glucose when the acetate/butyrate ratio increased from 0.083 to 0.406. This is because 1 mol glucose can produce 2 mol acetate and 4 mol H_2, while 1 mol glucose can produce 1 mol butyrate and 2 mol H_2.

3.4. *Hydrogen production in photo-fermentation*

The hydrogen production by *R. palustris* from acetate- and butyrate feedstocks of various concentrations in photo-fermentation is shown in Table 4. Optimal concentration (20 mM) of acetate yielded the peak specific HY of 1.80 mol H_2/mol acetate. The HPR steadily rose to 23.6 ml/l/h when the acetate

Table 3 – Hydrogen production and soluble metabolites byproducts in dark fermentation.

Glucose (g/l)	HY (mol/mol)	H_2 (vol %)	$R_{overall}$ (ml/l/h)	Kinetic model parameters				Soluble metabolites byproducts						
				H_m/ml	R_m (ml/h)	λ (h)	R^2	Ethanol (wt%)	Acetate (wt%)	Propionate (wt%)	Butyrate (wt%)	Valerate (wt%)	Caproate (wt%)	Acetate/Butyrate
10	1.32	56.86	35.6	543	13.2	19.8	0.9377	0.2	25.0	1.7	68.5	0.5	4.1	0.364
20	1.59	73.35	47.5	1108	15.2	20.5	0.9647	1.1	28.5	0.1	70.1	0.0	0.2	0.406
30	1.22	73.05	53.8	1493	16.7	21.5	0.9788	2.1	8.7	0.1	88.8	0.1	0.1	0.098
40	1.12	69.88	56.8	1569	18.5	25.6	0.9794	1.7	7.5	0.1	90.6	0.1	0.2	0.083

Table 4 – Hydrogen production by *R. Palustris* from acetate and butyrate in photo-fermentation.

Acetate/ mM	Butyrate/ mM	H_2 production/ ml	Maximum HPR (ml/l/h)	HY (mol/mol substrate)
10	–	75 ± 5	8.9	1.34
20	–	200 ± 8	19.6	1.80
30	–	276 ± 8	22.4	1.65
40	–	413 ± 10	23.6	1.47
–	15	403 ± 12	25.8	4.80
–	30	789 ± 15	26.9	4.70
–	45	606 ± 15	22.4	2.41
–	60	597 ± 14	19.1	1.78

Data are the averages (±standard deviation) of the values obtained in independent experiments conducted in triplicate ($n = 3$).

concentration increased to 40 mM, which was consistent with the previous report [21]. The peak specific HY of 4.80 mol H_2/mol butyrate was obtained at an optimal concentration (15 mM) of butyrate, which was higher by 29.7% than the 3.7 mol H_2/mol butyrate reported by Fang [22].

The original SMP from dark fermentation was not suitable to for direct reutilization as feedstock in photo-fermentation, because the high concentrations of acetate (5–36 mM) and butyrate (37–62 mM) seriously inhibit phototropic bacteria (*R. palustris*) and produce a low HY. Although the original butyrate concentration in SMP was higher than that of the acetate, their optimal concentrations for phototrophic hydrogen production were in reverse order. Therefore, the SMPs from dark fermentation were diluted to an optimal concentration of butyrate (15 mM) before reutilization as the feedstock in photo-fermentation. As shown in Table 5, the specific HY fell from 4.16 to 0.66 mol H_2/mol glucose in photo-fermentation when glucose concentration increased from 10 to 40 g/l. This could be attributed to the inhibition of excess ethanol, propionate, valerate, and caproate in the SMP. Fig. 4 shows that 82.1–92.3% of acetate and 98.3–99.8% of butyrate were removed during photo-fermentation.

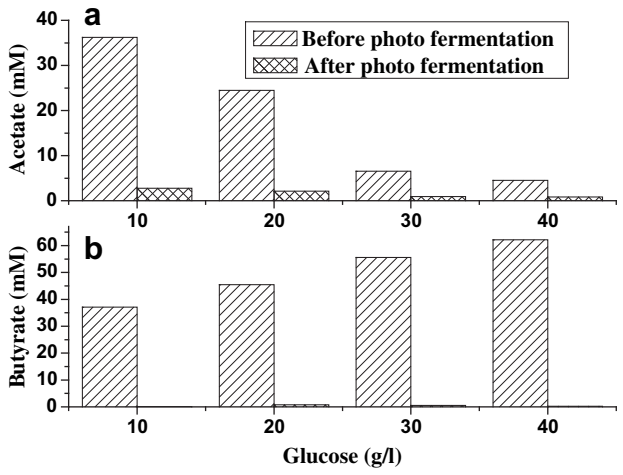

Fig. 4 – Removal ratios of acetate and butyrate in photo-fermentation.

Table 5 – Heat value and total energy conversion efficiencies in hydrogen production on combining dark- and photo-fermentation.

Glucose (g/l)	Heat value (KJ)	Heat value conversion efficiency										Input light energy (KJ)	Total energy conversion efficiency (%)
		Dark fermentation			Photo-fermentation			dark and photo-fermentation					
		H_2 (ml)	Heat value (KJ)	Specific HY (mol/ mol)	Heat value conversion (%)	H_2 (ml)	Heat value (KJ)	Specific HY (mol/ mol)	Heat value conversion (%)	H_2 (ml)	Heat value (KJ)	Specific HY (mol/ mol)	Heat value conversion (%)		
10	43.72	448 ± 6	4.84	1.32	11.1	1411 ± 20	15.24	4.16	34.9	1859	20.08	5.48	46.0	634.52	2.96
20	87.44	1078 ± 10	11.65	1.59	13.3	1241 ± 22	13.41	1.83	15.3	2319	25.05	3.42	28.6	634.52	3.47
30	131.16	1241 ± 15	13.41	1.22	10.2	1272 ± 20	13.74	1.25	10.5	2513	27.25	2.47	20.7	634.52	3.55
40	174..88	1519 ± 11	16.41	1.12	9.4	895 ± 15	9.67	0.66	5.5	2414	26.08	1.78	14.9	634.52	3.22

Data are the averages (±standard deviation) of the values obtained in independent experiments conducted in triplicate ($n = 3$).

The specific HYs from glucose in dark fermentation were lower than 3.0 mol H_2/mol glucose in many literatures [10,22]. Chen reported that the theoretical maximum HY from *C. butyricum* was 3.26 mol H_2/mol glucose in dark fermentation [23]. In this study, the specific HY markedly increased to 5.48 mol H_2/mol glucose on combining dark with photo-fermentation. This value was not only higher than the specific HYs in dark fermentation alone, but also higher than most specific HYs (2.4–2.8 mol H_2/mol glucose) combining dark- and photo-fermentation quoted in the literature [21,24,25]. Although Naoaki declared that the maximum HY was 1.4–5.6 mol H_2/mol glucose in a two-stage continuous bioreactor [24], this value was indirectly estimated based on photo-trophic HY from a synthetic cocktail of several pure chemicals (but not directly from actual dark fermentation residuals). Because the complex byproducts from dark fermentation experiments inhibited hydrogen production somewhat in photo-fermentation, the residual solutions were inevitably more difficult in reutilization than pure chemicals for simulating VFAs. In this study, the residual solutions from dark fermentation experiments were actually reutilized for hydrogen production in photo-fermentation experiments, which produced the maximum HY of 5.48 mol H_2/mol glucose in a two-phase fermentation experiment. However, this value was lower than the HYs obtained in a two-phase process co-immobilizing *Lactobacillus delbrueckii* and *Rhodobacter sphaeroides* or produced by an SLOF-illuminating system with clay addition (about 7.1 mol H_2/mol hexose) [2,26]. Therefore, some improvements such as cell immobilization and parameter optimization are beneficial in making the two-phase system more efficient in hydrogen production.

3.5. *Energy conversion efficiency*

The heat value and total energy conversion efficiencies are defined in Eqs. (5) and (6). The specific heat values of glucose and hydrogen were 2888 kJ/mol and 242 kJ/mol, respectively.

$$\text{Heat value conversion efficiency} = \frac{\text{Heat value of } H_2 \text{ produced (KJ)}}{\text{Heat value of substrate (glucose) (KJ)}} \times 100\% \quad (5)$$

$$\text{Total energy conversion efficiency} = \frac{\text{Heat value of } H_2 \text{ produced (KJ)}}{\text{Heat value of substrate(glucose) (KJ)} + \text{input light energy (KJ)}} \times 100\% \quad (6)$$

The heat value and total energy conversion efficiencies in hydrogen production from glucose by combining dark- and photo-fermentation were shown in Table 5. The conversion efficiency of heat value markedly increased from 9.4–13.3% in dark fermentation to 14.9–46.0% in combined dark- and photo-fermentation. The maximum conversion efficiency of heat value of 46.0% was 70% higher than 27.1% obtained from cassava starch in combined dark- and photo-fermentation [11], which implied that hydrolysis of starch into glucose was a key rate-limiting step in hydrogen production.

Incandescent lamps of 200 W/m^2 light intensity were used as input light resources in photo-fermentation. The effective area of light intercepted in each fermenter was 6.12×10^{-3} m^2. The duration of the photo-fermentation experiment being 144 h, the input light energy was calculated as follows: $200 \times (6.12 \times 10^{-3}) \times (144 \times 3600)/1000 = 634.52$ KJ. The heat value of hydrogen produced in combined dark- and photo-fermentation was in the range 20.08–27.25 KJ. If both the heat values of glucose feedstock (43.72–174.88 KJ) and input light energy were taken into account, the total energy conversion efficiency ranged from 2.96 to 3.55% in the two-phase system. This was higher than the total energy conversion efficiency of 1.93% reported by Chen [27], in which acetate was used for hydrogen production by *R. palustris WP3–5* in photo-fermentation. However, the utilization efficiency of input light energy was poor, and inhibited application of the two-phase system in industry. More efficient photo-bioreactor designs with renewable solar energy are needed to further raise hydrogen production and total energy conversion efficiency. The pure cultures, defined media, and glucose feedstock used in this study are difficult to implement on the industrial scale. Further study of the mixed bacteria, low-cost medium, and cellulosic biomass feedstock is necessary to make the two-phase system more practical and cost-effective.

4. Conclusions

Optimizing the combination of dark- and photo-fermentation enhanced hydrogen production from glucose as well as the energy conversion efficiency. The two-phase process dramatically raised not only the HY from 1.59 in dark fermentation to 5.48 mol H_2/mol glucose but also conversion efficiency of heat value from the range 9.4–13.3% in dark fermentation to 14.9–46.0%. The culture medium for *C. butyricum* in dark fermentation was optimized by the orthogonal experiments as the following: glucose 20 g/l, NaCl 3 g/l, $MgCl_2$ 0.1 g/l, $FeCl_2$ 0.1 g/l, K_2HPO_4 2.5 g/l, L-cysteine 0.5 g/l, vitamin solution 10 ml/l, and trace element solution 10 ml/l. The metabolite byproducts from dark fermentation, which mainly contained acetate and butyrate, were inoculated with *R. palustris* and reutilized to produce hydrogen in photo-fermentation. In photo-fermentation, the maximum HY obtained was 4.16 mol H_2/mol glucose and the maximum removal ratios of acetate and butyrate achieved were 92.3% and 99.8%, respectively.

However, the total energy conversion efficiency was low at 2.96–3.55% in the two-phase system, when both the heat value

of glucose feedstock and input light energy were taken into account. A more efficient photo-bioreactor design with renewable solar energy is necessary to further raise hydrogen production and the total energy conversion efficiency. Although pure cultures, defined medium, and glucose feedstock have been investigated, it is necessary to further study the mixed bacteria, low-cost medium, and cellulosic biomass feedstock to make the two-phase system more practical and cost-effective for application in industry.

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