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International Journal of Hydrogen Energy 31 (2006) 2223–2230
International Journal of HYDROGEN ENERGY
www.elsevier.com/locate/ijhydene

Phototrophic hydrogen production from glucose by pure and co-cultures of *Clostridium butyricum* and *Rhodobacter sphaeroides*

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Received 25 January 2005; received in revised form 12 February 2006; accepted 14 March 2006
Available online 8 June 2006

Abstract

Phototrophic hydrogen production from glucose by pure and co-cultures of *Clostridium butyricum* and *Rhodobacter sphaeroides* was studied in batch experiments. Results showed that in all batches hydrogen was produced after a lag phase of about 10 h; pure culture of *R. sphaeroides* produced hydrogen at rates substantially lower than *C. butyricum*. In co-culture systems, *R. sphaeroides* even with cell populations 5.9 times higher still could not compete with *C. butyricum* for glucose. In co-culture systems, *R. sphaeroides* syntrophically interacted with *C. butyricum*, using the acetate and butyrate produced by the latter as substrate for hydrogen production. Hydrogen production was ceased in all batches when the pH was lowered to the level of pH 6.5, resulting from the accumulation of fatty acids. It was also demonstrated in this study that fluorescence in situ hybridization (FISH) was an effective means for the quantification of the relative abundance of individual bacteria in a co-culture system.

Keywords: *Clostridium butyricum*; Co-culture; Glucose; Hydrogen; *Rhodobacter sphaeroides*

1. Introduction

Hydrogen production from wastewater has been investigated using both anaerobic heterotrophic bacteria, mostly *Clostridium* [1], and anoxygenic phototrophic bacteria, mostly *Rhodobacter* [2,3]. The former use organic matter as substrate, producing fatty acids as by-products, whereas the latter produce hydrogen directly from fatty acids. Among species of *Rhodobacter*, *Rhodobacter sphaeroides* (formerly known as *Rhodopseudomonas sphaeroides*) has been studied most widely for hydrogen production [2,4,5]. Of the genus *Clostridium*, *Clostridium butyricum* is the typical species [6]. It is capable of fermenting a variety of carbohydrates, including glucose, sucrose, starch and pectin into butyrate, acetate, carbon dioxide and hydrogen.

Enhanced hydrogen production from biomass or wastewater through the syntrophic interactions between anaerobic heterotrophs and anoxygenic phototrophs has attracted much research interests. Several pairs of co-culture have been studied for hydrogen production, including *C. butyricum* and *R. sphaeroides* for the conversion of glucose [7], *Klebsiella pneumoniae* and *Rhodospirillum rubrum* for glucose [8], *Cellulomonas sp.* and *Rhodobacter capsulatus* for cellulose [9,10], *Vibrio fluvialis* and *Rhodobium marinum* for starch [11], and *C. butyricum* and *Rhodobacter* for starch [12].

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0360-3199/$30.00
doi:10.1016/j.ijhydene.2006.03.005

However, interspecies relationship in the co-culture systems remains largely unknown.

Traditionally, bacteria may be identified and quantified by the plate culture method or by counting through microscopic examination. The plate culture method is time consuming, and thus cannot be applied for the real-time monitoring of population shift in co-culture systems. On the other hand, microscopic examinations are unable to distinguish bacteria that share similar morphology. Due to the rapidly developed 16S rDNA-based techniques in recent years, bacteria may now be identified with high degrees of specificity and be quantified accurately. The method of fluorescence in situ hybridization (FISH) using gene probes has been applied to quantify bacteria of mixed culture systems in chemical degradation [13] and hydrogen production [14].

In this research, the hydrogen production from glucose by the co-culture of *C. butyricum* and *R. sphaeroides* was investigated and compared with those of individual pure cultures. Glucose utilization, volatile fatty acids (VFA) accumulation, pH variation and shift of bacterial populations were monitored. The total cell numbers were quantified through the traditional microscopic counting method, whereas the relative abundances of *C. butyricum* and *R. sphaeroides* were analyzed by the FISH method.

2. Materials and methods

2.1. Bacterial strains and growth conditions

C. butyricum DSM 10702 and *R. sphaeroides* DSM 158 were purchased from DSMZ and used in the study. The basal solution used throughout this study comprised (in 1 l) 0.75 g K_2HPO_4, 0.85 g KH_2PO_4, 2.8 mg H_3BO_3, 0.75 mg $Na_2MoO_4 \cdot 2H_2O$, 0.24 mg $ZnSO_4 \cdot 7H_2O$, 2.1 mg $MnSO_4 \cdot 4H_2O$, 0.04 mg $Cu(NO_3)_2 \cdot 3H_2O$, 0.75 mg $CaCl_2 \cdot 2H_2O$, 2.0 mg EDTA, 0.2 g $MgSO_4 \cdot 7H_2O$, 11.8 mg $FeSO_4 \cdot 7H_2O$, 3.78 mg vitamin B1, 3.57 mg biotin, 5.25 mg *p*-aminobenzoic acid and 6.48 mg nicotin amide.

C. butyricum was cultivated in a medium, which was prepared by adding glucose ($9\,g\,l^{-1}$), yeast extract ($4\,g\,l^{-1}$) and peptone ($4\,g\,l^{-1}$) to the basal solution [7], at 30 °C in a serum bottle inside a dark anaerobic chamber (Forma Scientific, OH, USA). *R. sphaeroides* was on the other hand cultivated in another medium, which was prepared by adding $(NH_4)_2SO_4$ ($1.25\,g\,l^{-1}$), sodium succinate ($9.8\,g\,l^{-1}$) and yeast extract ($1\,g\,l^{-1}$) to the basal solution [7], anaerobically at 30 °C under illumination with an intensity of $200\,W\,m^{-2}$ (Radiometer IL 1400A, International Light, MA, USA).

2.2. Experimental conditions

Hydrogen production experiments were carried out in 200 ml flat tissue flasks (MA-60, Tsukuba Flat, Co., Tsukuba, Japan) filled with medium to the top, sealed with silicon stoppers [2] and submerged in a 30 °C water bath. The medium for all hydrogen production experiments was prepared by adding glucose (28 mM), glutamate (10 mM), sodium acetate (7.5 mM), peptone ($0.2\,g\,l^{-1}$), yeast extract ($0.2\,g\,l^{-1}$), and phosphate buffer (pH 7.8, 0.1 M) to the basal solution. Hydrogen produced from each flask was collected in a syringe through a silicon tube with a needle fitting in the stopper. A valve was connected to the needle to prevent air leakage during samplings. The flasks were illuminated by tungsten lamps (R80, GE Lighting, USA) at an intensity of $135\,W\,m^{-2}$, as measured by a radiometer (IL 1400A, International Light, USA).

Prior to the experiments, *R. sphaeroides* in a later stage of exponential growth phase was further cultivated for about 24 h in a so-called gL medium [7], containing glutamine (10 mM) and sodium lactate (36 mM) in the basal solution, to induce the hydrogen production activity. Both *R. sphaeroides* and *C. butyricum* were collected from respective culture media by centrifugation at 4000 rpm for 20 min, after suspending each pellet in a 5 ml hydrogen production medium. The cell number in each solution was counted under fluorescent microscope after staining cells with 4,6 diamidino-2-phenylindole (DAPI). The solutions were then transferred to five flasks, according to the designed cell number ratios. Two flasks contained pure individual cultures of *R. sphaeroides* ($1.27 \times 10^8\,ml^{-1}$) and *C. butyricum* ($0.63 \times 10^8\,ml^{-1}$), whereas the other three flasks contained co-cultures of *R. sphaeroides* and *C. butyricum*. One contained $0.95 \times 10^8\,ml^{-1}$ of *R. sphaeroides* and $0.16 \times 10^8\,ml^{-1}$ of *C. butyricum*; the other two were 0.64 and $0.32 \times 10^8\,ml^{-1}$, and 0.32 and $0.48 \times 10^8\,ml^{-1}$, respectively. The corresponding cell number ratios of *R. sphaeroides* and *C. butyricum* in the three co-cultures were 5.9:1, 2:1 and 0.67:1.

2.3. Chemical analysis

Hydrogen production was measured by the water displacement method after calibrating with pure hydrogen. The hydrogen content in the biogas was analyzed by a gas chromatograph (Hewlett Packard 5890 II, USA) equipped with a thermal conductivity detector and a 2 m × 2 mm column packed with Porapak N (80-100 mesh) [1]. Glucose was analyzed by the anthrone method [15]. VFA contents were analyzed by the

second gas chromatograph of the same model equipped with a hydrogen flame ionization detector and a 10 m × 0.53 mm fused-silica capillary column (HP-FFAP), following a procedure as previously described [1].

2.4. Bacterial analysis

At given time intervals throughout the hydrogen production experiments, solutions were taken from each flask for total cell counts after staining with DAPI and for the relative abundance analysis by FISH. The total bacterial cell number was counted by the following procedure. A 10–20 μl solution taken from the flask was mixed with an equal volume of 20 mg l^{-1} DAPI solution (Sigma Aldrich). After staining in dark for 10 min [16], the mixture was diluted to 10 ml with sterilized water (filtered through a 0.22 μm Millipore membrane). The bacterial cells in the diluted solution were transferred to a non-fluorescing membrane (Millipore) 22 mm diameter with 0.2 μm pores by vacuum filtration. The membrane was rinsed by another 10 ml sterilized water to remove residual DAPI. The membrane was air-dried at room temperature and mounted on a microscopic slide. A cover slide was mounted on the membrane with antifading mounting oil (Citifluor, London, UK). The bacterial cells on the membrane were counted under a Nikon E600 epi-fluorescence microscope at 1260× magnification. The cell number of the sample was calculated from the average count of four view fields times the area ratio between the membrane and the view field.

A 3–5 μl solution was taken from each flask for FISH analysis using two oligonucleotide probes. The EUB338 probe was used to target for *R. sphaeroides* and *C. butyricum* while the Chis150 probe was used to target for *C. butyricum.* The oligonucleotide probes were labeled in the 5′-end with either Cy3 or 6-carboxy-fluorescein (FAM) by Integrated DNA Technologies (Coralville, IA, USA). Mixed solution (0.1 ml) taken from the flask was added to 0.3 ml of a 4% (v/v) paraformaldehyde solution for fixation overnight. The bacterial cells were washed twice with a phosphate buffer (pH 7.4) by centrifugation followed by re-suspension. The cells were lastly re-suspended in a 0.4 ml mixture (1:1 by volume) of ethanol and a phosphate buffer (pH 7.2, 0.13 M NaCl and 10 mM Na_2HPO_4). The solution was coated on a welled slide, air-dried and dehydrated sequentially by ethanol solutions of 50%, 80% and 96% for 3 min each. The dehydrated sample was air-dried again, and hybridized with 1 μl each of the oligonucleotide probe solutions (100 ng μl^{-1}) in 10 μ1 of pH 7.2 hybridization formamide-containing buffer (0.9 M NaCl, 5% formamide, 0.01% SDS, 20 mM Tris-HCl). The hybridization was carried out at 46 °C in a sealed moisture chamber overnight, followed by incubation in a pH 7.2 washing buffer (0.07 M NaCl, 0.01% SDS, 20 mM Tris/HCl, 5 mM EDTA) at 48 °C for 30 min. The slide was then rinsed briefly with distilled water, air-dried and examined under a laser scanning confocal microscope (Model LSM 5 Pascal, Zeiss, Jena, Germany). The *R. sphaeroides* cells emitted green fluorescence from EUB338 labeled with FAM, and *C. butyricum* cells emitted yellow fluorescence from the combination of EUB338 labeled with FAM and Chis150 labeled with Cy3. Again, the cell numbers of individual bacterial species in each view field were manually counted. The average values of four view fields were used to calculate the relative abundances of the two bacteria in the co-culture.

The population of individual bacteria in each sample was calculated by the multiplication of the relative abundance obtained from the FISH observation and the total cell numbers obtained from the DAPI staining observation.

3. Results and discussion

Residual glucose, acetate and butyrate were detected in samples taken from all batches. But there was no detectable lactate, propionate, ethanol, methanol, propanol, butanol, *i*-butyrate, *i*-valerate, valerate and *i*-caproate. Fig. 1 illustrates the cumulative hydrogen production in the two pure culture and three co-culture batches. The co-culture having the *R. sphaeroides*:*C. butyricum* ratio of 5.9:1 had the highest hydrogen yield (0.60 ml ml^{-1} medium), seconded by the pure culture of *R. sphaeroides* (0.56 ml ml^{-1}) and the co-culture having the ratio of 2:1 (0.56 ml ml^{-1}), followed by the co-culture having the ratio of 0.67:1 (0.52 ml ml^{-1}).

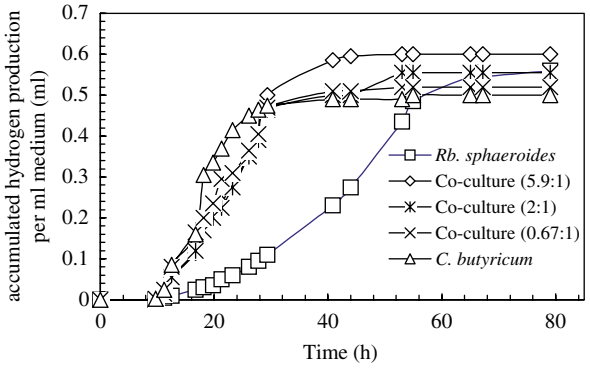

Fig. 1. Accumulated hydrogen production from two pure-culture and three co-culture batches.

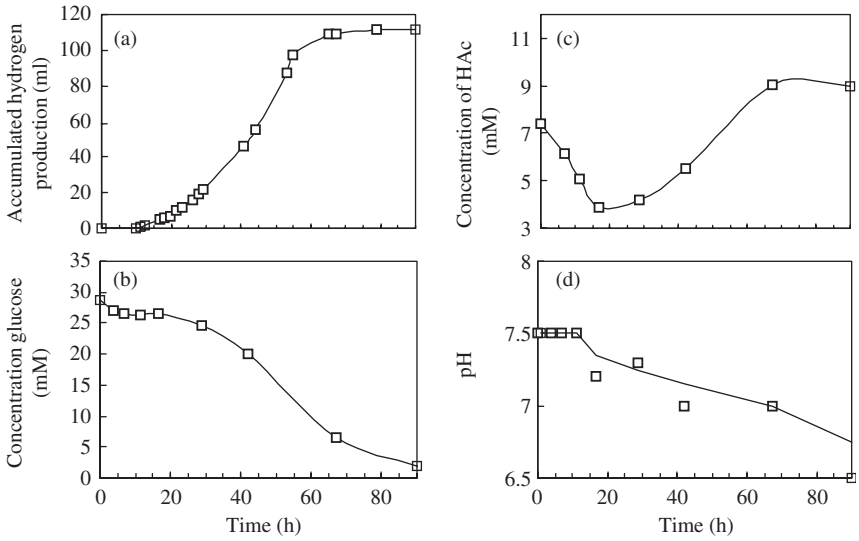

Fig. 2. Hydrogen production characteristics of pure *R. sphaeroides* culture: (a) accumulated hydrogen production, (b) glucose utilization, (c) acetate accumulation, and (d) pH shift.

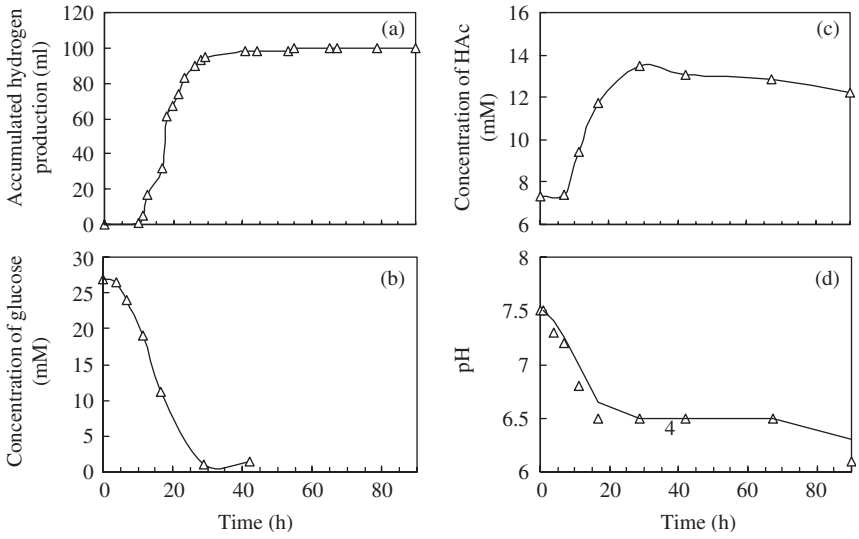

Fig. 3. Hydrogen production characteristics of pure *C. butyricum* culture: (a) accumulated hydrogen production, (b) glucose utilization, (c) acetate accumulation, and (d) pH shift.

The pure culture of *C. butyricum* had the lowest hydrogen yield of 0.50 ml ml^{-1}. In all batches, hydrogen was produced after a lag phase of about 10 h. Pure culture of *C. butyricum* produced hydrogen rapidly immediately after the lag phase, reaching the maximum rate of 4.92 ml-H_2 day^{-1} ml^{-1} by 18 h. On the other hand, pure culture of *R. sphaeroides* produced hydrogen slowly after the lag phase, and did not reach the maximum rate of 1.93 ml-H_2 day^{-1} ml^{-1} until the 55 h. Hydrogen production by the pure culture of *C. butyricum* was ceased after 40 h, but 80 h by the pure culture of *R. sphaeroides*. Fig. 1 illustrates that the three co-culture batches had nearly the same hydrogen production pattern. Thus, only the results of the co-culture batch with the *R. sphaeroides*:*C. butyricum* ratio of 5.9:1 are to be discussed in the following.

Figs. 2–4 illustrate the hydrogen production patterns for the pure *R. sphaeroides* culture, the pure

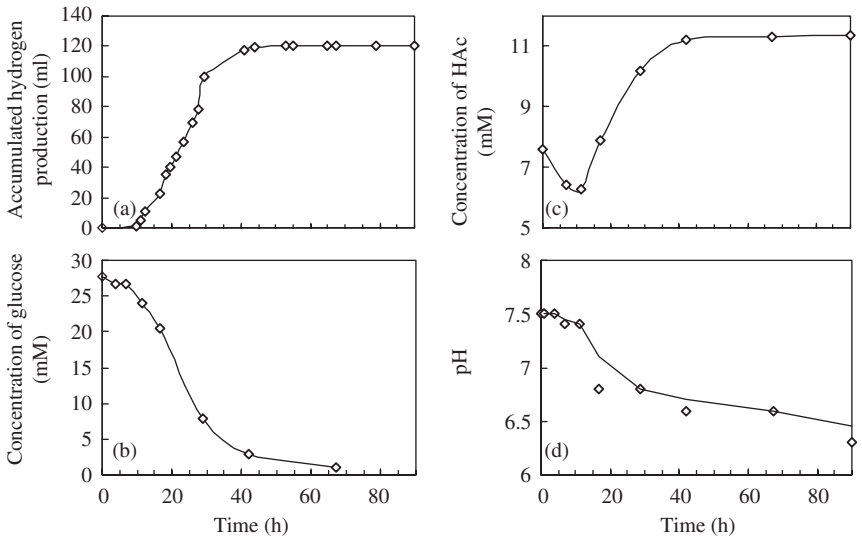

Fig. 4. Hydrogen production characteristics of co-culture of *R. sphaeroides* and *C. butyricum* at cell number ratio of 5.9:1: (a) accumulated hydrogen production, (b) glucose utilization, (c) acetate accumulation, and (d) pH shift.

C. butyricum culture, and the co-culture batch with the ratio of 5.9:1. Each figure shows results of (a) cumulative hydrogen production, (b) concentration of glucose, (c) concentration of acetate and (d) pH variations throughout the batch experiments. Fig. 5 illustrates the concentration variations of butyrate in the three batches.

Fig. 2 illustrates that during the first 17 h there was little hydrogen production (Fig. 2a) and glucose degradation (Fig. 2b), and yet the concentration of acetate decreased substantially from 7.4 to 3.9 mM (Fig. 2c). This seems to imply that acetate was used up for biomass production during this lag phase. Figs. 2b and c showed that in the first 17 h, *R. sphaeroides* used acetate at lower concentration (7.5 mM) while little glucose even at a higher concentration (27 mM) was utilized. Thus, it suggests that *R. sphaeroides* preferably consumed acetate initially as substrate over glucose without hydrogen production.

R. capsulatus had the similar feature [17], except *R. capsulatus* converts acetate into hydrogen according to the following reaction:

$$C_2H_4O_2 + 2H_2O \rightarrow 2CO_2 + 4H_2. \quad (1)$$

After 17 h, the pure culture of *R. sphaeroides* began to convert glucose into hydrogen (Figs. 2a and b) producing acetate and butyrate as by-products. However, the concentrations of acetate (Fig. 2c) and butyrate (Fig. 5) in the solution were substantially lower than those produced from glucose according to the following

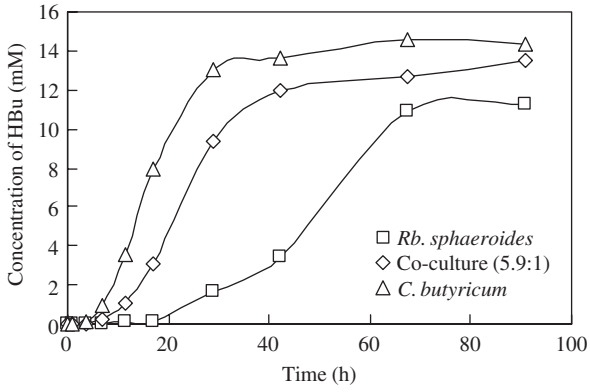

Fig. 5. Butyrate accumulation in pure and co-culture batches.

chemical reactions:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2C_2H_4O_2 + 2CO_2 + 4H_2, \quad (2)$$

$$C_6H_{12}O_6 \rightarrow C_4H_8O_2 + 2CO_2 + 2H_2. \quad (3)$$

Fig. 3 illustrates that after a 10 h lag phase, the pure culture of *C. butyricum* began to produce hydrogen rapidly (Fig. 3a), corresponding to a rapid decrease of glucose (Fig. 3b) and increase of by-products acetate (Fig. 3c) and butyrate (Fig. 5). Results in Figs. 3 and 5 also show that a total of 25.9 mM (from the starting 27.0 to 1.1 mM at 90 h) glucose was converted by *C. butyricum* into 5.5 mM acetate (from 7.4 to 12.9 mM) and 14.6 mM butyrate (from nil to 14.6 mM). According to Eqs. (2) and (3), producing 1 mol of either acetate or butyrate from

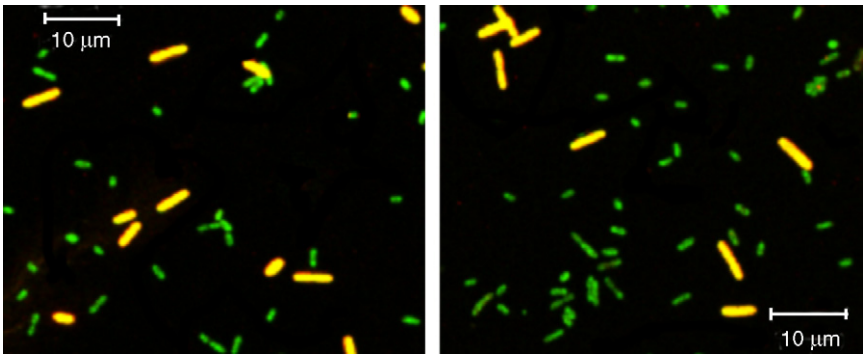

Fig. 6. FISH images of two co-culture samples: *C. butyricum* DSM 10702 (yellow and larger cells) and *R. sphaeroides* DSM 158 (green and smaller cells).

glucose results in the production of 2 mol of hydrogen. Thus, the theoretical hydrogen production from 200 ml of glucose solution to acetate and butyrate by *C. butyricum* was 200 ml ($0.2 \times 24.9 \times 2 \times (5.5+14.6)=200$). As compared to the measured hydrogen production volume of 100 ml, the yield was only 50%.

Fig. 4 illustrates that hydrogen was produced after a 10 h lag phase and the production was ceased after 40 h (Fig. 4a). Acetate concentration initially decreased from 7.5 to 6.3 mM in the first 10 h (Fig. 4c), probably consumed by *R. sphaeroides* as in the pure culture. The acetate concentration was subsequently increased leveling off at 11.2 mM. The increase of acetate concentration (Fig. 4c) was due to the production rate by *C. butyricum* surpassed the consumption rate by *R. sphaeroides*. The increase of acetate (Fig. 4c) and butyrate (Fig. 5) concentrations resulted in the lowering of pH to 6.6 at 40 h (Fig. 4d). Results in Fig. 4 seem to show that *R. sphaeroides* did not utilize acetate after 40 h when glucose had become depleted. The acetate concentration remained at 11.2 mM leveled from 40 h to the end of experiment at 90 h. This could be due to the lowering of pH from the initial pH 7.5 to pH 6.5 resulting from the accumulation of acetate and butyrate.

Previous studies have shown that pure culture of *R. sphaeroides* can produce hydrogen using glucose [2], butyrate and acetate [4] as substrates. Thus, *R. sphaeroides* in the co-culture may compete with *C. butyricum* for glucose, or syntrophically interact with *C. butyricum*, which converts glucose into butyrate and acetate for the consumption of *R. sphaeroides*. Results of this study seem to show that pure culture of *R. sphaeroides* consumed glucose at much slower rate (Fig. 2a) than *C. butyricum* (Fig. 3a). As a result, it is highly likely that in the co-culture system, *R. sphaeroides* cannot compete with *C. butyricum* for glucose, and it thus only grows syntrophically with the latter using fatty acids produced by the latter as substrate for hydrogen production.

Fig. 5 illustrates that the pure culture of *C. butyricum* produced more butyrate (14.3 mM) than co-culture (13.5 mM), which was in turn higher than *R. sphaeroides* (11.5 mM). In the pure culture of *R. sphaeroides*, degrading 1 mol of glucose produced 0.18 mol acetate and 0.42 mol butyrate. The corresponding values for the pure culture of *C. butyricum* were 0.21 mol acetate and 0.56 mol butyrate, and those for the co-culture were 0.19 mol acetate and 0.50 mol butyrate. The lower production of butyrate by the co-culture could be due to the consumption of butyrate by *R. sphaeroides*. Although *R. sphaeroides* can convert acetate and butyrate into hydrogen, the cease of hydrogen production in all batches was likely due to the lowering of pH to the level of pH 6.5.

Fig. 6 is a FISH image of a co-culture sample, illustrating the distinct differentiation between *R. sphaeroides* and *C. butyricum*. Based on total cell counts and the FISH images of samples taken from each batch at various time intervals, the population changes of *R. sphaeroides* and *C. butyricum* were examined. Fig. 7 illustrates the population change for the pure cultures of *R. sphaeroides* (Fig. 7a) and *C. butyricum* (Fig. 7b), and for both in the co-culture (Fig. 7c). Fig. 7a illustrates that the cell number of *R. sphaeroides* in the pure culture increased slowly in the initial 17 h by using low concentration of acetate. After 17 h, *R. sphaeroides* began to use the glucose to grow until reaching a stationary growth phase at about 80 h. Fig. 7b illustrates that, on the other hand, the cell number of *C. butyricum* in the pure culture increased only after a short lag phase of 3.8 h reaching the maximum at 18 h, and then began to decrease. Fig. 7c illustrates

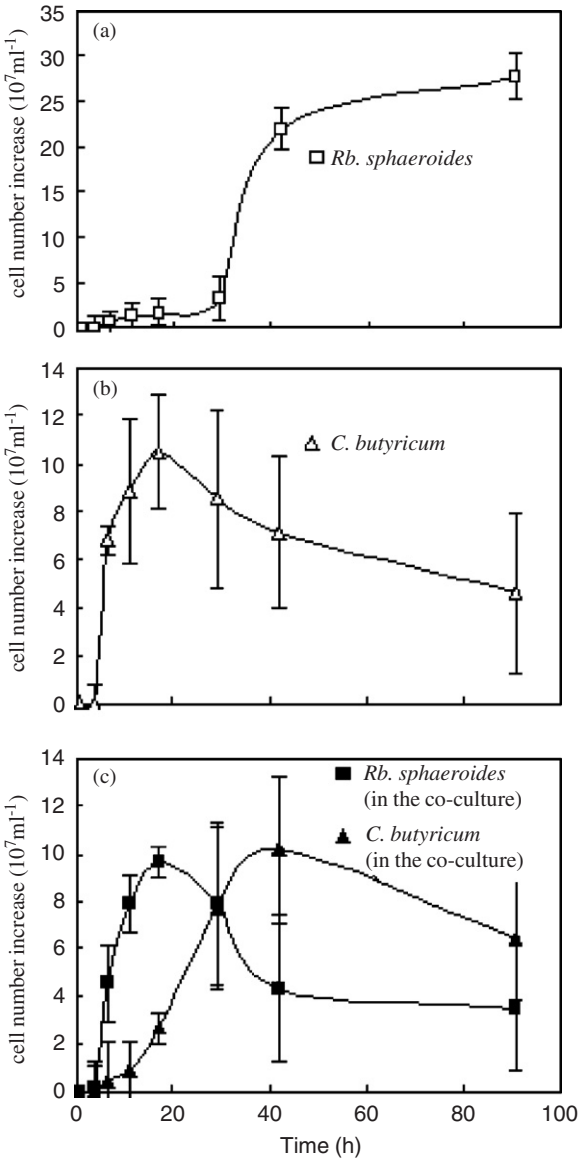

Fig. 7. Changes of bacteria populations in batches of: (a) pure *R. sphaeroides*, (b) pure *C. butyricum* culture, and (c) co-culture with cell number ratio of 5.9:1.

that the cell number of *R. sphaeroides* in the co-culture increased rapidly after a short lag phase of 3.8 h reaching the maximum at 18 h, and then began to decrease, most likely due to the lowering of pH to 6.5. The cell number of *C. butyricum* in the co-culture increased slowly after a short lag phase of 3.8 h reaching the maximum at 40 h, and then decreased due to the depletion of glucose. The discrepancy in growth behaviors of *R. sphaeroides* and *C. butyricum* in Figs. 7a–c suggests an interaction between these two species, the detail of which is unclear and deserves further investigation.

4. Conclusion

All batches produced hydrogen after a lag phase of about 10 h. Pure culture of *R. sphaeroides* produced hydrogen at rates substantially lower than *C. butyricum.* In co-culture systems, *R. sphaeroides* even with cell populations 5.9 times higher still could not compete with *C. butyricum* for glucose. *R. sphaeroides* syntrophically interacted with *C. butyricum*, using the acetate and butyrate produced by the latter as substrate for hydrogen production. Hydrogen production was ceased when the pH was lowered to the level of pH 6.5, resulting from the accumulation of fatty acids. The FISH method was effective in the quantification of the relative abundance of individual bacteria in a co-culture system.

Acknowledgment

The authors wish to thank the Hong Kong Research Grants Council for the financial support of this project (HKU7106/04E).

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