# Fermentative hydrogen production in packed-bed and packing-free upflow reactors

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**Abstract** Fermentative hydrogen production from a synthetic wastewater containing 10 g/L of sucrose was studied in two upflow reactors at 26°C for 400 days. One reactor was filled with packing rings (RP) and the other was packing free (RF). The effect of hydraulic retention time (HRT) from 2 h to 24 h was investigated. Results showed that, under steady state, the hydrogen production rate significantly increased from 0.63 L/L/d to 5.35 L/L/d in the RF when HRT decreased from 24 h to 2 h; the corresponding rates were 0.56 L/L/d to 6.17 L/L/d for the RP. In the RF, the hydrogen yield increased from 0.96 mol/mol-sucrose at 24 h of HRT to the maximum of 1.10 mol/mol-sucrose at 8 h of HRT, and then decreased to 0.68 mol/mol-sucrose at 2 h. In the RP, the yield increased from 0.86 mol/mol-sucrose at 24 h of HRT to the maximum of 1.22 mol/mol-sucrose at 14 h of HRT, and then decreased to 0.78 mol/mol-sucrose at 2 h. Overall, the reactor with packing was more effective than the one free of packing. In both reactors, sludge agglutinated into granules. The microbial community of granular sludge in RP was investigated using 16S rDNA based techniques. The distribution of bacterial cells and extracellular polysaccharides in hydrogen-producing granules was investigated by fluorescence-based techniques. Results indicated that most of the N-acetyl-galactosamine/galactose-containing extracellular polysaccharides were distributed on the outer layer of the granules with a filamentous structure.

**Keywords** Fermentation; granulation; hydrogen; phylogenetic analysis; upflow reactor

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## Introduction

Hydrogen is a promising alternative to fossil fuel. It produces water instead of greenhouse gases upon combustion. Hydrogen is commonly produced by either electrolytic, thermochemical, or radiolytic process, all of which are energy intensive. On the other hand, it has been demonstrated that hydrogen can also be produced sustainably from organic waste and wastewater by fermentation bacteria.

Most studies of bio-hydrogen production were conducted using the continuous stirred tank reactor (CSTR). The reactor under complete mixing conditions facilitates the release of biogas and the effective pH control. CSTR has been used for hydrogen conversion from synthetic wastewaters containing glucose (Lin and Chang, 1999), sucrose (Fang and Liu, 2004), starch (Lay, 2000), as well as actual sugar manufacturing wastewater (Ueno *et al.*, 1996) and bean curd manufacturing waste (Noike *et al.*, 2003). However, it is unable to maintain high levels of biomass due to sludge washout, especially when the hydraulic retention time (HRT) is lower than the bacteria generation time.

To retain high biomass concentrations in reactors, various techniques have been developed for hydrogen fermentation, including sludge immobilization (Wu *et al.*, 2003), utilization of upflow reactor (Yu *et al.*, 2002) and packed-bed reactor (Chang *et al.*, 2002). Among these techniques, upflow reactors have been extensively applied in the anaerobic wastewater treatment system with high efficiency. In such a reactor, sludge agglutinates into granules, resulting in the increase of biomass concentration and the reduction of sludge washout simultaneously.

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On the other hand, extracellular polymeric substances (EPS), the sticky materials secreted by bacterial cells, facilitate the aggregation of microbes, and thus play an important role in the development of granular sludge (Liu and Fang, 2002). Polysaccharides are the major component of EPS and their contents in anaerobic granule can be characterized by physical and chemical means (Liu and Fang, 2002). However, polysaccharides may also be stained *in situ* for microscopic characterization with lectins which have specific binding characteristics. Lectins labeled with fluorescent dyes can be used jointly with the DNA-targeting probes to reveal the distribution of both polysaccharides and bacterial cells in the granular sludge (Zhang and Fang, 2004). Although the distribution of polysaccharides in biofilm has been studied using lectin probes (Neu, 2000), limited information is available so far on the distribution of polysaccharides in hydrogen-producing granules.

This study was thus conducted to investigate the feasibility of dark fermentative hydrogen production from a sucrose-containing wastewater in two upflow reactors: one filled with packing rings (RP) and the other packing free (RF). The effects of HRT on continuous hydrogen production were investigated in both reactors. The microbial community of hydrogen-producing granular sludge was analyzed using the 16S rDNA-based molecular techniques, including polymerase chain reaction (PCR), denatured gradient gel electrophoresis (DGGE), cloning-sequencing and phylogenetic analysis. Furthermore, the distribution of both polysaccharides and bacterial cells in hydrogen-producing granules were evaluated.

## Materials and methods

### Seed sludge

The seed sludge of mixed cultures was taken from a CSTR, which had produced hydrogen by fermentation of sucrose at 26 °C and pH 5.5. The reactor, which had been operated for 1 year, contained 1.0 g-VSS/L and produced a biogas comprising 50% hydrogen. Degrading each gram of sucrose produced 0.16 L of hydrogen.

### Experiments of hydrogen production

A RF and a RP have been applied in this study for over 400 days. Both reactors had an effective volume of 2.8 L with a 2.0 L built-in gas-liquid-solid separator at the top. The RP was packed with 120 plastic Flexiring (Koch), which was 25 mm in both height and diameter with surface to volume ratio of 235 $m^2/m^3$.

The two reactors were operated at 26 °C. The feed solution in both reactors was composed of 10 g/L of sucrose, plus the following nutrients (in mg/L): $NaHCO_3$ 1250; $NH_4Cl$ 2500; $KH_2PO_4$ 250; $K_2HPO_4$250; $CaCl_2$ 500; $NiSO_4$ 32; $MgSO_4{\cdot}7H_2O$ 320; $FeCl_3$ 20; $Na_2BO_4H_2O$ 7.2; $Na_2MoO_4{\cdot}2H_2O$ 14.4; $ZnCl_2$ 23; $CoCl_2{\cdot}6H_2O$ 21; $CuCl_2{\cdot}H_2O$ 10; $MnCl_2{\cdot}4H_2O$ 30. The initial COD of the substrate was about 12,000 mg /L. The RF was operated at an HRT of 24, 20, 16, 12, 10, 8, 6, 4, 2 h sequentially, whereas the RP was operated at an HRT of 30, 24, 18, 14, 10, 8, 6, 4, 2 h. The feeding solution was controlled at pH 7.8, however, the effluent pH decreased to pH 3.8–5.0 with the decrease of HRT. At each HRT, the reactors were operated for 5 weeks to establish steady-state conditions, judging from the constant sucrose degradation, hydrogen production and effluent quality, before lowering the HRT.

The amount of biogas produced was recorded daily by the water replacement method. The composition of biogas was analyzed twice a week as described previously (Fang and Liu, 2004). Sucrose concentration was measured using the anthrone–sulfuric acid method (Gaudy, 1962), whereas the COD and VSS were measured according to the Standard Methods (APHA, 1992).

#### 16S rDNA-based microbial analysis

The microbial community of the hydrogen-producing granular sludge in the RP, which was found more effective than the RF in hydrogen production, was analyzed. Genomic DNA was extracted from the sludge at 30, 18, 14, 8, 4 and 2 h of HRT. 16S rDNA fragment was amplified by PCR using the primer set of 341FGC (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3′) and 518R (5′-ATT ACC GCG GCT GCT GG-3′) at the annealing temperature of 55 °C in an automated thermal cycler (GeneAmp® PCR 9700, Perkin-Elmer, Foster City, CA) (Muyzer *et al.*, 1993; Zhang and Fang, 2001). The PCR-amplified products were then screened using DGGE to investigate the microbial population shift under different HRT using 8% gel with 30% to 70% denaturant gradients (Muyzer *et al.*, 1993; Zhang and Fang, 2001). Electrophoresis was conducted in a 1 × TAE buffer solution at 200 V and 60 °C for 4 h. The bands on the gel were then stained with silver nitrate (Zhang and Fang, 2000).

The 16S rDNA of granular sludge sample of HRT 2 h was further analyzed. Firstly the 16S rDNA was amplified using the primer set of EUB8F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and EUB1501 (5′-GGT TAC CTT GTT ACG ACT T-3′). The PCR products were then cloned using the TA Cloning Kit (Invitrogen Corporation, Carlsbad, CA) following the manufacturer's instructions. A total of 23 clones were selected for the plasmid recovery and sequencing using EUB1501. Each DNA sequence was compared with the reference microorganism available in the GenBank by BLAST search (Altschul *et al.*, 1990). The obtained DNA sequences and their closet 16S rDNA sequences of reference microorganisms retrieved from the GenBank were aligned and checked manually using the BioEdit (Hall, 1999). A phylogenetic tree was then constructed using the neighbor-joining method (Satio and Nei, 1987) by MEGA 2.1 (Kumar *et al.*, 1993). Bootstrap re-sampling analysis (Felsenstein, 1985) for 500 replicates was performed to estimate the confidence of tree topologies.

#### Cell and polysaccharides *in situ* staining

The hydrogen-producing granules, 0.5 to 1 mm in diameter, were sampled from the RP at HRT 2 h to investigate the distribution of both bacterial cells and polysaccharides using the method described previously (Zhang and Fang, 2004). The lectin probe from *Erythrina cristagalli* labeled with fluorescein isothiocyanate (shortened as EC-FITC) (Sigma) was used to specifically stain *N*-acetyl-galactosamine/galactose in polysaccharide, whereas the DNA-targeting probes, propodium iodide (PI) was applied to stain bacterial cells. When excited by a laser at proper wavelengths, the EC-FITC probe emits green light, whereas PI emits red light. EC-FITC probe and PI were used together to examine simultaneously the distribution of both polysaccharides and bacterial cells. The probe working solutions were prepared using a pH 7.2 phosphate buffering saline (PBS; 0.13 mol $l^{-1}$ NaCl, plus 10 mmol $l^{-1}$ $Na_2HPO_4$) and stored at −20 °C before use. The concentration of the EC-FITC probe was 1 μmol $l^{-1}$, whereas PI was 30 μmol $l^{-1}$.

The hydrogen-producing granules were placed into wells on the glass slides. Each sample in the well was covered with 20 μL of a specific staining solution. The slides were then incubated in a moist chamber in the dark at room temperature for 60 min. These granules were subsequently rinsed with PBS three times to remove the residual staining solutions. Finally these stained granules were examined using a confocal laser scanning microscope (CLSM, LSM 5 Pascal, Zeiss, Jena, Germany).

## Results and discussion

### Production of hydrogen in RF

Wastewater containing 12,000 mg COD/L of sucrose was treated in the RF reactor at 26 °C and HRT varying from 24 h to 2 h. Extensive analysis were conducted at each HRT level under steady-state conditions. Figure 1 illustrates the HRT effect on: (a) sucrose conversion, (b) hydrogen content in biogas, (c) hydrogen production rate, and (d) hydrogen yield in the RF. Figure 1(a) illustrates that sucrose degradation increased from 88.6 ± 1.2% at HRT 2 h to 98.3 ± 0.9% at HRT 24 h. Figure1(b) shows that the hydrogen percentage was not sensitive to HRT in the range of 2–12 h, and declined from 38.0 ± 3.1% to 32.1 ± 2.7% as the HRT was further increased from 12 h to 24 h. The biogas was free of methane. Figure 1(c) illustrates that the hydrogen production rate significantly decreased from 5.35 ± 0.62 L-$H_2$/L/d to 0.63 ± 0.02 L-$H_2$/L/d when HRT increased from 2 h to 24 h. The hydrogen yield increased from 0.68 ± 0.08 mol-$H_2$/mol-sucrose at 2 h of HRT to 1.10 ± 0.05 mol-$H_2$/mol-sucrose at 8 h, and then decreased to 0.96 ± 0.04 mol-$H_2$/mol-sucrose at 24 h, as illustrated in Figure 1(d).

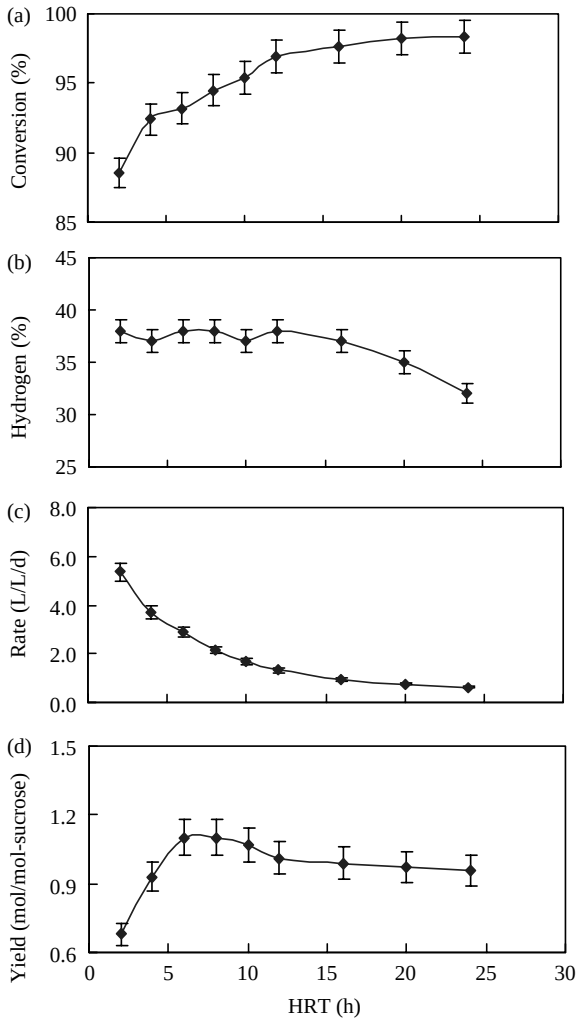


**Figure 1** Effect of HRT on (a) sucrose conversion; (b) hydrogen percentage; (c) hydrogen production rate and (d) hydrogen yield in RF

**Production of hydrogen in RP**

Figure 2 illustrates the HRT effect over the range of 2–30 h on hydrogen production: (a) sucrose conversion, (b) hydrogen content in biogas, (c) hydrogen production rate, and (d) hydrogen yield in the RP. Figure 2(a) shows that sucrose degradation increased from 89.2 ± 0.8% at HRT 2 h to 98.8 ± 0.9% at 24 h, and to 99.1 ± 0.8% at HRT 30 h. Figure 2(b) shows that hydrogen content in biogas was not sensitive to HRT at 2–8 h of HRT, but declined from 40.1 ± 2.2% to 33.3 ± 2.1% and 28.5 ± 1.9% as the HRT was further increased from 8 h to 24 h and 30 h, respectively. The biogas was free of methane. Figure 2(c) illustrates that the hydrogen production rate significantly decreased from 6.17 ± 0.39 L/L/d to 0.56 ± 0.04 L/L/d and 0.46 ± 0.04 L/L/d when HRT increased from 2 h to 24 h and 30 h, respectively. The hydrogen yield increased from 0.78 ± 0.07 mol/mol-sucrose at 2 h of HRT to 1.22 ± 0.13 mol/mol-sucrose at 14 h, and then decreased to 0.86 ± 0.08 mol/mol-sucrose and 0.88 ± 0.08 mol/mol-sucrose at 24 and 30 h, respectively, as illustrated in Figure 2(d).

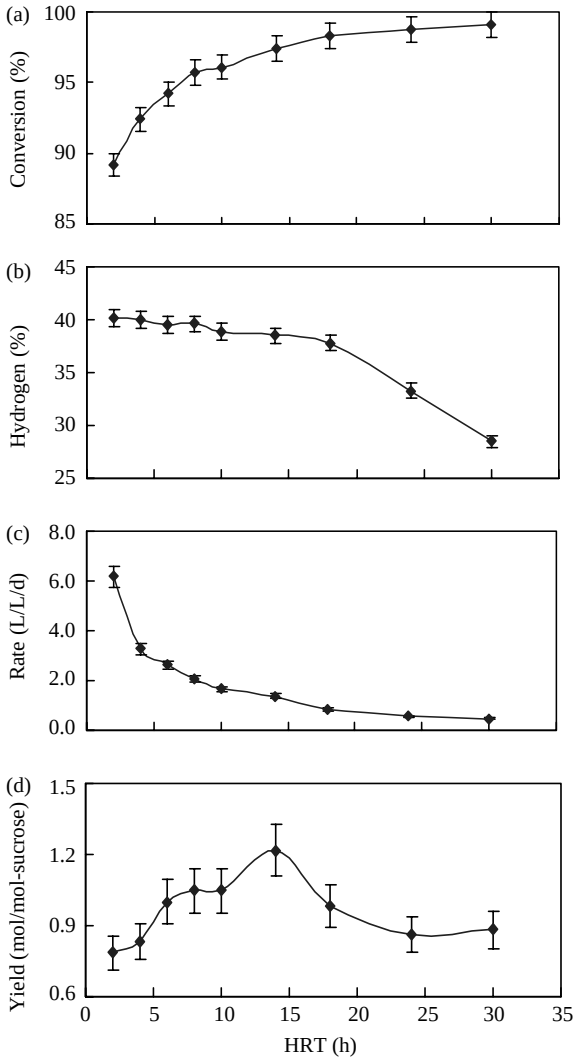


**Figure 2** Effect of HRT on (a) sucrose conversion; (b) hydrogen percentage; (c) hydrogen production rate and (d) hydrogen yield in RP

#### Sludge granulation in two reactors

Sludge granulation is important for the successful reactor operation. In both reactors, sludge agglutinated into granules after 2 months of operation, resulting in the increase of biomass concentration and the reduction of sludge washout. After 400 days of operation, the average VSS concentration in the RF increased up to 38 g/L, indicating that the upflow reactor is promising to establish hydrogen production systems with high biomass. Granule diameters varied from 1 to 3 mm in the RP, whereas the granules in the RF had a more uniform diameter of about 1 mm.

#### Microbial community analyses of sludge in RP

As RP was more effective than RF for continuous hydrogen production, the microbial populations in RP under different HRT were investigated by PCR-DGGE. Figure 3 is the DGGE image illustrating that varying the HRT from 30 h to 2 h had little effect on the microbial community.

As the HRT 2 h has the optimal hydrogen production rate, the microbial population of sludge at 2 h of HRT was further analyzed using the cloning-sequencing approach. Based on DNA sequence, 23 clones developed from this hydrogen-producing sludge were classified into 14 OTUs. The phylogenetic tree in Figure 4 illustrates that 9 of the 14 OTUs, G-HPB-25, -40, -55, -58, -59, -62, -63, -88, -89 formed a group with the strain *Clostridium* CGMCC1152 isolated from a hydrogen-producing system (AY833426). The intra-group similarity of the above species exceeded 98%, whereas inter-group similarity was below 94%. This indicates that these species may be a novel group which has not been described before.

The OTU G-HPB-32 is closely related to *Leuconostoc pseudomesenteroides* with 99% similarity. *Leuconostoc* species are used as the microbial seed for the production of many fermented dairy products, in particular for cultured buttermilk, sour cream, and ripened cream butter (Rattray *et al.*, 2003). It is also reported that *L. pseudomesenteroides* can cause nosocomial urinary tract infections (Cappelli *et al.*, 1999). However, it has never been reported in hydrogen production communities. The OTU G-HPB-66 is closely related to *Klebsiella ornithinolytica* with 99% similarity. Some *Klebsiella* sp. are fermentative hydrogen-producers (Solomon *et al.*, 1995; Tseng, 2004). A clone closely related to *Klebsiella ornithinolytica* (similarity 98%) has been found in a hydrogen-producing sludge previously (Iyer *et al.*, 2004).

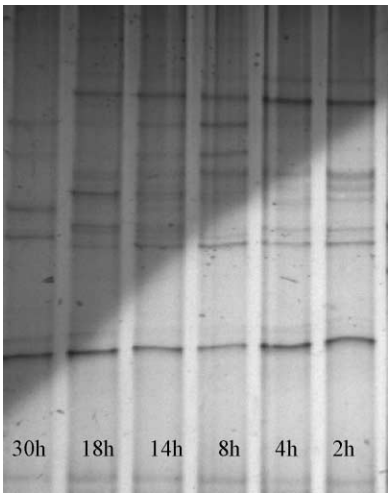


**Figure 3** DGGE profiles of hydrogen communities at various HRT

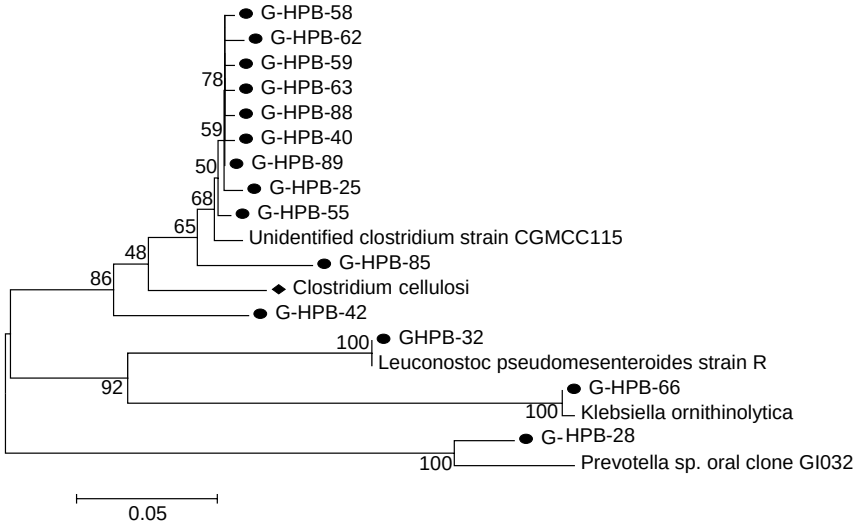


**Figure 4** Phylogenetic tree of the 14 OTUs and their close relatives based on almost full length 16S rDNA. The tree based on Jukes–Cantor distance was constructed using neighbor-joining algorithm with 1000 bootstrappings. The scale bar represents 0.05 substitution per nucleotide position. Numbers at the nodes are the bootstrap values. • OTUs obtained in this study; ◆ Known hydrogen-producing bacteria

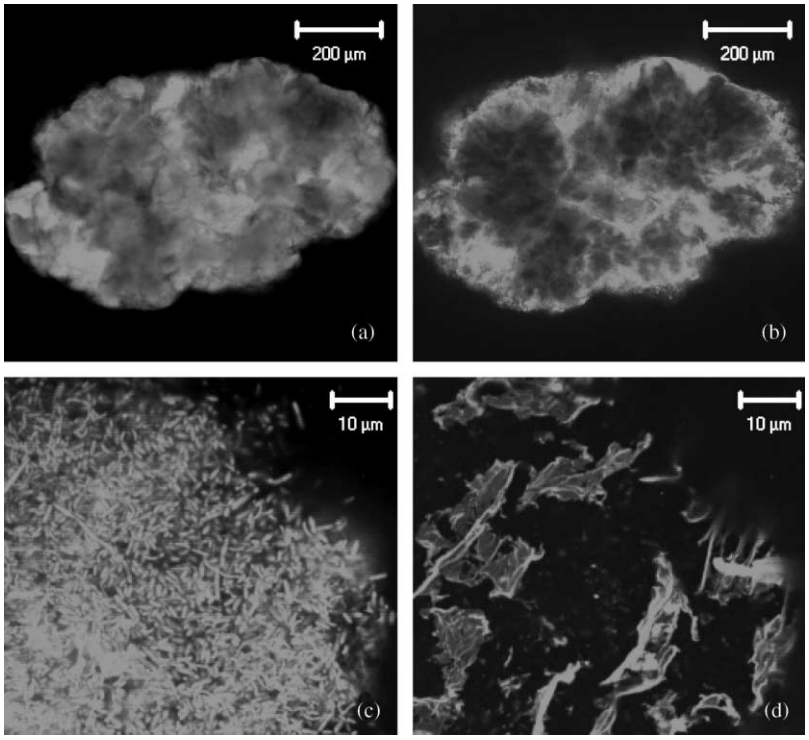


**Figure 5** Images of hydrogen-producing granules stained by (a) DNA-targeting PI and (b) *N*-acetyl-galactosamine/galactose-specific EC-FITC (bar = 200μm); (c) and (d) are the corresponding images under higher magnifications (bar = 10μm).

The OTUs G-HPB-42 and -85 were distantly related to *Clostridium cellulosi* with similarities of 94% and 95%, respectively. The OTU G-HPB-28 was related to an oral clone (*Prevotella* sp. GI032) with a similarity of 92%. Little information is available about them.



#### Cell and polysaccharide distribution in the hydrogen-producing granules

Figure 5 illustrates the images of the hydrogen-producing granule stained by the DNA-specific PI and the *N*-acetyl-galactosamine/galactose-specific EC-FITC probes. Figure 5(a) shows that the distribution of bacteria cells was rather uniform, whereas Figure 5(b) shows that *N*-acetyl-galactosamine/galactose-containing polysaccharides were mostly distributed at the surface of the granule, embodying the bacteria cells and forming a clear boundary. Some formed clusters inside the granule, suggesting that the large granule might be formed by the agglutination of small granules. Figures 5(c) and (d) illustrate that some filamentous polysaccharides may fill in the intercellular spaces inside the granule.

## Conclusions

Overall, packing enhanced the fermentative hydrogen production in upflow reactors. The maximum hydrogen production rates were 6.17 L/L/d in the reactor filled with packing rings and 5.35 L/L/d in the reactor without packing. The maximum hydrogen yields were 1.21 mol/mol-sucrose for the former reactor and 1.10 mol/mol-sucrose for the latter. In both reactors, sludge agglutinated into granules resulting in the increase of biomass concentration. Based on the 16S rDNA analysis, the clones developed from the hydrogen-producing granular sludge in the RP may be classified into 14 OTUs. Most of them were affiliated with the genus *Clostridium*. Results indicated that these species may be a novel group, and thus further study is warranted. *In situ* staining results showed that most of *N*-acetyl-galactosamine/galactose-containing polysaccharides were distributed in the outer layer of the granules with a filamentous structure, whereas the bacteria distributions were rather uniform.

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