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Isolation of Hydrogen-producing Bacteria from Granular Sludge of an Upflow Anaerobic Sludge Blanket Reactor

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Abstract H_2-producing bacteria were isolated from anaerobic granular sludge. Out of 72 colonies (36 grown under aerobic conditions and 36 under anaerobic conditions) arbitrarily chosen from the agar plate cultures of a suspended sludge, 34 colonies (15 under aerobic conditions and 19 under anaerobic conditions) produced H_2 under anaerobic conditions. Based on various biochemical tests and microscopic observations, they were classified into 13 groups and tentatively identified as follows: From aerobic isolates, *Aeromonas* spp. (7 strains), *Pseudomonas* spp. (3 strains), and *Vibrio* spp. (5 strains); from anaerobic isolates, *Actinomyces* spp. (11 strains), *Clostridium* spp. (7 strains), and *Porphyromonas* sp. When glucose was used as the carbon substrate, all isolates showed a similar cell density and a H_2 production yield in the batch cultivations after 12 h (2.24 - 2.74 OD at 600 nm and 1.02-1.22 mol H_2/mol glucose, respectively). The major fermentation by-products were ethanol and acetate for the aerobic isolates, and ethanol, acetate and propionate for the anaerobic isolates. This study demonstrated that several H_2 producers in an anaerobic granular sludge exist in large proportions and their performance in terms of H_2 production is quite similar.

Keywords: hydrogen, granular sludge, UASB, fermentation

H_2 is an efficient energy carrier with a high energy content per unit mass. It is considered to be the cleanest energy carrier because the combustion by-product is only water and it does not produce a green house gas [1]. It can also be used as a raw material for various industrial applications, which is gaining increasing attention [2].

Microbial H_2 production can be either photosynthetic or non-photosynthetic. Photosynthetic H_2 production is carried out by algae [3] or photosynthetic bacteria [4]. Non-photosynthetic or fermentative H_2 production is performed by facultative anaerobes [5,6] or obligate anaerobes [2,7]. The fermentative H_2 process generally has a faster production rate than the photosynthetic process and does not rely on the availability of light. However, the H_2 conversion yield (mol H_2/mol substrate) is lower than that obtained using the photosynthetic process [1,8]. There have been a large number of studies on fermentative H_2 producers with some emphasis on the H_2 production rate and yield from organic carbon [2,6,8,9]. Kumar and Das [8] reported that *Enterobacter cloacae* IIT-BT 08 has a high specific H_2 production rate of 29.6 mmol H_2 (g cell)$^{-1}$ h^{-1} and a high H_2 conversion yield of 2.2 mol H_2/mol glucose. Taguchi *et al.* [2] reported that the H_2 production yields of *Clostridium beijerinckii* AM21B isolated from termites ranged from 1.3 to 2.0 mol H_2/mol glucose. Oh *et al.* [5] reported that *Rhodopseudomonas palustris* P4, although originally isolated for CO-dependent H_2 production, could perform fermentative H_2 production utilizing various organic carbons.

In order to develop a cost-effective H_2 production process, the availability of efficient strains is very important. Thus far, the isolation of the H_2 producers has been mostly dependent on the H_2 production capability of each strain. This requires laborious experiments for growing the many individual cells under well-controlled conditions and measuring their H_2 production activity. This study focused on isolating new H_2-producing bacteria from the biogranules of a UASB (upflow anaerobic sludge blanket) reactor. The UASB reactor is currently being used to treat high-strength organic wastewater and is known to contain many acidogenic bacteria that are often H_2 producers. The anaerobic granules were homogenized, diluted, and plated on agar, and cultivated under aerobic or anaerobic conditions. Some colonies were isolated, identified by various biochemical tests, and finally examined for their individual H_2 production capability in a liquid culture.

The UASB reactor (working volume, 4.5 L) was operated at 35°C with a hydraulic retention time of 12 h in a continuous mode. The feed contents (per liter) were: 2,200 mg glucose, 50 mg of a beef extract, 55 mg of a yeast extract, 50 mg peptone, 50 mg urea, 2,800 mg

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$NaHCO_3$, 66 mg $Na_2HPO_4 \cdot 2H_2O$, 44 mg K_2HPO_4, 36 mg KH_2PO_4, 30 mg K_2SO_4, 6 mg $FeSO_4 \cdot 7H_2O$, 20 mg NH_4Cl, 20 mg $MgSO_4 \cdot 7H_2O$, 1.34 mg $CoCl_2 \cdot 6H_2O$, 76 mg $CaCl_2 \cdot 2H_2O$, 0.05 mg H_3BO_3, 0.11 mg $ZnSO_4$, 0.025 mg $Na_2MoO_4 \cdot 2H_2O$, 0.038 mg $MnSO_4$, 0.07 mg $CuSO_4 \cdot 5H_2O$, 1 mg EDTA, 0.07 mg $NiNO_3$, and 2 mg $AlCl_3 \cdot 2H_2O$ [10]. The granular sludge was dispersed into an aqueous suspension by vortexing it in PBS buffer (pH 7.2, 10 mM Na_2HPO_4, and 0.13% NaCl) for 5 min. The suspension was diluted serially, plated on an NB medium (pH 6.8, 0.3% beef extract, and 0.5% peptone) and incubated at 30°C under either aerobic or anaerobic conditions. The anaerobic conditions were produced by placing the plates in an acrylic desiccator container, and repeatedly vacuuming and purging the container with argon (Ar) gas (99.999%). After incubating for 24-36 h under either aerobic or anaerobic conditions, 72 colonies (36 of each type) were selected randomly and transferred to fresh NB plates at 12 colonies per plate.

The 72 colonies were tested for H_2 production, and 34 colonies (15 grown under aerobic conditions and 19 under anaerobic conditions) were selected as the H_2 producers. They were subsequently characterized by gram-staining, optical microscopy, the colony shape, the oxidase tests, and the catalase tests (oxidase and catalase kits, bioMrieux, France). The 34 colonies were also identified using an API kit (bioMrieux, France). H_2 production was examined in a mineral salt medium [11] containing 100 mM phosphate, 3 g yeast extract/L and 5 g glucose/L. A 165 mL serum bottle (working volume, 50 mL) was used. After inoculation, the bottle was flushed with Ar gas for 5 min and sealed with a 12 mm-thick butyl rubber septum and an aluminum cap. The cultivation was performed at 30°C for 12 h in a gyratory incubator with a shaking speed of 250 rpm. The inoculum was cultivated in the same bottle and transferred anaerobically during the late-exponential phase by a sterile hypodermic disposable syringe.

The biomass concentration was measured at 600 nm with a spectrophotometer (Lambda 20, Perkin-Elmer, USA). The H_2 gas was sampled from the headspace of the culture bottles with a 250 μL pressure-lock gastight syringe (1750SL, Hamilton Company, Reno, NV, USA) and measured by a gas chromatograph (GC) equipped with a thermal conductivity detector and a stainless steel column (6 x 1/8) packed with a Molecular Sieve 5A (80/100 mesh; Alltech, Deerfield, IL, USA). Ar was used as the carrier gas at a flow rate of 30 mL/min. The oven, injector and detector temperature were at 80°C, 90°C and 120°C, respectively. Organic acids and ethanol in the culture broth were also analyzed by a GC equipped with a flame ionization detector and a 0.25 mm (I.D.) x 30 m HP-INNOWax capillary column (Agilent Technologies, Forster, CA, USA). N_2 was used as a carrier gas in this case at a flow rate of 1 mL/min. The injection volume and split ratio were 1 μL and 30:1, respectively. The injector and detector were kept at 220°C and 260°C, respectively, while the column was held at 60°C for 2 min, heated to 200°C at 10°C/min

Table 1. Identity and H_2 production of the microorganisms isolated

Isolation condition	Colony group[1] (number)	Microorganism	Cell growth and H_2 production[2]						
			H_2 production (mL)	H_2 yield (mol/ mol glucose)	Final biomass (OD_{600})	Final pH	Major metabolites (mg/L)		
							Ethanol	Acetate	Propionate
Aerobic	A-1 (2)	*Aeromonas* spp.	29.7	1.13	2.38	6.21	1,062	647	nd
	A-2 (2)	*Aeromonas* spp.	28.4	1.02	2.24	6.20	820	621	nd
	A-3 (3)	*Aeromonas* spp.	29.8	1.10	2.32	6.22	984	461	nd
	A-4 (3)	*Pseudomonas* spp.	29.6	1.09	2.37	6.21	919	529	nd
	A-5 (2)	*Vibrio* spp.	30.6	1.22	2.44	6.21	810	421	nd
	A-6 (3)	*Vibrio* spp.	29.5	1.12	2.43	6.21	1,180	896	nd
Anaerobic	An-1 (2)	*Actinomyces* spp.	33.0	1.21	2.54	6.26	450	348	323
	An-2 (4)	*Actinomyces* spp.	32.4	1.15	2.39	6.24	514	325	312
	An-3 (3)	*Actinomyces* spp.	31.2	1.16	2.38	6.22	410	387	349
	An-4 (2)	*Actinomyces* spp.	31.2	1.18	2.46	6.23	449	412	357
	An-5 (6)	*Clostridium* spp.	32.2	1.15	2.74	6.24	634	446	440
	An-6 (1)	*Clostridium* spp.	32.7	1.17	2.33	6.22	383	333	305
	An-7 (1)	*Porphyromonas* sp.	29.7	1.08	2.60	6.21	391	275	272

nd, not detectable.

1) The 15 colonies (aerobic conditions) and 19 colonies (anaerobic conditions) were respectively classified into 6 and 7 groups according to the gram-staining, microscopic observations, the colony shape, the oxidase tests, and the catalase tests.

2) Cell growth and H_2 production of all strains was conducted for 12 h under anaerobic conditions.

and maintained at 200°C for 1 min.

Table 1 shows the isolated strains along with their growth and H_2 production rates. The strains were classified into 13 groups based on the various biochemical tests and microscopic observations. There were 6 groups in the aerobic isolates and 7 groups in the anaerobic isolates. Further identification by API analyses showed that the aerobic isolates were *Aeromonas* spp. (7 strains), *Pseudomonas* spp. (3 strains), and *Vibrio* spp. (5 strains), and the anaerobic isolates were *Actinomyces* spp. (11 strains), *Clostridium* spp. (7 strains), and *Porphyromonas* sp. It should be noted that, although 3 groups of *Aeromonas*, 2 groups of *Vibrio*, 4 groups of *Actinomyces*, or 2 groups of *Clostridium* were identified to be the same genus according to API tests, their biochemical characteristics were not identical. Among the aerobic isolates, *Aeromonas* spp. and *Vibrio* spp. belong to the γ-purple bacteria and *Pseudomonas* spp. belong to the β-purple bacteria. The photosynthetic or fermentative H_2 production by some purple bacteria has been well documented [12,13]. Among the anaerobic isolates, fermentative H_2 production by *Clostridium* spp. has been studied most extensively; while the H_2 production by *Actinomyces* spp. and *Porphyromonas* sp. to the best of the author's knowledge has not been detected. Further investigations including 16S rDNA analyses are currently underway.

Table 1 also summarizes the batch fermentation results with the new isolates when glucose was used as the carbon source. A high phosphate buffer of 100 mM and a relatively low glucose concentration of 5 g/L were employed in this experiment because the medium pH usually decreases drastically due to the accumulation of organic acids as fermentation proceeds and a lower pH suppresses H_2 production significantly (data not shown). The final pH of the culture broth was at approximately 6.2, indicating that the fermentation conditions were appropriate. Regardless of the screening conditions or the type of microbial species, all isolates could produce H_2 during anaerobic cultivation. The major by-products were ethanol and acetate for the aerobic isolates, and ethanol, acetate and propionate for the anaerobic isolates. No detectable propionate was found in the fermentation broth of the aerobic isolates, suggesting that there are distinctive differences in the carbon metabolism between the aerobic and anaerobic isolates.

The final cell density and H_2 production yields of all the species were approximately 2.24-2.74 A_{600} and 1.02-1.22 mol H_2/mol glucose, respectively. The cell density is primarily dependent on the initial glucose concentration and is not a key factor in evaluating the performance of a H_2 producer. In contrast, the H_2 production yield for glucose is the most critical in determining the economy of the biological production of H_2. Since one of the motivations of this study was obtaining some new isolates with a high H_2 production yield or at the least observing some significant variation in the H_2 yield among the isolates, observation of the same H_2 yield was somewhat disappointing. Nevertheless, it is believed that these results are important at least in the following two aspects. First, the H_2 yield (mol H_2/mol glucose) of the present isolates was not low and was generally similar to that of most other reported strains (the glucose concentration in the parenthesis indicates the initial concentration used in the experiments). The production yields are as follows: *Rhodopseudomonas palustris* P4, 1.33 (glucose, 5 g/L) [5]; *Enterobacter aerogenes* strain HO-39, 1.0 (glucose, 10 g/L) [6]; *E. cloacae* IIT-BT 08, 2.2 (glucose, 10 g/L) [8]; *Citrobacter intermedius*, 0.27-1.14 (glucose, 7.7 g/L) [14]; *C. freundii*, 1.29 (glucose, 7.7 g/L) [9]; *Clostridium beijerinckii* AM21B, 1.3-2.0 (glucose, 10 g/L) [2]; and *Cl. butyricum* strain SC-E1, 1.4–2.2 (glucose, 10 g/L) [15]. However, there remains the possibility of finding a superb strain from the 34 isolates by carefully optimizing the culture conditions such as the glucose concentration, temperature, and the H_2 partial pressure for each strain because the conditions giving the maximum H_2 yield tend to be species-specific. Secondly, and more importantly, most H_2 producers from the UASB reactor have a similar H_2-to-glucose yield and further efforts at trying to identify a high-yield strain from UASB granules are not rewarding. This suggests that further screening work of H_2 producers should be conducted with microbial consortia that are distinctively different from the UASB granules.

In conclusion, extensive screening for H_2-producing bacteria from anaerobic granular sludge was carried out. Among the 72 colonies chosen, 34 exhibited H_2-producing capability under anaerobic conditions with glucose as the carbon source. This suggests that the UASB granule is a rich source of fermentative H_2-producing bacteria.

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