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Production of Hydrogen and Volatile Fatty Acid by *Enterobacter* sp. T4384 Using Organic Waste Materials

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In a study of hydrogen-producing bacteria, strain T4384 was isolated from rice field samples in the Republic of Korea. The isolate was identified as *Enterobacter* sp. T4384 by phylogenetic analysis of 16S rRNA and *rpoB* gene sequences. *Enterobacter* sp. T4384 grew at a temperature range of 10–45°C and at an initial pH range of 4.5–9.5. Strain T4384 produced hydrogen at 0–6% NaCl by using glucose, fructose, and mannose. In serum bottle cultures using a complete medium, *Enterobacter* sp. T4384 produced 1,098 ml/l H_2, 4.0 g/l ethanol, and 1.0 g/l acetic acid. In a pH-regulated jar fermenter culture with the biogas removed, 2,202 ml/l H_2, 6.2 g/l ethanol, and 1.0 g/l acetic acid were produced, and the lag-phase time was 4.8 h. Strain T4384 metabolized the hydrolysate of organic waste for the production of hydrogen and volatile fatty acid. The strain T4384 produced 947 ml/l H_2, 3.2 g/l ethanol, and 0.2 g/l acetic acid from 6% (w/v) food waste hydrolysate; 738 ml/l H_2, 4.2 g/l ethanol, and 0.8 g/l acetic acid from *Miscanthus sinensis* hydrolysate; and 805 ml/l H_2, 5.0 g/l ethanol, and 0.7 g/l acetic acid from *Sorghum bicolor* hydrolysate.

Key words: Hydrogen, *Enterobacter* sp. T4384, food waste, *Miscanthus sinensis*, *Sorghum bicolor*

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Biological approaches are promising alternatives for fuel production as fossil fuel resources approach the limits of economical extraction [7]. As a fuel, H_2 is a nonpolluting, recyclable, and efficient energy source, and biologically produced H_2 is more environmentally friendly and less energy intensive than H_2 produced by thermochemical or electrochemical processes [4]. Both photosynthetic and fermentative organisms are known to produce biological H_2. Some algae and cyanobacteria produce H_2 photobiologically [1], and diverse fermentative bacteria produce H_2 during their fermentation processes, including strains of *Bacillus* [9], *Citrobacter* [30], *Clostridia* [10, 16], *Enterobacter* [8, 11, 23, 27], *Escherichia* [24], and *Rhodopseudomonas* [21]. Fermentative bacteria present particular advantages for biological H_2 production, such as the utilization of various carbon sources and raw materials, continuous H_2 production without light, valuable metabolites, and no oxygen limitation problems [4]. However, H_2 production using fermentation usually demands high-cost carbon sources such as glucose or starch, and therefore, a supply of a cheap carbon source, especially renewable resources, is needed for economic H_2 production.

The carbon source plays an important role in bio-H_2 production, and bacteria strains consuming a wide range of carbon sources would be attractive for future studies. In the Republic of Korea, owing to an abundance of food, a huge amount of food wastes are produced daily, which account for approximately 40% of the total amount of garbage produced every year. The generation of food waste reaches 11,237 T/day in the Republic of Korea, accounting for 23.2% of municipal solid waste [5]. Food waste is a carbohydrate-rich organic solid waste; therefore, it could

be used as a feedstock for fermentative H_2 production in the future. However, the NaCl content in food waste, which may be in the range 0–2% or even higher, can inhibit fermentative H_2 production. Thus, microorganisms should both consume diverse carbon sources and tolerate high contents of NaCl for the most effective production of H_2 from food waste. *Sorghum bicolor* and *Miscanthus sinensis*, fast-growing and drought-resistant perennial grasses performing C4-type photosynthesis, are potentially useful feedstocks of lignocellulosic biomass [29, 31]. Therefore, these grasses also can be used for bioenergy production after being hydrolyzed to monosaccharides.

Rice fields and sludge are good sources for bacteria with diverse metabolic functions. In this current study of biogas-producing bacteria collected from rice fields and sludge, a H_2-producing strain was selected among the isolates. The isolate, designated strain T4384, was studied for its taxonomic position, growth condition, and H_2 and volatile fatty acid production activities in complete medium. Finally, the production of H_2 and volatile fatty acid by strain T4384 was assessed using fermentable organic waste materials such as hydrolysates of food waste, *Miscanthus sinensis*, and *Sorghum bicolor*.

Materials and Methods

Microorganism and Culture Conditions

A bacterium, designated strain T4384, was isolated as a biogas producer from rice fields in the Republic of Korea. A complete medium (CM) was used for the selection of the biogas-producing strain and bacterial cultivation. The CM was composed as follows: 30 g D-glucose, 4 g yeast extract, 5 g malt extract, 5 g tryptone, 1.5 g K_2HPO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.1 g $MnSO_4 \cdot 7H_2O$, 0.015 g $FeSO_4 \cdot 7H_2O$, 0.1 g NaCl, and 0.25 g cysteine hydrochloride monohydrate dissolved in distilled H_2O to 1 L, pH 6.5. The medium for carbon source utilization was a minimal medium (MM) that was composed as follows: 30 g D-glucose, 1 g yeast extract, 0.5 g K_2HPO_4, 0.5 g KH_2PO_4, 2.2 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.01 g $MnSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, and 0.01 g NaCl dissolved in distilled H_2O to 1 L, pH 6.5.

The growth temperature was determined using CM plates incubated at 4°C, 10°C, 15°C, 25°C, 30°C, 35°C, 37°C, 40°C, 45°C, and 50°C. The optimal initial pH values suitable for cell growth and H_2 production were tested in MM broth after an adjustment of the pH with 1 M HCl or 1 M NaOH (pH 4.0–10.0 in 0.5 pH unit increments) after being sterilized by filtration. The concentration of NaCl for cell growth and H_2 production was measured in CM and MM broth containing different concentrations of NaCl. The utilization of carbohydrate for cell growth and H_2 production was tested with MM broth containing diverse carbon sources, such as cellulose, cellobiose, fructose, lactose, mannose, maltose, sucrose, or xylose, instead of glucose. Each broth medium was prepared in a serum bottle or a fermentation jar, purged with argon gas to create an anoxic environment, and cultured at 37°C after inoculation. For serum bottle cultures, 150 ml serum bottles containing 60 ml of medium were used for cell growth and H_2 and VFA production. A seed culture [2.5% (v/v)] was inoculated in each medium and cultivated for two days unless otherwise specified.

The effects of pH regulation and biogas removal during fermentation on cumulative H_2 production were tested in pH-controlled jar fermenter cultures using 5-L fermentation jars containing 2 L of CM broth after inoculation of 10% (v/v) seed culture. Biogas dissolved in the jar fermenter culture broth was removed *via* an argon purge for 10 min (flow rate 80 ml/min) every 2 h for 12 h.

Analytical Methods

Fermentation products were analyzed with gas chromatographs (GC, Model 6890N; Agilent Technologies) equipped with a thermal conductivity detector (TCD) or a flame ionized detector (FID). For analysis of CO_2 and H_2 in the gas phase, 100 µl of gas sample from the head space of the culture bottle was collected in a gas-tight syringe (Hamilton, Reno, NV, USA) and injected into a GC equipped with a TCD. Argon was used as a carrier gas at a flow rate of 20 ml/min. The operational temperatures of the oven, the injector port, and the detector were 50°C, 100°C, and 200°C, respectively. For the analysis of liquid-phase fermentation products such as ethanol and acetic acid, the culture broth was filtered with a 0.22 µm filter and injected into a GC equipped with FID and an HP-INNOWAX column (30 m × 320 µm × 0.25 µm; Agilent Technologies). Helium was used as the carrier gas at a flow rate of 1 ml/min. The oven temperature was programmed to increase from 50°C to 170°C at a rate of 1°C/min. The injector and detector temperatures were set to 250°C. The glucose concentrations in the culture broth were analyzed using a Reflect Quant Strip (Glucose Test Strip, Merck Co., Ltd.). The amount of gas produced in jar fermenter cultivation was measured by a Wet Gas Meter (Model W-NK-1A; Shinagawa Corp, Japan).

16S rRNA and *rpoB* Gene Sequence Analysis

DNA was extracted using a PowerSoil DNA kit (MO BIO Laboratories Inc., CA, USA). The 16S rRNA gene of strain T4384 was amplified by PCR with two universal primers that are specific for most bacteria: forward primer 27F, 5'-AGA GTT TGA TCC TGG CTC AG-3'; and reverse primer 1492R, 5'-TAC GGY TAC CTT GTT ACG ACT T-3' [13]. The *rpoB* gene of strain T4384 was amplified by PCR with two primers: forward primer CM_{7f}, 5'-AAC CAG TTC CGC GTT GGC CTG G-3'; and reverse primer CM_{31r}, 5'-CCT GAA CAA CAC GCT CGG A-3' [18]. The PCR product was purified using a QIAquick PCR purification kit (Qiagen) and sequenced using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), with an automated DNA sequencer (Model 377; Applied Biosystems). Using CLUSTAL X software [28], the 16S rRNA and *rpoB* gene sequences of strain T4384 were aligned with those of representatives of related taxa acquired from the Eztaxon server [3] and/or GenBank using the BLAST program (http://www.ncbi.nlm.nih.gov/blast/). A phylogenetic tree was constructed with the MEGA5 (Molecular Evolutionary Genetics Analysis) program [26] using the neighbor-joining method [22] based on distance-matrix data. The topology of the phylogenetic tree was evaluated with a bootstrap analysis of the neighbor-joining data based on 1,000 replications.

Kinetics of H_2 Production

The desired metabolic product in this study was H_2 in the gas phase. Therefore, the formation kinetics of H_2 were studied. A modified

Gompertz equation [14] was applied to simulate the accumulated gas production in a jar fermenter culture:

$$H = P_{max} \times \exp(-\exp(R_{max} \times e \div P_{max} \times (\gamma - t) + 1))$$

where H is the cumulative H_2 production per liter of reactor volume at cultivation time t (h), P_{max} is the potential maximum H_2 production (ml) per liter of reactor volume, R_{max} is the maximum rate of H_2 production (ml/l/h), e is 2.718281828, and γ is the lag-phase time (h).

Utilization of Organic Waste Materials

As fermentable organic waste materials for the production of H_2 and VFA by strain T4384, the hydrolysate samples of food waste, *Miscanthus sinensis*, and *Sorghum bicolor* were used as carbon sources. For utilization of food waste, dried food waste, which was supplied by E-Service Corp., Seoul, Republic of Korea, was suspended in water to make 6% and 12% (w/v) suspensions and was hydrolyzed with 1% (v/v) of a multi-enzyme complex (Viscozyme; Novozymes Corp.) at 37°C for 3 h. The enzyme hydrolysate of food waste was adjusted to pH 7 with 2 N KOH and used as a culture medium without any additional ingredient for bacterial growth. *Miscanthus sinensis* and *Sorghum bicolor* were decomposed using acid hydrolysis. To obtain acid hydrolysates, 20 g of dried *M. sinensis* or *S. bicolor* was reacted with 32 ml of 24 N sulfuric acid at 30°C for 1 h. Then, the solution was diluted with 128 ml of distilled water and finally heated to 105°C for 1 h for the hydrolysis of cellulose [2]. After cooling and filtration, the acid hydrolysate was neutralized to pH 7 with 2 N KOH. The concentrations of glucose in the neutralized acid hydrolysate were 46 g/l and 43 g/l from *M. sinensis* and *S. bicolor*, respectively. Strain T4384 was cultured on the hydrolysate of *M. sinensis* and *S. bicolor* containing the ingredients of the MM medium except glucose.

RESULTS AND DISCUSSION

Identification of H_2-Producing Bacteria

A H_2-producing strain was selected among isolates from rice fields and sewage sludge in the Republic of Korea. The isolate, designated strain T4384, showed the highest

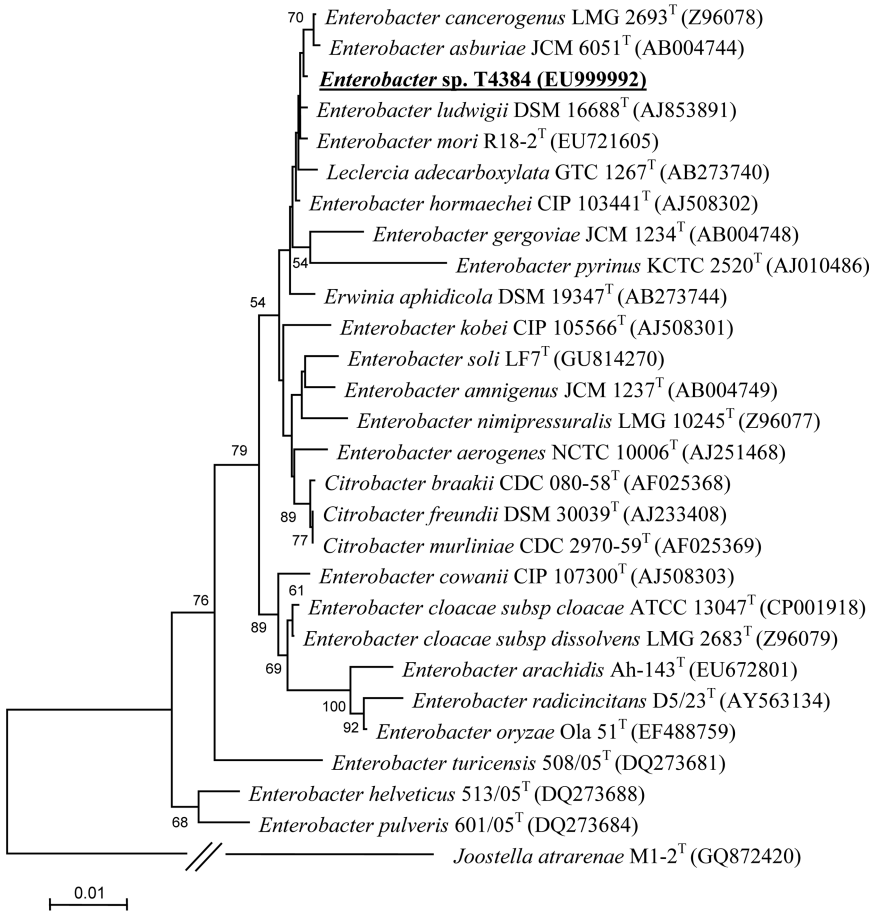

Fig. 1. Neighbor-joining phylogenetic tree derived from 16S rRNA gene sequence analysis of *Enterobacter* sp. T4384 and closely related organisms belonging to the Enterobacteriaceae of the class Proteobacteria.

The sequence of *Joostella atrarenae* M1-2T was used as an outgroup. The GenBank accession numbers of the 16S rRNA gene sequences are provided parenthetically. Bootstrap confidence values (based on 1,000 replications) greater than 500 are indicated as percentages at the branching points. The scale bar indicates 0.01 substitutions per nucleotide position.

level of gas production. Strain T4384 metabolized 19 g/l glucose, and produced 1,098 ml/l H_2, 4,261 ml/l CO_2, 4.0 g/l ethanol, and 1.0 g/l acetic acid over two days in a batch culture with CM broth.

A nearly complete 1,462-bp 16S rRNA gene sequence of strain T4384 was determined. In a phylogenetic study using 16S rRNA gene sequences, strain T4384 was found to belong to the genus *Enterobacter*, as seen in a neighbor-joining phylogenetic tree (Fig. 1). Strain T4384 represented the highest level of 16S rRNA similarity with *Enterobacter ludwigii* DSM 16688^T (99.65%), followed by *Enterobacter cloacae* subsp. *cloacae* ATCC 13047^T (99.31%), according to analysis using the Eztaxon server.

As with the 16S rRNA gene, the *rpoB* encoding RNA polymerase beta subunit is a universal gene, and the similarity of *rpoB* sequences has been used as a basis for phylogenetic analyses among some bacteria [18, 19]. For additional taxonomic study, the *rpoB* gene of strain T4384 was amplified, and a 1,005 bp partial sequence of the *rpoB* gene from strain T4384 was compared with that of type strains of the *Enterobacter* species. The *rpoB* gene sequence of strain T4384 was most closely related with that of *Enterobacter kobei* ATCC BAA260^T, GenBank Accession No. AJ566947, with 99.8% similarity. The *rpoB* gene sequence similarity with *E. ludwigii* DSM 16688^T and *E. cloacae* subsp. *cloacae* ATCC 13047^T was 98.2% and 97.4%, respectively.

According to the analysis of the two genes used for taxonomic study, the novel isolate T4384 was identified as *Enterobacter* sp. T4384; however, the most closely related type strains in the similarity analyses of the *rpoB* and 16S rRNA genes were not the same one. Therefore, as a future study, additional taxonomic study, such as a DNA–DNA relatedness test, may be needed for the designation of *Enterobacter* sp. T4384 as a species. The GenBank accession numbers for the 16S rRNA and *rpoB* gene sequences of strain T4384 are EU999992 and JN627207, respectively.

Effects of Carbon Source, Temperature, pH, and NaCl on the Growth of Strain T4384

Enterobacter sp. T4384 grew at 10–45°C, optimally at 37°C, and at pH 4.5–9.5, optimally at pH 6.0–7.0. Like other reports presenting the effect of pH on H_2 production [27], the pH of the growth medium influenced the production of H_2 by *Enterobacter* sp. T4384.

Enterobacter sp. T4384 utilized fructose, glucose, and mannose for the production of H_2, but not cellulose, cellobiose, lactose, maltose, sucrose, and xylose. *Enterobacter* sp. T4384 produced H_2 at concentrations of NaCl ranging from 0% to 6% (w/v), optimally at 1–2%. In CM broth containing 2% (w/v) NaCl, strain T4384 utilized 21.6 g/l glucose and produced 1,189 ml/l H_2, 5.6 g/l ethanol, and 1.1 g/l acetic acid (Table 2). It was reported that H_2 production by *Enterobacter aerogenes* HGN-2 and HT 34 was negatively influenced by NaCl concentrations above 1% (w/v) [8]. However, H_2 production by *Enterobacter* sp. T4384 was not inhibited at up to 6% (w/v) NaCl.

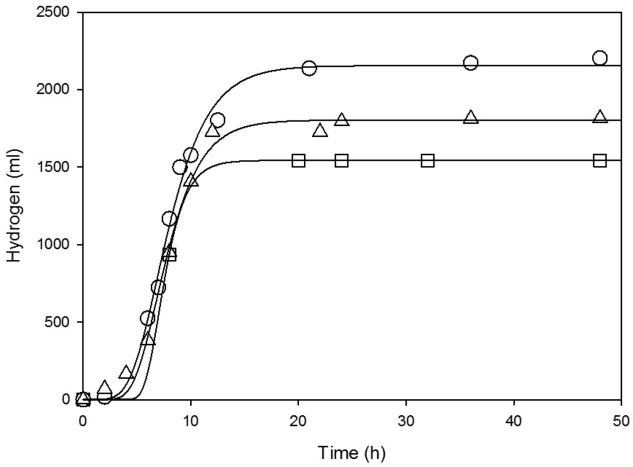

Fig. 2. H_2 production by *Enterobacter* sp. T4384 on complete medium at pH 5.5 and pH 6.5 with or without sporadic H_2 removal using argon purging.
H_2 production was plotted using a modified Gompertz equation. Symbols: ○; pH 6.5 with argon purging, △; pH 6.5, □; pH 5.5.

Effects of pH Regulation and Sporadic Gas Removal on Cumulative H_2 Production

Cumulative H_2 production by *Enterobacter* sp. T4384 was influenced by stable pH conditions and biogas removal during fermentation in batch cultures. *Enterobacter* sp. T4384 produced more H_2 in pH-regulated jar fermenter culture than in serum bottle culture. In jar fermenter cultivation, *Enterobacter* sp. T4384 produced 2,202 ml/l and 1,814 ml/l H_2 with or without sporadic argon gas purging in CM broth at pH 6.5, respectively (Fig. 2, Table 2), and intermittent argon purging improved H_2 production by 21.4%.

The enhanced H_2 production observed during sporadic purging is in accord with previous reports that showed increases in H_2 production resulting from decreased H_2 partial pressure or intermittent purging of culture broth by argon or nitrogen gas [15, 17, 21].

The fitted curves for H_2 production by *Enterobacter* sp. T4384 are shown in Fig. 2, and the parameters estimated for the formation of H_2 are summarized in Table 1. Purging increased the P_{max} value but not R_{max}, and at pH 6.5, P_{max} and R_{max} increased, but the lag phase time was decreased compared with the values at pH 5.5. All correlation coefficients of nonlinear analysis by modified Gompertz equations were over 0.99, suggesting that the equation was able to adequately describe H_2 production under these culture conditions.

In a review paper, Hawkes *et al.* [6] noted that reduced fermentation end products such as ethanol and other alcohols yield less H_2 because additional H atoms are

Table 1. H_2 production calculated using a modified Gompertz equation.

Culture conditions[a]	P_{max} (ml/l)	R_{max} (ml/l/h)	γ (h)	R^2
CM at pH 5.5	1,543	418	5.6	1.0000
CM at pH 6.5	1,802	299	4.6	0.9970
CM at pH 6.5 with purging[b]	2,154	308	4.8	0.9969

[a]Cells were cultured in a pH-regulated jar fermenter containing complete medium (CM).
[b]Biogas in culture broth was sporadically removed by argon gas purging during fermentation.

contained in ethanol compared with its corresponding acid. Kumar *et al*. [12] reported increased H_2 yield in a mutant in which ethanol production was decreased. It seems that the production of H_2 by *Enterobacter* sp. T4384 may increase if ethanol production in this strain is reduced. Meanwhile, H_2 production with immobilized cells is needed for continuous H_2 production [16]. Further fermentation studies using immobilized *Enterobacter* sp. T4384 are required to determine the optimal conditions for the highest and continuous levels of H_2 production.

Production of H_2 and VFA Using Organic Waste Materials

Enterobacter sp. T4384 produced H_2 from CM and MM containing 6% (w/v) NaCl. Food waste, an organic waste material, was used for the production of H_2 and VFA by *Enterobacter* sp. T4384. Food waste in the Republic of Korea typically contains high NaCl content [20]; as such, it is not suitable for H_2 production by strains that are inhibited by high NaCl.

Enterobacter sp. T4384 utilized food waste as a carbon source and produced H_2 and VFA. The hydrolysis of carbohydrate in food waste increased the production of metabolites by *Enterobacter* sp. T4384. From a 6% (w/v) food waste hydrolysate, strain T4384 produced 947 ml/l H_2, 3.2 g/l ethanol, and 0.2 g/l acetic acid. Only a slight increase in metabolic products was observed at 12% food hydrolysate (Table 2).

In addition, *M. sinensis* and *S. bicolor* were also used for H_2 production by *Enterobacter* sp. T4384. With acid hydrolysates of *M. sinensis* and *S. bicolor*, *Enterobacter* sp. T4384 produced 738 ml/l H_2 and 4.2 g/l ethanol and 805 ml/l H_2 and 5.0 g/l ethanol, respectively (Table 2). Even though the glucose content of an MM containing acid hydrolysate of *S. bicolor* was less than that of *M. sinensis*, more metabolites were produced from *S. bicolor*. This may be due to the presence of inhibitors, such as phenolic compounds, present in the acid hydrolysate [25].

The maximum and overall hydrogen productivities by *Enterobacter* sp. T4384 were 325 and 143 ml/l/h, at pH 6.5 with purging and without purging, respectively, in CM. These values are a bit lower compared with other species of *Enterobacter*, such as *Enterobacter asburiae* SNU-1. In the case of *Enterobacter asburiae* SNU-1, those productivities were 398 and 174 ml/l/h, respectively, in a complete medium [23]. The lower productivity of *Enterobacter* sp. T4384 in complete medium may be due to the production of ethanol and the difference of medium composition.

Table 2. Metabolites produced by *Enterobacter* sp. T4384 in diverse culture conditions using complete media and waste materials.

Culture conditions	H_2 (ml/l)	Ethanol (g/l)	Acetic acid (g/l)
CM containing 0% NaCl	1,098	4.0	1.0
CM containing 1% NaCl	1,172	5.4	1.1
CM containing 2% NaCl	1,189	5.6	1.1
Jar fermenter with purging[a]	2,202	6.2	1.0
Jar fermenter without purging[a]	1,814	5.2	0.8
6% Food waste	179	0.5	0.6
6% Food waste + enzyme[b]	947	3.2	0.2
12% Food waste	330	0.9	1.0
12% Food waste + enzyme[b]	992	4.3	0.5
Miscanthus sinensis[c]	738	4.2	0.8
Sorghum bicolor[c]	805	5.0	0.7

[a]10% (v/v) seed culture was inoculated into complete medium (CM) in a jar fermenter and pH was controlled at 6.5 during fermentation.
[b]Aqueous food waste suspension pretreated with 1% (v/v) Viscozyme.
[c]Acid hydrolysate of organic waste material was used instead of glucose in minimal medium.

Even though the amounts of H_2 and other metabolites produced by *Enterobacter* sp. T4384 using waste materials were less than those produced from CM, its NaCl tolerance and utilization of hydrolysates of food waste, *M. sinensis*, and *S. bicolor* make it a suitable bacterial strain for H_2 production using renewable organic waste materials.

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