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Water-insoluble material from apple pomace makes changes in intracellular NAD^+/NADH ratio and pyrophosphate content and stimulates fermentative production of hydrogen

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Apple pomace is one of the major agricultural residues in Aomori prefecture, Japan, and it would be useful to develop effective applications for it. As apple pomace contains easily fermentable sugars such as glucose, fructose and sucrose, it can be used as a feedstock for the fermentation of fuels and chemicals. We previously isolated a new hydrogen-producing bacterium, *Clostridium beijerinckii* HU-1, which could produce H_2 at a production rate of 14.5 mmol of H_2/L/h in a fed-batch culture at 37 °C, pH 6.0. In this work we found that the HU-1 strain produces H_2 at an approximately 20% greater rate when the fermentation medium contains the water-insoluble material from apple pomace. The water-insoluble material from apple pomace caused a metabolic shift that stimulated H_2 production. HU-1 showed a decrease of lactate production, which consumes NADH, accompanied by an increase of the intracellular pyrophosphate content, which is an inhibitor of lactate dehydrogenase. The intracellular NAD^+/NADH ratios of HU-1 during H_2 fermentation were maintained in a more reductive state than those observed without the addition of the water insoluble material. To correct the abnormal intracellular redox balance, caused by the repression of lactate production, H_2 production with NADH oxidation must be stimulated.

[**Key words:** Apple pomace; NAD^+/NADH; Pyrophosphate; Lactate; Hydrogen]

Apples and apple juice are the main farm products of Aomori prefecture, Japan; however, apple pomace (AP) including nonstandard apples is produced as a byproduct every year, and developing an effective use for it would be valuable. In this work, we focused on fermentative production of hydrogen (H_2) as an alternative energy resource. H_2 is widely recognized as a clean and efficient energy resource. It has the highest energy yield (143 GJ t^{-1}) of any common fuel (1) and is the only common fuel that is not chemically bound to carbon. Therefore, burning H_2 produces only water and does not contribute to the greenhouse effect. CO_2 emissions from human activities using fossil resources are the largest contributor to the greenhouse effect, and the reduction of CO_2 emissions and the sequestration of CO_2 are thus crucial global aims. One way for these aims to be accomplished is by transitioning society from petroleum-based energy production to production based on biomass. The appropriate production and application of biomass for energy production can help to reduce atmospheric CO_2 levels. H_2 is produced commercially by conventional production processes such as the steam reforming of natural gas and the electrolysis of water. However, these processes are highly energy-intensive and are not always suitable for the environment.

Biological production processes are carried out at ambient temperatures and pressures. Among the various H_2 production processes, biological production processes are known to be less energy intensive than chemical and electrochemical processes (2) and can be divided into fermentative production and photosynthetic production processes (3,4). AP is rich in fructose, glucose and sucrose, which are easily fermentable to H_2 by microorganisms such as Clostridia without any pretreatments. Clostridia such as *Clostridium butyricum, C. acetobutylicum* and *C. beijerinckii* have been known to be some of the strongest and most efficient H_2 producers (5–8). However, the varying compositions of different kinds of biomass often make it difficult to produce energy efficiently. We believe that the ability of microorganisms to use various carbon sources in their living environment (e.g., farmland, forestland, wetland) is evolving. Thus, we have previously screened bacteria from farmland in Aomori prefecture to identify new bacteria with a high ability to produce H_2 using AP as a carbon source. In the present work, we examined the effect of AP on fermentative H_2 production using a newly isolated H_2-producing anaerobe, *C. beijerinckii* HU-1.

MATERIALS AND METHODS

Bacterial strains and culture conditions The bacterium, *C. beijerinckii* strain HU-1, was isolated from farmland in Aomori, Japan. To isolate the bacteria responsible for H_2 production, we suspended 2 g each of several agricultural residues from farmlands into a sterile complex YTM medium containing 100 mM

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glucose as a carbon source. The YTM medium is composed of the following materials (per liter): yeast extract, 5.0 g; tryptone, 5.0 g; K_2HPO_4, 7.0 g; KH_2PO_4, 5.5 g; L-cysteine, 0.5 g; $(NH_4)_2SO_4$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; $CaCl_2 \cdot 2H_2O$, 0.021 g; $CoSO_4 \cdot 7H_2O$, 0.028 g; $FeSO_4 \cdot 7H_2O$, 0.0216 g; nicotinic acid, 2 mg; Na_2SeO_3, 0.172 mg; $NiCl_2 \cdot 6H_2O$, 0.035 mg and 10 mL of trace element solution. The trace element solution contains (per liter): $MnCl_2 \cdot 4H_2O$, 0.5 g; H_3BO_3, 0.1 g; $AlK(SO_4)_2 \cdot 12H_2O$, 0.017 g; $CuSO_4 \cdot 5H_2O$, 0.0015 g; Na_2EDTA, 0.5 g. Serial dilutions of these suspensions were plated and cultivated on YTM agar plates containing 100 mM glucose in an Anaero Pack (anaerobic cultivation system) (Mitsubishi Gas Chemical Co. Inc., Tokyo, Japan) at 30 °C. For plating, 1.5% agar was added to the medium. Several isolates were further examined for H_2 production in 35 mL test tubes containing 10 mL of YTM medium containing 100 mM glucose. The strain showing the best ability to produce H_2 among the isolates was selected. The biochemical and biophysical analysis were carried out by the TechnoSuruga Laboratory Co., Ltd. (Shizuoka, Japan). *C. beijerinckii* NCIMB8052 (ATCC51743) (9), as a comparative strain, was also routinely cultured at 30 °C on a YTM plate containing 100 mM glucose or a thioglycollate medium (TGC) plate (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan).

Determination of 16S rDNA sequences and phylogenetic analysis Genomic DNA was extracted and purified with an Illustra Bacteria Genomic Prep Mini Spin Kit (GE Healthcare UK Ltd., Buckinghamshire, UK) according to the manufacturer's instructions. To determine the 16S rDNA gene sequence, 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-CGGTTACCTTGTTACGACTT-3′) primers were used to amplify the approximately 1500-bp internal segment of the 16S rDNA gene. The nucleotide sequence of the gene was determined and registered in the DDBJ, EMBL and GenBank nucleotide sequence databases under accession no. AB771748. A search for similar sequences was done with the blastn program, and the matching sequences were retrieved from the database and aligned with the Clustal W program (10).

Evaluation of H_2-producing ability of *C. beijerinckii* HU-1 The effects of pH (pH 5.5, 6.0, 6.5, and 7.0), temperature (25 °C, 30 °C, and 37 °C) and agitation speed (50, 100, 150, and 200 rpm) on the H_2-producing ability were evaluated in fed-batch culture. The bacterium was precultured in a 35-mL test tube containing 10 mL of YTM. After the inoculation of approximately 10^5 spores, the test tube was sealed with a butyl rubber cap and heated at 80 °C for 5 min. After cool-down at 4 °C for 2 min, the test tube was incubated at 30 °C until it reached a mid-log phase (for about 28 h). The precultured broth (10 mL) was inoculated into YTM medium (1 L) in a BMJ-02 mini-jar fermentor (Able Co., Tokyo, Japan) with pH control. The medium in the reactor was purged with N_2 gas for 30 min to maintain an anaerobic condition at the beginning of cultivation. When the concentration of glucose during the cultivation decreased to around 20 mM, 50 mL of 1 M glucose solution was added.

The ability of HU-1 to produce H_2 from AP was evaluated in a batch culture under optimal conditions (pH 6, 37 °C, 150 rpm). AP (200 g, 81.5% moisture) was added to YTM medium (1 L), and the mixture was autoclaved at 121 °C for 20 min. The resultant YTM medium contained 42.9 mM fructose, 22.0 mM glucose, 6.7 mM sucrose, 2.3 mM mannose, 0.9 mM galacturonic acid, 0.4 mM xylose and 15.7 g (dry weight) of water-insoluble material. The sugar mixture that was prepared to supply fructose, glucose, sucrose, mannose, galacturonic acid and xylose at final concentrations of 42.9, 22.0, 6.7, 2.3, 0.9 and 0.4 mM, respectively, was designated as the model sugar mixture. The insoluble material from AP was prepared as follows. One liter of deionized water containing 200 g (wet weight) of AP was autoclaved and filtrated with filter paper (Advantec no. 2, Toyo Roshi Kaisha, Ltd., Tokyo, Japan). The residual material on the filter paper was washed twice with deionized water and used as the water-insoluble material from AP. The ability of HU-1 to produce H_2 from glucose (100 mM) was also evaluated in a batch culture under optimal conditions to compare the ability from apple pomace. Inoculum was prepared as described above.

Analytical methods The evolved H_2 was detected using a gas chromatography (7890 valve system, Agilent, Inc., CA, USA) equipped with Pora-pack Q and MS-5A columns (Agilent, Inc.) and a thermal conductivity detector (120 °C). The carrier gas was He with a flow rate of 3.5 mL/min, and the column oven temperature was 50 °C. The concentrations of acetic acid, butyric acid, lactic acid, glucose, fructose, sucrose and galacturonic acid were measured with a capillary electrophoresis (CE) system (7100 CE system, Agilent, Inc.) equipped with a CE standard capillary and basic anion buffer for HPCE (Agilent, Inc.). The electrophoresis conditions were as follows: electron voltage, 30 kV; column temperature, 27 °C; wavelength, 350 nm; detector, negative mode. Butanol, ethanol and acetone were measured with a gas chromatograph (7890A, Agilent, Inc.) equipped with an HP-PLOT/Q column (Agilent, Inc.) and a frame ionization detector (250 °C). The carrier gas was He with a flow rate of 5.0 mL/min. The program of the column oven temperature was as follows: initial temperature, 150 °C (for 5 min); final temperature, 250 °C (for 5 min); rate of increase, 10 °C/min. The cell concentration in the culture was determined by measuring the optical density at 600 nm using a spectrophotometer (U-1800, Hitachi High-Technologies Corporation, Tokyo, Japan).

NADH and NAD^+ were extracted from the culture directly as described previously (11). The extracted NAD^+ and NADH were quantified using a high-performance liquid chromatography (1200 series, Agilent, Inc.) equipped with a Zorbax Eclipse XDB-C18 column (4.6 mm in diameter, 150 mm in length, 0.5 μm particle size). The temperature of the column oven was 40 °C. We employed a gradient program of the mobile phase. Solvent A was 200 mM ammonium acetate and 1% CH_3OH in H_2O, and solvent B was 100% CH_3OH. Solvent B was increased from 0% to 10% in 10 min for separation, and then the column was washed by increasing solvent B to 100% for 15 min. The flow rate of the mobile phase was 1.0 mL/min, and the wavelength was 260 nm (for NAD^+) and 320 nm (for NADH). Pyrophosphate (PPi) was extracted from the cells as described (12). The extracted PPi was quantified using an EnzChek Pyrophosphate Assay Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Adenosine triphosphate (ATP) was extracted from the cells as described (13). The extracted ATP was quantified with an ENLITEN ATP Assay System (Promega Corporation, Madison, WI, USA) and AccuFLEX Lumi 400 (Hitachi Aloka Medical, Ltd., Tokyo, Japan).

RESULTS AND DISCUSSION

Characteristics of isolated H_2-producing strain The physiological characteristics of the isolated H_2-producing anaerobe strain are summarized in Table 1. The isolate was a gram-positive, rod-shaped bacterium. It was able to form endospores, and its growth strictly depended on an obligate anaerobic condition. Catalase and oxidase activities were not detected. These characteristics were in accordance with those of Clostridia (14). The oxidation ability of the strain on the various carbon sources, its hydrolysis activity on gelatin, esculin and starch, and its lipase activity were in accordance with those of *C. beijerinckii* (15). The strain could not utilize cellulose (Nacalai Tesque, Inc., Kyoto, Japan), carboxymethylcellulose (Nacalai Tesque, Inc.), xylan (Sigma−Aldrich Co., MO, USA) or apple pectin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) as a sole carbon source. The almost-complete sequence of the 16S rDNA from the strain was determined. The 16S rDNA sequence has 100% identity with that of *C. beijerinckii* E080 (accession no. JX267098), and 99% identities with those of *C. beijerinckii* NCIMB8052 (CP000721)

TABLE 1. Characteristics of the isolated bacterium.

Characteristics	Results
Gram stain	+
Cell form	Rod (0.7−0.8× 1.5−2.5 μm)
Spore formation	+
Mobility	+
Cultivation temperature (°C)	
30	+
37	+
45	−
Catalase	−
Oxidase	−
Acid/Gas productivity (from glucose)	+/+
Indole	−
Urease	−
Gelatin hydrolysis	−
Esculin hydrolysis	+
Milk coagulation	−
Lecithinase	−
Lipase (Tween80)	−
Starch hydrolysis	+
Oxidation of carbon source[a]	
D-Glucose	+
D-Mannitol	+
Lactose	+
Saccharose	+
Salicin	+
Maltose	+
D-Xylose	+
L-Arabinose	+
Glycerin	+
D-Cellobiose	+
D-Mannose	+
D-Melicitose	−
D-Raffinose	−
D-Sorbitol	+
D-Rhamnose	+
D-Trehalose	+

[a] Oxidation ability on various carbon sources was tested with an API20A kit (bioMeriéux, Lyon, France).