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Technical Note

Optimization of Hydrogen Production by Mannitol Utilizing a Bacterium Strain TM1

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This study investigated hydrogen (H_2) production by mannitol fermentation derived from a marine alga. Fermentative H_2 production from mannitol ($C_6H_{14}O_6$) by a recently isolated bacterium strain TM1 was analyzed under different conditions. After optimizing cafure condition, maximum yield of 2.9 mol H_2/mol $C_6H_{14}O_6$ was obtained at 37°C and pH 6.0. DNA sequences of oxidoreductase and dehydrogenase from strain TM1 showed 98% identity with that of *Klebsiella variicola* At-22.

Key words: hydrogen production, fermentation, sea alga, cultivation, mannitol

Introduction

There is a growing interest in issues related to environmental pollution, in particular, the problem of reduction of carbon dioxide levels. Fossil fuels, mainly oil, coal, and natural gas [1], supply approximately 79.8% of the world's energy requirement, resulting in the release of massive amounts of carbon dioxide into the atmosphere. Moreover, the amount of available fossil fuels is finite, and will eventually be depleted if usage continues at the current rate. Japan consumes approximately 5.1% of the global reserve of fossil fuel [2,3], and the vast demand for energy necessitates their import, raising additional concerns about energy loss during transport.

A concern arising from the ongoing development of neighboring countries is the increased pollution of oceans. Industrial waste and algal blooms currently float on the ocean surface, posing serious environmental and health problems. In addition, along the coastline of Yokohama City on the Kanazawa-hakkei sea, which is 1 km long and 60–200 m wide, approximately 1,000 tons (dry weight) of seaweed is washed ashore each year [4], which is equivalent to about two million tons for the entire Japanese coastline according to Investigative report of sea lettuce 2004 [5]. Incineration of the collected seaweed has become a valuable organic resource in Japan: 1 ton of seaweed can be processed to hydrogen (H_2) that can be converted into approximately 51.2 kWh of electrical energy, that is, approximately 20% of the country's energy requirement. Thus, exploiting an existing nuisance in this manner can alleviate the energy crisis as well as reduce coastline sludge. H_2 has recently gained much attention as a sustainable, alternative source of fuel. Unlike the use of crops, H_2 production from sea algae does not compete with food production, and can moreover contribute to the removal of nutrient-rich salts that are considered pollutants.

Seaweed is mainly composed of alginic acid and mannitol ($C_6H_{14}O_6$) and contains minimal amounts of cellulose. For example, the constituents of kelp (brown algae) by weight are alginic acid (7%), $C_6H_{14}O_6$ (8%), cellulose (1%), protein (1%), other (4%), and water (79%) [6]. Alginic acid is a highly viscous, minimally degradable material composed of glucuronic acid coupled to mannuronic acid with a molecular weight of 1,000–100,000. $C_6H_{14}O_6$ is a monosaccharide with a molecular weight of 182 and can be used by bacteria as an energy source. H_2 production by *Enterobacter aerogenes* using $C_6H_{14}O_6$ as a substrate has a maximum yield of approximately 1.6 mol H_2/mol $C_6H_{14}O_6$ [7,8], compared to 1 mol H_2/mol glucose [9]. This difference is largely due to the higher number of H atoms in $C_6H_{14}O_6$ compared to glucose. The metabolic pathway for H_2 fermentation from $C_6H_{14}O_6$ [10,11] is shown in Fig. 1. [12] $C_6H_{14}O_6$ present in seaweed can be readily extracted by simple grinding [13].

In this study, a bacterial isolate that produce high-yield of H_2 from mannitol as a substrate. Optimization of temperature and pH conditions for maximum H_2 production was performed. Conditions that promote rapid growth during seaweed culture were also optimized.

Materials and Methods

Sampling and culture condition of H_2-producing bacteria that uses $C_6H_{14}O_6$ as a substrate

Bacteria were collected from the water surface on the Misaki (Miura-shi, Kanagawa, Japan) shore and in the

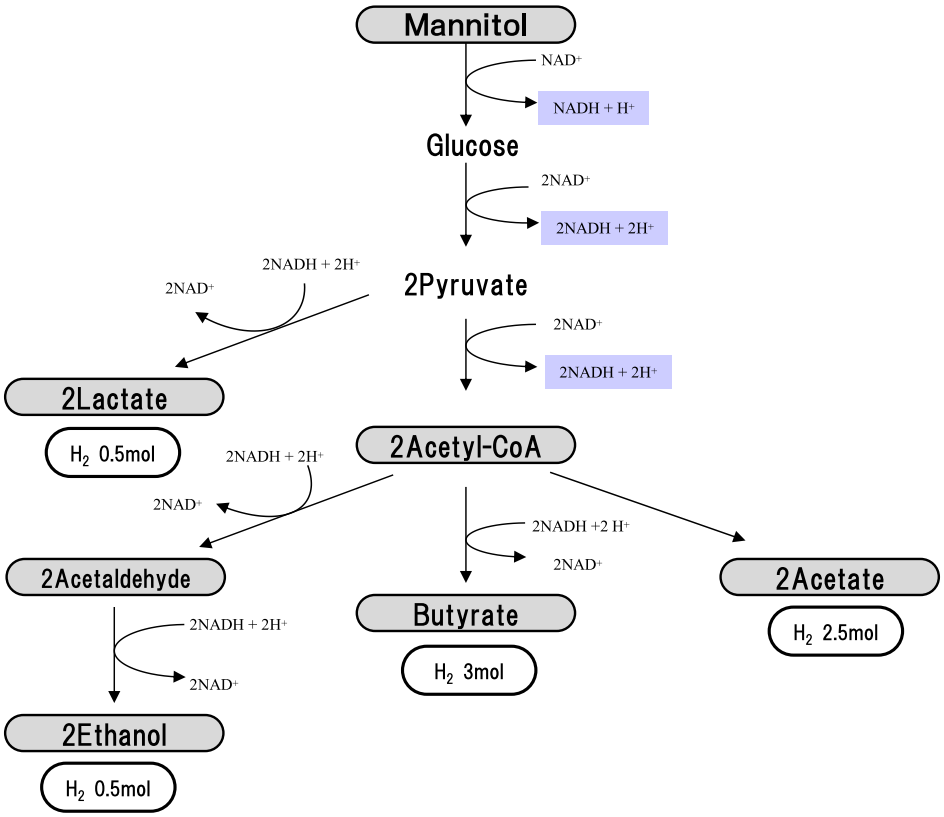

Fig. 1. Metabolic pathway of mannitol and possible hydrogen yields. (Hongo, A. 2011, p. 90)
The number of moles of hydrogen produced from 1 mole of a given substance is written under each product.

vicinity of the Iwa (Manazuru-machi, Kanagawa, Japan) shore; in addition, samples were obtained from sea mud from the Yokohama National University marine engineering side adjustment reservoir and a side channel ditch of the sewage treatment center. Each sample was inoculated using a sterile platinum loop and cultured in a test tube in semisolid nutrient medium (10 ml) containing 10 g/L $C_6H_{14}O_6$, 10 g/L casamino acid, 10 g/L dried yeast extract D-3, 2 g/L agar, (pH 6.0) at 37°C for 24 h. The test tube served as a simple gas generator that allowed quantification of the gas generated (Fig. 2). A small volume of the culture was transferred to medium containing 10 g/L $C_6H_{14}O_6$, 20 g/L casamino acid, 20 g/L trisodium citrate dihydrate, 5 g/L NaCl, 15 g/L agar, and 10 g/L dried yeast extract D-3, 2 g/L agar, (pH 6.0) and the cultures were incubated at 37°C for 24 h. H_2 sulfide produced as an off-gas was measured using a gas detector tube (No. 4LT; Gastec Corp, Ayase-City, Japan). Bacterial isolates that produced high levels of H_2 sulfide were excluded.

Optimization of culture condition

Experiments were performed to determine the optimal temperature and pH for bacterial culture. Isolated bacteria were cultured in approximately 10 ml of semisolid medium for 24 h in a simple vapor generator. The pH of cultures was adjusted to 5.0, 5.5, 6.0, 6.5, or 7.0 with phosphoric acid and sodium hydroxide. Cultures at each pH were incubated at 35°C, 37°C, 40°C, 42°C, and 45°C.

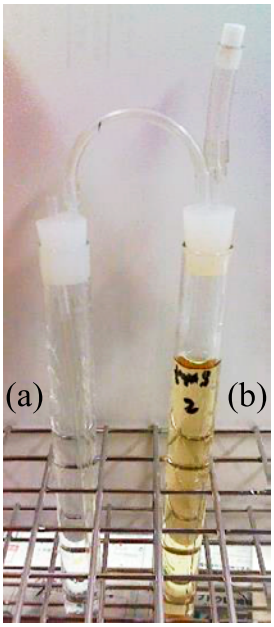

Fig. 2. Hydrogen production apparatus.
(a) Fermentative gas collection tube; and (b) test tube containing substrate and cell culture.

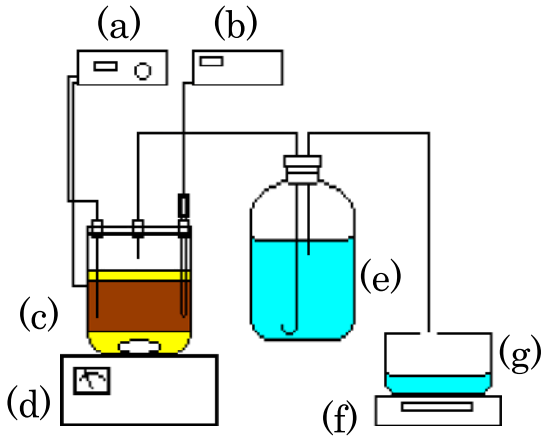

Fig. 3. Batch culture apparatus. (Hongo, A. 2011, p. 91)
(a) pH-measuring device (Fine pH controller SERIES-BFD-02, NISSIN TYPE CE108C-1.8BG); (b) temperature-measuring device (SHINO.LT100); (c) culture vessel; (d) stirring device (ASONE MAGNETIC STIRRER HS-4SP); (e) NaOH vessel; (f) electric scale (Sartorius BJ6100); and (g) measurement container.

Batch cultivation

Bacterial isolates were cultivated in 500 ml liquid medium consisting of 10 g $C_6H_{14}O_6$, 10 g/L casamino acid, 10 g/L dried yeast extract D-3, 0.3 g/L $CH_2CH(NH_2)COOHHClH_2O$, and 0.3 g/L fumaric acid. Batch cultivation was carried out at pH 6.0 and 37°C, which were determined as the optimal conditions for bacterial growth. H_2 production was quantified by determining the change in the absorbance of the culture at 560 nm, and by Hitachi RI Monitor detector and 655A-30 analyser (Hitachi High-Tech, Tokyo, Japan), as well as by gas chromatography (Shimadzu GC-3BT; Shimadzu Corp., Kyoto, Japan). Analysis of organic acids, using by liquid chromatography using a GL-C610H-S column (column temperature, 40°C; carrier, 0.1% phosphoric acid; flow rate, 0.5 ml/min). The batch cultivation apparatus is illustrated in Fig. 3. [14] For the downward displacement of water (Fig. 3e), sodium hydroxide was used to remove carbon dioxide from the generated gas.

Oxidoreductase and dehydrogenase DNA sequences

Bacteria screened in Section 2.1 were identified by sequencing the PCR products for the 16S rRNA gene. Sequencing was performed by Bio Matrix Research Inc. (Chiba, Japan). Portions of the DNA sequence were registered in DDBJ (accession number AB746174). The sequence data were used to perform a homology search of the DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/) using the Basic Local Alignment Search Tool to identify the isolates.

Results and Discussion

Isolation of H_2-producing bacteria

The TM1 and TMS8 strains were isolated after repeated transfer of medium. Results from experiments determining optimal temperature and pH conditions for TM1 and TMS8 were shown in Fig. 4(a) and 4(b), respectively. H_2 production was highest at 37°C and pH 6.0 for both TM1 and TMS8. A decline in H_2 production was observed at temperatures above 37°C. Maximum H_2 production was nearly twofold higher in TM1 than in TMS8.

Batch cultivation

Average of batch cultivation of TM1 hydrogen production resulted is 2900 mL/L, and maximum, minimum, and mean yields of 2.9, 1.8, and 2.1 mol H_2/mol $C_6H_{14}O_6$, respectively (Fig. 5). The maximum and mean absorbance values at 560 nm were 1.2 and 1.1, respectively. The latter corresponded to the lowest H_2 yield, indicating that there was likely to be correlation between bacterial concentration and gas production. The bacterial concentrations calculated from the absorbance measurements are shown in Fig. 5. Liquid chromatography showed that TMS8 and TM1 mainly produced lactic and butyric acids, respectively. However, in the results for TM1, an overlapping $C_6H_{14}O_6$ peak prevented

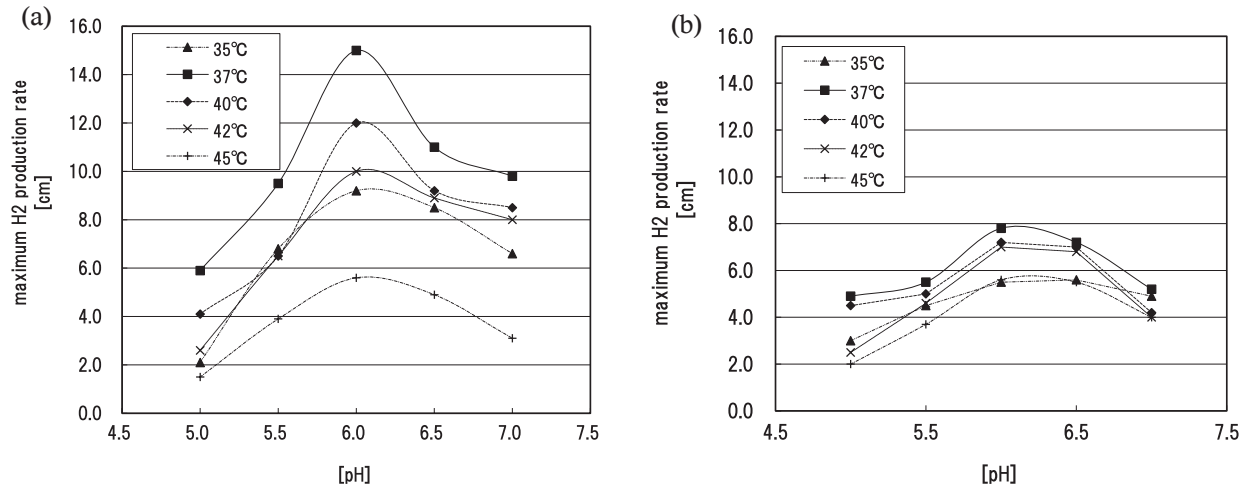

Fig. 4. Experimental results of the optimisation of culture conditions of TM1 (a) and TMS8 (b).

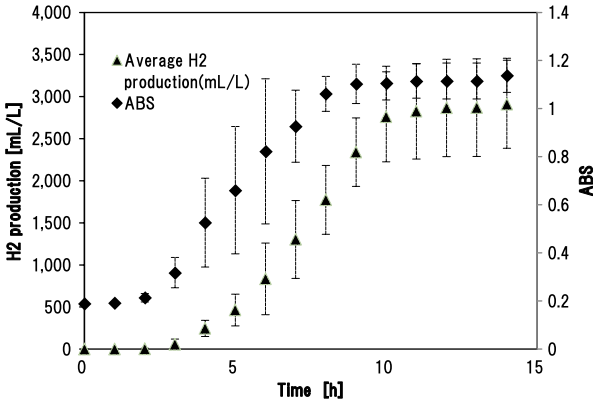

Fig. 5. Hydrogen production TM1 bacteria and Change in absorbance of TM1 bacteria over time.

the precise molecular quantification of butyric acid.

Analysis of TM1 oxidoreductase and dehydrogenase DNA sequences

DNA sequences of TM1 oxidoreductase and dehydrogenase was performed showed 98% identity with *Klebsiella variicola At-22* enzymes.

Summary

In this study, a new strain of bacterium, TM1, was isolated and was found to produce H_2 using mannitol as a substrate. The optimal conditions for obtaining a maximum H_2 yield of 2.9 mol H_2/mol $C_6H_{14}O_6$ with the bacterial culture were a temperature of 37°C and pH of 6.0.

When using *C. butyricum*, hydrogen yield is 2.3 mol H_2/mol According to "The Bacteria" in Wood, WA in the fermentation hydrogen production using the substrate glucose, a substrate mannitol According to the article by S. Tanisho, in the fermentation hydrogen production using hydrogen yield was 1.4 mol H_2/mol [13]. When used glucose as a substrate, maximum hydrogen yield 2.4 mol H_2/mol HN001 is a fungus with high hydrogen yield most in this laboratory. The isolated bacterial strain TM1 has been found that high hydrogen yield in the most bacteria was discovered until now.

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