Ethanol production from H₂ and CO₂ by a newly isolated thermophilic bacterium, **Moorella** sp. HUC22-1

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Received 24 June 2004; Revisions requested 20 July 2004; Revisions received 24 August 2004; Accepted 24 August 2004

**Key words:** alcohol dehydrogenase, ethanol production, H₂ and CO₂, intracellular pyridine nucleotide pool, **Moorella** sp.

**Abstract**

The thermophilic bacterium, **Moorella** sp. HUC22-1, newly isolated from a mud sample, produced ethanol from H₂ and CO₂ during growth at 55 °C. In batch cultures in serum bottles, 1.5 mM ethanol was produced from 270 mM H₂ and 130 mM CO₂ after 156 h, whereas less than 1 mM ethanol was produced from 23 mM fructose after 33 h. Alcohol dehydrogenase and acetaldehyde dehydrogenase activities were higher in cells grown with H₂ and CO₂ than those grown with fructose. The NADH/NAD⁺ and NADPH/NADP⁺ ratios in cells grown with H₂ and CO₂ were also higher than those in cells grown with fructose. When the culture pH was controlled at 5 with H₂ and CO₂ in a fermenter, ethanol production was 3.7-fold higher than that in a pH-uncontrolled culture after 220 h.

**Introduction**

Most anaerobic acetogenic bacteria (acetogens) gain energy by acetate production from H₂ and CO₂ with ATP synthesis (Drake et al. 2002). Although these bacteria usually produce only acetate as an end-product, several species of mesophilic acetogens, such as *Clostridium ljungdahlii* and *Acetobacterium woodii*, can produce ethanol (Buschhorn et al. 1989, Tanner et al. 1993). On the other hand, some studies of ethanol metabolism in thermophilic acetogens have been reported; for example, some strains of *Moorella thermoacetica* can use ethanol when nitrate is dissimilated (Fröstl et al. 1996). Resting cells and crude extracts of *M. thermoacetica* formed 14C-labeled ethanol from [5-14C]methyltetrahydrofolate, which is an intermediate in the acetyl-CoA pathway (White et al. 1987), and reduced many carboxylic acids to the corresponding alcohols in the presence of electron donors, such as CO and formate, together with artificial electron mediators, such as various viologens and cobalt sepulchrate (Simon et al. 1987). These reports suggest that some thermophilic acetogens also have an ethanol-producing pathway. To our knowledge, however, direct evidence of ethanol production by thermophilic acetogens and information about related enzymes during growth have not been reported to date. In this paper, we report that a thermophilic bacterium, **Moorella** sp. HUC22-1, isolated from a mud sample, produces ethanol together with acetate when grown on H₂ and CO₂ during growth.

**Materials and methods**

**Organism and medium composition**

**Moorella** sp. HUC22-1, newly isolated from a mud sample collected from underground hot water in Chiba, Japan, was used in this study. For the enrichment cultures, ATCC 1754 PETCH medium (http://www.atcc.org) was used as the basal medium. For the pure cultures, the basal medium had cysteine·HCl·H₂O at 0.3 g l⁻¹, and Na₂S·9H₂O was...
eliminated. A modified Hungate technique in combination with the serum bottle technique was used for the serum bottle experiments (Miller & Wolin 1974). The initial pH of the medium was adjusted to 6.3.

**Enrichment cultures and isolation of HUC22-1**

Approx. 1 g (wet wt) mud sample was cultured in the 25 ml medium with H2 and CO2 [80:20, (v/v)] at 55 °C for 2 weeks, the strain HUC22-1 was isolated as the highest producer of ethanol with H2 and CO2 by using the roll-tube method from the enrichment culture (Hungate 1969). Cell morphology and purity was examined using a phase-contrast microscope. The Gram stain was carried out by the method of Barrow & Feltham (1993).

**Batch culture with serum bottles**

To investigate the growth of strain HUC22-1, 720 ml serum bottles containing 150 ml basal medium were used. Sub-cultures were grown to an OD660 of 0.2 with H2 and CO2 or 1 with fructose and used as inoculum at 5% (v/v). Fructose, when was added to the medium, was at 5 g l\(^{-1}\). Bottles were incubated at 55 °C without shaking. When growth was on H2 and CO2 [80:20, (v/v)], bottles were flushed and brought to a final pressure of 0.19 MPa with a filter-sterilized gas mixture after inoculation. Bottles were incubated at 55 °C with shaking (135 strokes min\(^{-1}\)).

The utilization of substrates was tested in 30 ml anaerobic tubes containing 10 ml basal medium supplemented with autoclaved or filter-sterilized substrates. After the sub-cultures were inoculated with 5% (v/v), the tubes were incubated at 55 °C without shaking. Cell growth was then monitored turbidimetrically at 660 nm (OD660); 1 OD 660 unit = 0.46 g dry cell l\(^{-1}\). Cultures that increased their OD660 by more than 0.1, or those produced more acetate than that of the control culture, were considered as positive for substrate utilization. The control (final OD 660, < 0.05) did not contain any of the substrates tested.

**Batch culture with the fermenter**

Fermentation was performed with a system consisting of a 1 l jar fermenter (500 ml working volume; MJ-1, Able, Japan) and a 15 l Tedlar bag as a gas reservoir. A gas mixture consisting of H2 and CO2 [80:20, (v/v)] was filled into the headspace of the fermentor and the reservoir, and was circulated at a speed of 60 ml min\(^{-1}\) with the sparging from the bottom of the fermenter. The agitation speed and temperature were maintained at 500 rpm and 55 °C, respectively. The gas mixture in the reservoir was intermittently replaced with fresh gas mixture every 24 h. The culture was initiated by a 15% (v/v) inoculation with the sub-culture. The pH was monitored by a pH electrode, and controlled with a pH controller by the addition of 2 M NaOH or 2 M HCl.

**16S rRNA gene sequence determination**

Genomic DNA was extracted using a G NOME kit (Qiogene, Carlsbad, CA). Polymerase chain reaction (PCR) amplification of the 16S rRNA gene was performed using a Premix Taq kit (Takara Bio, Japan), the forward primer 27F (5'AGAGTTTGATCCTGGCTCAG-3', Escherichia coli, positions 8–27), and the reverse primer 1492R (5'GGCTACCTTGTTACGACTT-3', E. coli, positions 1510-1492) (Weisburg et al. 1991). The 1.5 kb fragment, purified using a DNA purification kit (Toyobo, Japan), was ligated into a pGEM-T easy vector (Promega, WI), and the product was used to transform E. coli DH5α. The E. coli cells, with recombinant plasmids, were selected on Luria–Bertani plates incorporating ampicillin, 5-bromo-4-chloro-3-indolyl-β-D-galactoside, and isopropyl thiogalactoside. The plasmids were extracted and purified using a Qiagen mini-prep kit (Qiagen, Germany). An ALFred DNA sequencer (Pharmacia Biotech., APB) was used for sequencing the entire DNA insert. The 16S rRNA gene sequence of the strain HUC22-1 has been deposited in the DNA Data Bank of Japan (DDBJ) nucleotide sequence database under the accession number AB127110.

**Preparation of cell-free extracts and enzyme assays**

Cells grown in a serum bottle to an OD660 of 0.2 with H2 and CO2, or to 1 with fructose, were harvested by centrifugation at 3000 × g for 10 min at 4 °C. The cells were washed twice and resuspended in 2 ml anaerobic 0.5 M potassium phosphate buffer, pH 7.5. After treating the cell suspension with lysozyme (1 mg ml\(^{-1}\)) and Triton X-100 [0.1% (v/v)] at 37 °C for 30 min, the cells were disrupted using an ultrasonic disintegrator. After 4 cycles of sonication (1 min bursts with 1 min cooling on ice), the cell lysates were centrifuged for 25 min at 4 °C and 20 000 × g to remove the unbroken cells and cell debris. The supernatant was used for the enzyme assays. All manipulations were carried out under N2.