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Glycerol acts as alternative electron sink during syngas fermentation by thermophilic anaerobe *Moorella thermoacetica*

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Moorella thermoacetica **is an anaerobic thermophilic acetogen that is capable of fermenting sugars, H_2/CO_2 and syngas (H_2/CO). For this reason, this bacterium is potentially useful for biotechnology applications, particularly the production of biofuel from CO_2. A soil isolate of *M. thermoacetica*, strain Y72, produces both ethanol and acetate from H_2/CO_2; however, the maximum concentrations of these two products are too low to enable commercialization of the syngas fermentation process. In the present study, glycerol was identified as a novel electron sink among the fermentation products of strain Y72. Notably, a 1.5-fold increase in the production of ethanol (1.4 mM) was observed in cultures supplemented with glycerol during syngas fermentation. This discovery is expected to aid in the development of novel methods that allow for the regulation of metabolic pathways to direct and increase the production of desirable fermentative compounds.**

[**Key words:** Anaerobe; Electron sink; Glycerol; *Moorella thermoacetica*; Syngas; Thermophilic]

Biofuels and other microbially derived compounds, such as ethanol, butanol, and lactate, are typically produced by either the direct fermentation of fermentable sugars present in sugar cane or sweet sorghum, or through the enzymatic or chemical hydrolysis of starch, cellulose, and hemicellulose. One of the major disadvantages for the utilization of cellulosic biomass as a substrate for biofuel production is the presence of a large proportion of non-degradable components, such as lignin (1–6). Recently, syngas fermentation, which is a combination of gasification and microbial fermentation processes, has attracted attention as an alternative approach for the bio-conversion of biomass to fuel and other bio-products. This approach has several advantages over conventional bio-conversion methods, including utilization of the entire spectrum of biomass components; reduced cost due to the elimination of pretreatment steps and enzyme addition; use of highly specific biocatalysts; and independence of the H_2/CO ratio for bioconversion (7).

A number of anaerobic bacteria, including *Acetobacterium woodii*, *Clostridium autoethanogenum*, *Clostridium carboxidivorans*, *Clostridium ljungdahlii*, *Peptostreptococcus productus*, and *Moorella thermoacetica*, are able to grow chemolithotrophically on CO and CO_2/H_2 to produce acetate (8–13). A few members of this anaerobic bacterial group, which are collectively referred to as acetogens, are able to function as biological catalysts for the conversion of syngas into volatile fatty acids and alcohols, particularly acetate and ethanol, through the Wood-Ljungdahl pathway (14). Notably, these biological catalysts produce liquid fuel more effectively than commonly used chemical catalysts, such as iron, copper, and cobalt (15,16).

Recently, we reported that a thermophilic bacterium, *M. thermoacetica* strain Y72, which was isolated from soil, produces acetate from fructose and H_2/CO_2 (17). Due to these properties, and because *M. thermoacetica* strain Y72 is highly amenable to genetic transformation, this acetogen is a promising candidate for the conversion of syngas into acetate, ethanol and other valuable chemicals (17). However, as the highest concentration of acetate produced from H_2/CO_2 by strain Y72 is reported to be 120 mM and ethanol has not been detected in cultures of strain Y72, it is necessary to dramatically increase ethanol production by this strain to enable commercialization of this syngas fermentation process (18).

To improve the ethanol productivity of strain Y72, we focused on approaches for increasing the flow of electrons towards ethanol. In growth-arrested cell cultures of class *Clostridia* bacteria, metabolism is shifted towards solvent compound production and away from biomass (cell carbon) production and acetogenesis (19). This type of metabolism is known as solventogenesis and is a characteristic property of Clostridial acetogens. In the growth-arrested state, excess electrons are generated and transported extracellularly as detrimental fermentative products or are consumed by solvent-producing reactions that can lead to the production of electron sink compounds. For example, *Clostridium perfringens* produces lactate in the presence of excess carbohydrates, suggesting that lactate is used as an electron sink to dispose of excess

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reducing power in excess substrate conditions (20). Thus, by controlling the concentration of electron sink compounds, it may be possible to direct metabolism towards the desired fermentation product, such as ethanol (21).

In several class *Clostridia* bacteria, electron sink compounds accumulate as fermentation by-products, such as organic acids (22) and ethanol (23). To date, however, ethanol production by strain Y72 has not been detected, suggesting that ethanol does not function as an electron sink in this species. Therefore, we hypothesized that a compound(s) other than ethanol functions as an electron sink during acetate production and solventogenesis in strain Y72.

In the present study, we report the identification of a novel electron sink during syngas fermentation by *M. thermoacetica* strain Y72, and describe a method for increasing the production of ethanol by supplementing the culture medium with the identified sink compound.

MATERIALS AND METHODS

Strains and media *M. thermoacetica* strain Y72, which was recently isolated from a soil sample collected from an agricultural field in Hiroshima, Japan (17), was used in this study. Strain Y72 was maintained as a 20% glycerol stock, which was used as inoculum for cultures used for measuring the ethanol-producing activity of strain Y72 (run-1). ATCC 1754 PETC medium (24) was used as the basal medium. For cultures, the basal medium contained 0.3 g/L cysteine·HCl·H_2O, and $Na_2S·9H_2O$ was eliminated. A modified Hungate technique in combination with the serum bottle technique was used for serum bottle experiments (25). For all cultures, the initial pH of the medium was adjusted to 6.3 with NaOH.

To investigate the fermentation of H_2/CO_2 (80:20, v/v) by strain Y72, 120-mL serum bottles containing 20 mL basal medium were used. After inoculation of the culture medium, the bottles were flushed with a filter-sterilized gas mixture of H_2/CO_2 and brought to a final pressure of 0.20 MPa. The bottles were then incubated at 55°C with shaking (190 strokes/min). For the measurement of ethanol-producing activity (run-1), strain Y72 was cultivated for 480 h (20 days). In the electron sink identification (run-2) and metabolic control experiments (run-3), strain Y72 was cultivated for 168 h. The cultivation solutions of run-1 and 2 were used as inocula for runs-2 and 3, respectively. In addition, in the metabolic control experiments, glycerol dissolved in distilled water was added at a final concentration of 20 mM to autoclaved basal medium in anaerobic vials.

Metabolite analysis Supernatants obtained by the centrifugation of culture samples were analyzed for acetate, ethanol, and glycerol by high-performance liquid chromatography (HPLC) using a LC-2000 HPLC (Jasco, Tokyo, Japan) equipped with a HPX87H column (Bio-Rad, Hercules, CA, USA). The column was eluted with 1 mM H_2SO_4 at a flow rate of 1.0 mL/min at 65°C. Detection of fermentation metabolites was performed using a RI-2031plus refractive-index (RI) detector (Jasco). Compounds were putatively identified according to retention time (RT) on the RI detector and from UV/visible spectra by comparison with relevant commercially available standards. The inorganic carbon (CO_2) concentrations were analyzed using a TOC-V meter (Shimadzu, Kyoto, Japan).

The organic carbon concentration in each HPLC fraction was measured by HPLC fractionation and total organic carbon (TOC) analysis. The fractionation of H_2/CO_2 fermentation products was performed using the same HPLC system as above with 1 mL/min of 1 mM HCl, and fractions were collected every minute. Fractionated samples were analyzed using a TOC meter and the organic carbon concentration, which was derived from H_2/CO_2 as the sole energy and carbon source, was determined. The putative electron sink candidate was identified using a linear ion trap (LIT) mass spectrometer (LTQ Orbitrap XL, Thermo Scientific, Bremen, Germany) at the Instrumental Analysis Division, Equipment Management Center, Creative Research Institution of Hokkaido University. The deprotonated compounds were produced from a 0.1% formic acid-based solution by electro-ionization. Confirmation and structural elucidation of the electron sink candidate was performed by LC/MS. MS experiments were performed using data-dependent scan techniques, in which the most abundant ions were selected automatically for fragmentation. In addition, changes of electron flow in response to the addition of glycerol were also investigated. The concentrations of acetate, ethanol, and glycerol were measured by HPLC and were converted to electron equivalents. Electron balance was calculated as described previously (26).

RESULTS AND DISCUSSION

Identification of fermentative metabolites Culture supernatants of *M. thermoacetica* strain Y72 grown in basal medium supplemented with 0.20 MPa H_2/CO_2 (80:20, v/v) for 20 days were analyzed by HPLC (run-1). The analysis revealed that a total of approximately 120 mM acetate was produced (Fig. 1). Additionally, ethanol was first detected on day 7 and reached a maximum concentration of 0.9 mM on day 20 (Fig. 1). The results from this experiment demonstrated that strain Y72 has ethanol-producing activity.

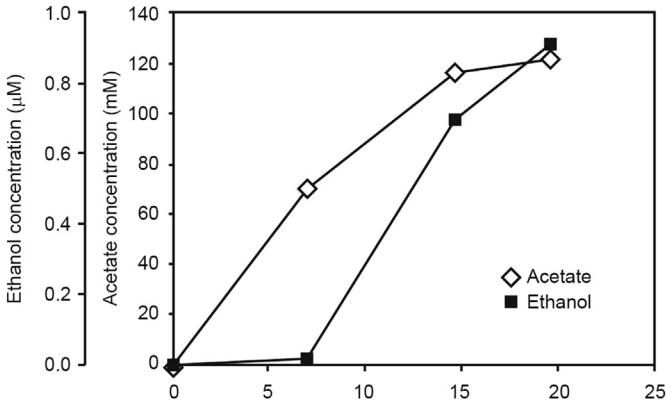

FIG. 1. Production of acetate and ethanol by *M. thermoacetica* strain Y72 during utilization of H_2/CO_2 (syngas fermentable conditions).

The cultivation solution from the above experiment was used as inoculum to establish cultures used for identification of the electron sink produced by strain Y72 during acetate production and solventogenesis (run-2). The concentration and production rates of acetate and ethanol in the culture supernatant of run-2 were measured and compared to those of run-1, revealing that the production rate of run-2 was increased and reached a steady state after

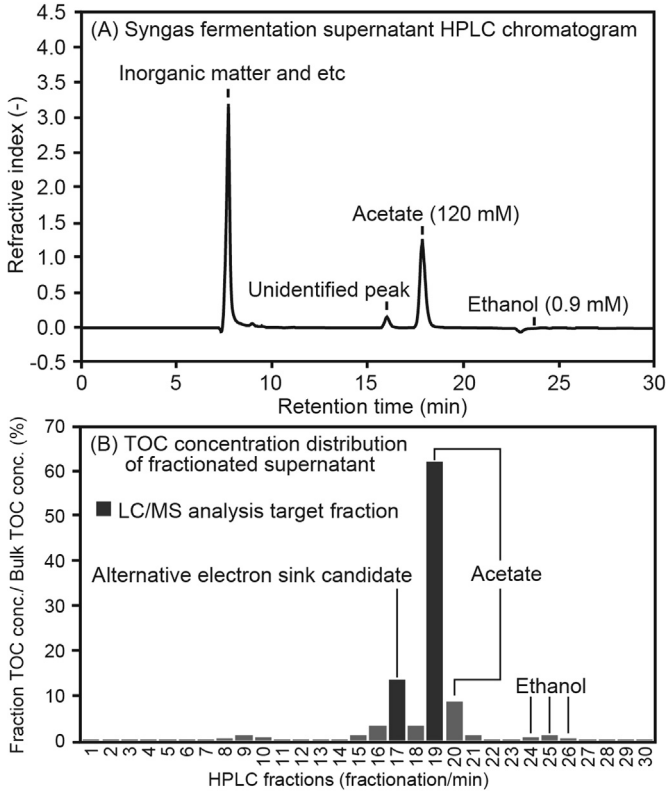

FIG. 2. Syngas fermentation products determined by HPLC fractionation. (A) HPLC chromatogram of the fermentation supernatant of strain Y72. (B) TOC concentration distribution within the fractionated supernatants.