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Kinetics of Strictly Anaerobic Ethanol Fermentation from Starch by *Clostridium thermohydrosulfuricum*

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Kinetics of anaerobic ethanol fermentation by *Clostridium thermohydrosulfuricum* were investigated for the one-step production of ethanol from starch. A mutant strain with a high ethanol yield was induced from C. *thermohydrosulfuricum*. The mutant, designated as ME4, produced anaerobically 6.1 g/l of ethanol, 3.1 g/l of lactate and 0.1 g/l of acetate from 20 g/l of starch at 68°C.

A thermophilic anaerobic bacterium, *Clostridium thermohydrosulfuricum*, which is capable of converting starch directly into ethanol, has been studied for the one-step ethanol production from starch (3, 4, 8, 9). The advantages of this bacterium are its ability to digest starch and low cell growth yield. Moreover, the recovery of ethanol produced during fermentation is easy for this microorganism which can grow at an extremely high temperature (80℃). However, two major undesirable problems are arising in the thermophilic anaerobic ethanol fermentation: low ethanol yield and a high concentration of by-products such as lactate and acetate (5). In order to produce ethanol effectively from starch and to minimize the production of by-products, we investigated the kinetics of ethanol fermentation using *C. thermohydrosulfuricum*. In addition, we induced a mutant with a capacity to produce a high concentration of ethanol from starch.

MATERIALS AND METHODS

Strain and Media

The thermophilic anaerobic bacterium, *C. thermohydrosulfuricum* ATCC 33223 was used throughout the study. This strain was grown in 26 ml anaerobic pressure tubes (Bellco, USA) containing complex medium (KM) and Ar (99.99%) gas in head space at 65℃. The KM medium described by Kannan *et al.* (7) consisted of (g/l): glucose, 20; yeast extract, 6; KH_2PO_4, 1.5; $Na_2HPO_4 \cdot 7H_2O$, 3.144; NH_4Cl, 0.5; and $MgCl_2 \cdot 6H_2O$, 0.384. The carbon source, glucose, was replaced with starch (KMS), lactate (KML), the mixture of starch and lactate (KMSL), and the mixture of lactate and acetate(KMLA). All of the anaerobic manipulations were handled in an anaerobic chamber (Coy, USA).

Induction of Mutants

Mutants with high ethanol yields were induced from *C. thermohydrosulfuricum* ATCC 33223 using a mutagen ethyl methane sulfonate (EMS) in the KMS medium. Cells harvested at log phase were centrifuged and transferred into the fresh KMS medium. EMS was added up to 1.0% (w/v) in the tubes, and the cells treated with EMS for 60 min were directly centrifuged and transferred into the fresh solid medium. The agar plates were incubated in the BBL Gas Pack jar at 60℃ for 7 days. The colonies developed were subcultured and analyzed for their ethanol production.

Fermentation

Batch fermentations were carried out at an initial pH of 8.0 without subsequent pH control in 5 l fermentor constructed for the high temperature fermentation. Samples drawn periodically from the fermentor were assayed for pH, optical density, glucose, ethanol, lactate and acetate.

Analysis

Ethanol and acetate were analyzed by a gas chromatography (Hewlett Packard, USA) with a flame ionization detector. The stainless steel column (2 meter) packed with porapak Q was used. The samples of culture broth

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were centrifugated at 1,300 rpm for 20 min and mixed with 0.25% isopropanol as an internal standard, and injected in 2 μl. The chromatogram was run at 170℃ with N_2 as a carrier gas at a flow gassing pressure of 40 psi.

Glucose and lactate were determined by a Glucose-Lactate Analyzer (YSI, USA). The optical density of the culture broth was measured at 600 nm by using a spectophotometer (Kontron, USA).

RESULTS AND DISCUSSION

Effect of Metal Ions on Ethanol Production

Most ethanol-producing Clostridia form similar end products. The proportion, however, is dependent on the particular species and the culture conditions (10, 11). *C. thermohydrosulfuricum* produces mainly ethanol, lactate, acetate, CO_2 and H_2 from various carbon sources such as pectin, xylose, cellobiose, glucose and starch. When starch was used as a carbon source in KM medium (designated as KMS medium), it was supposed that the growth of cells was limited. This is due to the low concentration of glucose in the early stage of the fermentation so as to make poor growth and ethanol production (Fig. 1). Whereas, under these condition, the lactate formation began at early exponential growth phase and its concentration was higher than any other end products.

A metal ion (i.e. Fe^{2+}) was known to increase the secretion and formation of enzymes for saccharifying and debranching of starch and to affect ethanol fermentation positively (1). In order to examine this, metal ions (Fe^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+}) were added into the KMS medium in the concentration of 50 mg/l each. Among these, Fe^{2+} was found to be one of the most effective ions in the ethanol production (Table 1). The ratio of ethanol to lactate in the end products increased to 0.5 in the KMS-Fe^{2+} from 0.3 in the metal ion free medium. Lactate, however, was produced in the same level in all cases including the control without metal ions. This suggested that the Fe^{2+} did not affect the enzymes engaged in the formation of lactate from pyruvate. However, it might have affected the activity of diastic enzymes (α-amylase, β-amylase, glucoamylase and pullulanase) as well as the cell growth. In fact, the amylase activity of the culture broth from KMS-Fe^{2+} medium was 12 times higher than that of the medium without Fe^{2+} (data not shown). The maximum optical density of the culture broth and ethanol concentration produced were higher in the KMS-Fe^{2+} medium than in the control (Fig. 2). Glucose formation rate seemed to be enhanced when

Table 1. Effect of metal ions on ethanol fermentation by *C. thermohydrosulfuricum* in KMS medium.

Medium	Ethanol (g/l)	Lactate (g/l)
KMS	1.3	6.4
KMS-Mg^{2+}	1.3	7.8
KMS-Fe^{2+}	3.4	6.4
KMS-Zn^{2+}	1.8	6.1
KMS-Mn^{2+}	1.7	6.3

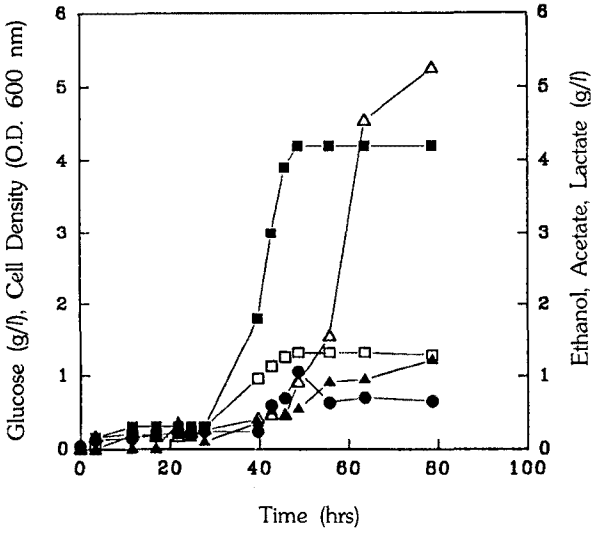

Fig. 1. End product formation by *C. thermohydrosulfuricum* in a pressure tube with KMS medium at 68°C. Glucose (△), cell density (●), ethanol (□), acetate (▲) and lactate (■)

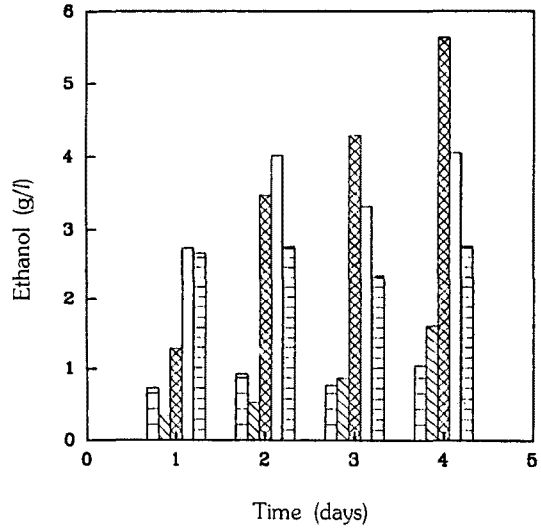

Fig. 2. Effect of Fe^{2+} on ethanol fermentation by *C. thermohydrosulfuricum* in a pressure tube with KMS medium at 68°C.

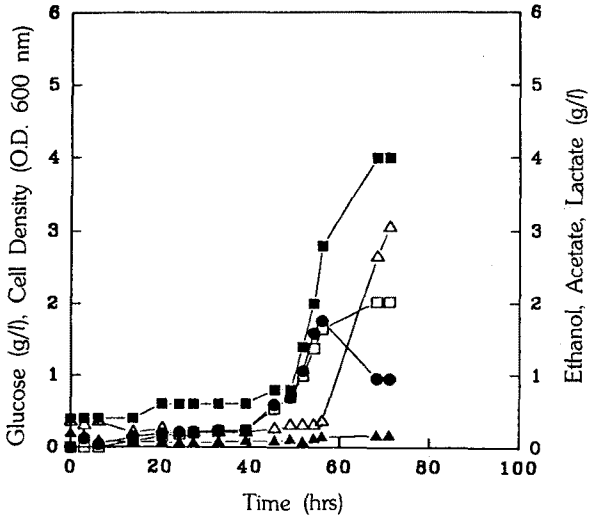

Fig. 3. Effect of lactate concentration on ethanol production by *C. thermohydrosulfuricum* in a pressure tube with KMS medium at 68°C.
Glucose (△), cell density (●), ethanol (□), acetate (▲) and lactate (■)

the stationary phase of the growth attained.

Effect of Lactate on Ethanol Production

Lactate is known to modulate the electron flow in the fermentation by anaerobic microbes (2). In order to measure the optimum concentration of lactate for high ethanol yield, fermentations were carried out using the KMS medium with lactate (KMSL) in various concentrations up to 50 g/l (Fig. 3). The ethanol concentration produced by *C. thermohydrosulfuricum* was greatly influenced by the concentration of lactate added. When the medium was supplemented with 20 g/l of lactate, ethanol was produced in a higher concentration (5.8 g/l) than that in the control (3.0 g/l) without lactate (KMS).

Induction of Mutants with High Ethanol Yield

In order to isolate the mutants with higher ethanol yield, *C. thermohydrosulfuricum* was mutagenized by the treatment with a mutagen (EMS). Among the mutants induced, a mutant *C. thermohydrosulfuricum* ME4 was found to have the highest ethanol yield (Table 2). The mutant produced 6.1 g/l of ethanol, 3.1 g/l of lactate and 0.1 g/l of acetate from KMS medium with 20 g/l of starch in a pressure tube at 68℃. *C. thermohydrosulfuricum* ME4 which has gained the ability to produce ethanol in higher yield than the parent strain seemed to be genetically stable. After the 6th subculture, in fact, the property of high ethanol yield was maintained reproducibly. The growth rate was slightly higher in the mutant (0.072 h^{-1}) than in the parent strain (0.062 h^{-1}), but the cell mass of the mutant was significantly lower than that of the parent strain at the maxium value. The significant differences between the parent and the mutant st-

Table 2. Comparison of end product formation produced by the parent and mutants of *C. thermohydrosulfuricum*.

Cell type	End products (g/l)		
	Ethanol	Lactate	Acetate
Parent	3.2	4.2	0.5
Mutant			
ME3	3.1	3.0	0.2
ME4*	6.2	3.1	0.1
ME5	3.8	3.2	0.1
ME6	–	2.6	–
MG6	4.8	2.6	0.1
MG2	3.5	2.5	0.1
NC3	4.9	3.1	0.1
OA6	3.8	3.0	0.1
OB1	3.5	3.2	0.1
OC4	4.3	3.0	0.1
OD1	4.0	3.0	0.1
OD3	5.2	3.3	0.1
OD5	4.9	3.1	0.1
OD6	1.8	3.4	0.1
PB1	4.7	3.2	0.1

Table 3. Comparison of the parent and a mutant of *C. thermohydrosulfuricum* for growth and ethanol production.

Metabolic feature	Parent (ATCC 33223)	Mutant (ME4)
Growth on[a]		
Lactose	++[b]	++
Fructose	+	0
Xylose	+	+
Cellobiose	++	–
Starch	0	0
Glucose	+	0
Ethanol production (g/l)		
Lactose	1.7	3.1
Fructose	1.9	1.7
Xylose	2.4	2.6
Cellobiose	1.9	2.1
Starch	1.0	1.1
Glucose	2.2	2.0

[a] Samples (10 ml) of KM complex medium with 0.5% (w/v) of various carbon sources were incubated at 68℃ for 43 hrs.
[b] Optical density was measured at 600 nm after 43 hours. ++; O.D.>1.0, +; O.D.>0.8, 0; O.D.>0.6, –; O.D.< 0.6

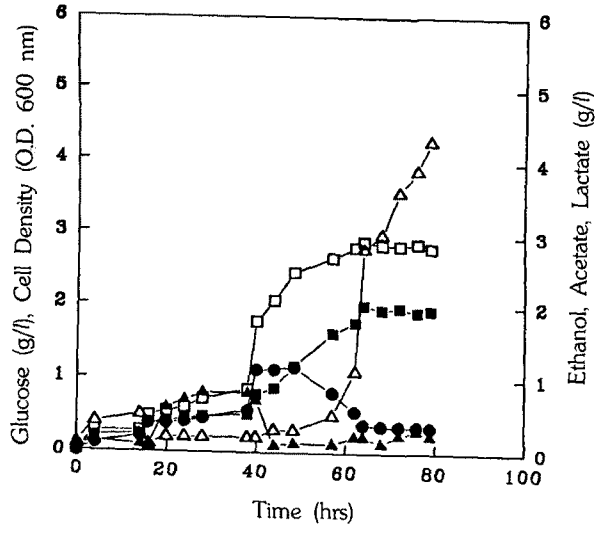

Fig. 4. End product formation by *C. thermohydrosulfuricum* ME4 in a 5 *l* fermentor with KMS-Fe^{2+} medium at 68°C.
Glucose (△), cell density (●), ethanol (□), acetate (▲) and lactate (■)

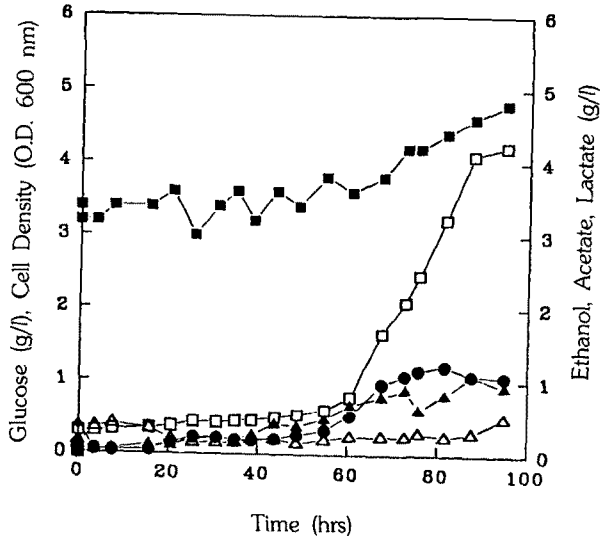

Fig. 5. End product formation by *C. thermohydrosulfuricum* ME4 in a 5 *l* fermentor at 68°C with KMS-Fe^{2+} medium reinforced with 3.4 g/*l* lactate.
Glucose (△), cell density (●), ethanol (□), acetate (▲) and lactate (■)

rain were observed in the yields of the end products according to carbon sources. Particularly from 5 g/*l* of lactose, 3.1 g/*l* of ethanol was produced by the mutant, whereas only 1.7 g/*l* of ethanol by the parent strain (Table 3).

Kinetics of Ethanol Fermentation by the Mutant ME4

Fig. 4 shows a typical fermentation profile for the mutant ME4 in a 5 *l* fermentor with the KMS medium reinforced with Fe^{2+} at 68℃. In a 5 *l* fermentor, the concentration of ethanol produced from the KMS-Fe^{2+} medium by the mutant was lower than in a pressure tube. This was most likely owing to the loss of ethanol by evaporation at a high temperature through the vent for the exit of N_2 gas which was equipped to maintain the anaerobic condition in the fermentor.

When the KMS medium with 50 mg/*l* of Fe^{2+} was reinforced with 20 g/*l* of lactate, the mutant produced 4.2 g/*l* of ethanol and 1.0 g/*l* of acetate (Fig. 5). However, the lag phase of the mutant in the medium with lactate was prolonged up to two times. It was supposed that the electrons discharged during the oxidation of lactate into pyruvate modulated the fermentation for solvent production by *Clostridium acetobutylicum* (6). Further investigation is needed to elucidate the role of lactate in association with the increased ethanol yield and the retardation of growth.

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