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Selection of an Ethanol Tolerant *Clostridium thermohydrosulfuricum* Strain

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An ethanol tolerant mutant was selected by successive transfers of *Clostridium thermohydrosulfuricum* ATCC 33223 into the media with progressively higher ethanol concentrations. The growth kinetics of the mutant were characterized under various growth conditions. Physiological differences such as enhanced growth, tolerance to various solvents, alteration of the optimum temperature and the ratio of end products during fermentation were noticed in the mutant.

An anaerobic and thermophilic bacterium, *Clostridium thermohydrosulfuricum* has a great potential for industrial ethanol production (3, 5). Thermoanaerobes, unlike *Saccharomyces* and *Zymomonas* species, can ferment directly the polysaccharides such as cellulose, hemicellulose and starch into ethanol. Thermophiles also possess a very high metabolic transformation rate. The growth of *C. thermohydrosulfuricum*, however, is strongly inhibited by ethanol at relatively low concentration (5 g/L).

Recently, Herrero and Gomez have studied the mechanism of ethanol tolerance in *C. thermocellum* (1). Solvents such as alcohols inhibited the membrane fluidity of bacteria and decreased the activity of enzymes which affected the metabolic pathways involved in the transformation of hexose into glyceraldehyde-3-phosphate.

In this study, the microbes were subcultured in the media with ethanol in order to induce ethanol tolerant mutants and a mutant, *C. thermohydrosulfuricum* 23YP, was induced. This note presents the fermentation characteristics of the mutant.

Microorganisms and Culture Conditions

C. thermohydrosulfuricum ATCC 33223 was used throughout the study. The organism was cultured at 65℃ using KMS medium and at an initial pH of 8.0 without subsequent pH control. The KMS medium consists of (g/L): starch, 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. Samples drawn periodically from the fermentor were assayed for pH, optical density, glucose, ethanol, lactic acid and acetic acid.

Stock cultures of *C. thermohydrosulfuricum* ATCC 33223 grown in KMS for 48 hr at 60℃ were stored at 4℃. Stock cultures of the mutant, *C. thermohydrosulfuricum* 23YP, grown for 48 hr at 60℃ in KMS medium which contained 50g of absolute ethanol per liter were also maintained at 4℃ and transferred into the same medium every 4 weeks.

Analysis

Ethanol and acetic acid were analyzed by using a gas chromatography (Hewlett Packard, USA) with a flame ionization detector. Glucose and lactic acid were determined by using the Glucose-Lactate Analyzer (YSI, USA). The optical density of the culture broth was measured as a growth parameter at 600 nm by using a spectophotometer (Kontron, USA).

Selection of an Ethanol Tolerant Mutant

Ethanol tolerant mutants were induced from *C. thermohydrosulfuricum* ATCC 33223 by sequential transfers of the culture in TYC medium with progressively higher amounts of ethanol at 55℃. The TYC medium was fortified from KMS medium by adding peptone (10 g/L), cystein (1 g/L) and 0.5% cellobiose instead of starch as a carbon source. After 25 subcultures, the growth of cells was active at 5% ethanol, thus, we could induce a mutant, *C. thermohydrosulfuricum* 23YP. The difference of surface between the parent and the mutant observed under electron microscope was considerable (Fig. 1). The surface of the mutant was rough and very bent.

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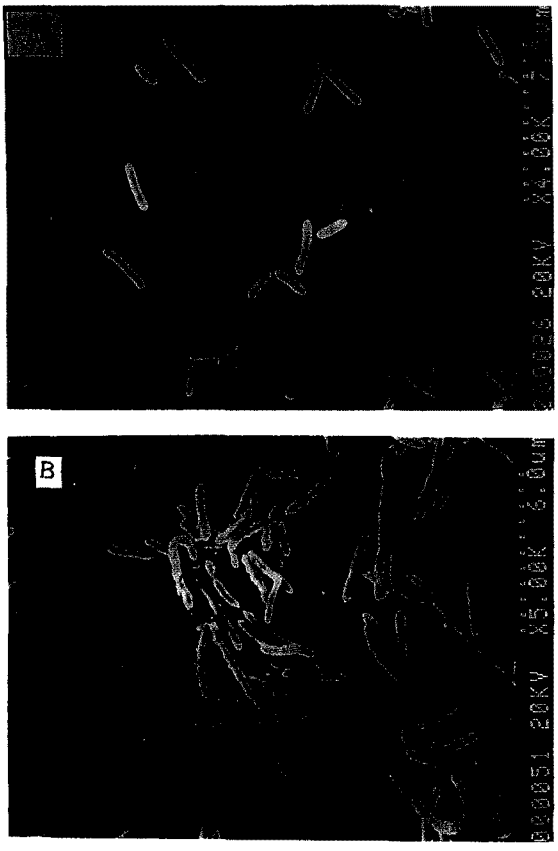

Fig. 1. Scanning electron micrograph of *C. thermohydrosulfuricum* strains.
(A) parent strain (×4,000) (B) ethanol tolerant mutant strain (×3,500) grown with 50 g/L of ethanol at 60℃.

Table 1. Influence of various solvents on the growth of the parent and the ethanol tolerant mutant strains of *C. thermohydrosulfuricum* 23YP[a].

Solvent concn. (%[wt/vol])	Parent (ATCC 33223)	Mutant (23YP)
None	0.88	0.90
Methanol		
1	0.87	0.86
2	0.85	0.97
5	0.81	0.90
Acetone		
1	0.86	0.97
2	0.93	0.97
5	0.99	1.16
Butanol		
1	0.50	0.81
2	0.17	0.77
5	0.20	0.79
Isopropanol		
1	0.80	1.04
2	0.71	0.98
5	0.20	0.16

[a] Both strains were grown in pressure tubes at 55℃ on KMS medium which contained 0.5% glucose instead of starch as a carbon source. Growth was measured as optical density at 600 nm after 50 hr culture.

Influence of Solvents on Growth and End Product Formation of the Mutant

Experiments were carried out to assess whether the basis for ethanol resistance of the mutant was related to enhanced solvent tolerance or not. In general, the ethanol tolerant mutant has a great tolerance to methanol, butanol and other alcohols (5). As a consequence, the ethanol tolerance of the mutant was investigated by means of adding 1, 2 and 5% of various solvents in KMS medium. Table 1 represents the results from the effects of various solvents on the growth of the parent and the mutant. Methanol and acetone had no effect on the growth of both parent and the mutant. The parent cells did not grow in the medium with butanol more than 2%, while the mutant cells were unaffected by butanol. In the case of isopropanol, the growth of the parent and the mutant cells was retarded with an increasing concentration of isopropanol. At a concentration of 5%, both parent and the mutant cells did not grow, but the mutant cells grew more actively than the parent and the control cells at a low concentration under 2%. It was found that the long-chain alcohols such as butanol and isopropanol were more toxic to the growth of the parent cells than to the mutant.

Influence of Temperature on Growth and End Product Formation of the Mutant

Because the ethanol tolerance of the mutant strain was affected by culture temperature, the growth rate and product yield of the mutant might be different from those of the parent. The growth rate and ethanol yield were investigated to see if they were related to the culture temperature. Culture temperature was controlled from 55 to 68℃ to determine the optimum temperature.

Figure 2 illustrates typical growth curves for 23YP at various temperatures in KMG medium. The mutant showed biphasic growth curves with two specific growth rates at 55, 65 and 68℃. At 60℃, however, the mutant displayed a monophasic growth curve with the highest specific growth rate of 0.12 h^{-1}. The optimum growth temperature for the ethanol tolerant mutant, 23YP, decreased to 60℃, whereas that of the parent cell was maintained at 68℃. Activation energy for the growth of *C. thermohydrosulfuricum* 23YP was 199.34 KJ/mole. Fig. 3 shows the fermentation profile of the mutant at

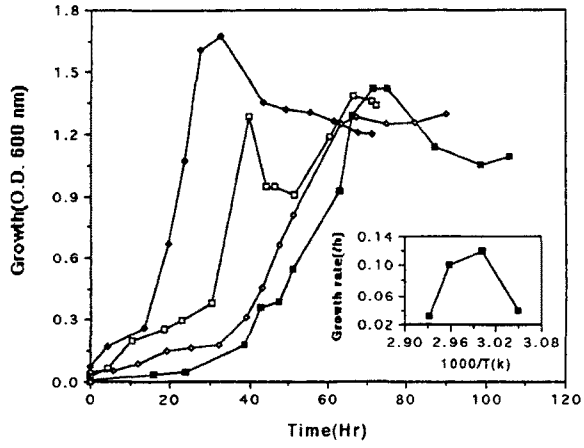

Fig. 2. Influence of different temperature and Arrhenius plot for growth rates of *C. thermohydrosulfuricum* 23YP in the KMS medium.
■; 55℃, ◆; 60℃, □; 65℃, ◇; 68℃

various temperatures. The mutant cell produced significantly lower concentration of acetic acid (0~0.4 g/L) than that of the parent (0.4~1.5 g/L). The highest ethanol concentration could be obtained at 60℃ (3.1 g/L). The physiological property for ethanol tolerance of *C. thermohydrosulfuricum* 23YP differed substantially from that reported for *C. thermocellum* in which its growth rate decreased at the ethanol concentration of less than 3.5% (1). On the contrary, the physiological property for ethanol tolerance was similar to that reported for *C. thermohydrosulfuricum* by Lovitt *et al.* (4). It was explained that *C. thermohydrosulfuricum* has unique membrane lipid component, C_{30} dicarboxylic acid, which was not found in *C. thermocellum* (2). Membranes containing this component as a major lipid may display strong alcohol tolerance because they are not subjected to the solvent disruption of the hydrophobic interactions between the side chains of free fatty acid which exist

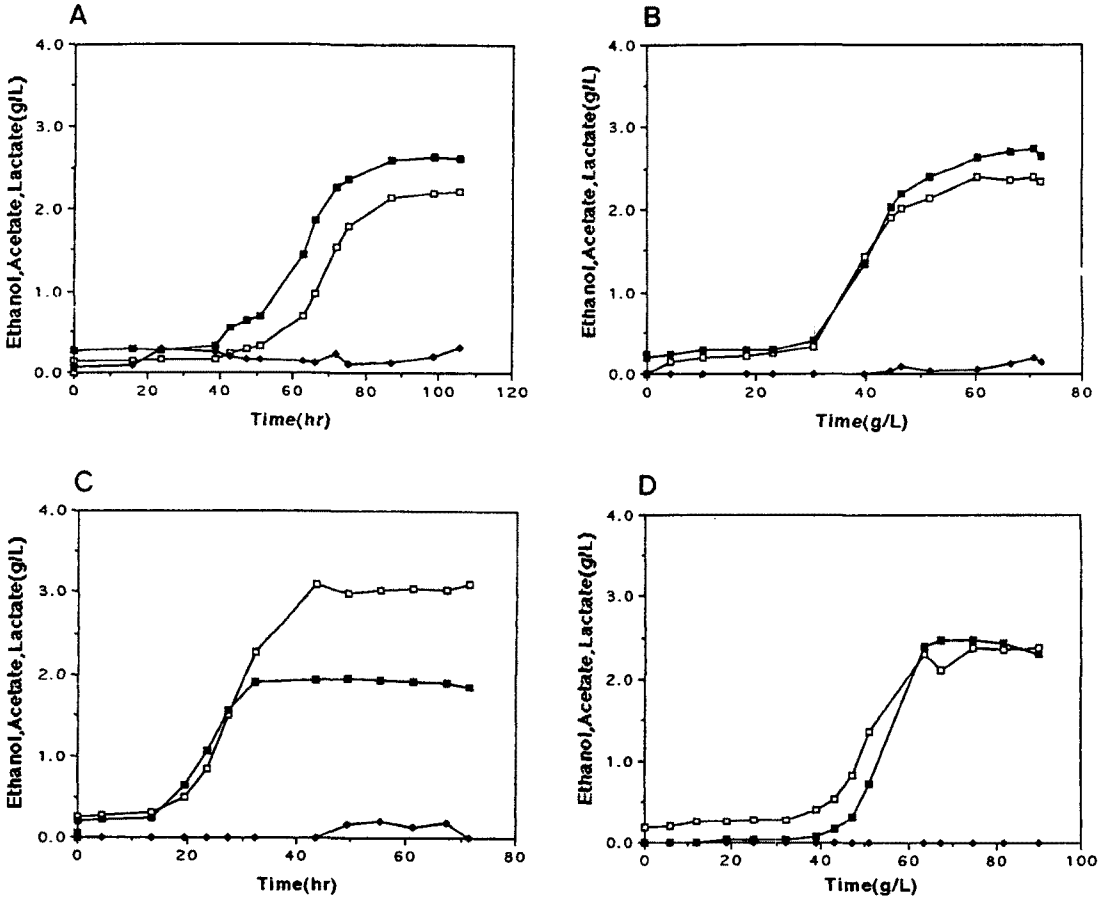

Fig. 3. End product formation of *C. thermohydrosulfuricum* 23YP at various temperatures. (A; 55°C, B; 60°C, C; 65°C, D; 68°C). Samples were harvested after 72 hr culture.
□; ethanol, ◆; acetic acid, ■; lactic acid

in normal lipids (4). Further work is needed to investigate the membrane composition of *C. thermohydrosulfuricum* 23YP. The relationship between the ethanol tolerant property and fatty acid composition of the membrane also remains to be clarified.

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