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Fermentation of Cellulose by a Newly Isolated Thermophilic *Clostridium* sp.

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A thermophilic, cellulolytic, gram negative, terminally sporulating and anaerobic bacterium was isolated from compost heap. Its colonies on solid medium containing cellulose were irregular, clear, translucent and had an undulated margin. The cells grown on the solid medium were rods of 0.5 to 0.7 μm wide by 3 to 6 μm long. All the properties insisted that this bacterium belonged to the genus *Clostridium*. Its optimum growth temperature was 60°C. This bacterium produced ethanol (45 mM), n-butanol (20 mM), acetic acid (23 mM) and n-butyric acid (25 mM) during 150 hr cultivation in a liquid medium containing 1 % cellulose as a sole carbohydrate source. The characteristic of n-butanol production may be a rare case in thermophilic cellulolytic anaerobes.

INTRODUCTION

First cellulolytic anaerobes were isolated from rumen of herbivorous animals. Most of them belonged to mesophilic *Clostridium* species, and their products were ethanol, acetic acid, butyric acid, lactic acid, formic acid and etc. (Hungate, 1960). Their cellulase activities were weak. Thermophilic anaerobes have also been studied by several workers (McBee, 1948; Lee and Blackburn, 1975; Weimer and Zeikus, 1977), because their degradation ability toward cellulose was stronger than that of mesophilic anaerobes.

In this work, we investigate on a newly isolated thermophilic cellulolytic anaerobe, especially its properties of solvent production.

MATERIALS AND METHODS

Media

The basal medium had the following composition per liter of deionized water: solution A (per 700 ml) ; K_2HPO_4, 2.2 g ; KH_2PO_4, 1.5 g ; $(NH_4)_2SO_4$, 1.3 g ; $FeSO_4 \cdot 7H_2O$, 6mg; $Na_2CO_3 \cdot 10H_2O$, 4g; yeast extract, 2g; polypepton, 5g; $CaCO_3$, 5g (only for liquid medium) and solution B (per 300 ml) : $MgCl_2 \cdot 6H_2O$, 1 g; $CaCl_2$, 0.15 g; cystein $HCl \cdot H_2O$, 0.5 g. The pH of each solution was adjusted to 7.0. Solutions A and B were separately sterilized at 1.2 kg/cm² for 20 min, and mixed aseptically just before use. Enriched liquid medium contained 5g of Avicel (microcrystalline cellulose, Asahi Chemical Industry Ltd.) and 10 g of filter

paper (Toyo Roshi Co. Ltd.) per liter of the basal medium contained 10 g of Avicel and 20g of agar per liter of the medium. Fermentation test medium contained 5g of various carbohydrates per liter of the basal medium. A larger scale liquid medium for the analysis of products contained 10 g of Avicel per liter of the basal medium.

Isolation of thenuophilic cellulolytic anaerobe

A thermophilic cellulolytic anaerobe was isolated from compost heap of the mixture of horse dung, rice strows and grass, collected from the Kasuya area of Fukuoka Prefecture.

One g of compost heap was suspended in 10 ml of 0.15 M NaCl solution. Then, 1 ml of the suspension was inoculated in 10 ml of enriched liquid medium containing 0.5 % Avicel and 1 % filter paper, and cultivated at 60°C. After 7 days, the filter paper-digested culture was inoculated in the fresh enriched liquid medium and cultivated as same above. After following 7 days, the filter paper-digested culture was diluted serially and each dilution was inoculated into solid medium of roll tube, according to Hungate (1944). Cellulose digesting colonies appeared on solid medium were picked up and their purity was certified by following the criteria of Hungate (1960). The isolates were preserved at -20°C in the culture medium plus 1% glycerine.

Anaerobic condition was maintained by passage of stream of N_2 gas. All anaerobic vessels were sealed with rubber stoppers.

Determination of cell growth

In liquid medium containing cellulose, cell growth was followed by measuring the contents of cell protein (Herbert et al., 1971), because the turbidity was not proportional to cell growth due to the existence of cellulose fibers. An aliquot of culture broth was taken out at desired intervals and centrifuged at 8, 000 $\times g$ for 20 min. The precipitate was washed with 0.15 M NaCl solution, and the cells in the precipitate were hydrolysed with 1 N NaOH at 100°C for 5min. Protein content in the hydrolysate was measured by the methods of Lowry et *al.* (1951).

In liquid media containing soluble carbohydrates, cell growth was followed by measuring the turbidity (OD_{660}) using electric photometer (Tokyo Koden Co. Ltd., Type 8 A).

Analysis of products

A 250 ml of liquid medium containing 1 % Avicel was used for the analysis of products. At desired time intervals, an aliquot of culture broth was taken out and centrifuged at 8,000 xg for 20 min. Its supernatant was subjected to the following analysis. The supernatant was applied on a small column (1 ml of volume) packed with Dowex 50 W X2 (100-200 mesh). The filtrate was analysed on the gas-chromatography (Hitachi Ltd., Type 163) which was equipped with glass U column (3 mm x2 m) packed with chromosorb 101 (Ottenstein and Bartley, 1971; Carlson, 1972). Amount of solvents and C_2-C_5 organic acids were calculated from their peak heights.

Products fermented from various carbohydrates were also analysed by gas-

chromatography, as described above.

Analysis of cellulase activities

Carboxymethylcellulase (CMCase : C_x activity) was measured as follows. One of the supernatant of culture broth was added to 1 ml of 1 % sodium carboxymethyl cellulose in phosphate buffer (0.1 M, pH 6.5) and incubated at 60°C for 5 hr, agitated by Monod shaker at 50rpm. The amount of reducing sugars liberated in the mixture was measured by the method of Somogyi (1952) and reading was taken at OD_{500nm} using by spectrophotometer (Hitachi Ltd., Type 124). Avicelase activity (C, activity) was measured by the same method as CMCase activity, using Avicel as substrate instead of CMC.

Analysis of residual cellulose

An aliquot of culture broth was centrifuged and the precipitate was hydrolysed, as described above. And then, a desired volume of conc. acetic acid was added to the precipitate in order to dissolve residual $CaCO_3$. The residual cellulose in the suspension was filtrated by 5 μm of pore size of membrane filter (Toyo Roshi Co., Ltd.). The filtrated cellulose was dried at 60°C for 12 hr, and weighed (Herbert *et al.*, 1971).

Electron microscopy

A 5 day-old culture grown on solid medium containing 1 % Avicel was used for electron microscopy. The cells were stained with 2 % phosphotungstic acid solution, and observed with JEM 100B electron microscope (Japan Electron Optics Lab. Ltd.).

RESULT

Morphology of colony and cells of strain AH-1

A thermophilic and cellulolytic anaerobe isolated from compost heap was named as strain AH-1. Strain AH- 1 made clear cellulolytic zone around the colonies on solid medium containing 1% Avicel, as shown in Fig. 1. The colonies and their cellulolytic zone were 1 mm and 2 mm in diameter, respectively. They were translucent, white color and irregular form with undulated margin. Under microscopic observation, the cells were straight or slightly curved rod, slightly motile and formed oval terminal endospore. Its size was 0.5 to 0.7 μm wide by 3 to 5 μm long. The cells were stained gram negatively.

Growth condition of strain AH-1

Strain AH-1 grew at high temperature, the range from 50 to 65°C, and no growth was observed below 45°C or above 70°C, as shown in Fig. 2. Highest growth was observed at 60°C. After 10 hr of lag phase, exponential growth phase continued for 20 hr in liquid medium containing 1 % Avicel at 60°C. In stationary phase, the cell protein was 0.32 mg per ml of culture broth at 60°C. The optimum pH for growth was 6.5 to 7.0. No growth occurred aerobically

Determination of biochemical properties of strain AH--l

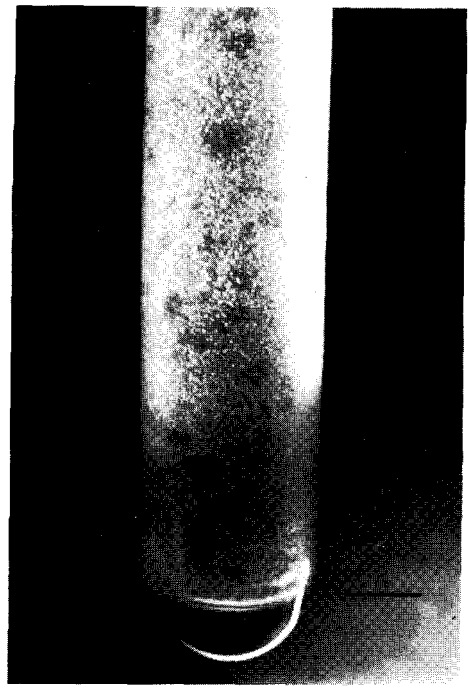

Fig. 1. Colonies of strain AH--l in role tube after 5 days of cultivation. Bar shows 1 cm.

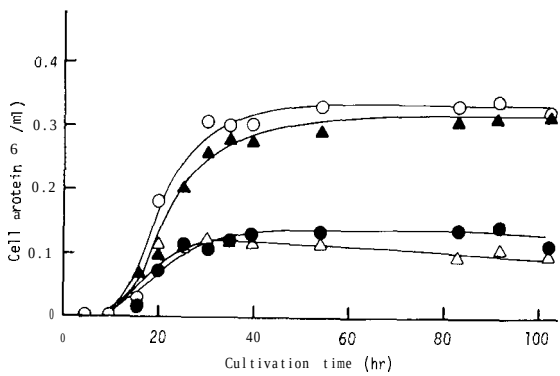

Fig. 2. Growth curve of strain AH–1 at various temperature. Strain AH-l was grown in a 250 ml of liquid medium containing 1 % Avicel. —△—, 50°C; —▲—, 55°C; —○—, 60°C; -.-, 65°C.

Biochemical properties of strain AH–1 were investigated as followed by the methods of Suzuki (1968). As shown in Table 1, strain AH 1 did not utilize sodium sulfate added into the liquid medium, not liquify gelatin and not reduce sodium nitrate through 5 days cultivation at 60°C. Strain AH-1 produced

Table 1. Properties of strain AH-l.

Tests	
Sulfate reduction	
Gelatin liquification	—
Nitrate reduction	—
Acetylmethylcarbinol production	
Indol production	+
Urease production	—
Catalase production	
Gram staining	

indol as the result of utillization of tryptophane that contained in polypepton.

Utilization of various carbohydrates by strain **AH-1**

Strain AH-l fairly fermented fructose, galactose, glucose, mannose, cellobiose, lactose, maltose, sucrose, melibiose, trehalose, dextrin, glycogen, starch, amygdalin, esculin, salicin, dulcitol and solbitol, though the cultivation times reached to the stationary phase were varied each other. The growth hardly occurred without carbohydrates, as shown in Table 2.

As shown in Table 3, strain AH-l produced ethanol, n-butanol, acetic acid and n-butyric acid from all carbohydrates fermented in 5 day of cultivation.

Cellulolytic activities

In a 250 ml of liquid culture containing 1 % Avicel, the cellulose degradation continued over 150 hr of cultivation, though the cell growth came to stationary phase at 50th hr. The cellulose was degraded to the extent of 80 % at 150th hr, as shown in Fig. 3. CMCase activity in the supernatant of the

Table 2. Utilization of carbohydrates by strain AH-l.

Carbohydrates	Growth[1)]	Carbohydrates	Growth[1)]
Monosaccharide		Polysaccharide	
L-(+)-Arabinose	+	Dextrin	++
D-(+)-Fructose	++	Glycogen	++
D-(+)-Galactose	++	Inulin	+
D-(+)-Glucose	++	Starch	++
D-(+)-Mannose	++	Glucoside	
L-(+)-Rhamnose	+	Amygdalin	++
D-(+)-Ribose	+	Esculin	++
D-(+)-Xylose	+	Salicin	++
Sorbose	+	Alcohol	
Disaccharide		Erythritol	—
Cellobiose	itt	Inositol	
Lactose	++	Mannitol	+
Maltose	++	Glycerol	—
Sucrose	++	Dulcitol	++
Melibiose	++	Sorbitol	ttt
Trisaccharide		Adonitol	—
Trehalose	++		
Raffinose	+	None	
Melezitose			

1) Growth was determined by its tnrbidity (OD_{660nm}) when the culture reached to stationary phase. ++, >0.4; ++, 0.2-0.4; +, <0.2; —, no growth.

Table 3. Products of strain AH-1 in liquid culture containing various carbohydrates. Strain AH-1 was cultivated in 10 ml of fermentation test medium containing 0.5% each carbohydrate at 60°C.

Carbohydrates	Products (mM)			
	Ethanol	*n*-Butanol	Acetic acid	n-Butyric acid
D-(+)-Fructose	24.7	1.7		10.7
D-(+)-Galactose	28.2	2.5	14.5 7.8	3.2
D-(+)-Glucose	23.3	1: 5	13.7	10.4
D-(+) --Mannose	24.4	1.2		11.3
L-(+)-Rhamnose	5.9	2.8	14.9 13.2 19.6	18.0
D-(+)-Ribose	12.7	3.0		6.4
D-(+)-Xylose	16.5	1.3	12.8	8.8
Cellobiose	27.0	2.2	15, 16.4 1	14.8
Lactose	25.3			21: 2
Maltose	29.5	3.2	14.4	14.1
Sucrose	47.1	1.9	8.2	2.8
Tre halose	31.5	4.9	8.9	29.6
Dextrin	28.5	2.6	11.4	12.8
Starch	26.5	0' 2.6	11.8	10.8
None	trace		trace	trace

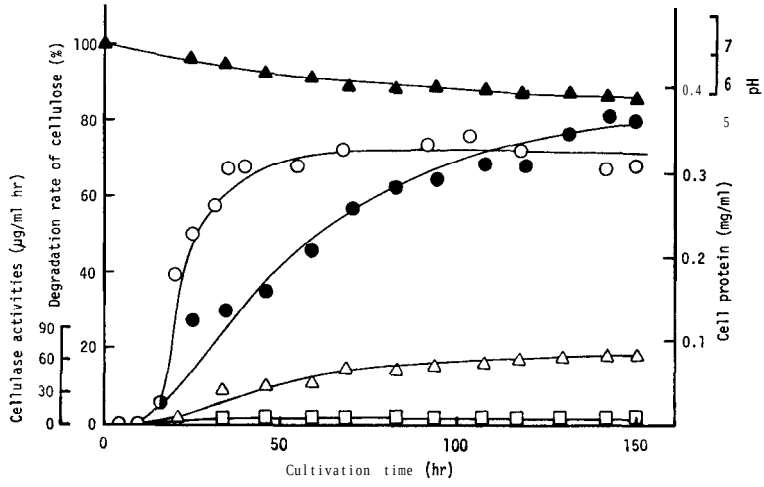

Fig. 3. Time-course of cellulose degradation and production of cellulose by strain AH-l at 60°C. Strain AH-l was grown in a 250 ml of liquid medium containing 1% Avicel. —o—, cell growth; —•—, degradation rate of cellulose; —▵—, CMCase activity; —□—, Avicelase activity; —▲—, pH.

culture broth increased proportionally with the cellulose degradation rate. Its activity was 64 μg of glucose per ml·hr at 150th hr of cultivation. Avicelase activity was one-tenth of CMCase activity and it did not increase through the cultivation. Avicelase of strain AI--l might be hardly released into the culture broth.

Products of strain AH-l in cellulose containing liquid medium

Strain AI-1 produced ethanol, n-butanol, acetic acid and n-butyric acid through the fermentation of cellulose. Ethanal and acetic acid were produced in the culture broth at the beginning of exponential growth phase, and gradually increased from early stationary phase to about 130 hr of stationary phase. n-Butyric acid and n-butanol were detected at early stationary phase, and increased until 130 hr of stationary phase, as shown in Fig. 4. At 150th hr of cultivation, the yields of products were as follows ; ethanol 45 mM (2.1 g/l), *n*-butanol 20 mM (1.5 g/l), acetic acid 23 mM (1.4 g/l) and n-butyric acid 25 mM (2.2 g/l).

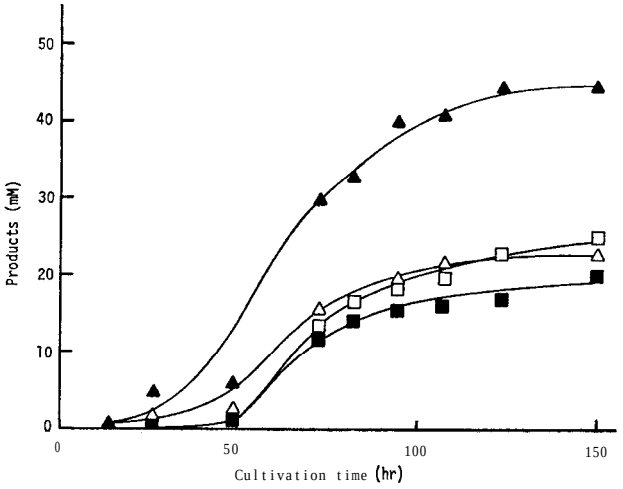

Fig. 4. Production of solvents and organic acids during cellulose fermentation by strain AH-l. Strain AH-1 was grown in a 250ml of liquid medium containing 1 % Avicel. —▲—, ethanol; —■—, n-butanol; —△—, acetic acid ; —□—, n-butyric acid.

DISCUSSION

A newly isolated thermophilic cellulolytic anaerobe, strain AH-l, was motile and spore forming rod, and this strain could not utilize sulfate. These properties implied that strain AI-1 belonged to genus *Clostridium*, not to *Desulfotomaculum* (Smith and Hobbs, 1974), compared with some thermophilic and cellulolytic clostridia already reported. Furthermore strain AH-l have similar morphological and biochemical properties, but differs in the utilization of carbohydrates and their products. C. *thermocellum* (McBee, 1948; Weimer and Zeikus, 1977; Taya *et al.*, 1978) cannot grow in the absence of special substrate, cellulose, hemicellulose or their components such as xylose and cellobiose. Its products are formic acid, acetic acid, lactic acid and ethanol, but n-butyric acid and n-butanol are not detected (McBee, 1948). Whereas strain AH-l can grow even in non-cellulosic carbohydrates such as fructose, lactose, glycogen and starch. And it produced ethanol, n-butanol, acetic acid and n-butyric acid from

those carbohydrates utilized. These properties and its biological properties that strain AH-l is different species from C. *thermocellum* that belong to group indicate V of genus ***Clostridium.*** And strain AH-l belong to group III, though no thermophilic *Clostridium* is described in Bergey's manual (Smith and Hobbs, 1974). On the other hand, C. *thermocellulaseum* (Enebo, 1951) and ***Clostridium*** sp. M7 (Lee and Blackburn, 1975) are thought to be similar species as those strains have wide variety of fermentative capacity. But the former do not produce n-butanol, and the latter is not investigated on its productivity of acids and solvents.

Strain AH-l degraded 8g of cellulose per liter of culture broth in 150 hr of cultivation. But its Avicelase activity was weak compared with CMCase activity. This weak Avicelase activity might be caused by the adsorption of Avicelase to undegraded cellulose or cell-boundary properties of the cellulases as described in the previous reports (Lee and Blackburn, 1975; Ng ***et al.,*** 1977).

The products of strain AH-1 from cellulose were ethanol, n-butanol, acetic acid and n-butyric acid. They are typical products of non-cellulolytic and mesophilic clostridia. In the case of thermophilic clostridia, n-butanol was hardly produced except for C. *thermobutyricum* (Enebo, 1951), but this strain cannot utilize cellulose as carbon source and its amount is very low compared with strain AH-1. Therefore, strain AH-1 is a unique thermophilic cellulolytic ***Clostridium,*** because of utilization of cellulose as substrate for n-butanol production.

In this work, we find out the possibility of solvent production using by thermophilic cellulolytic ***Clostridium.*** But the solvent productivity of strain AH-l is fairly low compared with that of mesophilic clostridia. Our current studies are aimed at solving this problem,

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