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Effect of Yeast Extract and Vitamin B_{12} on Ethanol Production from Cellulose by *Clostridium thermocellum* I-1-B

KANJI SATO,† SHINGO GOTO, SOTARO YONEMURA,‡ KENJI SEKINE, EMIKO OKUMA,§ YOSHIO TAKAGI, KOYU HON-NAMI,‖ AND TAKASHI SAIKI*

R & D Center, Alcohol Production Head Office, New Energy and Industrial Technology Development Organization, Inagehigashi 4-5-1, Chiba, Japan 281

Received 14 May 1991/Accepted 22 November 1991

Addition to media of yeast extract, a vitamin mixture containing vitamin B_{12}, biotin, pyridoxamine, and *p*-aminobenzoic acid, or vitamin B_{12} alone enhanced formation of ethanol but decreased lactate production in the fermentation of cellulose by *Clostridium thermocellum* I-1-B. A similar effect was not observed with *C. thermocellum* ATCC 27405 and JW20.

The single-step conversion of cellulosic biomass to ethanol by anaerobic bacteria may have economic advantages over the multiple-step process in which fungal cellulases and yeasts are used. The advantages and disadvantages of anaerobic bacterial fermentation have been discussed previously (2, 8, 15–17). One disadvantage of the anaerobic process is that significant amounts of organic acids, such as acetic acid and lactic acid, are produced in addition to ethanol. It is important to decrease the formation of organic acids to develop a practical process for direct conversion of biomass to ethanol. In an extensive screening program for superior thermophilic cellulolytic anaerobes, we isolated cellulolytic strain I-1-B of *Clostridium thermocellum* from Shibi Hot Spring in Kagoshima Prefecture, Japan. When this strain was grown in media containing high concentrations of yeast extract, it produced more ethanol and less lactate. In this paper we describe the effect of yeast extract and vitamin B_{12} on the ratio of fermentation products in *C. thermocellum* I-1-B cultures.

Yeast extract type D-3 (lot W106) was obtained from Daigoeiyo Chemical Co. Ltd., Osaka, Japan. Cellulose (cellulose powder C) and cellobiose were obtained from Toyo Rosi Co., Tokyo, Japan, and Kanto Kagaku Co., Tokyo, Japan, respectively. Pyridoxamine dihydrochloride was obtained from Sigma Chemical Co., St. Louis, Mo. D-Biotin, *p*-aminobenzoic acid, and vitamin B_{12} were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. All of the other chemicals which we used were reagent grade.

C. thermocellum I-1-B, which was isolated from a soil sample obtained from Shibi Hot Spring in Kagoshima Prefecture, has been deposited with the Fermentation Research Institute (Tsukuba) as Japan patent strain FERM P-9510. This strain will be described elsewhere. *C. thermocellum* ATCC 27405 was obtained from the American Type Culture Collection, Rockville, Md., through Sumisho Pharma Corp., Osaka, Japan. *C. thermocellum* JW20 (= ATCC 31549) was kindly provided by L. G. Ljungdahl, University of Georgia, Athens.

The anaerobic techniques of Hungate (6) were used in this study. The GS medium of Garcia-Martinez et al. (5) was modified slightly and was used as the basal medium. This medium contained (per liter of distilled water) 2.9 g of K_2HPO_4, 1.5 g of KH_2PO_4, 2.14 g of urea, 1 g of $MgCl_2 \cdot 6H_2O$, 0.15 g of $CaCl_2 \cdot 2H_2O$, 1.25 mg of $FeSO_4 \cdot 6H_2O$), 20.9 g of morpholinopropanesulfonic acid (MOPS), 0.5 g of cysteine hydrochloride, 0.5 g of $Na_2S \cdot 9H_2O$, and 2 mg of resazurin. To this medium we added 1.0% cellulose as a carbon source, and we varied the concentrations of yeast extract and vitamins. The standard vitamin mixture, which was sterilized by filtration, contained (per liter) 2,260 μg of pyridoxamine, 200 μg of biotin, 400 μg of *p*-aminobenzoic acid, and 200 μg of vitamin B_{12}. Varying amounts of the vitamin mixture were added to the culture media. The concentration of the vitamin mixture is expressed as the concentration of vitamin B_{12} below. The gas phase at the start of each culture was nitrogen (1 atm [1.01×10^2 kPa]). The pH of the medium was adjusted at 7.5 before autoclaving. Cultures were grown at 60°C without shaking.

Growth was measured by determining the optical density at 610 nm (OD_{610}). Cultures that contained residual cellulose were mixed in a Vortex mixer and allowed to stand for 45 min before the OD_{610} was measured.

Ethanol concentrations were determined by gas chromatography (10). Lactic acid, acetic acid, and formic acid concentrations were determined by high-performance liquid chromatography, using a model IC500 ion chromatograph analyzer (Yokogawa Denki Inc., Tokyo, Japan) equipped with a Yokogawa type SCS5-252 ion exclusion column (250 by 8 mm), a type PCS5-052 precolumn, and a conductivity detector.

Figure 1A shows the effect of yeast extract on the fermentation products of *C. thermocellum* I-1-B. As the yeast extract concentration increased in the medium, the production of ethanol increased; ethanol production reached a maximum at a yeast extract concentration of 1.4%. At concentrations above 1.4% ethanol production decreased. On the other hand, the formation of lactate was at the lowest level at a yeast extract concentration of about 1.4%. The production of acetate and the production of formate were at

* Corresponding author.

† Present address: Kashima-Kita Electric Power Corp., 16, Towada, Kamisu-machi, Kashima-gun, Ibaraki, Japan 314-02.

‡ Present address: Environmental Engineering Department, Institute of Technology, Shimizu Corp., 4-17, Etchujima 3-chome, Koto-ku, Tokyo, Japan 135.

§ Present address: Laboratory of Chemistry, Faculty of Home Economics, Kyoritsu Women's University, 1-710, Motohachioji, Hachioji, Tokyo, Japan 193.

‖ Present address: Global Environment Department, Engineering Research Center, Tokyo Electric Power Co., Inc., 2-4-1, Nishitsutsujigaoka Chofu-shi, Tokyo, Japan 182.

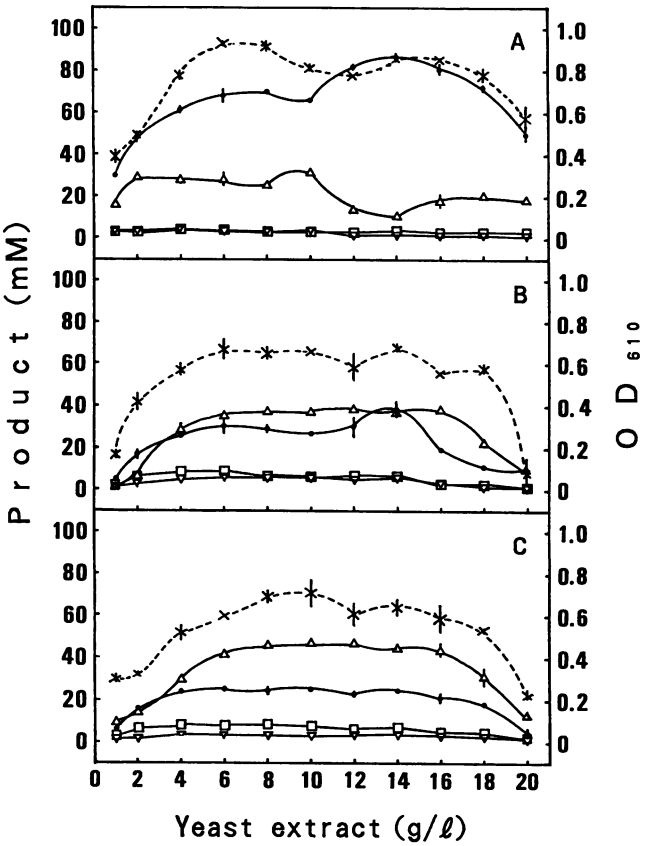

FIG. 1. Effect of yeast extract concentration on fermentation products in different strains of *C. thermocellum*. The results are means of values from duplicate cultures. Fermentation product concentrations and OD_{610} were measured after 7 days of culture at 60°C. Symbols: ●, ethanol; △, lactate; □, acetate; ▽, formate; ×, OD_{610}. (A) Strain I-1-B. (B) Strain ATCC 27405. (C) Strain JW20.

their low levels at all of the concentrations of yeast extract examined. At a yeast extract concentration of 1.4% the concentrations of ethanol, lactate, and total organic acids were 86.8, 10.4, and 16.9 mM, respectively, the ratio of ethanol to lactate was 8.35, and the ratio of ethanol to total organic acids was 5.14. Strain I-1-B did not grow on a medium that contained 0.6% yeast extract and no other carbon source.

The values for production of ethanol and organic acids by *C. thermocellum* ATCC 27405 and JW20 are plotted against yeast extract concentration as a variable in Fig. 1B and C, respectively. These strains produced much more organic acids than ethanol. At a yeast extract concentration of 1.4% the concentrations of ethanol, lactate, and total organic acids were 38.6, 37.3, and 50.6 mM, respectively, for strain ATCC 27405 and 24.8, 45.0, and 57.0 mM, respectively, for strain JW20. At this yeast extract concentration the ratios of ethanol to lactate were 1.03 for strain ATCC 27405 and 0.55 for strain JW20, and the ratios of ethanol to total organic acids were 0.76 for strain ATCC 27405 and 0.44 for strain JW20.

Figure 1 shows that the optimum concentration of yeast extract for ethanol production by strain I-1-B was about 1.4%. On the other hand, strains ATCC 27405 and JW20 needed about 0.6% yeast extract, and higher concentrations did not affect the ratios of ethanol to lactate.

TABLE 1. Effect of the vitamin mixture on the fermentation product ratios in *C. thermocellum* I-1-B[a]

Vitamin mixture concn (μg/liter)	Product concn (mM)				Ratio of:		OD_{610}
	Ethanol	Lactate	Acetate	Formate	Ethanol to lactate	Ethanol to acids[b]	
0	30.8	21.9	4.8	3.9	1.41	1.01	0.19
0.02	28.9	23.0	4.7	3.7	1.26	0.92	0.26
0.2	37.6	21.4	5.3	3.5	1.76	1.25	0.39
2	34.3	21.0	5.8	3.3	1.63	1.14	0.33
20	43.4	16.7	7.2	4.1	2.60	1.55	0.54
200	59.3	4.0	12.3	10.4	14.83	2.22	1.10
2,000	59.3	3.6	12.0	10.0	16.47	2.32	1.13

[a] The vitamin mixture (expressed as the concentration of vitamin B_{12}) was added to the basal medium containing 1.0% cellulose and 0.2% yeast extract. Fermentation product concentrations and OD_{610} were measured after 7 days of culture at 60°C. Each value is the average of values from two separate cultures.

[b] The acids included for these determinations were lactate, acetate, and formate.

As Table 1 shows, a vitamin mixture containing vitamin B_{12}, biotin, pyridoxamine, and *p*-aminobenzoic acid was as effective as yeast extract for increasing ethanol production and decreasing lactate production by strain I-1-B. Vitamin B_{12} at concentrations of more than 20 μg/liter (Table 2) had the same effect as the vitamin mixture.

The wild-type strains of *C. thermocellum* that have been studied so far produce about the same or higher concentrations of organic acids than ethanol from cellulose (1, 3, 4, 7, 9, 11–14). For example, strain ATCC 27405 has been reported to produce 25 mM ethanol, 10 mM lactate, and 7.5 mM acetate (7). Wang et al. (13) obtained mutants of strain ATCC 27405 that had ratios of ethanol production to acid production of 5:1, although the parent strain produced about equal amounts of ethanol and acetic acid on solka floc. Lynd described the instability of the mutant strains (8). Under our cultural conditions the ratio of ethanol to acidic end products for strain ATCC 27405 was 0.76 (Fig. 1B). Freier et al. (4) have reported that *C. thermocellum* JW20 produced 25.6 mM ethanol, 9.5 mM lactate, and 13.0 mM acetate when it was grown on 1% cellulose, giving a ratio of ethanol to lactate plus acetate of 1.14. In our experiments the ratio of ethanol to acids for strain JW20 ranged from 0.5 to 1. The

TABLE 2. Effect of vitamin B_{12} on the fermentation product ratios in *C. thermocellum* I-1-B[a]

Vitamin B_{12} concn (μg/liter)	Product concn (mM)				Ratio of:		OD_{610}
	Ethanol	Lactate	Acetate	Formate	Ethanol to lactate	Ethanol to acids[b]	
0	30.8	21.9	4.8	3.9	1.41	1.01	0.19
0.02	31.5	21.8	4.2	3.7	1.44	1.06	0.24
0.2	30.2	21.9	4.5	3.7	1.38	1.00	0.20
2	32.6	21.9	5.2	3.7	1.49	1.06	0.28
20	48.8	15.4	6.7	6.3	3.17	1.72	0.67
200	55.4	10.6	7.5	7.2	5.23	2.19	0.78
2,000	56.7	7.9	7.2	8.0	7.18	2.45	0.79

[a] Vitamin B_{12} was added to the basal medium containing 1.0% cellulose and 0.2% yeast extract. Fermentation product concentrations and OD_{610} were measured after 7 days of culture at 60°C. Each value is the average of values from two separate cultures.

[b] The acids included for these determinations were lactate, acetate, and formate.

ratio of ethanol to acidic end products seems to be very sensitive to cultural conditions.

The data shown in Fig. 1 and previous data (1, 3, 4, 7, 9, 11–14) demonstrate that strain I-1-B is superior to other wild-type strains of *C. thermocellum* for producing ethanol from cellulose. Tailliez et al. (11, 12) seem to have been successful in obtaining asporogenous mutants from strain NCIB 10682 that yield higher ratios of ethanol to acidic products. These authors reported a ratio of ethanol to acids of 2, which is much lower than the ratio obtained with strain I-1-B.

The effect of yeast extract or a vitamin mixture containing biotin, *p*-aminobenzoic acid, pyridoxamine, and vitamin B_{12} on increasing ethanol formation and decreasing lactate formation seems peculiar for strain I-1-B since such an effect was not observed with *C. thermocellum* ATCC 27405 and JW20. The effective component of the vitamin mixture appears to be vitamin B_{12} (Table 2). The increase in ethanol formation seems to be related to an increase in growth yield. Johnson et al. (7) reported that *C. thermocellum* ATCC 27405 requires the vitamins biotin, *p*-aminobenzoic acid, pyridoxamine, and vitamin B_{12} for growth and that the requirement for vitamin B_{12} or *p*-aminobenzoic acid is compensated for by methionine.

It should be noted that different yeast extract preparations from different pharmaceutical companies and even yeast extracts with different lot numbers from the same pharmaceutical company gave different results. This may have been due to differences in the vitamin contents of the various yeast extract preparations.

So far there is no explanation for the effects of vitamins or vitamin B_{12} on the fermentation end product ratios in *C. thermocellum* I-1-B.

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