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Specificity of Alcohol Dehydrogenase from *Clostridium acetobutylicum* ATCC 4259

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Alcohol dehydrogenase activity of *Clostridium acetobutylicum* ATCC 4259 was studied for its specificity against substrates in acidogenic and solventogenic cultures. The bacterium reduces propionate, valerate and caproate added to the medium to the corresponding alcohols. Acetaldehyde, propionaldehyde, butyraldhyde, pentanal, and hexanal were used as the substrates by alcohol dehydrogenase, and all were reduced to the corresponding alcohols with varying affinities and reaction velocities. Acetaldehyde showed the lowest affinity and lowest velocity while the other aldehydes showed similar K_m and V_{max} values. NADPH was used as the electron donor for the reduction of aldehydes. Alcohol dehydrogenase activity was low in acidogenic culture, and high in solventogenic culture.

Acetone-butanol-ethanol(ABE) fermentation is one of the oldest fermentation processes practiced in industry (14). Though there have been suggestions for the importance of alcohol dehydrogenase in ABE fermentation (7), few attempts have been made to elucidate the specificity and regulation of the enzyme.

Fogarty and Ward (5) examined the specificity of alcohol dehydrogenase of *C. acetobutylicum* NCIB 8049, with NADH as the electron donor. The enzyme activity was non-specific with regard to substrate, oxidizing primary and secondary alcohols.

Enzyme activities were compared between acidogenic and solventogenic cultures of *C. acetobutylicum* DSM 1732 (1), *C. acetobutylicum* NRRL B643 (15), and *C. beijerinckii* (6, 9). All the strains showed higher alcohol dehydrogenase activity in solventogenic culture than in acidogenic culture. The electron donor for these reactions was NADPH.

Though there is some evidence that butanol producing clostridia have separate alcohol dehydrogenases for ethanol and butanol (15), it is generally accepted that a single enzyme is reponsible for the production of ethanol and butanol. Allyl alcohol resistant mutants of *C. acetobutylicum* B643 showed coordinate reductions in activities of ethanol dehydrogenase and butanol dehydrogenase (16). For this reason it was hypothesized that this bacterium uses a single protein to produce butanol and ethanol. Hiu *et al.* (9) purified alcohol dehydrogenases of *C. beijerinckii* B592 and *C. beijerinckii* B593 and showed that a single alcohol dehydrogenase is involved in the productions of ethanol and butanol in *C. beijerinckii* B592, and ethanol, butanol and iso-propanol in *C. beijerinckii* B593.

In this report, *C. acetobutylicum* ATCC 4259 was used to study substrate specificity and electron donor of alcohol dehydrogenase activity.

MATERIALS AND METHODS

Chemicals

Reagent grade chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo) and Mallinckrodt (Paris, Ky). Gases were obtained from Matheson Scientific, Inc. (Joliet, Ill).

Bacterial Strain and Maintenance

C. acetobutylicum ATCC 4259 was maintained in preserved soil culture at room temperature (10).

Culture Conditions

Anaerobic conditions were maintained throughout medium preparation, culture, and cell harvest (10). For

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culture revival a few grains of soil culture was inoculated into 10 m*l* of CAB medium in an anaerobic pressure tube (Bellco Glass, Inc., Vineland, N.J.). The tube was subjected to a heat shock of 80℃ for 2 min before incubation at 34℃ for 48 hrs. The tube culture was used to inoculate 70 m*l* of CAB medium in a 150 m*l* serum vial, which was incubated at 34℃ for 24 hr for use as an inoculum, and for 3 days for fermentation experiment without shaking. An acidogenic culture was made using a Microferm fermentor (New Brunswick Scientific Co., Inc., Edison, N.J.) with a 7 *l* working volume at 34℃ for 16 hr. The culture was agitated at a speed of about 100 rpm and pH was maintained at 6.0± 0.1 by titrating the acids produced by 3 N NaOH using an automatic pH controller. A solventogenic culture was made using a 20 *l* carboy with a 10 *l* working volume at 34℃ for 24 hr without agitation. pH was not controlled with initial pH of 5.0. The growth was initiated by a 5% inoculum.

Quantification of Substrate and Fermentation Products

The glucose concentration was determined by the glucose oxidase method (Sigma Co.). Soluble fermentation products were analyzed by gas chromatographic methods using a Super Q column for ethanol and acetone, and a Chromosorb 101 column for fatty acids and alcohols other than ethanol (11).

Cell-free Extract Preparation and Enzyme Assay

Anaerobic procedures were used for the preparation of cell-free extract and enzyme assay (10). Cells were collected anaerobically by centrifugation using a KSB continuous flow system (Sorvall/Dupont, Wilmington, Del) and were resuspended in anaerobic distilled water, containing 3 mM dithiothreitol and 2 $\mu g \cdot ml^{-1}$ DNase, after the cells were washed with anaerobic saline solution containing 8.5 $g \cdot l^{-1}$ NaCl and 3 mM dithiothreitol. For the preparation of cell-free extract acidogenic cells were passed through a French press cell (American Instrument Co., Inc., Silver Spring, Md) at a pressure of 1,400 kg/cm^2 and solventogenic cells were incubated at 37℃ for 60 min with 50 $g \cdot ml^{-1}$ Mutanolysin (Sigma Co.) in 0.05 M phosphate buffer (pH 6.8). The enzyme treated cell suspensions were frozen at −70℃ and thawed before passing through the French press. Cell lyzates were centrifuged anaerobically at 10,000×g for 30 min. Supernatants were transferred to anaerobic glass vials for use as enzyme sources. The protein content was determined by the method of Lowry *et al.* (13) with bovine serum albumin as a standard.

Alcohol dehydrogenase activity was determined in both directions of alcohol oxidation and aldehyde reduction with oxidized and reduced NAD and NADP using 1.6 m*l* anaerobic cuvettes with 1 m*l* of reaction mixture spectrophotometrically at 334 nm ($\varepsilon = 6.1 \cdot mM^{-1} \cdot cm^{-1}$). The reaction mixture contained 0.1 M electron carrier and 20 mM substrate. The reaction was started by the addition of substrate to determine the electron carrier oxidation and reduction activities. The amounts of the electron carriers reduced and oxidized without substrates were subtracted from those with substrates to calculate enzyme activity. Ferredoxin, FAD and neutral red were tested as electron carriers for enzyme activity.

RESULTS

Fatty Acid Reduction by *C. acetobutylicum*

CAB medium with 30 $g \cdot l^{-1}$ glucose was made using anaerobic pressure tubes to which propionte, valerate or caproate were added before autoclave. To test the effects of electron flow modulators, 7% carbon monoxide in the headspace was added before autoclave and 5 mM neutral red after autoclave. The tubes were inoculated with a 24 hr old inoculum and incubated 96 hr before the fermentation products were anaylyzed by gas chromatography (Table 1). It was found that the fatty acids used in this study can be metabolized to alcohols, although they are not the metabolic products of glucose nor are they present in the natural fermentation system. This result shows that enzymes involved in the reduction of acetate and butyrate to ethanol and butanol can catalyze the reduction of the acids used in these experiments.

Glucose consumption was greatly reduced by the addition of fatty acids, especially in the fermentations added by caproate and valerate. Propionate at the concentration of 30 mM did not inhibit glucose consumption (data not shown). Valerate and caproate, at the same concentration, completely inhibited fermentation. Acids with higher carbon numbers showed higher degrees of inhibition. Glucose consumption was also inhibited by carbon monoxide and neutral red. This inhibition was significant in fermentation with added caproate. The electron modulators used in the study increased the reduction of acids added to alcohol.

Electron Carriers Used by Alcohol Dehydrogenase

Cell-free extracts were prepared from acidogenic cells grown at pH 6.0 for 16 hr, and solventogenic cells grown for 24 hr without pH control. Alcohol dehydrogenase activities were measured in both directions using acetaldhyde, ethanol, butyraldehyde and butanol as substrates, and NAD, NADH, NADP and NADPH as electron carriers (Table 2). The Highest activity was obtained in the reduction of butyraldehyde coupled with NADPH reduction. The reduction of butyraldhyde coupled with NADH was less than 10% of the NADPH coupled activity. The

Table 1. Reduction of added fatty acids during acetone-butanol-ethnol fermentation.

Acid Added (mM)	Electron Flow Modulator	Glucose Consumed (mM)	Acid Remaining (mM)	Alcohol Produced (mM)
				Propanol
Propionate (30)	None	107.5	18.4	10.6
	5 mM NR	95.3	16.0	14.3
	7% CO+5 mM NR	83.8	12.8	18.8
				Pentanol
Valerate (15)	None	59.3	4.1	7.3
	5 mM NR	57.1	2.3	10.9
	7% CO+5 mM NR	52.0	2.4	14.3
				Hexanol
Caproate (10)	None	43.8	9.3	0.2
	5 mM NR	14.4	9.4	0.3
	7% CO+5 mM NR	20.9	9.5	0.3

NR: Neutral Red
CO: Carbon monoxide
The cultures were made using an anaerobic pressure tube containing 10 ml of CAB medium at initial pH of 5.0 without shaking at 34℃ for 72 hr. Acids and carbon monoxide were added before and neutral red after autoclave. Acids and alcohols were analyzed by gas chromatographic methods.

Table 2. Alcohol dehydrogenase activities of acidogenic and solventogenic cultures.

Electron Carrier	Substrate	Activity (nM·mg protein^{-1}·min^{-1})	
		Acidogenic Culture	Solventogenic Culture
NADH	Acetaldehyde	2	9
	Butyraldehyde	11	8
NADPH	Acetaldehyde	20	72
	Butyraldehyde	71	247
NAD	Ethanol	1	1
	Butanol	1	1
NADP	Ethanol	3	6
	Butanol	1	18

The acidogenic culture was made in a pH controlled fermentor at 6.0 for 16 hrs. The solventogenic culture was made in a carboy without pH control at an initial pH of 5.0 for 24 hrs. For the preparation of cell-free extract, acidogenic cells were passed through a Frech press. Solventogenic cells were treated by Mutanolysin and freeze-thaw treatment before passing through the French press. Enzyme activities were measured at 37℃ using an anaerobic cuvette containing 0.1 M phosphate buffer (pH 7.8) 1 ml, 2 mM dithiothreitol, 5 mM substrate, and 0.3 mM electron carrier.

Table 3. NADPH-aldehyde reductase activities on different aldehydes.

Substrate	Activity (nM·mg protein^{-1}·min^{-1})	
	Acidogenic Culture	Solventogenic Culture
Acetaldehyde	24	72
Propionaldehyde	68	243
Butyraldehyde	77	247
Pentanal	78	261
Hexanal	72	335

Cell-free extract prepared from solventogenic culture was used. Enzyme activities were measured at 37℃ using an anaerobic cuvette containing 0.1 M phosphate buffer (pH 7.8) 1 ml, 2 mM dithiothreitol, 5 mM substrate, and 0.3 mM electron carrier. The concentration of pentanal and hexanal was 2.5 mM.

butanol oxidation activity was much lower than butyraldehyde reduction activity. Similar trends were observed in ethanol dehydrogenase activities but the activities were much lower than those of butanol dehydrogenase. The NADPH dependent alcohol dehydrogenase activities of the solventogenic culture were about 3.2 fold higher than the activities of the acidogenic culture. No activity was obtained in either direction with ferredoxin, FAD or neutral red (data not shown).

NADPH-aldehyde Reductase Activities

NADPH-aldehyde reductase activities were measured using acetaldehyde, propionaldehyde, butyraldehyde, pentanal and hexanal in solventogenic and acidogenic cultures. Table 3 shows the activities of acidogenic and solventogenic cultures. Acetaldehyde showed the lowest activity among the substrates used. Aldehydes with carbon numbers between 3 and 6 showed similar activities, which were about 3 times higher than the activity of acetaldehyde. All activities were lower in acidogenic culture than in solventogenic culture. The solventogenic

Table 4. Alcohol dehydrogenase activities of *Clostridium acetobutylicum* ATCC 4259.

Substrate	K_m (μM)	V_{max} (nM·mg protein^{-1}·min^{-1})
Acetaldehyde	565	87
Propionaldehyde	202	230
Butyraldehyde	235	238
Pentanal	221	2[illegible]3
Hexanal	227	276

culture showed a 3 to 4 fold increase in NADPH-aldehyde reductase activity, regardless of the carbon number of the substrate.

K_m and V_{max} Values of NADPH-aldehyde Reductase

A solventogenic cell-free extract was used to measure the K_m and V_{max} values of the NADPH-aldehyde reductase activity by a double reciprocal plot using acetaldehyde through hexanal (Table 4). In all assays a Michaelis-Menten kinetic pattern was observed. The apparent K_m value for acetaldehyde was 565 μM which is much higher than those for high carbon number aldehydes. The apparent K_m values for propionaldehyde, butyraldehyde, pentanal and hexanal were 202, 235, 221 and 227 μM, respectively. V_{max} values calculated in this study are similar to results shown in Table 3. They are 86, 230, 238, 228 and 276 nM NADH oxidized·mg protein^{-1}·min^{-1} for acetaldehyde, propionaldehyde, butyraldehyde, pentanal and hexanal, respectively.

DISCUSSION

Alcohol dehydrogenases are classified into 3 categories according to their specificities toward electron carriers. They are NAD dependent (E.C. 1.1.1.1), NADP dependent (E.C. 1.1.12) and non-specific (E.C. 1.1.1.71). *C. acetobutylicum* DSM 1732 possesses NAD dependent alcohol dehydrogenase (1). NAD was used to determine alcohol dehydrogenase of *C. acetobutylicum* NCIB 8049 (5). On the other hand, NADP dependent alcohol dehydrogenase is known in *C. acetobutylicum* NRRL B643 (15, 16) and in *C. beijerinckii* NRRL B592 (6, 9). This investigation showed that the alcohol dehydrogenase of *C. acetobutylicum* ATCC 4259 is NADP dependent. NADP dependent ADH, known in other anaerobes, has a higher activity against secondary alcohols than primary alcohols (2), but acetone is not reduced to isopropanol during fermentation by *C. acetobutylicum*. Solvent producing cells have been reported to be resistant to cell disintegration (1, 10). If the different alcohol dehydrogenases, among solvent producing clostridia, are not the result of insufficient cell disintegration, the differences in alcohol dehydrogenase specificity toward electron carriers might be a significant factor in classification of butanol producing clostridia.

Though it has been reported that *n*-propanol and *iso*-butanol are produced during ABE fermentation, probably by deamination and reduction of amino acids (5), to our knowledge, this is the first report showing the reduction of exogeneously added fatty acids to corresponding alcohols. Hartmanis *et al.* (8) suggested that a solventogenic culture uses acetoacetyl-CoA:acetate (butyrate) CoA-transferase for the uptake and activation of acetate and butyrate, which are produced and exported during the acidogenic phase. This same enzyme might be responsible for the uptake and activation of propionate, valerate, and caproate.

Enzyme assays showed that the alcohol dehydrogenase of *C. acetobutylicum* ATCC 4259 can reduce aldehydes with carbon numbers from 2 to 6, and that the enzyme shows its lowest affinity and lowest activity for acetaldehyde. These properties are similar to those of a non-specific alcohol dehydrogenase found in rat intestine, which has its highest activity on substrates with carbon numbers between 4 and 6 (4).

It was reported that ethanol is produced by an acidogenic culture maintained at pH 6.0 (1), and by a non-solvent producing mutant of P262 (D.T. Jones (1984) personal communication). Rogers and Hasen (15) reported that the difference between acidogenic and solventogenic cultures in specific ethanol dehydrogenase activities is less significant (about 2 to 4 fold increase) than butanol dehydrogenase activity, which increases over 16 fold as the culture becomes solventogenic in *C. acetobutylicum* NRRL B643. These results suggest that ethanol dehydrogenase activity is diffferent from butanol dehydrogenase activity.

On the other hand, allyl alcohol resistant mutants of *C. acetobutylicum* NRRL B643 showed coordinate reductions in ethanol dehydrogenase activity with that of butanol dehydrogenase (16). Based on this result the authors hypothesized that a single enzyme is responsible for the reduction of acetaldehyde and butyraldehyde. Alcohol dehydrogenases isolated from *C. beijerinckii* NRRL B592 and *C. beijerinckii* NRRL B593 showed ethanl dehydrogenase activity, as well as butanol dehydrogenase activity (9). In this investigation, the ratio of specific activities on each substrate of acidogenic culture, to those of solventogenic culture were similar. The activity of ethanol dehydrogenase in *C. acetobutylicum* ATCC 4259 increased 3.1 fold while activity of butanol dehydrogenase increased 3.2 fold as the culture became solventogenic. This result leaves the possibility of single enzyme for the production of ethanol and butanol. If

this is true the low affinity of alcohol dehydrogenase for acetaldehyde is one reason why the organism produces less ethanol than butanol.

Alcohol dehydrogenase activity from *C. acetobutylicum* ATCC 4259 is much higher in the direction of aldehyde reduction, than in the reverse direction. In the case of butanol dehydrogenase activity, aldehyde reduction activity was about 14 times higher than the reverse reaction, and acetaldehyde reduction activity was about 12 times greater than ethanol dehydrogenation. This property of alcohol dehydrogenase in *C. acetobutylicum* is different from alcohol dehydrogenase in *C. thermohydrosulfuricum*, the growth of which is inhibited by ethanol due to the reduction of the pyridine nucleotide pool by alcohol dehydrogenase with electrons from the alcohol (12).

The alcohol dehydrogenase of *C. beijerinckii* NRRL B592 showed a very low K_m value for butyraldehyde (6 μM) (9), while K_m values of 8.5 mM for butanol and 9.5 mM for butyraldehyde were measured in alcohol dehydrogenases of *C. acetobutylicum* NRRL B643 (16) and *C. beijerinckii* NRRL B593 (9), respectively. The observed K_m values for aldehydes with carbon numbers between 3 and 6 were about 0.2 mM in *C. acetobutylicum* ATCC 4259. The properties of clostridial alcohol dehydrogenase seem to be different from species to species. Indeed, the alcohol dehydrogenase of *C. beijerinckii* B593 reduces acetone, but isopropanol is not produced by *C. beijerinckii* B592 (9).

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