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Electron Flow Shift in *Clostridium acetobutylicum* Fermentation by Lactate

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Clostridium acetobutylicum **produced more butanol in the medium containing corn steep liquor (CSL) than in a complex medium without CSL. Addition of CSL to CAB medium increased sugar consumption by the bacterium. Similar results were obtained in the fermentation using CAB medium containing lactate. The ratio for the butanol produced to acetone of the control culture was 1.8, whilst that of the culture containing 44 mM lactate was 5.2. From these results it is hypothesized that lactate functions as an electron flow modulator in the fermentation. This finding has been utilized for the successful butanol fermentation of a non-corn based agricultural byproduct, palm oil waste.**

Sugar fermentation in *Clostridium acetobutylicum* involves a typical branched pathway producing acids (acetate and butyrate), neutral solvents (butanol, acetone and ethanol) and gases(hydrogen and carbon dioxide) (12). The fermentation pattern can be changed by modulating the electron metabolism of the bacterium. The butanol/acetone ratio was increased by using carbon monoxide, a hydrogenase inhibitor (6), and by supplying excess reducing equivalents by the electrochemical method (7).

Industrial acetone-butanol-ethanol (ABE) fermentation employed starch or molasses media containing CSL as an organic nitrogen source (11). A typical industrial ABE fermentation produced 6 parts butanol, 3 parts acetone, a part ethanol, and small amount of acetic and butyric acids with the complete utilization of carbohydrate added (12). Recent studies showed that *C. acetobutylicum* utilized only about two thirds of the glucose (45 g/l) added to a complex medium (6), whilst a complete carbohydrate utilization was achieved in a fermentation using a similar medium containing CSL (3). It was shown that the bacterium utilized the lactate present in CSL (3). Whey fermentation by *C. acetobutylicum* showed a much higher ratio for butanol produced to acetone than the industrial fermentation (9).

In this study attempts were made to find the role of lactate in ABE fermentation, and the results were applied to a fermentation based on palm oil residues, which do not contain lactate.

MATERIALS AND METHODS

Bacterial Strain and Culture Condition

Clostridium acetobutylicum KCTC 1037 (ATCC 42 59) was used throughout the study. The organism was maintained and cultivated under strictly anaerobic conditions using a complex CAB medium or clarification sludge with CAB ingredients as described previously (6). Clarification sludge was obtained from the Palm Oil Research Institute of Malaysia. The clarification sludge was autoclaved at 121 ℃ for 20 min and stored at 4 ℃ until use. A few grains of spore soil stock was inoculated into 10 ml of CAB medium (45 g/l glucose) in an anaerobic pressure tube (18×150 mm, Bellco glass Inc. Vineland, N.J.) to develop inocula. Soil cultures were pre-

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pared by the method of Walton and Martin (12).

Analytical Methods

Soluble fermentation products of *C. acetobutylicum* were analysed by gas chromatography (Varian 3300) according to the method described in a previous paper (8). Glucose was determined by the glucose oxidase method using a Sigma diagnostic kit (Cat. No. 510-A). Lactate was quantified according to Cottyn and Charles (2). Methylated samples were analysed in a similar way as the solvents.

Materials

Chemicals used in this study were of reagent grade. They were purchased from Sigma Chemical Co. (St. Louis, Mo) and other commercial sources. CSL was purchased from Sigma Chemical Co.

RESULTS

Fermentation Using CAB Medium Added by CSL

C. acetobutylicum KCTC 1037 was grown on CAB medium in the presence and absence of CSL to test the effects of CSL on the sugar utilization by the bacterium. As shown in Fig. 1 the culture containing 5% CSL utilized of glucose(Fig. 1b) whilst that without CSL utilized only about half of the sugar(Fig. 1a). Consequently higher butanol yield was obtained in the fermentation added by CSL than in the control. CSL is used as a nitrogen source in the fermentation. The increases in sugar utilization and butanol yield with CSL supplementation can be explained either by the increased organic nitrogenous compounds or by the addition of other CSL components to the culture. Since the complex CAB medium contained 4 g/l yeast extract and 1 g/l tryptone, it is more likely that the changes in the fermentation pattern were caused by the other CSL components rather than nitrogeneous compounds.

Fermentation of CAB Medium Containing by Lactate

In the corn starch process, corn is subjected to lactic acid fermentation, and the lactic acid produced ends up in CSL. In order to see if the effects of CSL was due to the lactate, fermentation was conducted using CAB medium containing 44 mM sodium lactate (Fig. 2). A complete sugar utilization was observed after 60 h fermentation. In addition to the increace in sugar utilization, the pattern of fermentation products was different from the previous fermentation. More butanol was pro-

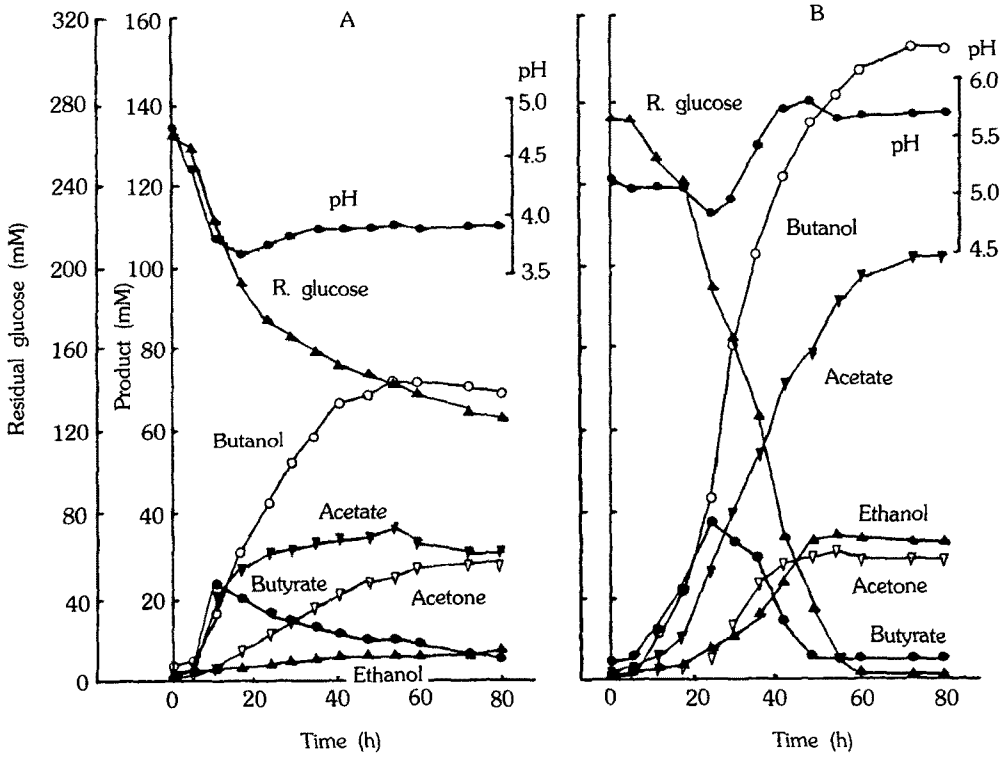

Fig. 1. Fermentation patterns of *C. acetobutylicum* in CAB medium in the presence and absence of CSL.
(A) CAB medium, (B) CAB medium containing 5% CSL.

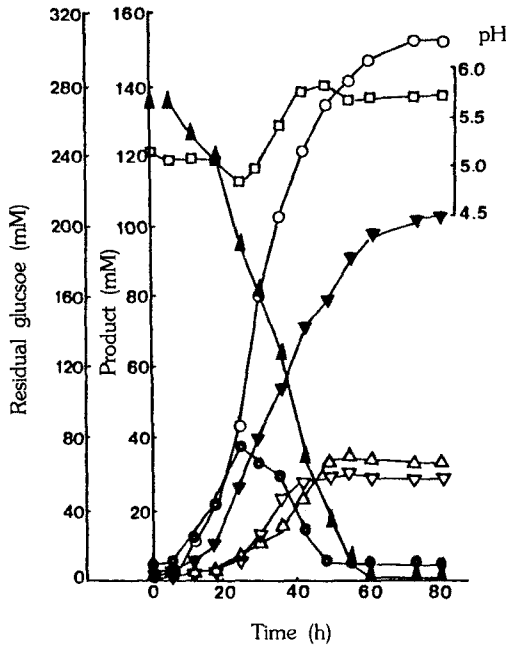

Fig. 2. Fermentation profiles of CAB medium with 44 mM lactate by *C. acetobutylicum*.
See Fig. 1 for the symbols.

duced at the expense of acetone and butyrate. The final pH was 5.7 which was much higher than that of the previous cultures. This indicates that lactate was converted to neutral solvents raising the culture pH.

Fermentation of CAB Medium Containing Different Amount of Lactate

Various concentrations of sodium lactate was added to the CAB medium before being fermented by *C. acetobutylicum*. The fermentation results are summarized in Table 1. Culture containing 44 mM lactate consumed the 250 mM glucose completely, whilst those added by more or less lactate left varying amounts of residual glucose. These results show that lactate at suitable concentrations stimulate the sugar utilization and that above this concentration lactate is toxic to the bacterium. The ratio for butanol to acetone increased with the increase in lactate added to the medium, indicating that lactate functions as an electron flow modulator in the bacterium.

Fermentation in the Presence of Lactate or Pyruvate

Lactate is oxidized to pyruvate before being metabolized by saccharolytic clostridia. Lactate and pyruvate are known not to be able to support the growth of *C. acetobutylicum* though lactate is consumed by the bacterium in the presence of fermentable sugar (3). In order to see if pyruvate modulates the fermentation as lactate does, fermentations were carried out using CAB medium containing sodium lactate or sodium pyruvate at the concentration of 44 mM. As shown in Table 2 lactate increased the sugar utilization and butanol yield. On the other hand fermentation in the presence of pyruvate produced less solvents than the control culture resulting in lower sugar utilization.

From this result it is hypothesized that the fermentation is modulated by the electrons released from the oxidation of lactate to pyruvate. Pyruvate may also have failed to modulate the fermentation because it was not transported into the cell.

Fermentation of Palm Oil Clarification Sludge Containing Lactate

Previous studies showed that clarification sludge from a palm oil mill is not a suitable substrate for butanol fermentation because of the low carbohydrate utilization and low butanol yield (8). Clarification sludge containing 44 mM sodium lactate and 25 g starch was used as a medium for *C. acetobutylicum*. Table 3 summarizes the fermentation results. About 66 mM butanol was produced by the culture containing lactate, whilst cultures without lactate produced much less solvent. These results indicate that lactate can be a substitute for CSL for butanol fermentation of non-corn based substrate to improve the solvent productivity.

Table 1. Effect of lactate concentration on acetone-butanol fermentation by *C. acetobutylicum*

Lactate Added (mM)	Products(mM)					Butanol/Acetone (mM)	Residual Glucose
	Acetate	Butyrate	Ethanol	Acetone	Butanol		
–	28.5	7.1	8.5	36.1	63.4	1.76	128.1
8.8	36.6	5.8	14.9	33.2	77.8	2.34	97.9
26.5	41.9	2.8	32.3	44.9	114.1	2.54	15.6
44.0	72.4	2.9	40.5	29.6	152.9	5.17	0.0
61.6	45.3	13.0	26.9	20.9	111.5	5.33	65.4
88.0	18.7	20.5	25.5	5.0	61.9	12.38	136.7

Fermentations were made for 72 hours before soluble products were quantified by gas chromatography.

Table 2. Effect of lactate and pyruvate on the fermentation by *C. acetobutylicum*

Substrate	Products (mM)					
	Acetate	Butyrate	Ethanol	Acetone	Butanol	Total as glucose
CAB	29.3	5.5	10.6	42.6	74.7	142.8[a]
CAB+Pyruvate	62.2	17.5	4.1	14.6	54.5	119.8
CAB+Lactate	97.1	10.6	23.8	40.6	131.5	243.2

Lactate and pyruvate concentrations were both 44 mM. Fermentation was made for 72 hours.
[a]The amount of glucose needed to produce the total fermentation products obtained.

Table 3. Fermentation by *C. acetobutylicum* of clarification sludge in the presence of CAB ingredients or lactate

Substrate	Products (mM)					Final pH
	Acetate	Butyrate	Ethanol	Acetone	Butanol	
Sludge	50.8	28.4	1.9	8.8	5.0	4.75
Slu.+Glucose	62.9	36.0	2.4	11.6	11.0	4.44
Slu.+Starch	61.4	42.9	2.1	9.3	7.5	4.56
Slu.+Sta.+CAB Ingred.	73.9	55.5	4.1	15.1	15.4	4.54
Slu.+Sta.+Lac.	82.3	61.5	11.8	21.4	66.5	5.09

Glucose and starch concentrations were both 25 g/l, and lactate concentration was 44 mM.
Slu: clarification sludge from a palm oil mill.
Sta: starch, CAB Ingred: CAB medium components, Lac: lactate

DISCUSSION

Clostridium acetobutylicum has a typical branched pathway (12). This bacterium takes advantage of its metabolic diversity to cope with the changing environment, especially changes in oxidation-reduction balance. This characteristic has been exploited to increase the yield of butanol at the expense of other products in studies to develop processes for converting biomass to an alternative energy source, because butanol has higher energy value than other fuels (3, 6, 7, 10). Results of this study showed that CSL increases the sugar consumption and the butanol yield in *C. acetobutylicum* fermentation.

Lactate was found to be the component of CSL modulating the fermentation. Lactate does not support the growth of the bacterium, though it is cometabolized with fermentable sugar (3). Lactate is known to modulate butanol fermentation by increasing butanol yield with a decreased acetone yield, but is not known to increase carbohydrate utilization (1, 4).

It is presumed that lactate is oxidized to pyruvate before entering into the normal carbon metabolism. The pyruvate/lactate half reaction has a redox potential of -0.19 volt, which is too high to reduce protons to hydrogen. It is hypothesized that acetoacetyl-CoA is used as the electron acceptor for the oxidation of lactate to pyruvate. Butyryl-CoA dehydrogenase has a redox potential of -0.015 volt. It is most probable that the oxidation of lactate is coupled to the reduction of crotonyl-CoA to butyryl-CoA. Since lactate is inhibitory to the bacterium (Table 1), the bacterium has to remove lactate efficiently consuming acetoacetyl-CoA. Consequently acetoacetyl-CoA is metabolized to produce mainly butanol and less acetone. Since crotonyl-CoA is efficiently reduced to butyryl-CoA in the presence of lactate, the concentrations of glycolytic intermediates including acetyl-CoA in the cell are kept low. Under this condition glycolysis is stimulated with the complete carbohydrate utilization. For the validation of this hypothesis, the existence of a lactate oxidizing enzyme has to be proven.

NADH dependant lactate dehydrogenase has been purified from *C. acetobutylicum* (5). This enzyme is unidirectional catalyzing only the reduction of pyruvate. A different lactate dehydrogenase system with an electron acceptor other than pyridine nucleotide is believed to be involved in lactate oxidation by the bacterium (1, 4), but it has not yet been found.

These discussions lead to the conclusion that the rate limiting step for the butanol production does not lie in the process of butyryl-CoA reduction to butanol but in the process of acetoacetyl-CoA reduction to butyryl-CoA. This finding has been utilized for the successful butanol

fermentation from a non-corn based agricultural by-product.

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