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Properties of Acetate Kinase Activity in *Clostridium thermocellum* Cell Extracts

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ABSTRACT

Acetate kinase (EC 2.7.2.1) is involved in the wasteful production of acetate during conversion of cellulose to ethanol by *Clostridium thermocellum*. The properties of this enzyme activity in *C. thermocellum* cell extracts were determined. Optimum enzyme activity was at 60°C and between pH 7.5 and 9.0. In the presence of air, acetate kinase was stable to temperatures up to 60°C, retaining 90% activity after 2 h, and was inactivated rapidly at higher temperatures. The enzyme exhibited a wide range of stability to pH (5.0–9.0) when incubated at 50°C for 2 h. As with other acetate kinases, a divalent cation, such as Mg^{2+}, was required for enzyme activity. Optimum activity was observed at 20 m*M* $MgCl_2$ when ATP was held constant at 10 m*M*. Acetate kinase activity was adversely affected by KCl, a salt commonly used in ion-exchange or affinity chromatography, with 0.3 *M* KCl inhibiting by 50%. These results will be important in optimizing the direct microbial conversion process of cellulose to ethanol using *C. thermocellum* in coculture with *Clostridium thermosaccharolyticum*.

Index Entries: *Clostridium thermocellum*; acetate kinase; direct microbial conversion; ethanol.

INTRODUCTION

In recent years, there has been a strong interest in converting cellulosic biomass to commodity chemicals and fuels, such as ethanol, by direct

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microbial conversion (DMC), in which cellulase synthesis, cellulose hydrolysis, and ethanol production are achieved within a single reactor *(1,2)*. The anaerobic, thermophilic bacterium *Clostridium thermocellum*, which is well-known for its impressive cellulase activity on crystalline *(3)*, is among the leading candidates for direct ethanol production from cellulose, especially in coculture with *Clostridium thermosaccharolyticum (4–7)*. Development of a practical DMC process for ethanol production utilizing both species, however, has been hindered by the low yield of ethanol, mostly because of the concomitant production of a major side-product, acetate (up to 40% in the case of *C. thermocellum*), along with a low level of lactate.

Phosphotransacetylase (PTA; EC 2.3.1.8) and acetate kinase (AK; EC 2.7.2.1) are the two enzymes responsible for acetate formation from acetyl-CoA in ethanol-producing clostridia. PTA catalyzes the conversion of acetyl-CoA to acetyl phosphate, which is then converted to acetate by AK. Understanding of the properties of PTA and AK in *C. thermocellum* and *C. thermosaccharolyticum* may provide information for reducing acetate synthesis and improving ethanol selectivity, which is required for the DMC process to become competitive in the biofuel industry. Here we report the properties of AK in *C. thermocellum* cell extracts.

MATERIALS AND METHODS

Bacterial Strain, Growth Medium, and Culture Conditions

The medium used for seed culture and main culture for *C. thermocellum* ATCC 27405 *(8)* was the complex GS medium *(9)*, which contains (per L): 10 g D-cellobiose, 6 g yeast extract (Difco, Detroit, MI), 1.5 g KH_2PO_4, 2.9 g K_2HPO_4, 2.1 g urea, 1 g $MgCl_2 \cdot 6H_2O$, 0.15 g $CaCl_2 \cdot 2H_2O$, 10 mg $FeSO_4 \cdot 7H_2O$, 1 g cysteine·HCl, 2 mg resazurin, 10 g morpholinopropane sulfonic acid (MOPS) buffer, and 3 g sodium citrate. Initial pH was 7.2.

Seed cultures were grown anaerobically (with N_2 as the gas phase) in Hungate tubes (Bellco, Vineland, NJ) containing 10 mL medium at 60°C for 21 h. After growth, 7.5 mL of the seed culture was transferred into each anaerobic shake flask containing 750 mL medium. The flasks were incubated anaerobically at 60°C for another 22 h when stationary phase was reached. The OD_{660} upon harvest was about 1.2.

Preparation of Cell-Free Extracts and Enzyme Assay

The main culture (1.5 L) was harvested by centrifugation at 5000 g, 4°C for 15 min. The cells were washed once with 50 m*M* Tris-HCl buffer (pH 7.4) containing 2 m*M* $MgCl_2$ and 2 m*M* dithiothreitol, then resuspended in 40 mL

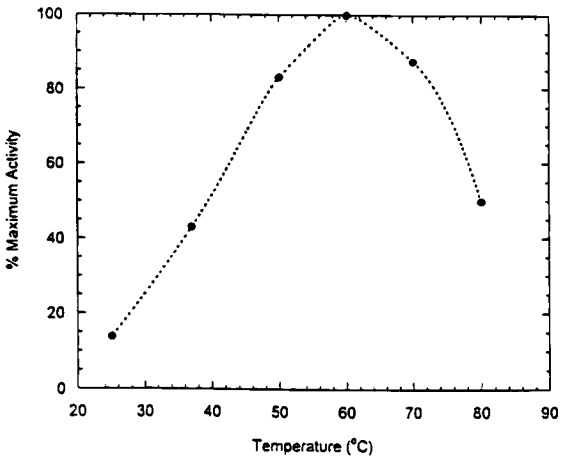

Fig. 1. Effect of temperature on the activity of acetate kinase in *C. thermocellum* cell extracts.

of the same buffer. The cell paste was disrupted in a French press at 1000 psi, followed by centrifugation at 30,000*g*, 4°C for 1 h to remove cell debris. The supernatant fluid was stored at –20°C until further analyzed.

AK activity was assayed by measuring the acetyl hydroxamate formed between the reaction product acetyl phosphate and hydroxylamine. The acetyl hydroxamate reacts with trivalent iron to give a colored complex, which can be monitored at OD_{520} *(10,11)*. One U of AK is the amount of enzyme catalyzing the formation of one μmol of acetyl phosphate per min at pH 7.2 and 50°C.

Total protein concentration was determined by the Bradford dye-binding method (Bio-Rad, Hercules, CA), using bovine serum albumin as standard.

RESULTS

Effect of Temperature on AK Activity and Stability

The effect of temperature on enzyme activity in cell extracts was examined from 24°C to 80°C, as shown in Fig. 1. The temperature for optimum activity was found to be 60°C, which is the same as that for *Clostridium thermoaceticum* pure AK *(10)*, and similar to those of purified enzymes from *Escherichia coli* (60–65°C) *(12)* and *Methanosarcina thermophila* (65°C) *(12)*. Very low activity (<15% of maximum activity) was observed for AK at 24°C; there was 50% of maximum activity at 80°C.

The effect of temperature on AK stability was studied by incubating the cell extracts at temperatures between –20°C and 80°C for 2 h. Figure 2

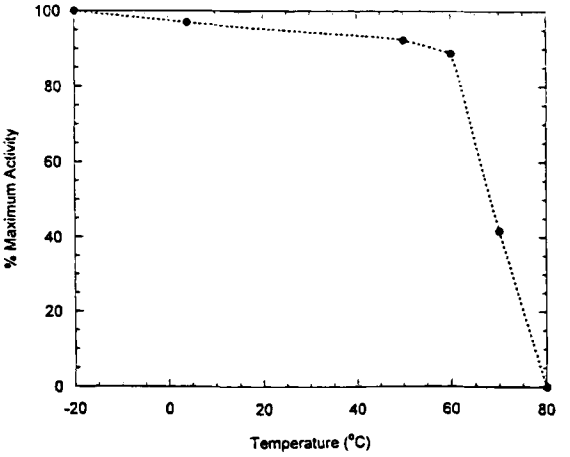

Fig. 2. Effect of temperature on the stability of acetate kinase in *C. thermocellum* cell extracts.

indicates that the enzyme was stable to heating up to 60°C, with only 10% loss in activity, but was completely inactivated at 80°C. The crude *C. thermocellum* AK appeared to be more thermostable than the purified AK of *C. thermoaceticum*, which remained stable up to 50°C, but lost 70% activity at 59°C after 30 min incubation *(10)*.

Effect of pH on Enzyme Activity and Stability

The influence of pH and AK activity is shown in Fig. 3: The optimum activity occurred between pH 7.0 and 9.0. Data for values above pH 9.0 were not measured because of a heavy precipitate, possibly $Mg(OH)_2$, which formed in the reaction mixtures during NaOH titration. The range observed is much broader than the optimum range of pH 7.0–7.4 in the case of *M. thermophila* pure AK *(11)*. At pH values below 6.0, the AK activity was almost negligible, a trend similar to that of the *M. thermophila* enzyme.

To analyze AK stability at various pH values, the cell extracts (pH 7.4) were diluted 10-fold in different buffers (Tris buffer between pH 5.0 and 9.0 and sodium carbonate buffer for pH 9.0 to 11.0). After incubating 2 h at 50°C, the remaining activity was measured and compared (Fig. 4). AK was completely stable between pH 5.0 and 9.0, and was inactivated rapidly at higher pH values.

Effect of Inorganic Salts on Enzyme Activity

As with other purified AKs studied to date, a divalent metal ion, such as Mg^{2+} or Mn^{2+}, is required for *C. thermocellum* AK activity. In this experi-

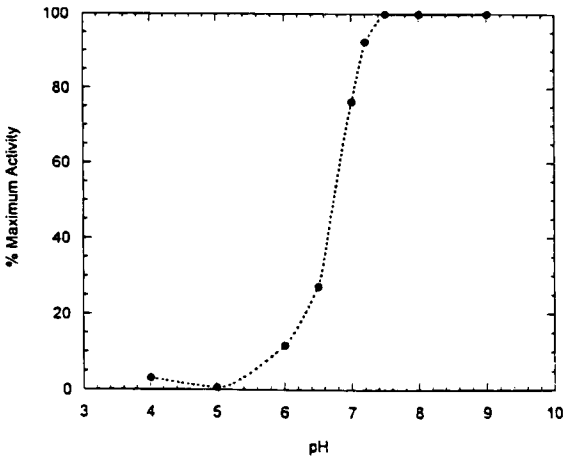

Fig. 3. Influence of pH on the activity of acetate kinase in *C. thermocellum* cell extracts.

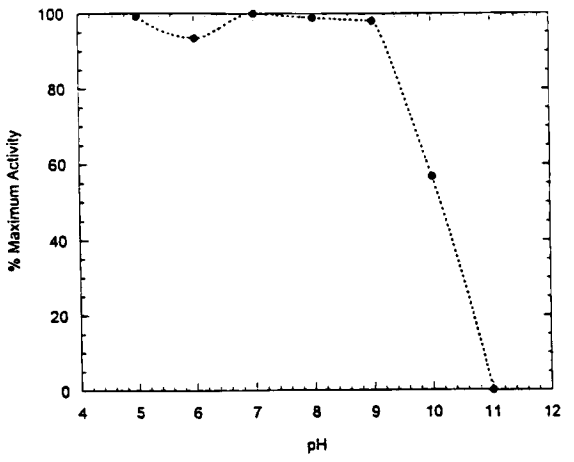

Fig. 4. Effect of pH on the stability of acetate kinase in *C. thermocellum* cell extracts.

ment, the ATP was kept at 10 m*M* while the $MgCl_2$ concentration was varied from 0 to 80 m*M*. The effect of $MgCl_2$ on AK activity is shown in Fig. 5. The optimum activity was observed at 20 m*M* $MgCl_2$, which corresponds to a $MgCl_2$: ATP ratio of 2, suggesting that optimum AK activity requires the interaction between Mg^{2+} and ATP, as well as that between Mg^{2+} and AK. This $MgCl_2$ ATP ratio of 2 is identical to that of the *E. coli* purified enzyme *(13)*, yet different from that of *M. thermophila*, in which an optimum $MgCl_2$· ATP ratio of 1 was reported *(11)*.

The effect of KCl, a salt frequently used in ion-exchange or affinity chromatography and in microbial growth media, on AK activity was also

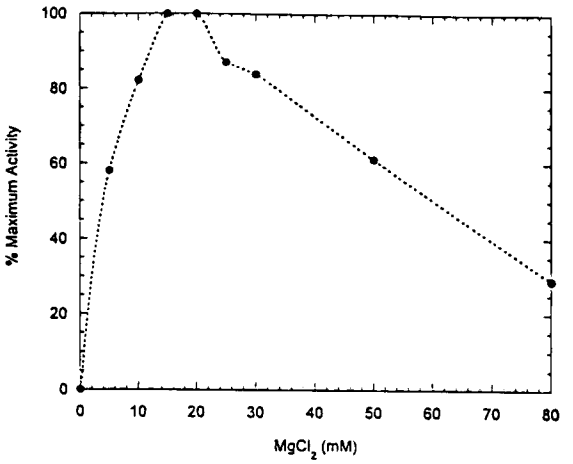

Fig. 5. Influence of $MgCl_2$ on the activity of acetate kinase in *C. thermocellum* cell extracts.

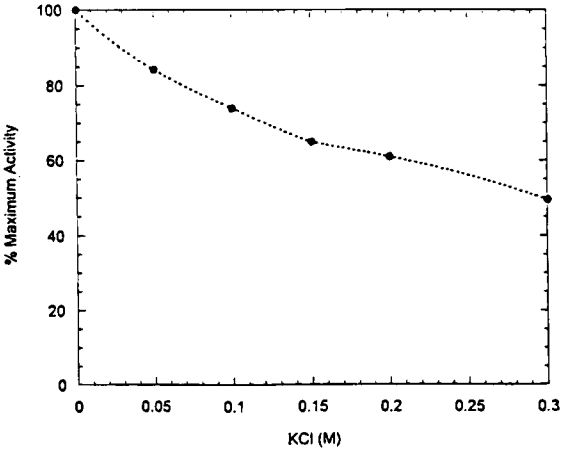

Fig. 6. Effect of KCl on the activity of acetate kinase in *C. thermocellum* cell extracts.

studied (Fig. 6). The AK activity decreased with increasing KCl concentrations, losing 50% activity in the presence of 0.3 *M* KCl. A similar result was reported for the purified AK of *M. thermophila (11).*

DISCUSSION

Today the world is being inundated with urban, agricultural, and industrial waste. Landfill space to handle this waste is becoming less available and its cost is rapidly rising. A potential solution to this problem is the conversion of wastes into motor fuel, i.e., ethanol, by a coculture of ther-

mophilic, anaerobic microorganisms (a cellulolytic strain and a saccharolytic strain) *(4–7)*. The authors favor the use of *C. thermocellum* as the cellulolytic strain and *C. thermosaccharolyticum* as the saccharolytic strain *(4)*. Together, these strains can attack cellulose and hemicellulose and convert the sugars produced to ethanol.

Attention has focused on the use of anaerobic thermophiles as "ethanologens" for the following reasons: Thermophiles are thought to be robust and contain stable enzymes; anaerobes generally have a low cellular growth yield, hence more of the substrate is converted to ethanol; thermophilic fermentations are less prone to detrimental effects of contamination; growth at higher temperatures may facilitate the removal and recovery of volatile products such as ethanol. Experience suggests that the advantages of cellulase production *in situ* and the high rates of growth on and metabolism of cellulose and hemicellulose are even more important.

In addition to solving a pollution problem, the clostridial coculture system is potentially capable of replacing petroleum with ethanol as a major liquid fuel using renewable photosynthetic biomass as feedstock. *C. thermocellum* breaks down cellulose with the formation of cellobiose as one of the main products. Cellobiose can be further utilized by the organism, and the final end products are ethanol, acetic acid, lactic acid, hydrogen, and carbon dioxide. The interest in this organism is a result of several factors in addition to those mentioned above. First, *C. thermocellum* can utilize lignocellulosic waste and generate ethanol; a rare property among living organisms. Second, for large-scale culture, anaerobiosis is an advantage, because one of the most expensive steps in industrial fermentations is that of providing adequate oxygen transfer, e.g., for cellulase production. The coculture process has great potential, but obstacles to commercialization exist. These are mainly the production of side-products such as acetate and lactate, which decrease the yield of ethanol and can act as weak uncouplers, and slow cell growth *(14)*. The mechanisms of acid production are clear: Acetate production involves the activity of phosphotransacetylase and AK and lactate formation involves lactate dehydrogenase. Acetate is a much more serious problem than lactate *(4)*.

Although strains have been obtained by mutagenesis followed by screening or selection *(15–18)*, which show decreased acid production or increased ethanol tolerance, genetic stability has been a problem *(19–20)*, and further work is needed. Thus far, attempts to use recombinant DNA technology to inactivate the gene encoding AK or that encoding phosphotransacetylase have failed. Thus, the authors have decided to pursue a physiological approach at the same time that they continue their genetic work. The basis of the physiological studies is to determine the properties of AK and phosphotransacetylase from *C. thermocellum* and *C. thernosac-*

charolyticum, in the hope of finding conditions under which one of these enzymes is inactive, yet ethanol production continues. The present study is the first step in that direction.

The AK properties determined in this work can be applied to process optimization of DMC for ethanol production. For example, Figs. 1, 3, 5, and 6 show that temperature, pH, $MgCl_2$, and KCl have strong effects on AK activity of *C. thermocellum*. Adjusting one or more of these variables in a DMC process may reduce AK activity, and thus acetate formation. For example, keeping pH at 6.0, conducting the fermentation under limiting Mg^{2+}, and adding 0.3 *M* or even higher concentrations of KCl could have a major effect on the performance of the coculture process *(4)*. Purification of AK from *C. thermocellum* is currently in progress in the authors' laboratory. The properties of pure AK might provide additional information for improving ethanol selectivity in *C. thermocellum*. Furthermore, knowledge of AK's N-terminal sequence could aid in the genetic approach, i.e., facilitate metabolic engineering to inactivate and/or attenuate the AK-encoding gene (*ack*), which should direct the carbon flow away from acetate and toward ethanol synthesis.

Studies on the properties and purification of AK of *C. thermosaccharolyticum*, as well as the phosphotransacetylase of both clostridia, are also proceeding.

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