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Purification and Characterization of Acetate Kinase from *Clostridium thermocellum*

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Abstract: Acetate kinase (EC 2.7.2.1), an enzyme involved in the wasteful production of acetate during conversion of cellulose to ethanol by *Clostridium thermocellum,* was purified 144-fold. The enzyme has an *Mr* of 84 kD by non-denaturing gradient gel electrophoresis, and an *Mr* of 46 kD when estimated with a denaturing gel; thus it appears to be a homodimer. Optimum enzyme activity occurs at 50°C and between pH 7.2 and 8.0. Acetate kinase is stable to temperatures up to 60°C, but is completely inactivated at 80°C after two h. The enzyme is stable between pH 7.0 and 9.0 when incubated at 50°C for two h. Optimum acetate kinase activity occurs at a $MgCl_2$:ATP ratio of 2:1, which indicates an interaction between Mg^{2+} and ATP and that between Mg^{2+} and acetate kinase. Enzyme activity is partially inhibited by KCl, an inorganic salt frequently used in chromatography and fermentation media, losing 60% activity in the presence of 0.2 M KCl. Sigmoidal enzyme kinetics were observed from the velocity plot of acetate kinase when either the acetate ($S_{0.5}$ = 285 mM) or ATP ($S_{0.5}$ = 11 mM) concentration was varied, suggesting cooperative binding of the two substrates.

INTRODUCTION

Production of ethanol and commodity chemicals by clostridia via direct microbial conversion (DMC), in which cellulase synthesis, cellulose hydrolysis and fermentation occur in a single bioreactor, has become an attractive topic in recent years [1-3]. A coculture of *Clostridium thermocellum*, a cellulase producer but incapable of xylose assimilation, and the xylose-utilizing *Clostridium thermosaccharolyticum*, is an excellent combination for direct production of ethanol from cellulose, the most abundant renewable substrate on earth [4-6]. However, this process has been prevented from

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commercialization due to its low ethanol yield, resulting from concomitant production of acetate and, to a lesser extent, lactate.

Genetic attempts to enhance ethanol yield by conventional approaches, such as chemical and UV mutagenesis and overexpression of genes encoding enzymes involved in the ethanol pathway (e.g. acetaldehyde dehydrogenase and alcohol dehydrogenase), suffered from stability problem during fermentations commonly employed for cheap bulk chemicals. An alternative is to inactivate genes involved in the wasteful production of acetate (e.g. *pta* and *ack*) by homologous recombination and gene knock-out [7, 8]. Genes *pta* and *ack*, which encode phosphotransacetylase (PTA; EC 2.3.1.8) and acetate kinase (AK; EC 2.7.2.1) respectively, are responsible for acetate formation from acetyl-CoA in clostridia. In this reaction, PTA catalyzes the conversion of acetyl-CoA to acetyl phosphate, which is then converted to acetate by AK with the release of ATP as an important energy source.

Information on the N-terminal sequence of purified AK will enable the design of a homologous gene probe for *ack* cloning via PCR, which in turn will allow inactivation of the *ack* gene by homologous recombination to redirect carbon flux from acetate to ethanol production. Furthermore, understanding the properties of AK can improve the DMC process even without genetic manipulation by revealing conditions which might reduce acetate formation, thus enhancing ethanol yield and selectivity. Here we report on the purification and characterization of AK from *C. thermocellum* and compare its properties to those of AK in crude extracts [9].

RESULTS AND DISCUSSION

Purification: AK from *C. thermocellum* was purified 144-fold to a specific activity of 490 μmoles ADP formed/min/mg of protein using anion exchange and heparin affinity chromatography (see Experimental). A typical purification of AK is summarized in Table 1. It is interesting to note that unlike AK from *Methanosarcina thermophila* which binds strongly to the ATP (the substrate) affinity column [10], the *C. thermocellum* AK did not bind to any ATP columns tested (including one with ATP attachment to the agarose

matrix via its ribose hydroxyls, Sigma #A6888; N-6 attachment, Sigma #A9264; and another ribose hydroxyls attachment, Sigma #A4793), nor to an AMP column (ribose hydroxyls attachment, Sigma #A8895) or an ADP column (C-8 attachment, Sigma #A2810). The *C. thermocellum* enzyme, however, did interact unexpectedly with a heparin column as well as Pharmacia's red dye (Procion Red HE-3B, #17-0528) and BioRad's blue dye (Affi-Gel B, #53-7302) affinity columns (data not shown).

Table 1. Purification of acetate kinase from *Clostridium thermocellum*.

	Total activity (U*)	Total Protein (mg)	Specific Activity (U/mg)	Purification (fold)	Recovery (%)
1. Cell extract	377	112	3.4	1	100
2. HiTrap Q anion-exchange eluent	181	15.6	11.6	3.4	48
3. HiTrap Heparin eluent	49	0.1	490	144	13

*One unit (U) of acetate kinase is the amount of enzyme catalyzing the formation of one μmole of acetyl phosphate per min at pH 7.2 and 50°C.

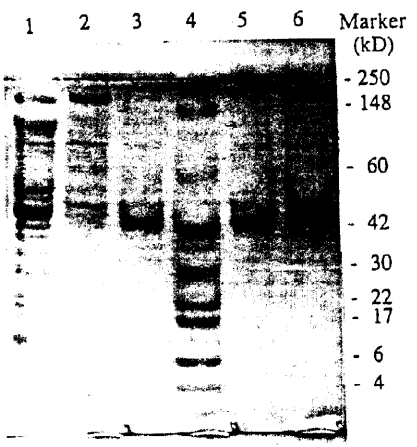

Fig. 1. SDS-polyacrylamide gel of *Clostridium thermocellum* AK at each stage of purification. *Lane 1*, cell extract; *lane 2*, HiTrap Q fraction; *lanes 3, 5 and 6*, HiTrap Heparin fraction; *lane 4*, molecular weight markers (Novex).

The purity of the purified fraction was examined by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. It can be seen in lanes 3, 5 and 6 on a 10% gel (Fig. 1)

that the heparin-purified fraction contained mainly AK (46 kD) along with a minor impurity (52 kD). Identification of AK on the gel was achieved by a parallel native gel of the purified fraction coupled with activity assay of the two excised protein bands (data not shown). The AK purity was estimated to be 90% based on densitometry scanning of the SDS-polyacrylamide gel. We believe that the protein impurity could be reduced to 5% or less if columns larger than those described in Experimental are used.

Molecular weight estimation: The molecular weight of AK was estimated using polyacrylamide gels under denaturing (with SDS) and native conditions (without SDS). An *Mr* of 46 kD was calculated for the denatured AK from the SDS-polyacrylamide gel (Fig. 1), whereas an *Mr* of 86 kD was determined for the native enzyme on a 4 to15% gradient non-denaturing gel (BioRad #161-0902) (data not shown). These results indicate that the *C. thermocellum* AK is a homodimer, similar to those of *Escherichia coli* and *Salmonella typhimurium*, each with a subunit *Mr* of 40 kD [11]. AKs from *Clostridium acetobutylicum* and *M. thermophila* are also homodimers. AK of *C. acetobutylicum* has been isolated from two different strains, DSM 1731 [12] and P262 [13]. The dimeric AK from strain DSM 1731 had an *Mr* of 87-94 kD (based on gel filtration and non-denaturing gel), with a subunit *Mr* of 43 kD, while the P262 enzyme had an *Mr* of 78 kD and a subunit *Mr* of 42 kD. Similarly, a molecular weight in the range of 87-94 kD was reported for the *M. thermophila* AK, which consists of two 53 kD subunits [10].

Effect of temperature on enzyme activity and stability: Fig. 2 indicates that the optimum temperature for activity is 50°C. This optimum temperature is lower than the value of 60°C which we observed for *C. thermocellum* crude extracts [9], and also lower than those reported for *Clostridium thermoaceticum* (60°C) [14], *E. coli* (60-65°C) [15], and *M. thermophila* (65°C) [15].

The influence of temperature on AK stability was examined by incubating the purified enzyme at temperatures from -20°C to 80°C for 2 h (Fig. 2). The enzyme retained full activity up to 60°C, lost 55% of its activity at 70°C and was inactivated completely at 80°C. This thermostability is virtually the same as previously determined with cell extracts

of *C. thermocellum* [9], and better than that from *C. thermoaceticum*, in which a 70% loss in activity was reported after 30 min incubation at 59°C [14].

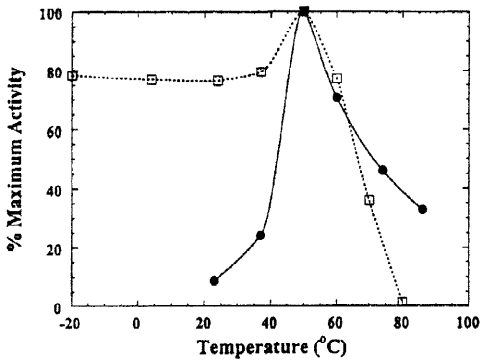

Fig. 2. Effect of temperature on the activity (•) and stability (□) of purified acetate kinase of *Clostridium thermocellum*.

Effect of pH on enzyme activity and stability: AK showed an optimum range from pH 7.2 to 8.0 and with only a 15% loss in activity at pH 10 (Fig. 3). Previously we found the activity of cell extracts to be optimal at pH 7.5 to 9.0 [9]. The *C. thermocellum* purified AK exhibited a wider optimum range than that of *M. thermophila*, which had an optimum range of pH 7.0-7.4 and lost more than 60% activity at pH 9.0 [10]. An optimum pH of 8.0 was described for AK in *C. acetobutylicum* strain P262 [13].

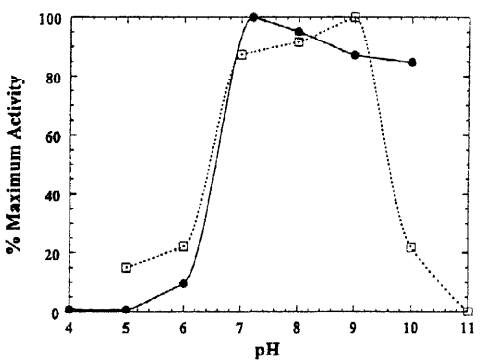

Fig. 3. Effect of pH on the activity (•) and stability (□) of purified acetate kinase of *Clostridium thermocellum*.

The stability of AK at various pH values was studied by diluting the purified enzyme 10-fold in various buffers. Depending on the pH ranges, two different buffers were used: Tris buffer for pH 5.0 to 9.0 and sodium carbonate for pH 9.0 to 11.0. After 2-h incubation at 50°C, the residual enzyme activity at various pH values was determined. AK was quite stable between pH 7.0 to 9.0, but was inactivated rapidly outside this range (Fig. 3). In contrast, crude extracts were stable from pH 5.0 to 9.0 [9]. The greater stability of extracts in the range of pH 5.0 to 7.0 could be due to protection of the enzyme by cell components.

Effect of metal ions on enzyme activity: A divalent metal ion, Mg^{2+} or Mn^{2+}, is required for AK activity in many organisms [10]. The effect of Mg^{2+} on enzyme activity was examined by varying the $MgCl_2$ concentration from 0 to 50 mM while keeping ATP constant at 10 mM (Fig. 4). The optimum $MgCl_2$ concentration was found to be 20 mM, giving a $MgCl_2$:ATP ratio of 2, which suggests that optimum AK activity requires an interaction between Mg^{2+} with ATP as well as that between Mg^{2+} with AK. The same optimum $MgCl_2$:ATP ratio of 2 was observed for crude AK from *C. thermocellum* [9], and for purified AK from *E. coli* [16], while a ratio of 1 was reported for purified *M. thermophila* AK [10].

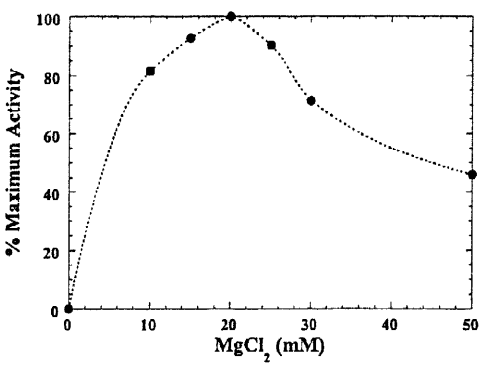

Fig. 4. Influence of $MgCl_2$ on the activity of purified acetate kinase of *Clostridium thermocellum*.

The effect of the monovalent metal ion K^+, which is commonly found in ion-exchange or affinity chromatography and fermentation media, on AK activity was also

studied. As shown in Fig. 5, the activity decreased linearly as the KCl concentration was increased from 0 to 0.2 M, at which only 40% activity was observed. The purified enzyme appeared slightly more susceptible to K^+ inhibition than the crude enzyme [9]. The degree of inhibition of purified AK from *C. thermocellum* is similar to that of the *M. thermophila* enzyme [10].

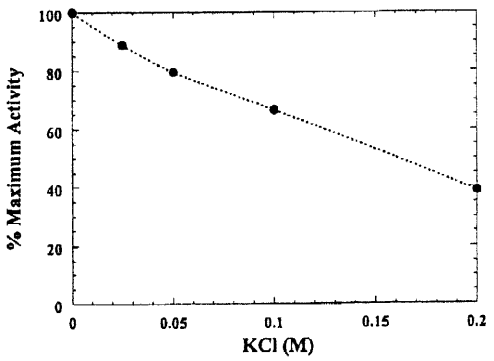

Fig. 5. Effect of KCl on the activity of purified acetate kinase of *Clostridium thermocellum*.

Kinetic properties: The kinetic properties for the two substrates, acetate and ATP, were evaluated by monitoring the enzyme activity at various substrate concentrations. Sigmoidal kinetics were observed from the velocity plot of AK (Fig. 6A) when the acetate concentration was varied between 25 to 500 mM (with ATP kept constant at 10 mM). The $S_{0.5}$ value (the substrate concentration at which half of the maximum enzyme activity (V_{max}) is observed) was estimated from the double-reciprocal Lineweaver-Burk plot to be 285 mM (Fig. 6B). Similar sigmoidal enzyme kinetics were observed when ATP was varied from 1.5 to 25 mM (Fig. 7A), from which an $S_{0.5}$ of 11 mM was calculated (Fig. 7B). The above sigmoidal kinetics suggest a cooperative binding for the two substrates. Hill coefficients of 1.94 and 1.78 were obtained for acetate and ATP respectively (Figs. 6C and 7C), suggesting two interacting substrate sites [17].

Various kinetics have been reported for AKs from several clostridial species and *M. thermophila*. For example, the *C. thermoaceticum* AK exhibited typical Michaelis-Menten kinetics with acetate (K_m = 135 mM), and sigmoidal kinetics ($S_{0.5}$ = 1.64 mM) when ATP concentration was varied [14]. A K_m of 160 mM was obtained for acetate with AK from

C. acetobutylicum strain P262 [13], while apparent K_m values of 73 and 0.37 were reported for acetate and Mg-ATP respectively with AK from *C. acetobutylicum* DSM 1731 [12]. The AK in *M. thermophila* revealed Michaelis-Menten kinetics for both acetate (K_m = 22 mM) and ATP (K_m = 2.8 mM) [10]. It appears that AK affinity to acetate in all three clostridia was considerably lower than that in *M. thermophila*.

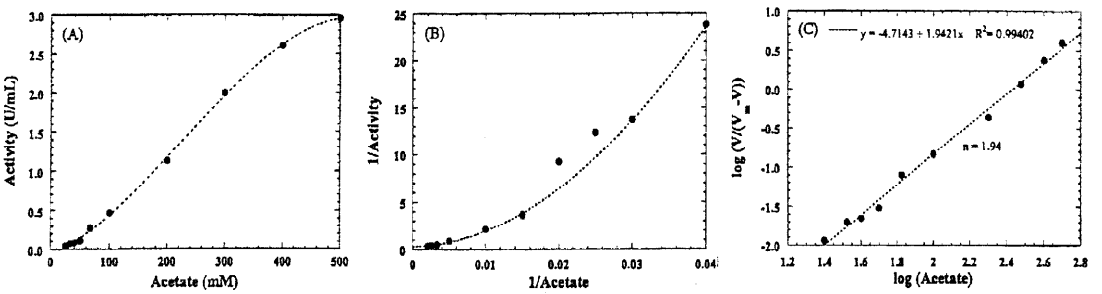

Fig. 6. (A) Reaction velocity curve, (B) Lineweaver-Burk plot, and (C) Hill plot for purified acetate kinase from *Clostridium thermocellum* at various acetate concentrations.

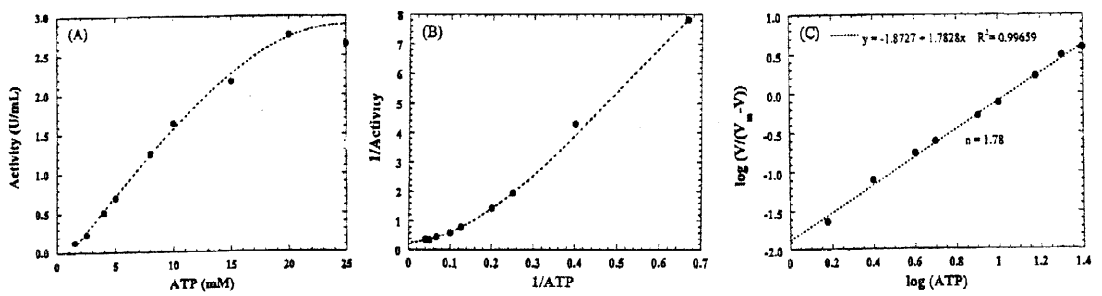

Fig. 7. (A) Reaction velocity curve, (B) Lineweaver-Burk plot, and (C) Hill plot for purified acetate kinase from *Clostridium thermocellum* at various ATP concentrations.

The results from this study may be important for optimization of DMC processes using *C. thermocellum*. For example, since the AK activity was profoundly affected by parameters such as pH, temperature and $MgCl_2$ (demonstrated in Fig. 2, 3 and 4), the ethanol yield and selectivity in a DMC process might be improved by adjusting these parameters to reduce AK activity and hence acetate production. In addition, the N-terminal sequence of AK can be deduced with the purified enzyme, which will allow the

design of a homologous gene probe for *ack* cloning via PCR. With the cloned gene, the carbon flux toward acetate might be redirected to ethanol synthesis by inactivating or attenuating *ack* through homologous recombination.

EXPERIMENTAL

Bacterial strain, growth medium and culture condition: *Clostridium thermocellum* ATCC 27405 was grown in the complex GS medium [18] supplemented (per liter) with 2 mg pyridoxamine·HCl, 0.2 mg d-biotin, 0.4 mg *p*-aminobenzoic acid, and 0.2 mg vitamin B_{12} [9]. Culture conditions were those used by Lin *et al* [9].

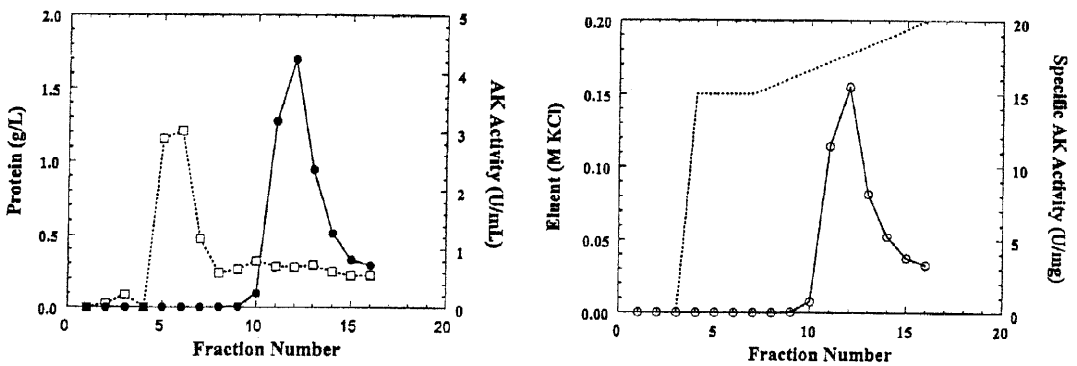

Fig. 8. Purification of acetate kinase from *Clostridium thermocellum* on a HiTrap Q anion-exchange column: (□) protein concentration, (•) acetate kinase activity, (---) eluent concentration, (o) specific acetate kinase activity.

Preparation of cell-free extracts, protein and enzyme assays: The preparation of cell-free extracts, and assays of total protein and AK activity were as described previously [9].

Purification of acetate kinase: AK from *C. thermocellum* was purified via anion-exchange chromatography and heparin affinity chromatography. All steps were carried out aerobically at 4°C. In a typical experiment, two 5-mL HiTrap Q anion-exchange columns (Pharmacia #17-1154) connected in series were equilibrated with 5 column volumes (50 mL) of Buffer C (10mM Tris-HCl, pH 7.2, containing 2 mM $MgCl_2$ and 2 mM dithiothreitol). Cell-free extract (10 mL, containing 377 U total activity and 112 mg total

protein) was loaded onto the columns at 0.6 mL/min, followed by a 100-mL linear gradient elution of KCl (0.15-0.2 M in Buffer C) at 0.8 mL/min (Fig. 8). Three fractions with high AK activity (#11, #12 and #13, 10 mL each) were pooled and desalted with Buffer C using Pharmacia desalting columns, giving 42 mL of total desalted pooled samples. About one half of this desalted solution (22.5 mL, containing 97 U total activity and 8.4 mg total protein) was applied to two 5-mL HiTrap Heparin affinity columns (Pharmacia #17-0407) connected in series at 0.6 mL/min. The AK was eluted with 60 mL of 0-0.15 M KCl gradient in Buffer C at 0.6 mL/min volume (Fig. 9). Of the eluted samples, Fraction #11 contained the highest specific activity of 490 U/mg protein (with a total activity of 26.9 U and 55 μg total protein in 6 mL total volume). The same procedures were repeated for the remaining 20 mL desalted samples using the same HiTrap Q and Heparin columns.

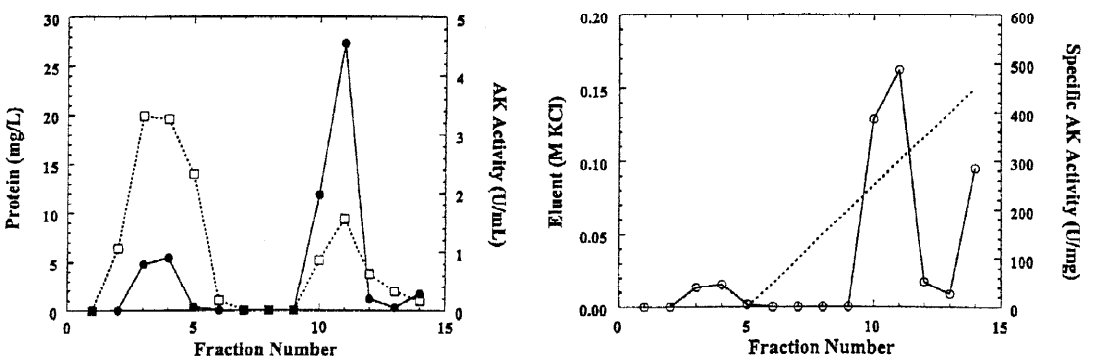

Fig. 9. Purification of acetate kinase from *Clostridium thermocellum* using a HiTrap Heparin affinity column: (□) protein concentration, (•) acetate kinase activity, (---) eluent concentration, (o) specific acetate kinase activity.

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REFERENCES

1. Jones, D. T.; Woods, D. R. Solvent Production. In *Clostridia*; Minton, N. P.; Clarke, D. J., Eds.; Plenum, New York, 1989; pp. 105-144.
2. Hogsett, D. A.; Ahn, H.-J.; Bernardez, T. D.; South, C. R.; Lynd, L. R.. *Appl. Biochem. Biotechnol.* **1992**, 34/35, 527-541.
3. South, C. R.; Hogsett, D. A.; Lynd, L.R.. *Appl. Biochem. Biotechnol.* **1993**, 39/40, 587-600.
4. Venkateswaran, S.; Demain, A. L. *Chem. Eng. Commun.* **1986**, 45, 53-60.
5. Demain, A. L.; Lynd, L. R. Turning garbage into motor fuel: fanciful dream or feasible scheme? In *Genetics, Biochemistry and Ecology of Lignocellulose Degradation*; Shimadu, K.; Hoshino, S.; Ohmiya, K.; Sakka, K.; Karita, S. Eds.; University Publishing Co., Ltd., Tokyo, 1994, pp. 573-583.
6. Demain, A. L.; Klapatch, T. R.; Jung, K. H.; Lynd, L. R. *Ann. N.Y. Acad. Sci.* **1996**, 782, 402-412.
7. Green, E. M.; Boynton, Z. L.; Harris, L. M.; Rudolph, F. B.; Papoutsakis, E. T.; Bennett, G.N. *Microbiology* **1996**, 142, 2079-2086.
8. Boynton, Z. L.; Bennett, G. N.; Rudolph, F. B. *Appl. Environ. Microbiol.* **1996**, 62, 2758-2766.
9. Lin, W. R.; Lee, C. C.; Hsu, J. J.; Hamel, J. F.; Demain, A. L. *Appl. Biochem. Biotechnol.* **1998**, 69, 137-145.
10. Aceti, D. J.; Ferry, J. G.. *J. Biol. Chem.* **1988**, 263, 15444-15448.
11. Fox, D. K.; Roseman, S. *J. Biol. Chem.* **1986**, 261, 13487-13497.
12. Winzer, K.; Lorenz, K.; Durre, P. *Microbiology* **1997**, 143, 3279-3286.
13. Diez-Gonzalez, F.; Russell, J. B.; Hunter, J. B. *Arch. Microbiol.* **1997**, 166, 418-420.
14. Schaupp, A.; Ljungdahl, L. G. *Arch. Microbiol.* **1974**, 100, 121-129.
15. Latimar, M. T.; Ferry, J. G.. *J. Bacteriol.* **1993**, 175, 6822-6829.
16. Anthony, R. S.; Spector, L. B. *J. Biol. Chem.* **1971**, 246, 6129-6135.
17. Monod, J.; Wyman, J.; Changeux, W. *J. Mol. Biol.* **1965**, 12, 88-118.
18. Johnson, E. A.; Sakajoh, M.; Halliwell, G.; Madia, A.; Demain, A. L.. *Appl. Environ. Microbiol.* **1982**, 43, 1125-1132.