Microbiological Aspects of Acetate Oxidation by Acetic Acid Bacteria, Unfavorable Phenomena in Vinegar Fermentation

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Several strains of acetic acid bacteria belonging to the genus Acetobacter, showing strong acetate oxidation, were screened and their microbiological aspects in acetate oxidation were investigated. When all available carbon and energy sources were exhausted and only acetic acid remained in the late stationary phase, the bacteria started to consume the acetic acid that had been accumulated in the culture medium for vinegar fermentation. They grew rapidly, showing the second stationary phase and a typical biphasic growth curve was observed. The cells from the first growth phase were acid tolerant, while the cells from the second growth phase turned over to become acid sensitive. However, no distinct acetate oxidation took place when oxidizable ethanol and other available carbon sources still remained in the culture medium. Moreover, no apparent acetate oxidation was observed in vinegar mash in which more than 4.5% of acetic acid was allowed to accumulate. There was a threshold in acetate concentration since the most selected strains oxidized acetate when the final concentration of acetic acid accumulated was less than 3.7%. When only acetic acid was administrated as the sole carbon and energy sources, the organisms finally used acetic acid after a long lag time. The lag time was shortened by the addition of a small amount of readily usable energy source, such as ethanol. From enzymatic analysis, only acetyl-CoA synthetase increased much among the enzymes concerning acetyl-CoA formation from acetate, while the enzyme activities of acetate kinase and phosphotransacetylase were not changed significantly. The enzyme activities of isocitrate lyase and malate synthase also increased significantly in the cells when acetate was consumed. These results indicate that acetic acid is converted to acetyl-CoA by acetyl-CoA synthetase to put acetate into the TCA cycle as well as to the glyoxylate cycle allowing the bacteria to grow rapidly on acetic acid after ethanol exhaustion. Taking together with growth experiments and enzymatic data accumulated, it was strongly suggested that cells different in physiological characteristics from the first growth phase emerged in the second growth phase.

Key words: acetate oxidation; acetic acid bacteria; Acetobacter aceti; acetyl coenzyme A synthetase; vinegar fermentation

Acetic acid bacteria belonging to the genus Acetobacter are used for vinegar production, while Gluconobacter strains are strong in ketogenesis and thus favorable for gluconic acid and ketogluconic acids production, L-sorbose fermentation for vitamin C production, and other oxidative fermentations.1) Recently, several specified strains of the genus Acetobacter have been one of the current topics as biocellulose producers.2) Mankind has obtained vinegar from ethanol-containing solutions by natural fermentation for centuries without understanding the nature of the process. The first description of microbial vinegar fermentation was made by Pasteur in 1862.3) He recognized that mother of vinegar was a mass of living organisms that caused acetic acid fermentation. Scientific approaches had never been done about vinegar fermentation as well as other oxidative fermentations in which acetic acid bacteria are involved, until Nakayama indicated two novel cytochromes in acetic acid bacteria in the early 1960s, which catalyzed oxidation of alcohol and aldehyde, respectively.4,5) Nevertheless, vinegar fermentation had been believed to occur by the action of cytosolic NAD(P)-dependent enzymes. It was in the late 1970s when a membrane-bound alcohol dehydrogenase was isolated from acetic acid bacteria in which a quinoprotein dehydrogenase involving PQQ as the primary cofactor besides cytochrome components had the primary function in vinegar fermentation.6,7)

According to the latest issue of Borgey’s Manual of Systematic Bacteriology,8) all strains belonging to the genus Acetobacter oxidize acetic acid into carbon dioxide and water. “Over-oxidation of acetate” or “acetate peroxidation” has been used synonymously with acetate oxidation designating the phenomena of aerobic acetate anabolism by acetic acid bacteria. An intensive consumption of acetic acid is always accompanied by a corresponding increase in

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Abbreviations: Acetyl-CoA, acetyl coenzyme A; ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; PQQ, pyrroloquinoline quinone; AMP, ADP, ATP, adenosine 5’-monophosphate, 5’-triphosphate; TCA cycle, tricarboxylic acid cycle; IFO, Institute for Fermentation, Osaka; SKU, Faculty of Science, Kasetsart University.
cell mass of the organisms. In the southeast Asian countries and also other tropical areas, coconut juice is used as the most popular source for home-made vinegar, yielding a lower acetic acid content than rice vinegar produced from sake or sake lees. A serious problem frequently happens with such coconut vinegar, that acetic acid is readily oxidized further by many acetic acid bacteria. Acetate oxidation is also seen even in the temperate countries. Experiences accumulated for centuries have allowed us to terminate vinegar fermentation when a small amount of ethanol still remains in the vinegar mash, that has been thought to be the safest way to avoid acetate oxidation. Vinegar fermentation allowing acetic acid accumulation to be more than 4% has become an alternative way to overcome acetate oxidation. In usual commercial vinegar production, the final concentration of acetic acid is controlled at 4 to 5%. Selected microorganisms which show the least acetate oxidation have been currently used for vinegar fermentation, though a prolonged incubation after ethanol exhaustion sometimes causes acetate oxidation more or less. Therefore, it is important to clarify the microbiological aspects of acetate oxidation by acetic acid bacteria, because few microbiological and biochemical investigations on acetate oxidation have been reported, in spite of its basic and practical significance. In this report, several aspects of acetate oxidation by acetic acid bacteria are described. A preliminary enzymatic investigation on acetate oxidation is also conducted briefly.

Materials and Methods

Chemicals. Yeast extract, ATP, ADP, AMP, hexokinase (EC 2.7.1.1), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), malate dehydrogenase (EC 1.1.1.37) were kindly supplied by Oriental Yeast Co. (Tokyo, Japan). Acetyl-CoA, isocitrate, citrate synthase (EC 4.1.3.7), and malate synthase (EC 4.1.3.2) were products from Sigma (St. Louis, MO, U.S.A.). All other chemicals used were guaranteed grade from commercial sources.

Bacterial strains, culture media, and growth conditions. Mesophilic acetic acid bacteria* used in this study were generously supplied by the Institute for Fermentation, Osaka (IFO). Acetobacter methanolicus JCM 6891, a methylothrophic acetic acid bacterium, was from the Japan Collection of Microorganisms (JCM). Thermotolerant acetic acid bacteria belonging to A. rancens subsp. pasteurianus, A. lovaniensis subsp. lovaniensis, A. acetii subsp. liquefaciens, and A. xylinum subsp. xylinum were from Kasetsearch University, Thailand. All acetic acid bacteria were maintained on agar slants, which were prepared by adding 15 g of agar and 5 g of CaCO₃ to the potato medium consisting of 5 g of glucose, 20 g of glyceral, 10 g of yeast extract, 10 g of Polypepton, and 150 ml of potato extract per liter of tap water.

Basal medium for seed culture contained 5 g each per liter of glucose, glyceral, yeast extract, and Polypepton in tap water and was sterilized by autoclaving at 120°C for 30 min. In a seed culture, ethanol and acetic acid were added aseptically to 2% (v/v) and 1% (w/v), respectively. The main culture medium was prepared by reducing the contents of yeast extract and Polypepton to 2 g per liter while ethanol was increased to 4%. Seed culture was made under shaking on a rotary shaker at 200 rpm or a reciprocal shaker at 120 strokes/min. Temperature and cultivating period are indicated in the legends of individual experiments. To the main culture medium in a jar fermenter (Mitowa Bio System, Kudamatsu, Japan) in 1 liter, 0.01 mol/L NaOH in a 3-liter side-arm flask, 10% of the seed culture was inoculated by aseptic transfer. Bacterial growth was followed by measuring turbidity at 660 nm by a photometer or by a Klett-Summersone photoelectric colorimeter with a red filter. In case of culture in a jar fermenter, agitation speed was set at 1000 rpm and aeration was controlled to be 0.2vvm.

Selection of acetic acid bacteria capable of oxidizing acetate. The tested strains were inoculated into 1 ml of the potato extract medium in a 10-ml test tube. The medium was supplemented by adding either 2 or 4% of acetic acid, or 0 or 6% of ethanol or sterile coconut juice. The cultures were statically incubated at 30°C or 37°C. After 3, 5, and 7 days of incubation, several loopfuls of the cultures were streaked onto the YPG agar plate (0.5 g of yeast extract, 0.5 g of Polypepton, 1.0 g of glycerol, 0.5 g of CaCO₃, and 1.5 g of agar per 100 ml of medium) containing 3% ethanol or a coconut juice agar plate containing 3% ethanol and 3 mg of bromocresol purple per 100 ml of medium. The colonies that appeared to show halos on the YPG agar plate or yellow color on the coconut juice agar plate, both indicating acid production, were isolated. After prolonged incubation on a coconut juice agar slant for over 100 h, color changes of bromocresol purple by alkaline reaction appeared along the clones among the tested strains indicating a potenti acetate oxidation.

Preparation of crude enzyme solution. All operations were done at 4°C unless otherwise stated. Cells were harvested by centrifugation at 9000 x g for 10 min, and washed twice with ice-cold 0.1 M Tris-HCl, pH 8.0. The washed cells were resuspended at about 1 g of wet cells per 2 ml in the same buffer and passed through a French pressure cell press (American Machine Co., Silver Spring, Md., U.S.A.) at 16,000 psi. After centrifugation at 9000 x g for 10 min to remove intact cells, the supernatant, crude cell-free extract, was centrifuged at 68,000 x g for 90 min to separate the cytosolic fraction from the cytoplasmic membrane.

Analyses. Acetic acid and glucose in the culture medium were measured by a high pressure liquid chromatograph (HPLC, Hitachi, L-6000) using Shodex sugar column SH 1011 (8 x 300 mm) with 0.01 M H₂SO₄ as a mobile phase at a flow rate of 1 ml/min as described previously. A refractometer (Hitachi, L-3300) was attached to the HPLC as a detector. Acidity of the culture media was measured by titration with 0.5 N NaOH using phenolphthalein as pH indicator. Ethanol and glycerol in the culture medium were measured enzymatically.

Enzyme assays. Acetate kinase (EC 2.7.2.1) was measured by the reverse reaction using acetylphosphate as the primary substrate in the presence of ADP and inorganic phosphate as described by Nishimura and Griffith. The ATP generated was measured by the coupling reaction with hexokinase and glucose-6-phosphate dehydrogenase. Acetyl-CoA synthetase (EC 6.2.1.1) was similarly measured by the reverse reaction using acetyl-CoA as the primary substrate in the presence of AMP and pyrophosphate under similar conditions as in the assay of acetate kinase. The forward reaction of acetyl-CoA synthetase was measured by a published method of Frenkel and Kitchens. Phosphotransacetylase (EC 2.3.1.8) was measured by the reverse reaction using acetylphosphate as the substrate under essentially the same conditions as described by Klotzsch and Iseose. Malate synthase (EC 4.1.3.2) was measured by a published method described by Yoshida et al. Malate synthase activity was determined by measuring the formation of oxaloacetate with acetyl-CoA as described by Weitzman, an aliquot of the reaction mixture (0.1 ml) was taken as the substrate for malate dehydrogenase (EC 1.1.1.37) at pH 10.0 in the presence of NAD and the initial rate of NADH formation was measured at 340 nm by a photometer. Alcohol dehydrogenase (ADH) (EC 1.1.1.31) and aldehyde dehydrogenase (ALDH) (EC 1.2.99.3) were measured by the method described by Amano and Ohnishi* using cytochrome c as the cytochrome oxidase and NADH or NADPH as the coenzyme. All enzyme assays used in this study except for malate synthase, one unit of enzyme activity was defined as the amount of enzyme catalyzing formation or consumption of one μmol of reaction product or substrate per min. All enzyme assays were done at 25°C unless otherwise stated. Protein content was measured by a modified method of Lowry et al with bovine serum albumin as a standard.

Results and Discussion

Selection of acetic acid bacteria having potent acetate oxidation

Acetate oxidation, which causes the loss of acetic acid in the medium, was observed with many strains. A. methanolicus was selected from the strains of mesophilic acetic acid

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* To differentiate from the thermotolerant acetic acid bacteria from Thailand, the term, mesophilic, is tentatively used for the strains from IFO and A. methanolicus, though all strains dealt with in this paper are typical mesophiles.
bacteria. Among the thermotolerant acetic acid bacteria from Thailand, 35 of 129 strains showed potent acetate oxidation. Three strains of *A. rancens* subsp. *pasteurianus* SKU 1102, SKU 1106, and SKU 1111 were selected for further experiments. The course of acetate oxidation was examined with the selected strains during the growth on a medium containing 2% ethanol and 1% acetic acid which allowed the final acetic acid accumulation to be 3%. They were compared with mesophilic strains from IFO under the same conditions. It was clear that acetate oxidation was dramatically observed with *A. methanolicus* and the three strains of *A. rancens* subsp. *pasteurianus* (Fig. 1), while no appreciable acetate oxidation was observed with the strains from IFO, such as *A. acti* IFO 3283 and IFO 3284, and *A. rancens* IFO 3298, all of which are well known in actual vinegar fermentation (Fig. 2). When examined in the culture medium, allowing them to accumulate 3% acetic acid at the stationary phase, *A. methanolicus* and the three strains of *A. rancens* subsp. *pasteurianus* came to the first stationary phase after ethanol exhaustion at about 20 to 40 h of cultivation. The first stationary phase lasted subsequently for more than 60 h. After 100 h of cultivation, the concentration of acetic acid in the medium rapidly decreased reciprocally to the increase in turbidity of culture medium, showing the second stationary phase of the growth caused by a complete exhaustion of acetic acid accompanied with some pH shift, such as from pH 2.8 to 3.5 (data not shown). They showed a typical biphasic growth. The course of glucose consumption observed with *A. rancens* subsp. *pasteurianus* SKU 1111 was plotted in Fig. 1. A peak of glucose disappeared from the chart paper of HPLC when checked with the culture medium at the beginning of the second stationary phase. Glycerol dehydrogenase activity was not detected with the same culture medium (data not shown). Glucose and glycerol in other cultures were also deduced to have been exhausted before the second growth came to the early stationary phase.

Contrary, as can be seen in Fig. 2, a single phase of growth was extensively observed with the strains from IFO without significant acetate oxidation, even though the cultivation was prolonged for more than 170 h. Acetic acid accumulated in the culture medium was maintained at a constant level throughout the experiment. Glycerol is a better carbon source for the bacterial growth than glucose in genus *Acetobacter*, since the EMP pathway and the TCA cycle have a normal function in *Acetobacter*, unlike the genus *Gluconobacter* in which the TCA cycle functions incompletely. Other available carbon sources that might come from yeast extract and Polypeptone must have been consumed similarly. The second bacterial growth seems to take place by consuming acetic acid exclusively. The latest issue of Bergey's Manual of Systematic Bacteriology indicates that all strains belonging to the genus *Acetobacter* use acetic acid as a carbon source. Acetyl-CoA synthetase and also isocitrate lyase, which are enzymes directly related
to acetate use, might be regulated by the balance of carbon sources available, as discussed later. Anyway, it is interesting to check what kinds of expression regulation in acetate oxidation are valid in the genus *Acetobacter*.

**Effects of initial ethanol concentration on acetate oxidation**

It is important to check the effects of initial ethanol concentration on acetate oxidation. Growth curves of *A. rancens* subsp. *pasterianus* SKU 1111 and *A. methanolicus* were measured in the presence of different initial concentrations of ethanol as shown in Fig. 3. In the figure, the course of acetic acid fermentation is followed only by growth curves of individual organisms. However, it was confirmed that the initially added ethanol had already been converted to acetic acid by the end of the first exponential phase (20 to 40 h of incubation) as deduced from the data shown above. It is very interesting to see that the growth curves became biphasic when the initial ethanol concentration was limited below 3%. This means that acetate oxidation must take place if the amounts of acetic acid accumulated is controlled to be less than 3.7%, though the length of the first stationary phase depends on the initial ethanol concentration. Thus, beginning of the second growth was delayed more with 3% ethanol than with 1% or 2% of initial ethanol concentration. Growth curves observed with 4% and 5% of ethanol showed no second stationary phase, though *A. rancens* subsp. *pasterianus* SKU 1111 is a typical acetate oxidizer (Fig. 3A). If vinegar mash allows to accumulate the final acetic acid concentration to more than 4.5%, any serious acetate oxidation would no longer occur, even if it is incubated with a potent acetate oxidizer. Similar results were also observed with *A. methanolicus* as shown in Fig. 3B. Similarly to the case of *A. rancens* subsp. *pasterianus* SKU 1111, *A. methanolicus* showed acetate oxidation when incubated with 1% and 2% ethanol in the presence of initially added 1% acetic acid. The growth curve observed with 3% initial ethanol concentration showed a single phase of growth, which means the final concentration of acetic acid came to nearly 4%, even though the turbidity went up gradually after prolonged incubation, over 160 h. The growth curve obtained with 4% ethanol gave a single phase throughout the experiment. Judging from the combined data, these results strongly support the conclusion that the critical point for acetate oxidation exists between 3.7% and 4.5% of acetic acid accumulated. At the moment, it is unclear what kinds of regulation occur in the presence of more than 4.5% acetic acid. One speculation can be proposed, that the accumulated acetic acid in the culture medium can exist as two forms, dissociated and undissociated. If the undissociated form of acetic acid increases, it can be out of the mediated transport system and diffuse through the bacterial membrane, causing inhibitory to bacterial respiration.

**Effects of initial concentrations of acetic acid and ethanol on acetate oxidation**

A question arose whether acetate oxidation is controlled by the initial ethanol concentration. Different combinations of acetic acid and ethanol allowing 3% for the final acetic acid accumulation were added at the initial stage of growth of *A. rancens* subsp. *pasterianus* SKU 1111. As can be seen from Fig. 4, the initial ethanol concentration gave a significant effect to the length of the first lag phase. When ethanol was added to 1.5% and acetic acid to 1.5%, the length of the first stationary phase lasted for nearly 100 h, and when the initial ethanol concentration gradually decreased to 1 to 0.5%, the length of the first stationary phase also became shorter. The first stationary phase almost disappeared when tested with the combination of 2.5% acetic acid and 0.5% ethanol. On the other hand, when 3% acetic acid was administered as available carbon and energy sources, exhaustion of acetic acid appeared after 120 h of incubation. These results indicate that a small amount of readily usable energy source, ethanol in this case, has an important role in the initiation of acetate oxidation. To check the role of ethanol in acetate oxidation, an additional experiment was done as shown in Fig. 5. To the medium containing acetic acid, ethanol was added to 0.2% during the course of acetate use. It was shown clearly that the addition of a small amount of energy source increased acetate usage allowing the bacteria to grow on the medium. It can be suggested that ethanol plays an important role as oxidizable substrate generating energy that supported the initial part of microbial growth in a stage where the TCA cycle and NADH dehydrogenase system are not predominant. 

In all experiments so far described in this paper, bacterial growth was followed by reading the turbidity of the culture medium as a reference to cell number. As has been reported by Takemura et al., it must be noted that the turbidity does not mean the total viable cells. Different from those used by Takemura et al., the acetic acid bacteria used in this study were always exposed to acidic conditions, at least, in the presence of 1% acetic acid. The viable cell count experiments during the culture showed a good accuracy.
Fig. 4. Effects of Initial Concentrations of Acetic Acid and Ethanol on Acetate Oxidation.

A. rancens SKU 1111 was incubated with various combinations of acetic acid and ethanol as indicated. A2.0 + E1.0 means that acetic acid and ethanol were initially added to 2.0% and 1.0%, respectively. The fate of acetic acid (open circles) and changes in bacterial growth measured by the turbidity (closed squares) were followed for the period as indicated. Incubation was done in a jar fermentor in 1 liter of the main culture medium in which the concentrations of acetic acid and ethanol varied as indicated.

Fig. 5. Effects of Readily Usable Energy Source as a Starter for Acetate Oxidation.

A. rancens SKU 1111 was incubated in a medium containing 3% acetic acid as carbon and energy sources. To the culture medium, 0.2% ethanol was added aseptically as indicated. Incubation was done in a jar fermentor in 1 liter of the main culture medium. In this figure, acetic acid concentration (open circles) and bacterial growth as turbidity (closed squares) are shown. Ethanol concentrations are not shown. Top frame, ethanol was added when incubation was started. Middle frame, ethanol was added after 51 h incubation. Bottom frame, control (ethanol was not added throughout the incubation).

with the course of turbidity (data not shown). The majority of the cells in the first stationary phase showed acid tolerance and grew on a plate containing 3% of acetic acid, while the acid tolerance decreased very much with the cells from the second stationary phase. The physiological properties of acetic acid bacteria might have been much changed yielding two different cell types in the two growth phases (M. Taniguchi et al., unpublished observations). In our previous data, a change of the culture conditions, from static to shaking or vice versa, results in a change of the cell type, which is related to the change in the terminal oxidase from cytochrome $a_1$ to cytochrome $o$ in A. aceti. 25) The cell types from the two different stationary phases have to be differentiated to identify the cells.

Enzyme activities in acetate oxidation

Since many isolated strains showed acetate oxidation extensively, it was important to see what kind of enzyme activity is increased in acetate oxidation. In addition to acetyl-CoA formation via pyruvate dehydrogenase complex through catabolism of carbon sources, acetyl-CoA is formed by two different ways from acetate. One route is a direct acetylation of CoA catalyzed by acetyl-CoA synthetase as shown in Fig. 6. Acetyl-CoA formation by a coupling reaction of acetate kinase and phosphotransacetylase is an alternative route. Acetyl-CoA thus formed is then combined with oxaloacetate to form citric acid and incorporated spontaneously into the TCA cycle. Another route of acetyl-CoA assimilation is seen in malate formation by combining with glyoxylate from isocitrate by the action of isocitrate lyase.

The enzyme activities concerning acetate oxidation were assayed with the enzyme solutions prepared from “young cells” and “aged cells” of A. rancens subsp. pasteurianus SKU 1111 as shown in Table. The young cells were harvested from the main culture medium after 60 h of incubation that corresponded to the early stage of the first stationary phase. On the other hand, the aged cells came from 180 h of incubation corresponding to the second stationary phase. It was very interesting to see that there were no significant increases in enzyme activities of acetate kinase and phosphotransacetylase between the two types of cells, while the enzyme activities of acetyl-CoA synthetase and isocitrate lyase were significantly increased in the aged cells. Thus, acetic acid accumulated outside the cells might have been used directly as a substrate for acetyl-CoA synthetase followed by incorporation of resulted acetyl-CoA into the TCA cycle and glyoxylate cycle. Malate synthase also became clearly detectable in the cell-free extract from the aged cells, though the enzyme assay was rather qualitative in this study. As can be readily guessed from the growth curves in Fig. 2, no significant data
ALDH would have no physiological roles in the cells any longer. Alternatively, the decrease in specific activity of ADH and ALDH in the aged cells would reflect the increase of ADH-deficient mutants in the prolonged cultures as reported by Takemura et al. They found a novel insertion sequence, IS1380, causing genetic instability that produced spontaneous mutants at high frequency that were deficient in ethanol oxidation because of the loss of ADH activity. To check the possibility of occurrence of such an IS element, the total DNA was extracted with the acetic acid bacteria from Thailand and analyzed by hybridization with IS1380 DNA fragment to investigate the distribution of the insertion sequence. Among the 129 isolates, 36 isolates showed the hybridization signals with the IS1380 DNA probe both on the chromosomal and plasmid bands. However, no hybridization with IS1380 was observed with the strains used in this study, A. rancens subsp. pasteurianus SKU 1102, SKU 1106, and SKU 1111 (G. Theeragool et al., unpublished observations). Recently, Matsushita et al. reported the formation of inactive ADH when G. suboxydans was grown in acidic and high-aeration conditions. It is interesting to see that a similar phenomenon can be observed in A. acetii.

From the observations in this study, another interesting question came up, whether the metabolic activities and physiological characteristics are identical between the young cells and the aged cells. It is also exciting to check why there is such a strict change in cellular characteristics, for example, the young cells are ethanol tolerant as well as acetic acid tolerant while the aged cells are rather sensitive to ethanol and acetic acid (K. Matsushita et al., unpublished observations). This is strongly related to the facts that ethanol oxidizing cells are predominant in earlier growth phase and acetate assimilating cells are abundant in the second stationary phase. Since acetic acid bacteria are well known to be readily mutated during cultivation by extrachromosomal elements such as insertion sequences, it really sounds like this might lead us to outstanding findings in microbial physiology by pursuing this interesting phenomenon.

The acetate oxidation by many strains of Acetobacter shows many exciting aspects in biochemistry of carbohydrate as well as in energy metabolism in aerobic bacteria, because ethanol is the primary substrate and acetic acid is the direct oxidation product by the organisms. Enzymes involved in the acetate oxidation are not strengthened while ethanol remains in the culture medium and the oxidative fermentation yielding acetic acid becomes predominant. However, once ethanol is gone from the culture medium, the enzymes related to acetate oxidation including the enzymes in the TCA cycle become predominant, and oxidize acetate to carbon dioxide and water, which brings the cells to the phase of acetate anabolism. Thus, it is obvious that ethanol is the key substrate for metabolic transduction from oxidative fermentation to respiration. What has been discussed about the results obtained in this study is completely different from the methanol oxidase system in methylotrophic bacteria, in which formaldehyde is directly fixed to either serine or hexulose-phosphate.

With respect to acetate oxidation, further screening to isolate a thermotolerant strain which shows no serious acetate oxidation like some mesophilic strains has to be
done. It would be helpful to stabilize the coconut vinegar fermentation without any loss of acetic. On the other hand, the target enzymes activated in acetic oxidation must be confirmed to be acetyl-CoA synthetase and isocitrate lyase. This point would require comparison by means of mRNA levels for the enzymes, when the organism is growing at the first growth phase and in the second phase.

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