Inhibition of Acetate Ester Biosynthesis in Banana (*Musa sapientum* L.) Fruit Pulp under Anaerobic Conditions

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The effect of anaerobic conditions on acetate ester biosynthesis in ripened banana pulp was investigated. Incubation of the pulp in less than 1% O_2 resulted in a significant reduction in the formation of ethyl acetate. Regardless of the presence of a large amount of endogenous ethanol and the remaining exogenous isobutyl alcohol after complete anaerobic incubation with the pulp, the production of acetate ester decreased. The effect of addition of pyruvate, isobutyl alcohol, acetate, and methyl hexanoate on acetate ester formation in 100% N_2 was also investigated. The addition of pyruvate and isobutyl alcohol to the pulp gave lower acetate esters in N_2 than in air, whereas the pulp incubated with acetate and isobutyl alcohol produced more acetate ester in both conditions. Therefore, the lack of acetyl CoA, or more precisely acetate, in the tissue is the main reason for the inhibition of acetate ester formation under anaerobic conditions. The activity of β-oxidation measured by incubation with methyl hexanoate was detected only in the samples incubated in air. The formation of acetyl CoA, derived from pyruvate through mitochondria and through β-oxidation, was inhibited by anaerobic conditions, which suggests that mitochondrial activity and/or β-oxidation are essential for ester biosynthesis.

KEYWORDS: Banana; acetyl CoA; acetate esters; ethanol; anaerobic conditions

INTRODUCTION

Aroma is an important contributor to the quality of fruits and influences consumer acceptability. Banana mainly produces volatile esters, such as isoamyl acetate and isobutyl acetate, which contribute to the characteristic odor of banana by combining low molecular size alcohols and acetate. Gilliver and Nursten (*1*) and Ueda and Ogata (*2*) reported that the acid moiety of acetate esters is derived from acetyl CoA in banana fruit. Acetyl CoA and alcohol are then converted to acetate esters by alcohol acyl transferase (AAT), which has been identified and partially purified in ripened banana fruit (*3*).

In higher plants, acetyl CoA is formed from different sources, such as pyruvate and fatty acids. Murphy and Stumpf (*4*) and Liedvogel and Stumpf (*5*) demonstrated that free acetate derived from pyruvate via acetyl CoA in the mitochondria moves rapidly to the chloroplast, in which this acetate is utilized to form acetyl CoA in spinach leaf cell. Some acetyl CoA is produced from pyruvate via acetaldehyde and then used for terpene biosynthesis in the diseased tissues of sweet potato (*6*). Bartley et al. (*7*) reported that acetate esters, such as pentyl and propyl acetates, are formed from fatty acids via β-oxidation of added methyl heptanoate in apples. In animals, cytosolic acetyl CoA is synthesized from citrate, which is formed in the tricarboxylic acid (TCA) cycle in mitochondria.

Aroma production often fluctuates when fruits are sealed in plastic film bags that create a modified atmosphere (MA) during storage or transport. Most commodities require at least 1% oxygen in MA or controlled atmosphere (CA) storage to prevent anaerobic metabolism (*8*). Pesis et al. (*9*, *10*) showed that feijoas and strawberries produced distinctive amounts of ethyl acetate, ethyl butyrate, and ethanol following 98% nitrogen treatment for 24 h. Apples packaged in low-density polyethylene bags increased the production of ethyl alcohol and ethyl esters during storage at 8 °C for 11 weeks (*11*). Greenish-yellow banana packed in polyethylene bags produced a high concentration of ethyl alcohol, acetaldehyde, and ethyl acetate, resulting in a reduction of the typical banana aroma (*12*).

A low oxygen concentration around fresh fruits and vegetables sometimes creates anaerobic conditions under which mitochondria are inactivated, resulting in the prevention of oxidative phosphorylation. However, the effect of less than 1% oxygen or anaerobic conditions on ester biosynthesis in banana is not completely understood.

In this research, the effects of aerobic and anaerobic conditions on acetate ester biosynthesis in banana fruit pulp were investigated.

MATERIALS AND METHODS

Yellow-ripened bananas (*Musa sapientum* L. cv. Cavendish) were purchased from a retail market and further ripened at room temperature to a fully ripened stage, in which they showed some small brown spots.

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Fully ripe bananas, which easily form esters, were used in the experiments unless otherwise noted.

Acetate Ester Formation under Various Oxygen Concentrations. The effect of low oxygen concentrations on acetate ester formation was determined by the following method. Banana pulp (5 g) was cut into small pieces, approximately 3 × 10 × 20 mm, and placed in a 100 mL Erlenmeyer flask. The flask was then kept in a 20 × 30 cm gas barrier bag laminated with nylon (15 μm) and polypropylene (70 μm) (Toyo Canning Co., Tokyo, Japan) with a silicon cap. After that, the bag was vacuumed and flushed with pure nitrogen gas to create anaerobic conditions in the flask, and the bag was sealed with a Multivac A 300 vacuum-sealing machine (Sepp Haggenmuller KG, Germany). Eight samples were prepared for making various oxygen concentrations of the atmosphere inside the bags by injection of various amounts of air, except one sample, which was left under completely anaerobic conditions. All of the samples were placed at 20 °C for 18 h. The sample in a perforated laminated bag was used as a control. After 18 h, oxygen concentrations in the bags were measured to identify the atmospheric oxygen concentrations (0%, 0.29%, 0.36%, 0.57%, 0.9%, 1.29%, 2.92%, and 21%) in the flasks, by gas chromatography (GC; Yanaco G 80). All the flasks were then sealed with silicon caps and further incubated in a water bath at 30 °C for 1 h. After incubation, the volatile compounds in the headspace gas of the flasks and the oxygen concentration inside the flasks were detected by GC (Shimadzu 8A and Yanaco G 80). This experiment was similarly conducted two more times with banana pulp from different hands.

Gas Chromatography. The volatile compounds were measured by GC (Shimadzu 8A) equipped with a flame ionization detector (FID) and poly(ethylene glycol) (PEG) 9000 column (1 m × 3 mm). The flow rate of the nitrogen carrier gas was 27 mL/min. The column temperature was 90 °C. Oxygen concentrations were measured with a Yanaco G 80 model gas chromatograph equipped with a thermal conductivity detector (TCD) and a column (1 m × 3 mm) containing molecular sieve 5A. The flow rate of the argon carrier gas was 40 mL/min. The column temperature was 60 °C. The volatile compounds of banana were identified by comparing the retention times of GC peaks with those of authentic standards.

Acetate Ester Formation by Addition of Pyruvate. For the determination of the relation between pyruvic acid and acetate ester formation under aerobic and anaerobic conditions, 10 g of banana pulp was cut into small pieces (3 × 10 × 20 mm) and put into a 100 mL Erlenmeyer flask. One milliliter of a 0.1 M potassium phosphate buffer (pH 5.5) with 0.7 M mannitol, together with 0.05 mM dithiothreitol (DTT), 50 mM isobutyl alcohol, and 0.05 mM sodium pyruvate, was placed in a micro tube (1.5 mL) attached inside the flask, and then placed into the laminated bag. After the bag was purged with nitrogen gas to create an anaerobic environment, the reaction mixture in the tube was added to the fruit pulp, and the flask was sealed with a silicon cap. The flasks were incubated in a water bath at 30 °C for 4 h. The volatiles that had accumulated in the headspace of the flask were determined by GC. A sample sealed in an aerobic condition was incubated as a control.

Ester Formation by Addition of Alcohol and Acid. To measure the formation of acetate or butyrate esters by the addition of both alcohol and acid, one gram of banana pulp was cut into small pieces (2 × 5 × 5 mm) and placed into a warburg vessel, which contained 1 mL of a 0.1 M potassium phosphate buffer (pH 5.5) with 0.7 M mannitol and 0.05 mM DTT. After mild suction to remove the air in the pulp, 50 mM isobutyl alcohol, 1 mM adenosine triphosphate (ATP), and 5 mM potassium acetate or sodium butyrate were placed in the side pocket of the vessel. They were then transferred to the laminated bags and sealed under recharging nitrogen gas, and the vessels were then sealed with silicon caps. Immediately after the addition of the reaction mixture to the fruit pulp, the pulp was incubated for 4 h at 30 °C in a water bath. The volatile compounds in the headspace of the vessels were determined by GC. The sample sealed in air was used as a control.

Ester Formation by Addition of Methyl Hexanoate. For the determination of β-oxidation of fatty acid under aerobic and anaerobic conditions, 10 g of banana pulp was incubated with 5 μL of methyl hexanoate placed on a piece of filter paper (1 cm^2) in a 100 mL Erlenmeyer flask. The procedures for anaerobic treatment were identical
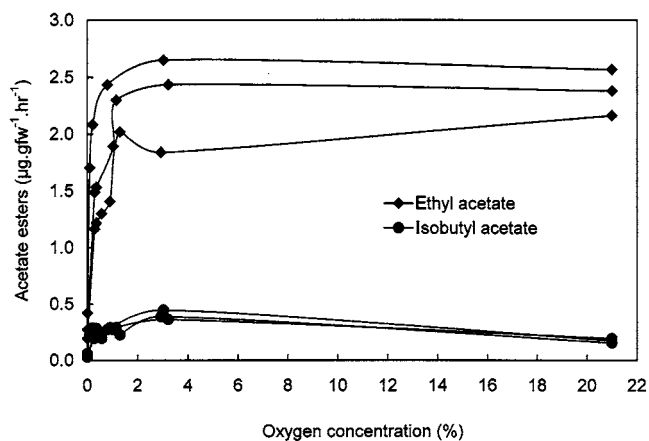

Figure 1. Effects of various oxygen concentrations on the formation of acatate esters in banana pulp. fw, fresh weight.

to those described above with the vacuum sealing machine. After incubation for 16 h at 30 °C, the volatiles in the headspace of the flask were detected by GC. For this experiment, yellow bananas without brown spots were used to distinguish the desirable esters, such as butyl acetate or ethyl butyrate, from other endogenous esters.

RESULTS AND DISCUSSION

Effects of Various Oxygen Concentrations on Acetate Ester Formation. The ethyl acetate and isobutyl acetate formation of banana pulp incubated for 18 h in low-oxygen or anaerobic conditions was lower than in that incubated in air (**Figure 1**). Incubation of banana pulp in concentrations of less than 1% oxygen resulted in a significant reduction of ethyl acetate and a moderate decrease in isobutyl acetate. Endogenous isobutyl alcohol concentration is lower than ethyl alcohol in banana pulp. Ethyl acetate and isobutyl acetate share acetyl CoA as one of their substrates. As the ethanol levels are much higher, a large amount of acetyl CoA is used by the ethanol to produce ethyl acetate, and this reaction limits the availability of acetyl CoA for the biosynthesis of isobutyl acetate. Therefore, the production of isobutyl acetate did not show a significant difference between in air and low-oxygen conditions. Generally, ethyl alcohol formation increased at a greater rate in fruits stored in low-oxygen conditions (*10*, *12*, *13*). Pesis et al. (*9*) reported that higher levels of ethyl alcohol were accumulated in anaerobically stored feijoa. Likewise, banana also produced a considerable amount of ethyl alcohol under anaerobic conditions (**Figure 2**). However, a gas chromatogram (**Figure 2**) showed that, regardless of the presence of endogenous ethyl alcohol after incubation for 18 h under anaerobic conditions, ethyl acetate formation is lower than in banana pulp incubated in air. The production of other acetate esters, such as butyl acetate, isobutyl acetate, and isoamyl acetate, was also lower than that in air.

Furthermore, when the pulp was incubated with exogenous isobutyl alcohol under anaerobic conditions, isobutyl acetate formation was reduced when compared to that of pulp incubated in air despite the presence of remaining isobutyl alcohol after incubation for 4 h (**Figure 3**). The results suggest that the metabolic pathway leading to acetate esters, such as acetate supply or acetyl CoA formation, must have been suppressed by low-oxygen or anaerobic conditions.

Effect of Addition of Pyruvic Acid on Acetate Ester Production. The effect of the addition of sodium pyruvate on acetate ester formation is shown in **Figure 4**. When banana pulp was incubated in the presence of both isobutyl alcohol and
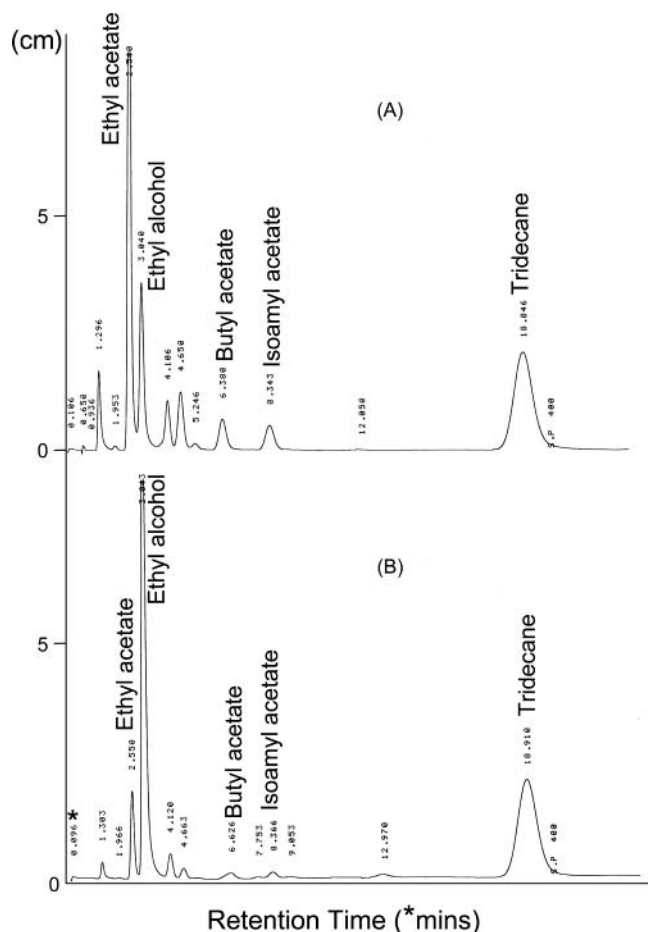
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Figure 2. Gas chromatogram of the formation of acetate esters in banana pulp under aerobic (A) and anaerobic (B) conditions. Tridecane was used as an internal standard.
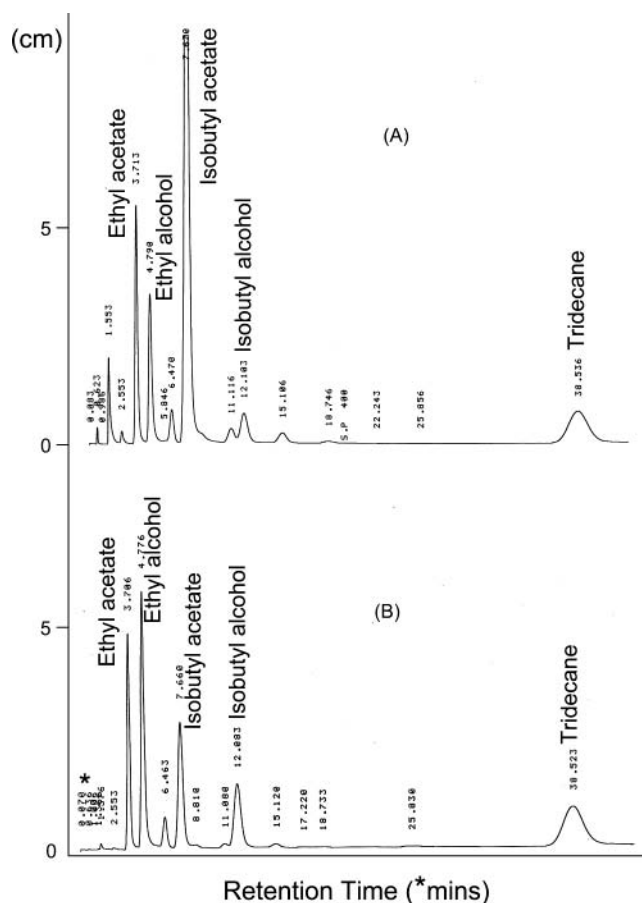

Figure 3. Gas chromatogram of the formation of isobutyl acetate in banana pulp resulting from the addition of isobutyl alcohol under aerobic (A) and anaerobic (B) conditions. Tridecane was used as an internal standard.

sodium pyruvate in air, higher levels of isobutyl acetate production were detected than in the sample without sodium pyruvate under the same conditions. Increasing the levels of ester production indicated that some of the acid parts of acetate esters are derived from pyruvate. On the other hand, when the reaction mixture was included with both isobutyl alcohol and sodium pyruvate under anaerobic conditions, the production of isobutyl acetate ester was almost the same as it was without sodium pyruvate under the same conditions (**Figure 4B**). The reduction in the production of isobutyl acetate in banana pulp incubated with isobutyl alcohol and sodium pyruvate under anaerobic conditions suggested that sodium pyruvate may not pass into mitochondria and that the metabolites, such as acetate, do not leak out from mitochondria. Pyruvate is generally formed from glycolysis and converted to acetyl CoA and carbon dioxide (CO_2) by pyruvate dehydrogenase in mitochondria. Liedvogel and Stumpf (*5*) reported that acetyl CoA derived from pyruvate is hydrolyzed to free acetate by mitochondrial hydrolase and moved to the chloroplast, where acetate is utilized to form aceyl CoA in spinach leaf. In addition, acetyl CoA itself cannot directly pass through the inner mitochondrial membrane, although acetylcarnitine is formed from acetyl CoA and the carnitine can pass through the membrane (*14*). In the intermembrane space, acetylcarnitine is converted to acetyl CoA and then changed to acetate by acetyl CoA hydrolase, and acetate then leaks out from the mitochondria (*15*, *16*). Acetate could be converted to acetyl CoA by acetyl CoA synthetase in the cytosol.

Effects of Added Substrates and Cofactors on Ester Biosynthesis under Anaerobic Conditions. Acetate ester

formation by the addition of isobutyl alcohol, potassium acetate, and ATP under aerobic and anaerobic conditions is shown in **Figure 5**. When the pulp was incubated with isobutyl alcohol alone in nitrogen (0% O_2) for 4 h, the production of isobutyl acetate was lower than that in banana pulp incubated in air. Rudell et al. (*17*) also reported that exogenous 1-pentanol-derived biosynthesis of apple fruit was inhibited under anoxic conditions. The sample, in the presence of potassium acetate alone, does not show any significant difference in the formation of acetate esters in either aerobic or anaerobic conditions (**Figure 5**). However, when the pulp was incubated with isobutyl alcohol and potassium acetate for 4 h under aerobic or anaerobic conditions, higher productions of acetate ester were detected than with other treatments. Even in anaerobic conditions, the production of acetate ester reached half the production that was obtained in aerobic conditions (**Figure 5**). In the experiment when sodium butyrate was used as an acid source instead of potassium acetate in the presence of isobutyl alcohol, the formation of isobutyl butyrate also increased under both aerobic and anaerobic conditions (**Figure 6**). The results demonstrated that added acetate or butyrate passes through the cell membrane and is converted to an activated form, acyl CoA, which is then combined with alcohols to produce esters. Furthermore, AAT activity is not inhibited by short-term anaerobic storage.

The addition of ATP in each experiment did not show any positive effect on acetate or butyrate ester formation (**Figures 5** and **6**). The formation of acyl CoA requires ATP because acetyl CoA synthetase is ATP-dependent. In this research, we found that, without the addition of ATP, acetate or butyrate ester
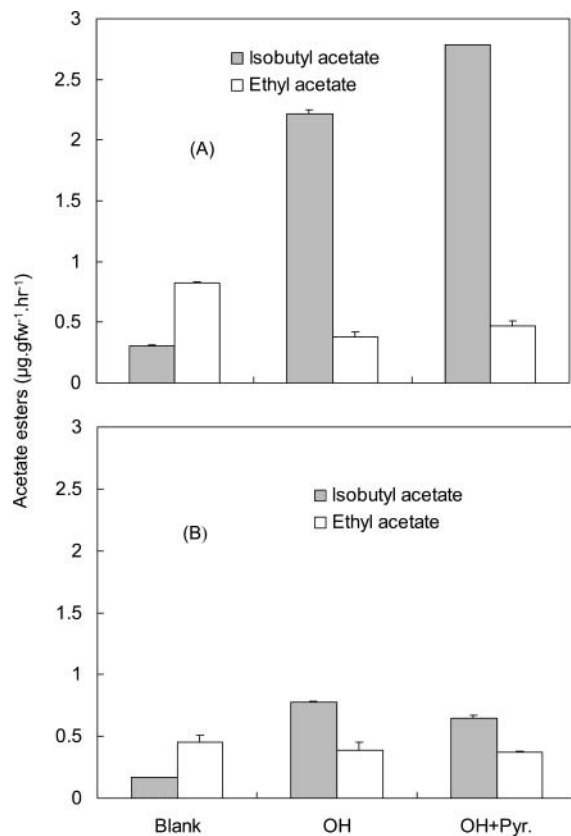
Figure 4. Acetate ester formation in banana pulp resulting from the addition of sodium pyruvate (pyr) and isobutyl alcohol (OH) under aerobic (A) and anaerobic (B) conditions. Error bars represent SE ($n = 3$). fw, fresh weight.
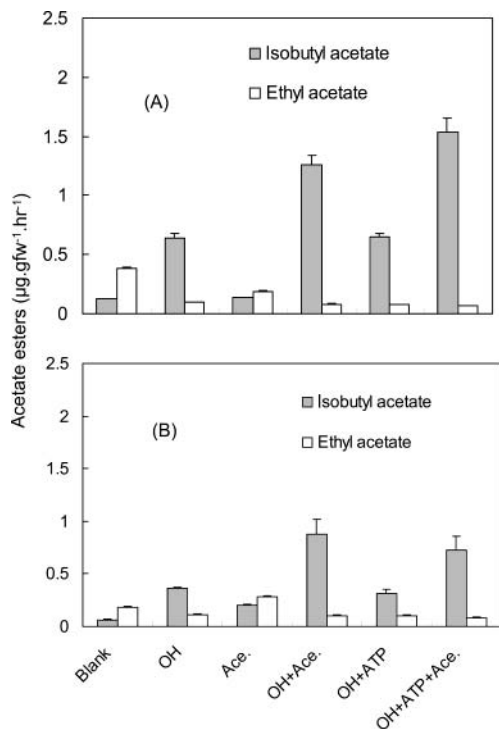

Figure 5. Acetate ester formation in banana pulp resulting from the addition of isobutyl alcohol (OH), acetate (ace), and ATP under aerobic (A) and anaerobic (B) conditions. Error bars represent SE ($n = 3$). fw, fresh weight.

production occurred in banana pulp in the presence of alcohol and acid under anaerobic conditions in which ATP could not be produced in mitochondria (**Figures 5** and **6**). The results indicate that added ATP does not penetrate the cells or that a
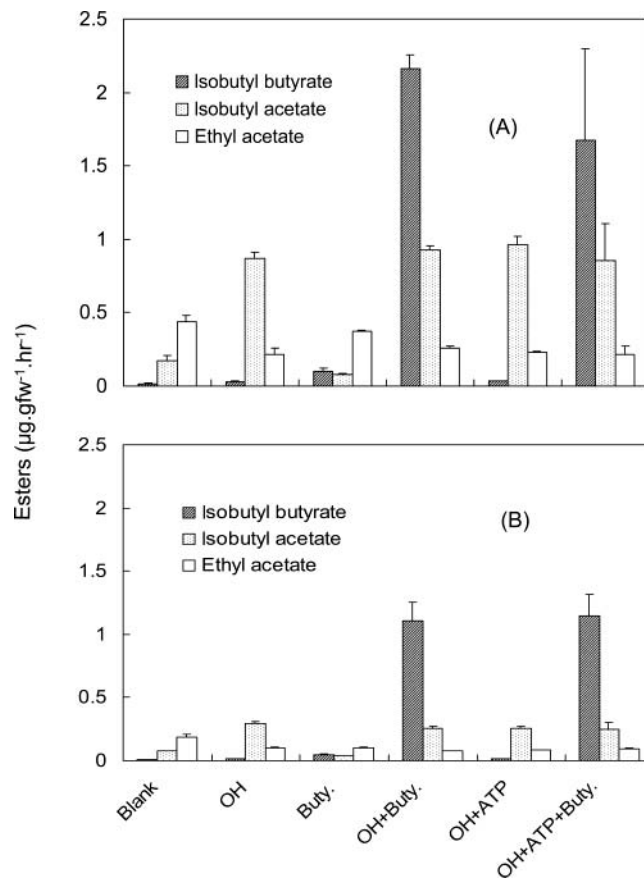

Figure 6. Ester formation in banana pulp resulting from the addition of isobutyl alcohol (OH), butyrate (buty), and ATP under aerobic (A) and anaerobic (B) conditions. Error bars represent SE ($n = 3$). fw, fresh weight.

fair amount of ATP seems to be present or is produced by glycolysis in the cytosol for acetyl CoA formation.

Cytosolic acetyl CoA can be synthesized not only from pyruvate via mitochondria but also from acetaldehyde in higher plants. Diseased root tissues of sweet potato produce acetyl CoA for terpene biosynthesis, mainly from pyruvate via acetaldehyde (*6*). Low-oxygen storage of fruits induces the accumulation of acetaldehyde by pyruvate decarboxylase and a great amount of ethanol production as a result of anaerobic respiration (*18*). As shown in **Figure 2**, a high concentration of ethanol was detected after banana pulp was stored in anaerobic conditions. Ethanol and acetyl CoA are the substrates for ethyl acetate formation. However, despite the presence of anaerobically produced ethanol in the pulp, the formation of ethyl acetate was significantly reduced after banana pulp was stored anaerobically (**Figure 2**). If acetyl CoA can be produced from acetaldehyde in banana, then the production of ethyl acetate should increase in anaerobic conditions. The results suggest that ethanol or pyruvic acid is not converted to acetyl CoA via acetaldehyde in the cytosol, at least in anaerobic condition (**Figure 8**). Therefore, acetyl CoA must be derived from pyruvate through the mitochondria in banana. Some cytosolic acetyl CoA is formed from citrate that leaked out of the mitochondria as a result of ATP−citrate lyase (*19*).

β-**Oxidation of Fatty Acid under Anaerobic Conditions.** High levels of ethyl acetate, butyl acetate, ethyl butyrate, and methyl butyrate were detected when banana pulp was incubated with methyl hexanoate (MH) under aerobic conditions (**Figure 7A**),while small amounts of those esters, except ethyl acetate, were detected without the addition of MH in the same conditions. The increase of butyl acetate, ethyl butyrate, and
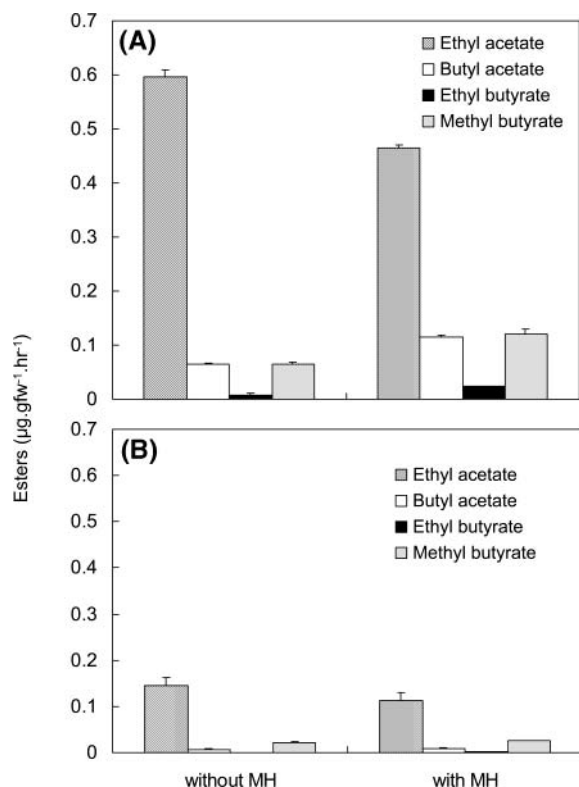
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Figure 7. Ester formation in banana pulp with or without methyl hexanoate (MH) in air (A) and N_2 (B). No bars shown, not detected. Error bars represent SE ($n = 3$). fw, fresh weight.
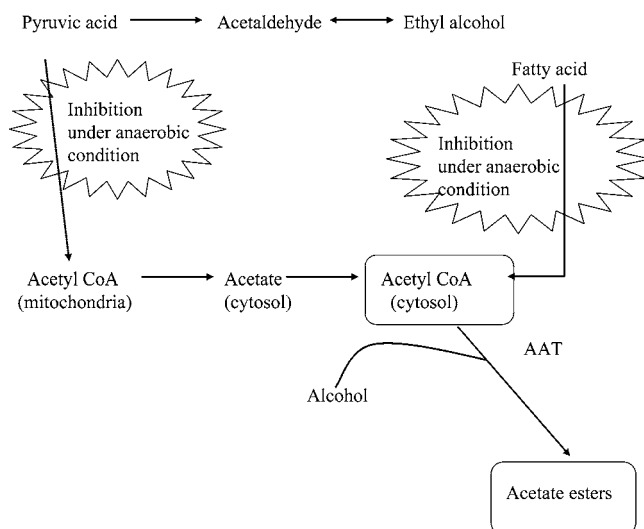

Figure 8. Proposed pathways for acetate ester biosynthesis and inhibition under anaerobic conditions in banana pulp. AAT, alcohol acetyltransferase.

methyl butyrate with MH in air indicates that these esters were derived from the added MH. After incubation for 16 h, the oxygen concentration in the atmosphere in each flask was checked by GC, and an oxygen concentration in excess of 5% remained. Higher levels of butanoate and acetate ester under aerobic conditions indicated that hexanoic acid, which is hydrolyzed from methyl hexanoate by esterase in the fruit pulp, completed one or two cycles of the β-oxidation pathway. It has been reported that when the methyl ester of a middle-chain fatty acid, methyl heptanoate, was supplied to Cox's Orange Pippin apples, acetate esters, such as pentyl and propyl acetate, were formed by β-oxidation, and acetyl CoA was formed with each cycle of β-oxidation of the substrate fatty acid (*7*). β-Oxidation

that occurs in glyoxysomes and oxygen is required for oxidation (*20*). Mitochondrial β-oxidation has also been demonstrated in plants (*21*). When the pulp was incubated with or without methyl hexanoate under anaerobic conditions (N_2), a much smaller amount of ethyl acetate than in aerobic conditions, and negligible amounts of butyl acetate and methyl butyrate, were detected because of the suppression of β-oxidation in the anaerobic conditions (**Figure 7B**). The results show that β-oxidation of fatty acid supports the production of acetate esters during ripening of bananas.

It has been reported that the activity of alcohol acetyltransferase (AAT) was affected and weakened by low oxygen concentrations during long-term storage of fruits, such as CA storage, and acetate ester production was decreased (*22, 23*). However, the results here (**Figures 5** and **6**) show that, in the presence of both alcohol and acid, the AAT activity was not markedly affected by anaerobic conditions for the ester production of banana. This indicates that short-term anaerobic conditions during shipping or transportation do not affect AAT activity but that acetyl CoA formation is affected by anaerobic conditions within a few hours. The mitochondrial activities and β-oxidation of fatty acids are the main pathways of acetyl CoA formation, and both pathways are oxygen-dependent. The suppression of these activities must be responsible for the inhibition of ester formation. The possible pathways of acetate ester synthesis and the inhibition of this synthesis under anaerobic conditions are shown in **Figure 8**.

Most fruits, including bananas, which are packed in plastic bags for maintaining quality, lose their characteristic odor when oxygen concentrations decrease to around 1%. An increase of ethyl alcohol and ethyl acetate denatured the fruit aroma, as many authors have indicated (*24, 25*). When oxygen levels were dropped below 1% oxygen or under completely anaerobic conditions, depression of acetate ester production was observed because of a lack of the acids for ester formation as we indicated, although ethyl alcohol production was still high. Furthermore, the great increase in the concentration of ethyl alcohol caused by anaerobic respiration and a decrease in acetate esters may alter the flavor of banana fruit.

Conclusion. This study clearly shows that acetate ester formation is inhibited under anaerobic conditions because acetyl CoA derived from pyruvate via mitochondria and β-oxidation of long-chain fatty acids cannot be produced without oxygen. Therefore, when bananas are sealed in plastic bags during transport, storage, or display in supermarkets, there is a possibility that anaerobic conditions will be created in the bags, which inhibit the production of acetate ester because of the lack of acetate.

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