# *Rhodococcus* sp. may convert ethylene to acetaldehyde to slow ripening in climacteric fruit.

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**ABTRACT:**

Pre-Print Update. Previous studies suggested *Rhodococcus rhodochrous* and *Bacillus licheniformis* cells converted ethylene to a nitrile compound to delay the effects of ripening, (Perry, G. Nov. 9, 2017). However, there may be an alternative compound that plays a more significant role in induced Rhodococcus and Bacillus ability to delay ripening. It has been known for years that Rhodococcus can convert the alkyne compound acetylene to acetaldehyde and potentially ethanol as a secondary product (DeBont, 1980).

This pre-print re-examines the prior data to determine if the tri-phasic system previously discussed in 2017, induced bacteria to convert ethylene and/or propylene into acetaldehyde (a primary product), ethanol (a secondary product), and acetonitrile (a product of ethanol and a subsequent ammoxidation reaction). The acetaldehyde may delay the effects of ripening and inhibit fungal growth, while the nitrile by products enhance early plant development including germination and root elongation. Experimental results suggest an inducible monooxygenase or dioxygenase like enzyme is required to facilitate this process.

## I. INTRODUCTION:

The U.S.D.A. Economic Research Service estimates over 34.6 % of the loss is directly related to unwanted climacteric ripening leading to eventual spoilage, deterioration, mechanical injuries, sprouting, and/or physiological disorders in produce (15% of these effects are linked to ripening) (Grolleaud 2002; Kader 2005; Barth et al., 2010). Climacteric ripening can be initiated in organic bananas with less than 124 mg/m$^3$ ethylene (Rasori *et al.,* 2002; Theologis 2004). Current methods to deter ripening include scrubbers and filters, and application of 1-MCP (Morretti *et al.,* 2002; Singh *et al*., 2008). 1-MCPis the most effective, but permanently alters the fruit physiological process and taste, (Sisler *et al*., 2001). Other alternatives may include acetaldehyde. Preliminary finding suggests the aldehyde may inhibit ethylene production by inhibiting ACC synthase and ACC oxidase enzymes (Podd and Staden, 1998).

A new emerging frontier of biological inhibitors may offer a more cost effective, ecofriendly, and organic alternative to prior chemical inhibitors. Research suggest Rhodococcus and Bacillus species maybe the most appealing candidates to biosynthesize compounds that may inhibit fruit ripening and delay flower senescence (Pierce et *al.* 2008 & Perry, 2011). Rhodococus is capable of expressing the oxygenase and oxidase enzymes required to produce an acetaldehyde that can delay fruit ripening and an acetonitrile to that can ehance early plant development (DeBont *et al.,* 1974; DeBont &Perk, 1980; Germon and Knowles, 1988; Saeki *et al.,* 1999). The present report investigates whether *R. rhodochrous DAP 96253* cultured on alkane hydrocarbon (i.e.ethylene/propylene) can induce a monooxygenase like enzyme to convert ethylene to acetaldehyde that inhibits ripening and potentially fungal infection (Perry, 2011).

## I. MATERIALS & METHODS:

### Growth & Preparation:

*Rhodococcus rhodochrous* DAP 96253 (ATCC 55899) were stored in 30 % glycerol stock solution, at - 80 ˚C. Step 1, glycerol stock of *R. rhodochrous DAP 96253* cells were transferred to 100 mL of Difco® Nutrient Broth and cultured for 3 d at 30 ˚C at 120 rpm (Shaking Incubator; Orbit 19 mm). Step 2, cells were transferred to Difco® Nutrient Agar for 3 d at 30 ˚C. Step 3, cells were transferred from nutrient agar to Yeast Extract Malt Extract Agar (YEMEA) supplemented with or without cofactors urea and cobalt and cultured for 3 d at 30 ˚C. *YEMEA Media* (g/L): (Bacto Agar 20 g; Glucose 4 g; Malt Extract 10 g; Yeast Extract 4 g; (Cofactors – Urea 7.5 g; $CoCl_2$ 0.201 g) (Dietz *et al.,*1980; Pierce *et al.,* 2008).

### Ethylene Induction:

Resuspend 2 - 3 g of *R. rhodochrous DAP 96253* in 15 mL of (1X) PBS buffer. Inoculate a 1 L tri-phasic flask. Tri- phasic System: 300 mL of Bacto Agar (300 mL $dH_2O$ and 14 g Bacto Agar), 300 mL Minimal Salts Media (No Glucose); 150 mL Hydrocarbon Gas for 4 d at 30 ˚C with shaking at 120 rpm (Shaking Incubator; Orbit 19 mm) (Shadowen *et al.,* 1989). M9 Stock: 64 g $Na_2HPO_4$-$7H_2O$, 15 g $KH_2PO_4$, 2.5 g NaCl, 5.0 g $NH_4Cl$ in 1 L distilled $H_2O$ and (autoclave), 200 mL M9 Stock, 2 mL of 1 M $MgSO_4$ (sterile), & 100 uL of 1 M $CaCl_2$ (sterile) in 1 L).

**Enzyme Assays:**

Rhodococcus cells were assayed using both a nitrile hydratase (NHase) and Cyanidase (CDH) assay. Standards, 2336 mg/m$^3$ acrylonitrile and 57.3 mg/m$^3$ potassium cyanide. The NHase and cyanidase method were used according to Perry, 2011. Ammonium concentrations were determined using a colorimetric assay (Fawcett *et al.,* 1960; Lambert *et al.,*1975).

## II. RESULTS:

### Dry Weight Comparison

*R. rhodochrous DAP 96253* previously cultured on cofactors (urea and cobalt) verses Rhodococcus previously cultured without cofactors, displayed a significant difference in biomass when grown on ethylene/propylene as a sole carbon source. Cofactors increased biomass by 83%, (Perry, 2011; Cumming et al. 2007), SEE Table 1

| | Initial TDW | Final TDW | Biomass Increase |
|---|---|---|---|
| No Cofactors | 243 mg (± 20 mg) | 285 mg (± 13 mg) | 42 mg (± 15 mg) |
| Cofactors (Cobalt &Urea) | 198 mg (± 1 mg) | 275 mg ( ± 3 mg) | 77 mg ( ± 2 mg) |

**Table 1: Growth on Ethylene as a Sole Carbon Source**: No Cofactors contained (4 g/L) glucose, Cofactors contained (4 g/L) glucose, (200 mg/L) cobalt, and (7.5 g/L) urea. Rhodococcus cells cultured with 124 mg/m$^3$ ethylene for 4 d at 30 °C at 120 rpm (Shaking Incubator; Orbit 19 mm). Calculations obtained from (Barreto and Howland, 2008 economics software), (Perry, 2011). Means (*M*) with Standard Deviations (*SD*) for two conditions: n= 14, n=14. The ( ± ) symbol represents the typical difference between the data points and their *M*. No Co-Factor Samples, *M* = 42 mg, S.D. =15 mg. Cofactors Samples, *M* = 77 mg mean, S.D. = 2 mg. A *p*-value of ≤ 0.01 %, suggests the addition of cofactors made a significant difference in the data. Cofactors increased biomass by 83 %.

## NHase and Cyanidase Activity

Nitrile hydratase (NHase) and Cyanidase activity was measured after Rhodococcus cells were cultured on ethylene/propylene as a sole carbon source for 3 days. Hydrocarbons ethylene and propylene induced NHase and cyanidase activity, See Table 2 & Table 3.

| | Initial NHase | Final NHase after Growth on Hydrocarbon |
|---|---|---|
| No Cofactors | 43 U (± 10.58 U) | 75 U (± 22.37 U) |
| Cofactors (Cobalt &Urea) | 4 U (± 6.65 U) | 27 U (± 2.52 U) |

**Table 2: Comparison of Enzymatic Assays (NHase Activity):** No Cofactors contained (4 g/L) glucose, Cofactors contained (4 g/L) glucose, (200 mg/L) cobalt, and (7.5 g/L) urea. Rhodococcus cells cultured with 124 mg/m$^3$ ethylene for 4 d at 30 °C at 120 rpm (Shaking Incubator; Orbit 19 mm). Units 1 uM of acrylonitrile converted to 1 uM acrylamide in 1 min, pH 7.2 at 30 °C, (Perry, 2011). Means (*M*) with Standard Deviations (*SD*) for two conditions: n= 40, n= 40. The ( ± ) symbol represents the typical difference between the data points and their *M*. No Co-Factor Samples, *M* = 75 U, S.D. =22.37 U. Cofactors Samples, *M* = 27 U mean, S.D. = 2.52 U. A *p*-value of ≤ 0.01 %, suggests the addition of cofactors made a highly significant difference in the data. Rhodococcus sp. cultured with cofactors increased NHase activity by 1000 %.

| | Initial CDH Activity | Final CDH after Growth on Hydrocarbon |
|---|---|---|
| No Cofactors | N.D. | 1.33 U (± 0.03 U) |
| Cofactors (Cobalt &Urea) | 5 U (± 2.88 U) | 3 U (± 1.20 U) |

**Table 3: Comparison of Enzymatic Assays (CDH Activity):** No Cofactors contained (4 g/L) glucose, Cofactors contained (4 g/L) glucose, (200 mg/L) cobalt, and (7.5 g/L) urea. Rhodococcus cells cultured with 124 mg/m$^3$ ethylene for 4 d at 30 °C at 120 rpm (Shaking Incubator; Orbit 19 mm). Units 1 uM of KCN converted to 1 uM formic acid in 1 min, pH 7.2 at 30 °C. N.D. means not detectable, (Perry, 2011). Means (*M*) with Standard Deviations (*SD*) for two conditions: n= 15, n= 15. The ( ± ) symbol represents the typical difference between the data points and their *M*. No Co-Factor Samples, *M* = 1.33 U, S.D. = 0.03 U. Cofactors Samples, *M* = 3 U mean, S.D. = 1.20 U. A *p*-value of ≥ 0.05 %, suggests the addition of cofactors makes a significant difference in the data. Rhodococcus sp. cultured with cofactors decreased CDH activity.

**Delay Fruit Ripening:**

*Rhodococcus rhodochrous DAP 96253* cells cultured with cofactors (cobalt-0.201 g/L & urea-7.5 g/L) and hydrocarbons displayed an enhanced ability to delay fruit ripening, (SEE Figure 1). However, Rhodococcus cultured only on hydrocarbons without cofactors was also able to delay ripening, (SEE Figure 2).

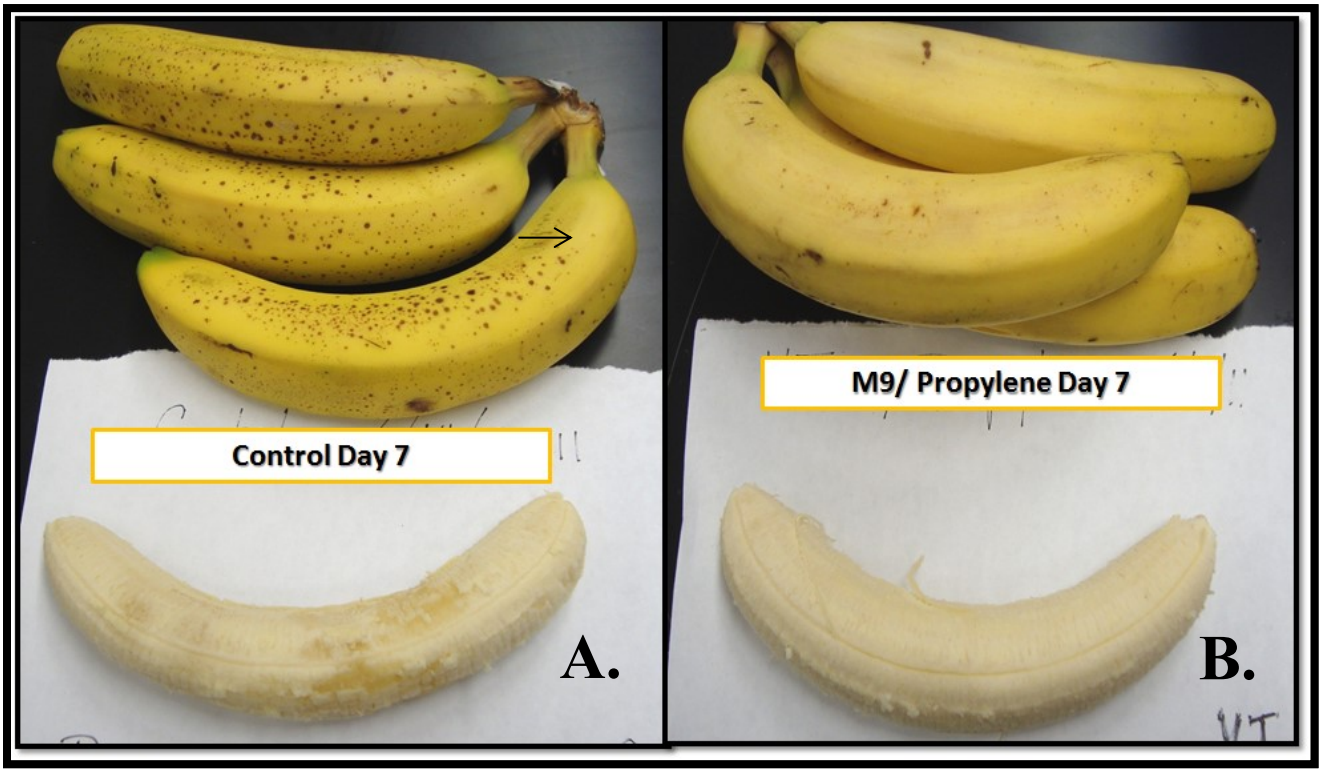


**Figure 1: Effect of Cofactors / Hydrocarbon on Delayed Ripening Capabilities:** A) Control bananas after 7 days (no cells). B) Bacteria cultured on hydrocarbon and cofactors.

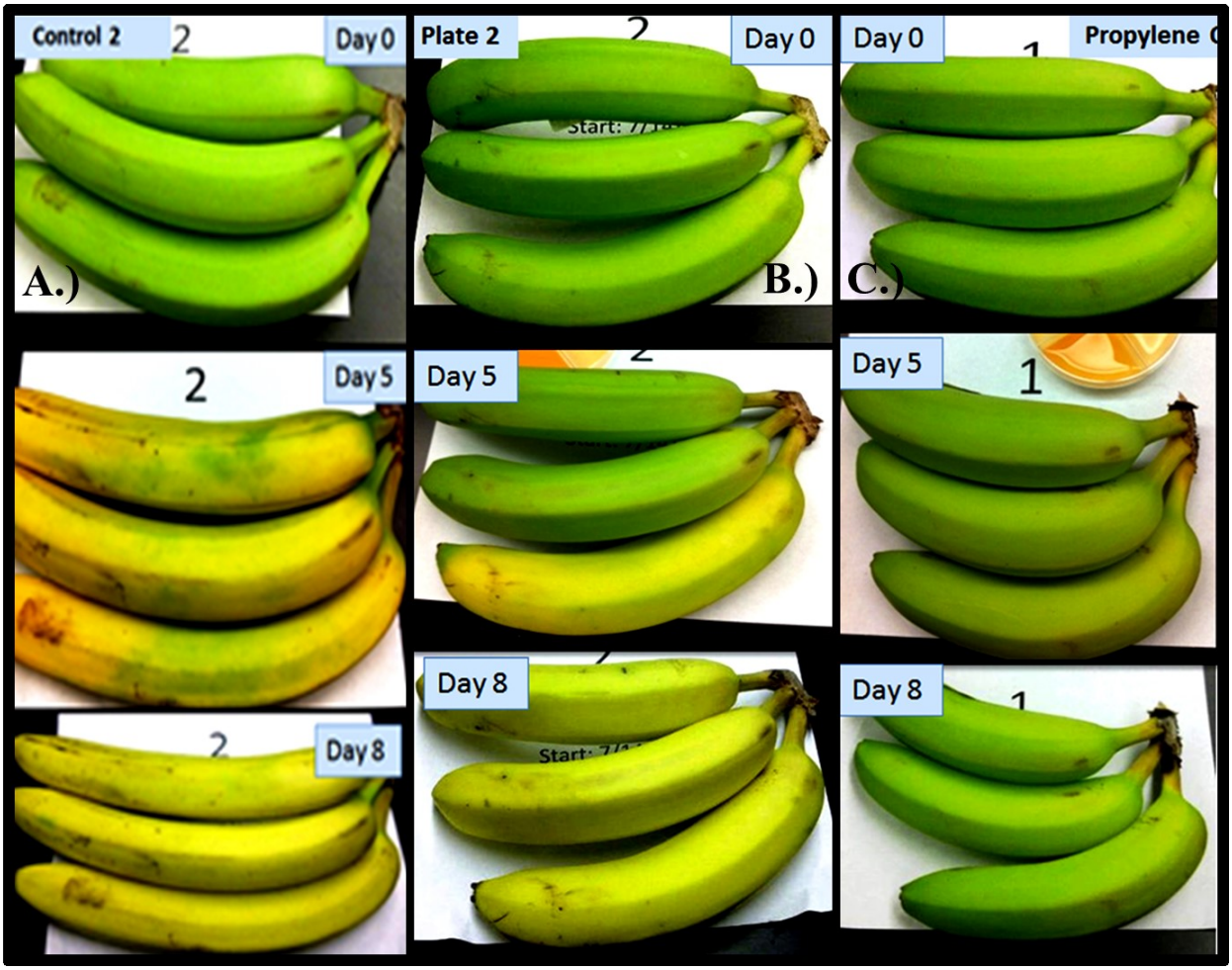


**Figure 2: Hydrocarbon on Delayed Ripening Capabilities:** Organic unripen green bananas were stored in sealed plastic bins with *Rhodococcus rhodochrous DAP 96253* cells for 8 days. Bacteria were re-used for a second time. . (A.) Control. (B.) Bacteria Cofactors Only. (C.) Bacteria Hydrocarbons Only, (Perry, 2011).

## GC/MASS SPEC Analysis

The headspace was compared for bacteria cultured with cofactors and ethylene to bacteria cultured with cofactors and with ethylene. The results displayed different images. Cells cultured with cofactors and ethylene displayed peaks at retention times at 7.0 min (Ethanol), 7.1min (Acetonitrile), and 12.4 min (butanol).

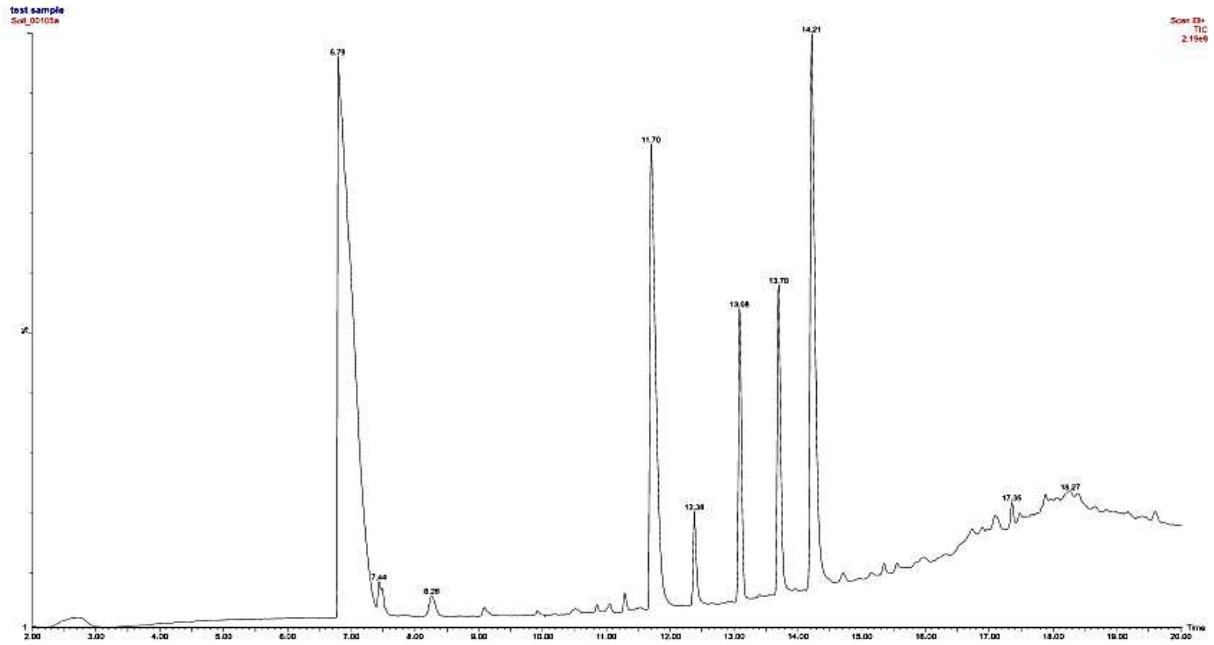


**Figure 3: GC Mass Spec Image:** Bacteria induced with cofactors and ethylene. Ethanol/Acetonitrile present.

## III. DISCUSSION:

The data suggest Rhodococcus is capable of being induced to metabolize ethylene/propylene by adding cofactors cobalt and urea to the media as previously reported in literature, (Hartmans *et al.,* 1991; Elsgaard 1998; Elsgaard 2000; Perry, 2011). This induction method may be facilitated by a monooxygenase like enzyme that converts ethylene into an epoxide (Ensign *et al.,* 2003; Perry 2016). The epoxide is highly reactive and unstable and in an aqueous solution the compound can be converted into an acetaldehyde then ethanol, (SEE Figure 5). These steps are not novel and are accomplished frequently in industrial manufacturing processes, but under high temp and pressure conditions. Rhodococcus robust genomic plasmid and enzyme potential may have the ability to accomplish the same conversion process with a lower energy barrier requirement at significantly lower temperatures. Such a process may have significant industrial applications.

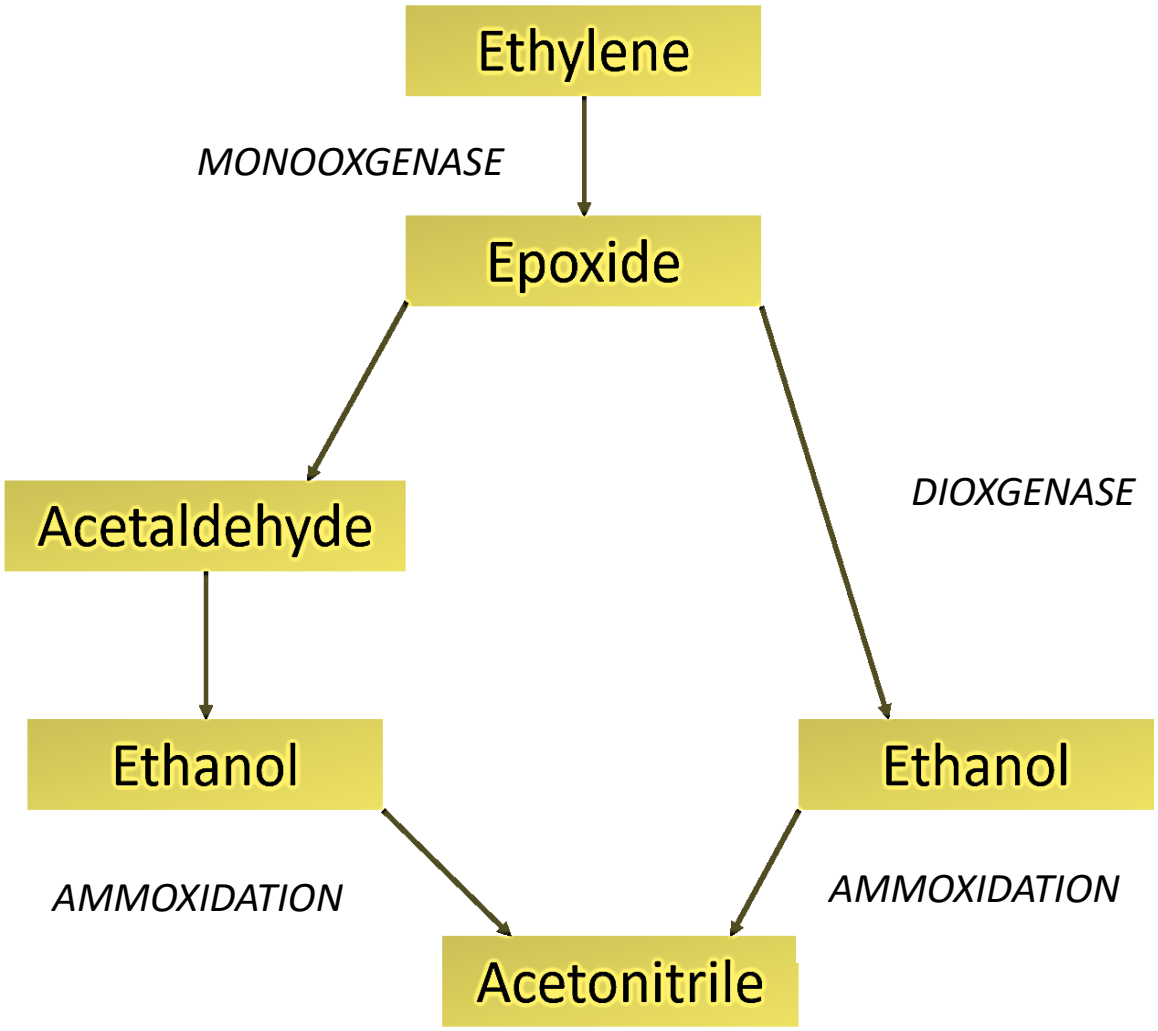


**Figure 5: Proposed Pathway:** Pathway is proposed based on GC Mass Spec data and enzyme assay results. To formulate a more conclusive proposed pathway additional data and findings are required.

## IV. CONCLUSION:

Rhodococcus and Bacillus maybe be a cost-effective means to produce aldehydes, ketones, and nitriles that control plant development and senescence. Preliminary findings from this work showed promising results. However, there is a need for additional studies to verify and quantify the total concentration of organically synthesized acetaldehyde and/or acetonitrile is required to inhibit ripening in climacteric fruits.

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