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Enhancing the Expression of Enzymes Used to Degrade Hydrocarbons and Cyanohydrins in *Rhodococcus* sp. DAP 96253 by Using Inducers such as Cobalt, Urea, and Propylene Gas; Also Enhances the Ability of the Bacteria to Delay the Ripening of Several Fruit Species

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ENHANCING THE EXPRESSION OF ENZYMES USED TO DEGRADE HYDROCARBONS AND CYANOHYDRINS IN *RHODOCOCCUS* *sp. DAP 96253* BY USING INDUCERS SUCH AS COBALT, UREA, AND PROPYLENE GAS; ALSO ENHANCES THE ABILITY OF THE BACTERIA TO DELAY THE RIPENING OF SEVERAL FRUIT SPECIES.

by

GUENEVERE DIANE PERRY

Under The Direction of George E. Pierce

ABSTRACT

Recent studies have shown that *R. rhodochrous DAP 96253* has the ability to delay the ripening of many climacteric fruit, by potentially degrading volatile compounds released by plant cells during the ripening process. *Rhodococcus rhodochrous DAP 96253* cells were cultured on YEMEA medium supplemented with inducers, (16mM cobalt and 125mM urea), that over-expressed nitrile hydratase (NHase) and amidase (AMDase) enzymes. Cells were cultured on propylene/ ethylene as sole carbon source to induce alkene monooxygenase (AMO) like activity. Induced *R. rhodochrous DAP 96253* cells displayed an 83% increase in final total dry weight compared to cells previously cultured on non-induced medium.

Induced *R. rhodochrous DAP 96253* cells displayed a 53-85% increase in NHase activity after exposure to propylene/ethylene, and cells displayed a 24-53% increase in NHase activity after exposure to fruit. Non-induced *R. rhodochrous DAP 96253* cells displayed a 1-5% increase in NHase activity after propylene/ethylene, and cells displayed an 18-38% increase in NHase

activity after exposure to fruit. Propylene/ethylene induced nitrilase activity in non-induced *R. rhodochrous* DAP 96253 cells.

Experimental results suggest that *R. rhodochrous* DAP 96253 may use NHase, amidase, nitrilase, and AMO like activity to delay ripening of climacteric fruit. *Rhodococcus rhodochrous* 96253 cells cultured on propylene/ethylene and cofactors (16mM cobalt and 125mM urea) displayed improved ability to delay ripening of fruit.

INDEX WORDS:

Rhodococcus rhodochrous DAP 96253, Nitrile Hydratase (NHase) (E.C.4.2.1.84), Amidase (E.C. 3.5.1.4), Cyanide Dihydratase (CDH) (E.C. 3.5.5.-), Nitrilase (E.C. 3.5.5.1), Alkene Monooxygenase (AMO), Ethylene gas, Propylene Gas

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GUENEVERE DIANE PERRY

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

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December 2011

DEDICATION

I would like to thank Dr. Pierce and Dr. Crow for being great mentors, and providing me with the tools I needed to complete my degree. I would like to thank Dr. Baumstark, you are not only an amazing scientist but I have really admired your dedication to your work and to the individuals that have worked for you. Working on the GSU Bio Bus program will always be some of my best memories of my journey here at Georgia State. Thank you for giving me a chance and hiring me and supporting me throughout my Master's program, and believing enough in me to recommend me for my doctorate. I will always be greatly indebted, thank you. I would like to thank Dr. Gilbert, for giving great advice to me in regards to being a better student but also as a growing scientist. I would also like to thank all of my lab mates, it has been a memorable experience and I have really learned a lot from working with everyone.

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LIST OF ABBREVIATIONS:

Acry	Acrylonitrile
AMO.....	Alkene Monooxygenase
Benzo.....	Benzonitrile
CDW.....	Cell Dry Weight
CH.....	Cyanide Hydratase
CDH.....	Cyanide Dihydratase
EC.....	Epoxide Carboxylase
MW.....	Molecular Weight
NHase.....	Nitrile Hydratase
YEMEA.....	Yeast Extract Malt Extract Media

INTRODUCTION

Postharvest Loss and Fruit Ripening:

An estimated \$750 million of harvested fruits and vegetables is lost each year due to the deterioration, increased respiration, ethylene production, mechanical injuries, water stress, sprouting, physiological disorders, and pathological breakdown. In general, this is referred to as (post-harvest loss; Kader 2005). The U.S.D.A. Economic Research Service suggests that in developed countries 12% of produce is lost between harvest and the final retail consumer site. The report further states that 19.6% of this loss can be attributed to spoilage (caused by microbial growth) and 15% to effects linked to ripening (Grolleaud 2002; Kader 2005; Barth *et al.*, 2010). Prevention/ reduction of post-harvest loss is of great concern. Deteriorated produce has reduced nutrition and many Americans are developing a desire for a healthier diet; a trend that has led to a 32.6% increase in fresh vegetable consumption and a 25.8% increase in fresh fruit consumption. It has been predicted that this trend will continue and the numbers of people preferring fresh produce to processed fruits and vegetables will continue to grow (Grolleaud 2002; Kader 2005). Reducing post-harvest losses would eliminate losses attributed to man power, fertilization, pesticides, and refrigeration cost spent on crops that were loss on transport to consumer sites.

The ripening of fruit, due to ethylene production, is one of the major contributors to post-harvest losses. Plant cells produce ethylene gas that results in physiological and physical changes in the fruit, these changes can cause the fruit to become more susceptible to bruising and shrinkage during handling or transportation to consumer sites (Grolleaud 2002; Burg 2004).

Physiological Effects of Fruit Ripening:

Fruit ripening, in climacteric fruit, occurs when ethylene gas binds to ETR complexes (ethylene receptors) found on the surface of the fruit cells. This binding causes a cascade signal that initiates and accelerates the ripening process (Blankenship 2001; Morretti *et al.*, 2002; Burg 2004; Reed 2011). Ethylene binds to truncated ETR1 proteins embedded in the surface membranes of plant cells; the number of ethylene receptors found on the surface of a plant cell is influenced by abiotic and biotic signals linked to climate changes, levels of atmospheric oxygen, or the presence of microbes (Rasori *et al.*, 2002; Trobacher 2009). The binding of ethylene molecules to ETR1 proteins causes a kinase reaction that prevents the degradation of elongated internode (*EIN*) genes. The expression of *EIN* genes causes an immediate increase in cellular respiration, and utilization of soluble sugars during glycolysis (such as glucose formed from the conversion of sucrose to glucose and fructose). The transcription and expression of *EIN* is regulated by CTR1. CTR1 negatively regulates the signal pathway that initiates fruit ripening. Mutation of the CTR1 gene allows for the over-expression of ethylene gas, (Figure 1) (Theologis 2004; Trobacher 2009).

Fruit ripening causes physical changes that include a decrease in the fruit's firmness, change in texture, darkening of the exterior hue, and an overall decrease in the fruit's mass. Biochemical changes occur which affect the ratio of sugars found in fruit tissues, resulting in an increase in fructose levels (Elsgard *et al.*, 1998; Blankenship 2001; Morretti *et al.*, 2002; Basher 2003).

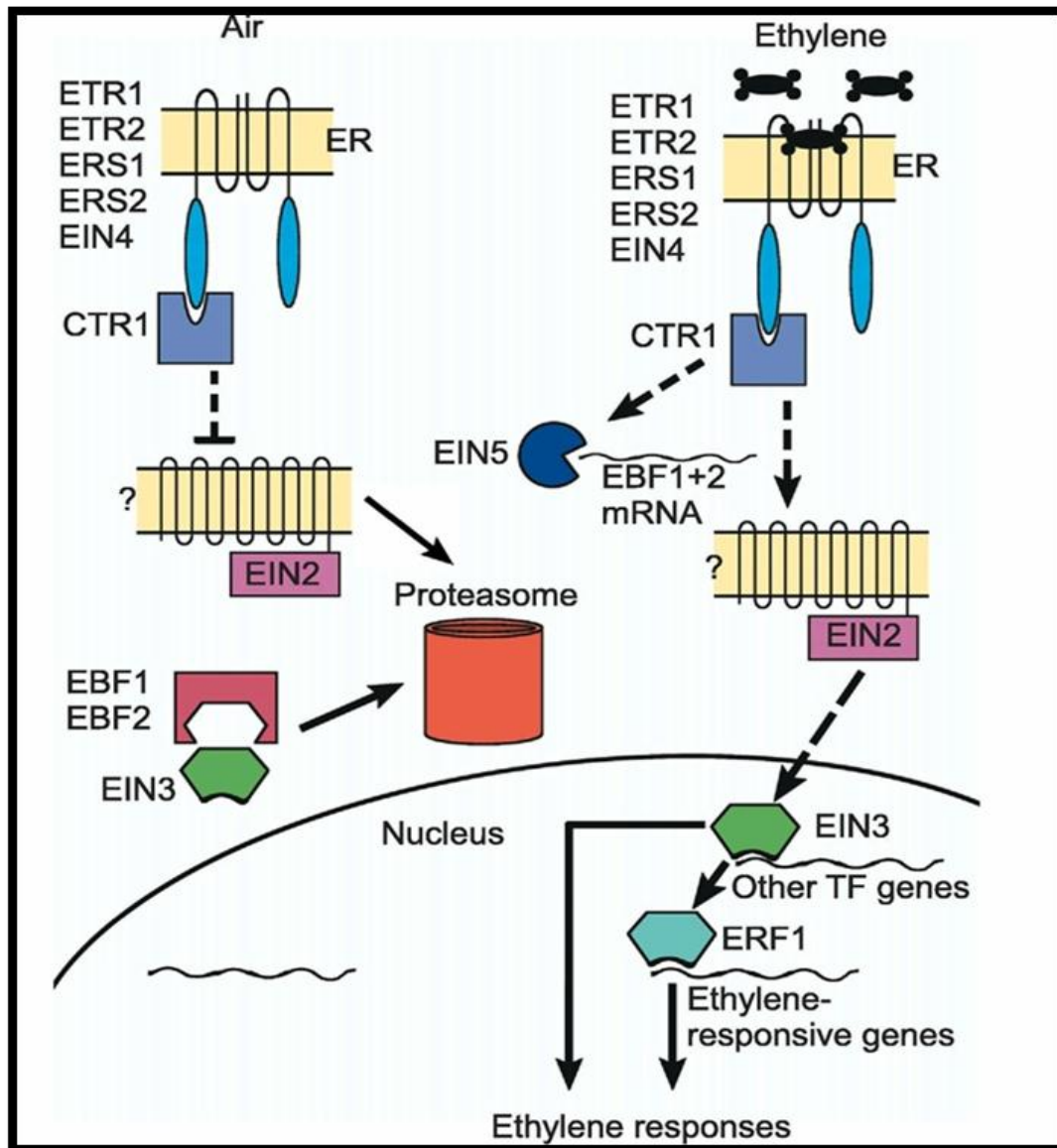


Figure1: Ethylene Response Pathway: Proposed pathway for signal transduction and gene regulation in an *Arabidopsis sp.* (mustard) plant, initiated by the docking of an ethylene gas molecule on an ETR protein complex on the plant cell surface. (Trobacher 2009)

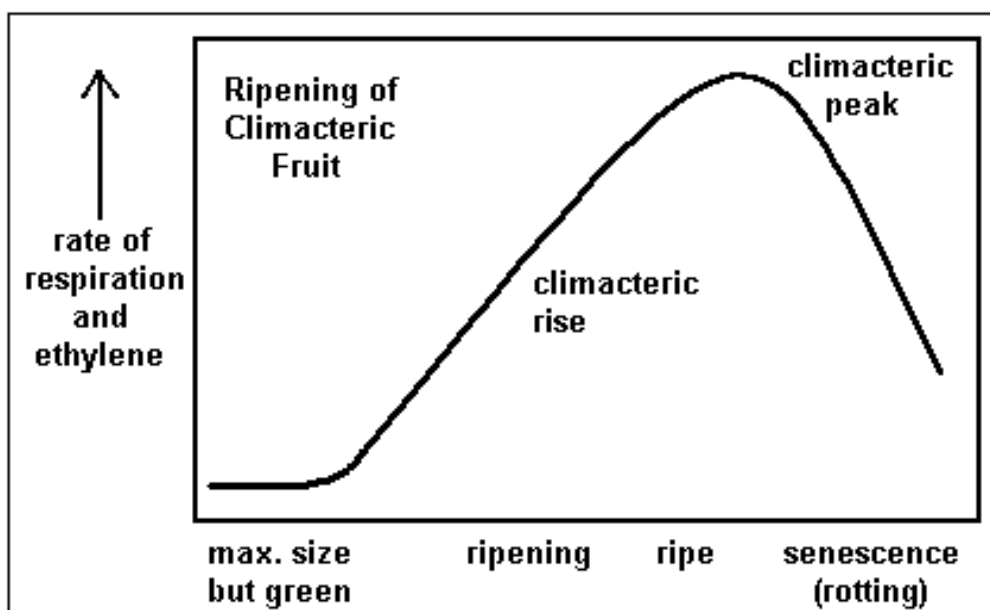


Figure 2: Cellular respiration response to ripening by climacteric fruit. The progression of ripening in climacteric fruit and the effect on fruit ripening on the plant cells rate of cellular respiration. (Reed 2011)

The increase in fructose concentration causes an increase in the sweetness of the fruit and the formation of a syrup-like substance found in fruits such as peaches, mangos, and watermelon (Sisler 2001; Bashir *et al.*, 2003; Burg 2004). Metabolic activity decreases as the cell reaches a rotting (or senescence) stage of development, (Figure 2) (Blankenship 2001; Morretti *et al.*, 2002; Burg 2004; Reed 2011).

Current Methods to Delay Fruit Ripening

Scrubbers, filters, and chemicals such as aminoethoxyvinylglycine hydrochloride (Aviglycine[®]) or 1-methylcyclopropene (1-MCP) are currently used by whole sale growers also use MCP to delay fruit ripening and decrease post-harvest losses (Elsgard *et al.*, 1998; Blankenship 2001). 1-MCP acts as an analog of ethylene, irreversibly inhibiting the binding of ethylene to ETR complex, while Aviglycine[®] temporarily prevents the biosynthesis of ethylene (Blankenship 2001; Sisler *et al.*, 2001; Morretti *et al.*, 2002; Singh *et al.*, 2008).

Recent research has shown that induced cells of *Rhodococcus rhodochrous* DAP 96253 can delay the ripening of the fruit. This can be applied to fruit transportation to reduce post-harvest loss (Pierce *et al.* 2008; Pierce *et al.*, 2011). *Rhodococcus rhodochrous* DAP 96253 may be a cost effective approach which represents an all-natural alternative to preserving fruit without chemically altering the fruit.

Understanding How Microbes Affect Plant Physiology and Ethylene Production

Plant cells produce ethylene gas by converting methionine to S-adenosylmethionine (SAM). SAM is converted to 1-aminocyclopropane-1-carboxylate (ACC) via ACC synthase, and ACC is converted to ethylene via the ACC oxidase, (Figure 3) (Chen *et al.*, 1991; Yang *et al.*,

1998; Karthinkeyan *et al.*, 2004; Arshad *et al.*, 2007). Many bacterial species present in the soil, including *Pseudomonas*, *Achromobacter*, and *Rhodococcus* species use ACC deaminase to degrade ACC into ammonium and α -ketobutyrate. The degradation of ACC halts ethylene production and prevents the binding of ethylene to cell receptors (Sheehy 1991; Hontezas *et al.*, 2005; Govindasamy *et al.*, 2008).

DeBont *et al.*, (1974) initially observed ethylene degradation during a study that exposed soil samples to gaseous hydrocarbons; the soil samples displayed an accumulation of nitrogen. It was proposed that the soil bacteria were degrading methane or ethylene gas and using the by-products of cellular respiration for nitrogen fixation (Kranter *et al.* 1982; Ginkel *et al.* 1987). Recent literature has shown that ethylene degradation by bacteria present in the soil can affect developmental changes experienced by climacteric plants such as the plants ability to germinate, to elongate its roots, and to ripen (Kranter 1997; Elsgard *et al.*, 1998; Elsgard 2000; Hontezas *et al.*, 2005; Arshad *et al.*, 2007; Govindasamy *et al.*, 2008). Many soil microbes, such as *Rhodococcus rhodochrous* and *Xanthobacter* strain Py2, can degrade ethylene or cyanide directly (Hartsman *et al.*, 1991; Kranter 1997; Small *et al.*, 1997; Hontezas *et al.*, 2005).

Elsgard (2000) isolated and propagated a mixed culture of cells capable of degrading ethylene. The mixed culture was placed into a closed system; 117 ppm of an ethylene in air was constantly flushed through the system. The cells were able to degrade over 90% of the ethylene in a 24 to 72 hour period. The mixed culture was able to degrade more ethylene gas from the system the longer the cells were exposed to the ethylene in air, the culture also performed better when the cells were initially exposed to a low concentration of ethylene (i.e. 20 ppm) then gradually exposed to higher concentrations of ethylene in air (i.e. 100 ppm) (Elsgard *et al.*, 1998;

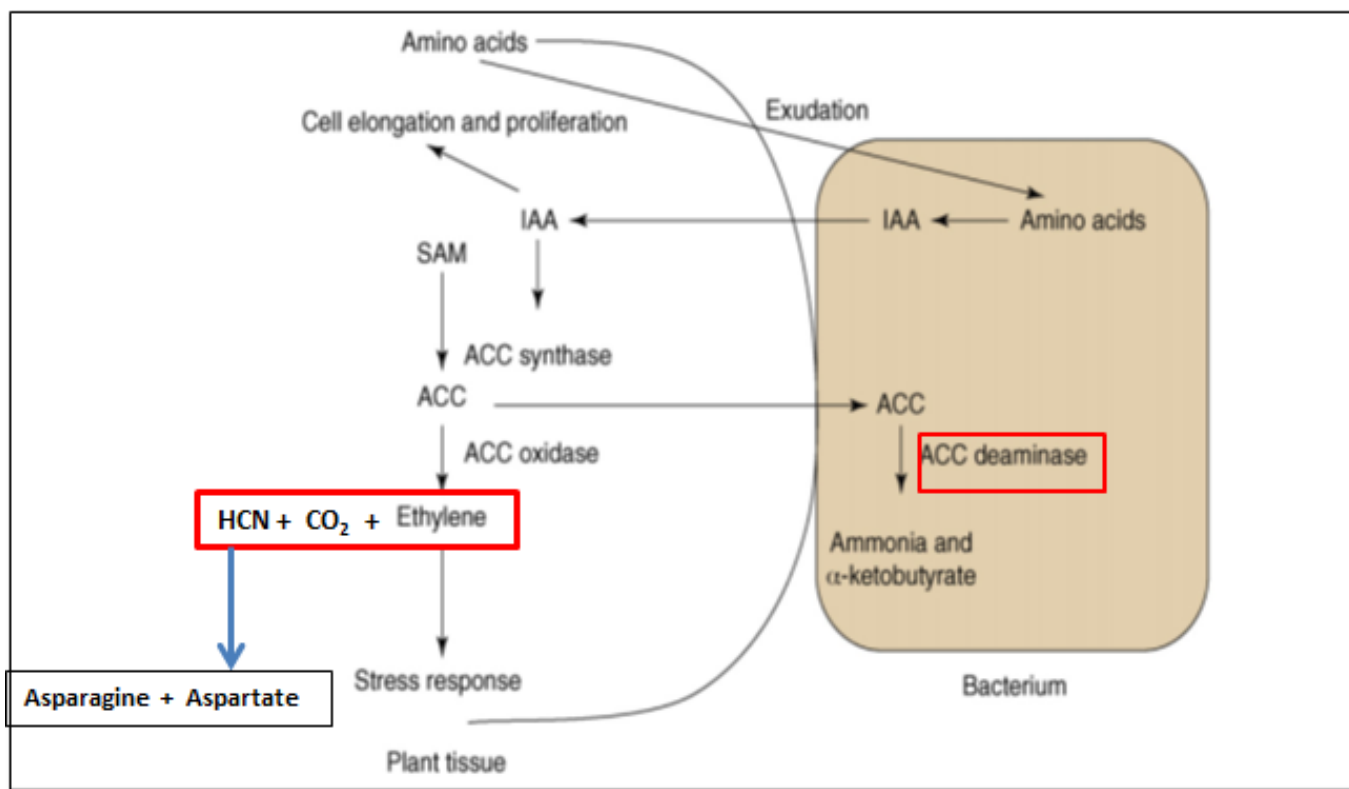


Figure 3: Plant bacterial interactions involved in ethylene production and degradation.
(Yang *et al.*, 1998; Arshad *et al.* 2007)

Elsgard 2000). Recent literature suggests that alkene monooxygenase (AMO) and epoxide carboxylase (EC) may be the enzymes involved in ethylene degradation (Ensign *et al.*, 2003).

Rhodococcus rhodochrous DAP 96253 the microorganism and delayed fruit ripening

In very recent studies it has been shown that when *Rhodococcus rhodochrous* DAP 96253 cells were cultured on YEMEA medium supplemented with inducers (16mM cobalt and 125mM urea), the cells displayed the ability to delay the ripening of a variety of climacteric fruits and vegetables (Pierce *et al.* 2008; Pierce *et al.*, 2011). The cells were cultured on YEMEA induced medium containing glucose or trehalose as a primary carbon to overexpress NHase and amidase enzymes, and to facilitate the stabilization of the NHase enzyme (Pierce *et al.*, 2008; Pogorelova *et al.*, 1996).

Understanding *R. rhodochrous* DAP 96253

Members of the genus Rhodococcus are Gram-positive, aerobic soil bacteria that belong to the family *Nocardiaceae*. The rhodococci classified as Actinobacteria, the cell morphology and protein expression may depend on the cells developmental stage of growth (Goodfellow *et al.*, 1977). The genus *Rhodococcus* includes over thirty recognized species, with two pathogenic species *Rhodococcus equi* and *Rhodococcus fascians* (Tsukamura *et al.*, 1978; Gürtler *et al.*, 2004). Considerable research has shown that enzymes harvested from *Rhodococcus rhodochrous* species can be used for the bioconversion or biodegradation of a wide range of toxic pollutants and waste chemicals, including aliphatic and aromatic hydrocarbons (Saeki *et al.*, 1999). It has been proposed that the genes required to express the nitrile degrading enzymes may account for 10% of the total genome of *Rhodococcus rhodochrous* cells (Sunairi *et al.*, 1993; Saeki *et al.*,

1999). *Rhodococcus rhodochrous* DAP 96253 when induced with cobalt and urea has the ability to delay the ripening of fruit, this may be due to organism's metabolic versatility, containing a variety of hydrolyzing enzymes that can degrade volatile compounds such as ethylene or cyanide gas released by plant cells during the ripening process.

Potential Enzymes Involved in Fruit Ripening

Previous studies demonstrated that *R. rhodochrous* DAP 96253 cells cultured on YEMEA supplemented with cobalt and urea (Induced Medium) showed increased levels of NHase, amidase, nitrilase, and cyanidase activity. These rhodococcal cells were also capable of delaying fruit ripening; however the pathway or enzymes used by the cells to delay the ripening were unknown. Research conducted for this dissertation shows that *R. rhodochrous* DAP 96253 cells exposed to fruit resulted in increased expression of NHase, amidase, and nitrilase activity, hence these enzymes will be discussed further.

Nitrile Hydratase

Nitrile hydratase (NHase) is a multi-meric enzyme, that is composed of an α and β subunit. NHase contains three important cysteine sulfur residues, two of the cysteine residues must be oxidized to sulfinic and sulfenic acid during post-transcriptional modifications (Pogorelova *et al.*, 1996; Nojiri *et al.*, 2000). One of the oxidized cysteine residues removes a proton from a nucleophilic water molecule to attack the carbon nitrile forming an amide shown in Figure 4 (Kobayashi *et al.*, 1998; Nojiri *et al.*, 2000; Hopmann *et al.*, 2006). NHase hydrolyzes aromatic or aliphatic nitrile compounds to their respective amide, and then amidase converts the amide into its respective acid and ammonia (Kobayashi *et al.*, 1998).

NHase genes have been found in many soil bacterium species including: *Pseudomonas*, *Brevibacterium*, and *Rhodococcus* (Kobayashi *et al.*, 1991). *Rhodococcus JI* strain has two forms of the NHase, a high (**H**-NHase) and low (**L**-NHase) molecular mass form. The **H**-NHase form prefers aliphatic substrates, while the **L**-NHase prefers aromatic substrates. Heavy and low mass NHase can be induced using different inducers during cell cultivation, the **H**-NHase can be induced with an aliphatic compound (such as acrylonitrile) while **L**-NHase would need to be cultivated on an aromatic nitrile (Nagasawa *et al.*, 1991; Masaki *et al.*, 2000; Miyanaga *et al.*, 2003). NHase can contain either an iron or cobalt metal in the catalytic center. Recent literature suggest the metal ion acts as a electrostatic stabilizer for the intermediate form of the substrate, lowering the reaction barrier needed for the complete conversion process to occur (Nagasawa *et al.*, 1991; Hopmann *et al.*, 2006). Expression of NHase protein in cells can be observed by identifying the expression of the NHase α and β subunits on a SDS gel. In *Rhodococcus JI* NHase contains an α and β subunit that is encoded as *nhhA* and *nhhB* genes (for **H**-NHase) and *nhlA* and *nhlB* genes (for **L**-NHase) (Kobayashi *et al.*, 1991). The subunits have a molecular mass range of 22-27kDa on a standard SDS gel (α -23kDa and β -27kDa in *R. rhodochrous DAP 96253*) (Masaki *et al.*, 2000; Miyanaga *et al.*, 2003; Ganguluy *et al.*, 2005).

NHase activity was previously induced in *R. rhodochrous DAP (96253 and 96622)* by culturing cells in the presence of toxic substances such as acrylonitrile, acrylamide, acrylonitrile, cyanoglycosides, or methacrylamide. Varying concentrations of cobalt and urea can also act as inducers for NHase (Nagasawa *et al.*, 1991; Masaki *et al.*, 2000; Miyanaga *et al.*, 2003).

Amidase

Amidase cleaves aliphatic amides by removing a proton from a nucleophilic water molecule to attack the amide group producing a carboxylic acid and a free ammonia molecule (Figure 4) (Kotlova *et al.*, 1999). Amidase can belong to four different families; the nitrile/ cyanide hydratase, acyl transferases, ureases, or GGSS motif families (Kotlova *et al.*, 1999; Pertsovich *et al.*, 2005). *Rhodococcus rhodochrous* is proposed to contain an amidase that belongs to the nitrile/ cyanide hydratase family, and converts aliphatic amides and not aromatic amides (Pertsovich *et al.*, 2005). Amidase belong to a family that contains a catalytic triad residue that is also seen in nitrilase (Pace *et al.*, 2009). The function, molecular weight, and structure of amidase are dictated by the enzymes sub-family. Amidase has been purified from many soil microbes including *Rhodococcus*, *Pseudomonas*, *Sulfolobus*, and *Brevibacterium*. Amidase purified from *Rhodococcus rhodochrous* strains display a molecular weight range of 40-60kDa on a SDS gel (Hirrlinger 1996; Kotlova *et al.*, 1999; Pertsovich *et al.*, 2005). Amidase purified from *Rhodococcus rhodochrous* DAP 96253 subunits display a molecular mass range of 40kDa \pm 2kDa (Ganguly *et al.*, 2005).

Nitrilase

Nitrilase is an enantiomer-selective enzyme that hydrolyzes aromatic and aliphatic nitrile compounds. Nitrilase contains a thiol group, two cysteine residues, and a disulfide bridge (Kobayashi *et al.*, 1990; Kobayashi *et al.*, 1992). The thiol group adds two nucleophilic water molecules that donate two protons used to attack the nitrile group shown in Figure 4 (Stevenson *et al.*, 1992; Gerasimova *et al.*, 2004; Gupta *et al.*, 2010).

Nitrilase is a sulfhydryl enzyme similar to aliphatic amidases. The nitrilase protein is encoded on the *nitA* gene (Komeda *et al.*, 1996). Expression of the nitrilase protein can be identified on a SDS gel with subunits that have a molecular mass range of 40±5 kDa (Kobayashi *et al.*, 1990; Kobayashi *et al.*, 1992; O'Reilly *et al.*, 2003).

Some literature suggests that nitrilase and amidase are transcribed from the same genes, posttranslational modifications of the translated protein forms either amidase or nitrilase. Other literature suggests that nitrilase is encoded by a separate *Nit* gene that is inducible by aromatic compounds and requires posttranslational modifications at residue 327 in order to become active (Kobayashi *et al.*, 1990; Kobayashi *et al.*, 1992; O'Reilly *et al.*, 2003; Thuku *et al.*, 2007) The active form of the amidase forms a dimer, but the active form of nitrilase form six dimmers composed of the same polypeptide sequence (Thuku *et al.*, 2007). The protein is pH and temperature sensitive, optimum pH range is 6.0-9.0 and optimum temperature range is 30-55°C (Pace *et al.*, 2001; O'Reilly *et al.*, 2003).

The Nit enzyme can be found in many rhizosphere bacteria and fungi such as *Rhodococcus*, *Pseudomonas*, *Brevibacterium*, *Fusarium*, and *Aspergillus* species (Koboyashi *et al.*, 1990; Martinkova *et al.*, 2009). Studies have demonstrated that nitrilase activity can be induced by culturing *R. rhodochrous* cells with aromatic nitrile compounds like benzonitrile (Kobayashi *et al.*, 1992).

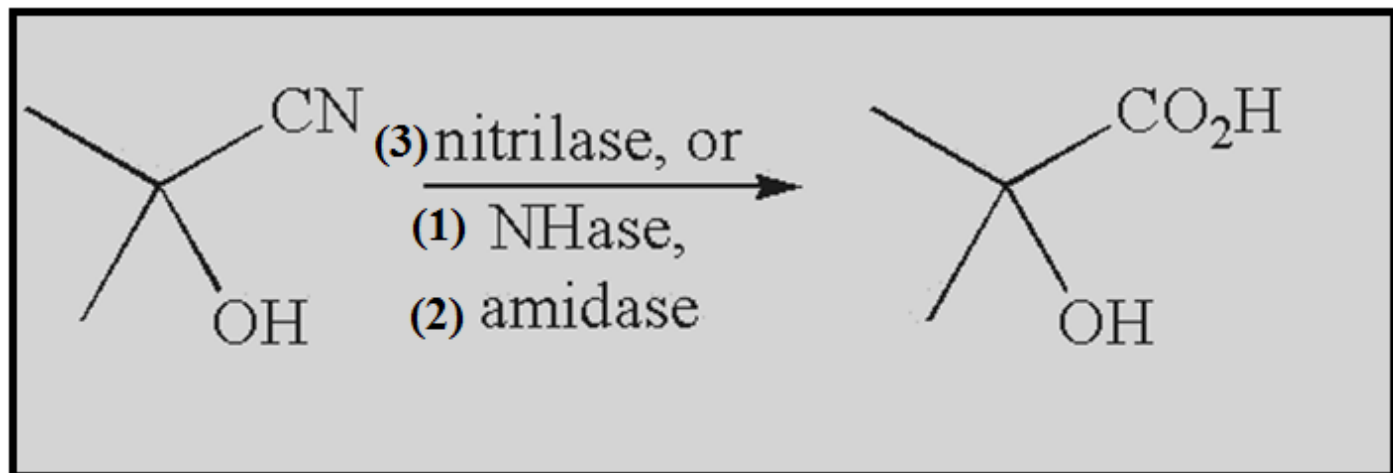


Figure 4: Catalytic conversion of a nitrile compound.

Enzymes (1) & (2): Nitrile Hydratase and amidase enzymes converting an aliphatic nitrile to an acid (Kobayashi *et al.*, 1998; Kotlova *et al.*, 1998).

Enzyme (3): Nitrilase adds two water molecules to produce an acid and ammonia molecule (Gerasimova *et al.*, 2004).

Cyanide Dihydratase

Cyanide dihydratase (CDH) is a nitrile hydrolyzing enzyme that converts cyanide compounds to formic acid and ammonia and is shown in Figure 5 (O'Reilly *et al.*, 2003; Gupta *et al.*, 2010). CDH (cyanidase or cyanide nitrilase) does not form an amide by-product and is therefore not a member of the nitrilase/ cyanide hydratase super-family. The enzyme is found in many soil microbes because plants release hydrogen cyanide, cyanide inhibits cytochrome C function during cellular respiration, leading to cell death. CDH helps the microbes convert cyanide to formic acid and ammonium that can be transported into other pathways to form other less toxic compounds or amino acids (Jandhyala *et al.*, 2003; Gupta *et al.*, 2010).

CDH protein in *B. pumilus* C1 is encoded on the *cynD* gene (Jandhyala *et al.*, 2003). The protein has no known metal cofactors, but displays an increase in activity when placed in the presence of the heavy metals Sc^{3+} , Cr^{3+} , Fe^{3+} , and Th^{3+} , independent of metal concentrations (Jandhyala *et al.*, 2003; Gupta *et al.*, 2010). Purified CDH has a molecular mass range of 40 ± 6 kDa on a SDS gel (O'Reilly *et al.* 2003; Gupta *et al.*, 2010). CDH is pH sensitive and has an optimum pH range of 6-10 (Gupta *et al.*, 2010). Recent literature suggests that CDH is not inducible, *Pseudomonas stutzeri* cells were cultured in the presence of cyanide and in the absence of cyanide. Cultures displayed similar enzymatic efficiency at degrading the cyanide substrate (Gupta *et al.*, 2010).

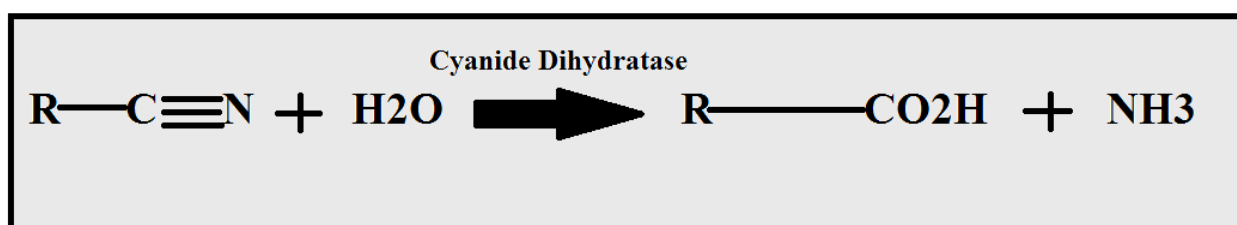


Figure 5: Cyanide Dihydratase converts a nitrile compound. CDH adds one water molecule to produce formic acid and an ammonia molecule. (Gupta et al., 2010)

Potential Involvement of an Oxygenase

Alkene monooxygenase (AMO) is a 3-component di-nuclear iron protein that adds O₂, NADH, and H⁺ into the reaction to convert propylene/ethylene into a chiral epoxide structure (Ensign 1995; Ensign *et al.*, 2003). Component I is composed of an epoxygenase that attacks the alkene double bond and inserts a free oxygen molecule; component II is NADH dependent reductase (NDR) that removes a proton from NADH to attach to the remaining oxygen molecule forming water and NAD⁺ (Figure 6). Component III is a coupling protein (CP), the function is unknown but the protein is essential for substrate conversion (Ensign 1995; Small *et al.*, 1997; Gallagher *et al.*, 1997). AMO is encoded by a four gene operon *amoABCD* and has a molecular mass range of 14-53kDa (epoxygenase- 35 & 53kDa; NDR- 40kDa; CP- 14kDa) (Gallagher *et al.*, 1992; Smith *et al.*, 1999; Perry *et al.*, 2006).

Rhodococcus rhodochrous DAP 96253 cells cultured on YEMEA medium (supplemented with inducers cobalt and urea) displayed an increased ability to degrade propylene/ ethylene gas. This increased ability to metabolize propylene/ethylene as a sole carbon source suggests an increased expression or increased enzymatic activity of alkene monooxygenase (AMO) enzymes, the cells also had increased delayed fruit ripening capabilities.

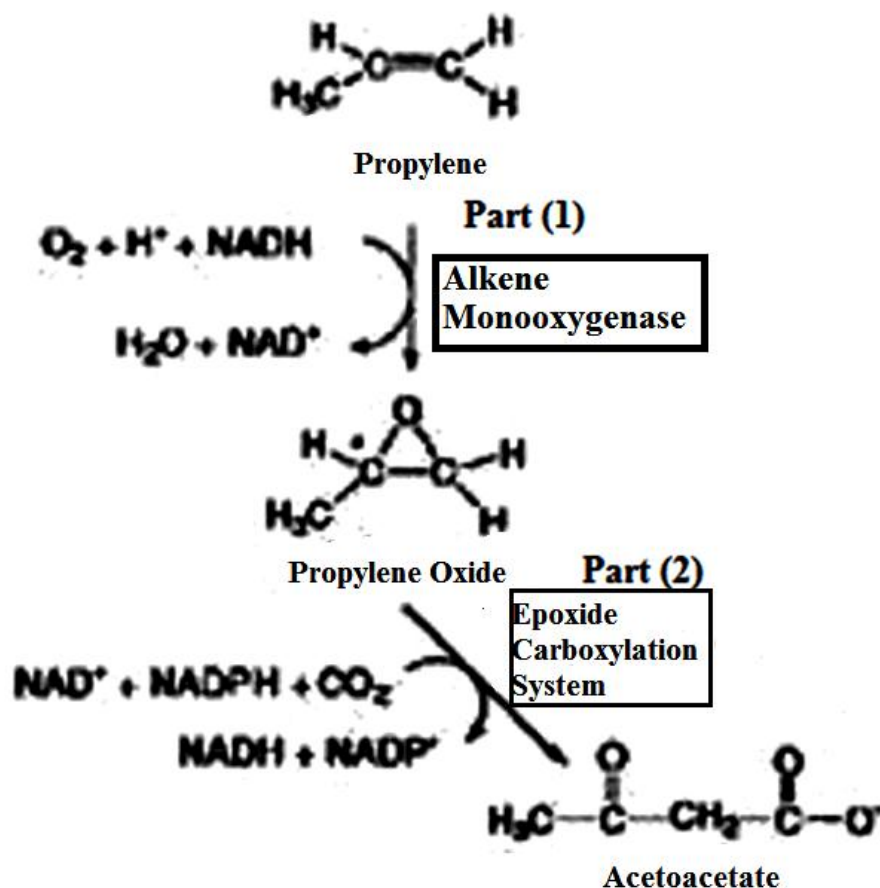


Figure 6: Propylene/ Ethylene conversion pathway.

Pathway (1): AMO enzyme converts propylene gas to propylene epoxide using NADH as a proton donor (Ensign 1995; Small *et al.*, 1997). Enzymes 3 components are used to convert propylene to propylene epoxide. Component I uses O_2 , component II uses NADH, and component III function is unknown.

Pathway (2): Carboxylation pathway converting propylene epoxide to acetoacetate. Pathway is dependent on a disulfide reductase, a zinc dependent alkytransferase, and two different dehydrogenase enzymes (Ensign *et al.*, 2003).

Hypothesis:

A post-harvest loss of produce is a major concern for many farmers and produce distributors.

Rhodococcus rhodochromus DAP 96253 has been shown to delay fruit ripening, when cells were cultured on YEMEA media used to induce NHase and amidase activity. There was a need to improve induced *R. rhodochromus* DAP 96253 cells ability to improve cells efficiency and improve cells ability to consistently delay ripening of climacteric fruit.

I hypothesized that if *R. rhodochromus* DAP 96253 cells were cultured on propylene/ethylene gas the cells would over express the AMO and EC enzymes. The over-expression of AMO would improve the cells ability to degrade ethylene released by plants, preventing the ethylene from re-binding to ethylene receptors found on the plant cell. This would prevent the fruit from initiating further ethylene production and delay fruit ripening process. The over expression of AMO would also improve cells ability to consistently delay fruit ripening. The objectives for this dissertation work are listed below.

Objectives

The objectives of this dissertation work were as follows;

- (1) Determine if *R. rhodochromus* DAP 96253 displayed growth on propylene/ ethylene gas as sole carbon source. (The presence of an AMO like activity provides a potential mechanism to degrade ethylene, potentially providing the cells the ability to delay ripening of climacteric fruits).

- (2) Determine if the use of propylene/ ethylene gas could induce over-expression of a AMO like enzyme and increased AMO like activity in *R. rhodochrous DAP 96253*. Also to identify enzymes other than AMO that displayed increased expression or enzymatic activity when cultured on propylene/ ethylene gas as sole carbon source.
- (3) Determine if YEMEA medium supplemented with cobalt and urea as inducers were also capable of inducing AMO expression and increased AMO activity in *R.rhodochrous DAP 96253*.
- (4) Identify enzymes that showed increased expression or increased enzymatic activity when exposed to ripening fruit.
- (5) To determine if increased NHase expression in *R. rhodochrous DAP 96253* gives cells the ability to deal with HCN released by plant cells, potentially increasing cells ability to delay fruit ripening.
- (6) Determine if over-expression of potential target enzymes would result in increased delayed ripening capabilities for *Rhodococcus rhodochrous DAP 96253*, this would result in reduced post-harvest loss and increased efficiency in produce storage.

MATERIALS AND METHODS:

Microorganism

Rhodococcus rhodochrous DAP 96253 (ATCC 55899) was obtained from the American Type Culture Collection (ATCC) located in Vienna, VA. The bacterial strains were stored in a 30% glycerol stock solution, at -80°C. A 1ml aliquot of (30% glycerol stock of *R. rhodochrous* DAP96253) cells were transferred to 100ml of Difco® Nutrient Broth (Becton, Dickinson, & Co.; Sparks, MD) in a 250ml flask and grown for three days at 30°C with shaking at 120 rpm. Cells were transferred to Difco® Nutrient Agar (Becton, Dickinson, & Co.; Sparks, MD) for an additional three days, cells from NA plates were used to inoculate Yemea Extract Malt Extract (YEMEA) media supplemented with or without inducers (Dietz *et al.*, 1980; Pierce *et al.*, 2009).

Table 1: YEMEA Medium

(Media Components (g/L))						
Media Type	CoCl ₂	Urea	Bacto Agar	Glucose	Malt Extract	Yeast Extract
INDUCED	0.201	7.5	20	4	10	4
NON INDUCED	-	-	20	4	10	4

YNI- YEMEA Medium Non-Induced

YI- YEMEA Medium Supplemented with Inducers, (Dietz *et al.*, 1980)

Chemical Materials

Ethylene in air (100ppm) (Air Gas Co.; Mableton, GA); Propylene gas (99.0% minimum) (Air Gas Co.; Mableton, GA); Acrylonitrile (99.0% minimum) (Sigma Aldrich; St. Louis, MO.) ; 1-

Aminocyclopropane-1-Carboxylate (ACC) (Sigma Aldrich; St. Louis, MO.); Benzonitrile (Sigma Aldrich; St. Louis, MO.); Potassium Cyanide (JT Baker; Phillipsburg NJ); Sodium Phenate (Sigma Aldrich; St. Louis, MO.); Sodium Nitroprusside (Sigma Aldrich; St. Louis, MO.); Proto Gel (30% Acrylamide/ 0.85 bisarylamide) (National Diagnostics; Atlanta, GA.); Tris (hydroxymethyl) aminomethane (Tris) (GE© Healthcare; Amersham, UK); Sodium Dodecyl Sulfate (SDS) (GE© Healthcare; Amersham, UK); Glycerin (JT Baker; Phillipsburg NJ); Ammonium Persulfate (Fisher Scientific; Bridgewater, NJ); Tetramethylethylenediamine (TEMED) (GE© Healthcare; Amersham, UK); Dithiothreitol (DTT) (GE© Healthcare; Amersham, UK); Triza Base (Sigma-Aldrich; St. Louis, MO).

NHase/ Amidase Induction

R. rhodochrous DAP 96253 cells were cultured on YEMEA induction medium supplemented with inducers cobalt and urea shown in Table 1. The cells were cultured on YEMEA induced plates for 6-7 days at 30°C, the cells were harvested and assayed for NHase and other enzyme activity according to standard assay protocol. Samples that were not used immediately were temporarily stored at -20 °C.

Rhodococcus rhodochrous DAP 96253 Cells Cultured on Propylene/Ethylene:

R. rhodochrous DAP 96253 cells grown on YEMEA induced and YEMEA non-induced plates were scraped from YEMEA plates and suspended in 15ml of (1X) PBS buffer (0.8% NaCl, 0.02% KCl, 0.02M PO₄, and pH 7.2), (DeAngelis, 2007). The cell suspension was used to

inoculate a 1L biphasic flask (the mini-flask contained a solid agar phase and liquid minimal salts media phase); each run was for 3-6 days at 30°C with shaking at 120 rpm.

1L Biphasic Flask

Biphasic flask (Figure 7) contained 300ml agar (300ml dH₂O and 14g Bacto Agar, Difco©), 300ml of minimal salts media (preparation protocol shown in appendix), and propylene/ ethylene gas (15-20% available headspace) (Shadowen *et al.*, 1989). The mini flask consisted of a rubber stopper and a small reservoir (used to help control pressure build up by collecting overflow gases or media). The flask's tubing was capped with mini valves to allow insertion of gases or media into the closed gas system.

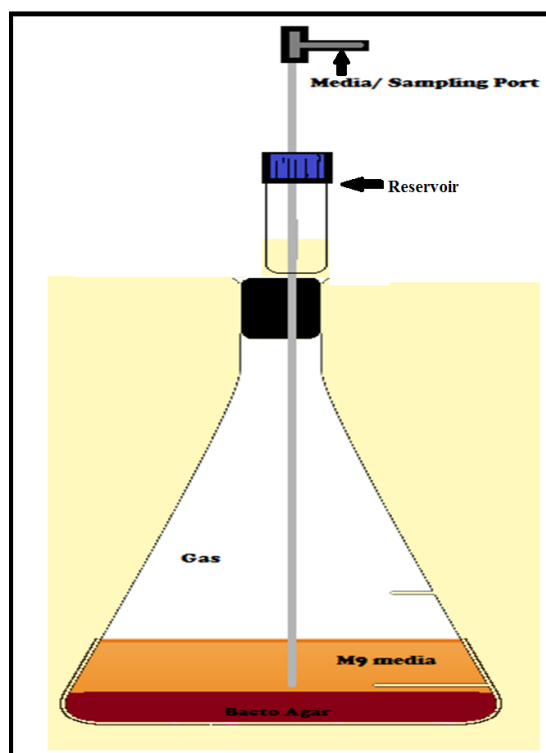


Figure 7: Propylene/Ethylene 1L Biphasic Flask.

Delayed Fruit Ripening

Rhodococcus rhodochrous DAP 96253 cells were cultured on YEMEA induced (YI) and YEMEA non-induced (YNI) plates for 6-7 days at 30°C, the cells were scraped into 10ml of 1X PBS buffer then transferred to a biphasic flask, the cells were grown in the flask on propylene/ethylene gas for 3-6 days. *Rhodococcus rhodochrous* DAP 96253 cells were collected into 50ml centrifuge tubes and centrifuged for 10 min at 13,000rpm (Beckman Centrifuge; Brea, CA). The supernatant was decanted and sample pellets were transferred to pre-weighed micro-centrifuge tubes for enzymatic assays. Sample pellets were re-suspended into 30ml of M9 minimal salts medium, followed by vortexing for 40-60 sec to ensure complete cell re-suspension and then poured into empty Petri plates. The Petri plates containing rhodococcal cells were placed into containers (brown paper bags or sealed plastic bins) containing organic fruits (apples, bananas, and peaches). The containers were sealed and left on a bench top at room temperature for 4-23 days. To harvest *R. rhodochrous* DAP 96253 cells after exposure to propylene/ethylene, cells were pipetted into 50ml centrifuge tubes and centrifuged for 10 min at 13,000 rpm (Beckman Centrifuge; Brea, CA). The supernatant was decanted and enzyme assays were conducted on sample pellets. Samples that were not used immediately were collected into cryogenic vials and stored at -80C. Containers were opened periodically during run to take images of the fruit and to collect cell samples. Images of fruits were captured using a digital camera (Sony “Cyber Shot”, 7.2 mega pixels, automatic flash).

NHase Assay:

NHase activity was quantified using 1000 ppm of a acrylonitrile solution as substrate. Individual reaction mixtures were made by pipetting 9ml of 1000 ppm acrylonitrile standard into 15ml centrifuge tubes. *R. rhodochrous* DAP 96253 samples were prepared by scraping 50mg of cells into a 1.5ml eppendorf tube that contained 1ml of (1X) PBS buffer (pH 7.2). The sample suspension was vortexed for 30-60sec to ensure complete cell re-suspension. The cell suspension was added to the 9ml reaction mixture and gently shaken for 2min. Duplicate samples of each reaction mixture were transferred to clean 1.5ml eppendorf tubes. The samples were centrifuged for 2min at 13,00rpm (Eppendorf (Fisher); Westbury, NY) and the supernatant pipetted into clean 1.5ml eppendorf tubes, 10 μ l of a working Amidase solution (0.25units/ μ l; 1:50 dilution; Sigma Aldrich; St. Louis, MO) was added to supernatant to ensure complete conversion of amide by-product into ammonia and acid. Samples were vortexed for 30sec and placed into a 37°C incubator for 30min.

Ammonium concentrations were determined using a colorimetric assay (Fawcett *et al.*, 1960). Reaction reagents were added to 1ml of sample in a 15ml glass tube in the following order and volumes; 2ml of sodium phenate [7.5g Phenol, 240ml ddH₂O, 23.4ml of 4N NaOH], 3ml of 0.1% sodium nitroprusside, 3 ml of 0.02N sodium hypochlorite (Clorox, 6.15%). The reaction mixture was vortexed for 1sec and incubated at room temperature in the dark for 30 min.

Samples were diluted to appropriately to fall within range of the standard absorbances, 200 μ l of the diluted samples were pipetted into a 96 well plate. Absorbances of the diluted samples were read using a spectrometer (Wallac 1420 Victor, multi well plate reader; Waltham,

MA) for 10sec at 620nm. One unit of NHase is defined as the conversion of 1 μ M of AN per minute per mg dry weight (units/mg cdw) of cells at 30 °C, pH 7.

Aliphatic/Aromatic Nitrilase Assay

Nitrilase activity was quantified using 1000 ppm of acrylonitrile (AN) or 1000 ppm of benzonitrile (BN) solution as substrate. Individual reaction mixtures were made by pipetting 9ml of 1000 ppm nitrile standard into 15ml centrifuge tubes. *R. rhodochrous* DAP 96253 samples were prepared by scraping 50mg of cells into a 1.5ml eppendorf tube that contained 1ml of (1X) PBS buffer (pH 7.2). The cell suspension was vortexed for 30-60sec to ensure complete cell re-suspension. The cell suspension was added to the 9ml reaction mixture and gently shaken for 2-10 min. Duplicate samples of each reaction mixture were transferred to clean 1.5ml eppendorf tubes. The samples were centrifuged for 2min at 13,00rpm (Eppendorf (Fisher); Westbury, NY) and ammonia concentrations determined.

Ammonium concentrations were determined using a colorimetric assay (Fawcett and Scott 1960). Reaction reagents were added to 1ml of sample in a 15ml glass tube in the following order and volumes; 2ml of sodium phenate [7.5g Phenol, 240ml ddH₂O, 23.4ml of 4N NaOH], 3ml of 0.1% sodium nitroprusside, 3 ml of 0.02N sodium hypochlorite (Clorox, 6.15%). The reaction mixture was vortexed for 1sec and incubated at room temperature in the dark for 30 min.

Samples were diluted to appropriately to fall within range of the standard absorbances, 200 μ l of the diluted samples were pipetted into a 96 well plate. Absorbances of the diluted samples were read using a spectrometer (Wallac 1420 Victor, multi well plate reader; Waltham,

MA) for 10sec at 620nm. One unit of NHase is defined as the conversion of 1 μ M of AN per minute per mg dry weight (units/mg cdw) of cells at 30 °C, pH 7.

Cyanidase “Cyanide Dihydratase”CDH Assay

CDH activity was quantified using 50 ppm of KCN solution as substrate. Individual reaction mixtures were made by pipetting 9ml of 50 ppm KCN solution into 15ml centrifuge tubes. *R. rhodochrous DAP 96253* samples were prepared by scraping 50mg of cells into a 1.5ml eppendorf tube that contained 1ml of (1X) PBS buffer (pH 7.2). The cell suspension was vortexed for 30-60sec to ensure complete cell re-suspension. The cell suspension was added to the 9ml reaction mixture and gently shaken for 2-10 min. Duplicate samples of each reaction mixture were transferred to clean 1.5ml eppendorf tubes. The samples were centrifuged for 2min at 13,00rpm (Eppendorf (Fisher); Westbury, NY) and ammonia concentrations determined.

Ammonium concentrations were determined using a colorimetric assay (Fawcett *et al.*, 1960). Reaction reagents were added to 1ml of sample in a 15ml glass tube in the following order and volumes; 2ml of sodium phenate [7.5g Phenol, 240ml ddH₂O, 23.4ml of 4N NaOH], 3ml of 0.1% sodium nitroprusside, 3 ml of 0.02N sodium hypochlorite (Clorox, 6.15%). The reaction mixture was vortexed for 1sec and incubated at room temperature in the dark for 30 min.

Samples were diluted to appropriately to fall within range of the standard absorbances, 200 μ l of the diluted samples were pipetted into a 96 well plate. Absorbance of the diluted samples were read using a spectrometer (Wallac 1420 Victor, multi well plate reader; Waltham, MA) for 10sec at 620nm. One unit of NHase is defined as the conversion of 1 μ M of AN per minute per mg dry weight (units/mg cdw) of cells at 30 °C, pH 7.

Alkene Monooxygenase Assay:

AMO activity was quantified using 99% propylene substrate. Frozen *R. rhodochrous* DAP 96253 cells were removed from -20°C or -80°C storage and thawed at room temperature, 25mg of thawed cells were scrapped into a 1.5ml eppendorf tube and 500µl of M9 media containing 2% glucose (pH 7.4) was added to the tube. Samples were vortexed for 60sec to ensure cell re-suspension and placed into a 30°C water bath for 1 min to equilibrate cells.

The individual reaction mixtures were prepared by pipetting 500µl of cell suspension into a 2ml GC vial. Vials were crimped sealed with silicon/ Teflon® crimp caps, 700µl of propylene gas was added to the sealed vials, and samples were placed in a 30°C bath for 10 min, 150µl of head space was removed at variable time periods and analyzed on the GC using manual injection. GC parameters were modified from standard AMO assay protocols (Table 2) (Small *et al.*, 1995; Smith *et al.*, 1997). Peaks were compared to propylene and propene oxide standards, standards were prepared using 99% propylene and propylene oxide (Table 3).

Table 2: AMO Assay GC Parameters:

Carrier gas and flux:	Temp. °C Range Injection=1 Detector=2 Column= 3	Injector Volume:	Colum and GC (FID):
1- Helium= 2 ml min ⁻¹ 2- Hydrogen= 45 ml min ⁻¹ 3- Air= 450 ml sec ⁻¹	Temp range: (-) 60°C to 350°C 1. Injector= temp 150°C 2. Detect temp=200°C 3. Column= temp200°C	50µL of gas phase 1 µL of liquid phase	Supelco SPB-1: (non-polar methylsilicone column) 30.0M X 0.25mm; Hewlard Packard 5890 series Autosampler GC (FID)

(Small *et al.*, 1997; Smith *et al.*, 1999)**Table 3: AMO Standard Preparation:**

AMO Assay Standards:			
<u>Standard:</u>	<u>Propylene</u>	<u>Propene Oxide(2mM)</u>	<u>M9 (2% Glucose)</u>
Standard-1	700µL	-----	500µL
Standard-2	---	150µL	500µL

(Small *et al.*, 1997; Smith *et al.*, 1999)

Dry Weight and Loss on Drying

R. rhodochrous DAP 96253 cells were harvested from YEMEA plates and scrapped into a 50ml centrifuge containing 15ml of (1X) PBS buffer (0.8% NaCl, 0.02%KCl, 0.02M PO₄, and pH 7.2). The tube was vortexed for 60sec to ensure cell re-suspension, 2ml aliquots of samples were pipetted into clean 1.5ml eppendorf tubes and then transferred to the hot porcelain containers. Dry weight samples were collected before and after exposure to propylene/ ethylene gas. Dry weight analysis was used to give an accurate representation of increases in biological matter.

To calculate total dry weight (TDW) for *R. rhodochrous DAP 96253* samples, two porcelain containers (43 x 37 x 2mm; max temp. 1150°C; glazed bottom; Fisher Scientific; Bridgewater, NJ) were preheated in an oven for 2 hrs. at 105°C. The porcelain containers were removed from the oven and weighed while hot for 10sec, the weight was recorded as (W4). Dry weight samples (2ml) was added to porcelain containers and weighed; weight was recorded as (W6). Porcelain containers were placed back into oven for 20 hrs. at 105°C. Containers were removed from the oven and weighed while hot for 10sec; the weight was recorded as (W7).

$$(\% \text{ Loss of Drying} = \frac{W6 - W4}{W7} \times 100)(7.5)$$

- a) W4 = tare weight of hot container in grams
- b) W6= weight of sample and hot container in grams
- c) W7 = dry weight of sample and container in grams.

RESULTS:

Comparison of TDW for *R. rhodochrous* DAP 96253 cells cultured on propylene gas:

Total dry weights (TDW) for *R. rhodochrous* DAP 96253 cells were recorded before and after cells were exposed to propylene/ethylene gas. TDW was used to compare the ability of YEMEA induced and YEMEA non-induced cells to metabolize propylene/ ethylene during a 3-6 day incubation period. The average initial dry weight for YEMEA induced cells was 198mg (± 1 mg) and the average initial dry weight for YEMA non-induced cells was 243mg (± 20 mg). The average final dry weight for YEMEA induced cells was 275mg (± 3 mg), and the average final dry weight for YEMEA non-induced cells was 285mg (± 13 mg).

The initial and final dry weights were compared for both YEMEA induced and YEMEA non-induced samples to identify total net gain in dry mass over a 3-6 day culture period (Table 4). YEMEA induced cells displayed an overall **83%** increase in dry mass compared to Non-induced cells cultured for same time period with same substrate. YEMEA induced cells were more efficient at degrading propylene/ethylene than YEMEA non-induced cells.

Table 4: Comparison of net growth on propylene for *Rhodococcus rhodochrous* DAP 96253

Samples:	Avg.Net Gain	p-Value
YEMEA Induced (Co & Urea)	(77 mg ± 2 mg)	≤0.01%
YEMEA Non-Induced	(42 mg ± 15 mg)	≤0.01%

YEMEA (YNI) contained (4g/L) glucose, YEMEA (YI) contained (4g/L) glucose, (200mg/L) cobalt, and (7.5g/L) urea. Propylene exposed cells received 180- 200ml 99.0% pure propylene gas, and glucose cells received (2g/L). All experiments used 99.0% pure propylene gas as sole carbon source. Calculations obtained from (Barreto and Howland, 2008 economics software).

Enzyme Assays

Rhodococcus rhodochrous DAP 96253 cells were initially cultured on YEMEA medium supplemented with inducers (cobalt-0.201g/L & urea-7.5g/L) or YEMEA medium that contained no inducers. Cells were then transferred to biphasic flasks (containing M9 & propylene/ethylene) or shake flasks (containing M9 media & 2% glucose), cells were exposed to fruit. *Rhodococcus rhodochrous* DAP 96253 cells were assayed for NHase, Nit, and CDH activity before and after propylene/ethylene or fruit exposure.

NHase Activity

Rhodococcus rhodochrous DAP 96253 cells were assayed for NHase activity before and after propylene/ethylene exposure or fruit exposure (Table 5 & Table 6).

YI-P cells and YI-2% cells both displayed an overall increase in NHase activity, but YI-2% cells displayed a significant decrease in NHase activity when removed from a rich carbon source and exposed to fruit. YNI-P cells and YNI-2% cells both displayed a constant increase in NHase activity after exposure to propylene and the fruit, but YNI-P cells displayed the greatest overall increase (1100%).

Nitrilase Activity

Rhodococcus rhodochrous DAP 96253 cells were assayed for Nitrilase activity before and after propylene/ethylene or fruit exposure (Table 5 & Table 6).

YI-P cells displayed an overall 195% increase in Nitrilase activity, but Nitrilase activity actually decreased when cells were removed from YEMEA induced plates. Nitrilase activity increased again when cells were exposed to fruit, but YNI-P cells displayed a constant increase in Nitrilase activity after exposure to propylene and fruit.

CDH activity:

Rhodococcus rhodochrous DAP 96253 cells were assayed for CDH activity before and after propylene/ethylene exposure or fruit exposure (Table 5 & Table 6).

YI-P and YI-2% cells both displayed an overall decrease in CDH activity after being cultured in the presence of gas and exposed to fruit. The cells activity decreased when cells were removed from YEMEA induced plates, but activity increased slightly when YI-P and YI- 2% cells were exposed to fruit.

YNI-P cells and YNI-2% cells both displayed an overall increase in CDH activity after being cultured in the presence of gas and exposed to fruit, but YNI-P cells displayed the greatest increase in CDH activity (150%).

Table 5: Comparison of Enzymatic Assays after Propylene/Ethylene Gas Exposure

Sample	NHase Assay (25mM ACN)		NHase and/or Nitrilase Assay (25mM Acry)		CDH Assay (50ppm KCN)	
	Initial Activity	Final Activity	Initial Activity	Final Activity	Initial Activity	Final Activity
YI-Prop	(43 ± 10.58)	(75 ± 22.37)	(57 ± 7.78)	(48 ± 0.71)	(5 ± 2.88)	(3 ± 1.20)
YI-2%	(43 ± 10.58)	(140 ± 8.66)	-----	-----	(5 ± 2.88)	(5 ± 1.45)
YNI-Prop	(4 ± 6.65)	(27 ± 2.52)	N.D.	(1 ± 0.71)	N.D.	(1.33 ± 0.03)
YNI-2%	(4 ± 6.65)	(6 ± 0)	-----	-----	N.D.	N.D.5RY

(**YI-P**) *R. rhodochrous* DAP 96253 cells were cultured on YEMEA with inducers (cobalt-0.201g/L & urea-7.5g/L), transferred to biphasic flask with M9 media and propylene/ethylene, and then exposed to fruit. (**YNI-P**) cells were cultured on YEMEA with no inducers, transferred to biphasic flask with M9 media and propylene/ethylene, and then exposed to fruit. (**YI-2%**) cells were cultured on YEMEA with inducers (cobalt-0.201g/L & urea-7.5g/L), transferred to flask with M9 media and 2% glucose, and then exposed to fruit. (**YNI-2%**) cells were cultured on YEMEA with no inducers, transferred to flask with M9 media and 2% glucose, and then exposed to fruit. (**SI**) slight increase, unable to give percentage does to initial activity range ≥ 0 .

*Units 1uM of acrylonitrile converted to 1 uM acrylamide in 1 min, pH 7.2 at 30°C.

*Units 1uM of KCN converted to 1 uM formic acid in 1 min, pH 7.2 at 30°C.

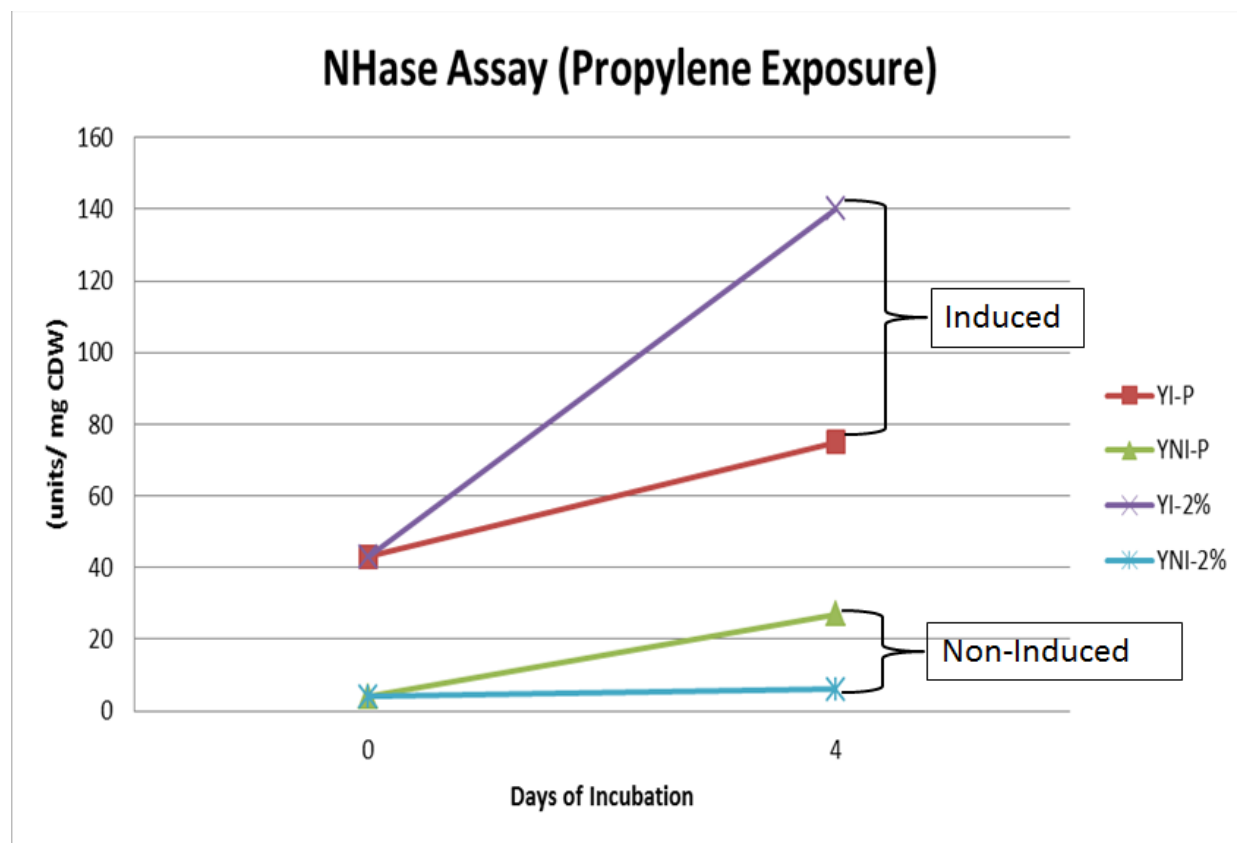


Figure 8: NHase activity after propylene exposure. (YI-P) *R. rhodochrous* DAP 96253 cells were cultured on YEMEA with inducers (cobalt-0.201g/L & urea-7.5g/L), transferred to biphasic flask with M9 media and propylene/ethylene, and then exposed to fruit. (YNI-P) cells were cultured on YEMEA with no inducers, transferred to biphasic flask with M9 media and propylene/ethylene, and then exposed to fruit. (YI-2%) cells were cultured on YEMEA with inducers (cobalt-0.201g/L & urea-7.5g/L), transferred to flask with M9 media and 2% glucose, and then exposed to fruit. (YNI-2%) cells were cultured on YEMEA with no inducers, transferred to flask with M9 media and 2% glucose, and then exposed to fruit.

*Units 1uM of acrylonitrile converted to 1 uM acrylamide in 1 min, pH 7.2 at 30°C.

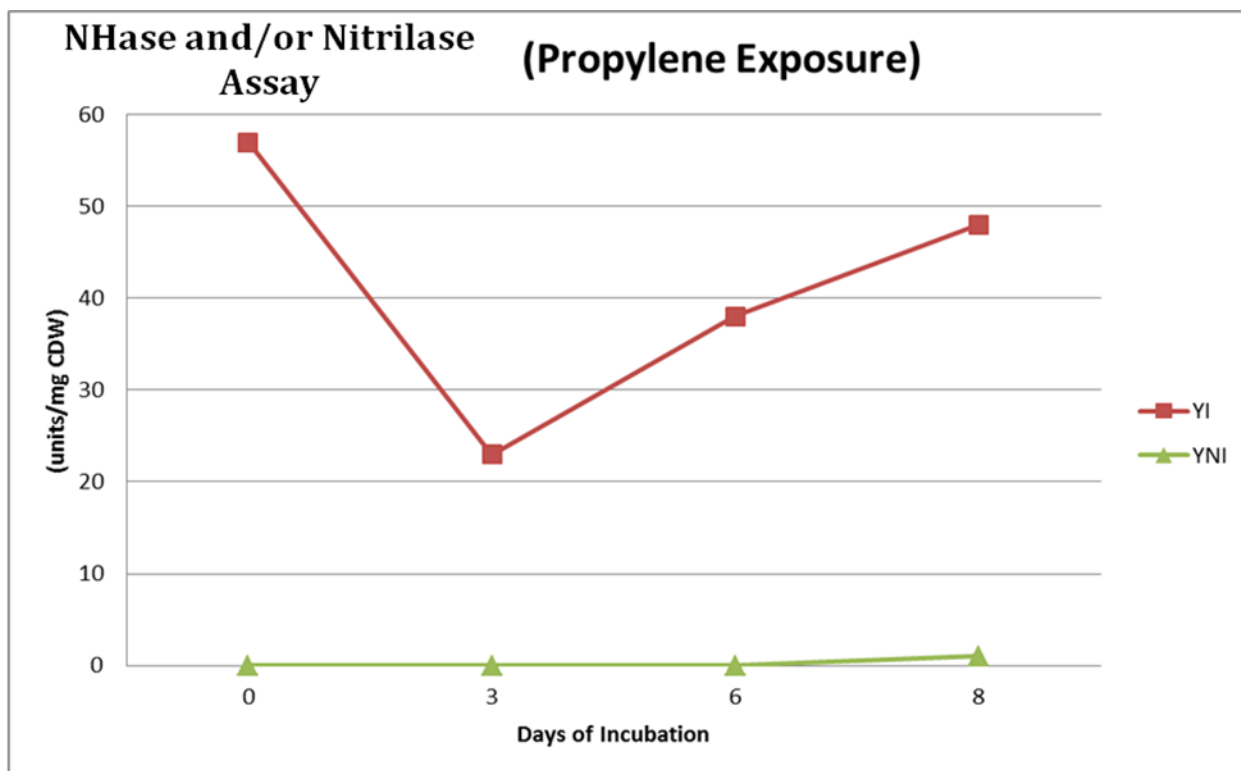


Figure 9: Nitrilase activity after propylene exposure. (YI-P) *R. rhodochrous* DAP 96253 cells were cultured on YEMEA with inducers (cobalt-0.201g/L & urea-7.5g/L), transferred to biphasic flask with M9 media and propylene/ethylene, and then exposed to fruit. (YNI-P) cells were cultured on YEMEA with no inducers, transferred to biphasic flask with M9 media and propylene/ethylene, and then exposed to fruit. (YI-2%) cells were cultured on YEMEA with inducers (cobalt-0.201g/L & urea-7.5g/L), transferred to flask with M9 media and 2% glucose, and then exposed to fruit. (YNI-2%) cells were cultured on YEMEA with no inducers, transferred to flask with M9 media and 2% glucose, and then exposed to fruit.

*Units 1uM of acrylonitrile converted to 1 uM acrylamide in 1 min, pH 7.2 at 30°C.

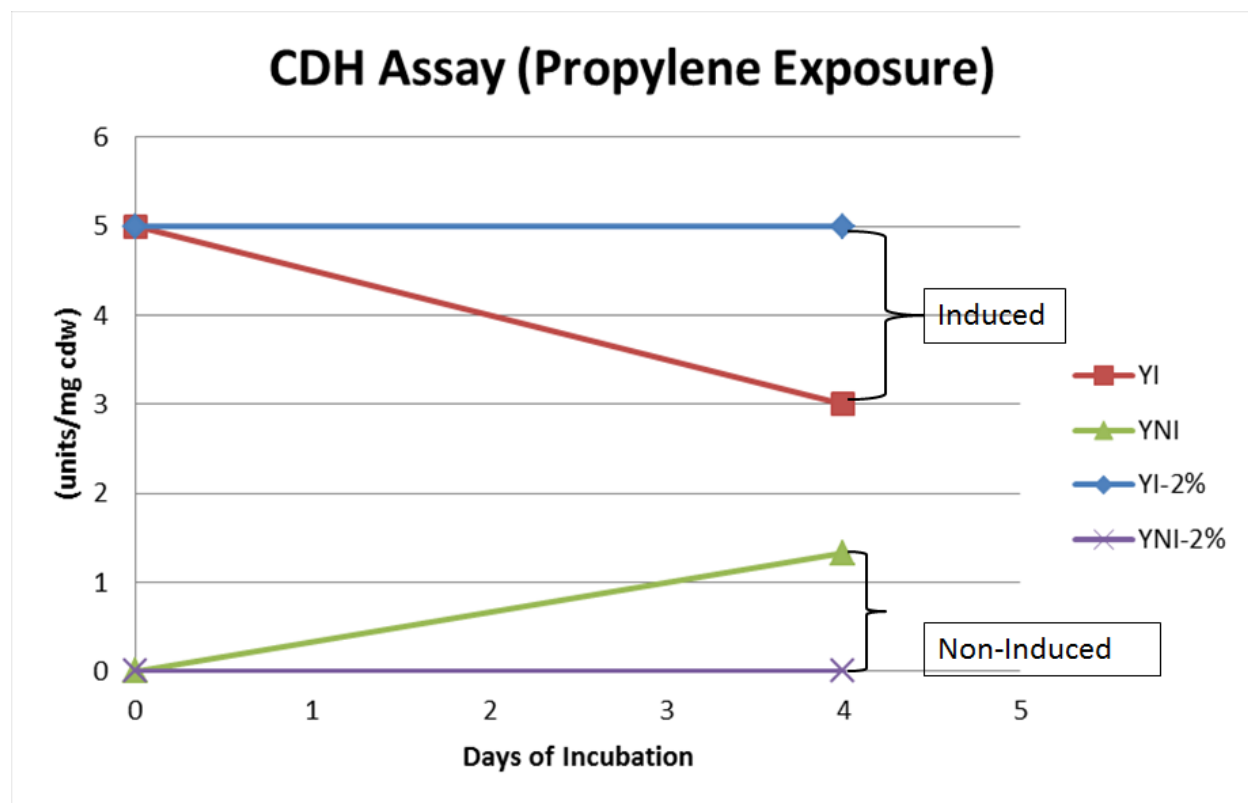


Figure 10: CDH activity after propylene exposure. (YI-P) *R. rhodochrous* DAP 96253 cells were cultured on YEMEA with inducers (cobalt-0.201g/L & urea-7.5g/L), transferred to biphasic flask with M9 media and propylene/ethylene, and then exposed to fruit. (YNI-P) cells were cultured on YEMEA with no inducers, transferred to biphasic flask with M9 media and propylene/ethylene, and then exposed to fruit. (YI-2%) cells were cultured on YEMEA with inducers (cobalt-0.201g/L & urea-7.5g/L), transferred to flask with M9 media and 2% glucose, and then exposed to fruit. (YNI-2%) cells were cultured on YEMEA with no inducers, transferred to flask with M9 media and 2% glucose, and then exposed to fruit.

*Units 1uM of KCN converted to 1 uM formic acid in 1 min, pH 7.2 at 30°C.

Table 6: Comparison of Enzymatic Assays after Exposure to Fruit

Sample	NHase Assay (25mM ACN)		NHase and/or Nitrilase Assay (25mM Acry)		CDH Assay (50ppm KCN)	
	<u>After</u>	<u>After Fruit</u>	<u>After</u>	<u>After Fruit</u>	<u>After</u>	<u>After Fruit</u>
	<u>Propylene</u>	<u>Exposure</u>	<u>Propylene</u>	<u>Exposure</u>	<u>Propylene</u>	<u>Exposure</u>
YI-P	(75 ± 22.37)	(96 ± 17.0)	(48 ± 0.71)	(111 ± 7.78)	(3 ± 1.20)	(4 ± 1.20)
YI-2%	(140 ± 8.66)	(85 ± 1.4)	-----	-----	(5 ± 1.45)	(5 ± 0.577)
YNI-P	(27 ± 2.52)	(48 ± 10.5)	(1 ± 0.71)	(4 ± 1.41)	(1.33 ± 0.03)	(3.33 ± 0.03)
YNI-2%	(6 ± 0)	(10 ± 0.7)	-----	-----	N.D.	(2 ± 0.577)

(**YI-P**) *R. rhodochrous* DAP 96253 cells were cultured on YEMEA with inducers (cobalt-0.201g/L & urea-7.5g/L), transferred to biphasic flask with M9 media and propylene/ethylene, and then exposed to fruit. (**YNI-P**) cells were cultured on YEMEA with no inducers, transferred to biphasic flask with M9 media and propylene/ethylene, and then exposed to fruit. (**YI-2%**) cells were cultured on YEMEA with inducers (cobalt-0.201g/L & urea-7.5g/L), transferred to flask with M9 media and 2% glucose, and then exposed to fruit. (**YNI-2%**) cells were cultured on YEMEA with no inducers, transferred to flask with M9 media and 2% glucose, and then exposed to fruit. (**SI**) slight increase, unable to give percentage does to initial activity range ≥ 0 .

*Units 1uM of acrylonitrile converted to 1 uM acrylamide in 1 min, pH 7.2 at 30°C.

*Units 1uM of KCN converted to 1 uM formic acid in 1 min, pH 7.2 at 30°C.

N.D. – Not Detectable

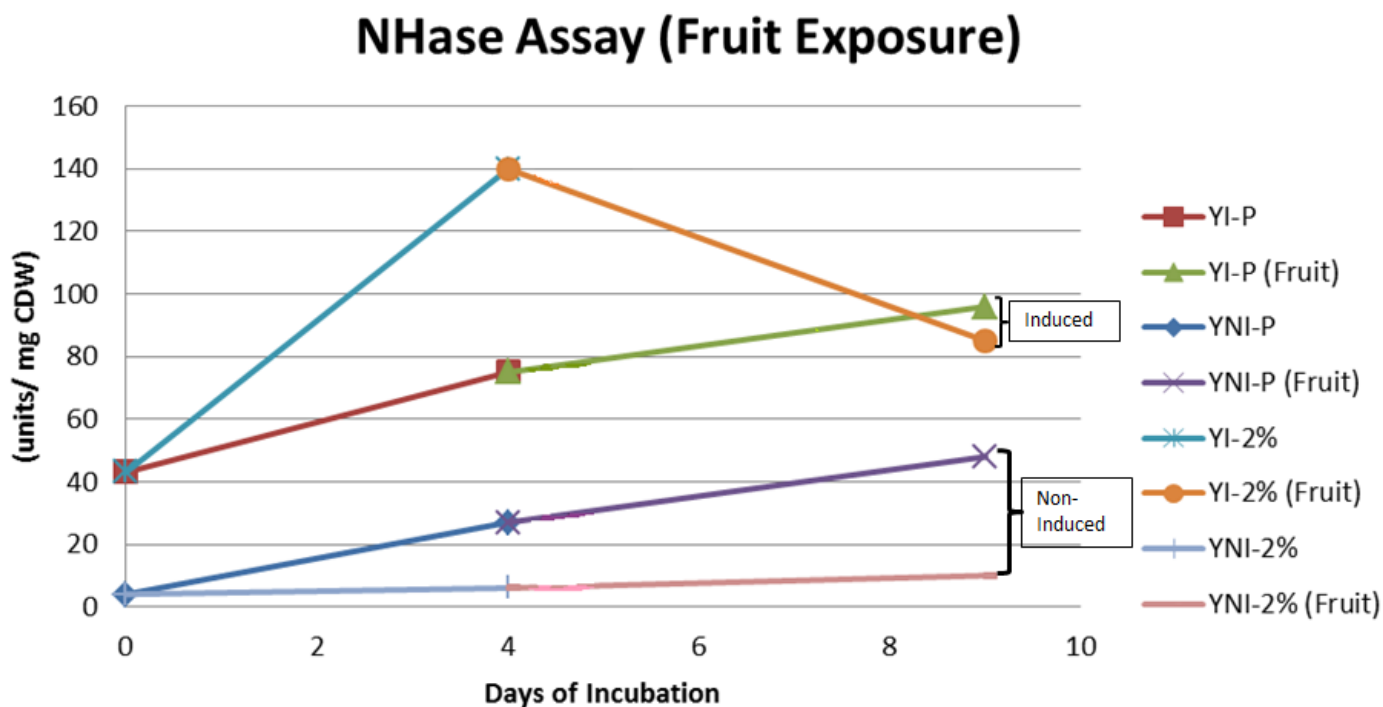


Figure 11: NHase activity after fruit exposure. (YI-P) *R. rhodochrous* DAP 96253 cells were cultured on YEMEA with inducers (cobalt-0.201g/L & urea-7.5g/L), transferred to biphasic flask with M9 media and propylene/ethylene, and then exposed to fruit. (YNI-P) cells were cultured on YEMEA with no inducers, transferred to biphasic flask with M9 media and propylene/ethylene, and then exposed to fruit. (YI-2%) cells were cultured on YEMEA with inducers (cobalt-0.201g/L & urea-7.5g/L), transferred to flask with M9 media and 2% glucose, and then exposed to fruit. (YNI-2%) cells were cultured on YEMEA with no inducers, transferred to flask with M9 media and 2% glucose, and then exposed to fruit.

*Units 1uM of acrylonitrile converted to 1 uM acrylamide in 1 min, pH 7.2 at 30°C.

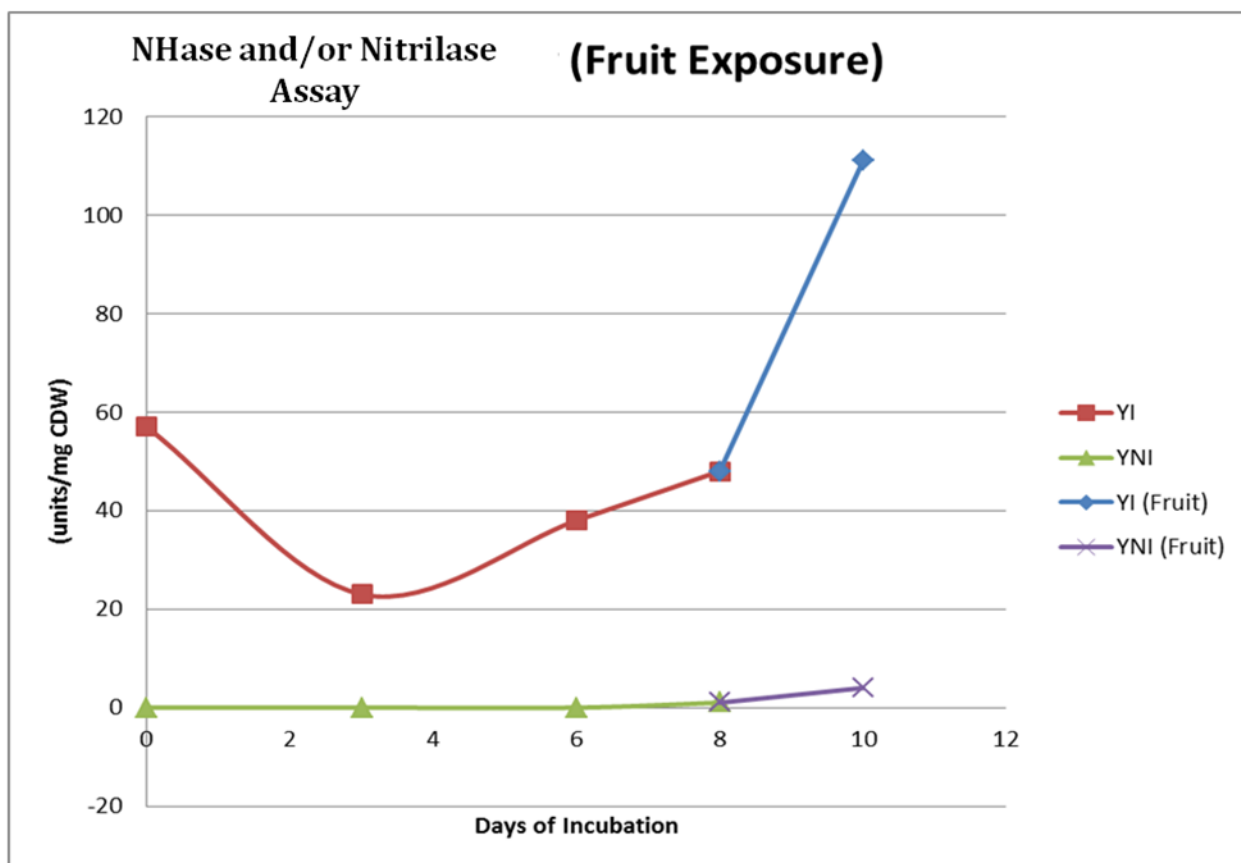


Figure 12: Nitrilase activity after fruit exposure. (YI-P) *R. rhodochrous* DAP 96253 cells were cultured on YEMEA with inducers (cobalt-0.201g/L & urea-7.5g/L), transferred to biphasic flask with M9 media and propylene/ethylene, and then exposed to fruit. (YNI-P) cells were cultured on YEMEA with no inducers, transferred to biphasic flask with M9 media and propylene/ethylene, and then exposed to fruit. (YI-2%) cells were cultured on YEMEA with inducers (cobalt-0.201g/L & urea-7.5g/L), transferred to flask with M9 media and 2% glucose, and then exposed to fruit. (YNI-2%) cells were cultured on YEMEA with no inducers, transferred to flask with M9 media and 2% glucose, and then exposed to fruit.

*Units 1uM of acrylonitrile converted to 1 uM acrylamide in 1 min, pH 7.2 at 30°C.

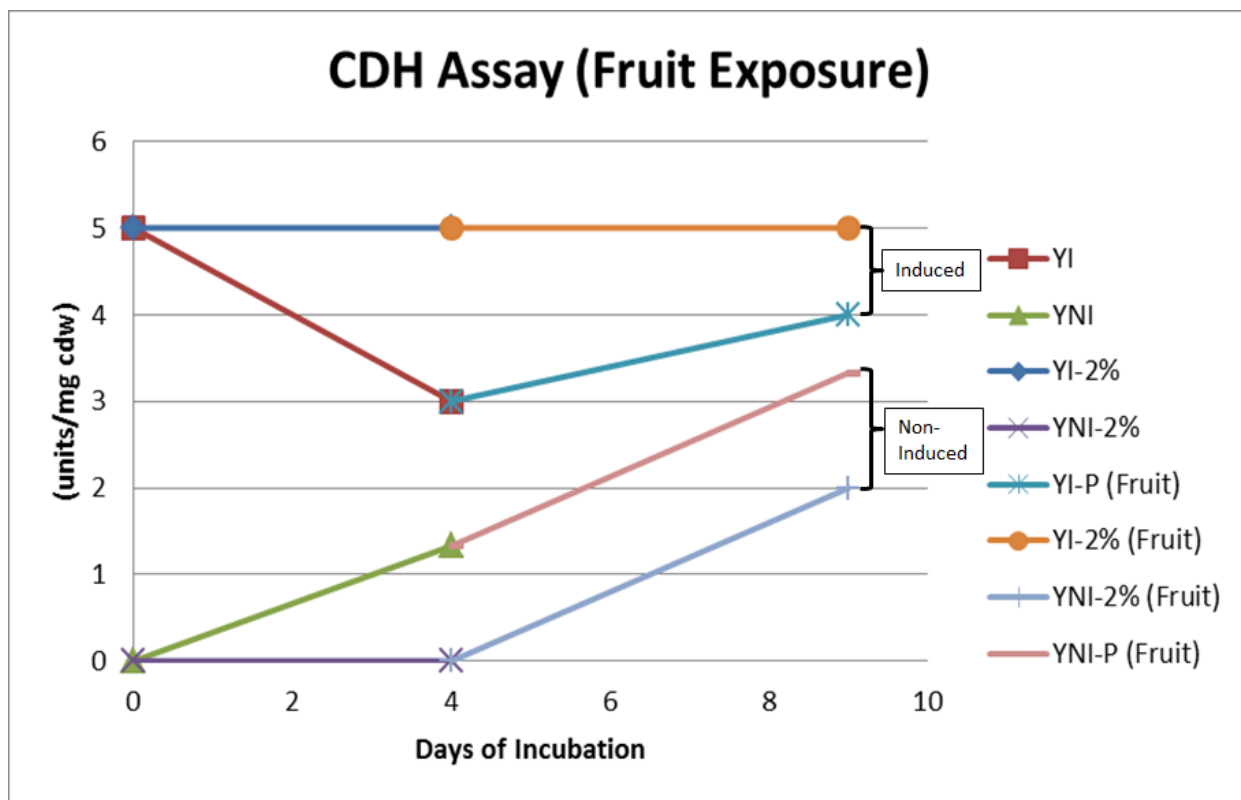


Figure 13: CDH activity after propylene exposure. (YI-P) *R. rhodochrous* DAP 96253 cells were cultured on YEMEA with inducers (cobalt-0.201g/L & urea-7.5g/L), transferred to biphasic flask with M9 media and propylene/ethylene, and then exposed to fruit. (YNI-P) cells were cultured on YEMEA with no inducers, transferred to biphasic flask with M9 media and propylene/ethylene, and then exposed to fruit. (YI-2%) cells were cultured on YEMEA with inducers (cobalt-0.201g/L & urea-7.5g/L), transferred to flask with M9 media and 2% glucose, and then exposed to fruit. (YNI-2%) cells were cultured on YEMEA with no inducers, transferred to flask with M9 media and 2% glucose, and then exposed to fruit.

*Units 1uM of KCN converted to 1 uM formic acid in 1 min, pH 7.2 at 30°C.

Aliphatic Nitrilase Induction in *R. rhodochrous* DAP 96253

Rhodococcus rhodochrous DAP 96253 cells were assayed for nitrilase activity before and after propylene/ethylene exposure or fruit exposure (Table 5 & Table 6). Nitrilase assays used 1000ppm AN/BN as substrate.

Nitrilase Assay (Acrylonitrile as Substrate)

YNI-P cells and YNI-Cof had a slight increase (SI) in activity after being cultured on propylene. YI-P cells displayed an overall 7% decrease in nitrilase activity after being cultured in the presence of propylene.

Nitrilase Assay (Benzonitrile as Substrate)

YNI-P cells had a slight increase in nitrilase activity after being cultured on propylene, but YNI-Cof and YI-P cells displayed an overall decrease in nitrilase activity after being cultured in the presence of propylene.

Table 7: Comparison of Nitrilase activity after Propylene Gas Exposure (10min Reaction)

Sample	NHase and/or Nitrilase Assay (25mM ACN)			Only Nitrilase Assay (10mM Benzonitrile)		
	<u>Initial Activity</u>	<u>Final Activity</u>	<u>Increase Nit Activity</u>	<u>Initial Activity</u>	<u>Final Activity</u>	<u>Increase Nit Activity</u>
YI-P	(31 ± 0.0)	(29 ± 0.05)	(-) 6%	(6 ± 0.0)	(1 ± 0.05)	(-) 83%
YNI-P	(0 ± 0)	(2 ± 1.0)	SI	(0.1 ± 0.0)	(1 ± 1.0)	SI
YNI-Cof	(0 ± 0)	(11 ± 6.11)	SI	(0 ± 0)	(0 ± 0)	0%

YI-P) *R. rhodochrous* DAP 96253 cells were cultured on YEMEA with inducers (cobalt-0.201g/L & urea-7.5g/L), transferred to biphasic flask with M9 media and propylene/ethylene, and then exposed to fruit. **(YNI-P)** cells were cultured on YEMEA with no inducers, transferred to biphasic flask with M9 media and propylene/ethylene, and then exposed to fruit. **(YNI-Cof)** cells were cultured on YEMEA with no inducers, transferred to biphasic flask with M9 media with inducers (cobalt-0.201g/L & urea-7.5g/L) and propylene/ethylene, and then exposed to fruit. **(SI)** slight increase, unable to give percentage does to initial activity range ≥ 0 .

*Units 1uM of acrylonitrile/benzonitrile converted to 1 uM acrylamide/benzamide in 1 min, pH 7.2 at 30°C.

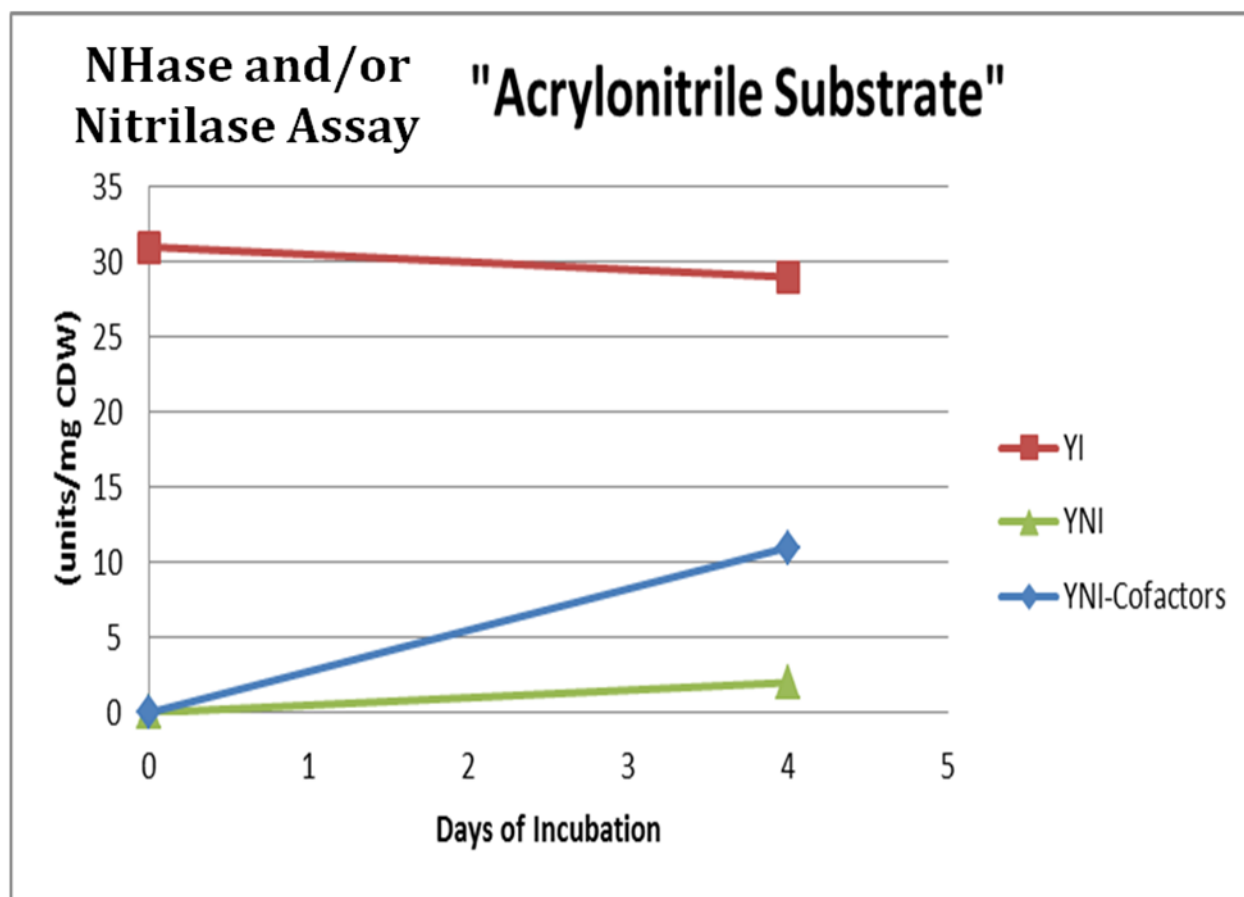


Figure 14: Nit activity after propylene exposure. Nit activity after propylene exposure. **YI-P)** *R. rhodochrous* DAP 96253 cells were cultured on YEMEA with inducers (cobalt-0.201g/L & urea-7.5g/L), transferred to biphasic flask with M9 media and propylene/ethylene, and then exposed to fruit. **(YNI-P)** cells were cultured on YEMEA with no inducers, transferred to biphasic flask with M9 media and propylene/ethylene, and then exposed to fruit. **(YNI-Cof)** cells were cultured on YEMEA with no inducers, transferred to biphasic flask with M9 media with inducers (cobalt-0.201g/L & urea-7.5g/L) and propylene/ethylene, and then exposed to fruit.

*Units 1uM of acrylonitrile/benzonitrile converted to 1 uM acrylamide/benzamide in 1 min, pH 7.2 at 30°C.

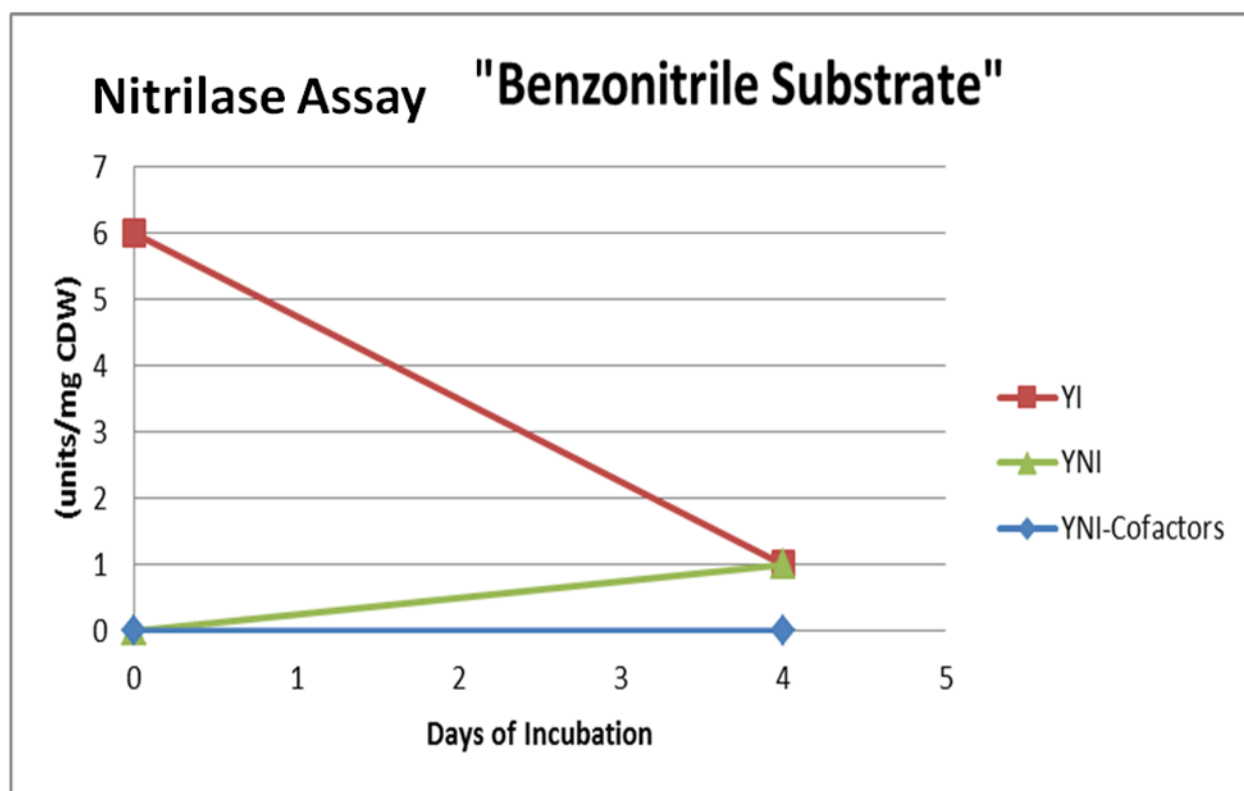


Figure 15: Nit activity after propylene exposure. YI-P) *R. rhodochrous* DAP 96253 cells were cultured on YEMEA with inducers (cobalt-0.201g/L & urea-7.5g/L), transferred to biphasic flask with M9 media and propylene/ethylene, and then exposed to fruit. (YNI-P) cells were cultured on YEMEA with no inducers, transferred to biphasic flask with M9 media and propylene/ethylene, and then exposed to fruit. (YNI-Cof) cells were cultured on YEMEA with no inducers, transferred to biphasic flask with M9 media with inducers (cobalt-0.201g/L & urea-7.5g/L) and propylene/ethylene, and then exposed to fruit.

*Units 1uM of acrylonitrile/benzonitrile converted to 1 uM acrylamide/benzamide in 1 min, pH 7.2 at 30°C.

Stability Enzyme Assays After Fruit exposure.

Rhodococcus rhodochrous DAP 96253 cells were assayed for NHase and CDH activity before, during, and after fruit exposure (Table 8 & Table 9).

Stability of NHase After Fruit Exposure

YI-Cof cells displayed an increase in NHase activity after being exposed to fruit for 6 days, but YI and YNI cells displayed a decrease in NHase activity. YNI cells displayed the largest overall increase in NHase activity (113%), over the 12 day run.

Stability of CDH After Fruit Exposure

YI-Cof, YI, and YNI cells all displayed an increase in CDH activity after being exposed to fruit for 6 days, but activity decreased or stabilized in all three sample sets after 9 days incubation with fruit. YNI cells displayed this largest overall increase in CDH activity (567%), over the 12 day run.

Table 8: Comparison of NHase stability during Fruit Ripening Experiments.

<u>Sample</u>	<u>NHase Assay:</u>								
	<u>Initial Activity</u>	<u>Day 6 Activity</u>	<u>NHase Activity Increase</u>	<u>Day 6 Activity</u>	<u>Day 9 Activity</u>	<u>NHase Activity Increase</u>	<u>Day 9 Activity</u>	<u>Day 12 Activity</u>	<u>NHase Activity Increase</u>
YI-Cof	72	109	53%	109	83	(-) 24%	83	57	(-) 31%
YI	73	45	(-) 38%	45	62	38%	62	73	18%
YNI	8	7	(-) 13%	7	8	13%	8	17	113%

(YI-Cof) *R. rhodochrous* DAP 96253 cells were cultured on YEMEA with inducers (cobalt-0.201g/L & urea-7.5g/L), transferred to biphasic flask with M9 media with inducers (cobalt-0.201g/L & urea-7.5g/L) and propylene/ethylene, and then exposed to fruit. **(YI)** cells were cultured on YEMEA inducers (cobalt-0.201g/L & urea-7.5g/L) then exposed to fruit. **(YNI)** cells were cultured on YEMEA with no inducers then exposed to fruit.

*Units 1uM of acrylonitrile converted to 1 uM acrylamide in 1 min, pH 7.2 at 30°C.

Table 9: Comparison of CDH stability during Fruit Ripening Experiments.

<u>Sample</u>	<u>CDH Assay:</u>								
	<u>Initial Activity</u>	<u>Day 6 Activity</u>	<u>CDH Activity Increase</u>	<u>Day 6 Activity</u>	<u>Day 9 Activity</u>	<u>CDH Activity Increase</u>	<u>Day 9 Activity</u>	<u>Day 12 Activity</u>	<u>CDH Activity Increase</u>
YI-Cof	2	7	150%	7	3	(-) 40%	3	16	433%
YI	6	3	50%	3	3	0%	3	17	467%
YNI	0	3	SI	3	3	0%	3	20	567%

(YI-Cof) *R. rhodochrous* DAP 96253 cells were cultured on YEMEA with inducers (cobalt-0.201g/L & urea-7.5g/L), transferred to biphasic flask with M9 media with inducers (cobalt-0.201g/L & urea-7.5g/L) and propylene/ethylene, and then exposed to fruit. **(YI)** cells were cultured on YEMEA inducers (cobalt-0.201g/L & urea-7.5g/L) then exposed to fruit. **(YNI)** cells were cultured on YEMEA with no inducers then exposed to fruit. **(SI)** slight increase, unable to give percentage does to initial activity range ≥ 0 .

*Units 1uM of KCN converted to 1 uM formic acid in 1 min, pH 7.2 at 30°C.

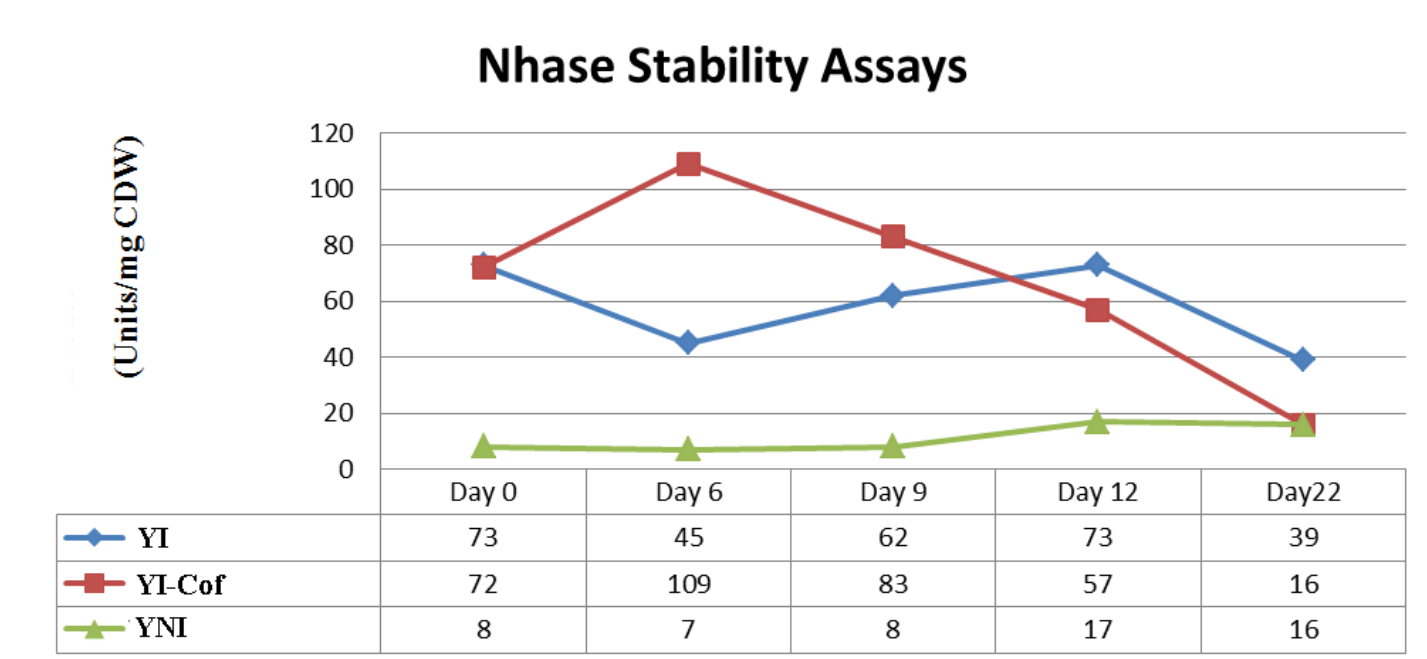


Figure 16: NHase activity after fruit exposure. (YI-Cof) *R. rhodochrous* DAP 96253 cells were cultured on YEMEA with inducers (cobalt-0.201g/L & urea-7.5g/L), transferred to biphasic flask with M9 media with inducers (cobalt-0.201g/L & urea-7.5g/L) and propylene/ethylene, and then exposed to fruit. (YI) cells were cultured on YEMEA inducers (cobalt-0.201g/L & urea-7.5g/L) then exposed to fruit. (YNI) cells were cultured on YEMEA with no inducers then exposed to fruit.

*Units 1uM of acrylonitrile converted to 1 uM acrylamide in 1 min, pH 7.2 at 30°C.

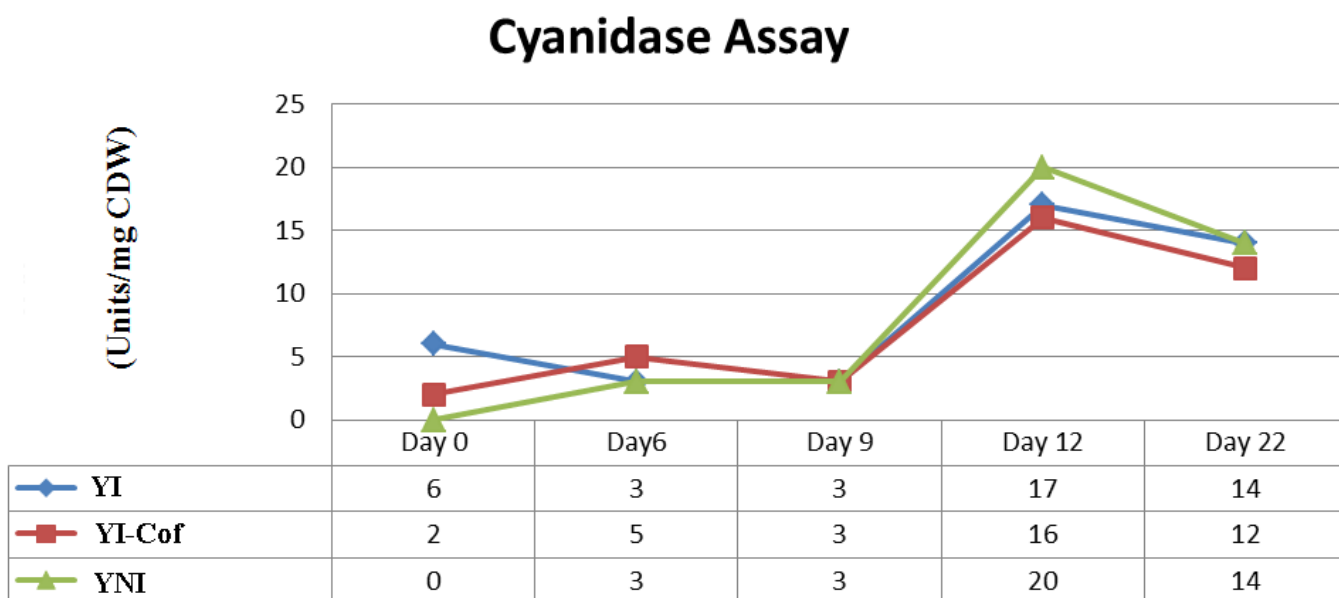


Figure 17: CDH activity after fruit exposure. (YI-Cof) *R. rhodochrous* DAP 96253 cells were cultured on YEMEA with inducers (cobalt-0.201g/L & urea-7.5g/L), transferred to biphasic flask with M9 media with inducers (cobalt-0.201g/L & urea-7.5g/L) and propylene/ethylene, and then exposed to fruit. (YI) cells were cultured on YEMEA inducers (cobalt-0.201g/L & urea-7.5g/L) then exposed to fruit. (YNI) cells were cultured on YEMEA with no inducers then exposed to fruit.

*Units 1uM of acrylonitrile converted to 1 uM acrylamide in 1 min, pH 7.2 at 30°C.

Comparing *R. rhodochrous* DAP 96253 cells cultured on YEMEA Induced medium or YEMEA Induced media and Propylene/Ethylene

R. rhodochrous DAP 96253 cells were placed into plastic bin containers and exposed to organic (US) Granny Smith apples for 17 days. Images were captured before and after fruit were exposed to *R. rhodochrous* DAP 96253 cells.

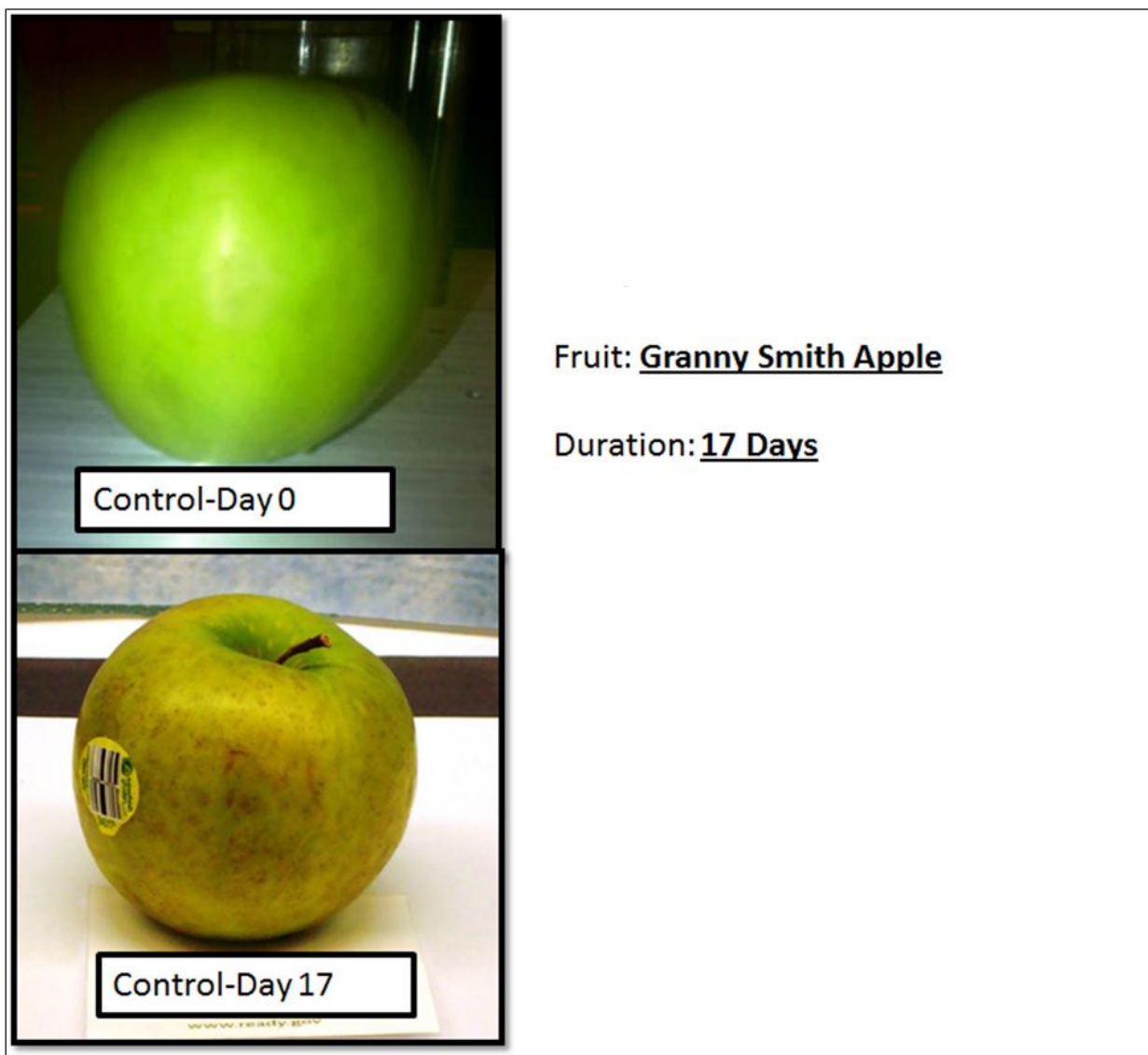


Figure 18: Comparison of Control Apples; Control images from 1st day and 17th day.

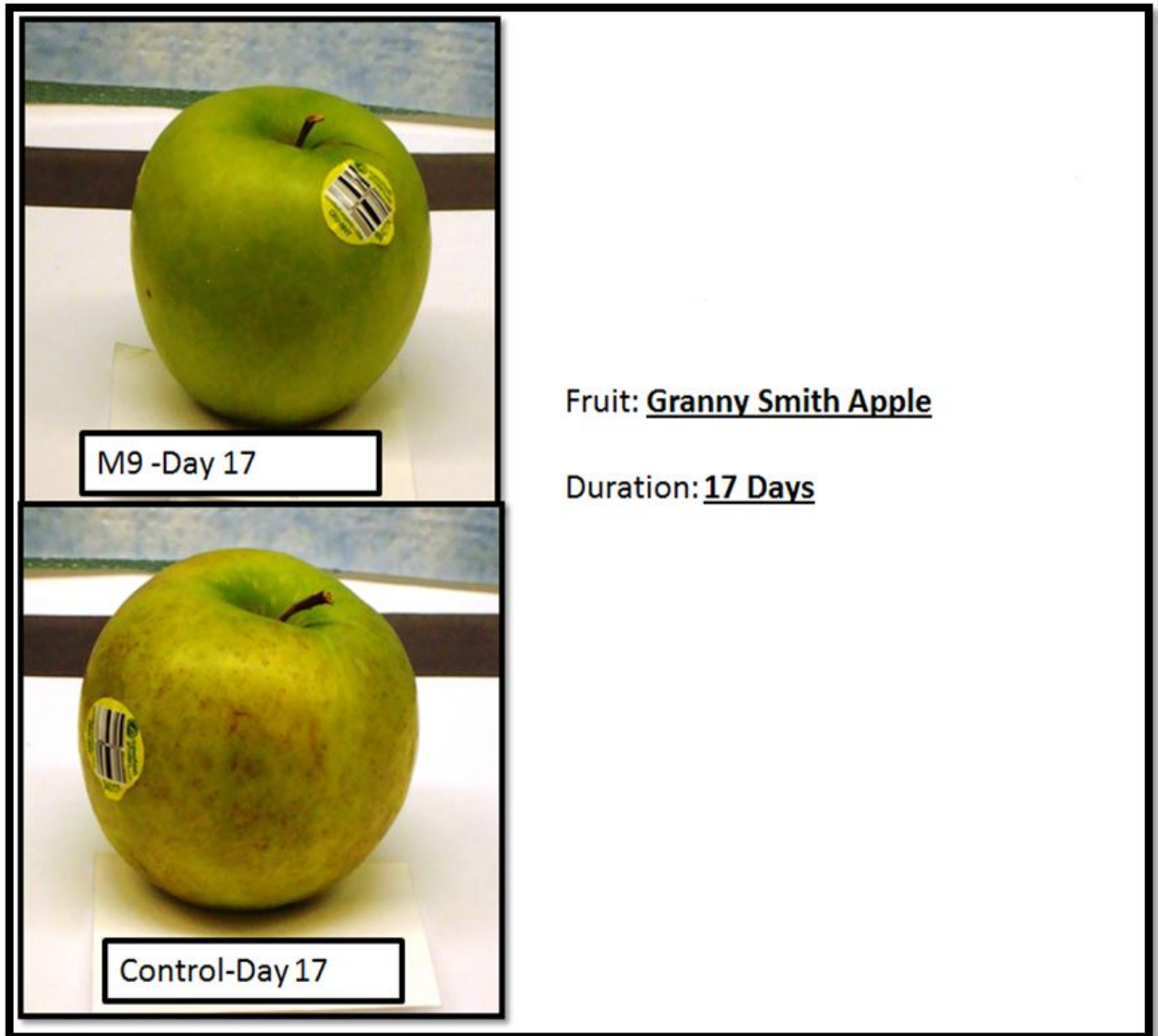


Figure 19: Effect of rhodococcal cells on apples; Control apple and (M9) apple. The (M9) apple was exposed to *R. rhodochrous* DAP 96253 cells that were cultured on YEMEA with inducers (cobalt-0.201g/L & urea-7.5g/L), transferred to a biphasic flask with M9 media and propylene/ethylene, and then exposed to fruit. Images from Day 17 of run.

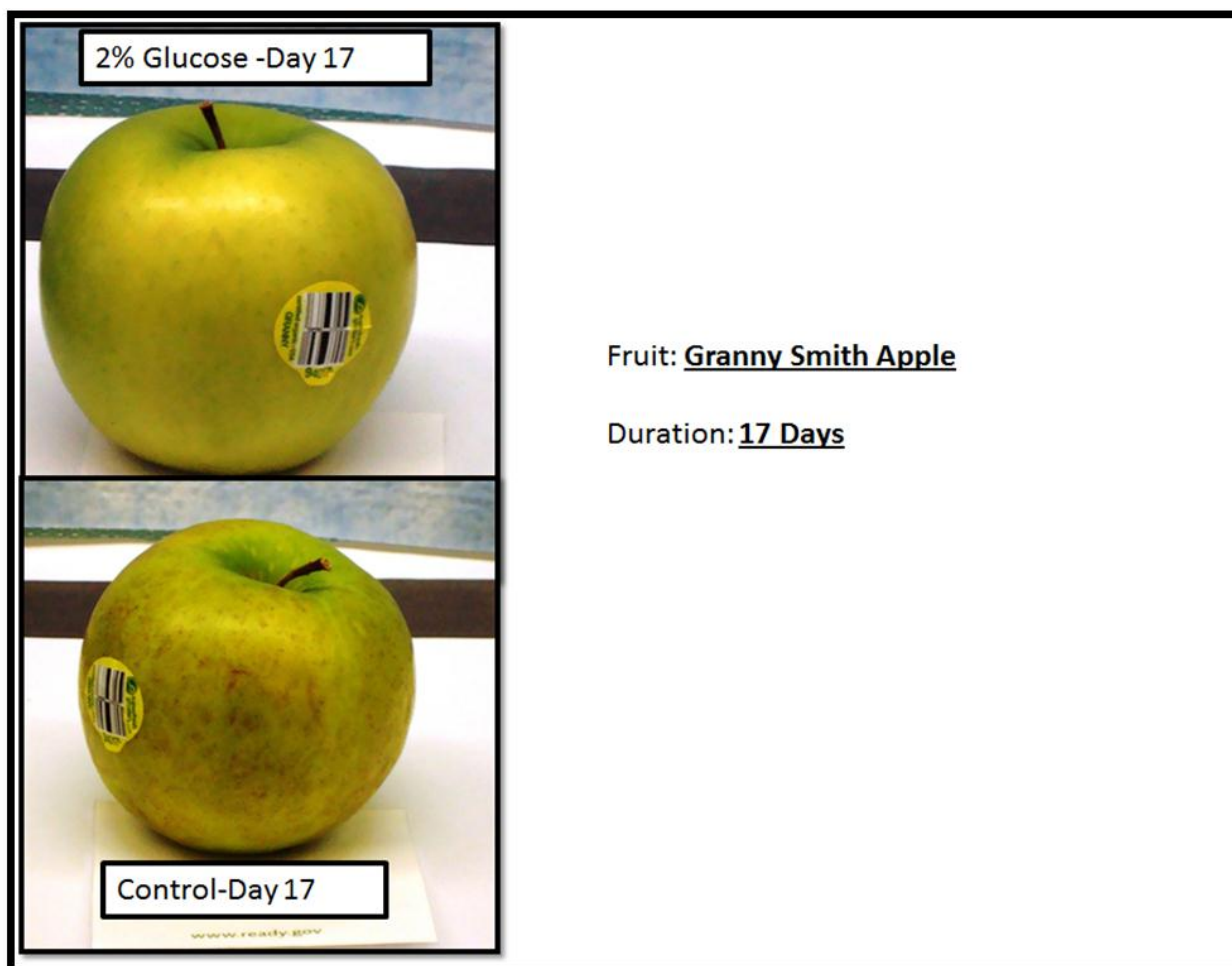


Figure 20: Effect of rhodococcal cells on apples; Control apple and (2% Glucose) apple. The (2% Glucose) apple was exposed *R. rhodochrous* DAP 96253 cells that were cultured on YEMEA with inducers (cobalt-0.201g/L & urea-7.5g/L), transferred to shake flask with M9 media (that contained 2% glucose), and then exposed to fruit. Images from Day 17 of run.

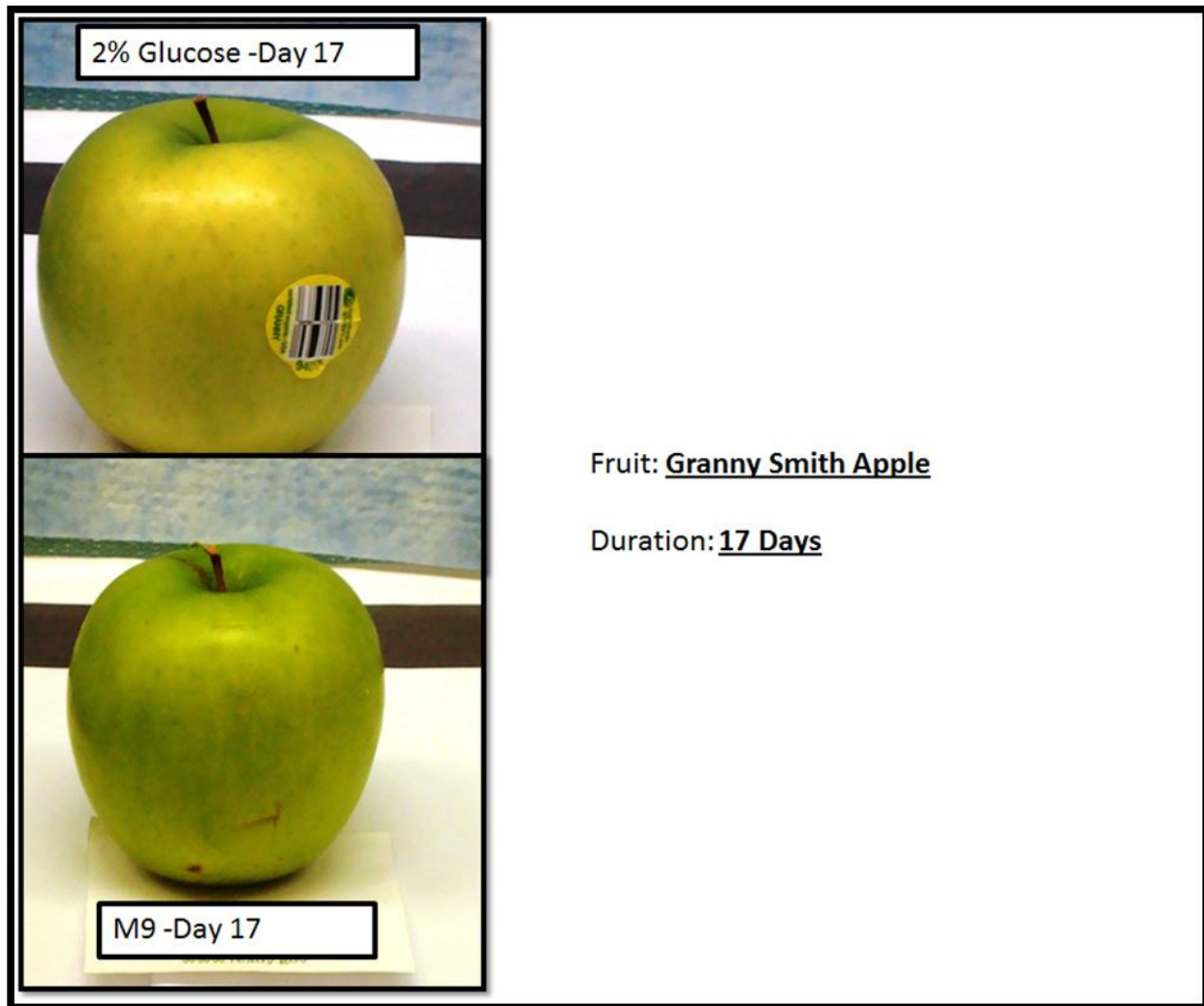


Figure 21: Effect of rhodococcal cells on apples; Comparison of (M9) apple and (2% Glucose) apple. The (M9) apple was exposed to *R. rhodochrous* DAP 96253 cells that were cultured on YEMEA with inducers (cobalt-0.201g/L & urea-7.5g/L), transferred to a biphasic flask with M9 media and propylene/ethylene, and then exposed to fruit. The (2% Glucose) apple was exposed to *R. rhodochrous* DAP 96253 cells that were cultured on YEMEA with inducers (cobalt-0.201g/L & urea-7.5g/L), transferred to shake flask with M9 media (that contained 2% glucose), and then exposed to fruit. Images from Day 17 of run.

18.2 Comparing effects to delayed ripening; using air tight sealed plastic containers

Sealed plastic bin containers were used in this experiment as opposed to brown paper bags. *Rhodococcus rhodochrous* DAP 96253 cells were exposed to organic bananas grown in S. America. The bananas and cells were placed into the plastic bins at 23°C for 7 days. Images were captured before and after fruits were exposed to *R. rhodochrous* DAP 96253 cells. Experiments showed that the use of containers instead of brown paper bags was more efficient.

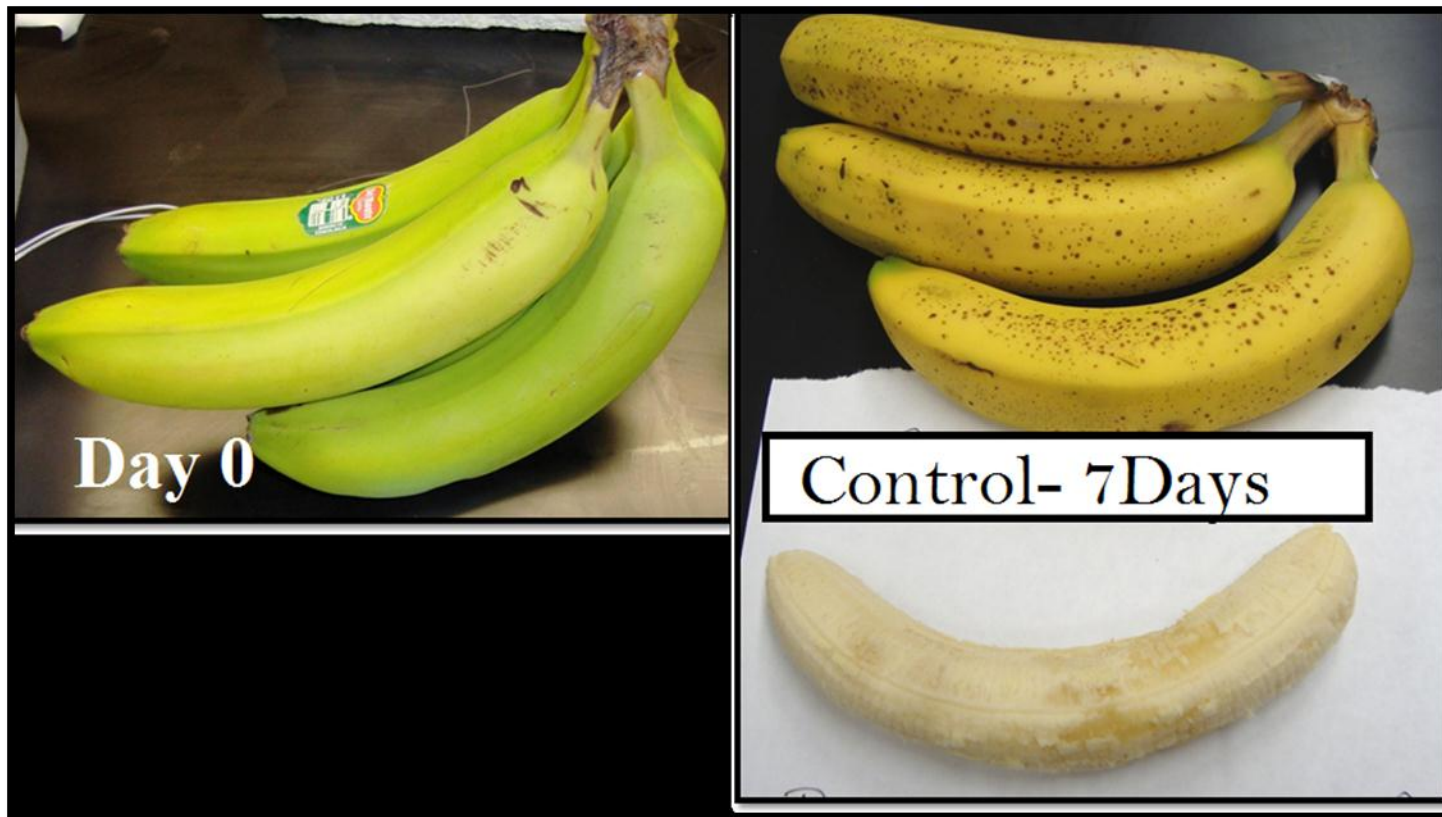


Figure 22: Control bananas on day 0 and day 7; Bananas were exposed to 30ml of minimal salts media only.

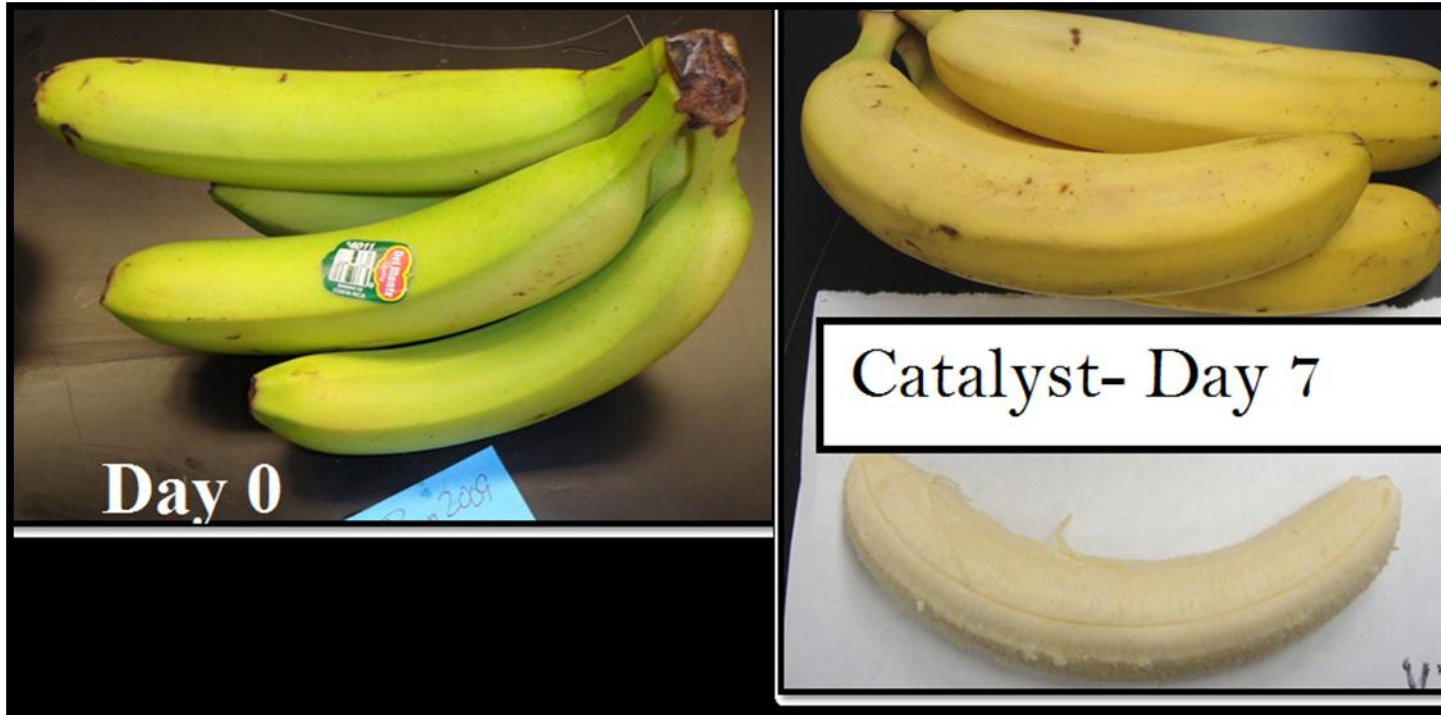


Figure 23: Effect of YI-P cells on bananas; Bananas were exposed to (YI-P) cells. (YI-P) were *R. rhodochrous* DAP 96253 cells that were cultured on YEMEA with inducers (cobalt-0.201g/L & urea-7.5g/L), transferred to a biphasic flask with M9 media and propylene/ethylene, and then exposed to bananas.

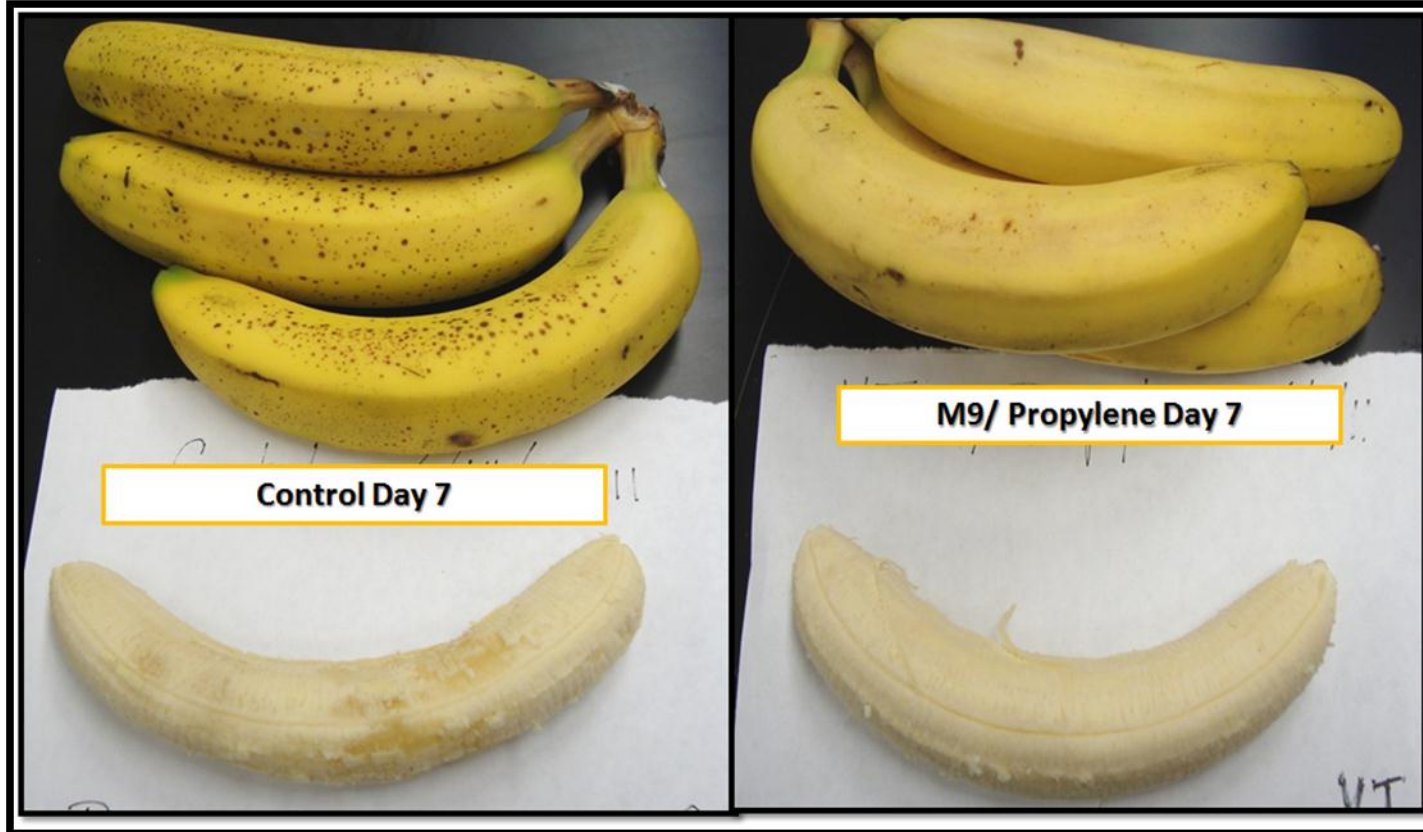


Figure 24: Comparison of control and bananas exposed to YI-P cells on Day 7; Control bananas were exposed to 30ml of M9 media only. **(YI-P)** bananas were exposed to *R. rhodochrous* DAP 96253 cells that were cultured on YEMEA media with inducers (cobalt-0.201g/L & urea-7.5g/L), transferred to a biphasic flask with M9 media and propylene/ethylene, and then exposed to bananas.

Comparing delayed ripening abilities of YI cells cultured with or without propylene

R. rhodochrous DAP 96253 cells were exposed to green organic bananas grown in S. America. The bananas and cells were placed into the plastic bins at 23°C for 7 days.

Images were captured before and after fruits were exposed to *R. rhodochrous* DAP 96253 cells. Bananas were exposed to a 100 ppm ethylene/ air gas mixture to expedite ripening process on day 3 of each phase. Experiment was used to determine if *R. rhodochrous* DAP 96253 ability to delay fruit ripening improves the longer the cells are exposed to the fruit.

Phase 1: Images of 11 Day Run

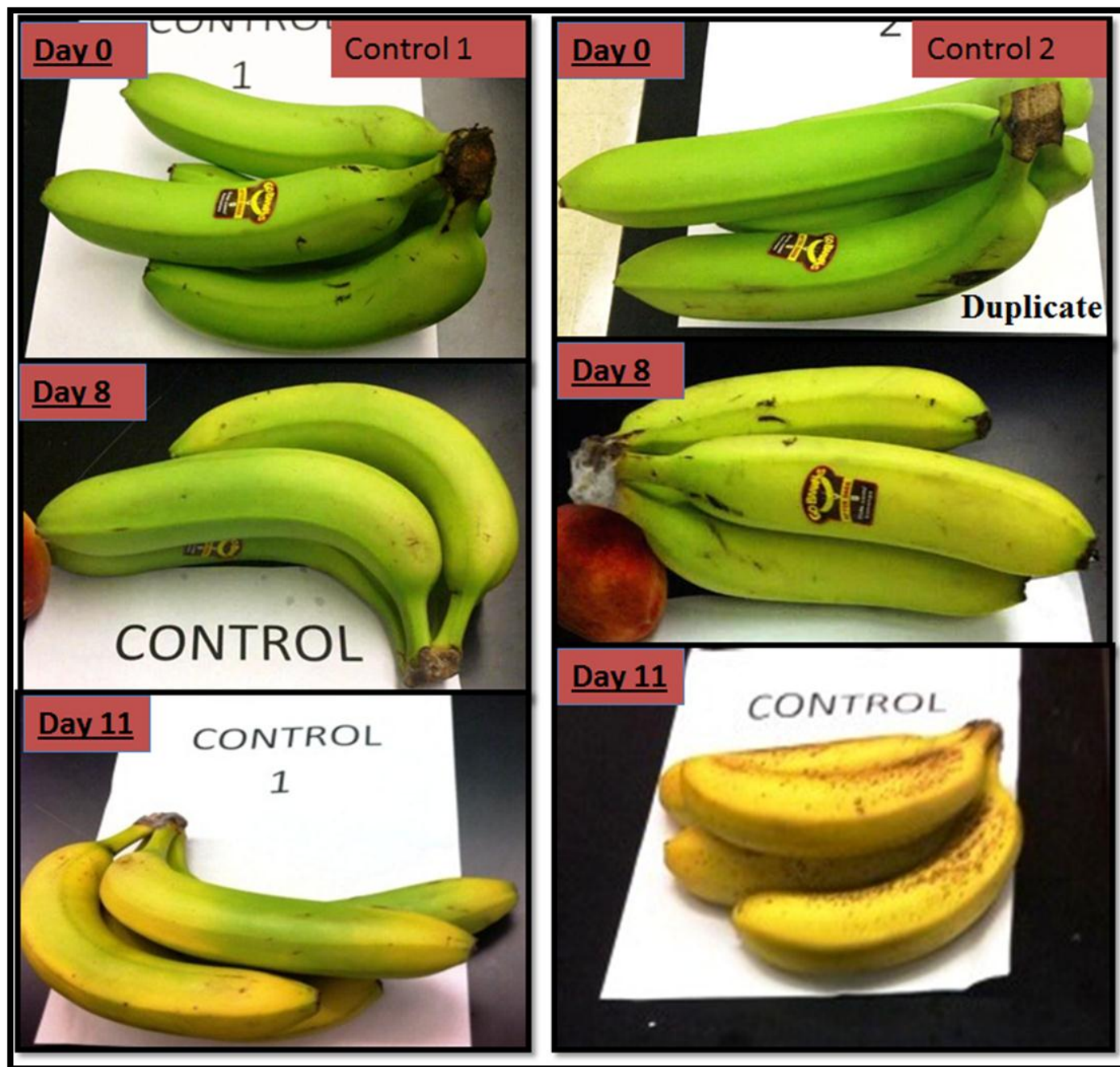


Figure 25: Comparison of duplicates initial fruit exposure (PHASE 1); Control bananas were to exposed to 35ml of M9 media only.

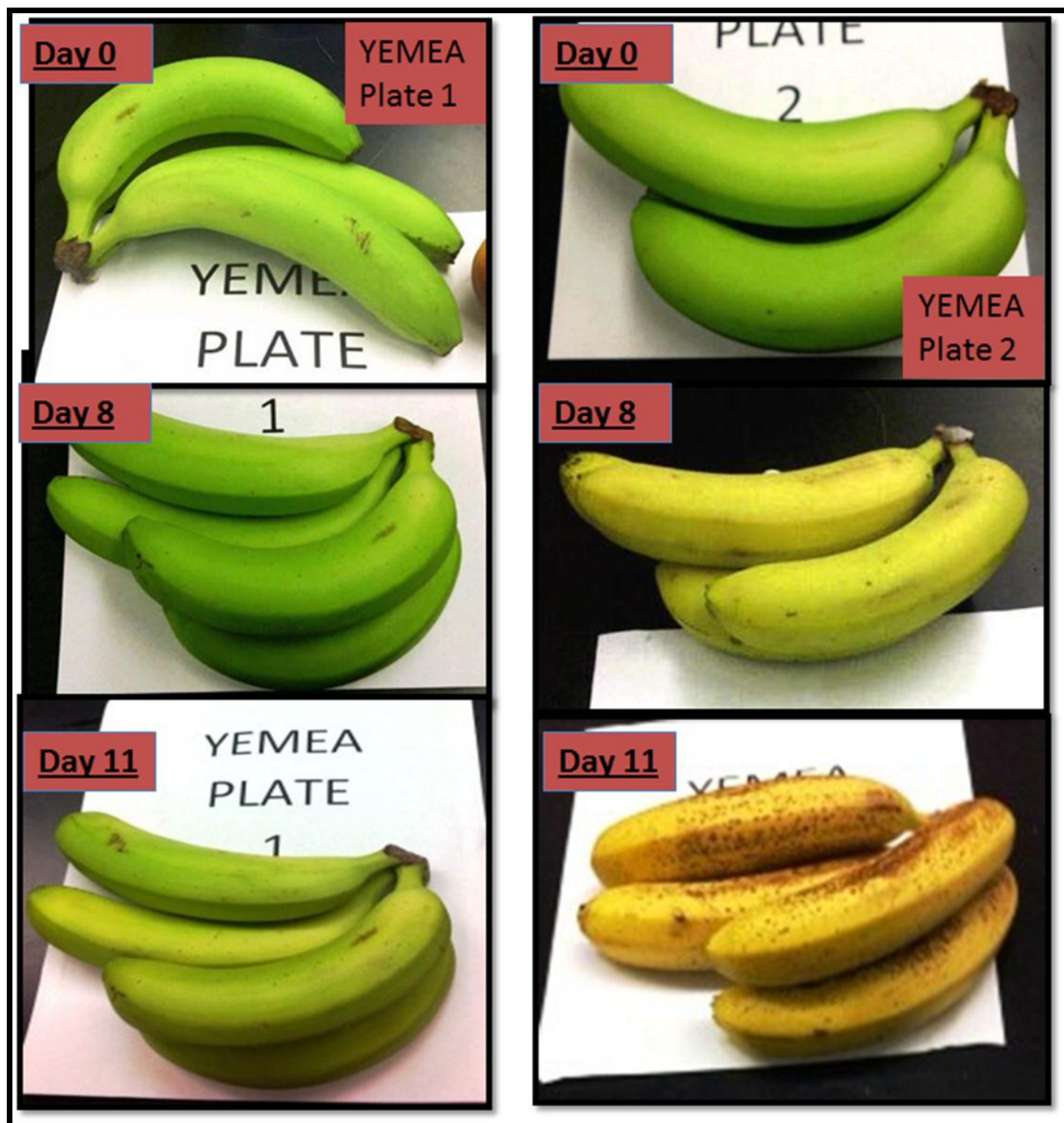


Figure 26: Comparison of duplicates for initial fruit exposure (PHASE 1); (YI) bananas were exposed to *R. rhodochrous* DAP 96253 cells that were cultured on YEMEA media with inducers (cobalt-0.201g/L & urea-7.5g/L) then exposed to bananas.

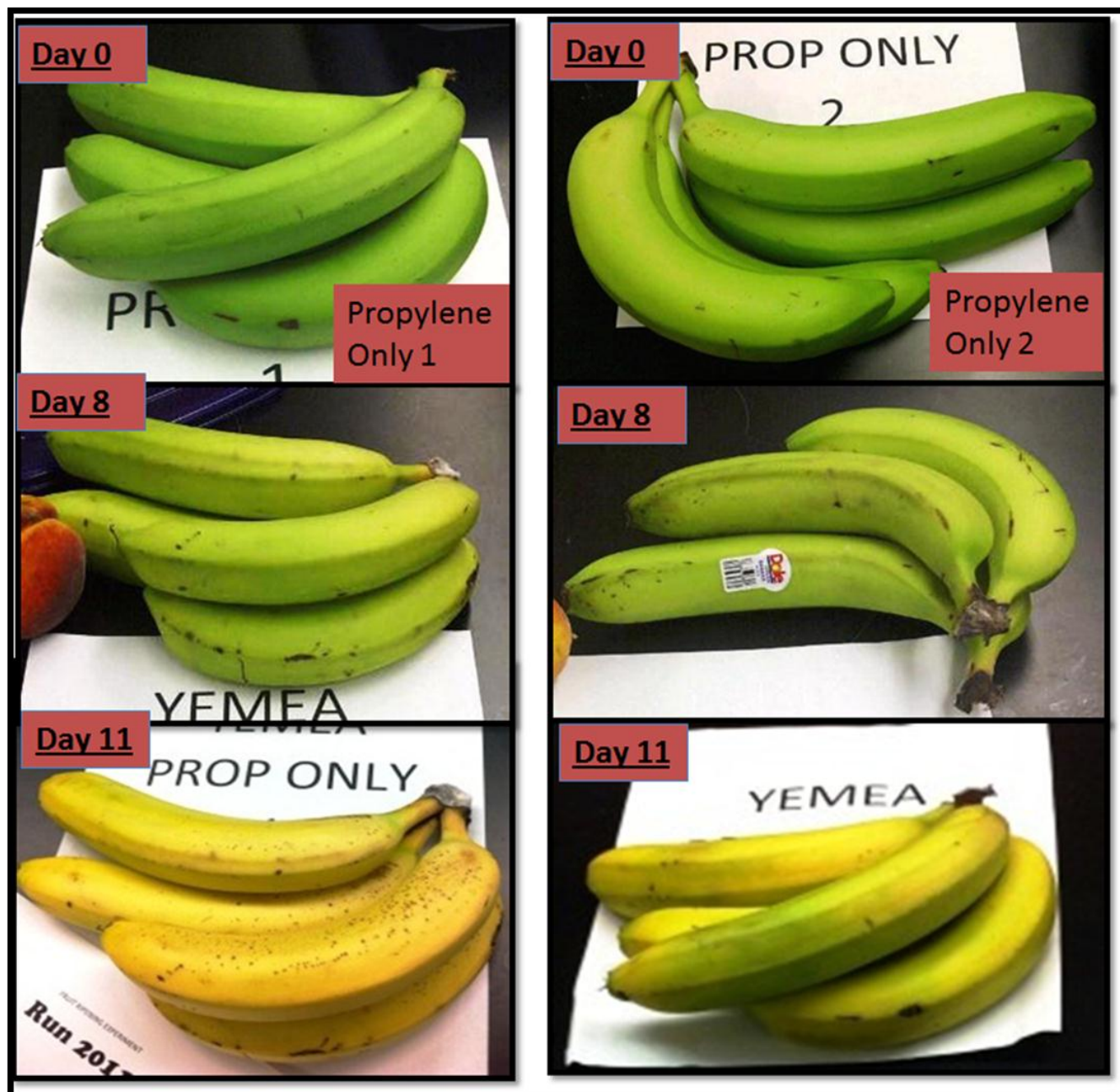


Figure 27: Comparison of duplicates for initial fruit exposure (PHASE 1); (YI-P) bananas were exposed to *R. rhodochrous* DAP 96253 cells that were cultured on YEMEA media with inducers (cobalt-0.201g/L & urea-7.5g/L), transferred to a biphasic flask with M9 media and propylene/ethylene, and then exposed to bananas.

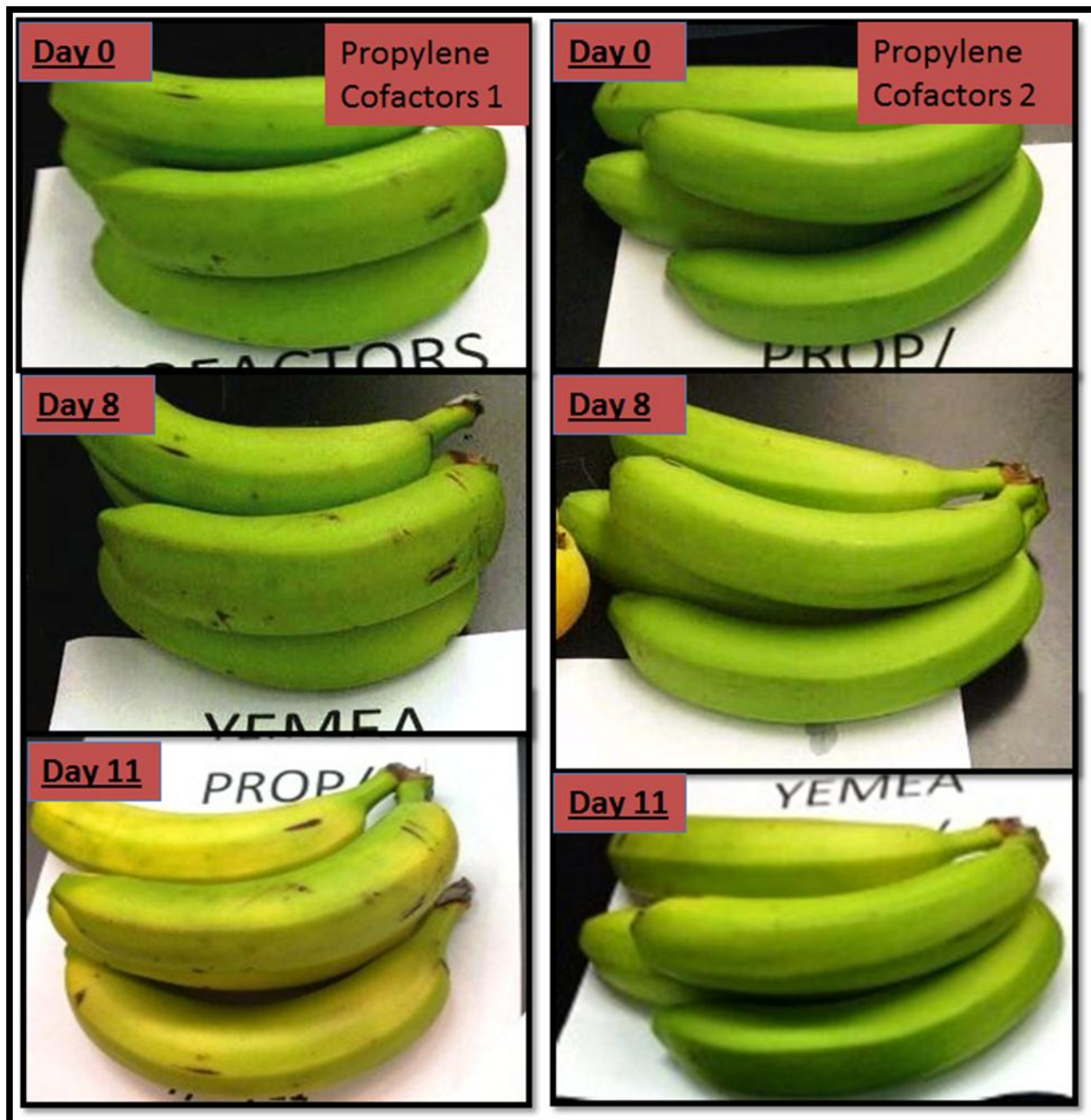


Figure 28: Comparison of duplicates for initial fruit exposure (PHASE 1); (YI-Cof) bananas were exposed to *R. rhodochrous* DAP 96253 cells that were cultured on YEMEA media with inducers (cobalt-0.201g/L & urea-7.5g/L), transferred to a biphasic flask with M9 media with inducers (cobalt-0.201g/L & urea-7.5g/L) and propylene/ethylene, and then exposed to bananas.

Phase 2 Images (8 Day Run w/ Recycled Cells)



Figure 29: Comparison of duplicates second fruit exposure (PHASE 2); Control bananas were exposed to 35ml of M9 media only.

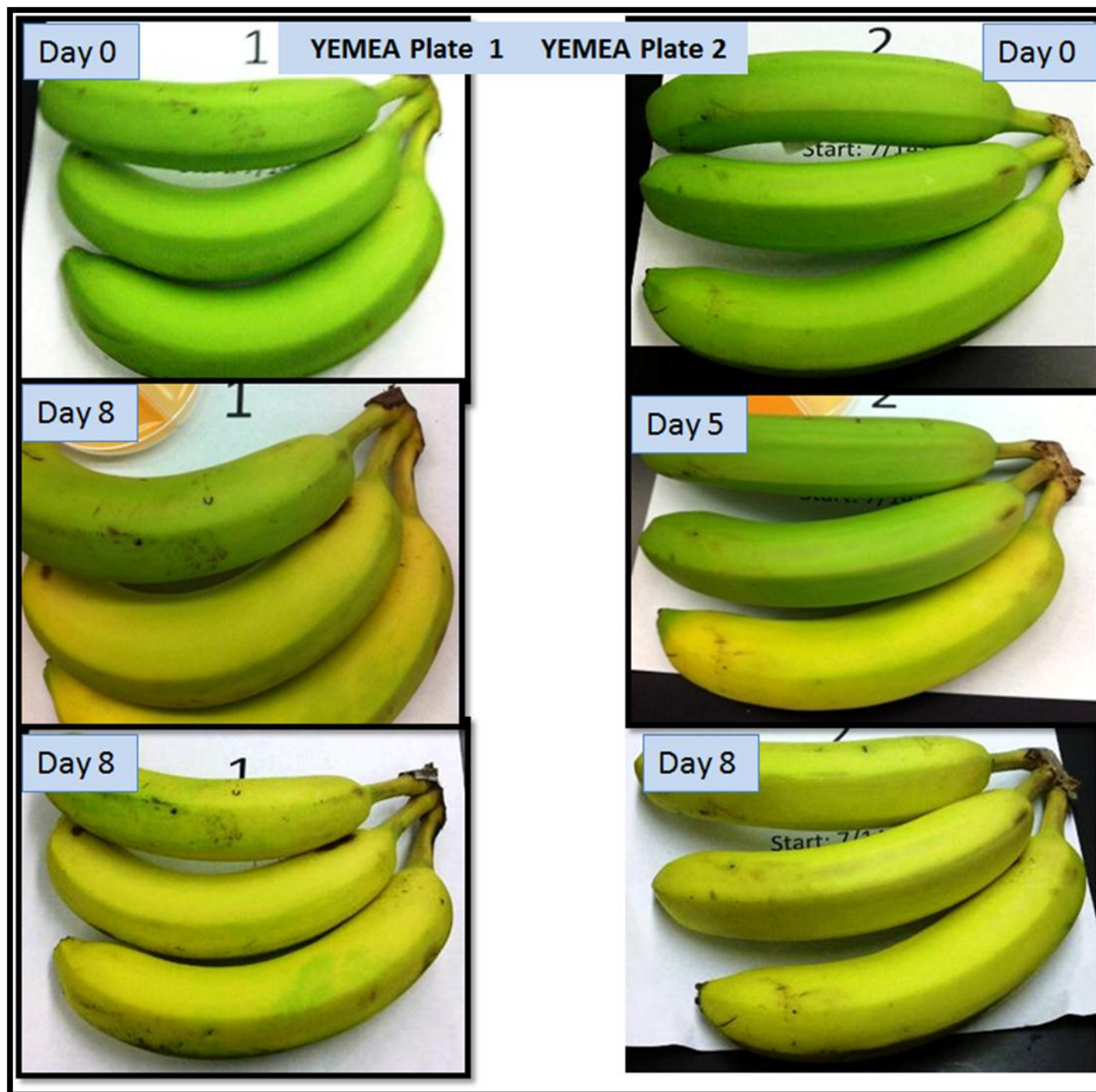


Figure 30: Comparison of duplicates for second fruit exposure (PHASE 2); (YI) bananas were exposed to *R. rhodochrous* DAP 96253 cells that were cultured on YEMEA media with inducers (cobalt-0.201g/L & urea-7.5g/L) then exposed to bananas.

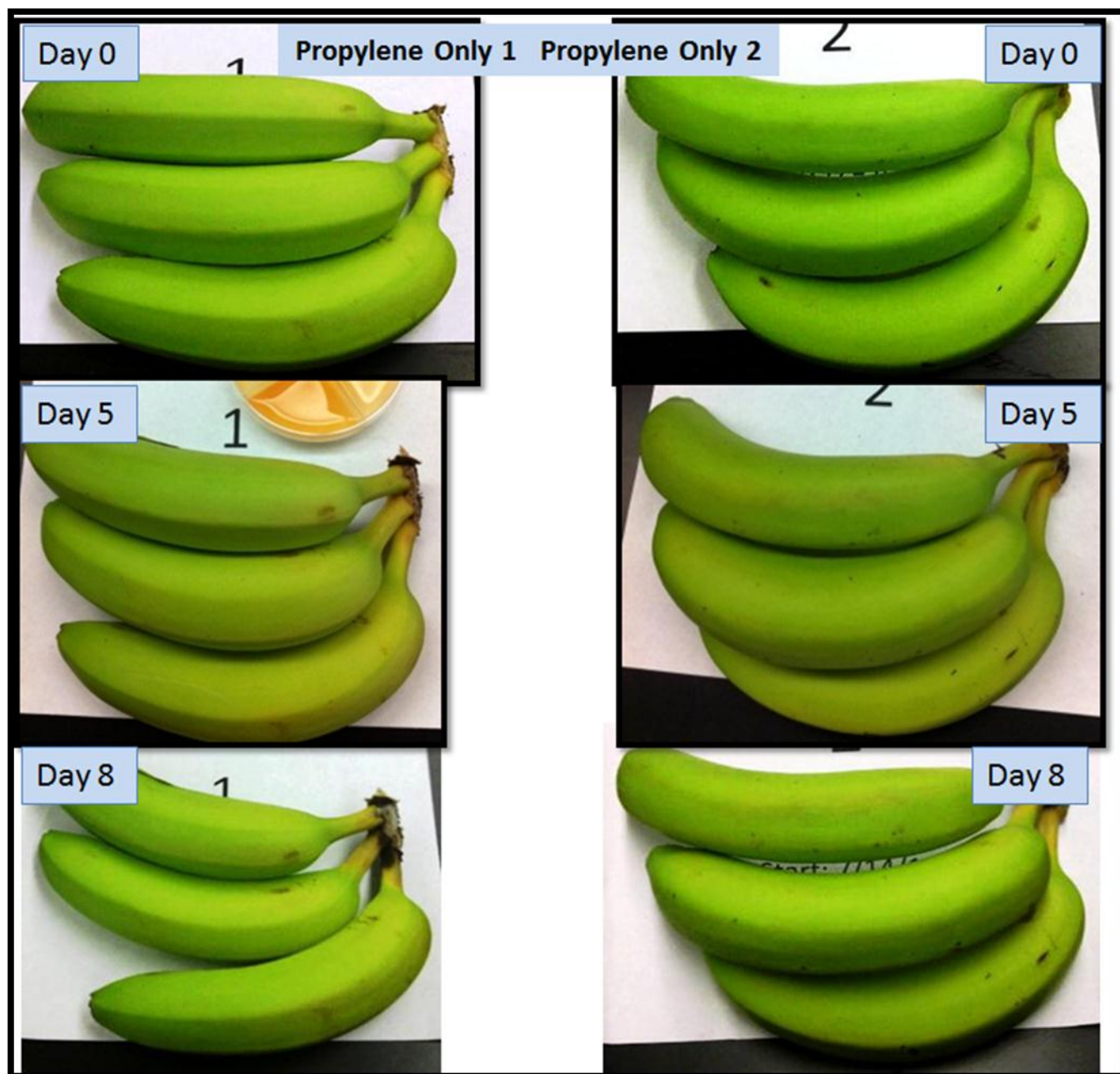


Figure 31: Comparison of duplicates for second fruit exposure (PHASE 2); (YI-P) bananas were exposed to *R. rhodochrous* DAP 96253 cells that were cultured on YEMEA media with inducers (cobalt-0.201g/L & urea-7.5g/L), transferred to a biphasic flask with M9 media and propylene/ethylene, and then exposed to bananas.

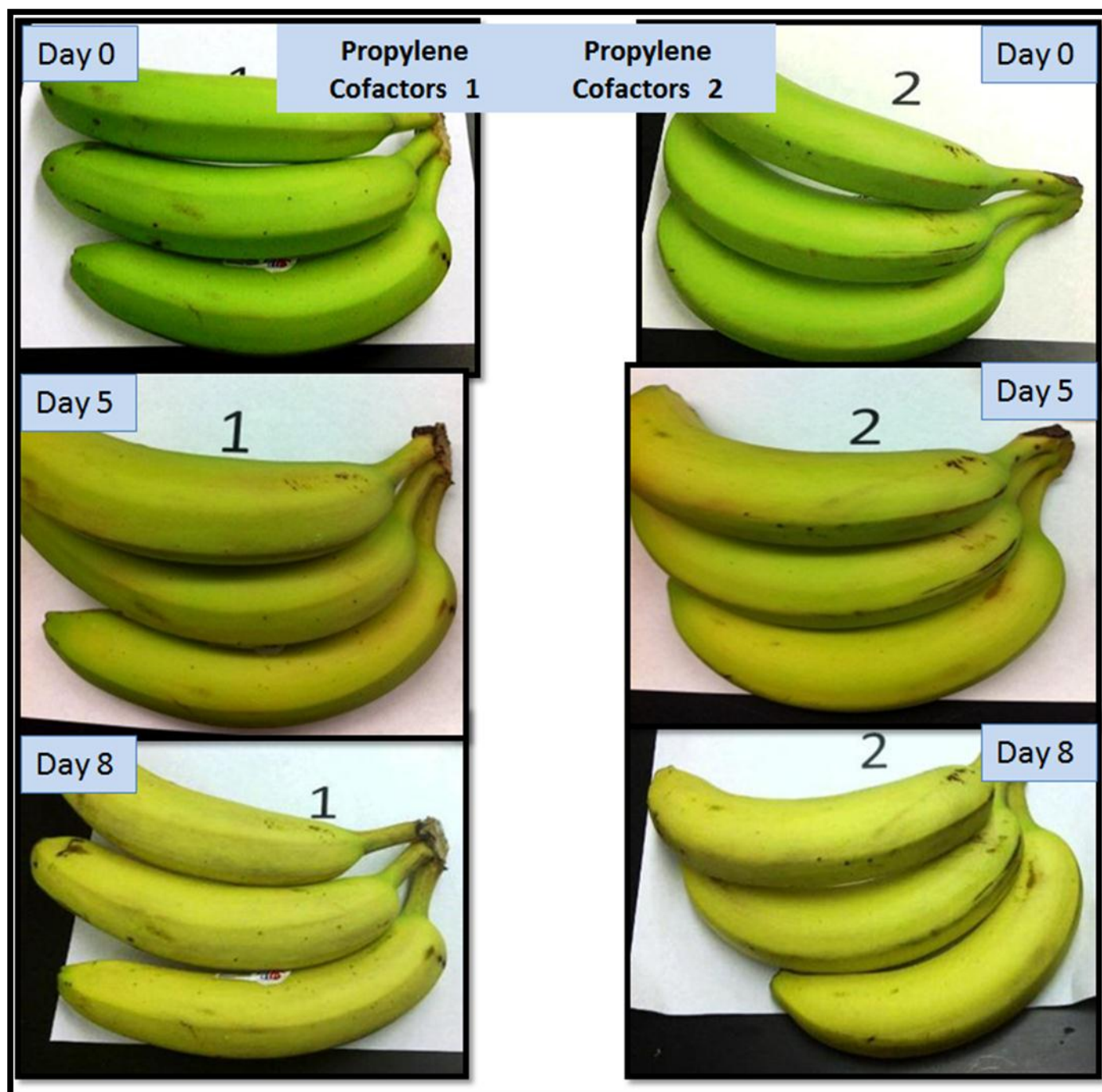


Figure 32: Comparison of duplicates for second fruit exposure (PHASE 2); (YI-Cof) bananas were exposed to *R. rhodochrous* DAP 96253 cells that were cultured on YEMEA media with inducers (cobalt-0.201g/L & urea-7.5g/L), transferred to a biphasic flask with M9 media with inducers (cobalt-0.201g/L & urea-7.5g/L) and propylene/ethylene, and then exposed to bananas.

DISCUSSION:

Propylene/ Ethylene Degradation by Using Rhodococcal Cells Grown on Induced YEMEA

Pierce *et al.* (2011) showed that *Rhodococcus rhodochrous* DAP 96253 cells cultured on YEMEA medium (supplemented with cobalt and urea) are able to delay the ripening of selected climacteric fruits, whereas non-induced cells were not as successful in delaying fruit ripening. Climacteric plants synthesize and release a number of volatile compounds, including ethylene, cyanide, and species-specific aroma chemicals into their surrounding environments. Many microorganisms can metabolize these compounds, and this can affect plant development (Teranishi *et al.*, 1993; Elsgard 2000).

Rhodococcus rhodochrous DAP 96253 cells cultured on YEMEA supplemented with inducers (cobalt and urea) medium were far more efficient at degrading propylene/ethylene gas than cells cultured on YEMEA alone. Induced cells displayed an 83% increase in total dry mass compared to non-induced cells (Table 4). The cobalt and urea inducers used in the YEMEA medium appear to induce AMO like enzymes that are capable of oxidizing propylene/ethylene to biodegradable intermediates (Smith *et al.*, 1997; Allen *et al.*, 1998; Ensign *et al.*, 2010).

Literature suggests that ethylene degrading enzymes such as AMO like enzyme are located on a linear plasmid in strains of *R. rhodochrous*, and that the AMO genes located on the plasmid are expressed when the cells are cultured on propylene and/or ethylene as sole carbon source (Saeki *et al.*, 1999; Perry *et al.*, 2006). The plasmids containing the AMO enzyme can be lost when cells are repeatedly cultured on a glucose rich medium (Gallagher *et al.*, 1997; Saeki *et al.*, 1999).

YEMEA supplemented with inducers (cobalt and urea) could possibly create a heavy-metal stress on *R. rhodochrous* DAP 962532 cells as shown by Kamnev et al. (2004). This causes the cells to retain and replicate plasmids, which could result in the retention of an AMO like enzyme. If this is the case for *R. rhodochrous* DAP 962532 then AMO activity (which is retained when cells are cultured on YEMEA supplemented with cobalt and urea) would be easily induced and expressed when the cells are cultured on propylene gas. This may give *R. rhodochrous* DAP 962532 cells cultured on YEMEA supplemented with inducers (cobalt and urea) media a competitive edge for propylene/ ethylene degradation.

Propylene Gas Acts as an Inducer for NHase, CDH, and Nitrilase

Propylene and/or ethylene induced NHase (and/or nitrilase) activity in *R. rhodochrous* DAP 92653 cells. *Rhodococcus rhodochrous* DAP 96253 cells cultured on YEMEA only medium with propylene/ethylene (YNI-P) displayed a 1-48 (unit/mg CDW) increase in NHase activity, whereas cells cultured on YEMEA supplemented with 2% glucose (YNI-2%) showed only a 1-10 (unit/mg CDW) increase in NHase activity (Table 5; Figure 8; Figure 9). Both the YEMEA-E/P and Y--2% cells were cultured without added metal inducers. This suggests therefore that increases in NHase activity are probably due either to nitrilase or NHase that may be induced using endogenous iron present in sugars used in the medium. Nitrilase does not require a metal cofactor but the enzyme is still capable of converting a nitrile compound into an acid and ammonia by product in the absence of amidase (Gupta *et al.*, 2010). *Rhodococcus rhodochrous* DAP 96253 cells cultured on YEMEA displayed an increase in nitrilase activity using benzonitrile as the substrate, while *R. rhodochrous* DAP 96253 cells cultured in the

presence of NHase inducers (cobalt and urea) displayed a decrease in benzonitrile specific nitrilase.

Induced *R. rhodochrous* DAP 96253 cells cultured on propylene and/or ethylene displayed a decrease in cyanide dihydratase (CDH) activity, but non-induced *R. rhodochrous* DAP 96253 cells displayed a slight increase in CDH activity (Table 5; Figure 10). The results suggest that propylene acts as an inducer for CDH, but YEMEA medium supplemented with inducers (cobalt and urea) is a better inducer of CDH. This may be due to the fact that YEMEA induced medium is supplemented with metals that may increase the activity of CDH.

The results from these experiments suggests that *R. rhodochrous* DAP 96253 cells cultured on induction medium with propylene/ethylene may convert propylene/ethylene into an epoxide ring. The epoxide may interact with HCN taken up by the cell to form corresponding cyanohydrins, which in the case of an ethylene epoxide will re-arrange to acrylonitrile (a known inducer of NHase in *R. rhodochrous* DAP 96253 cells) (Figure 33) (DeBont *et al.*, 1974; Ensign *et al.*, 1995; Pierce *et al.*, 2011). The over-expression of NHase/nitrilase is not attributed to increased translation of general housekeeping proteins; instead propylene/ethylene appears to cause an enzyme specific induction of certain nitrile hydrolyzing enzymes.

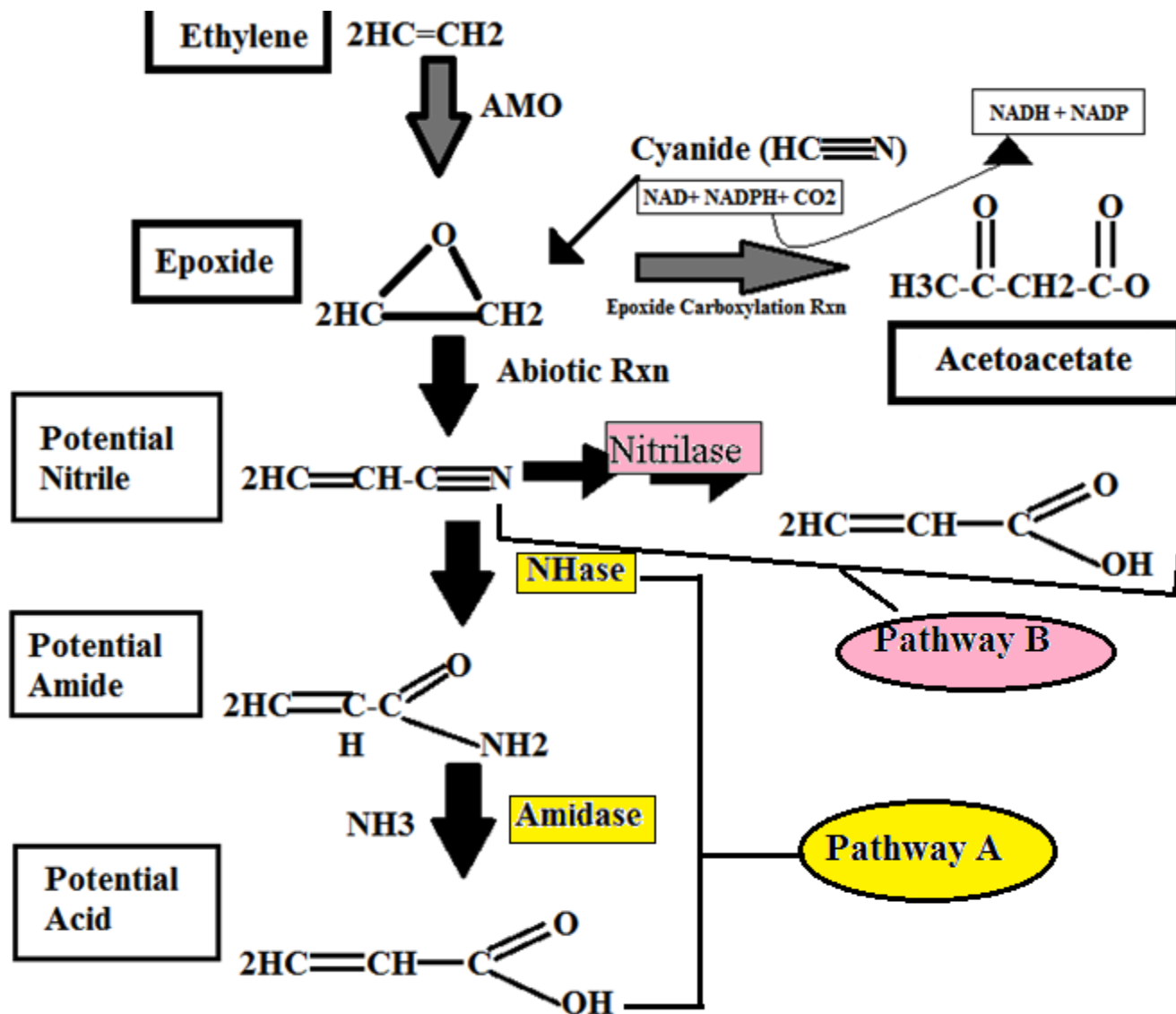


Figure 33: Modified Potential Ethylene Degradation pathway used by *R. rhodochrous* DAP 96253

Pathway (A): Used by *R. rhodochrous* DAP 96253 that were previously cultured on YEMEA induced media and induced for NHase and amidase activity.

Pathway (B): Used by *R. rhodochrous* DAP 96253 that were previously cultured on YEMEA non-induced media in the absence of inducers (cobalt and urea). (Pierce *et al.*, 2011)

Fruit Acts as an Inducer for NHase, CDH, and Nit enzymes

Rhodococcus rhodochrous DAP 96253 cells when exposed to bananas showed marked increase in NHase, nitrilase, and CDH activity. The increases in enzymatic activity may be attributed to volatile compounds released by the fruit (such as HCN, ethylene, or other volatiles compounds released by the fruit) that act as inducers for the enzymes (Table 6; Figure 11; Figure 12; Figure 13). If these enzymes can be induced in cells grown on YEMEA supplemented with the inducers cobalt and urea, and exposed to propylene and/or ethylene, then we can assume that the volatile gases produced by climacteric plants make at the very least ethylene + HCN. Plants (such as bananas) are able to react with and induce both AMO and nitrile metabolic activity.

Exposure of induced *R. rhodochrous* DAP 96253 cells to propylene/ethylene only induced NHase/nitrilase activity, but not CDH activity. However exposure to fruit resulted in increased CDH activity for induced and non-induced cells, CDH activity also increased the longer the cells were exposed to the fruit (Table 6; Table 9).

Climacteric fruits, vegetables, and flowers release ethylene and cyanide during the climacteric. CDH converts HCN to formic acid and ammonia (Figure 34) (Pierce *et al.*, 2011). When HCN reacts with appropriate substrates (such as ethylene epoxide) cyanohydrins can be formed. Cyanohydrins represent excellent substrates for NHase/Amidase (Figure 33; Figure 34) (Pierce *et al.*, 2011).

Fruit exposure appears to be optimal inducer for NHase, CDH, and nitrilase in *R. rhodochrous* DAP 96253 cells, the fruit recruits rhodococcal cells to delay ripening by supplying cells with volatile substrates that can act as enzyme inducers.

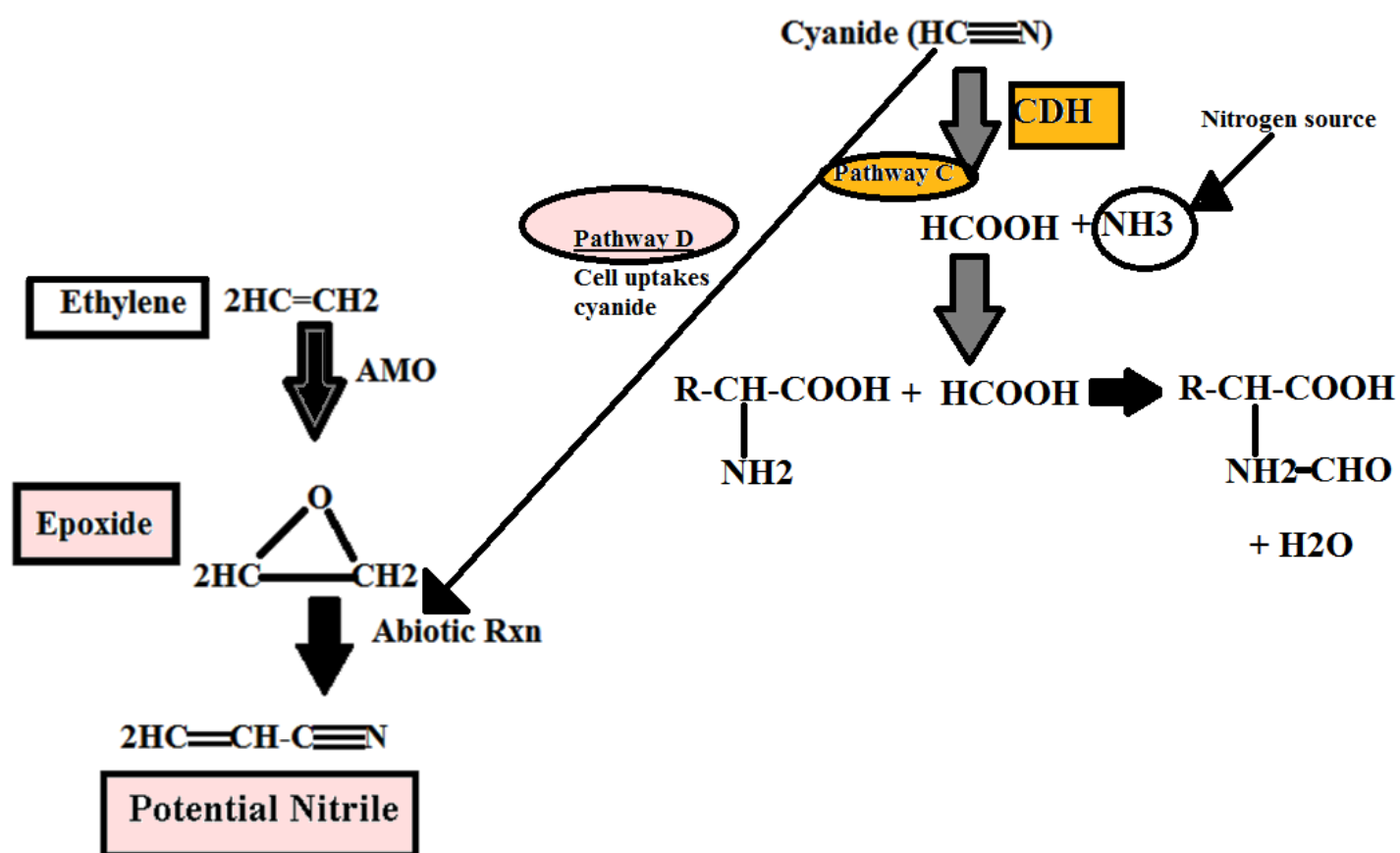


Figure 34: Modified Potential Ethylene Degradation pathway used by *R. rhodochrous* DAP 96253

Pathway (C): Used by *R. rhodochrous* DAP 96253 that were previously cultured on YEMEA induced or YEMEA non-induced media. CDH is used by cell to convert HCN into formic acid and ammonia; the products are used to make amino acids and nucleic acids in the cell.

Pathway (D): Cells uptake cyanide gas and abiotic reaction occurs and free nitrile is attached to epoxide compound forming a new nitrile compound. (Pierce *et al.*, 2011)

Comparing Fruit Ripening Results

Pierce *et al.* (2011) determined that *R. rhodochrous* DAP 96253 cells cultured on YEMEA media supplemented with inducers (cobalt and urea) had the ability to delay the ripening of climacteric fruits, however there were some variations. These variations may be attributed to differences treatment or handling of fruit; the variations may also be due to the absence of enzymes essential to enhance delayed ripening, such as an AMO like enzyme.

R. rhodochrous DAP 96253 cells were cultured on propylene/ethylene as sole carbon source and then exposed to fruit (organic apples and bananas). The controls displayed variations in the rates that the fruit ripened (Figure 25; Figure 29). YI cells exposed to fruits also displayed variations in the rate the fruit ripened, the variations were reduced when YI cells were harvested and exposed to new fruit for a second time (Figure 26; Figure 30).

When *R. rhodochrous* DAP 96253 cells were harvested and re-exposed to fruit for a second time; these cells displayed an increased ability to delay ripening (at similar rates in both duplicates). The initial fruit exposure may have induced expression of an enzyme that may play an essential role in delaying fruit ripening or served to stabilize enzyme activities. *Rhodococcus rhodochrous* DAP 96253 cells in the initial experiments utilized the ethylene released by the plants. The metabolization of ethylene suggests the cells were induced to express a AMO like enzyme; this may also lead to further induction of other enzymes. Results suggest the initial fruit exposure induced an AMO like enzyme; this increased the cells ability to delay fruit ripening during the second exposure. *R. rhodochrous* DAP 96253 cultured on propylene (YI-P) cells provide more reliable results than YI cells, but these cells also improved on delaying ripening during second exposure to fruit (Figure 27; Figure 31).

R. rhodochrous DAP 96253 cells cultured on propylene as a sole carbon source showed the greatest ability to delay fruit ripening (for those conditions employed). The results suggest that *R. rhodochrous* DAP 96253 cells cultured on propylene and/ or ethylene may induced cells to express AMO like activity, this enzyme induction may facilitate improved degradation of ethylene, which resulted in better looking fruit. Cells cultured on propylene and inducers (cobalt and urea) performed better than *R. rhodochrous* DAP 96253 cells induced on propylene alone. The inducers (cobalt and urea) helps the cells over express heavy mass Co-NHase.

R. rhodochrous DAP 96253 cells not cultured in the presence of inducers (propylene, cobalt, and urea) or cells harvested from nature are not as efficient at delaying the ripening of climacteric fruits. The cells are not exposed to the three inducers in high enough concentrations to force induction of CDH, nitrilase, or NHase essential for delaying ripening.

Inhibiting Microbial Growth may Enhance Delayed Ripening Ability of Cells

Previous experiments demonstrated that induced *R. rhodochrous* DAP 96253 cells had antifungal capabilities; fruit exposed to induced rhodococcal cells displayed little or no fungal growth on fruit (Pierce *et al.*, 2011). The presence of microbial species on fruit surfaces can affect the number of ethylene receptors produced by fruit cells, ripening is greatly influenced/impaired by the number of ethylene receptors on plant cells, this affects the rate that ethylene rebinds to ETR receptor complex (Trobacher *et al.*, 2009). Reduction in ethylene receptors (ETR) on plant cells will result in fewer ethylene molecules binding to ETR complexes, which will lead to a decrease in the rate at which fruit ripens. Induced *R. rhodochrous* DAP 96253 cells may also delay fruit ripening by controlling the microbial populations usually found on fruit.

CONCLUSION:

The results showed that *R. rhodochrous* DAP 96253 cells are capable of growing on propylene or ethylene as a sole carbon source. The results also showed that culturing the cells on propylene and/or ethylene induces enzymes (such as NHase and nitrilase) that are correlated with delayed the ripening of climacteric fruit. Culturing *R. rhodochrous* DAP 96253 cells on propylene and/or ethylene improved the cells ability to consistently delay fruit ripening. The results also showed that the fruit itself may play an essential role in *R. rhodochrous* DAP 96253 cells ability to delay ripening. Fruit continuously induces enzymes needed to delay fruit ripening (such as NHase, amidase, nitrilase, and CDH), the fruit also appears to be more efficient in inducing enzyme activity than propylene/ethylene or YEMEA supplemented media.

FUTURE DIRECTIONS:

1. Upscale potential AMO induction techniques to larger scale fermentation, using propylene gas and cofactors (CoCl₂ and urea).
2. Validate the presence of AMO enzyme, an AMO assay should be performed on *R. rhodochrous* DAP 96253 cells by using a GC to analyze the cells ability to convert propylene to propylene oxide.
3. Determine if exposing *R. rhodochrous* DAP 96253 cells to propylene gas changes the configuration of NHase enzyme; determine if potential configuration changes improve or alter enzymatic activity.
4. Identifying what components used in the YEMEA Induced plates (supplemented with CoCl₂ and urea) induce aliphatic nitrilase activity (capable of hydrolyzing acrylonitrile to an acid and ammonia). Also perform in vitro experiments with purified aliphatic nitrilase enzyme to compare activity to activity cited in previous literature to determine overall efficiency of this enzyme.
5. Use of 2D gel electrophoresis to compare the expression of proteins of cells cultured in YEMEA induction medium, YEMEA Non-Induction medium, propylene gas exposure, and fruit exposure. This technique will allow increase ability to search to expression of proteins essential for delaying fruit ripening that were not assayed for.

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APPENDIX:**Minimal Salts Media**

1. Make M9 salts
2. To make M9 Salts (**M9 Stock**) aliquot 800ml H₂O and add
 - 64g Na₂HPO₄-7H₂O
 - 15g KH₂PO₄
 - 2.5g NaCl
 - 5.0g NH₄Cl
 - Stir until dissolved
 - Adjust to 1000ml with distilled H₂O
 - Sterilize by autoclaving
3. Autoclave 700ml of distilled H₂O
4. Add 200ml of M9 salts (**M9 Stock**)
5. Add 2ml of 1M MgSO₄ (sterile)
6. Add 100ul of 1M CaCl₂ (sterile)
7. Adjust to 1000ml with distilled H₂O

M9 media contains no carbon source, unless stated otherwise. (Author Unknown 2005)

Reagent Preparation for Ammonia AssaysA. Sodium phenate solution

25 g Phenol (Sigma- Aldrich Co., St. Louis, MO) added to 800 ml water and 78 ml 4N sodium hydroxide (Sigma- Aldrich Co., St. Louis, MO) solution.

B. Sodium Nitroprusside Solution (0.1%)

1 g of Sodium Nitroprusside (Sigma- Aldrich Co., St Louis, MO) added to 100 ml DI water (stock solution). 1 ml of stock solution was added to 99ml DI water.

C. Sodium hypochlorite (0.02N)

2.44 ml of 6.15% Chlorox® made up to 100 ml with DI water.