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Induction of Cyanide Metabolizing Enzymes and Production of Antifungal Compounds by Rhodococcus Species

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INDUCTION OF CYANIDE METABOLIZING ENZYMES AND PRODUCTION OF ANTI-FUNGAL COMPOUNDS BY *RHODOCOCCUS* SPECIES.

by

KATHARINE SWENSEN

Under the Direction of Dr. George E. Pierce

ABSTRACT

Rhodococcus is a soil microbe known for its metabolic versatility. Cyanide is one of many compounds that *Rhodococcus* can detoxify. This work identified several inducers which affect the activity of two cyanide-metabolizing enzymes produced by *R. rhodochrous* DAP 96253, cyanidase and β -cyanoalanine synthase-like enzyme. Chemical inducers were added to growth media and cells were tested for the ability to transform cyanide to ammonia or hydrogen sulfide as a quantifiable measure of cyanidase or β -cyanoalanine synthase-like activity, respectively.

Urea-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 has been shown to inhibit germination of selected fungal spores. By varying supplements in growth media, several new compounds were identified which also enable *Rhodococcus* to inhibit germination.

This inhibition is achieved through non-contact co-culture between organisms. Properties of antifungal activity were studied for each supplement. It was shown that supplemented *R. rhodochrous* DAP 96253 is able to inhibit germination of single or mixed cultures the selected fungi, *Aspergillus niger*, *Aspergillus fumigatus*, and *Penicillium* sp. Depending on the supplemental compound added to growth media, spores were unable to recover after a maximum 48 hour exposure to *R. rhodochrous* DAP 96253. *Rhodococcus* effectively inhibits germination of spores when actively growing on agar or when removed from the plate.

Lastly, the effects of co-culturing three strains of YEMEA-grown *Rhodococcus* sp. with supplemented, YEMEA-grown *R. rhodochrous* DAP 96253 were examined to determine if supplemented *R. rhodochrous* DAP 96253 affected unsupplemented *Rhodococcus* sp. When co-cultured with cobalt- and urea-supplemented or just urea-supplemented YEMEA-grown *R. rhodochrous* DAP 96253, the three strains tested, *R. rhodochrous* DAP 96253, *R. rhodochrous* 96622, and *R. erythropolis* 47072 exhibited cyanidase enzyme levels comparable to those seen in directly induced cells. Additionally, after co-culture with urea-supplemented YEMEA-grown *R. rhodochrous* DAP 96253, YEMEA-grown cultures became inhibitory to germination of spores of *A. niger*. Overall, these findings showed that, with proper supplementation, *R. rhodochrous* DAP 96253 can be inhibitory to selected fungal species and is simultaneously able to stimulate antifungal activity in other, unsupplemented *Rhodococcus* sp.

INDEX WORDS: *Rhodococcus*, Cyanide, Cyanidase, β -cyanoalanine synthase, Fungal inhibition, rhododoccal volatiles

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by

KATHARINE SWENSEN

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Georgia State University

2013

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KATHARINE SWENSEN

Committee Chair: George E. Pierce

Committee: Sidney A. Crow, Jr

Eric S. Gilbert

Electronic Version Approved:

Office of Graduate Studies

College of Arts and Sciences

Georgia State University

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LIST OF ABBREVIATIONS

ACC.....	1-aminocyclopropane-1-carboxylate
ACCD.....	1-aminocyclopropane-1-carboxylate deaminase
ACN.....	Acrylonitrile
AMD.....	Amidase
AMO.....	Alkene monooxygenase
β -CAS.....	β -cyanoalanine synthase
CAR/PDMS..	Carboxy/Polydimethylsiloxane
CoCl ₂	Cobalt Chloride
CDW.....	Cellular dry weight
CNase.....	Cyanidase
CO ₂	Carbon dioxide
CYS.....	Cysteine
FID.....	Flame ionized detector
GC.....	Gas chromatography
Et-epoxide..	Ethylene epoxide
H ₂ S.....	Hydrogen sulfide
KCN.....	Potassium cyanide
HCN.....	Hydrogen cyanide
MET	Methionine
NaCl.....	Sodium Chloride
NB..	Nutrient broth
NH ₃ ..	Ammonia

NHase.....Nitrile hydratase
SAM.....S-adenosyl methionine
SPME..Solid phase microextraction
YEMEA..Yeast extract malt extract agar

1. INTRODUCTION

1.1 Background

Post-harvest loss destroys up to 50% of produce grown around the world (El-Gaouth, 1997). Of this, an estimated 25% is attributed to microbial deterioration, which may begin prior to harvest or during picking and storage (Droby, 2006). Climacteric fruits such as tomatoes, peaches, and bananas follow an ethylene-mediated ripening process, resulting in many metabolic adjustments which vary throughout the stages of ripening and senescence. Typically during the ripening process, stored starches are metabolized to their constituent sugars (Byrne *et al.*, 1991; Moneruzzamen *et al.*, 2008). The resultant decrease in pH as well as compromised tissue integrity, make produce more susceptible to fungal infection (Pitt and Hocking, 1997).

The damage that fungal infection causes is well characterized. *Aspergillus* can have detrimental effects on the appearance, flavor, and texture of fruits and vegetables (Perrone, 2007). A decline in these qualities makes the product less attractive to consumers, leading to economic loss. Species of *Penicillium* and *Aspergillus* are two of the primary organisms responsible for yam rot, which can begin prior to harvest but does not become visible until much later (Okigbo and Emeka, 2010). The ability of both organisms to grow under diminished moisture contents makes them especially persistent in contamination of stored harvest products (Tsitsigiannis, 2012).

Fungal crop damage extends beyond climacteric fruits and vegetables. In some tropical and subtropical regions, up to 50% of harvested grains are contaminated by fungi including *Aspergillus*, *Penicillium*, and *Fusarium* species (Hall, 1970; Magan and Aldred, 2007). In addition to the threat of product damage, many fungi produce dangerous spores and mycotoxins.

Multiple species of *Aspergillus*, *Penicillium*, and *Fusarium* have been shown to produce toxins which can be fatal to pigs, cows, and poultry when contaminated feedstock is ingested (Scott, 1964). Fungi are not only problematic in tropical zones, but have historically posed a significant threat to livestock in many other regions, such as Canada, the United States and Britain.

Current methods for reducing fungal load in grain storage involve complex, multi-stage management of pre- to post-harvest handling and storage. For example, pre-harvest plant stresses must be limited and proper sanitization employed during harvest. Water used for field irrigation may require regular testing to monitor and control microbial load (Matthews, 2006). Post-harvest conditions such as water availability, temperature, packaging, and many other factors must also be carefully regulated (Choudary and Kumari, 2010). When elimination of mycotoxins is unsuccessful, binding agents such as bentinol or polyvinyl-pyrrolidone may be added to feedstock to reduce detrimental health effects of mycotoxins (Trenholm *et al.*, 1989).

Chemically-derived fungicides and treatment by UV-irradiation are commonly employed to reduce post-harvest fungal impact. With repeated use, chemical fungicides decrease in efficacy, as the targeted organisms develop resistance mechanisms. Furthermore, optimal parameters for chemical application to fruit vary depending on the type of organisms present. The type of organisms present is not always immediately evident, thereby impacting the success of the chemical treatment (Zahavi *et al.*, 2007). Chemically treated produce is not widely accepted by the public and there is a growing interest in organic, and “green” treatment of fruits and vegetables.

Additional factors contributing to post-harvest loss include storage and handling conditions. Upon sustaining tissue injury, many plants begin releasing ethylene and other ripening-related signaling molecules. The same may occur if plants are depleted of moisture or subjected to fluctuations in temperature or humidity.

Multiply induced cells of *Rhodococcus rhodochrous* DAP 96253 have been shown to not only interact with plant ripening signal molecules, but also to exhibit antimicrobial activity, thereby establishing a dual-faceted approach to delaying the ripening of climacteric fruits, while protecting against fungi (Pierce, 2011).

1.1.1 Characteristics of rhodococcal species

The rhodococci species are Gram-positive, aerobic, non-motile microorganisms that undergo a coccus-to-rod lifecycle. Colony morphology varies based on nutrient composition. Most species of *Rhodococcus* produce either an orange or yellow pigment (Goodfellow, 1989) and can be isolated from soil, oceanic environments, and animal feces.

While some strains of *Rhodococcus* are known pathogens, such as *Rhodococcus equi* in animals and *Rhodococcus fascians* in plants, most are environmental saprophytes, many of which are useful industrially. Virulence in *R. equi* and *R. fascians* is conferred by extra-chromosomal elements, a linear plasmid in *R. fascians* and a circular plasmid in *R. equi* (Crespi *et al.*, 1992; Duquesne *et al.*, 2010). Industrial interest in *Rhodococcus* stems from their metabolic versatility and associated potential for degradation of hazardous materials (Kobayashi and Shimizu, 1998).

R. rhodochrous DAP 96253, when induced with aliphatic nitriles, shows nitrile hydratase (NHase), amidase (AMDase), cyanidase (CNase), β -cyanoalanine synthase-like enzyme (β CAS-like enzyme), 1-aminocyclopropane-1-carboxylate (ACC) deaminase, and alkene monooxygenase- (AMO) like activity. The primary enzymes of interest are described in detail, in the following sections.

1.2 Nitrile Hydratase

Nitrile hydratases are multi-subunit enzymes that hydrolyze nitrile and cyanohydrin compounds to their respective amides, which can be further hydrolyzed to an acid and ammonia by the enzyme, amidase (Figure 1) (Nagasawa and Yamada, 1990).

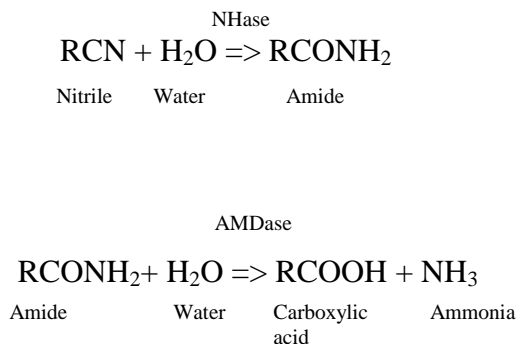


Figure 1: Mode of action of Nitrile Hydratase and Amidase (Nagasawa and Yamada, 1990).

Currently, two types of nitrile hydratases are recognized, those containing an iron cofactor and those with a cobalt cofactor. Some organisms are able to produce both forms of the enzyme, depending on which metal is available (Kobayashi and Shimizu, 1998). The NHase of *Rhodochrous* DAP 96253 can contain a cobalt cofactor when induced by supplementation of growth media with CoCl_2 (Pierce, unpublished).

Nitrile hydratase consists of equal numbers of the subunits, α and β . The quantity of α and β subunits present, as well as the size of each subunit, varies among organisms; however, β is usually larger (Banerjee *et al.*, 2002; Nagasawa and Yamada, 1990; Endo and Watanabe, 1989). The metal cofactor associates with the α subunit (Kobayashi and Shimizu, 1998).

Watanabe *et al.* (1987) identified yeast extract as the preferred basal media for optimal growth and production of NHase by *Rhodococcus* sp. N-774.

Kobayashi and Shimizu (1998) showed nitrile hydratase induction with urea and other amides. In *R. rhodochrous* DAP 96253, NHase activity is induced by addition of CoCl_2 to growth media (Pierce, unpublished).

1.3 β -cyanoalanine synthase

Cyanide is present in plants in several forms that are described below. In plants, cyanide can be detoxified via the enzyme β -cyanoalanine synthase. β -CNA synthase enables plants to not only detoxify cyanide, but also recycle its nitrogen for use in amino acid generation.

Bacteria that produce β -cyanoalanine are believed to do so using the enzyme *O*-acetylserine sulfhydrylase (Castric and Conn, 1971; Dunnill and Fowden, 1965). *R. rhodochrous* DAP 96253 has been shown to produce a β -cyanoalanine synthase-like enzyme (Pierce, unpublished).

The information about the mode of action of β -CNA synthase comes from the characterization of purified enzyme from plants. Cyanide acts as a nucleophile and is double-bonded at the sulfhydryl moiety (-SH) of L-cysteine, thereby converting HCN to β -cyanoalanine and H_2S (Larsen *et al.*, 2004; Machingura and Ebbs, 2010) (Figure 2). Serine is an alternate substrate for β -CNA synthase in plants.

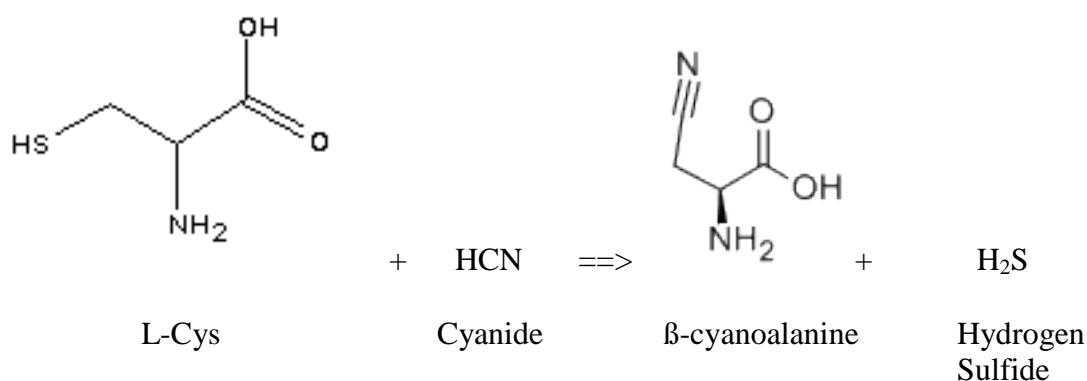


Figure 2: Enzymatic reaction of β-cyanoalanine synthase (Akopyan *et al.*, 1975; Larsen *et al.*, 2004).

In addition to detoxifying cyanide, β-CNA synthase enables organisms to utilize cyanide as a source of nitrogen. Organisms may incorporate reduced nitrogen into amino acid synthesis. For example, hydrolysis of β-cyanoalanine can produce the amino acid asparagine and/or aspartic acid (Hatzfield *et al.*, 2000; Knowles, 1976).

1.4 Cyanidase

A member of the nitrilase superfamily, cyanidase is a bacterial enzyme that hydrolyzes cyanide to formate and ammonia (Figure 3).

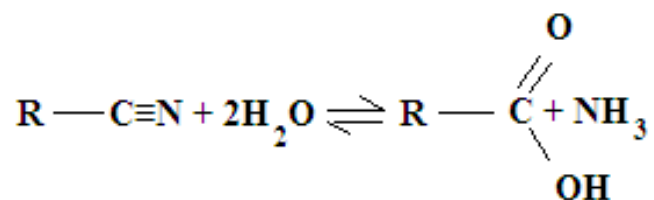


Figure 3: Pathway through which cyanide is degraded by cyanidase (Jandhyala *et al.*, 2003).

A relationship between cyanidase and amidase has been suggested (Kobayashi *et al.*, 1998; Pertsovich *et al.*, 2005). Both cyanidase and amidase directly hydrolyze their substrates to ammonia and acid with no intermediary.

The substrates of each, amides and nitriles, are structurally comparable in that both have a carbon-nitrogen bond. Kobayashi *et al.* (1998) identified a rhodococcal amidase with the ability to hydrolyze selected nitriles directly to ammonia and acid. Further work by Kobayashi *et al.*, (2008) suggests that amidase and nitrilase enzymes in *Rhodococcus rhodochrous* J1 share a common intermediate and one enzyme is inhibited by the substrate of the other.

Presently, there is little information about rhodococcal cyanidase-like activity. Jandhyala *et al.* (2005) investigated optimal conditions for cyanidase from *Bacillus pumilus* and *Pseudomonas stutzeri* AK61 and found that pH 7-8 and temperatures between 37°C-42°C are optimal for activity.

1.5 Cyanide

Cyanide is a simple compound consisting of a carbon triple bonded to nitrogen ($C\equiv N$). Cyanide can be present in the environment in various forms, including as a solid salt that is water-soluble and readily volatilizes. Cyanide toxicity is due to a very strong affinity for the iron moiety of the protein cytochrome c oxidase. This results in inhibition of the electron transport chain and ATP generation (Wantanabe *et al.*, 1998).

Cyanide is produced by thousands of ethylene-synthesizing plant species. Some notable examples include cassava, lima beans, almonds, peaches, and bananas. It has several roles in plant chemistry and may be present as cyanogenic glycosides, cyanohydrins, β -cyanoalanine, or given off by ripening plants in the form of HCN.

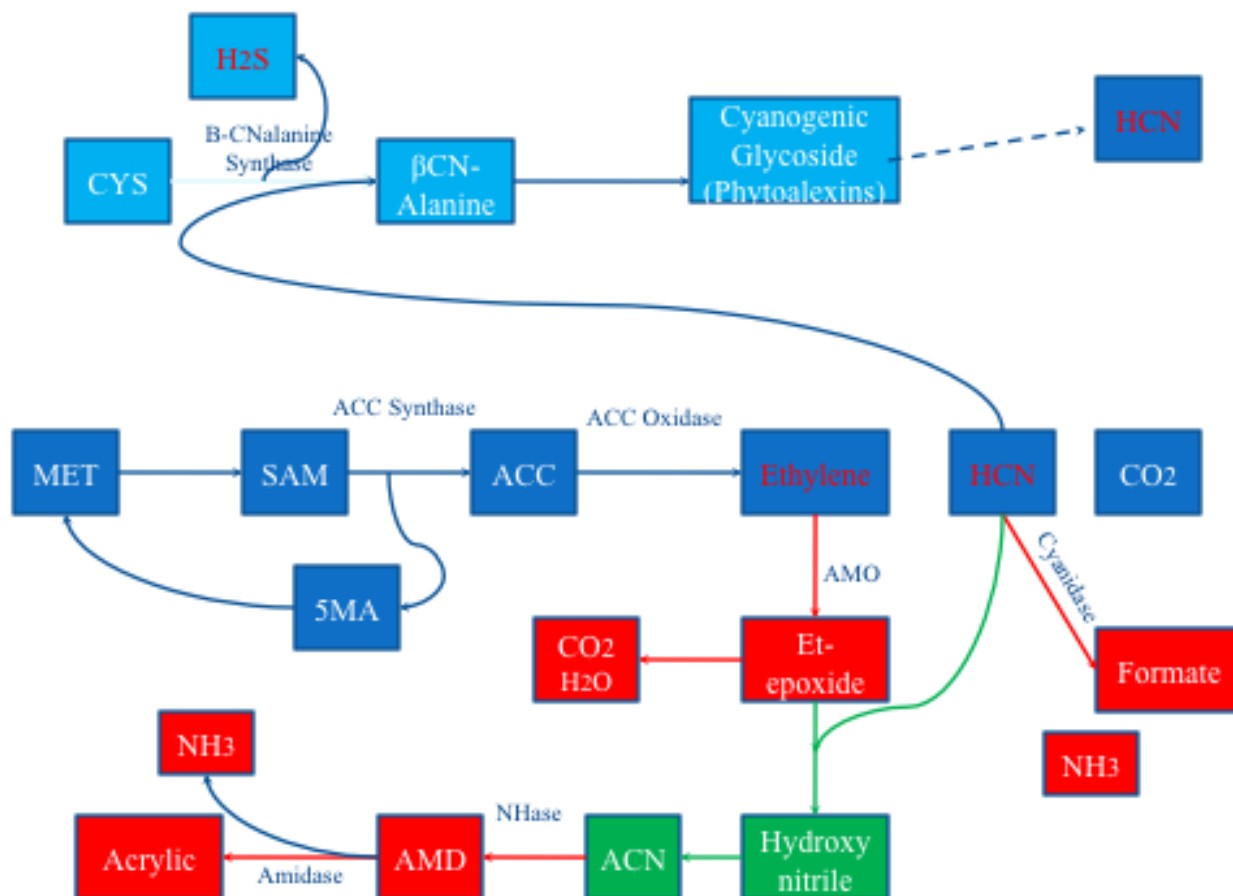


Figure 4: Schematic showing the possible integration of selected *R. rhodochrous* DAP 96253 enzymes and plant metabolism (Pierce, 2011).

1.6 Ethylene/Cyanide Pathway

Ethylene (C₂H₄) is a gaseous plant hormone associated with ripening of climacteric fruits. Some phenotypic changes attributed to the climacteric burst of ethylene include the softening of fruit tissue, color maturation, and increased sugar content as starches are converted to sugar (Theologis, 1992). In plants, ethylene is produced from the amino acid, methionine, which is metabolized to S-adenosylmethionine (SAM). SAM has many uses in a plant, including cell wall and membrane synthesis, cell division, and more.

Prior to ethylene production, SAM can either be recycled back to methionine or converted into 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor for ethylene with cyanide and carbon dioxide (Amir, 2010; Peiser *et al.*, 1984, Yang, 1984) (Figure 4).

Once produced, cyanide can be subjected to one of several possible fates. It may be transformed to cyanoalanine by the enzyme β -CNA synthase and later incorporated into cyanogenic glycosides. Cyanide can be hydrolyzed to formate and ammonia by a variant of the enzyme cyanidase, or may undergo an abiotic reaction with ethylene epoxide to form cyanohydrins (Figure 4). *R. rhodochrous* DAP 96253 has been shown to have both cyanidase and β -CNA synthase-like activity (Pierce, unpublished).

1.7 Cyanohydrins

Naturally present in plants, there are several pathways by which cyanohydrins can be formed. Cyanohydrins can be generated enzymatically by hydrolysis of cyanogenic glycosides (Xu *et al.*, 1988). In the absence of enzymatic catalysis, cyanohydrins can be formed chemically by a nucleophilic reaction between cyanide and the electrophilic carbonyl group of aldehydes and ketones. The previously described plant hormone, ethylene, can be oxidized to an epoxide, ethylene oxide (Figure 5).

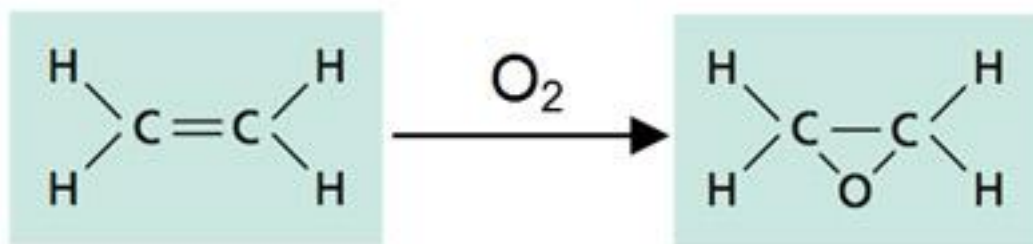


Figure 5: Oxidation of ethylene to ethylene oxide (Taiz and Zeiger, 2010).

Ethylene oxide is an unstable ring structure with an affinity for nucleophilic compounds (Marczynski *et al.*, 1995). In the presence of cyanide, ethylene oxide may be converted to ethylene cyanohydrin, $\text{OHC}_2\text{H}_4\text{CN}$. Ethylene cyanohydrin can be dehydrated to form acrylonitrile, which is a substrate for the nitrile hydratase of *Rhodococcus rhodochrous* DAP 96253. *R. rhodochrous* DAP 96253 and 96622 have been shown to degrade acetone cyanohydrin (Pierce, unpublished). Additionally, *Rhodococcus erythropolis* NCIMB 11540 produces nitrile hydratase with activity against some cyanohydrins (Osprian *et al.*, 2003).

1.8 Delayed Fruit Ripening

When multiply induced, cells of *Rhodococcus rhodochrous* DAP 96253 have been shown to delay the ripening of selected climacteric fruits including, but not limited to, bananas, peaches, avocados, and apples (Pierce *et al.*, 2011) (Figure 6). The progression of ripening, which includes changes in appearance, tissue firmness, sugar content, and pH, was delayed by as much as 10 days when peaches were stored in close proximity to *Rhodococcus rhodochrous* DAP 96253 (Pierce *et al.*, 2011). It was noted that the longest delay in fruit ripening was caused by cells of *R. rhodochrous* DAP 96253 that were induced for high levels of NHase (Pierce *et al.*, 2011).



Figure 6: Control peaches (left) and peaches stored with induced *R. rhodochrous* DAP 96253 (right). Picture taken after 7 days (Pierce, unpublished).

1.9 Fungal Inhibition

Studies of rhodococcal ability to delay fruit ripening conducted at Georgia State University led to the observation that control fruit showed substantially more fungi than experimental fruits. Based on these observations, experiments were conducted, which showed that induced cells of *R. rhodochrous* DAP 96253 were able to inhibit spores germination of various filamentous fungi including *Fusarium* species, *Cladosporium* species, *Penicillium* species, and *Aspergillus* species (Pierce, unpublished). Non-contact culture set-ups suggest that fungal inhibition may be achieved through rhodococcal production of volatile compounds.

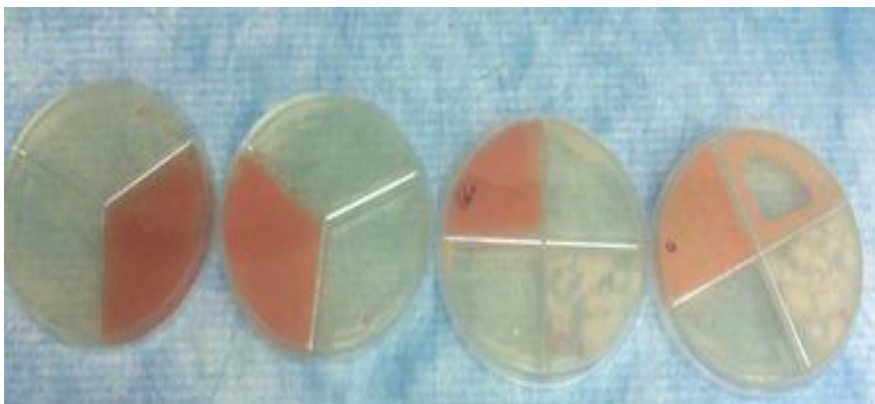


Figure 7: Fungal inhibition by *Rhodococcus* after 7 days. *Rhodococcus* was grown on media supplemented (from left to right) with urea only, cobalt and urea, cobalt only, and no supplements in proximity to the same concentration of *Fusarium* spores under all conditions.

1.10 Purpose of the Study/Rationale

This is a multi-phase work intended to identify compounds that increase activity of the cyanide metabolizing enzymes, cyanidase and β -cyanoalanine synthase-like enzyme in *R. rhodochrous* DAP 96253, as well as study inhibition of fungal spore germination. As growth supplements were examined for their effect on these enzymes, a correlation was identified with the induction of rhodococcal inhibition of fungal germination. These observations led to experimentation to simultaneously promote cyanidase and β -CAS activity as well as production of antifungal compounds. Through these experiments, various aspects of fungal inhibition were studied and the properties of *Rhodococcus* that cause this inhibition were investigated.

The information obtained from this work has great industrial potential. Inducing rhodococcal enzymes that transform cyanide could serve as a preliminary step in developing a biodegradation catalyst. Optimizing the organism for degradation of the compounds of interest prior to immobilization and application is a critical step for an efficient degradation model. *Rhodococcus* could become an environmentally friendly, cost-effective platform for biocatalysis of cyanide-containing compounds.

Additionally, the ability of *Rhodococcus* to inhibit fungal germination has far-reaching potential both industrially and agriculturally. This work demonstrates that induced *R. rhodochrous* DAP 96253 is highly effective at inhibiting germination of selected fungal species. Because *Rhodococcus* is a soil microbe, it is a very sensible choice for use as a biocontrol agent throughout the various stages of farming and harvesting agricultural products. It is already present and able to survive in that environment. Additionally, environmental strains of rhodococci are typically not pathogenic to humans, making them an optimal organism for agricultural application. The ability of induced *Rhodococcus* to activate uninduced cells implies its capacity to generate extensive cultures of antifungal organisms through use of minimal concentrations of induction compounds. This work was completed with the following aims:

1. To identify the most effective inducers of cyanidase and β -cyanoalanine synthase like enzyme in *R. rhodochrous* DAP96253.
2. To identify growth media supplements that enable *Rhodococcus* to inhibit germination of selected fungal species.
3. To evaluate the effects of supplemented *Rhodococcus* on selected fungal species, as well as unsupplemented *Rhodococcus* sp.
4. To characterize rhodococcal volatiles using gas chromatographic analysis.

2. MATERIALS AND METHODS

2.1 Microbiological

Yeast extract malt extract agar (YEMEA) was used as a base component for the media employed in this study (Dietz and Thayer, 1980).

YEMEA broth consists of 4 g yeast extract, 10 g malt extract, and 4 g glucose (Becton Dickson and Co, Sparks, MD) made up to 1 L with dH₂O. 20 g agar was added to make YEMEA plates. Additional chemicals were added to basic YEMEA for induction experiments (Table 1).

The microorganisms used, *Rhodococcus rhodochrous* DAP 96253 (ATCC 55899), *Rhodococcus rhodochrous* 96622 (ATCC 55898), and *Rhodococcus erythropolis* (ATCC 47072) were acquired from the American Type Culture Collection (ATCC).

Stocks of each organism were made by combining 500µl from a nutrient broth culture with 500µl of 60% glycerol and stored at -80°C until revival by inoculation in 75 ml of Nutrient Broth (NB). Culture growing in NB was incubated at 30°C, shaking, for 48 hours. After 48 hours, Nutrient Agar (NA) plates were inoculated with 100 µl of the NB culture. Inoculated NA plates were incubated at 30°C for 48 hours, at which time cells were scraped into YEMEA broth. YEMEA plates were inoculated by spreading 100 µl of the broth suspension on each plate and incubated at 30°C for 7 days.

The fungi used, *Aspergillus niger* AEM 97-001, *Aspergillus fumigatus* AEM 97-002, and *Penicillium* sp AEM 97-003, were initially obtained from indoor air samples and stored as part of the Georgia State Culture Collection, maintained by Dr. Sidney Crow, Jr. Prior to spore harvesting, these cultures were grown on nutrient agar plates until production of spores was visible. Spores were harvested and aliquots stored at -20°C in 0.9% NaCl until use in antifungal assays.

Table 1: Concentration and manufacturer of chemicals added to YEMEA for induction experiments.

Chemical Inducers	Concentration	Supplier
Cobalt + Urea	0.85 mM cobalt, 200 mM urea	Cobalt: EMD Chemicals Gibbstown, NJ Urea: Fisher Scientific Fairlawn, NJ
Urea	200 mM	Fisher Scientific Fairlawn, NJ
Methacrylamide	40 mM	Sigma-Aldrich St. Louis, MO
Potassium Nitrate	200 mM	Mallinckridt St. Louis, MO
Acetamide	200 mM	Fisher Scientific Fairlawn, NJ
Methyl carbamate	200 mM	Sigma-Aldrich St Louis, MO
Methyl urea	200 mM	Sigma-Aldrich St Louis, MO
Ethyl urea	200 mM	Sigma-Aldrich St Louis, MO
Melamine	24 mM	Sigma-Aldrich St Louis, MO
Allantoin	200 mM	Sigma-Aldrich St Louis, MO
Hydantoin	200 mM	Sigma-Aldrich St Louis, MO
Histidine	200 mM	Sigma-Aldrich St Louis, MO
Phenylacetaldehyde	200 mM	Sigma-Aldrich St Louis, MO
Glutamine	7 mM	Sigma-Aldrich St Louis, MO
Methacrylamide	22 mM	Sigma-Aldrich St Louis, MO
Cobalt + Urea + Methacrylamide	0.85 mM cobalt, 200 mM urea, 22 mM methacrylamide	Cobalt: EMD Chemicals Gibbstown, NJ Urea: Fisher Scientific Fairlawn, NJ Methacrylamide: Sigma-Aldrich St. Louis, MO
Cobalt + Urea + Methacrylamide	0.85 mM cobalt, 200 mM urea, 40 mM methacrylamide	Cobalt: EMD Chemicals Gibbstown, NJ Urea: Fisher Scientific Fairlawn, NJ Methacrylamide: Sigma-Aldrich St. Louis, MO
Nickel	200 μ m	Sigma-Aldrich St Louis, MO
Iron	200 μ m	Sigma-Aldrich St Louis, MO
Hydroxyphenyla- cetamide	200 mM	Sigma-Aldrich St Louis, MO
Potassium Cyanide	50, 100, 150 μ m	JT Baker Phillipsburg, NJ
Asparagine	8 mM	Sigma-Aldrich St Louis, MO
Glutamine + As- paragine	7 mM Gln, 8 mM Asn	Both: Sigma-Aldrich St Louis, MO
Glutamine + Urea	7 mM gln, 200 mM urea	Gln: Fisher Scientific Fairlawn, NJ Urea: Sigma-Aldrich St Louis, MO
Potassium Nitrate + Urea	200 mM each	KNO ₃ : Mallinckridt St. Louis, MO Urea: Fisher Scientific Fairlawn, NJ
Serine	9 mM	Sigma-Aldrich St Louis, MO
Zinc Sulfate	100 μ m, 200 μ m	Fisher Scientific Fairlawn, NJ
Lead	100 μ m, 200 μ m	Fisher Scientific Fairlawn, NJ
Cobalt + Urea+ Iron	0.85 mM cobalt, 200 mM urea, 100 μ m iron	Cobalt: EMD Chemicals Gibbstown, NJ Urea: Fisher Scientific Fairlawn, NJ Iron: Sigma-Aldrich St Louis, MO

2.1.1 Spore harvest

Three nutrient agar plates were inoculated with 100 µl of spores of *Aspergillus niger* AEM 97-001, *Aspergillus fumigatus* AEM 97-002, or *Penicillium* sp AEM 97-003 and grown until visibly sporulated. Spores were harvested by twice pipetting 25 mL of sterile Conidia Harvest Solution (see appendix) over the plate and using a sterile glass rod to disperse the spores into solution. Approximately 50 mL of solution was collected in a 50 mL Falcon centrifuge tube. This was centrifuged for 10 minutes at 3000 rpm (International Equipment Company, United States). The supernatant was discarded and the pellet resuspended in 50 mL of 0.9% NaCl. The pellet was washed repeatedly in 50 mL of fresh 0.9% NaCl until supernatant was clear.

The solution was then filtered through a sterile funnel containing sterile glass wool to remove mycelia. Filtered solution was collected in a fresh tube. The spore solution was counted by adding 10 µl per well to a hemocytometer and viewed on a Nikon Optiphot-2 microscope. The suspension was diluted until a concentration of 1×10^4 spores/mL was achieved. The solution of 1×10^4 spores/mL was aliquoted into 1 mL portions and stored in sterile cryovials at -20°C .

2.1.2 Co-culture set up

R. rhodochrous DAP 96253 was grown on a 35 mm diameter YEMEA plate for 7 days at 30°C prior to co-culture set up. One week after inoculation of *Rhodococcus*, 1×10^2 spores of selected fungi were inoculated on a 35 mm diameter Petri dish containing YEMEA. In order to achieve non-contact, shared airspace conditions, one bacteria plate and one fungal plate, with covers removed, were placed in a sterile 100mm diameter Petri dish and the dish sealed with parafilm. Co-cultures were returned to a 30°C incubator for 48 hours prior to visual evaluation of results.

2.1.3 Spore recovery assays

A variation of the above described co-culture was employed to determine if 1×10^2 spores of *Aspergillus niger* AEM 97-001 would recover after non-contact exposure to supplemented YEMEA-grown *Rhodococcus*. Sterile Whatman® filter paper, cut into a circle (30 mm diameter) was placed over the surface of an YEMEA plate prior to inoculation with 1×10^2 spores. The spore inoculum was then co-cultured with *Rhodococcus* grown on YEMEA supplemented with urea or methylcarbamate or acetamide or methacrylamide for 24, 48, or 72 hours. At each time interval, the spore inoculum was removed from the co-culture, the filter paper aseptically transferred to a fresh YEMEA plate, and returned to the 30°C incubator for 72 hours. Plates were screened visually for germination after removal from co-culture.

2.1.4 Directed airflow

Cells of *R. rhodochrous* DAP 96253 were inoculated on YEMEA plates, parafilm individually, and incubated at 30°C for 7 days. Plates were then assembled in a one-directional flow cell system. Four one-liter jars were connected with 0.25-inch diameter Eastman Poly-Flo® tubing (Kingsport, TN). The first three jars contained up to 3 (100 x 60mm) Petri plates of urea-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 (Figure 1, appendix). The final jar in the series contained one YEMEA plate inoculated with approximately 5×10^2 spores of *A. niger* AEM 97-001. Filtered air was drawn through the tubes at 6 cc's/minute using a Masterflex (Vernon Hills, IL) peristaltic pump. After exiting the final jar, remaining volatiles were passed through Norprene® (Rochester, NY) 0.25-inch tubing and outgassed into a flask containing 25 ml of dH₂O.

2.2 Quantification of Enzyme Activity

2.2.1 Nitrile Hydratase

The nitrile hydratase assay was done using a variation of the ammonia production assay described by Fawcett and Scott (1960). 50 mg of rhodococcal cells, wet weight, were suspended in 1 ml of 50 mM Phosphate Buffer (PB) and vortexed thoroughly. This was added to 9 ml of 1000 ppm acrylonitrile for 2 minutes. After two minutes, 1 ml was removed from the reaction solution and acidified using 10 μ l of 2N H₂SO₄ to halt continued NHase activity. The sample was centrifuged at 13,000 rpm for 2 minutes and the supernatant removed. The pH was neutralized using 10 μ l of 8N NaOH. Commercially obtained amidase (Sigma-Aldrich, St Louis, MO) was added to the supernatant and incubated at 37°C for 30 minutes. After incubation, the sample was placed in a glass test tube, followed by the addition of 2 ml of 0.3M sodium phenate, 3 ml of 0.1% sodium nitroprusside, and 3 ml of 0.02 N sodium hypochlorite (see appendix). Test tubes were vortexed and incubated in the dark for 30 minutes.

Optical density of samples was measured spectrophotometrically, using a Wallac Victor 1420 microplate-reader at 630 nm (Perkin Elmer, Waltham, MA). Units of enzyme activity were then calculated as the rate of conversion of AN/minute/mg cell dry weight. The activity presented for each inducer is the average of triplicate assays.

2.2.2 Cyanidase-like activity

Cyanidase-like activity was also determined according to the Fawcett and Scott (1960) assay for ammonia production. A wet weight of 50 mg of rhodococcal cells was scraped from plates and suspended in 1 ml 50 mM Phosphate Buffer (PB), then reacted with 9 ml of 1000 ppm potassium cyanide (KCN) for 2 minutes. After the reaction, 1 ml was removed, acidified to about pH 3-4 with 10 μ l of 2N H₂SO₄ and centrifuged at 13,000 rpm for 2 minutes.

As described above, 10 μ l of 8N NaOH was used to neutralize the sample prior to its addition to a reaction mixture of 3 ml of 0.3M sodium phenate, 2 ml 0.1% sodium nitroprusside, and 2 ml 0.02N sodium hypochlorite. Test tubes were vortexed and incubated for 30 minutes at 25°C. Commercially-obtained ammonia(Sigma-Aldrich, St Louis, MO) was diluted in water in a concentration range of 1-10 ppm to generate a standard for each assay. Optical density was determined using a Wallac Victor (Perkin Elmer, Waltham, MA) micro plate reader.

2.2.3 β -Cyanoalanine Synthase-like enzyme

β -Cyanoalanine Synthase-like enzymatic activity was measured using the assay described by Ezzi and Lynch (2002). 50 mg (wet weight) of cells were suspended in 1 ml of pH 7, 50 mM PB. A sodium sulfide (Acros Organics, Geel, Belgium) standard was prepared for each assay. 500 μ l of each substrate, KCN and Cysteine (Sigma Life Sciences, Japan), were added to the cell suspension, vortexed, and incubated at 30°C for 20 minutes. Cells were removed from reaction solution by centrifugation (Eppendorf, Hamburg, Germany) at 13,000 rpm for 2 minutes.

Supernatant was then added to 500 μ l of 0.02M N,N-dimethyl-p-phenylene diamine sulfate and 500 μ l of 0.03M FeCl₃ (see appendix). Samples were incubated in the dark for 25 minutes. Color change was quantified by measuring optical density at 600 nm with a Wallac Victor micro plate reader (Perkin-Elmer, Hamburg, Germany).

2.3 Volatile Analysis

2.3.1 Headspace Sampling

Three 60x15 mm plates of *Rhodococcus* were placed in gas tight 250 ml Bellco (Vine-land, NJ) spinner flasks and left on the bench top overnight. Spinner flasks were equipped with septum-sealed sampling ports.

A 500 μ l gas-tight syringe (Hamilton, CO, Reno, NV) was used to remove 500 μ l of headspace from the jars and injected onto the GC. Gas chromatography was performed according to the method below.

2.3.2 Solid Phase Microextraction (SPME) Sampling

Three 60x15 mm plates of *Rhodococcus* were placed in gas tight 250 ml Bellco (Vine-land, NJ) spinner flasks and left at ambient temperature overnight. The SPME fibers used were 75 μ m, 24 gauge, Carboxen/Polydimethylsiloxane (Car/PDMS) (Sigma-Aldrich, St Louis, MO). Fiber holders were inserted into sealable sampling ports on the above described sampling system and fibers were extended for a one hour sampling period prior to analysis using Gas Chromatography (GC). After sampling, fibers were retracted into manual fiber holder and remained closed until GC injection.

2.3.3 Gas Chromatography

GC analysis was performed using a Perkin-Elmer (Waltham, MA) Autosystem XL with built-in autosampler and flame ionized detector (FID). Programs were run with TotalChrom Workstation version 6.2.1. A 75m x 0.530 mm DB 624 column from J & W Scientific (Folsom, CA) was used. SPME fibers were placed in the GC injection port, which was set to 250°C, and remained in the port for the duration of each run. Oven temperature started at 80°C for one minute before ramping to 180°C at a rate of 20°C /min. The temperature was held at 180°C for 10 minutes. Gas flow for air and hydrogen were 450.0 ml/min and 45.0 ml/min, respectively. The carrier gas used was helium with a flow rate was 2 ml/min.

3. RESULTS

3.1 Enzyme Induction

Experiments to evaluate cyanide-metabolizing enzymes were developed using YEMEA as a base media and various chemical additives for potential inducers. Enzyme activities were calculated as units/mg cdw from these units, converted to a percentage. This percentage is given in the table below. Many of the compounds used for enzyme induction experiments were also tested for conferring antifungal properties on *Rhodococcus*. Those results are also listed in the following tables.

Table 2a: Normalized enzyme activities showing the effects of selected compounds on NHase, AMDase, ACC Deaminase, CNase, and β -CAS-like enzyme in *R. rhodochrous* DAP 96253.

Inducer	NHase	AMDase	ACCD	CNase	β -CAS-like enzyme	Spore Inhibition
	Percent					
0.85 mM cobalt, 200 mM urea	100 (65*)	100 (39**)	78	58 \pm	80	partial
200 mM Urea	49	81	100 (35***)	58	90	yes
40 mM Methacrylamide	25	47	74	75	100*****	yes
200 mM Acetamide	<1	33	26	100***	90	yes
200 mM Methylcarbamate	<1	47	26	75	60	yes
Unsupplemented YEMEA	1	0	0	0	90	no
200 mM Potassium nitrate	1	42	61	92	90	no
200 mM Methyl urea	1	100	8	<1	80	no
200 mM Ethyl urea	1	19	<1	<1	70	no
24 mM Melamine	<1	0	0	1	80	no
200 mM Allantoin	17	74	71	50	100	no
200 mM Hydantoin	9	2	2	<1	60	no
200 mM Histidine	1	2	2	<1	70	no
200 mM Phenylacetaldehyde	15	<1	<1	<1	90	no

*65 units/mg cdw Nitrile Hydratase is considered 100% activity

**36 units/mg cdw amidase is considered 100% activity

*** 23 units/mg cdw ACC Deaminase is considered 100% activity

****12 units/mg cdw cyanidase is considered 100% activity

*****10 units/mg cdw β -CAS-like enzyme is considered 100% activity

\pm shaded red columns indicate p-value <0.05

Table 2b: Normalized enzyme activities showing the effects of selected compounds on NHase, AMDase, ACC Deaminase, CNase, and β -CAS-like enzyme in *R. rhodochrous* DAP 96253 (continued).

Inducer	NHase	AMD	ACCD	Cyanidase	β -CAS-like enzyme	Spore inhibition
	Percent					
7 mM Glutamine	9	33	70	83	80	no
0.4% Methacrylamide	11	17	65	66	100	no
Cobalt+Urea+0.4% Methacrylamide	80	78	83	66	80	no
Cobalt+Urea+0.7% Methacrylamide	85	62	83	66	80	no
200 μ m Nickle	23	0	4	0	90	no
200 μ m Iron	15	31	22	0	90	no
7 mM Glutamine, 8 mM Asparagine	3	36	57	100	90	no
200 mM Hydroxyphenylacetamide	4	1	<1	<1	40	not tested
50 μ m KCN	0	0	0	0	90	not tested
100 μ m KCN	0	0	0	0	90	not tested
150 μ m KCN	0	0	0	0	90	not tested
8 mM Asn	3	5	61	42	50	not tested
7 mM Glutamine, 200 mM Urea	31	94	58	58	80	not tested
200 mM KNO ₃ , 200 mM Urea	25	84	66	66	90	not tested
9 mM Serine	1	1	4	1	90	not tested
100 μ m Zinc	0	0	0	0	90	not tested
200 μ m Zinc	0	0	4	0	80	not tested
100 μ m Pb	1	0	0	1	90	not tested
200 μ m Pb	1	0	0	1	80	not tested
0.85 mM Cobalt, 200 mM Urea, 200 μ m Iron	68	83	57	75	90	not tested

3.2 Antifungal activity

3.2.1 Inhibition of individual and mixed fungal cultures

Table 3: Effect of supplemented YEMEA-grown *R. rhodochrous* DAP 96253 on germination of 1×10^2 spores of selected fungal species separately and as a mixed culture.

Inducer	Inhibits germination of 1×10^2 spores of <i>A. niger</i>	Inhibits germination of 1×10^2 spores of <i>A. fumigatus</i>	Inhibits germination of 1×10^2 spores of <i>Penicillium</i>	Inhibits germination of 3×10^2 spores of mixed cultures
200 mM Urea	Yes	Yes	No	Partial
200 mM Methylcarbamate	Yes	Yes	Yes	Yes
200 mM Acetamide	Yes	Yes	Yes	Yes
22 mM Methacrylamide	Yes	Yes	No	Partial

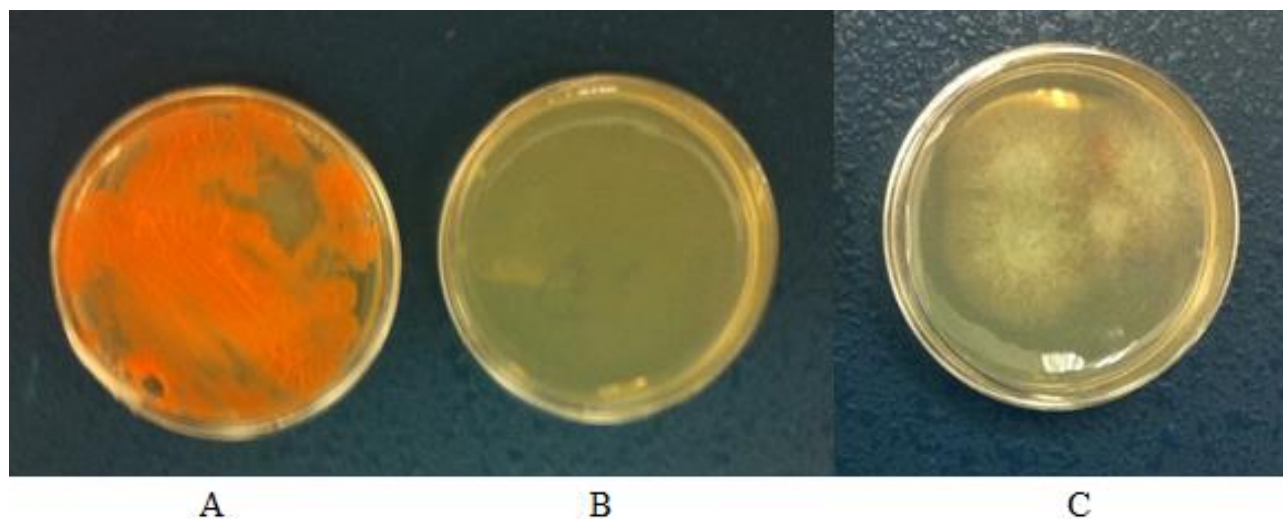


Figure 8a: Effect of urea-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 on germination of a standardized spore challenge of *A. fumigatus*.

a) urea-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 b) standardized spore challenge of *A. fumigatus* exposed to *Rhodococcus* c) standardized spore inoculum of *A. fumigatus* on spore germination media (control)

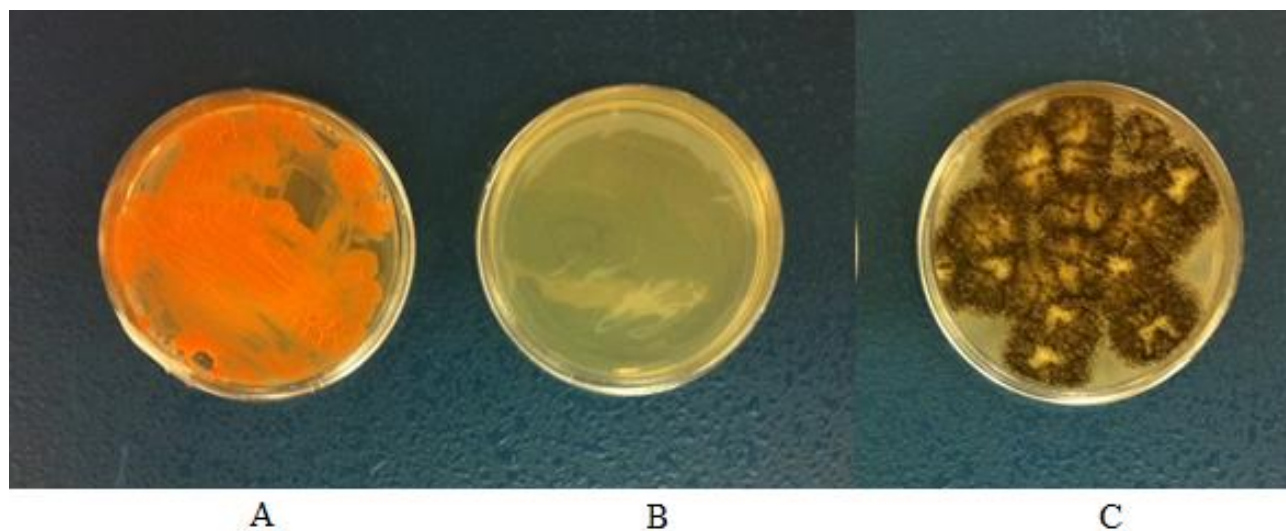


Figure 8b: Effect of urea-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 on germination of a standardized spore challenge of *A. niger*.

a) urea-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 b) standardized spore challenge of *A. niger* exposed to *Rhodococcus* c) standardized spore inoculum of *A. niger* on spore germination media (control)

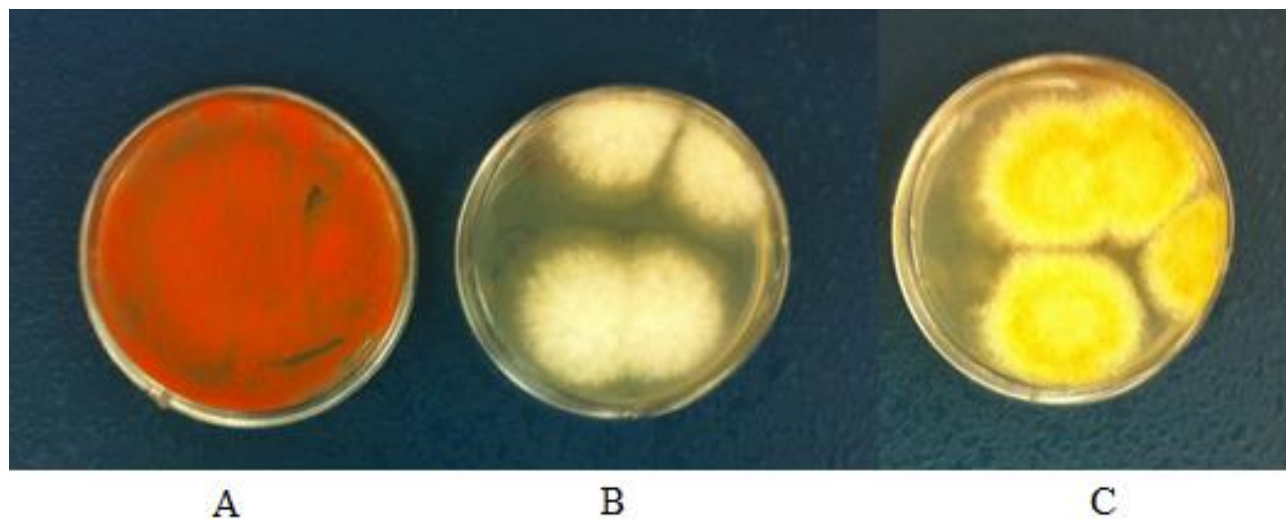


Figure 8c: Effect of urea-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 on germination of a standardized spore challenge of *Penicillium* sp.

a) urea-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 b) standardized spore challenge of *Penicillium* sp. exposed to *Rhodococcus* c) standardized spore inoculum of *Penicillium* on spore germination media (control)

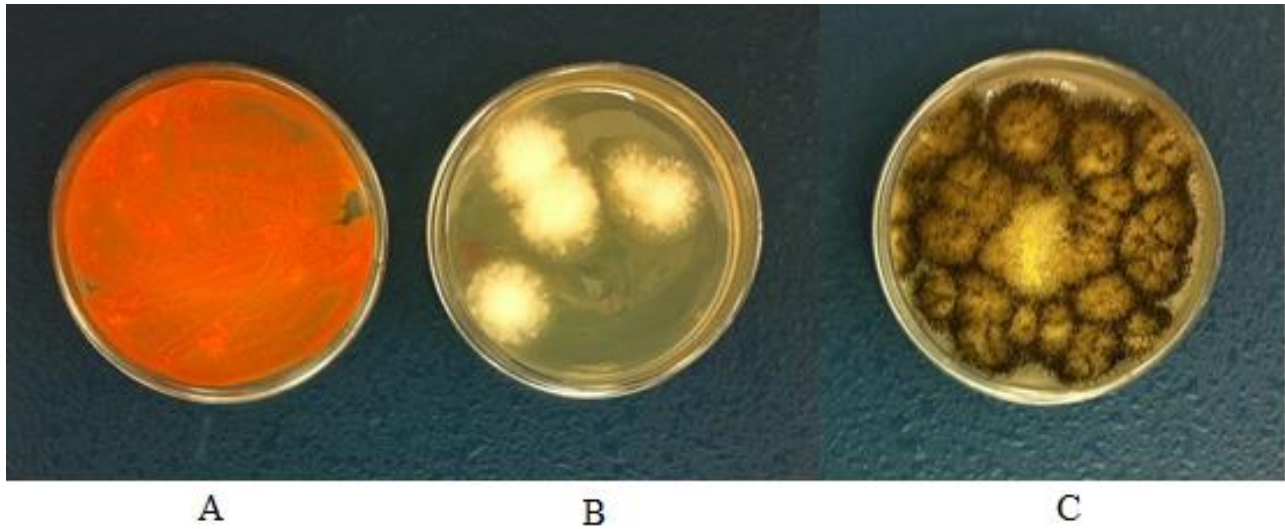


Figure 8d: Effect of urea-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 on germination of a standardized spore challenge of *A. niger*, *A. fumigatus*, and *Penicillium* sp. together. a) urea-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 b) standardized spore challenge of *A. niger*, *A. fumigatus*, and *Penicillium* sp. exposed to *Rhodococcus* c) standardized spore inoculum of *A. niger*, *A. fumigatus*, and *Penicillium* sp. on spore germination media (control)

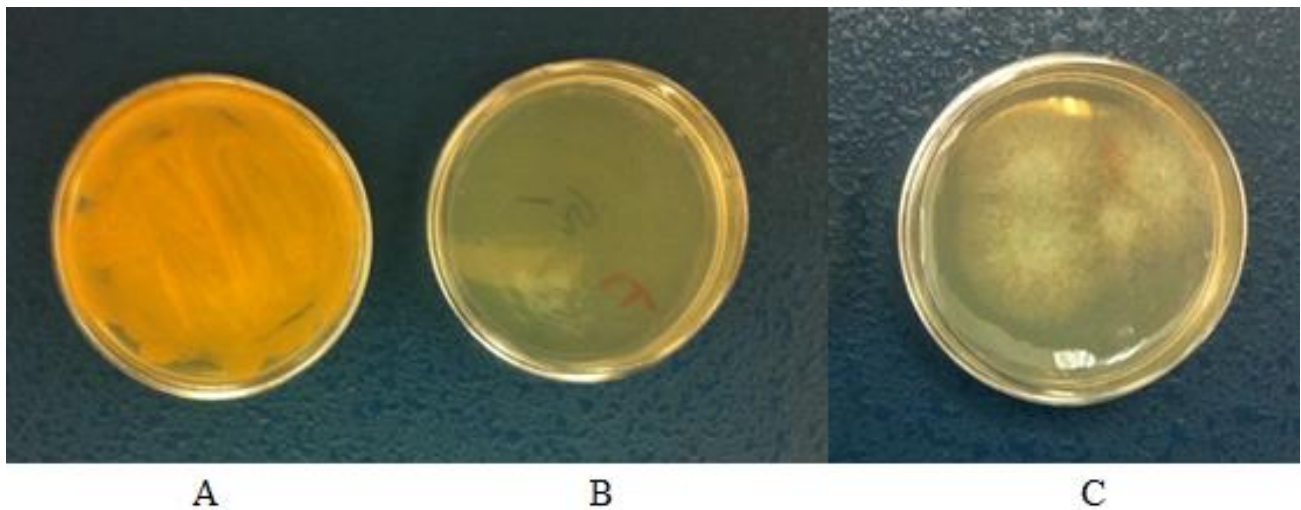


Figure 9a: Effect of methylcarbamate-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 on germination of a standardized spore challenge of *A. fumigatus*. a) methylcarbamate-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 b) standardized spore challenge of *A. fumigatus* exposed to *Rhodococcus* c) standardized spore inoculum of *A. fumigatus* on spore germination media (control)

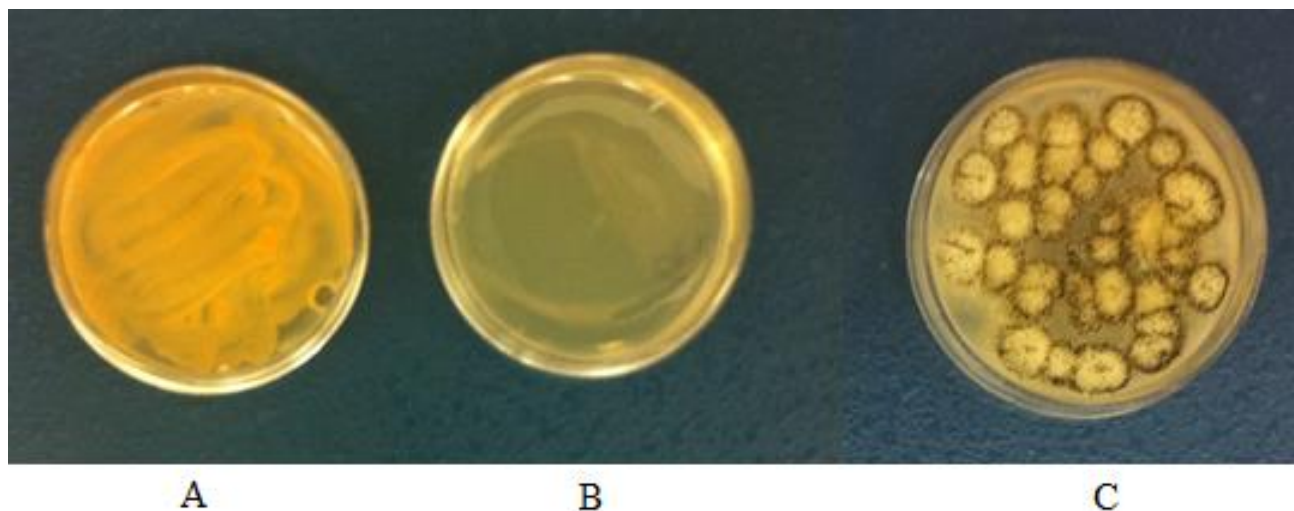


Figure 9b: Effect of methylcarbamate-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 on germination of a standardized spore challenge of *A. niger*.
 a) methylcarbamate-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 b) standardized spore challenge of *A. niger* exposed to *Rhodococcus* c) standardized spore inoculum of *A. niger* on spore germination media (control)

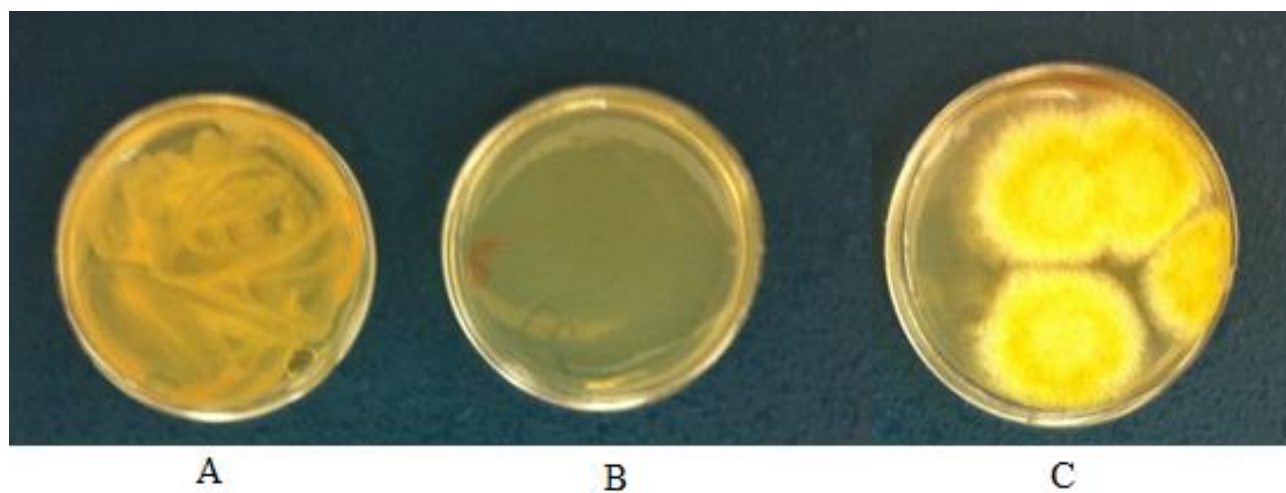


Figure 9c: Effect of methylcarbamate-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 on germination of a standardized spore challenge of *Penicillium* sp.
 a) methylcarbamate -supplemented YEMEA-grown *R. rhodochrous* DAP 96253 b) standardized spore challenge of *Penicillium* sp. exposed to *Rhodococcus* c) standardized spore inoculum of *Penicillium* on spore germination media (control)

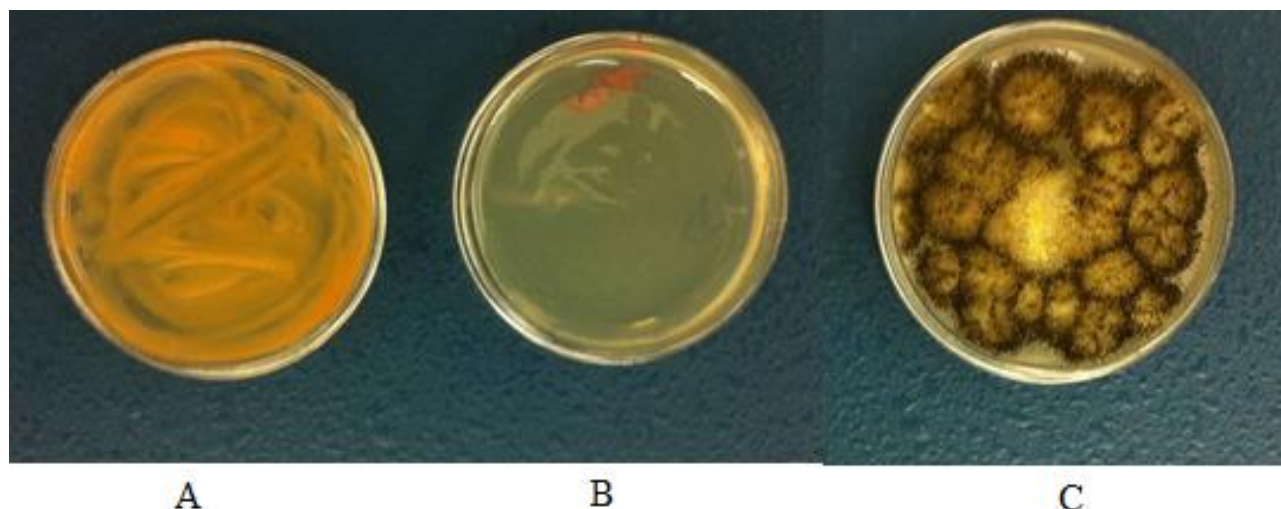


Figure 9d: Effect of methylcarbamate-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 on germination of a standardized spore challenge of *A. niger*, *A. fumigatus*, and *Penicillium* sp. together.

a) methylcarbamate-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 b) standardized spore challenge of *A. niger*, *A. fumigatus*, and *Penicillium* sp. exposed to *Rhodococcus* c) standardized spore inoculum of *A. niger*, *A. fumigatus*, and *Penicillium* sp. on spore germination media (control)

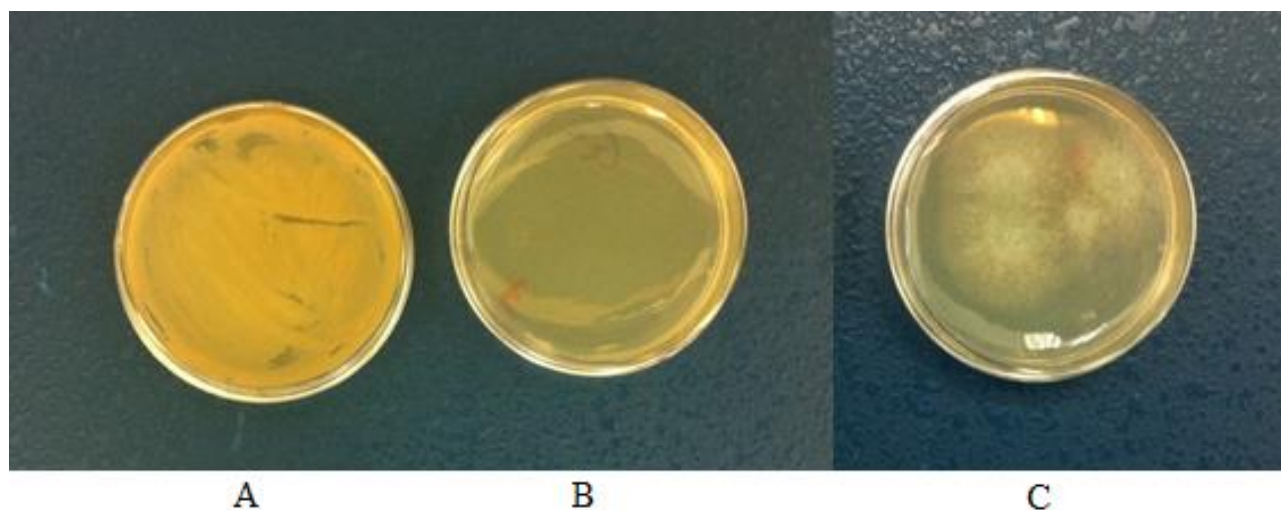
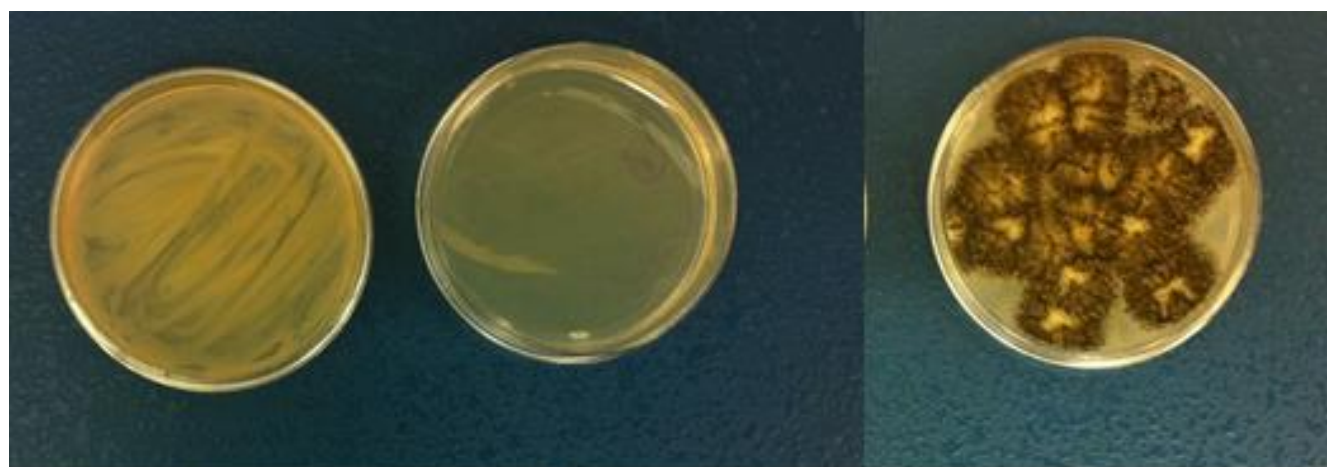


Figure 10a: Effect of acetamide-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 on germination of a standardized spore challenge of *A. fumigatus*.

a) acetamide-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 b) standardized spore challenge of *A. fumigatus* exposed to *Rhodococcus* c) standardized spore inoculum of *A. fumigatus* on spore germination media (control)



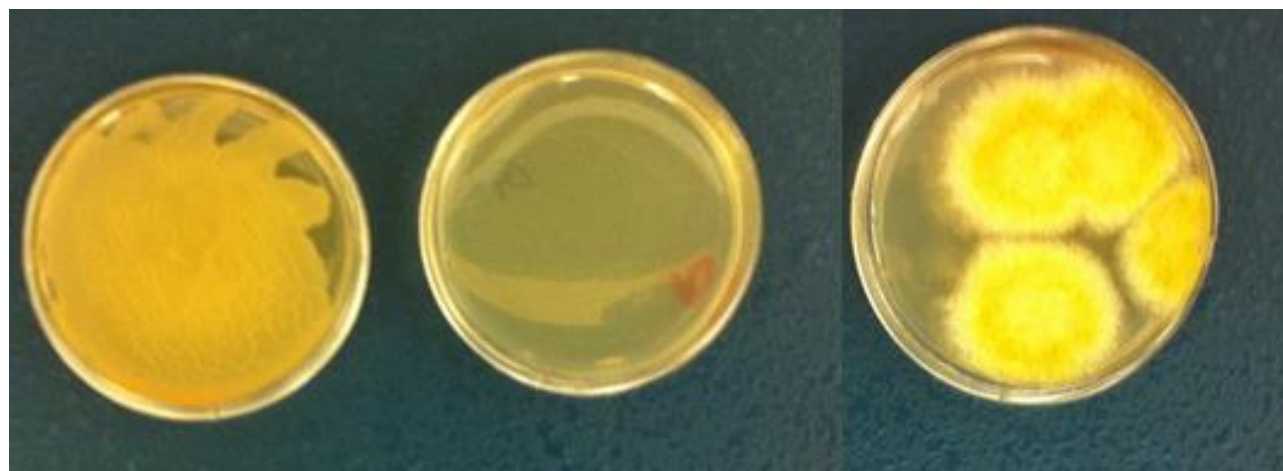
A

B

C

Figure 10b: Effect of acetamide-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 on germination of a standardized spore challenge of *A. niger*.

a) acetamide-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 b) standardized spore challenge of *A. niger* exposed to *Rhodococcus* c) standardized spore inoculum of *A. niger* on spore germination media (control)



A

B

C

Figure 10c: Effect of acetamide-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 on germination of a standardized spore challenge of *Penicillium* sp.

a) acetamide-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 b) standardized spore challenge of *Penicillium* sp. exposed to *Rhodococcus* c) standardized spore inoculum of *Penicillium* sp. on spore germination media (control)

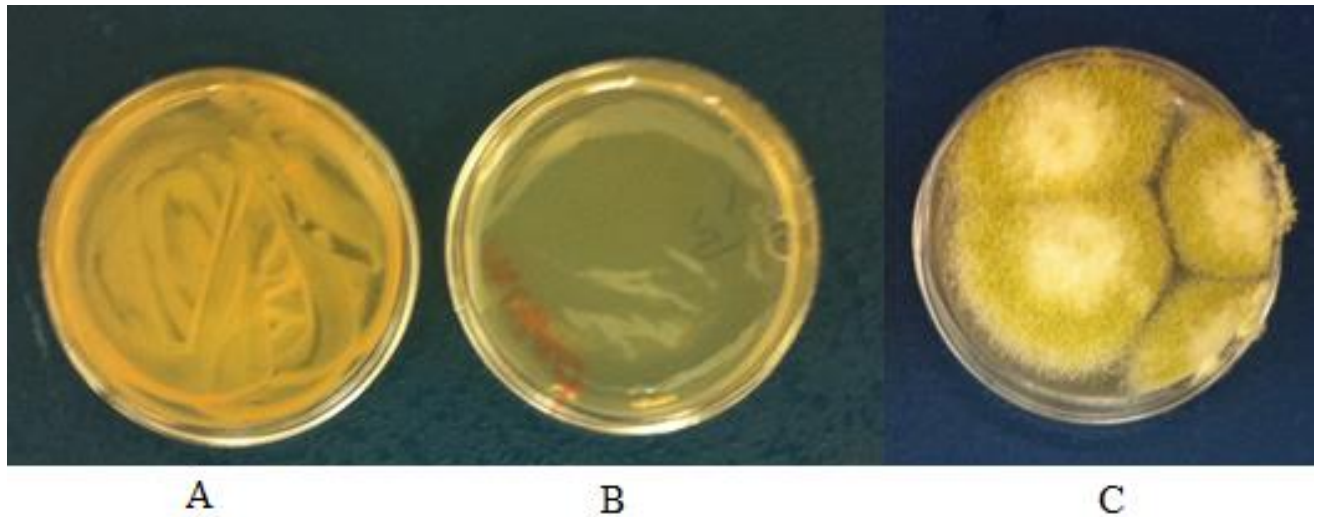


Figure 10d: Effect of acetamide-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 on germination of a standardized spore challenge of *A. niger*, *A. fumigatus*, and *Penicillium* sp. together.

a) acetamide-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 b) standardized spore challenge of *A. niger*, *A. fumigatus*, and *Penicillium* sp. exposed to *Rhodococcus* c) standardized spore inoculum of *A. niger*, *A. fumigatus*, and *Penicillium* sp. on spore germination media (control)

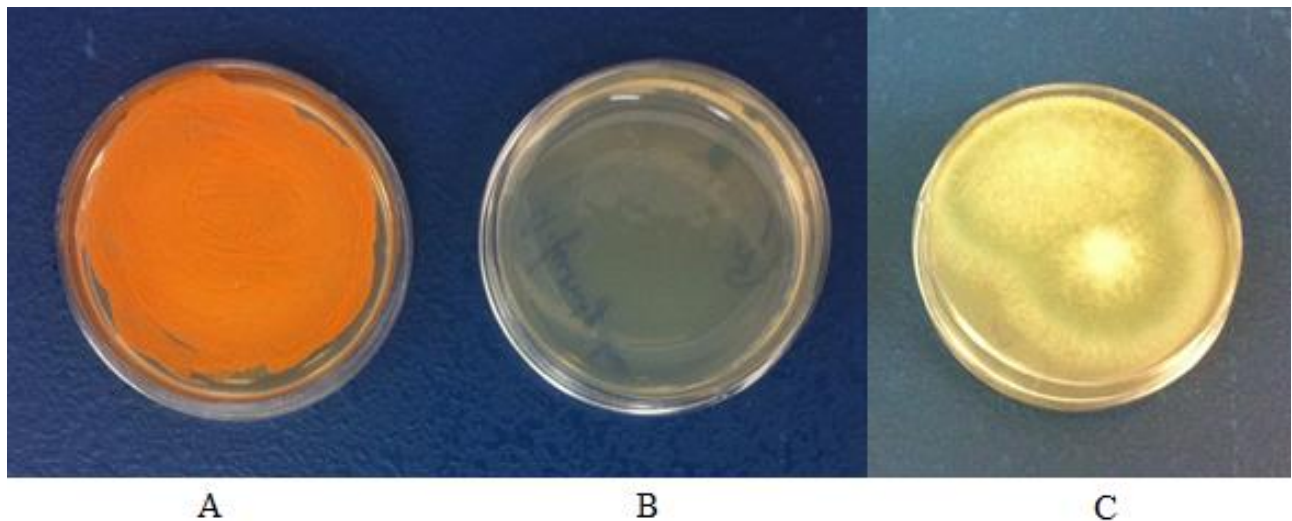


Figure 11a: Effect of methacrylamide-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 on germination of a standardized spore challenge of *A. fumigatus*.

a) methacrylamide-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 b) standardized spore challenge of *A. fumigatus* exposed to *Rhodococcus* c) standardized spore inoculum of *A. fumigatus* on spore germination media (control)

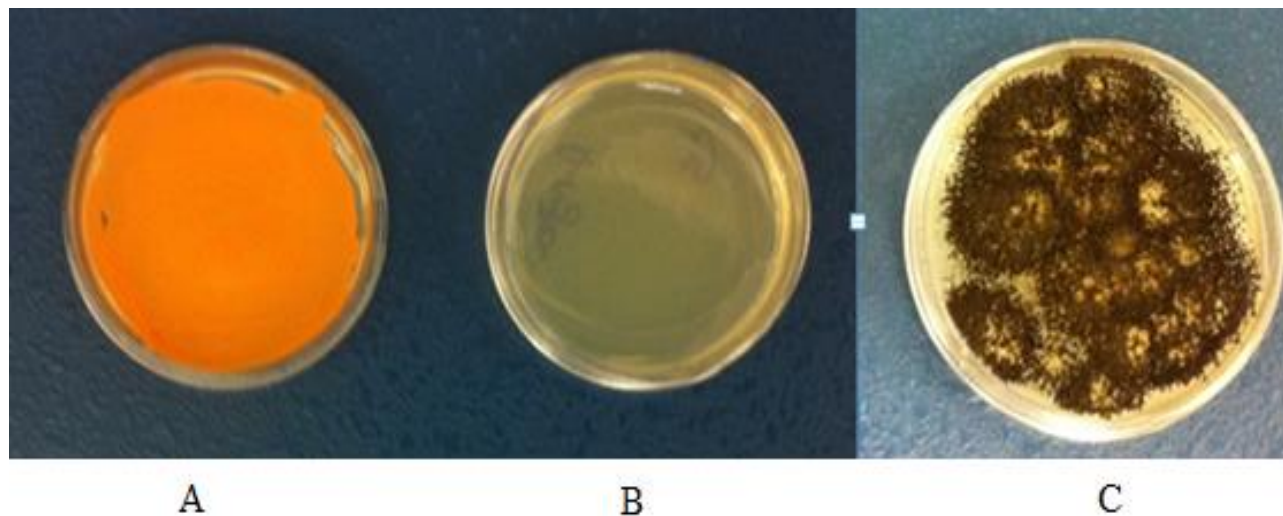


Figure 11b: Effect of methacrylamide-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 on germination of a standardized spore challenge of *A. niger*.
 a) methacrylamide-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 b) standardized spore challenge of *A.niger* exposed to *Rhodococcus* c) standardized spore inoculum of *A. niger* on spore germination media (control)

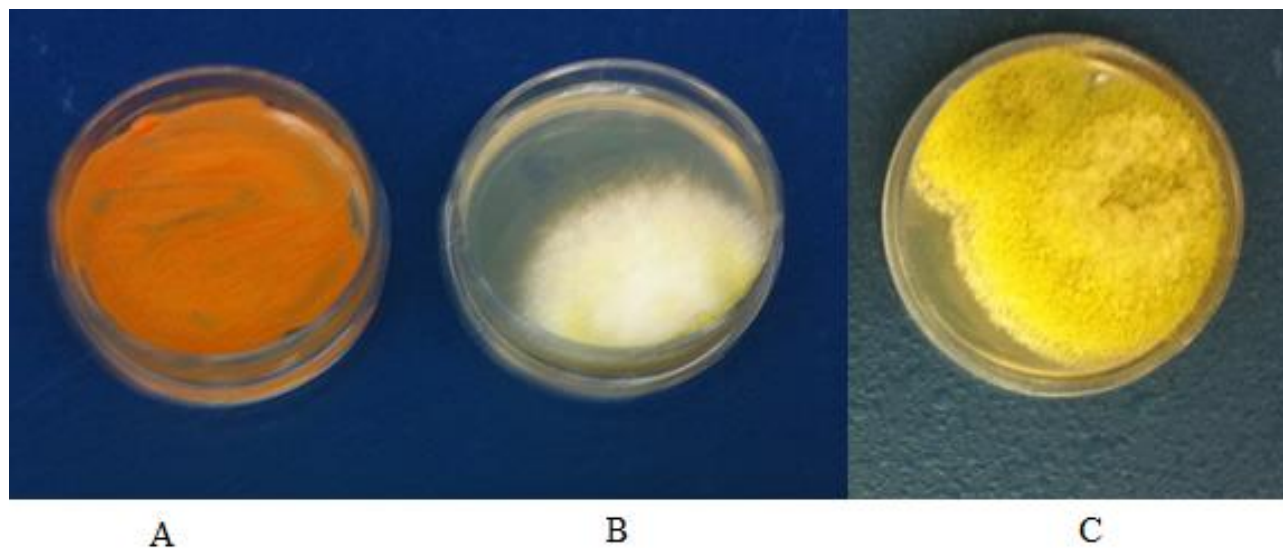


Figure 11c: Effect of methacrylamide-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 on germination of a standardized spore challenge of *Penicillium* sp.
 a) methacrylamide-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 b) standardized spore challenge of *Penicillium* sp. exposed to *Rhodococcus* c) standardized spore inoculum of *Penicillium* sp. on spore germination media (control)

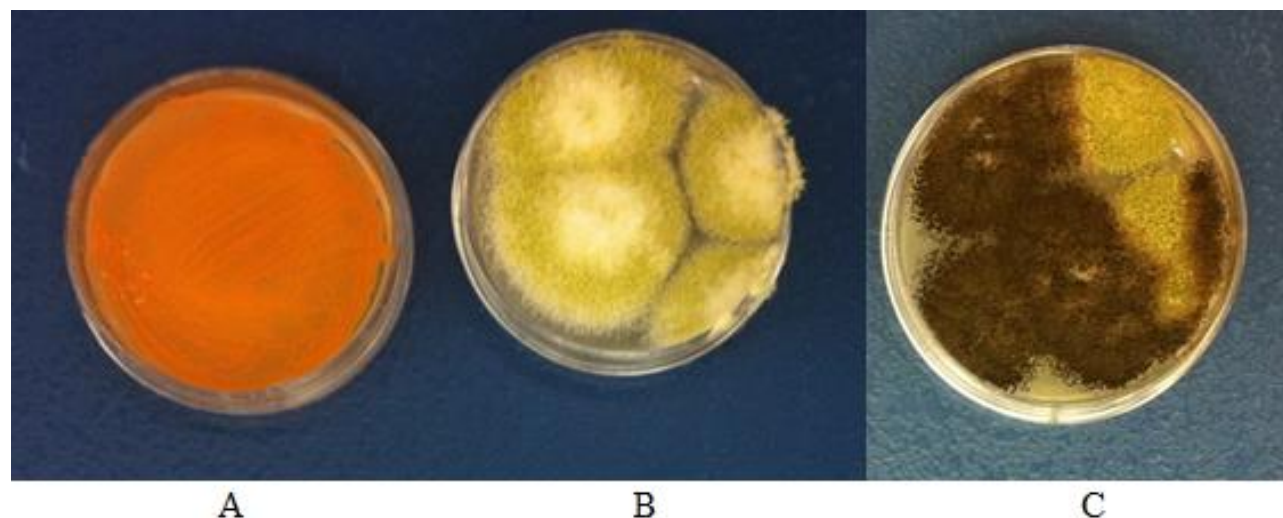


Figure 11d: Effect of methacrylamide-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 on germination of a standardized spore challenge of *A. niger*, *A. fumigatus*, and *Penicillium* sp. together.

a) methacrylamide-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 b) standardized spore challenge of *A. niger*, *A. fumigatus*, and *Penicillium* sp. exposed to *Rhodococcus* c) standardized spore inoculum of *A. niger*, *A. fumigatus*, and *Penicillium* sp. on spore germination media (control)

3.2.2 Recovery assays

Table 4: Exposure of *A. niger* to cells of urea-, methylcarbamate-, or acetamide-supplemented, YEMEA-grown *R. rhodochrous* DAP 96253.

YEMEA Supplementation	Germination of 1×10^2 <i>A. niger</i> spores after 24 hours co-culture	Germination of 1×10^2 <i>A. niger</i> spores after 48 hours co-culture	Visible germination of 1×10^2 <i>A. niger</i> spores after 76 hours co-culture
200 mM urea	Yes	No	No
200 mM methylcarbamate	No	No	No
200 mM acetamide	Yes	No	No
20 mM methacrylamide	Yes	No	No

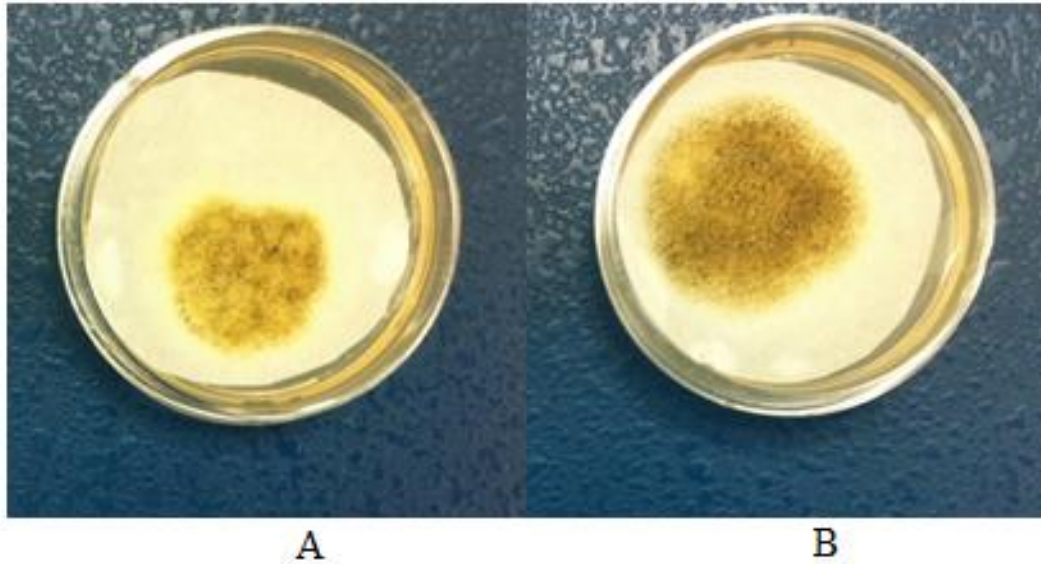


Figure 12a: Effect of a 24 hour exposure to urea-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 on a standardized spore challenge of *A. niger*.

a) standardized spore challenges of *A. niger* exposed to urea-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 for 24 hours b) standardized spore inoculum of *A. niger* 24 hours after inoculation (control)

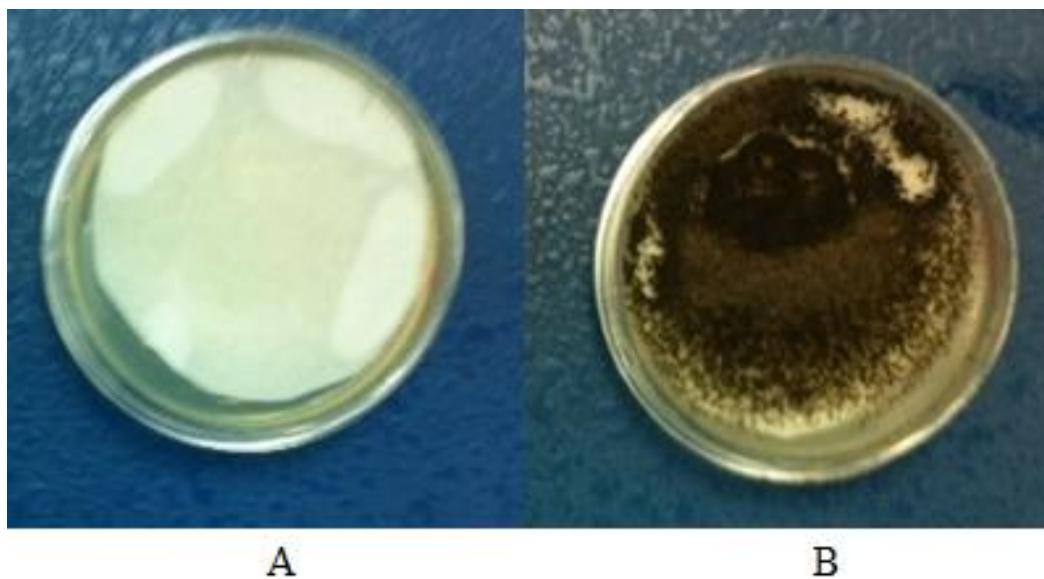


Figure 12b: Effect of a 48 hour exposure to urea-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 on a standardized spore challenge of *A. niger*.

a) standardized spore challenges of *A. niger* exposed to urea-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 for 48 hours b) standardized spore inoculum of *A. niger* 48 hours after inoculation (control)

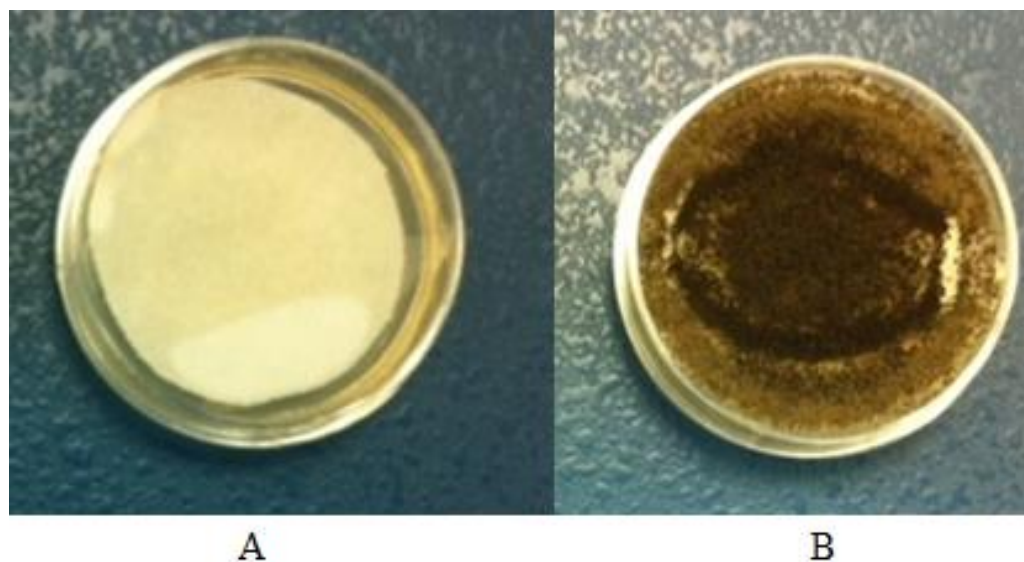


Figure 12c: Effect of a 72 hour exposure to urea-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 on a standardized spore challenge of *A. niger*.
 a) standardized spore challenges of *A. niger* exposed to urea-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 for 72 hours b) standardized spore inoculum of *A. niger* 72 hours after inoculation (control)

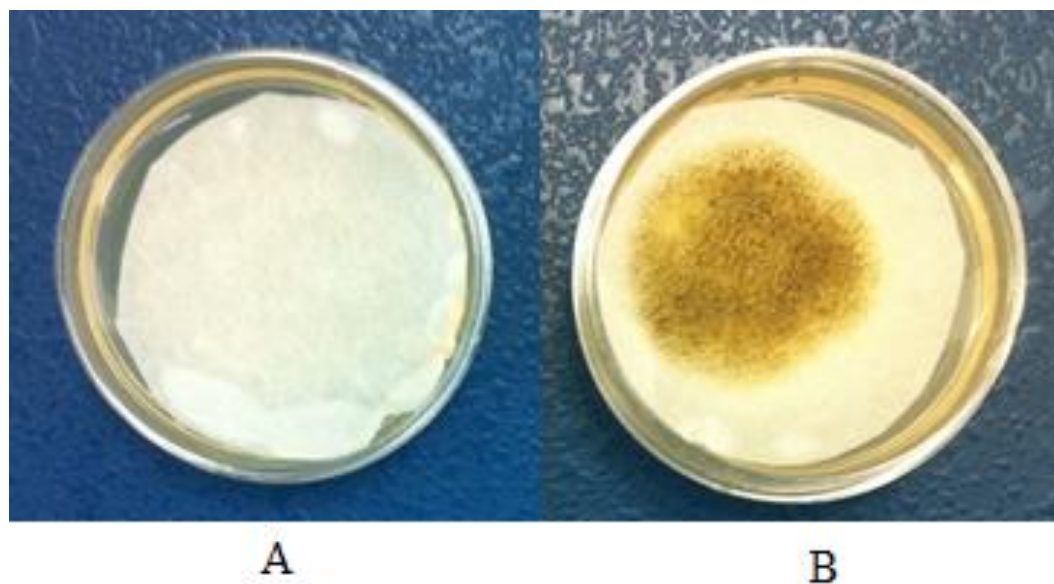


Figure 13a: Effect of a 24 hour exposure to methylcarbamate-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 on a standardized spore challenge of *A. niger*.
 a) standardized spore challenges of *A. niger* exposed to methylcarbamate-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 for 24 hours b) standardized spore inoculum of *A. niger* 24 hours after inoculation (control)

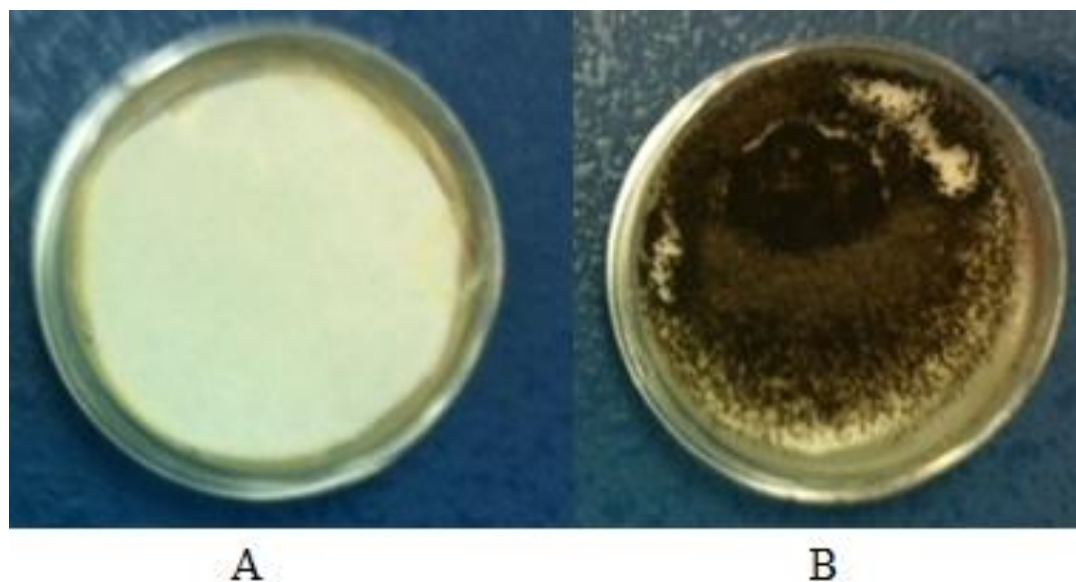


Figure 13b: Effect of a 48 hour exposure to methylcarbamate-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 on a standardized spore challenge of *A. niger*.
 a) standardized spore challenges of *A. niger* exposed to methylcarbamate-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 for 48 hours b) standardized spore inoculum of *A. niger* 24 hours after inoculation (control)

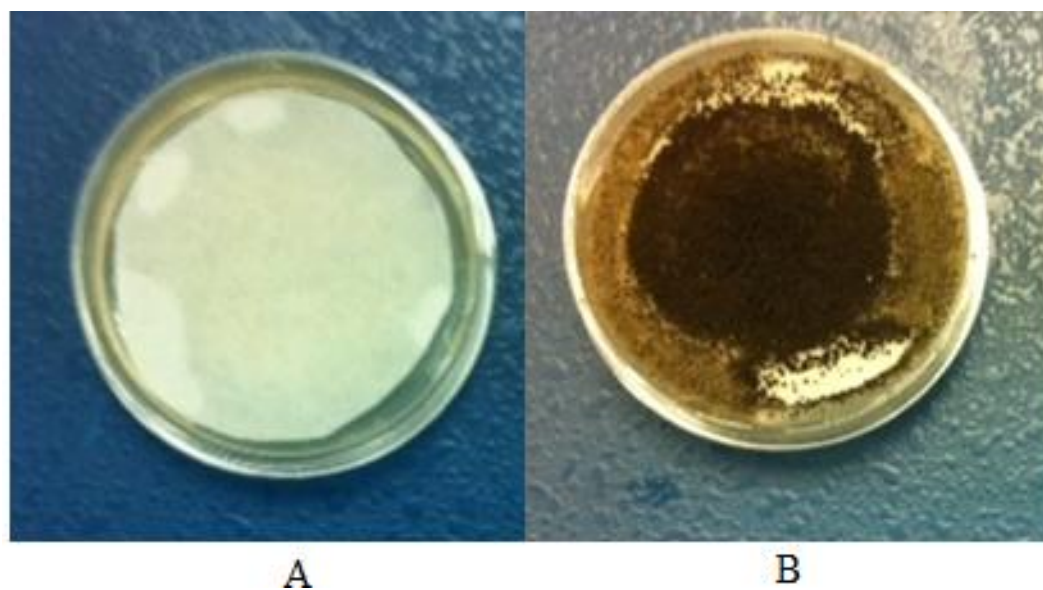


Figure 13c: Effect of a 72 hour exposure to methylcarbamate-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 on a standardized spore challenge of *A. niger*.
 a) standardized spore challenges of *A. niger* exposed to methylcarbamate-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 for 72 hours b) standardized spore inoculum of *A. niger* 72 hours after inoculation (control)

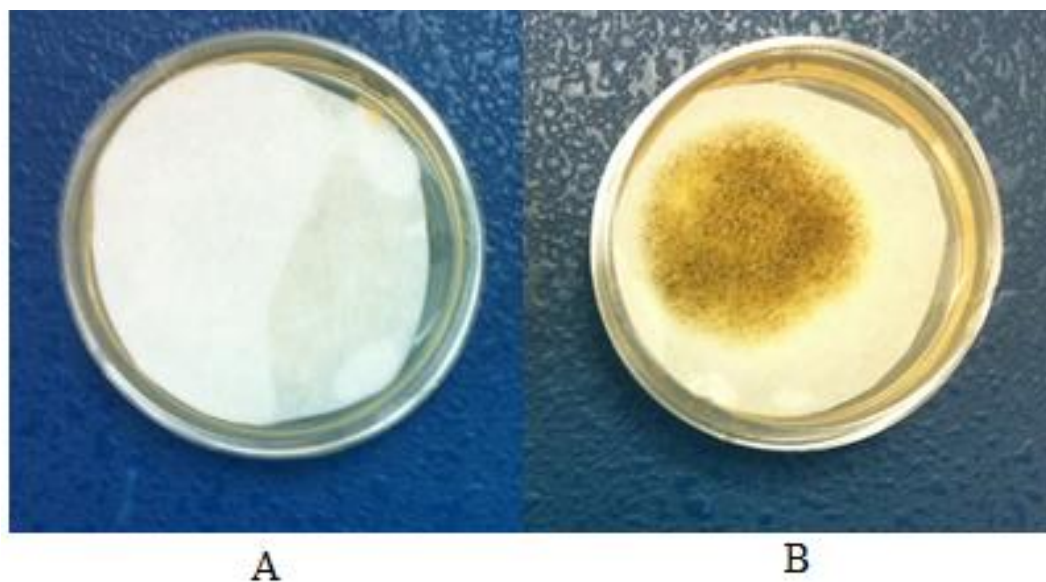


Figure 14a: Effect of a 24 hour exposure to acetamide-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 on a standardized spore challenge of *A. niger*.
 a and b) standardized spore challenges of *A. niger* exposed to acetamide-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 for 24 hours c) standardized spore inoculum of *A. niger* 24 hours after inoculation (control)

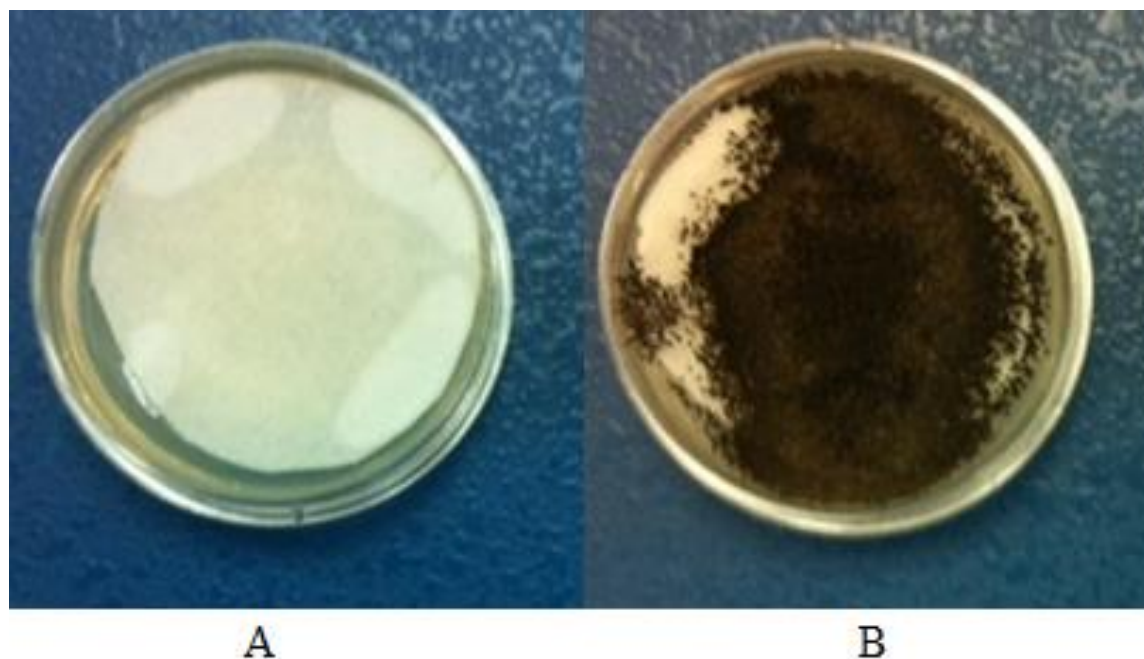


Figure 14b: Effect of a 48 hour exposure to acetamide-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 on a standardized spore challenge of *A. niger*.
 a and b) standardized spore challenges of *A. niger* exposed to acetamide-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 for 24 hours c) standardized spore inoculum of *A. niger* 48 hours after inoculation (control)

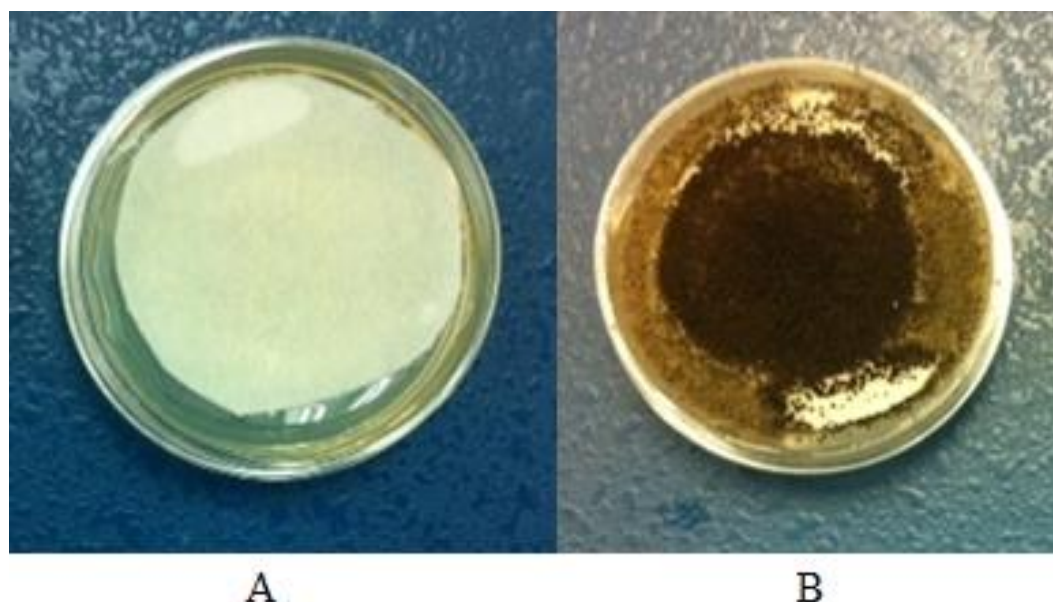


Figure 14c: Effect of a 72 hour exposure to acetamide-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 on a standardized spore challenge of *A. niger*.
 a and b) standardized spore challenges of *A. niger* exposed to acetamide-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 for 24 hours c) standardized spore inoculum of *A. niger* 72 hours after inoculation (control)

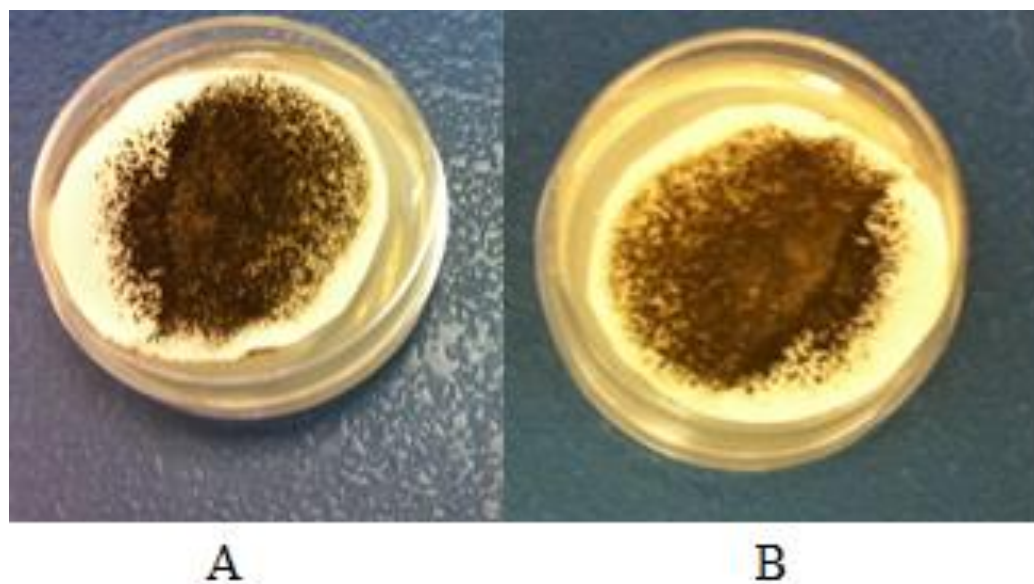


Figure 15a: Effect of a 24 hour exposure to methacrylamide-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 on a standardized spore challenge of *A. niger*.
 a) standardized spore challenges of *A. niger* exposed to methacrylamide-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 for 24 hours b) standardized spore inoculum of *A. niger* 24 hours after inoculation (control)

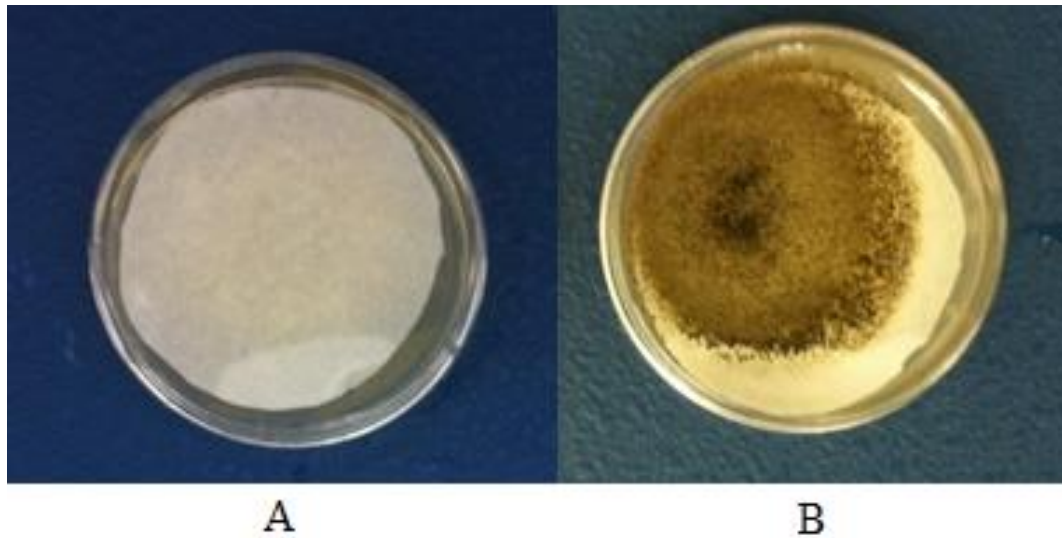


Figure 15b: Effect of a 48 hour exposure to methacrylamide-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 on a standardized spore challenge of *A. niger*.
 a) standardized spore challenges of *A. niger* exposed to methacrylamide-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 for 24 hours b) standardized spore inoculum of *A. niger* 48 hours after inoculation (control)

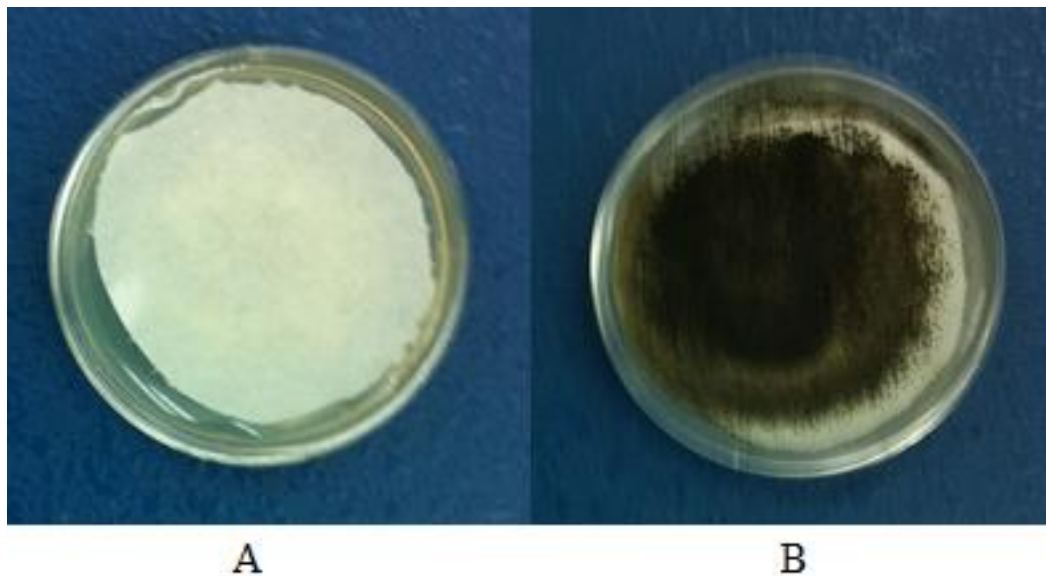


Figure 15c: Effect of a 72 hour exposure to methacrylamide-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 on a standardized spore challenge of *A. niger*.
 a) standardized spore challenges of *A. niger* exposed to methacrylamide-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 for 24 hours b) standardized spore inoculum of *A. niger* 72 hours after inoculation (control)

3.2.3 One directional airflow

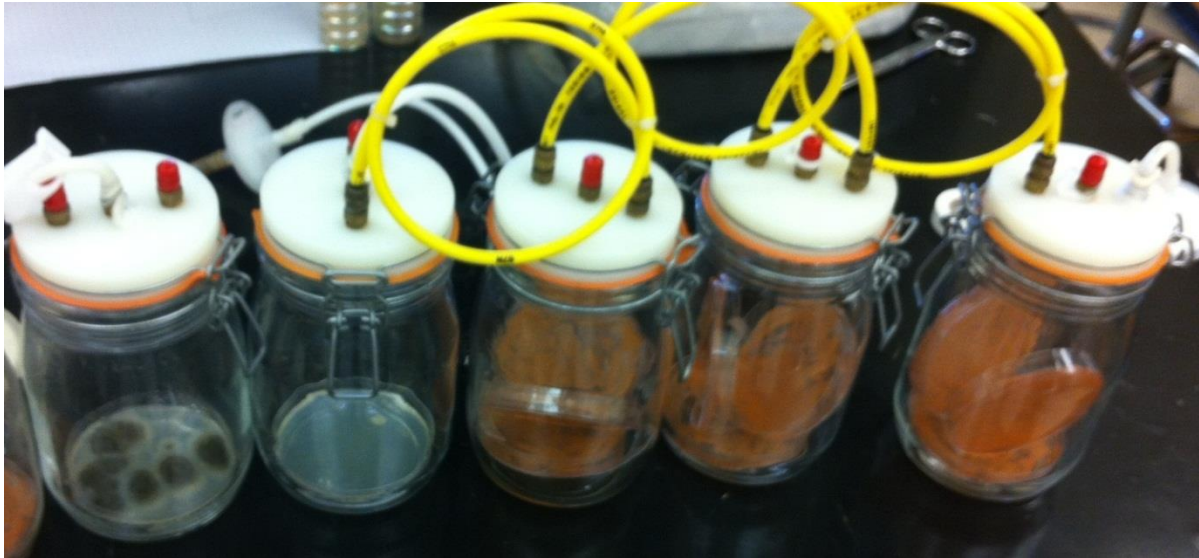


Figure 16a: Inhibition of germination of 5×10^2 *A. niger* spores with one-directional airflow of rhodococcal volatiles. Jar to the far left contains control *A. niger* inoculum.

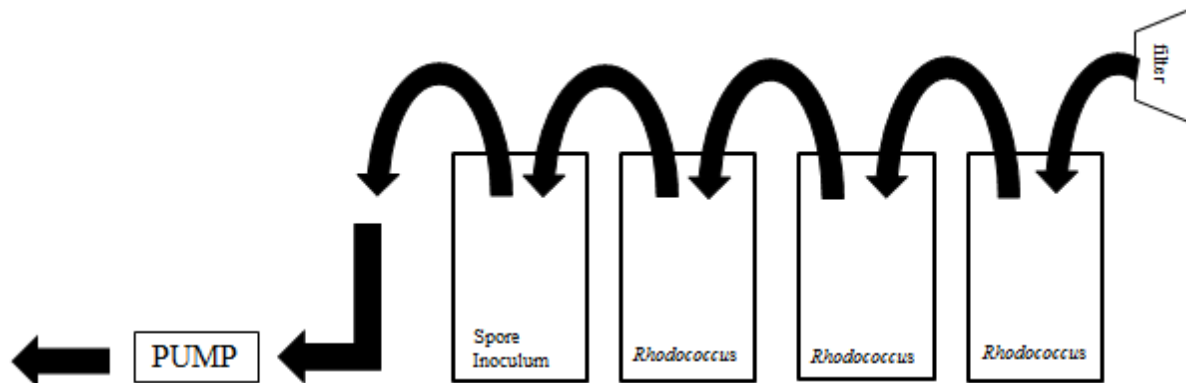


Figure 16b: Schematic showing directed airflow.

3.2.4 Antifungal activity of *R. rhodochrous* DAP 96253 scraped from plates

Table 5: Effect of *R. rhodochrous* DAP 96253 scraped from YEMEA plates on fungal germination.

YEMEA supplemented with	Weight of cells scraped	Inhibition of germination of 1×10^2 <i>A. niger</i> spores
0.85 mM Cobalt and 200 mM urea	200 mg	Yes
0.85 mM Cobalt and 200 mM urea	150 mg	Partial
0.85 mM Cobalt and 200 mM urea	100 mg	No
200 mM Urea	200 mg	Yes
200 mM Urea	150 mg	Partial
200 mM Urea	100 mg	No
No supplementation	250 mg	No

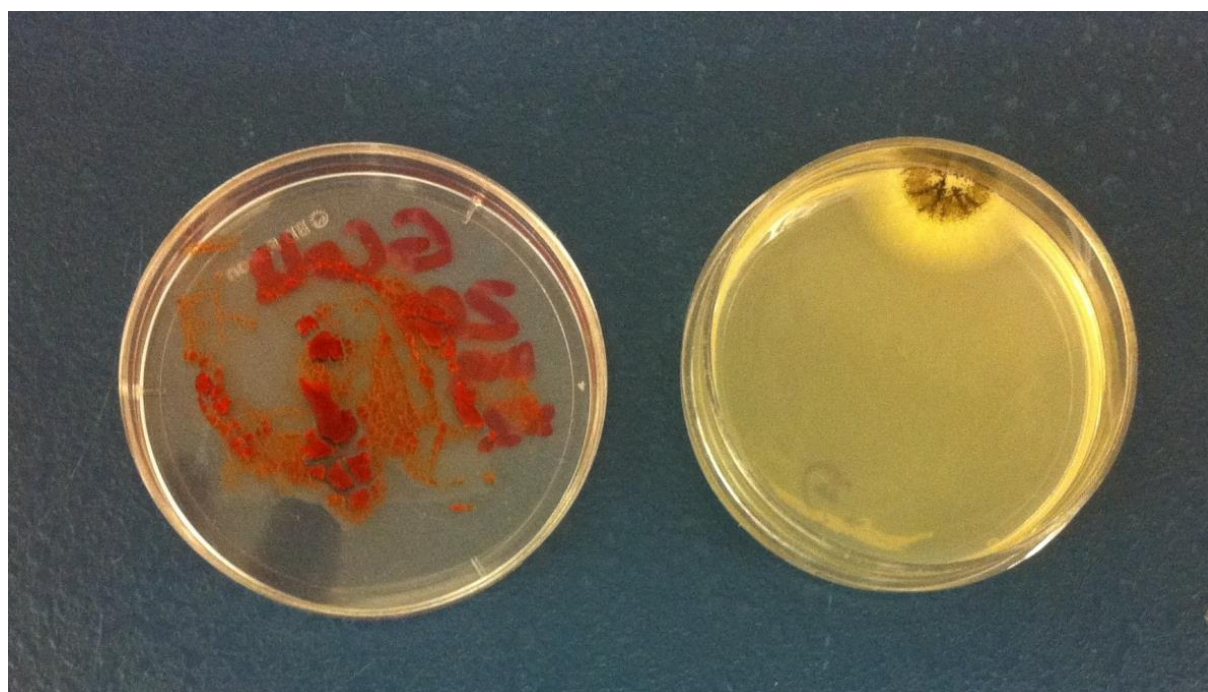


Figure 17a: 200 mg of scraped, cobalt- and urea-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 co-cultured with a standardized *A niger* spore challenge.



Figure 17b: 150 mg of scraped, cobalt- and urea-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 co-cultured with a standardized *A. niger* spore challenge.



Figure 17c: 100 mg of scraped, cobalt- and urea-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 co-cultured with a standardized *A. niger* spore challenge.



Figure 17d: Standardized spore inoculum of *A. niger* (control).

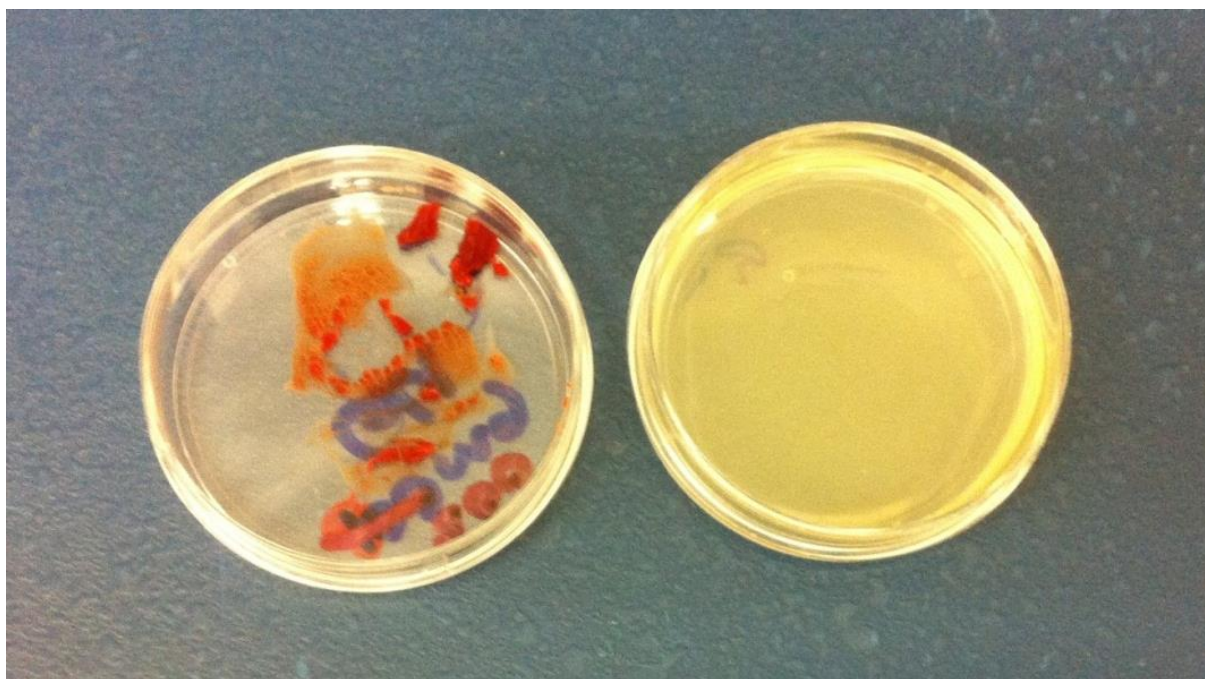


Figure 18a: 200 mg of scraped, urea-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 co-cultured with a standardized *A. niger* spore challenge.



Figure 18b: 150 mg of scraped, urea-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 co-cultured with a standardized *A niger* spore challenge.



Figure 18c: 100 mg of scraped, urea-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 co-cultured with a standardized *A niger* spore challenge.



Figure 18d: Standardized spore inoculum of *A. niger* (control).



Figure 19a: 250 mg scraped, YEMEA-grown *R. rhodochrous* DAP 96253 co-cultured with a standardized *A. niger* spore challenge.



Figure 19b: Standardized spore inoculum of *A. niger* (control).

3.3 Recruitment of induction conditions

Table 6: Effect on cyanidase activity and fungal inhibition of coculturing urea- or cobalt- and urea-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 with YEMEA-grown *R. rhodochrous* 96253, *R. rhodochrous* 96622 , and *R. erythropolis* 47072 (images below table).

Sample	Cyanidase activity (units/mg cdw)	Inhibition of germination of $10^2 A. niger$
Uninduced <i>R. rhodochrous</i> 96253	<1	No
Uninduced <i>R. rhodochrous</i> 96253, co-cultured with <i>R. rhodochrous</i> 96253 grown on YEMEA induced with urea	11	Yes
Uninduced <i>R. rhodochrous</i> 96253, co-cultured with <i>R. rhodochrous</i> 96253 grown on YEMEA induced with cobalt and urea	<1	Partial
Uninduced <i>R. rhodochrous</i> 96622	2	No
Uninduced <i>R. rhodochrous</i> 96622, co-cultured with <i>R. rhodochrous</i> 96253 grown on YEMEA induced with urea	9	Yes
Uninduced <i>R. rhodochrous</i> 96622, co-cultured with <i>R. rhodochrous</i> 96253 grown on YEMEA induced with urea and cobalt	11	Partial
Uninduced <i>R. erythropolis</i> 47072	1	No
Uninduced <i>R. erythropolis</i> 47072, co-cultured with <i>R. rhodochrous</i> 96253 grown on YEMEA induced with urea	11	Yes
Uninduced <i>R. erythropolis</i> 47072, co-cultured with <i>R. rhodochrous</i> 96253 grown on YEMEA induced with urea and cobalt	7	Partial
<i>R. rhodochrous</i> 96622 grown on YEMEA induced with urea does not recruit cyanidase-like activity or antifungal properties in uninduced <i>R. rhodochrous</i> DAP 96253 or <i>R. erythropolis</i> 47072		
Uninduced <i>R. rhodochrous</i> 96253, co-cultured with <i>R. rhodochrous</i> 96622 on YEMEA induced with urea	<1	No
Uninduced <i>R. erythropolis</i> 47072, co-cultured with <i>R. rhodochrous</i> 96622 on urea-induced YEMEA	<1	No



Figure 20a: YEMEA-grown *R. rhodochrous* DAP 96253 co-cultured with a standardized spore challenge of *A. niger* on spore germination media.

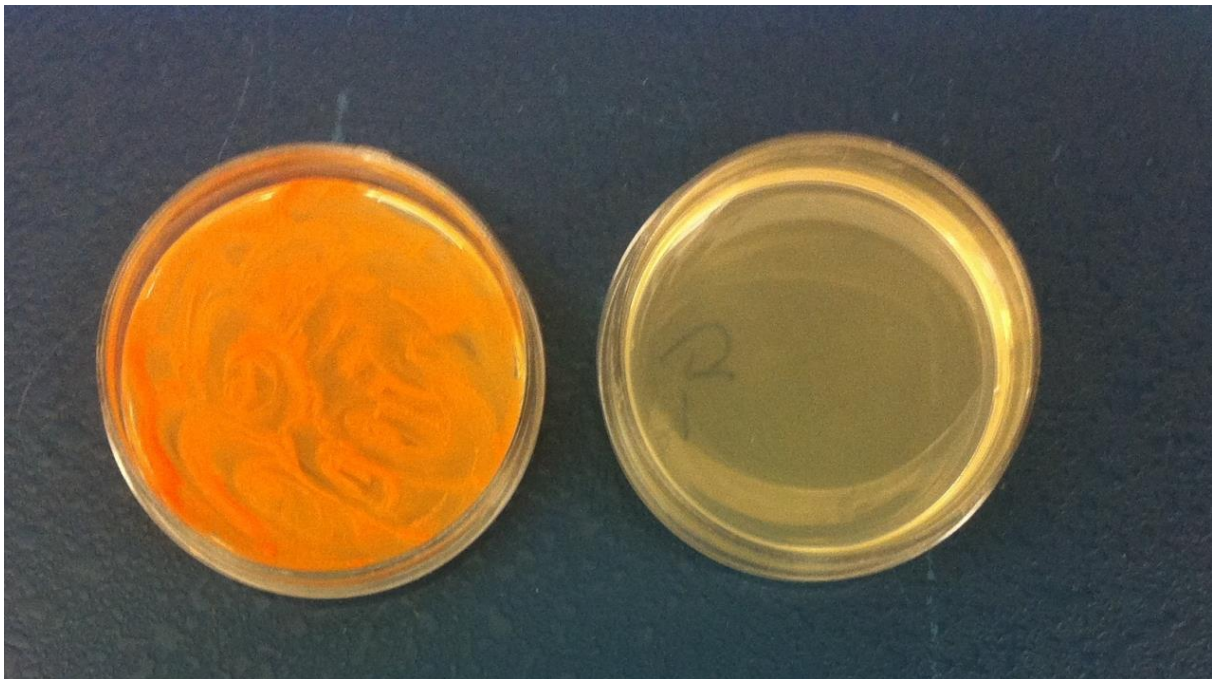


Figure 20b: YEMEA-grown *R. rhodochrous* DAP 96253 co-cultured with urea-supplemented YEMEA-grown *Rhodococcus rhodochrous* 96253. Shown here after co-culture a standardized challenge of *A. niger* spores on germination media.



Figure 20c: YEMEA-grown *R. rhodochrous* DAP 96253, co-cultured with cobalt- and urea supplemented YEMEA-grown *R. rhodochrous* DAP 96253 Shown here after co-culture with a standardized challenge of *A. niger* spores on germination media.



Figure 20d: Standardized spore inoculum of *Aspergillus niger* (control).



21a: YEMEA-grown *R. rhodochrous* 96622, co-cultured with a standardized spore challenge of *A. niger* on germination media.



Figure 21b: YEMEA-grown *R. rhodochrous* 96622 co-cultured with urea-supplemented YEMEA-grown *R. rhodochrous* 96253. Shown here after co-culture with a standardized spore challenge of *A. niger*.



Figure 21c: YEMEA-grown *R. rhodochrous* 96622 , co-cultured with cobalt- and urea-supplemented YEMEA-grown *R. rhodochrous* 96253. Shown here after co-culture with a standardized spore challenge of *A. niger* on germination media.

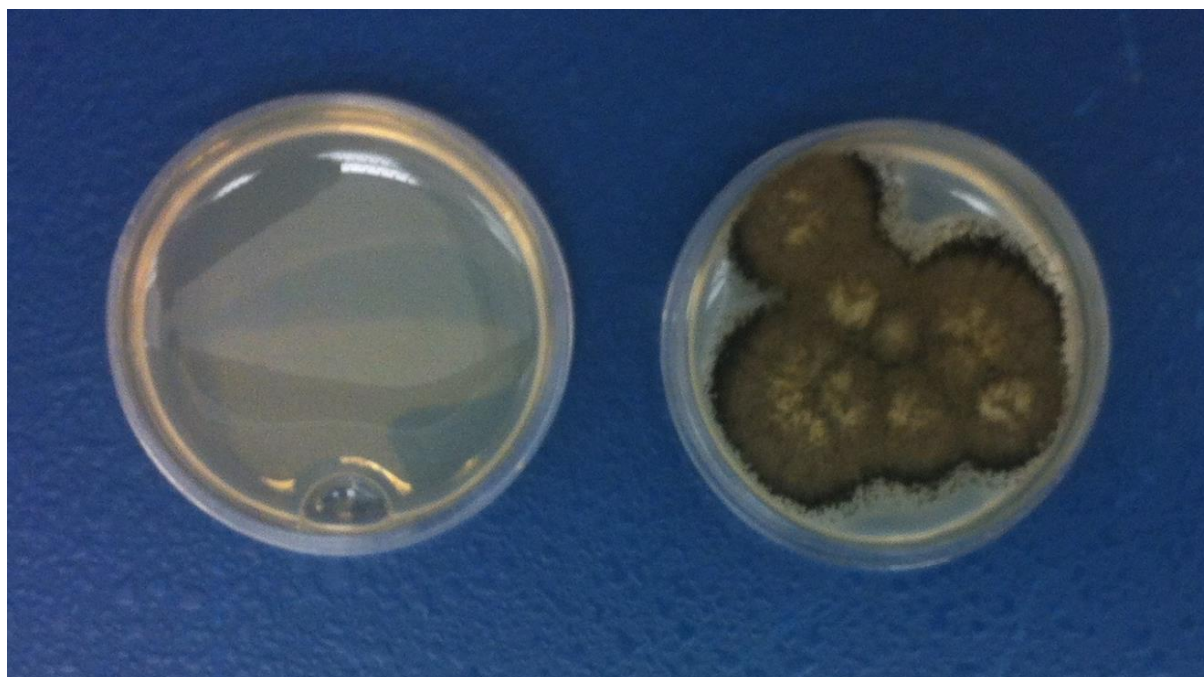


Figure 21d: Standardized spore inoculum of *Aspergillus niger* (control).



Figure 22a: YEMEA-grown *R. erythropolis* 47072 co-cultured with a standardized spore challenge of *A. niger* on spore germination media.



Figure 22b: YEMEA-grown *R. erythropolis* 47072, co-cultured with urea-supplemented YEMEA-grown *R. rhodochrous* 96253. Shown here after co-culture with a standardized spore challenge of *A. niger* on germination media.



Figure 22c: YEMEA-grown *R. erythropolis* 47072, co-cultured with cobalt- and urea-supplemented YEMEA-grown *R. rhodochrous* 96253. Shown here after co-culture with a standardized spore challenge of *A. niger* on germination media.



Figure 22d: Standardized spore inoculum of *Aspergillus niger* (control).

Table 7: Nitrile hydratase activity of YEMEA-grown *R. rhodochrous* DAP 96253 before and after co-culture with urea- and cobalt-urea-supplemented YEMEA-grown *R. rhodochrous* DAP 96253.

Sample	Nitrile Hydratase activity units/mg cdw
YEMEA-grown <i>R. rhodochrous</i> DAP 96253	2
YEMEA-grown <i>R. rhodochrous</i> DAP 96253 co-cultured with <i>R. rhodochrous</i> DAP 96253 grown on urea-supplemented YEMEA	3
YEMEA-grown <i>R. rhodochrous</i> DAP 96253 co-cultured with <i>R. rhodochrous</i> DAP 96253 grown on cobalt-supplemented YEMEA	2

3.4 Gas Chromatographic analysis of Rhodococcal volatiles

3.4.1 Headspace sampling

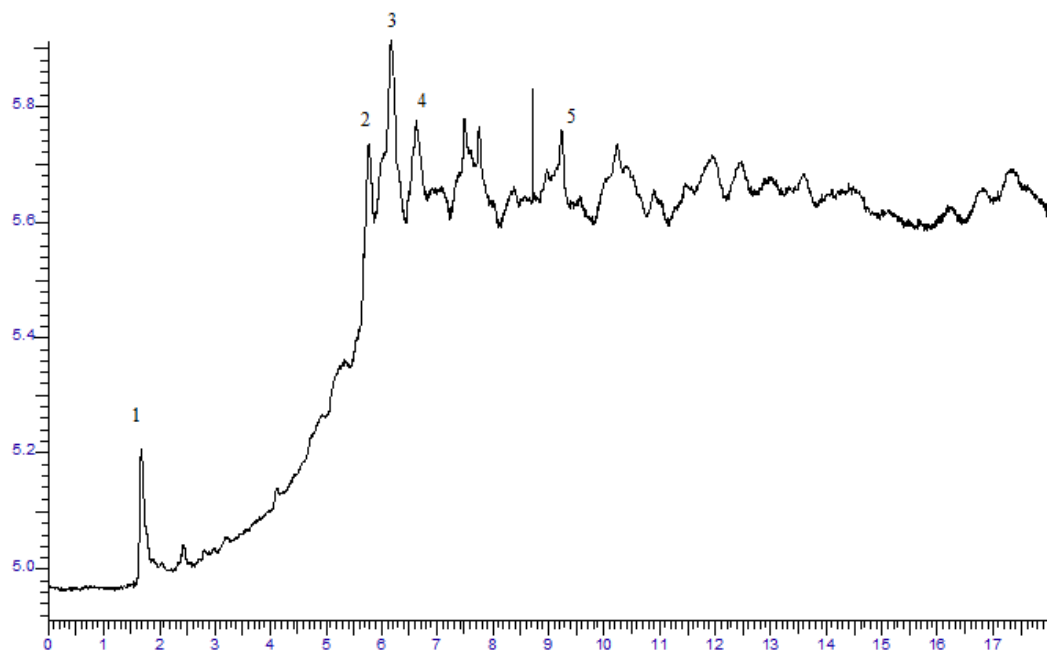


Figure 23: GC analysis of headspace from urea-supplemented *R. rhodochrous* DAP 96253.

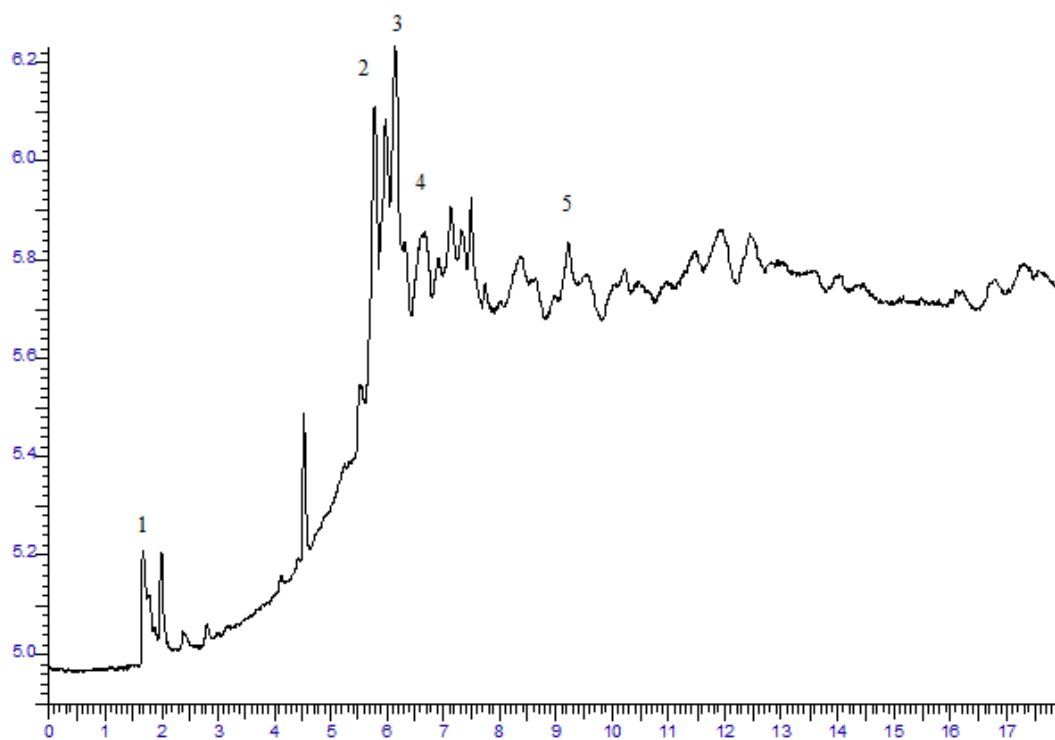


Figure 24: GC analysis of headspace from methylcarbamate-supplemented *R. rhodochrous* DAP 96253.

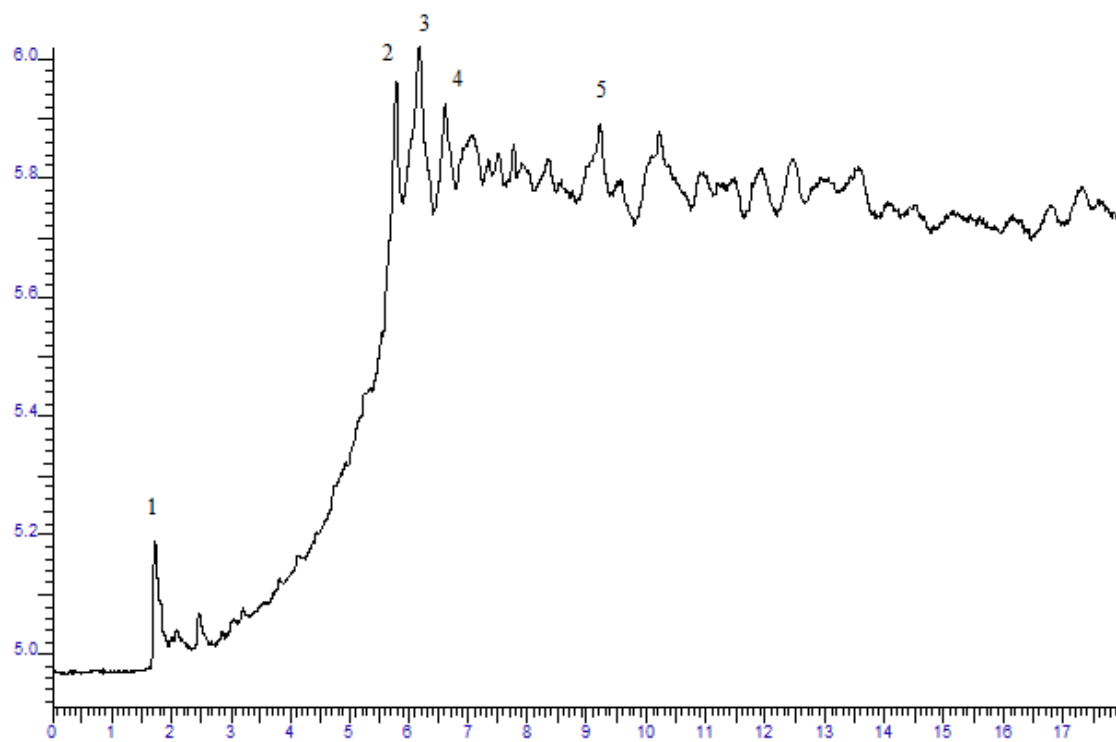


Figure 25: GC analysis of headspace from acetamide-supplemented *R. rhodochrous* DAP 96253.

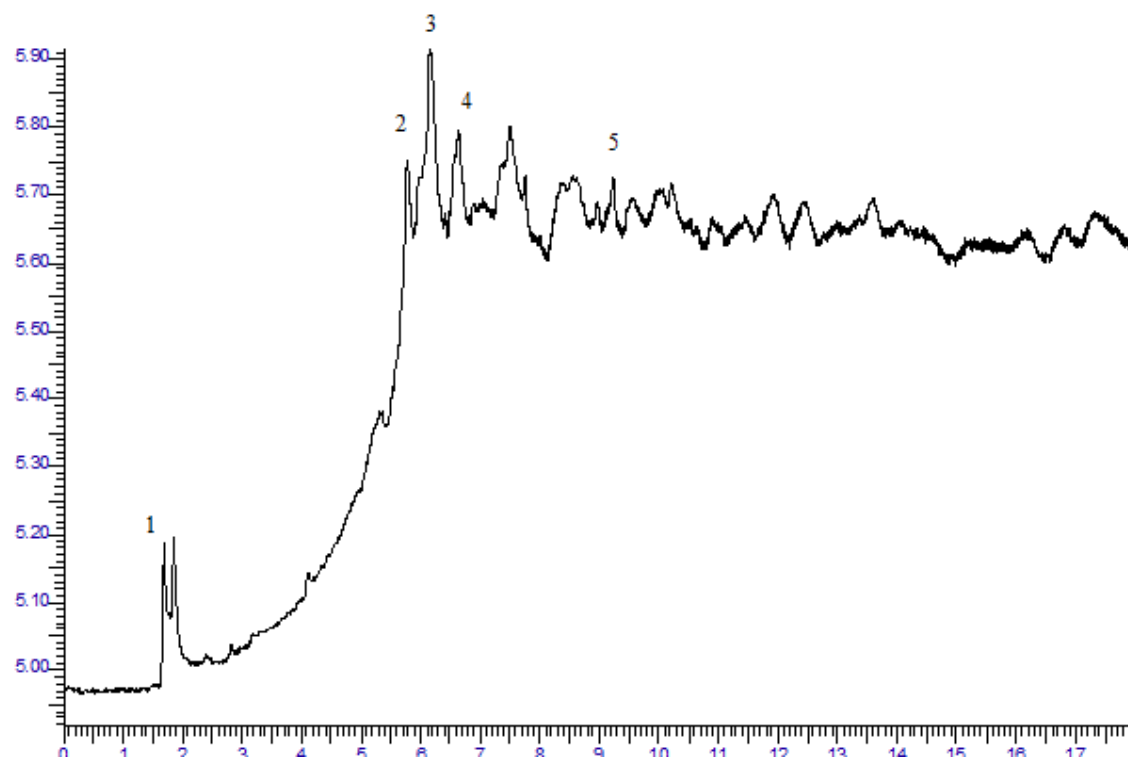


Figure 26: GC analysis of headspace from methacrylamide-supplemented *R. rhodochrous* DAP 96253.

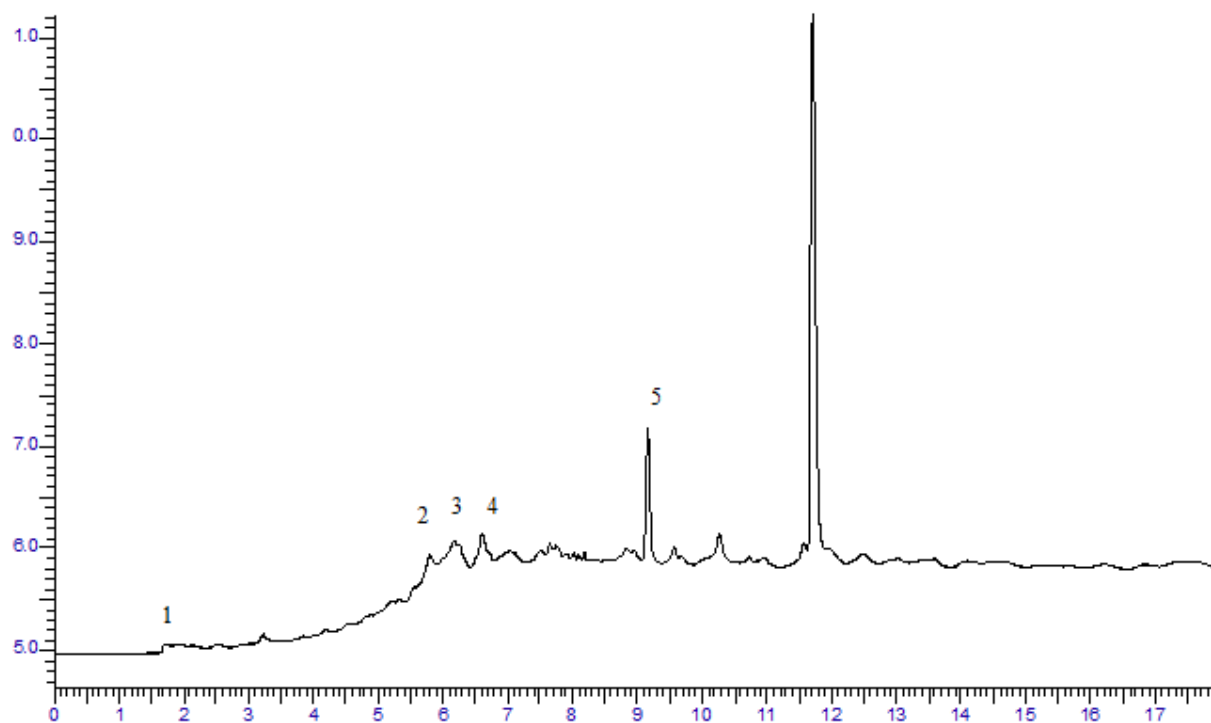


Figure 27: GC analysis of headspace from *R. rhodochrous* DAP 96253 grown on YEMEA supplemented with cobalt and urea.

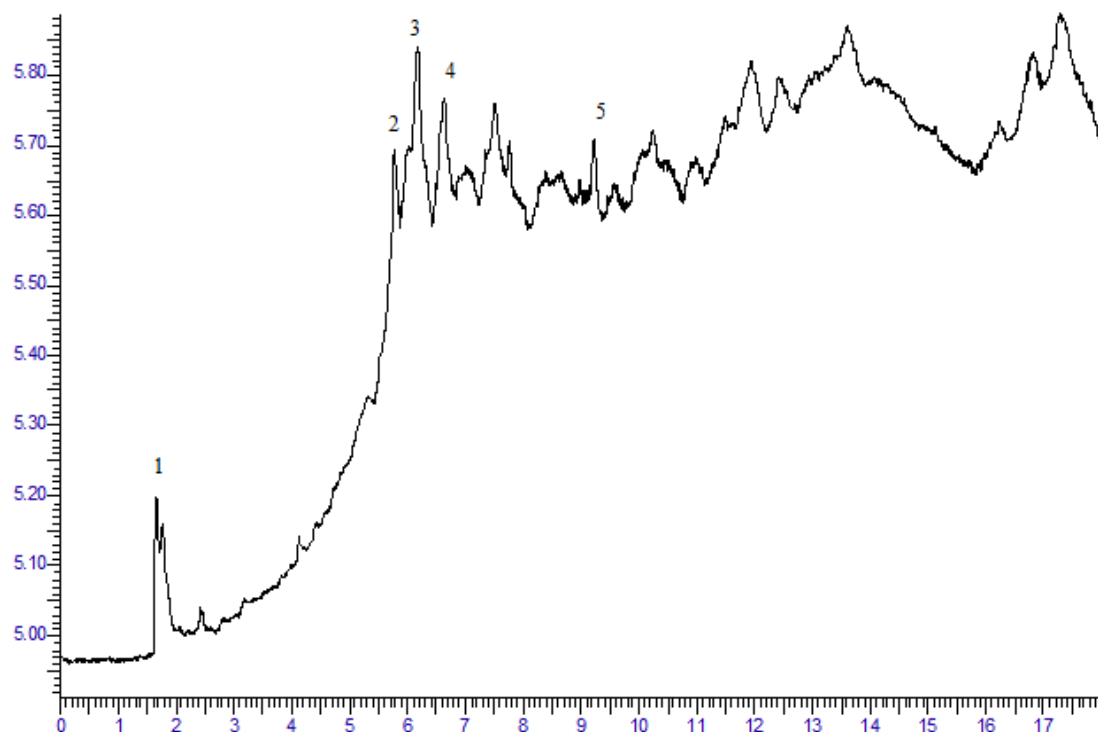


Figure 28: GC analysis of headspace from YEMEA grown *R. rhodochrous* DAP 96253.

Table 8: Comparison of area counts for common headspace peaks in common between *Rhodococcus* grown under various induction conditions.

Supplement	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
Retention Time	1.68	5.78	6.16	6.63	9.22
	Relative Percent				
Urea	19	100*	100*	68	.3
Methylcarbamate	56	70	13	0	5
Acetamide	0	42	48	41	41
Methacrylamide	100*	42	33	39	.1
YEMEA only	0	65	31	54	.2
Cobalt + Urea	25	46	4	100*	100*

*100% indicates that the peak was largest for the supplement listed. Remaining percentages for each growth condition are calculated as a percentage of the largest.

3.4.2 Solid Phase Microextraction

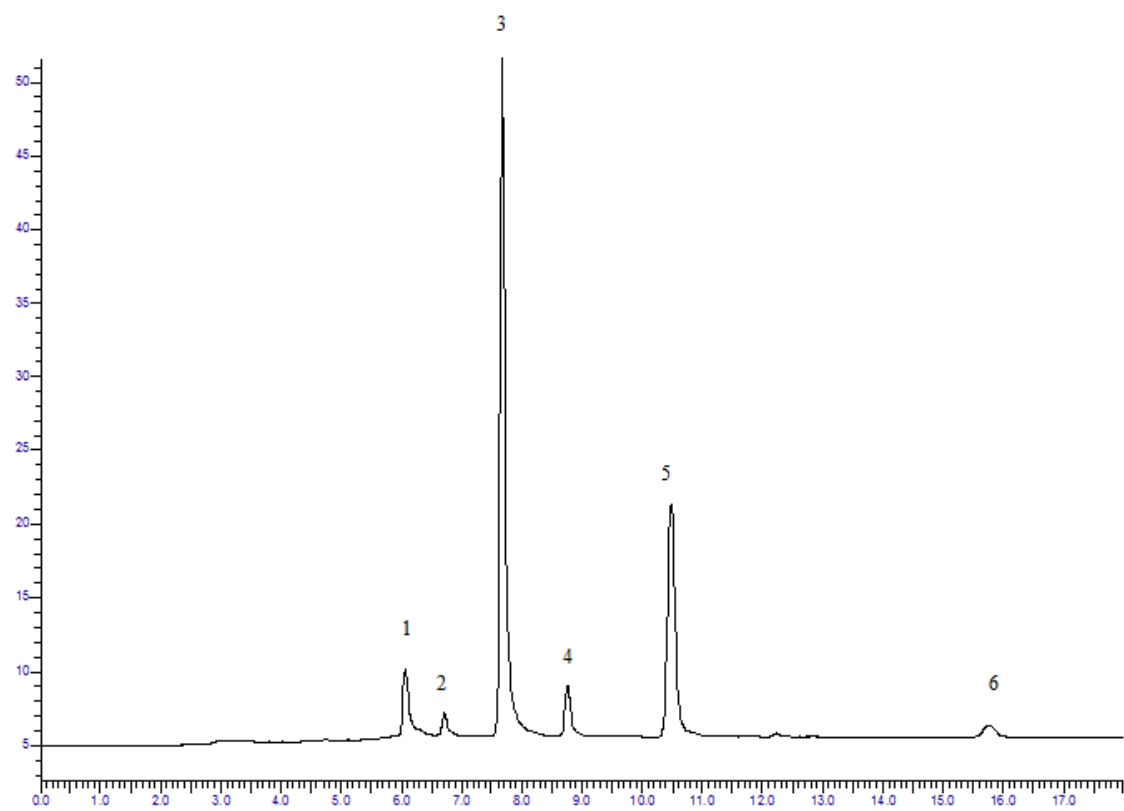


Figure 29: GC/SPME analysis of *R. rhodochrous* DAP 96253 supplemented with urea.

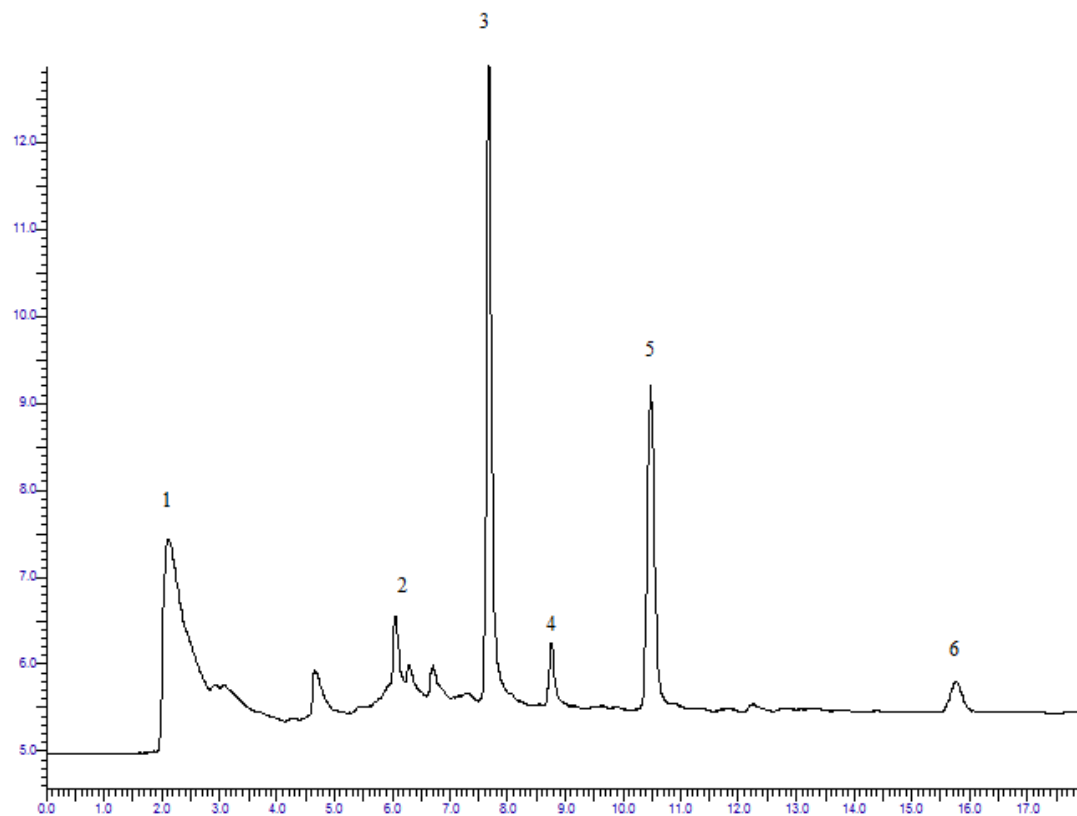


Figure 30: GC/SPME analysis of *R. rhodochrous* DAP 96253 supplemented with methylcarbamate.

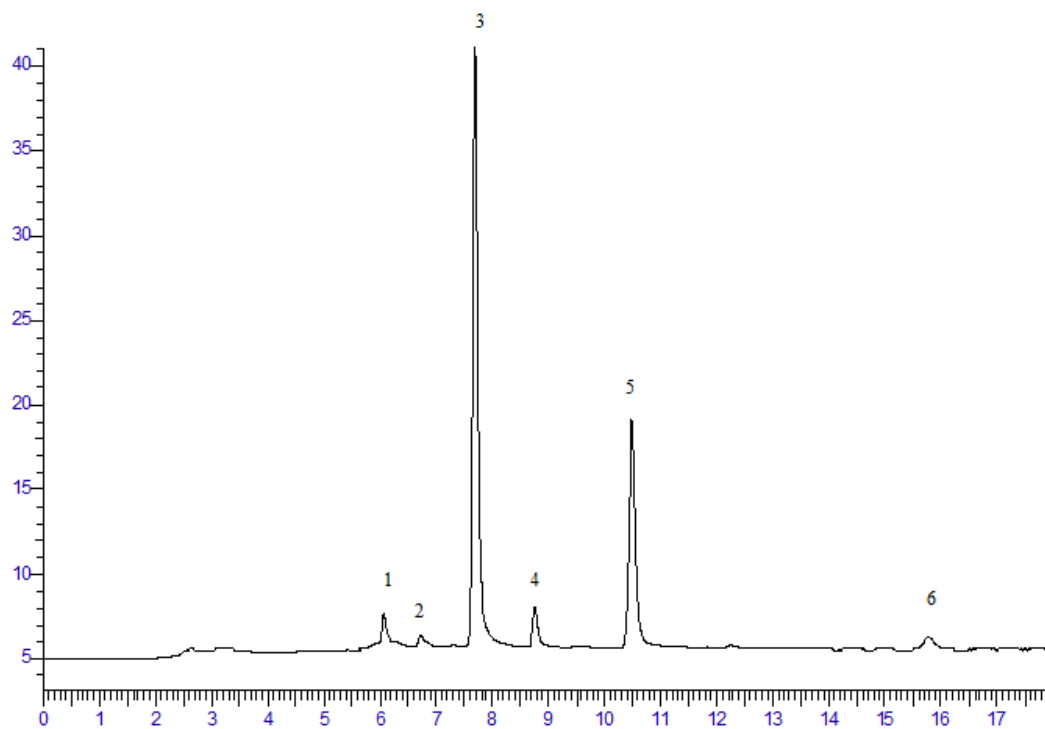


Figure 31: GC/SPME analysis of *R. rhodochrous* DAP 96253 supplemented with acetamide.

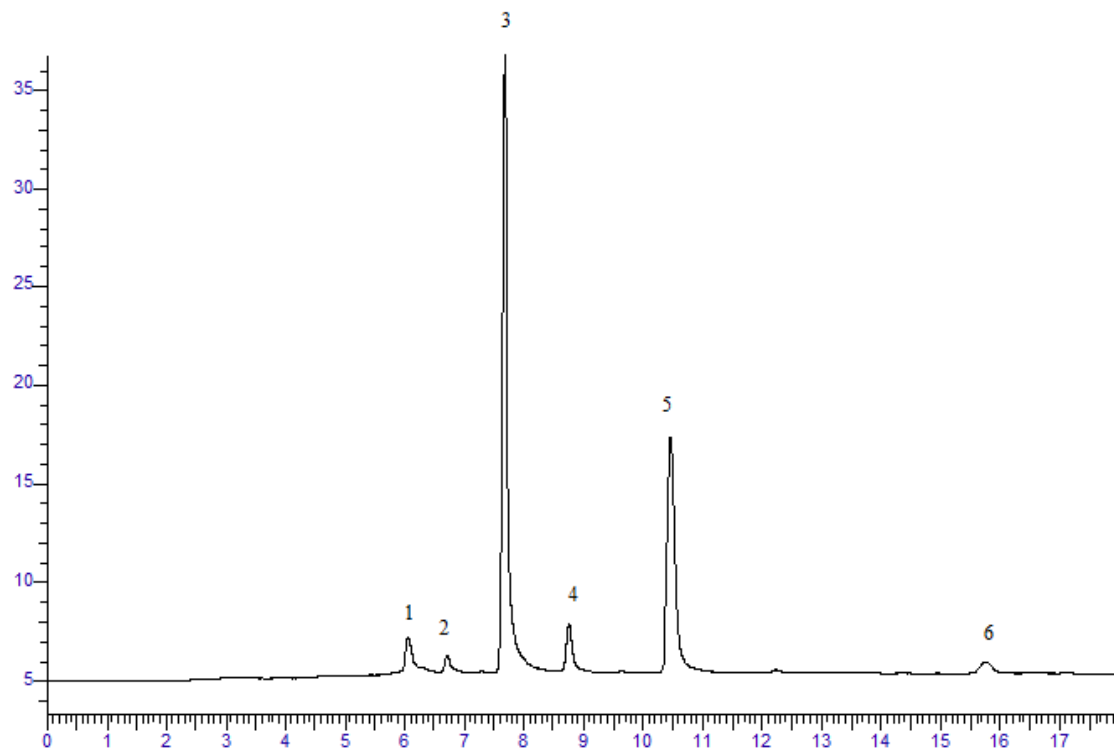


Figure 32: GC/SPME analysis of *R. rhodochrous* DAP 96253 supplemented with methacrylamide.

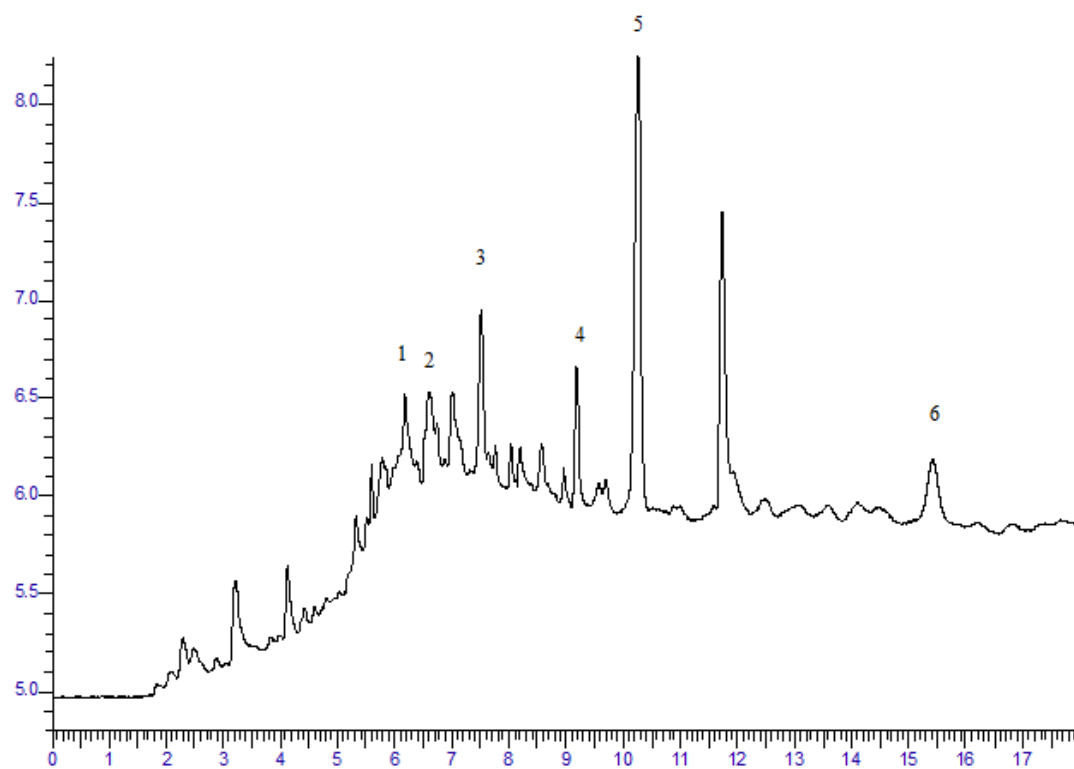


Figure 33: GC/SPME analysis of cobalt- and urea-supplemented *R. rhodochrous* DAP 96253.

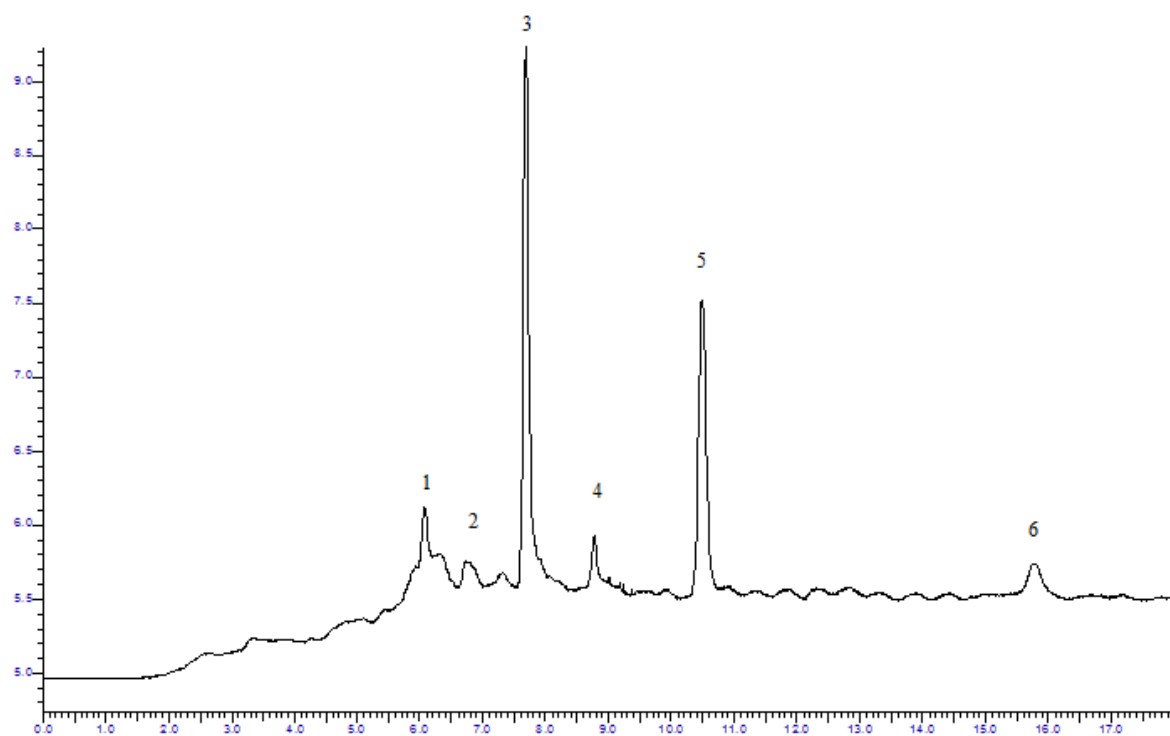


Figure 34: GC/SPME analysis of YEMEA grown *R. rhodochrous* DAP 96253.

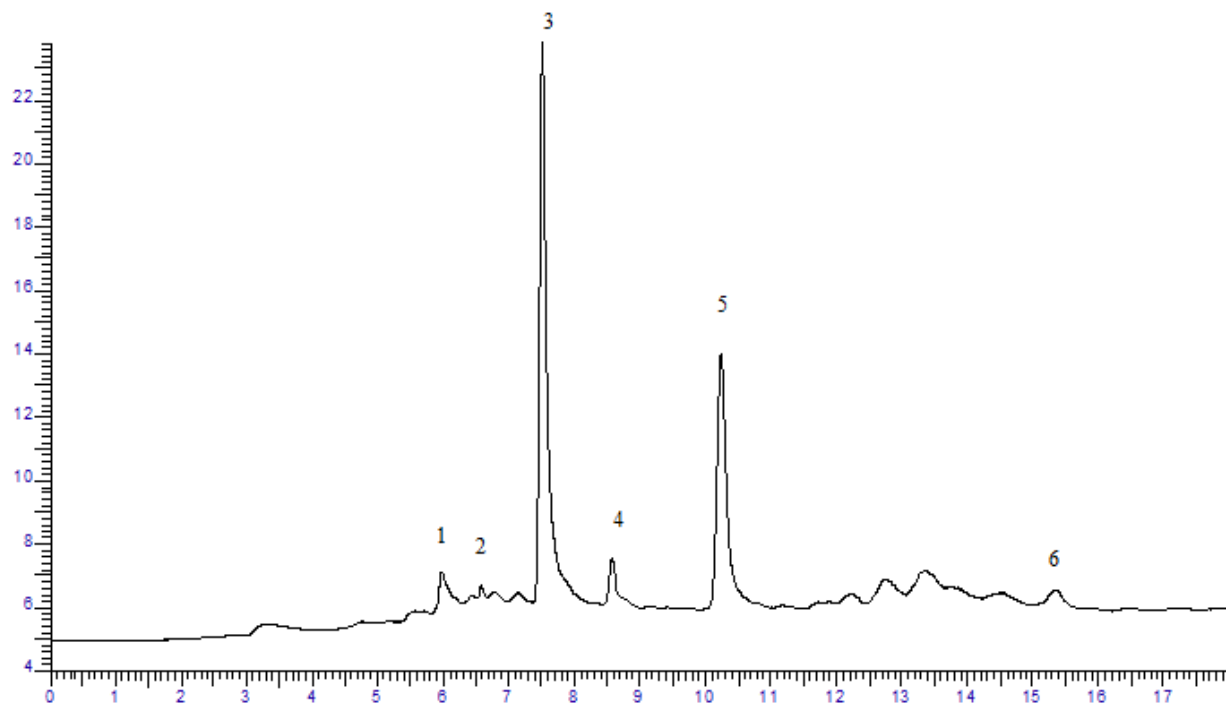


Figure 35: GC/SPME analysis of YEMEA-grown *R. rhodochrous* DAP 96253 after co-culture with urea-supplemented *R. rhodochrous* DAP 96253.

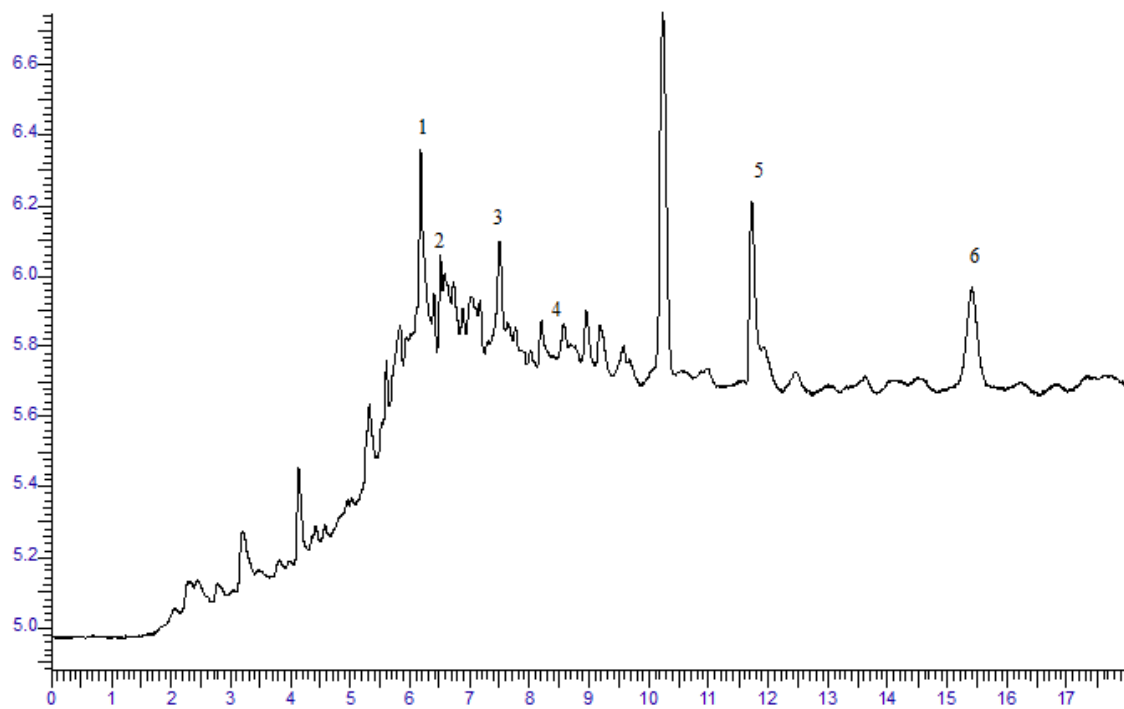


Figure 36: GC/SPME analysis of YEMEA-grown *R. rhodochrous* DAP 96253 after co-culture with cobalt- and urea-supplemented *R. rhodochrous* DAP 96253.

Table 9: Comparison of area counts for GC/SPME analysis of *Rhodococcus* grown with various media supplements.

Supplement	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6
Retention Time	6.05	6.72	7.69	8.75	10.48	15.70
	Relative Percent					
Urea	100*	53	92	100*	100*	34
Methylcarbamate	46	37	41	63	62	42
Acetamide	93	100*	100*	92	96	64
Methacrylamide	47	45	62	69	72	4
Cobalt + Urea	27	20	3	21	47	100*
YEMEA grown after co-culture with urea-supplemented	38	5	56	56	70	52
YEMEA grown after co-culture with cobalt- and urea-supplemented	14	16	2	6	16	0
YEMEA only	11	3	8	8	12	3

*100% indicates that the peak was largest for the supplement listed. Remaining percentages for each inducer are calculated as a percentage of the largest.

4. DISCUSSION

4.1 Cyanide Metabolism

Many microorganisms are known to degrade cyanide, and may do so through a suite of enzymes (Banerjee *et al.*, 2002). Cyanidase, a member of the nitrilase superfamily, has been well characterized in *Pseudomonas* species and, in some strains, may be induced through media supplemented with cyanide (Watanabe *et al.*, 1998). The metabolism of cyanide through cyanidase activity is inducible in *R. rhodochrous* DAP 96253. *R. rhodochrous* DAP 96253 cells grown on YEMEA alone do not show cyanidase activity and initial induction levels, 7 units/mg cdw, were only seen when *Rhodococcus* was grown in the presence of urea.

Addition of KCN to the growth media did not enhance rhodococcal cyanidase activity, as seen in some *Pseudomonas* strains (Watanabe *et al.*, 1998). However, additional chemicals were tested and higher levels of cyanidase activity were achieved. The most successful inducers of cyanidase were 200 mM acetamide as well as 7 mM glutamine + 8 mM asparagine, each resulting in 12 units/mg cdw, or 100% cyanidase activity (Table 2).

Acetamide was investigated as a potential inducer of cyanidase because of its similarity in structure to urea. The chemical formulas of acetamide and urea are C_2H_5NO and CH_4N_2O , respectively. Glutamine and asparagine were originally examined as inducers of nitrile hydratase activity and subsequently found to increase cyanidase activity as well (Ganguly, dissertation, 2007).

β -cyanoalanine synthase has been most widely reported as a plant enzyme (Akopyan *et al.*, 1975). Some bacteria have been shown to produce β -cyanoalanine, but may do so using the enzyme *O*-acetylserine sulfhydrylase (Castric and Conn, 1971; Dunnill and Fowden, 1965). Given the substrates KCN and Cysteine, *Rhodococcus* produces H_2S , which can also be measured colorimetrically, and is indicative of a β -CAS-like activity (Ezzi and Lynch, 2002). YEM-EA-grown *R. rhodochrous* DAP 96253 has around 90% of the maximum β -CAS-like activity identified. It was expected that addition of KCN to growth media would induce both CNase and β -CAS-like activity, however, this result was not observed. Another supplement examined, serine, is a substrate of β -CAS in plants (Hatzfield *et al.*, 2000) and therefore was expected to induce β -CAS-like activity. The β -CAS-like activity of serine-supplemented YEMEA grown *Rhodococcus* was equivalent to that of cells grown on un-supplemented YEMEA.

None of the supplements examined in this work resulted in a statistically significant increase in β -CAS-like activity in *R. rhodochrous* DAP 96253, suggesting that the gene for β -CAS-like enzyme may be constitutively expressed, or that this work has not identified appropriate inducers.

4.2 Fungal inhibition

Antagonism between organisms is a widely known phenomenon and has become the basis for the field of biological controls (El-Ghoauth, 1997). At present, there are several EPA-registered bacterial control agents commercially available, and much work being done to identify more organisms with this potential (Kim *et al.*, 1997). Several strains of *Rhodococcus erythropolis* were shown to produce several different antibiotics with efficacy against a wide range of Gram positive bacteria (Kitigawa and Tamura, 2008). *Rhodococcus equi* has been shown to inhibit germination of *Aspergillus flavus*, although very little is known about how this is achieved (Reddy *et al.*, 2010). *R. rhodochrous* DAP 96253 grown on appropriately supplemented YEMEA is inhibitory to several fungal species. Initially added to *Rhodococcus* growth media as a potential inducer of nitrile hydratase activity, urea was later identified as the first supplement that enabled *R. rhodochrous* DAP 96253 to inhibit fungal germination (Drago, dissertation, 2007; Pierce, unpublished). Further research was conducted to analyze the effect of supplementing YEMEA with other compounds similar in structure to urea.

Conditions shown to be most effective for antifungal work were YEMEA induced with 200 mM urea or 200 mM methylcarbamate or 200 mM acetamide, or 40 mM methacrylamide. The chemical formulas for methylcarbamate and methacrylamide are $C_2H_4NO_2$ and C_4H_7NO , respectively; these are fairly consistent with the structure of acetamide and urea (C_2H_5NO and CH_4N_2O). All of the effective supplements have a carbonyl carbon with at least one amino side group in their structure.

Methylcarbamate or acetamide-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 were most effective at inhibiting germination of *A. niger*, *A. fumigatus*, *Penicillium* sp., as well as the mixed fungal culture. This work showed fungal inhibition by *Rhodococcus* can be achieved without direct contact between organisms. Although work done by Reddy *et al.* (2010) has previously shown inhibition of *Aspergillus* by a *Rhodococcus* species, those organisms were placed in direct contact. Current employment of biological control agents typically requires contact between the plant of interest, the pathogen, and the agent (Bleve *et al.*, 2006; Jones and Samac, 1996).

4.2.1 Germination recovery assays

It is well known that many antimicrobial compounds have a minimum bactericidal concentration, below which, growth of the target organism will resume upon removal from the compound (Andrews, 2001). For this work, a parallel assay was designed to determine minimum exposure time required for the antimicrobial-producing organism, *Rhodococcus*, to prevent growth of the target organism. Initial recovery assays were performed by directly inoculating the germination media with spores, then removing the entire agar plate from co-culture after exposure. Using this assay, spores were completely unable to recover, even after a minimal exposure of 24 hours.

However, when spores were moved to fresh YEMEA that had not previously been exposed to *Rhodococcus*, spores became viable after short-term exposure. This indicates that, in the initial set up, compounds given off by *Rhodococcus* were diffusing into the agar on which the spores had been inoculated, and exposure to the inhibitory compounds continued after separation from co-culture. To circumvent this problem, spores were inoculated on filter paper, which facilitated their transport to fresh agar after a 24, 48, or 72 hour exposure to induced *Rhodococcus*.

After transfer to fresh agar, *A. niger* spores were incubated at 30°C for an additional 48 hours. Visual screening indicated that spores were able to germinate after removal from a 24 hour co-culture with urea-induced *R. rhodochrous* DAP 96253 (Table 5).

24 hour exposure to methylcarbamate-, acetamide-, or methacrylamide- supplemented *R. rhodochrous* DAP 96253 was sufficient to prevent germination of spores. These results were confirmed by continuing the assays for 48- and 72-hour exposures, and with each subsequent exposure, the spores remained unviable. After 48 hours of co-culture with urea-induced *Rhodococcus*, spores could not recover. A subsequent 72-hour exposure to urea-induced *Rhodococcus* also rendered the spores unable to recover (Table 5). These results suggest greater efficacy of volatiles produced by methylcarbamate-, acetamide-, or methacrylamide- supplemented YEM-EA-grown *R. rhodochrous* DAP 96253 than those produced by urea-supplemented YEMEA-grown cells.

4.2.2 Directed airflow

A directed airflow system was used to determine if *R. rhodochrous* DAP 96253 continually produced inhibitory compounds, or only did so in response to the presence of fungi. The setup was designed to allow a continuous circulation of air and replenished supply of rhodococcal volatiles to an inoculum of 5×10^2 *A. niger* spores, without backflow of air from the spores to the *Rhodococcus*. The resultant inhibition of germination indicated that the antifungal compounds given off by *Rhodococcus* are done so without back-and-forth communication between cultures (Figure 16).

4.2.3 Recruitment

Some species of *Rhodococcus* can impact plant growth cycles through volatile signaling. For example, *R. fascians* may produce phytohormones, while simultaneously degrading plant signaling molecules, resulting in leafy-gall formation in infected plants (Goethals *et al.*, 2001). *R. fascians* has also been shown to produce volatiles that are attractive to the fly, *Wohlfahrtia magnifica* (Khoga *et al.*, 2002). However, less is known about volatile signaling between rhodococcal species. The work presented in this dissertation identified a unique type of rhodococcal interspecies signaling.

Experiments were completed to determine if urea-supplemented, YEMEA-grown *R. rhodochrous* DAP 96253 was inhibitory to other strains of YEMEA-grown *Rhodococcus*. Specifically, the effects on YEMEA-grown *R. rhodochrous* DAP 96253, *R. rhodochrous* 96622, and *R. erythropolis* 47072 were evaluated. In previous studies by Kitagawa and Tamura (2008), it was shown that most strains of *R. erythropolis* were susceptible to the antibiotics produced by other strains of *R. erythropolis*.

Urea-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 showed no inhibition of the same, or other strains of YEMEA-grown *Rhodococcus*. After being co-cultured with YEMEA + urea-grown *Rhodococcus*, all YEMEA-grown strains were tested for cyanidase activity and exhibited levels comparable to those of the urea-supplemented YEMEA-grown *R. rhodochrous* DAP 96253. Cyanidase activity of YEMEA-grown *R. rhodochrous* DAP 96253 went from <1 unit/mg cdw to 11 when recruited by urea-supplemented YEMEA-grown cells. When related to Table 2, 11 cyanidase units/mg cdw is equal to approximately 92% of the maximum induction achieved through chemical supplements.

Cyanidase activity of *R. rhodochrous* 96622 is 2 units/mg cdw when grown on YEMEA and 9 when recruited by YEMEA + urea-grown *R. rhodochrous* DAP 96253. YEMEA-grown *R. erythropolis* 47072 typically has 1 unit/mg cdw cyanidase activity, which is increased to 11 units/mg cdw by co-culture with YEMEA + urea-grown *R. rhodochrous* DAP 96253. These results for *R. erythropolis* 47072 are noteworthy because this organism will not grow in the presence of urea, so could not previously be induced for cyanidase activity or fungal inhibition.

When cobalt- and urea-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 cells were co-cultured with YEMEA-grown *R. rhodochrous* DAP 96253, no increase in cyanidase activity was seen, however, cyanidase activity of 9 units/mg cdw was seen in YEMEA-grown *R. rhodochrous* 96622 after co-culture with cobalt- and urea-supplemented YEMEA-grown *R. rhodochrous* DAP 96253. YEMEA-grown *R. erythropolis* 47072 exhibited cyanidase activity of 7 units/mg cdw by co-culture with cobalt- and urea-supplemented YEMEA-grown *R. rhodochrous* DAP 96253.

In addition to examining the effect on cyanidase activity, cobalt- and urea-induced *R. rhodochrous* DAP 96253 was also tested for its ability to recruit antifungal activity in the three YEMEA-grown strains. In all cases, only partial inhibition of fungi was seen (Figure 20c, 21c, 23c.). The “recruitment” of *Rhodococcus* species by cobalt- and urea- or urea-supplemented YEMEA-grown *R. rhodochrous* DAP 96253 further elucidates the mechanism by which the cultures interact. Without direct contact, one culture is able to activate another through volatile interactions. Mold inhibition and cyanidase activities are stimulated in YEMEA-grown *Rhodococcus* by simply sharing airspace with cobalt- and urea- or urea-supplemented YEMEA-grown *R. rhodochrous* DAP 96253.

This suggests that not only are urea- or cobalt-and urea-supplemented YEMEA-grown cultures giving off volatile signaling compounds, but also that YEMEA-grown cultures are receptive to these compounds and respond by increasing cyanidase activity or fungal inhibition. This approach to studying induction of fungal inhibition is unique and has not been previously reported in literature.

Although *R. rhodochrous* 96622 cells grown on urea-induced YEMEA is inhibitory to germination of *A. niger* spores, they were unable to recruit cyanidase activity or antifungal production in any of the above mentioned strains. As seen in Table 6, even after co-culture with urea-supplemented YEMEA-grown *R. rhodochrous* 96622, YEMEA-grown *Rhodococcus* continued to have low levels of cyanidase and no effect on germination of spores. *R. erythropolis* 47072 did not grow on YEMEA supplemented with urea, so its ability to recruit YEMEA-grown cultures could not be tested.

As indicated by Table 7, Nitrile hydratase activity of YEMEA-grown *R. rhodochrous* DAP 96253 does not increase after co-culture with cobalt-supplemented or urea-supplemented YEMEA grown *R. rhodochrous* DAP 96253.

4.3 Gas Chromatography

Gas chromatographic characterization of microbial volatiles is a commonly employed technique with wide-ranging applications. Uses include investigating bacterial induction of plant growth (Farang *et al.*, 2006), understanding the mechanism by which some microorganisms inhibit growth of others (Yuan *et al.*, 2012), as well as identification of pathogenic organisms (Larsen *et al.*, 1978). Some biologically produced antimicrobial volatiles that have been identified previously include alcohols, ketones, aldehydes, acids, and benzenes (Strobel *et al.*, 2001; Utama *et al.*, 2002; Yuan *et al.*, 2012).

For this work, several columns that have been used previously to examine bacterial volatiles were tested with rhodococcal volatiles, including Poropak Q (Larssen *et al.*, 1978), and DB Wax (Xu *et al.*, 2011). Additionally, SPB 20 and SPB 100 were tested with rhodococcal samples. Ultimately, the DB 625 was chosen for analysis as this column provided the most consistent results. The DB 624 stationary phase consists of 6% Cyanopropyl-phenyl, 94% dimethyl polysiloxane, and is of midpolarity.

4.3.1 Headspace sampling

Gas chromatographic headspace analysis is a common method for investigating microbial volatiles (Labows *et al.*, 1980; Larsson *et al.*, 1978; Khoga *et al.*, 2002). For the headspace analysis completed in this work, samples were run on the GC according the method described previously. An ethylene standard was run prior to analysis of each sample. Peak profiles were similar among all conditions tested, urea-, methylcarbamate-, acetamide-, methacrylamide-supplemented YEMEA, and YEMEA only. Compounds with similar retention times were present in all 5 samples. However, several issues were encountered with headspace sampling. It was difficult to consistently collect repeatable amounts of the volatiles present by using a gas-tight syringe. This led to inconsistent area-counts among duplicates of the same samples. Compounds that came off after 5 minutes were difficult to discern from the baseline and were present in very small amounts. Overall, the chromatograms were difficult to read and volatiles could not be collected in uniform concentrations among samples. For these reasons, SPME sampling was used to supplement the headspace chromatographic analysis.

4.3.2 Solid Phase Microextraction

Farag *et al.* (2006), Strobel *et al.* (2001), Yuan *et al.* (2012), and others have utilized solid phase microextraction for concentration of microbial volatiles prior to GC analysis. Due to the previously described problems with direct headspace injection, SPME was used to improve GC analysis for this work. Yuan *et al.* (2012) used carboxen/polydimethylsiloxane (Car-PDMS) fibers for analysis of *Bacillus amyloliquefascians* volatiles. Car-PDMS fibers exhibit a bipolarity that allows sampling of a wide range of low molecular weight compounds. For this work, samples were drawn from airtight flasks containing three 60x15 mm plates of *Rhodococcus*. This size plate was chosen used because it was size-appropriate to the sampling container. Three plates were added to each container with the aim of concentrating sufficient volatiles for sampling.

SPME profiles were developed for the volatile compounds given off by *R. rhodochrous* DAP 96253 grown in the presence of urea, methylcarbamate, acetamide, methacrylamide, as well as uninduced and recruited cells. It was noted that several compounds with shorter retention times were seen in headspace but were lost from SPME analysis. This included 3 peaks that came off before 5 minutes. However, SPME sampling provided a much clearer depiction of the primary peak profiles among samples. Chromatographic profiles were similar for all of the conditions tested, with major peaks coming off at the same time in each sample. Duplicates of the same samples were consistent in retention time and peak area

Concentrations of compounds were higher in all supplemented conditions and recruited cultures than in YEMEA-only (Table 9). An interesting trend emerges when analyzing the volatiles given off by urea-supplemented or cobalt- and urea-supplemented *Rhodococcus* as compared to those given off by YEMEA-grown *Rhodococcus* recruited by these supplemented cultures. The concentrations from urea-supplemented YEMEA-grown cells are typically higher than those from cobalt- and urea- supplemented YEMEA-grown *Rhodococcus*. These results parallel the efficacy in antifungal assays with *Rhodococcus* supplemented the same way, implying a correlation between the volatiles studied and fungal inhibition.

YEMEA-grown *Rhodococcus* recruited by urea-induced *Rhodococcus* has a higher concentration of volatiles than cobalt- and urea-supplemented YEMEA-grown *Rhodococcus*, which was expected because *Rhodococcus* recruited by urea-supplemented YEMEA-grown cells are capable of fully inhibiting germination of spores, whereas cobalt- and urea-supplemented YEMEA-grown *Rhodococcus* exhibit only partial inhibition. In other words, the inhibition of fungal spore germination and the concentration of the volatiles studied can be ranked as follows, from high to low: urea-supplemented YEMEA-grown *Rhodococcus*, YEMEA-grown *Rhodococcus* recruited by urea-supplemented YEMEA-grown *Rhodococcus*, cobalt- and urea-supplemented YEMEA-grown *Rhodococcus*, YEMEA-grown *Rhodococcus* recruited by cobalt- and urea- supplemented YEMEA-grown *Rhodococcus*.

5. CONCLUSION

This work identified several supplements which increased activity of cyanidase in *R. rhodochrous* DAP 96253. Increased enzyme activity was determined using colorimetric assays. The degradation of cyanide has many applications to the field of microbiology. Cyanide degrading enzymes can be utilized in the decontamination of cyanide-containing wastes. Additionally, bacterial ability to metabolize cyanide could impact interactions with climacteric plants, which release cyanide during ripening.

Some of the compounds tested for induction of enzymes also induced rhodococcal production of antifungal compounds. When grown with these compounds, *R. rhodochrous* DAP 96253 was shown to inhibit germination of *Aspergillus fumigatus*, *Aspergillus niger*, and *Penicillium* sp. Inhibition was achieved by proximity exposure; the only contact between the two organisms was through a shared airspace. This is indicative of volatile inhibition by *Rhodococcus*.

In the field of biological controls, microorganisms are becoming an increasingly interesting option for control of pathogenic species. Many of the currently commercially available biological control agents function through contact inhibition, which would not be necessary if utilizing *Rhodococcus*.

Some unique volatile interactions were also seen between *R. rhodochrous* DAP 96253 and other rhodococcal species. When un-supplemented *Rhodococcus* sp. were exposed to appropriately supplemented *R. rhodochrous* DAP 96253, the un-supplemented cultures developed cyanidase activity, as well as fungal inhibition that was comparable to the activities seen in directly supplemented *R. rhodochrous* DAP 96253.

The mechanism of enzyme induction through volatile signaling between *Rhodococcus* species has not previously been described in the literature. Additionally, the ability of supplemented *R. rhodochrous* DAP 96253 to activate production of antifungal compounds in un-supplemented *Rhodococcus* is a novel induction mechanism that has not been described previously.

Overall, the work presented here identified methods for inducing cyanidase activity in *R. rhodochrous* DAP 96253. This work also showed that β -cyanoalanine synthase activity was not inducible with any of the chemicals tested. Furthermore, this work characterized the antifungal properties of *Rhodococcus* by investigating methods for exposure to the fungal target. Information was gained about the duration of exposure, to rhodococcal volatiles, that was required for total inhibition of fungal germination. Additionally, methods for inducing the production of antifungal volatiles in several rhodococcal strains were determined.

6. FUTURE DIRECTIONS

Further work should be completed to determine if *R. rhodochrous* DAP 96253 supplemented with methylcarbamate, acetamide, or methacrylamide is able to activate increased cyanidase activity and production of antifungal volatiles in un-supplemented *Rhodococcus*. Additionally, *R. rhodochrous* 96622 and *R. erythropolis* 47072 should be tested for induction of antifungal activity by growth in the presence of methylcarbamate, acetamide, or methacrylamide. Mass spectrometry should be employed to identify the antifungal volatiles given off by *Rhodococcus*, and determine if the volatiles are the same for each growth condition.

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APPENDIX

Enzyme Quantification

Sodium phenate: 25 g of Phenol (Fisher Scientific, Fair Lawn, NJ) was melted into 800 ml of H₂O using a 50°C water bath. 78 mL of 4N Sodium Hydroxide (Fisher Scientific, Fair Lawn, NJ) was added to the solution

0.1% sodium nitroprusside: 1 g of Sodium Nitroprusside (Sigma-Aldrich, St Louis, MO) added to 100 ml DI water (stock solution). 1 ml of stock solution was added to 99 ml of distilled H₂O

0.02 N sodium hypochlorite: 2.44 ml of 6.15% Chlorox® made up to 100 ml with DI water

Reagent used to assay for H₂S

0.3 M FeCl₃ in 1.2 M HCl: 1 g FeCl₃ (Sigma-Aldrich, St Louis, MO) was dissolved into 205 ml of 1.2 M HCl (Mallinckrodt, Phillipsburg, NJ)

0.02 M N,N-dimethyl-p-phenylene diamine sulfate: 4.68 g diamine sulfate (Sigma-Aldrich, St Louis) dissolved in 1 L of 7.2 M HCl

Reagents used for spore harvesting

Conidia harvesting solution: 250µL Tween (Sigma-Aldrich, St Louis, MO) and 4.5 g NaCl (Fisher Scientific, Fairlawn, NJ) were added to 500 ml dH₂O

0.9% NaCl: 18 g NaCl was dissolved in 2 L dH₂O.

One-directional air flow

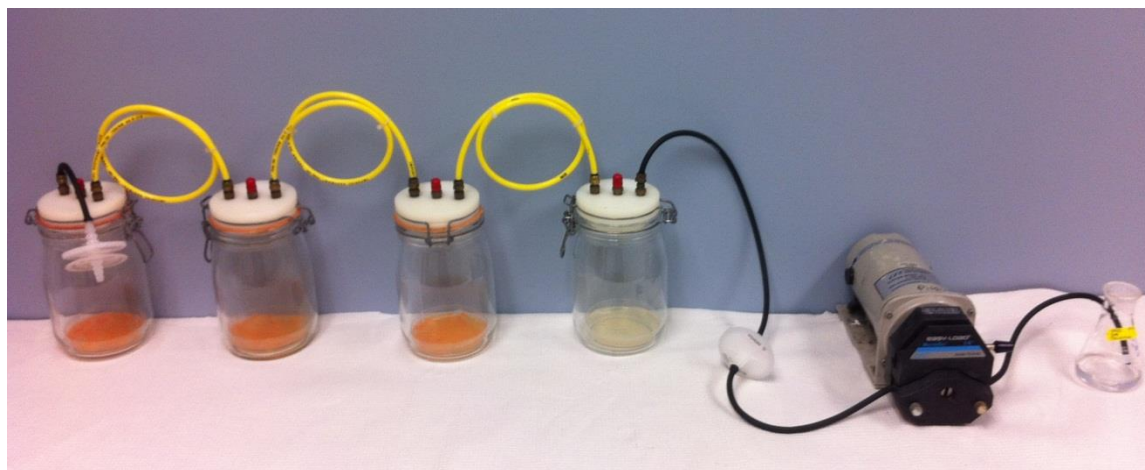


Figure 37: One-directional airflow through sealed jars containing plates of *Rhodococcus* followed by one plate inoculated with 5×10^2 spores. Outgas collected in flask with 25 ml dH₂O.

Gas Chromatographic analysis



Figure 38: 250 mL Bellco (Vineland, NJ) Spinner Flask set-up used for SPME sampling.