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Process Improvements to Fed-batch Fermentation of Rhodococcus rhodochrous DAP 96253 for the Production of a Practical Fungal Antagonistic Catalyst

Courtney Barlament

PROCESS IMPROVEMENTS TO FED-BATCH FERMENTATION OF RHODOCOCCUS RHODOCHROUS DAP 96253 FOR THE PRODUCTION OF A PRACTICAL FUNGAL ANTAGONISTIC CATALYST

by

COURTNEY BARLAMENT

Under the Direction of George Pierce, PhD

ABSTRACT

Recent evaluations have demonstrated the ability of the bacteria *Rhodococcus rhodochrous* DAP 96253 to inhibit the growth of molds associated with plant and animal diseases as well as post-harvest loss of fruits, vegetables and grains. Pre-pilot-scale fermentations (20-30L) of *Rhodococcus rhodochrous* DAP 96253 were employed as a research tool with the goal of producing a practical biological agent for field-scale application for the management of white-nose syndrome (WNS) in bats and post-harvest fungal losses in several fruit varieties. Several key parameters within the bioreactor were evaluated for the potential to increase production efficiency as well as activity of the biocatalyst. These parameters included elapsed

fermentation time, dissolved Oxygen, and carbohydrate concentration of which increased carbohydrate concentration at the time of harvest was shown to have a negative impact on the catalyst activity. In addition, process improvements including utilization of a liquid inoculum, an autoinduction feed strategy, and increased glucose concentration in the feed medium increased fermentation yields to 100-150g/L, while the biocatalyst efficiency was increased from previous work. To increase production efficiency, a multi-bioreactor scheme was developed that used a seed bioreactor and subsequent production tank, which doubled run yields per production cycle. Amidase, cyanidase, urease, and alkene-monoxygenase activity were monitored throughout the study as potential indicators for the multi-faceted mechanism of fungal antagonism. Of these amidase, cyanidase, and urease were demonstrated to be more elevated in cells that showed antifungal activity than those that did not. This study represents the first example of a reproducible pre-pilot plant-scale biomanufacturing process for a contact-independent biological control agent for established and emerging fungal pathogens of plants and animals, and facilitates large-scale production for broad application.

INDEX WORDS: Biomanufacturing, Fermentation, *Rhodococcus rhodochrous* DAP 96253, Biocatalyst, Fungal antagonism, Bioreactor, Process improvements

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COURTNEY BARLAMENT

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COURTNEY BARLAMENT

Committee Chair: George Pierce

Committee: Sidney Crow Jr.

Eric Gilbert

Electronic Version Approved:

Office of Graduate Studies

College of Arts and Sciences

Georgia State University

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DEDICATION

You know that you can find it, it's out of your reach

They told us to stand in our place

That's what they teach

Don't stand too close now, you might get burned

Don't show that you know much at all

Just what you have earned

I'm searching for answers

To the questions that I can't define

I keep fallin' backwards, lookin' forwards but always behind

You've seen what they offered, they tasted the wine

They know what to make you believe, they take their time

They hide in your shadow, they burn too close

They're here but can't see them at all, such a great hoax

-*The String Cheese Incident*

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1 INTRODUCTION

1.1 A candidate for fermentation: *Rhodococcus rhodochrous*

Nocardiaceae is a family of Gram-positive, aerobic bacteria that are commonly found in soil and water. Of this family, The genus Rhodococcus is currently composed of 30 species that are known for being metabolically diverse and capable of both economical and environmental valuable biotransformation reactions (Bell *et al.,* 1998; Larkin *et al.,* 2005).

Due to its metabolic diversity and lack of pathogenicity to humans, *R. rhodochrous* is an outstanding candidate for biomanufacturing and use as a biocatalyst. *R. rhodochrous* is commonly used as a catalyst for industrial applications such as wastewater treatment, because it degrades complex hydrocarbons and nitriles via multienzyme systems (Bhalla *et al.*, 1992; Rodrigues *et al.,* 2015). Additionally, it has been utilized for the production of several commodity chemicals such as lipids, pharmaceuticals, and acrylamide (Kobayashi *et al.*, 1990; Shields-Menard *et al.*, 2015).

1.2 *R. rhodochrous* DAP 96253 and the delay of ripening in climacteric fruit

R. rhodochrous strain DAP 96253 (*R. rhodochrous*) cells induced under the conditions described in US patents 7,531,343 and 7,531,344 demonstrate the ability to delay the ripening of selected climacteric fruit in a contact-independent fashion. When placed in close proximity the ripening process of several fruit varieties (e.g. bananas and peaches) are delayed by several days or more. In addition, it was noted that fruit exposed to induced cells (live or dead) also inhibited the development of mold. Although the exact mechanism currently is not yet fully elucidated, it is hypothesized that the mechanism for indirect delayed ripening involves the enzymatic degradation of volatile plant hormones that aid in the ripening process, and thus delays the natural ripening process of fruits (primary and secondary).

1.3 *R. rhodochrous* DAP 96253 as fungal disease management tool

When properly induced *R. rhodochrous* DAP 96253 cells have been shown to extend the shelf life of selected fruit, when placed in close proximity, through the enzymatic degradation of plant ripening hormones and the production of inhibitory volatile compounds, and represents an additional mode of action. In fruits and vegetables, trauma caused by harvesting and handling techniques may injure the plant and allow fungal species normally present on the surface of the plant to enter the injury site, colonize, and render the plant inedible by mold. Post-harvest loss of fruits and vegetables amounts on average to 40% of all spoiled produce, and this could be modulated by better storage, handling, and fungal disease management techniques (Harvey, 1978). While chemical control agents are currently used to control fungal pathogens found in soil, the downstream effects of these applications on the ecosystem can result in environmental damage, fungicide-resistant species, and harmful effects on humans. A biological control option represents a better approach for fungal control in soils and on edible fruit because it could be utilized while conserving the inherent microenvironment of soils and would undeniably pose less of a threat to human health.

Through *in vitro* and *in vivo* experimentation the contact-independent antagonism of induced *R. rhodochrous* DAP 96253 cells towards select fungi has been demonstrated. Accordingly, *R. rhodochrous* is being developed as a biocontrol agent for fruits, the fungal plant pathogen *Botrytis cinerea,* and in parallel work is being conducted with *Pseudogymnoascus destructans,* the causative agent of White-Nose syndrome in bats (Cornelison *et al.*, 2014).

1.4 Fermentation

In order to provide a commercially viable fermentation product to combat various fungal pathogens, biomass production has to be further developed with the target of improving the yield, performance, stability, economical efficiency, consistency, and practical application techniques of the catalyst. When improving the overall efficiency for the production and commercialization of a biocatalyst, it is paramount to transition from small-scale to a pilot or plant scale production technique. The size and state of inoculum have a dramatic effect on yields from the bioreactor and should be improved for the appropriate scale of production (Drago, 2006). Within the bioreactor, the temperature (˚C), pH, dissolved oxygen (%O_2), stir rate (rpm), airflow (LPM), glucose concentration (g/L), feed rate (mL/min), inducer concentration, mass of inoculum (g), and Elapsed Fermentation Time (EFT) are crucial factors influencing the yield, stability, and performance of the resulting catalyst (Kim *et al.*, 2001).

Improving biomass yield and efficacy is critical to producing a practical commercial biological catalyst for an application. Enzyme stabilities also must be taken into account when designing fermentations as enzymes are the catalyst of biological reactions. Cells grown under a patented set of conditions have demonstrated enhanced activities of ammonia-producing enzymes as well as MO, and it is hypothesized that all of these may play a role in the mechanism of fungal antagonism (Pierce *et al.,* 2014). Several enzymes are elevated upon induction in the bioreactor. Of these, amidase, cyanidase, urease, and 1-HMO have been pursued in this work.

1.5 Post-production

1.5.1 Storage

Once harvested from the bioreactor, a stable storage procedure is critical for industrial catalysts to preserve their activity and integrity over time and cold chain methods should be

determined in order to decrease the amount of wasted product. Storing in cooler temperatures will result in stable activity of cells for an extended time period, however refrigeration costs must be taken into account (Legett, 2014). Cell integrity must be taken into account when using whole cell catalysts, so ice crystal formation when freezing and thawing must be evaluated.

1.5.2 Immobilization

Whole cell catalysts are immobilized in order to enhance stability and allow for repeated or extended use of the biocatalyst. Edible coatings such wax, alginate, and cellulose are commonly used on fruits, so could be used for immobilized catalyst for controlling mold on fruits (Tapia *et al.,* 2008). (See Pierce and Crow U.S. Published Applications 2016 0021890, 00213039).

1.5.3 Application techniques

Lastly, in order to maximize the utility of the biocatalyst, multiple application techniques should be investigated in order to consider possible delivery techniques such as contact dependent or independent. Under certain conditions less catalyst could be used for appropriate treatment and could minimize costs associated with production by maximize the potential efficiency of applications. Of potential application techniques, direct and indirect-contact, membranes, and immobilized cells have been evaluated in this study for fungal inhibition.

1.6 A diverse plant pathogen: *Botrytis cinerea*

B. cinerea is a global plant pathogen with a wide host diversity (>200 species) and is known to cause pre- and post-harvest fungal infections resulting in huge economic losses from crop damages rendered during growing, handling, and storage. *B. cinerea* alone causes about

20% of harvest loss in all affected species throughout the world, and estimated losses for all French vineyards amount to up to 15-40% of all grapes harvested (Awasthi, 2015). Multiple other lucrative agricultural industries are negatively impacted by *B. cinerea* including berry growers, vegetable farms, and the cut flower industry. The disease caused by *B. cinerea* is currently impossible to treat and difficult to manage because the spores can lay dormant in desiccated fruit or in soils during the winter months, and reemerge every growing season. In areas of high humidity and rainfall, it is especially difficult to manage the disease because *B. cinerea* thrives in high humidity and the excess moisture serves as a source of spore transmission between plants (Figure 1.1).

The current methods of disease management in soils include modifications of growing techniques, removal of obviously infected plants, and a heavy rotation of multiple chemical fungicides with various modes of action. These techniques may lower fungal burdens on plants, but they will inherently result in fungicide-resistant cultivars of *Botrytis* and other fungal pathogens as they adapt to the chemicals.

Once fruits, vegetables, and flowers are harvested, *B. cinerea* continues as a threat, because harvest and handling techniques inevitably wound the plant and create a germination site for spores present on the flesh. Commonly used post-harvest control methods include using fungicides sprayed directly on products, or storing plants in chemically- fungistatic atmospheres (Hammer *et al.*, 1990). Currently, there are no biological control options being utilized against *B. cinerea* in the agricultural industry for pre-harvest or post-harvest fungal disease. *R. rhodochrous* represents a biological control option to control or inhibit post-harvest infection in fruit, and has been further investigated in this work.

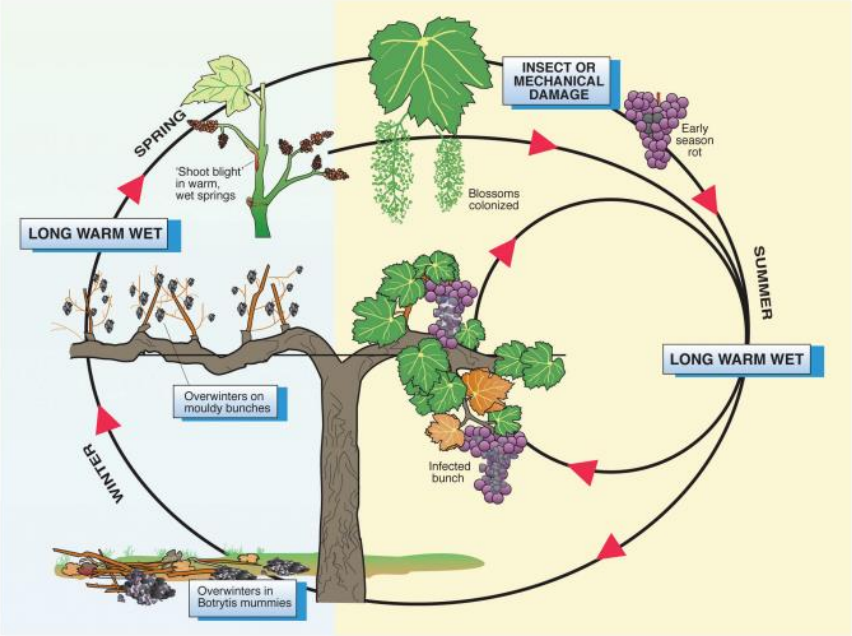

Figure 1.1 The disease cycle of B. cinerea on grapes
Diagram taken from Nicholas *et al.*,1994, Grape Production Series Number 1: Diseases and Pests, Winetitles.

1.7 White-Nose Syndrome in Bats and *Pseudogymnoascus destructans*

In recent years, major declines in the populations of North American bats can be attributed to white-nose syndrome (WNS), and to date around 5.7 billion bats have died of this disease. Currently, there are seven species of bats that have been diagnosed with the disease, and

five more species that have been found with the fungus present. *Pseudogymnoascus destructans*, determined as the causative agent in 2011, causes mortality in bats not only by the colonization of the muzzle, but by disrupting the physiological functions of the bat wings by destroying tissue essential for internal temperature and moisture control (Lorch *et al.*, 2010). Bat wings represent the majority of the exposed surface area of a bat and are integral for homeostasis and thermoregulation (Reeder & Cowles, 1951). Bats infected with *P. destructans* experience the loss of the ability to control internal temperature, because the wing tissue becomes eroded (appendix C).

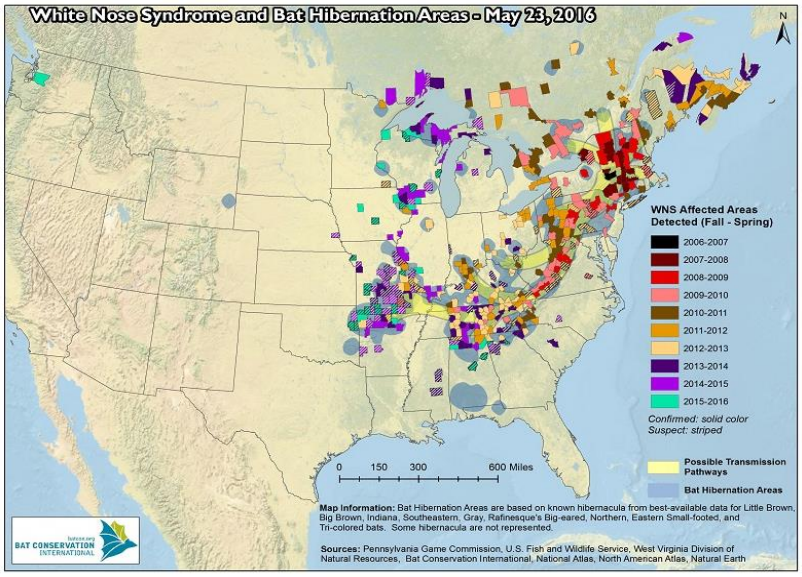

Figure 1.2 The spread of WNS in North America since initial 2006 case
Map courtesy of Bat Conservation International. (May 23, 2016).

WNS was first observed in 2006 near Albany, New York and has traversed the United States moving towards the West with increasing intensity every year. As bats are known to consume up to their body weight of pest insects on a single summer night, the loss of bats in the ecosystem will disrupt the food and lumber industries, among others, that rely heavily on the insecticidal properties of bats. Currently, there are no commercial prophylactics or therapeutics

known to prevent or treat WNS in bats, however known bacterial VOC's, derived from work on fungistatic soils, have been shown to be fungistatic and fungicidal to *P. destructans in vitro* (Cornelison *et al.*, 2013). In the *in vitro* VOC work, several chemical control options were presented that have shown to decrease mycelial extension and inhibit or slow spore germination of *P. destructans*. Currently, only chemical control techniques have been proposed, however a biological control method could provide a safe treatment alternative for the bats, as the health effects of chemical treatment are still unknown. Induced *R. rhodochrous* DAP 96253 cells have demonstrated contact-independent fungal antagonism of *P. destructans in vitro* and on bat tissue explants and represents a safe treatment method for bats (Cornelison *et al.*, 2014). The production of this biological control option was further investigated in this work (Appendix E, Appendix F).

1.8 Enzymes hypothesized to be mechanistically involved in fungal antagonism

R. rhodochrous DAP 96253 cells grown under the conditions described in US patents 7,531,343 and 7,531,344 have demonstrated significantly higher nitrile hydratase, amidase, cyanidase, and urease activities than those previously seen in other rhodococci. These enzymes are all involved in ammonia evolution and could play a role in the contact-independent antifungal activity of induced cells of *R. rhodochrous* (Pierce *et al*., 2014).

Table 1. Enzyme Activity (Units/Mg-Cell Dry Weight) of Three *Rhodococcus* Strains When Grown on Inducing Medium[a]

	NHase	AMIDASE	CYANIDASE	ACC DEAMINASE	*B* CAS-LIKE)	ISOCITRATELYASE	OXIDO-REDUCTASE
R. erythropolis **ATCC47072**	0	12	7	7	22	ND[b]	ND
R. rhodochrous **DAP 96622**	40	9	7	7	14	ND	ND
R. rhodochrous **DAP 96253**	210	28	8	15	8	48	present

[a]Except for isocitrate lyase, all units are μmoles/min/mg-cell dry weight. For isocitrate lyase, units are nmoles/min/mg-cell dry weight;
[b]ND = not determined

Figure 1.3 Comparative enzyme activities of several species of rhodococci
Taken from Pierce *et al.*, 2014

Additionally, 1-hexene specific alkene monooxygenase (1-HMO) has been studied for it's role in the mechanism for the delay of ripening and it was noticed that it too was significantly elevated upon induction. We have many enzymes that exert activity against alkenes and AMO is just one (List of 19 monooxygenases (MO)'s: see appendix H). MO related activity is hypothesized to be involved in the production of fungal antagonistic volatile compounds, and also the degradation of plant volatile hormones. Primary alcohols, as can other oxidized compounds, could be formed by a product of MO, and various aldehydes and acids that have demonstrated fungicidal or fungistatic activity on various fungal pathogens (Fernando *et al.*, 2005).When produced with flask and petri plate production techniques (small-scale), induction is correlated with fungal antagonistic VOC production, and elevated amidase, cyanidase, urease, and 1-HMO activity of the cells, but the aim of this research was to elucidate and further examine conditions surrounding induction at a larger scale (20 and 30L bioreactors) with several aims taken into account such as increasing yield and pertinent enzyme activities, increasing catalyst stability, lengthening storage times, experimenting with multiple application techniques, while decreasing the mass of application and minimal inhibitory mass (MIM). All of these aims were improved while scaling appropriately to accommodate plant-scale operations in the future.

1.9 1-Hexene specific alkene monooxygenase (1-HMO) and related MOs

Alkene monooxygenase (AMO) is an industrially relevant enzyme that holds utility for the production of chiral specific epoxide pharmaceuticals, where chemical synthesis methods fail to achieve the high standards of biologically-mediated chirality (Besse & Veschambre, 1994). Up to 20 monooxygenases have been identified in *R. rhodochrous* DAP 96253 and specifically AMO (Appendix H) . AMO is the first step in the pathway for alkene metabolism where it stereoselectively carries out the oxygenation of a double carbon bond to yield a chiral epoxide and water. (Smith *et al.*,1999). The epoxide then gets converted to a β-keto acid via an epoxide carboxylase, and then is utilized as a carbon source. During this process, alcohols could potentially be formed by the reduction of the epoxide. Multiple biologically-produced alcohol-containing compounds have demonstrated fungistatic and/or fungicidal activities. Induction of *R. rhodochrous* and an increase in MO activity has been demonstrated in this work, so it is possible that several MOs could play a role in the mechanism of fungal antagonism, via the production of volatile organic compounds by the production and reduction of epoxides.

1.10 Rhodococcal enzymes involved in ammonia-evolution

Many Rhodococcal enzymes produce ammonia as a byproduct of catabolism and nitrile hydratase, asparaginase II, ACC deaminase, amidase, cyanidase, and urease are among those. In previous work on *R. rhodochrous* and the delay of ripening, induction was correlated to increased activity of these enzymes, but no studies have been conducted in the scaled-up production (20 and 30L bioreactors) to increase these valuable enzyme activities or establish correlations between production conditions and activities. Ammonia fungal antagonism is well

characterized and demonstrates a method that induced *R. rhodochrous* could be utilized to control pathogenic plant and animal fungal pathogens (Depasquale & Montville, 1990). Scanning electron microscopy (SEM) of treated fungal cultures has indicated plasmolysis of the fungi when exposed to ammonia. (Becker-Ritt *et al.*, 2007).

1.10.1 Urease

Urease (urea amidohydrolase) is group of nickel-dependent metalloenzymes that belong in the family of amidases. Bacterial urease is a multimer composed of two or three subunits, and is responsible for the catalysis of urea into carbon dioxide (CO_2) and ammonia (NH_3) (Callahan *et al.*, 2005). The structure of plant, fungal, and bacterial ureases are highly conserved and all known ureases contain at least 50% homology to each other (Krajewska, 2009). In bacteria, ammonia production has evolutionarily allowed bacteria to produce unique microbial niches, such as the gut. *Helicobacter pylori* is known to produce ammonia in the human gastrointestinal system (GI) via ureases, and serves as a virulence factor, for *H. pylori*, by alkalizing the surrounding tissues (Hazell, 1990). In fungistatic soils, bacteria produce ammonia that helps to control the growth of surrounding filamentous fungi and could serve to aid in competition for nutrients in oligotrophic conditions such as the soil.

1.10.2 Amidase

R. rhodochrous DAP 96253 contains nitrile hydratase that biotransform nitriles into their corresponding acid and ammonium (NH_4^+) by utilizing nitrile hydratase and then amidase respectively (Nagasawa *et al.*, 1991; Nagasawa *et al*., 1993). While nitrile hydratase converts a nitrile to an amide, amidase catalyzes the conversion of amides into their corresponding carboxylic acids and NH^{4+}. Rhodococcal amidase has demonstrated stabile activity for extended

periods of time when immobilized through multiple methods such as wax, calcium alginate beads, and cross-linked gluteraldehyde and polyethylenimine (Wang, 2013).

1.10.3 Cyanidase

R. rhodochrous DAP 96253 contains cyanidase, an enzyme responsible for the hydrolysis of cyanide into NH_3 and acid. Environmental cyanide can be found at wastewater treatment facilities and in the groundwater and can pose several risks to human health. Rhodococci were first studied in mid-1900s for their ability to convert 3-cyanopyridine to nicotamide with high efficiency and stability. Under inducing conditions, cyanidase has demonstrated elevated activity and could be utilized in detoxifying cyanide produced from biological sources.

2 MATERIALS & METHODS

2.1 Culture acquisition and inoculum preparation

R. rhodochrous DAP 96253, ablanked 2010 culture was started from a glycerol stock stored at -80˚C by transferring 1mL of the glycerol stock to 75mL nutrient broth (NB) in a 250mL flask. The culture was incubated at 30˚C while shaking at 150 revolutions per minute (RPM) for 2 days. 30mL of cell suspension from the 48hr nutrient broth culture (NB) was inoculated into 2L 1X modified R_3A broth flask (mR_3AB: 15g/L glucose; urea concentrations varied throughout experimentation) and incubated for 4 days shaking at 150 RPM at 30˚C. After incubation, the inoculum flask contents were transferred into a sterile 3L bottle, containing a fermentation top, and pumped into the bioreactor via manual edition through a peristaltic pump and masterflex tubing (Cole Palmer, Vernon Hills, IL.). A sample was taken and plated on NA to check for contamination.

2.2 Media and Solution Preparation

2.2.1 Fermentation corrective solutions

1N HCl and 2N NaOH both prepared within 1 day of use, and 20% by volume Antifoam 204® were prepared with reagents purchased from Sigma-Aldrich.

2.2.2 Preparation of 2L R. rhodochrous 96253 fermentation seed inoculum (R3A 1X Flask Medium)

Part 1:

1. A 4L clean flask was obtained and filled to 200mL with ddH_2O
2. While stirring, the following chemicals were added: 3g casamino acids (BD Medical Supplies), 3g cottonseed hydrolysate (BD Medical Supplies), 3g soluble starch (BD Medical Supplies), 3g proyield cotton CNE50M (Friesland Campina), 0.3g $MgSO_4$ (JT Baker), 1.8g K_2HPO_4 (EMD Millipore).
3. The chemicals were dissolved using a stir bar and a stir plate and the final volume was brought to1.5L.
4. The mixture was autoclaved for 30min at 121°C and cooled to room temperature.

Part 2:

1. A clean 1L flask was obtained and filled to 200mL with ddH_2O.
2. While stirring, the following chemicals were added: 60g dextrose (Fisher Chemical), 1.52g Sodium Pyruvate (Fisher Chemical), 32g urea (Fisher Chemical) and dissolved up to 500mL with ddH_2O.

3. The solution was filtered with a 0.2µm filter and poured into part 1.

2.2.3 R3A 10X Batch Medium

Part 1:

1. A clean 2L bottle was obtained and filled to 500mL with ddH_2O .
2. While stirring, the following chemicals were added: 14.9g casamino acids, 14.9g cottonseed hydrolysate, 14.9g soluble starch, 14.9g yeast extract technical, 1.5g $MgSO_4$, 9g K2HPO_4.
3. The chemicals were dissolved and the final volume was brought to1.5L.
4. The mixture was autoclaved for 30min at 121°C and cooled to room temperature.

Part 2.

1. A clean 1L flask was obtained and filled to 200mL with ddH_2O
2. While stirring, the following chemicals were added: 14.9g dextrose, 7.6g Sodium Pyruvate, and160g urea
3. The solution was diluted up to 500mL with ddH_2O.
4. The solution was filtered with a 0.2µm bottle top filter into cooled part 1.

2.2.4 R3A 15X Batch Medium

Part 1:

1. A clean 2L bottle was obtained and filled to 500mL with ddH_2O .

2. While stirring, the following chemicals were added: 22.35g casamino acids, 22.35g cottonseed hydrolysate, 22.35g soluble starch, 22.35g yeast extract technical, 2.25g $MgSO_4$, 13.5g $K2HPO_4$.
3. The chemicals were dissolved and the final volume was brought to1.5L.
4. The mixture was autoclaved for 30min at 121°C and cooled to room temperature.

Part 2:

1. A clean 1L flask was obtained and filled to 200mL with ddH_2O
2. While stirring, the following chemicals were added: 22.35g dextrose, 11.4g Sodium Pyruvate, and240g urea
3. The solution was diluted up to 500mL with ddH_2O.
4. The solution was filtered with a 0.2µm bottle top filter into cooled part 1.

2.2.5 8L YEMEA feed medium

Part 1.:

1. A clean 10L bottle was obtained and filled to 1500L with ddH_2O.
2. While stirring, the following chemicals were added: 256g of yeast extract technical and 62.4g of cottonseed hydrolysate were added.
3. The solution was diluted up to 5L with ddH_2O and autoclaved for 30min in a 10L bottle.

Part 2:

1. A clean 4L flask was obtained and filled to 1L with ddH_2O
2. While stirring, the following chemicals were added: 638g of dextrose and 96g urea

3. The solution was diluted up to 3L with ddH_2O and filtered into cooled part 1 with a0.2µm Sartopore2-150^{TM} filter (Sartorius-stedim) to yield 8L feed medium.

2.2.6 1X YEMEA flask medium

YEMEA (L^{-1}); 10g glucose, 4g yeast extract, 10g malt extract, 16g Urea.

2.3 Vessel preparation and set-up

Three sterilize in place (SIP) Sartorius-Stedim (Goettingen, Germany) vessels were utilized for this work. Before each use the vessel was rinsed with 10L ddH_2O and 250 RPM stir rate for 10min. The vessel was then prepared by inserting and mounting the probes, septa, and disposable inlet and exhaust filters. The pH (Hamilton EasyFerm Plus® arc 120 P/N#242091) and pO_2 (Mettler Toledo InPro® 6050/12/120 P/N#52200891) probes were assembled on the vessel ports and the pH probe was calibrated using pH 4 and pH 7 buffers prior to the addition of the water in the vessel. The vessel was sterilized in place (SIP) at 121°C for 45 minutes by the plant steam generated by the building boiler (cooling valve closed and air filter in fermentation mode (gassing headspace) and stirrer at 300 RPM). Once the vessel cooled to the fermentation temperature of 30°C, the triple inlet connectors (Sartorius-stedim P/N# 993 057/6) were screwed in to the vessel top reserve ports and the masterflex tubing (Cole Palmer C-flex tubing 1/8" x ¼" item# EW-06424-67) was attached to each media or corrective solution. The R3A batch media was manually pumped into the vessel via a peristaltic pump (Cole Palmer system model no. 7553-80 1-100 RPM) and all tubes were primed with their respective peristaltic automatic pumps on the bioreactor (added until filled entire tube leading to the vessel to remove air bubble) with media or the corrective solution (1N HCl, 2N NaOH, and 20% antifoam 204). The DO

probe 0% was calibrated during autoclaving (boiling water has DO of 0%) or after autoclaving by the addition of Nitrogen to the vessel (completely gas out air and calibrate for 0% air). The 100% DO was calibrated once the vessel was completing set up but prior to inoculation (stirrer at max to fully aerate the media). 100% DO is based on air, not pure Oxygen. A sample was taken and plated on NA (incubated at 37ºC) to check for contamination.

2.4 Fermentation run

Temperature and pH values were set to 30ºC and 7 respectively. DO was maintained at 30% saturation, by cascade control with agitation (Rushton turbines) (minimum value of 150rpm and a maximum value of 450rpm) and air supplementation (gas mix) with the stirrer responding first and no pure Oxygen addition because the stir rate is adequate. The airflow was set to 5 or 7.5 if the run was a 10L or 15L batch respectively. A substrate feed profile was set up for the addition of 2X YEMEA (Table 2). The glucose concentration was monitored throughout the run via the YSI glucose analyzer 2700 (see below) and the feed profile was adjusted accordingly to maintain glucose values of around 1.5g/L glucose. The fermentation parameters were monitored on the bioreactor display as well as on an additional computer Biopat® MFCS/WIN (proprietary software of Sartorius for data acquisition, control, and monitoring). The run was initiated by automatic addition of a liquid inoculum through a peristaltic pump and 0.5mm masterflex tubing.

2.4.1 Sampling

Samples were collected in sterile 15m conical tubes at previously determined time points throughout the fermentation run through a sterile sampling port. The procedure was to steam the port with pharmaceutical grade steam from the pure steam generator, before and after each use to

not contaminate the vessel or the sample (Paul Mueller Company, Springfield, MO). Once daily, enough paste was harvested to set up *R. rhodochrous* co-cultures and enzyme assays that tested the relationship between elapsed fermentation time and fungal antagonism.

2.4.2 Glucose monitoring

Glucose concentrations were measured offline by manually injecting a sample into the YSI 2700S stat glucose analyzer (Yellow Springs Instruments, Inc., Yellow Springs, OH). During the run the reading was utilized to help modulate the glucose concentration at 1.5 ± 0.5g/L via the feed profile and addition of the feed medium (containing maltose and/or glucose).

2.4.3 Optical density monitoring

Once a sterile sample was taken during the run, the optical density (OD_{600}) was measured offline by an Eppendorf Biophotometer plus® (Eppendorf, Hamburg, Germany). The spectrophotometer was only accurate when the sample absorbance read <2.0nm, so 1:10, 1:100, and 1:1000 dilutions were prepared to check the OD_{600} of the highly dense samples from the bioreactor. Additionally, an Optek automatic OD reader was utilized to track the optical density in the vessel in real time (TT electronics, Perry, OH).

2.4.4 Transfer of seed inoculum

The production bioreactor was fitted with a single inlet connector (Sartorius-stedim P/N#883 054/1) attached to 5mm.. masterflex tubing (see above). The ports on the seed bioreactor were sterilized and the sterile masterflex tubing side was attached to the harvest port. The seed was transferred over to the production bioreactor at a rate of 1L/minute.

2.5 Fermentation harvest and storage

Immediately concluding the fermentation run, the cells were pumped into the pilot-scale Carr Powerfuge® via Cole Palmer Masterflex tubing (see above) and a Cole Palmer peristaltic pump (see above) (Carr separations, Medfield, MA). The cells were centrifuged at 12,000 RPM and the media was collected in buckets and disposed of after treatment with 12M bleach (250mL bleach per 5L bucket). The resulting paste was scraped from the sterile powerfuge bowl and were wrapped in aluminum foil and were placed at 4°C in a 4L plastic container (Tupperware). For long-term storage experiments, 40g aliquots in 50mL conical tubes (BD Falcon) and rapidly frozen in liquid nitrogen and then stored at -20°Cor at -80°C and aliquots were also maintained at4°C . Frozen cells were thawed on ice for 30min. prior to use, or prior to performing enzyme assays.

2.6 Spores and mycelial extension inhibition assays

A 50 mm x15mm polystyrene petri plate was used to assess the contact-independent antifungal activity of the fermentation paste. 1g (packed wet weight) of paste was put into the lid of a 35mm x 10mm petri plate and this is turn was placed into the larger plate. For spore assays, Sabaroud Dextrose Agar (SDA) plates were inoculated with 10µL of a 10^5 spore solution (in phosphate buffered saline solution (PBS) pH 7.2) to yield a plate with 10^3 of select spore fungal species. The SDA plate was placed in the larger petri dish and the plate was sealed with parafilm. All assays were completed in triplicate and controls without *R. rhodochrous* were run. Pictures were taken concluding the run. For assays that assessed mycelial inhibition, a mycelial plug was placed in the middle of 35mm x 10mm petri plate.

2.7 Temperature range for fungal antagonism

The temperature range for fungal antagonism was tested at multiple temperatures. Multiple pathogens were tested and the temperatures tested were 4°C, 15°C, 25°C, and 30°C. Spore and mycelial inhibition assays were carried out for multiple pathogens and the 4 temperatures aforementioned.

2.8 Catalyst box preparation

R. rhodochrous DAP 96253 was cultivated using fed-batch fermentation and stored at 4^{o}C until utilized. A suspension was prepared of 5g (packed wet weight) fermentation paste and diluted to a final volume of 20mL with LUSTR 295® (Decco Us, coatings) and vortexed until it became a homogenous solution using a Vortex-Genie 2 (Scientific industries inc., Bohemia, NY). A Badger model detail 200 airbrush was used to spray the catalyst suspension on a box (Badger Air-Brush Co., Franklin Park, IL.). A volume of 20mL catalyst mixture was used for a 25lb. peach box. The box was allowed to dry in the biological safety hood for 1.5hr. before use.

2.9 Catalyst membrane preparation

R. rhodochrous was cultivated using fed-batch fermentation. The harvested cell paste was stored at 4°C, until it was suspended in1x phosphate buffered saline (PBS). Fermentation paste was weighed and then a proportionate amount of PBS was added to suspend the cells in the following ratio (1g cells: 2mL PBS). The cell suspensions were aliquoted into 1mL increments into 33mm Millipore absorbent pads inside of 35mm Petri dishes. The Petri dishes were covered

with 5µm cellulose-acetate membranes and sealed with acrylic. The membranes were used for direct inhibition assays in addition to fruit trials for fungal management.

2.10 Fermentation paste dialysis membrane preparation

Cellulose acetate dialysis membranes were cut into 3in.x5in. and soaked in ddH_2O for 2 minutes prior to use. Cell paste was loaded into the membrane with a metal utensil and the spread evenly to increase surface area of the cells.

2.11 Fungal management on fruit

2.11.1 Peaches

To test the control of native fungi on peaches, several peach boxes were coated in wax-immobilized cells and filled with peaches. Concluding the experiment, moldy peaches were removed, counted and compared for the control and treated groups. Peaches were kindly provided by: Lane Orchards (50 Lane rd. Fort Valley, GA) and Dickey Orchards (3440 Musella rd. Musella, GA).

2.11.2 Tomatoes

For the tomato trial, catalyst containers were tested against Brown Grape variety tomatoes. Tomatoes were received in clamshell containers that were either 1 (Phase 4) or 2 (Phase 2) days post-harvest. Phase 2 and 4 tomatoes were evaluated in the original clamshell containers. Fruits were not rinsed and no undesirable fruits were removed. Catalyst membranes were added to each treated container, control containers received 5 membranes with only 1X PBS. Tomatoes were incubated at room temperature or 12°C (chilled). The chilled containers

were removed from refrigeration after 7 days and stayed at room temperature for the remainder of the trial.

2.11.3 In vivo evaluation of the contact-independent antifungal activity with red, seedless grapes (Vitis vinifera)

Red, seedless grapes were purchased from local retailers. Twenty-four grapes of similar size and ripening stage were selected and individually cut to retain stems. The grapes were washed with ddH_2O and pre-treated by soaking in a 0.5% sodium hypochlorite solution for 5 minutes. The stems were then gently removed, followed by inoculation with *Botrytis cinerea* conidia (10 µl of a 10^5 conidia mL^{-1} solution in 0.9% NaCl) under the same stem site (~2mm) using a sterile pipet tip. Twelve inoculated grapes were placed in a sterile airtight jar (1L) as the untreated control, while the other twelve were placed in sterile airtight jar (1L) with 5g (wet weight) fermentation paste. Evaluations were conducted at 25 °C.

2.11.4 Strawberries (B. cinerea inoculation)

A transfer tube was utilized to remove a 0.5cm plug from each strawberry, and *B. cinerea* mycelia plugs were replaced into the bores. *R. rhodochrous* (4g wet weight) was placed into the same airspace for the three treatment strawberries. Images were captured at 2 and 3 days. There was no attempt to remove native strawberry flora before inoculating with *B. cinerea*

2.11.5 Strawberries (native flora)

6 packs of strawberries with no visual bruising, injury, or mold were selected from the supermarket. Each container was dunked 3 times in a 200ppm hypochlorite solution and

left to dry for 2 hours. 2 containers were set in an untreated box and utilized as controls. 2 containers were placed in catalyst boxes, and 2 containers were placed in an untreated box with a Rhodococcus dialysis membrane.

2.12 Fungal management of bats

Field trials were conducted at 4 locations in the United States 2 sites in Missouri and 2 sites in Kentucky. The bats were collected and placed in coolers in close proximity (no direct-contact) to *R. rhodochrous* paste for 72 hours (appendix D), and then released into the cave for the remainder or torpor. Bats mean body masses for treated and control groups were collected initially and before release. In a wing health study of treated bats, bats were monitored for signs of disease and images were captured on multiple time points to demonstrate wing health of the treated and untreated groups.

2.13 Structure of exposed spores

The structure of spores exposed to Rhodococcal volatiles were examined using SEM. The spores were prepared by sputter coating on gold.

2.14 Enzyme Assays

Enzyme activities were measure for amidase, cyanidase, and urease via the evolution of ammonia, and spectrophotometric analysis as described by Ganguly (2005).

Using a modified method by Fawcett and Scott (1960), enzyme activities were determined by detecting the production of ammonia. Where, 9ml of substrate (defined in appendix I) was mixed with 1mL of cell suspension (40mg of cells to 1ml of phosphate buffer), after two minutes, 1ml of the mixture was pipette into a microcentrifuge tube and centrifuged for

2 minutes at 13,000 RPM. The supernatant was removed with a pipette and transferred to a second microcentrifuge tube. 1ml of the supernatant was pipetted into a test tube and the following reagents were added in the following order; 2mL of Sodium phenate, 3mL of 0.01% Sodium nitroprusside and 3mL of 0.15% Sodium hypochlorite. The reaction was incubated at room temperature in the dark for 30 minutes. All test tubes were vortexed for 30 seconds and 200µL was pipette in triplicate into a 96 well plate and the absorbance was read at 600nm via a Victor Multilabel Counter Reader (Wallac, Turku, Finland). One enzyme unit was defined as the the conversion of uM of NH^3 per minute per mg of cells (dry weight) at room temperature, pH 7.4. All materials are detailed in appendix I.

AMO activity was determined by using the NBP assay as described by McClay *et al* (2000) and spectrophotometric analysis (Cheung *et al.,* 2013). Where, 10mmol·L^{-1} of 1,2-epoxyhexane solution was made as stock solution, then 1, 2, 3, 4 and 5mmol·L^{-1} of 1,2-epoxyhexane solution was made by taking 100, 200, 300, 400 and 500 µL of the stock solution added to 900, 800, 700, 600 and 500µL of acetone, correspondingly. Next, 50µL of each concentration of 1,2-epoxyhexane solution was transferred into outer vials with 5mL of phosphate buffer (sodium salts, 50 mM, pH 7), respectively. 5 mL of *R. rhodochrous* DAP 96253 cell suspension (0.02 g·mL^{-1}) in phosphate buffer (sodium *salts,* 50 mM, pH 7.0) was transferred into a 40mL amber glass vial (outer vial). 200µmol of 1-hexene was added to the cell samples. 500µL of 4-(4-nitrobenzyl) pyridine (NBP) solution (100 mmol·L^{-1}) in ethylene glycol was added to a 4mL transparent glass vial (inner vial) and placed into the outer vial which was crimp-sealed with a Teflon-faced butyl rubber stopper. For controls, a blank (5 mL of phosphate buffer only) and (0g cells control) were included. All the 2-vial sets were incubated at 30°C shaking at 150 RPM for 24 hours. Lastly, 500µL of triethylamine solution (1:1 v/v) in acetone

was added to each inner vial and the absorbance was immediately read at 600nm via a BioPhotometer *plus* (Eppendorf).

2.15 Formulation of calcium alginate and fermentation paste beads

Calcium-alginate bead formulations were prepared by methods described by Wu *et al.* (2002) and modified to those described by Wang (2013). The modifications made within this work included using induced fermentation paste.

3 RESULTS

3.1 Process development of seed inoculum

In order to further develop the first phase of the production scheme, flask media were evaluated to grow the inoculum in a formulation not previously investigated. In production schemes utilized in previous work for increased Nitrile hydratase, the use of plate-derived cells generated desired yields (Drago, 2006). Initial experiments on liquid seed inoculum compared shake flask growth of *R. rhodochrous* in R3A (comparable to the batch-medium in the bioreactor) with the shake flask grown YEMEA broth (comparable to the feed-medium in the bioreactor). Under the conditions employed, the R3A supported a higher density growth of *R. rhodochrous* (around 12 OD_{600}) versus YEMEA (around 3 OD_{600}) when both grown at 30°C at 225rpm for 5 days in a 1L flask (Fig. 3.1). The R3A seed inoculum was scaled to 2L and the resulting seed inoculum was 37g of pH 7, OD_{600} 17 cell suspension (Table 3.1). This seed culture preparation method was used for further work investigating a reduction in the time associated with seed culture preparation.

Previously, there were a multitude of steps involved in the production of the inoculum seed that was be used to inoculate the bioreactor. In this previous method, a 30% glycerol stock

was used to inoculate a 75mL nutrient broth (NB) shake flask and was incubated for 2 days. This cell suspension was used to inoculate nutrient agar (NA) plates, and then 3 days later these plate cells were scraped from the NA plates and spread on another plate media (gU) and incubated for an additional week. The entire process was 12 days and the gU plates had to be scraped and resuspended in PBS and then put into a syringe for direct injection into the vessel (Fig. 3.2). The procedure developed in this study utilized the first two steps of the previous method, but then used NB to inoculate another flask that was directly pumped into the bioreactor 4 days later for a total 6 day seed preparation process (Fig. 3.2).

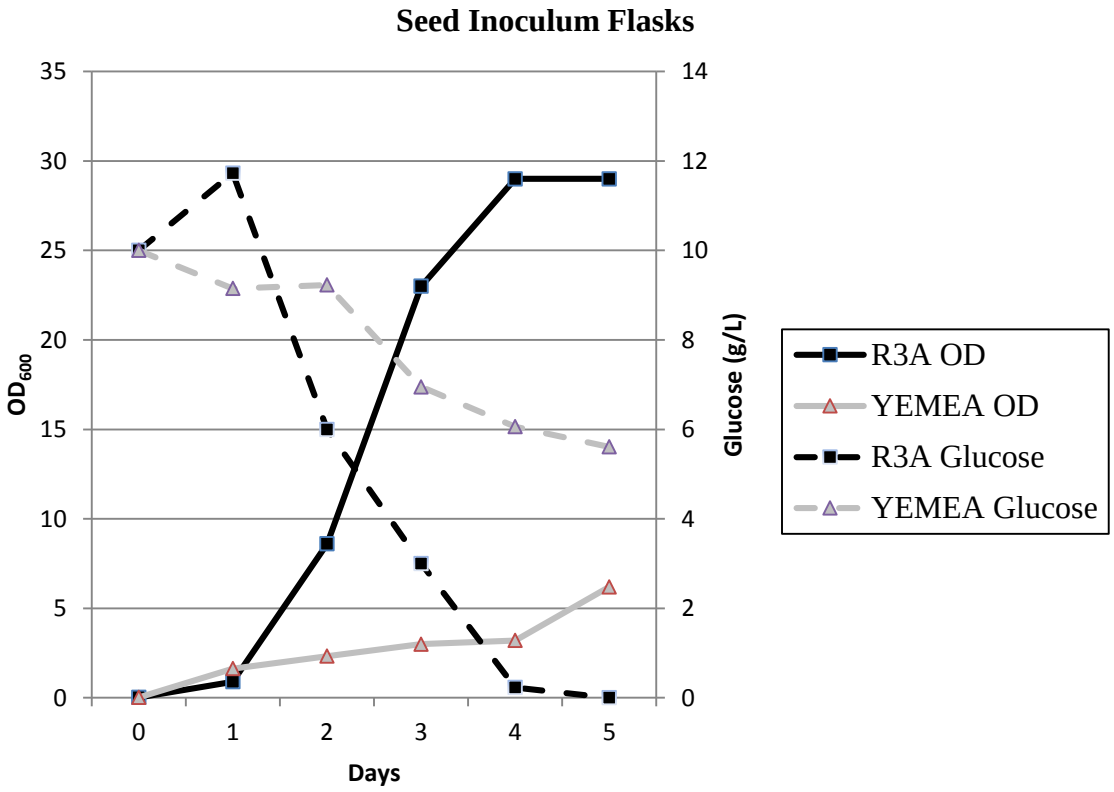

Figure 3.1 Seed inoculum development
Each timepoint is the mean of the triplicate flasks. **Solid lines**; OD_{600}, **Gradient lines**; Glucose concentration(g/L), **Black line**; R3A, **Grey line**; YEMEA).

Table 3.1 Inoculum flask

Seed culture features	
Flask volume	4L
R3A Volume	2L
pH	7
OD_{600}	17
Yield (g)	35

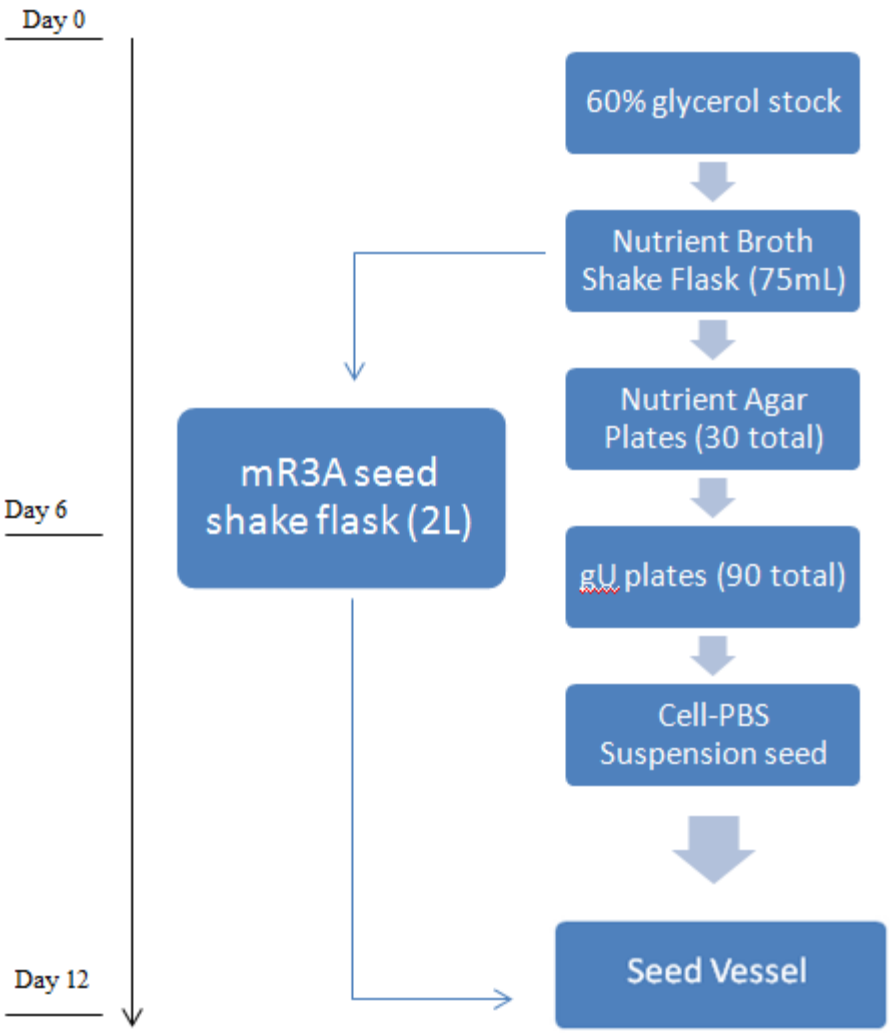

Figure 3.2 Development of 6-day seed inoculum

3.2 Process development: Utilization of multiple bioreactors for increased productivity

In the original production methodology, which aimed to produce acrylamide from acrylonitrile, the bioreactor was inoculated with the cell suspension scraped from plates and the

fed-batch fermentation run was harvested after 48 hours and harvested through the Carr pilot powerfuge yielding between 0.5-1kg (Fig. 3.3) (Drago,2006).

In this work, in order to increase efficiency, multiple bioreactors were utilized from the same original seed culture. The 20L seed bioreactor was inoculated with the mR3A seed and incuated for 24hrs. Once the cells adapted and started to utilize the feed medium, a 1L seed was transferred to the 30L production bioreactor. Both fed-batch fermentation runs were run side-by-side and harvested with the Carr pilot powerfuge at the conclusion of the run to yield between 1-2kg per bioreactor (Fig 3.4). The fermentation run yields increased gradually over time with the modifications that were made in the production scheme (Fig. 3.5).

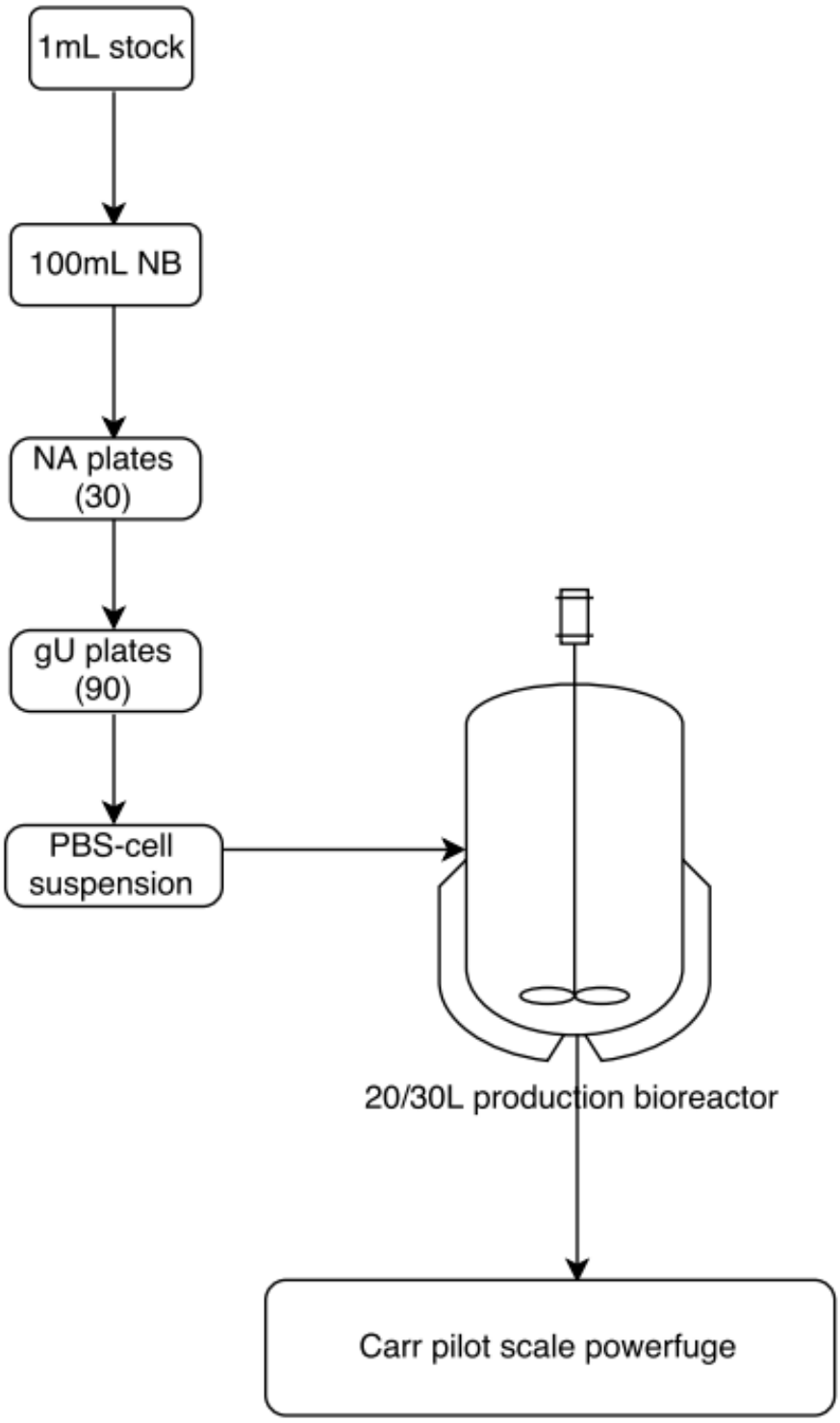

Figure 3.3 Initial production flowchart

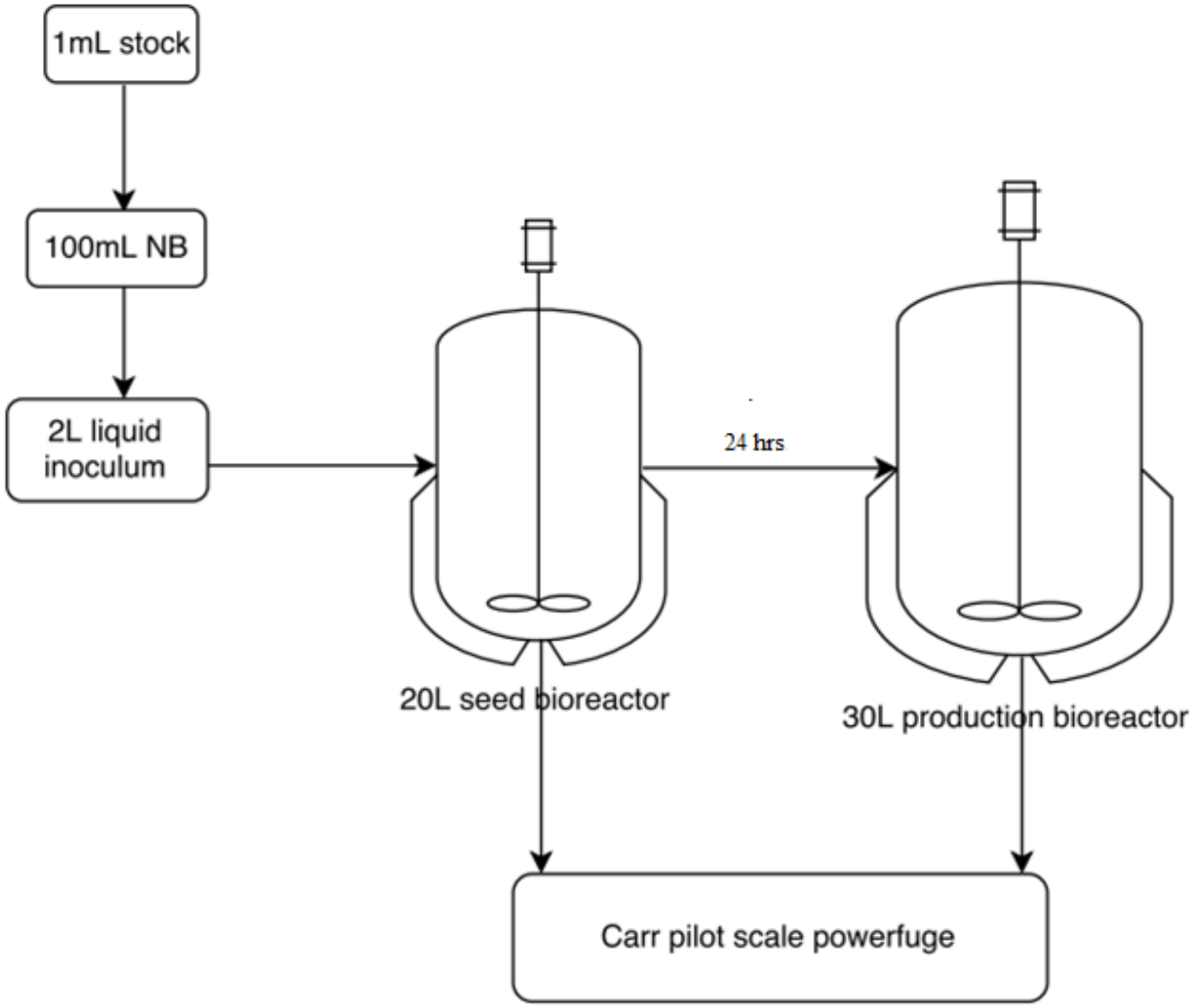

Figure 3.4 Production flowchart developed in this work

3.3 Process development: Defining features in the bioreactor resulting in increased productivity and performance of the catalyst (induction techniques, glucose, and maltose concentrations)

In previous work, inducer was pulse-fed at 0 EFT and again at 24 EFT regardless of cell densities present in the bioreactor (Drago, 2006). To further refine this process for fungal applications, an autoinduction technique was utilized and the urea was constant at 0 EFT to the end of the feed schedule. The urea was added to the YEMEA and R3A and the feed was

controlled in order to maintain around 1.5g/L glucose, and 16g/L urea.. Cells autoinduced showed antifungal properties and contamination risks associated with additional reagent addition were minimized (data not shown).

Several concentrations of urea were tested within the bioreactor. When 16g/L was utilized as the inducer, the cells were demonstrated to have antifungal activity in all but one run, so this was used for runs to evaluate other variables. The maltose concentration was 384g in one + run, while 0g in all other runs that showed fungal antagonism (Table 3.2). The glucose concentration ranged from 334.1g to 866.1g in runs resulting in paste showing robust fungal antagonism (+) (Table. 3.2). In runs that resulted in no fungal antagonism (-) the maltose and glucose concentrations were 768g and 334.1g respectively (Table. 3.2). In runs that were slightly fungal antagonistic (/), multiple concentrations of maltose and glucose were utilized and there were no obvious trends (Fig. 3.2). Yields from the bioreactor varied, but dropped with the removal of maltose from the feed medium, and then increased over the study with an increase in glucose concentration in the feed medium (Figure 3.2).

RUN	Volume	Glucose (total)	Urea (total)	Yield (g/L)	Yield (g/L)	(g cells/ g glucose)
60616	17.7	653	0	79	1400	2
50216	17.7	653	288	85	1495	2
41816	17.7	865	288	65	1146	2
40416	17.7	866	216	113	2000	2
32116	17.7	773	342	57	1000	1
20116	17.7	655	288	112	1978	3

Figure 3.5 Yields from consecutive runs

3.4 Dissolved O_2 in the bioreactor

Dissolved Oxygen (%O_2) set points were defined as 30% of saturation for all runs, but deviated as much as 30% higher depending on O_2 usage of the cells in the bioreactor (never dropped lower than 20% saturation due to stir rate). The dissolved Oxygen at the initiation of every fermentation run begun at100% ±10 (calibrated with media right before inoculation). The trends in final dissolved oxygen (%) were as follows: fungal antagonistic (**+)**; avg. 77.6, min. 54.8, max. 98.7; slight fungal antagonism (**/)**; avg. 64.2, min. 56.2, max. 80; no fungal antagonism (-); avg. 33.8, min. 29.4, and max. 45 (Table 3.3). The entire runs can be seen on the bioreactor dissolved Oxygen plots. For the runs showing fungal antagonism, the dissolved Oxygen dropped in response to glucose within the first stage of the run, but increased before harvesting (in response to glucose exhaustion) to above 40% for all batches (Fig. 3.6-3.7). For the runs that showed no fungal antagonism, the DO remained relatively low at the point of harvest (below 60% for all) (Fig. 3.8). For the runs showing slight antifungal capacities, the DO for all 3 runs was less than 80% when harvested (Fig. 3.7). The data on Oxygen saturation at harvest, shows that the mean for DO was higher for the antifungal cells (+) and the slightly antifungal cells (/) than those demonstrating no fungal antagonism (Table 3.3).

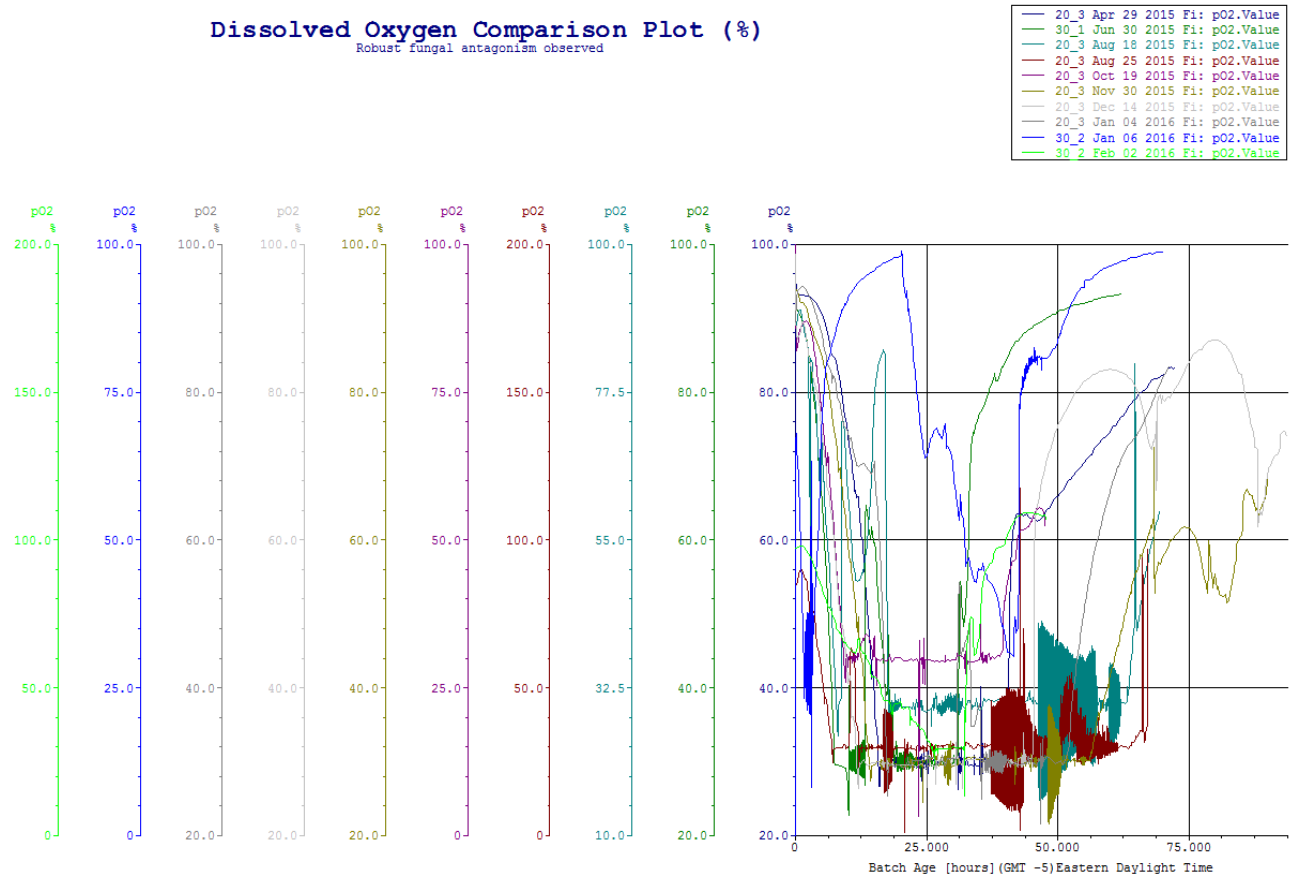

Figure 3.6 Dissolved Oxygen plots for runs with robust antifungal activity (+)

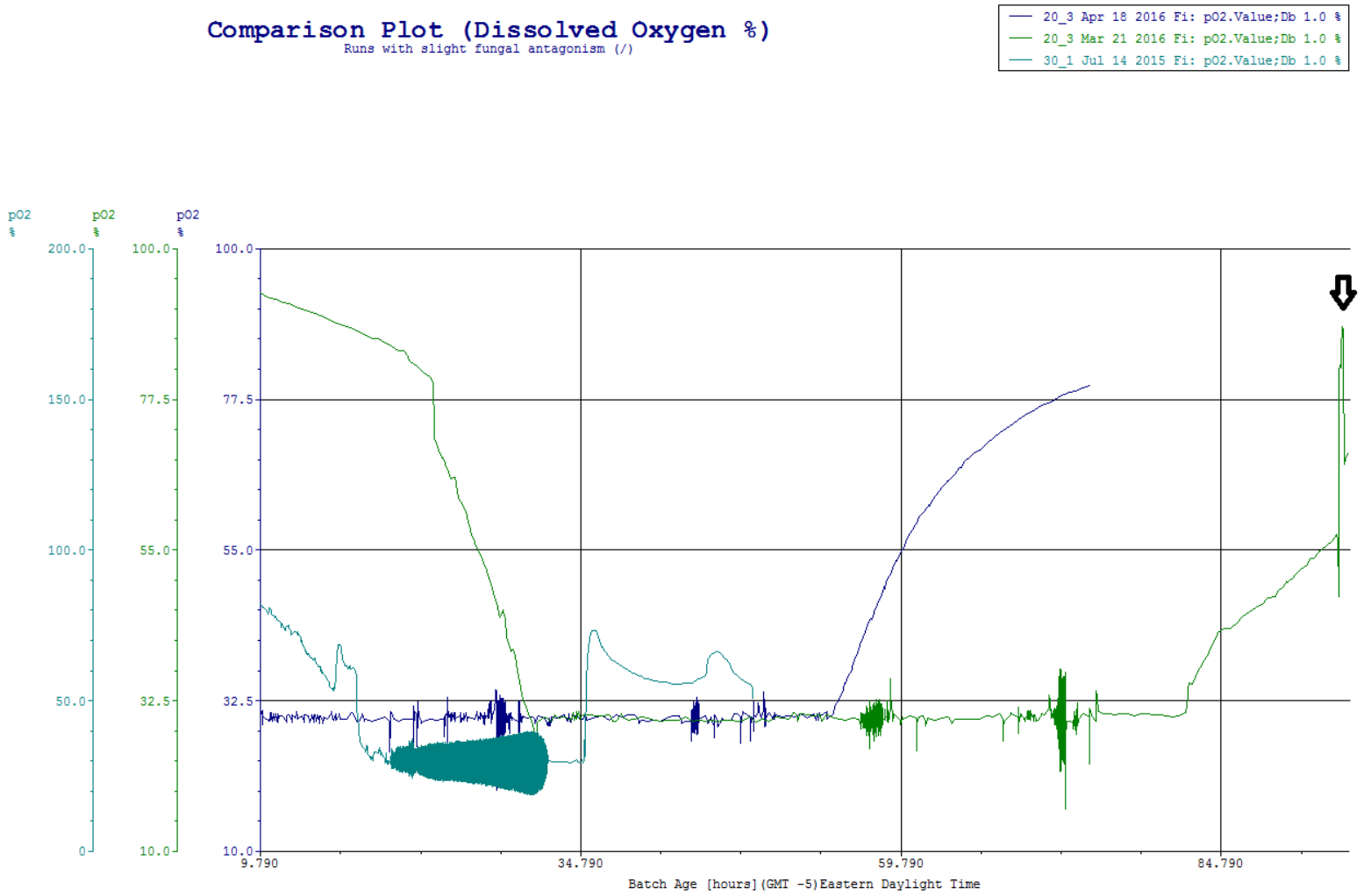

Figure 3.7 Dissolved Oxygen plots for runs with slight antifungal activity (/)
Arrow: harvested and failed to turn data acquisition software off (false positive)

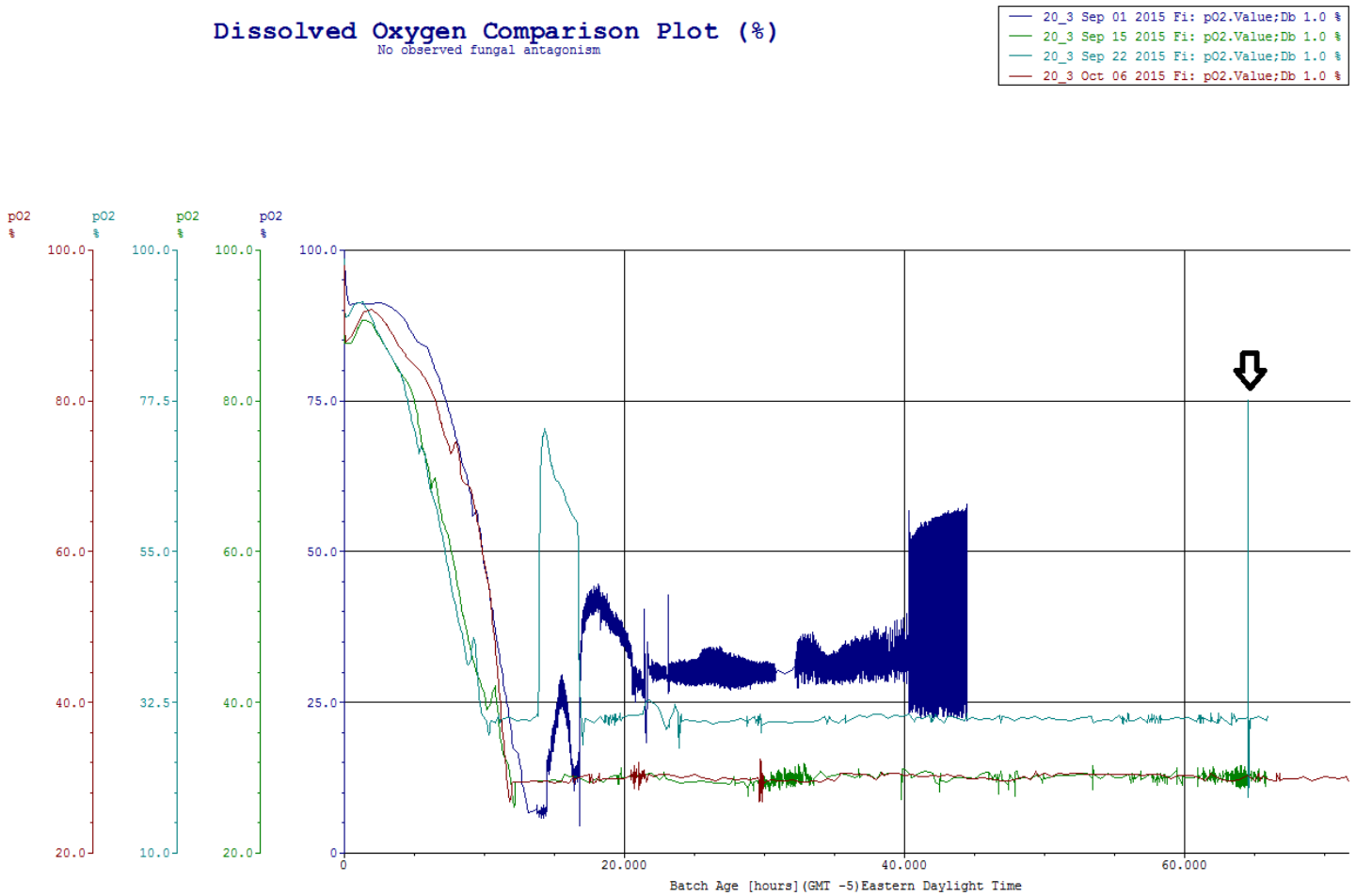

Figure 3.8 Dissolved Oxygen plots for runs resulting in paste with no antifungal activity (-)
Arrow: harvested and failed to turn data acquisition software off (false positive)

Table 3.2 Bioreactor data from runs
Spore inhibition of *P. destructans* and *B. cinerea* **(+)**; *B. cinerea* spores only **(/)**; neither spores inhibited **(-)**. Units: **AMO** (nmol of 1,2-epoxyhexane · hour)/g (cdw). **Amidase, Urease, Cyanidase** (uM NH^3/min/mg (cdw)). **Free ammonia** (mg/mL supernatant/40mg cells)

RUN	**Fungal Activity**	**AMO**	**Amidase**	**Urease**	**Cyanidase**	***Free ammonia**	**EFT**	**DO at harvest**	**Glucose**	**Maltos**
42915	+	12	23	6	1	6	72.2	**83.1**	334.1	
63015	+	141	153	10	9	2	62.1	**93.3**	341.6	
71415	/	10	140	25	3	25	48	**56.5**	341.6	76
81815	+	11	36	11	8	2	69.5	**56**	334.1	
82515	+	36	-	0	0	1	67.2	**96.1**	334.1	
90115	-	6	26	6	0	5	44.3	**45.6**	334.1	76
91815	-	69	60	0	0	0	66	**29.8**	334.1	76
92315	-	24	37	0	0	3	65.9	**30**	334.1	76
100615	-	204	24	0	0	0	71.6	**29.4**	334.1	76
101915	+	-	30	0	0	4	47.2	**54.8**	334.1	
102615	-	192	25	0	0	0	49.6	**30.4**	334.1	76
113015	+	159	-	-	-	-	90.0	**67.3**	334.1	38
121415	+	67	422	21	0	32	93.7	**74.9**	493.7	
10416	+	190	-	-	-	-	71.1	**83.6**	653.3	
10616	+	117	-	-	-	-	70.0	**98.7**	660.8	
20216	+	29	12	0	0	3	94.0	**68.2**	652.9	
32116	/	15	3	0	1	1	93.3	**56.2**	772	
40416	+	18	86	54	46	13	94	**34.7**	866.1	
41816	/	27	37	8	1	4	75	**80**	478	
50216	+	-	76	26	26	11	94	69	653	
60616	-		0	5	2	0	91	33	653	

3.5 Elapsed fermentation time (EFT)

Cells from runs were harvested at different times ranging from 2-4 days EFT. The + cells average harvest time was 73.31 (max. 94.02, min. 47.22).. The / cells average harvest time was 72 (max. 93.3, min. 48), and the – cells average harvest time was 57.88 (max. 71.60, min. 44.28) (Tables 3.2 and 3.3).

3.6 Bioreactor variables with little variability between all runs

Throughout all fermentation runs there were several factors that remained extremely constant in the bioreactor throughout the study (±0.5/ unit). These variables include the temperature (30°C), airflow (5LPM (20L bioreactor) or 7.5LPM (30L bioreactor), and pH 7. The stir rate (proportional-integral-derivative (PID) loop responsive to $\%PO_2$) fluctuated between 150 and 450rpm for every run (data not shown).

3.7 Enzymes activities at harvest

Previous work on *R. rhodochrous* showed several enzymes to be elevated upon induction, so immediately following the harvest, enzyme activities were assayed (Fig 1.3). Enzymes tested included AMO, amidase, cyanidase, and urease. Triplicates were averaged to yield average enzyme units and are given in the table. The data on enzymes tested, shows that the mean for urease, amidase and cyanidase activities were higher for the antifungal cells (+) and the slightly antifungal cells (/) than those demonstrating no fungal antagonism. Additionally, free ammonia assayed on the cells was increased in the cells showing antifungal properties (+,/) than those that did not (-) (Tables 3.2).

3.8 Product assessment against B. *cinerea*

B. cinerea spores (on a cellulose membrane) were exposed to fermentation paste in a shared-airspace assay for 24hours and demonstrated atypical spherical shape upon exposure to *R. rhodochrous* fermentation paste (Fig. 3.8). In an experiment testing inhibitory effects of fermentation paste harvested from multiple time points (at 1 and 0.5g per shared airspace assay), 0.5g and 1g of fermentation paste were inhibitory for EFT 45.40, EFT 68.70, and EFT 93.65 harvests (Fig. 3.9). Fermentation paste was consistently inhibitory to *B. cinerea* spores on SDA, with little variability between batches (minimal dose of 0.5g) (Fig. 3.10). For contact-independent assays against *B. cinerea* mycelia, fermentation paste demonstrated inhibitory effects on mycelia plugs consistently when utilizing more 0.2-1g paste (Fig. 3.11).When *B. cinerea* plugs were placed on SDA plates containing various amount of fermentation paste (direct and indirect contact), there was slowed mycelia extension when using as little as 0.1g (Fig.3.12-3.13).

For testing inhibition on plant models directly, *B. cinerea* was inoculated into grape-stem bores and was inhibitory upon visual inspection (Fig. 3.14). On strawberries, *B. cinerea* plugs were inhibited completely in the treatment group, while mycelial extension and proliferation of native strawberry fungi was seen to be increased only in the untreated group (Fig.3.15).

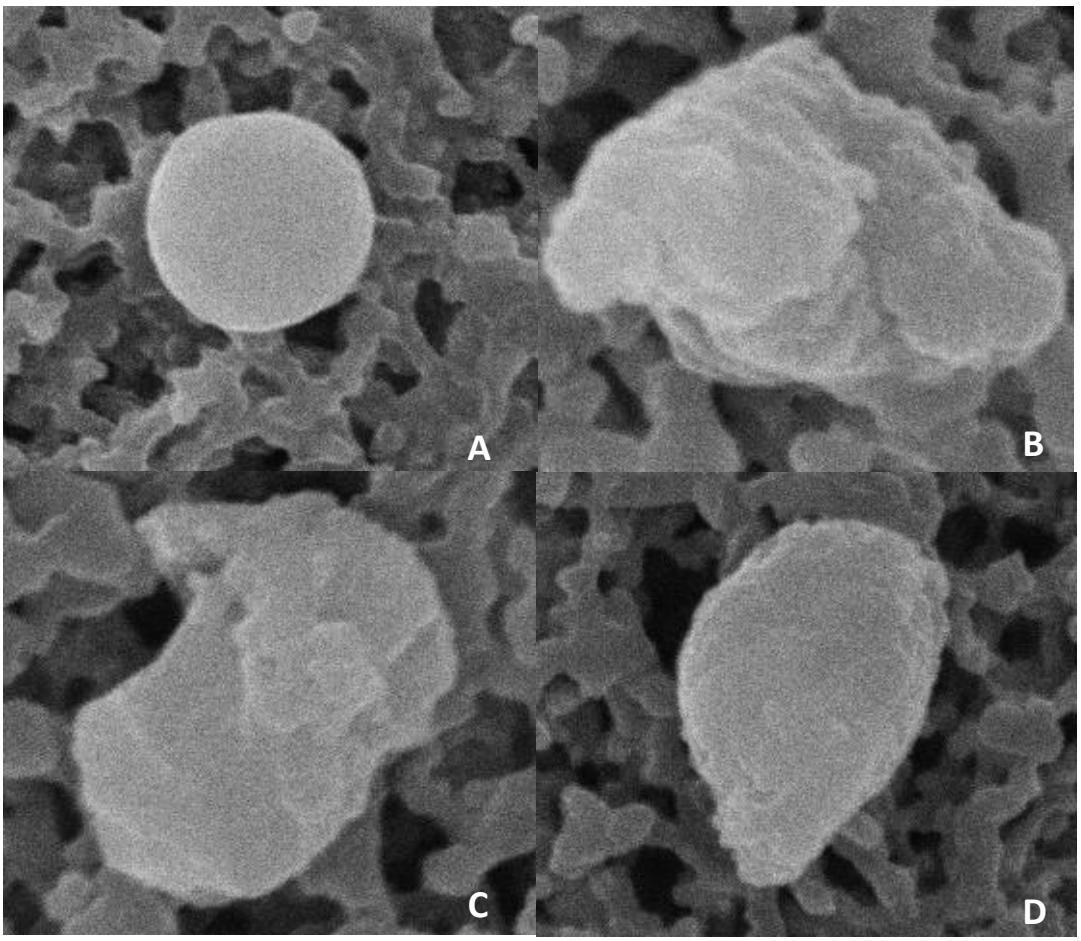

Figure 3. 1 Scanning electron micrograph of B. cinerea spores (sputter-coated in gold)
Exposed to 1g of fermentation paste for 20hours on a membrane (B,C,D) and control (A). Generously conducted by John Neville, GSU core facilities.

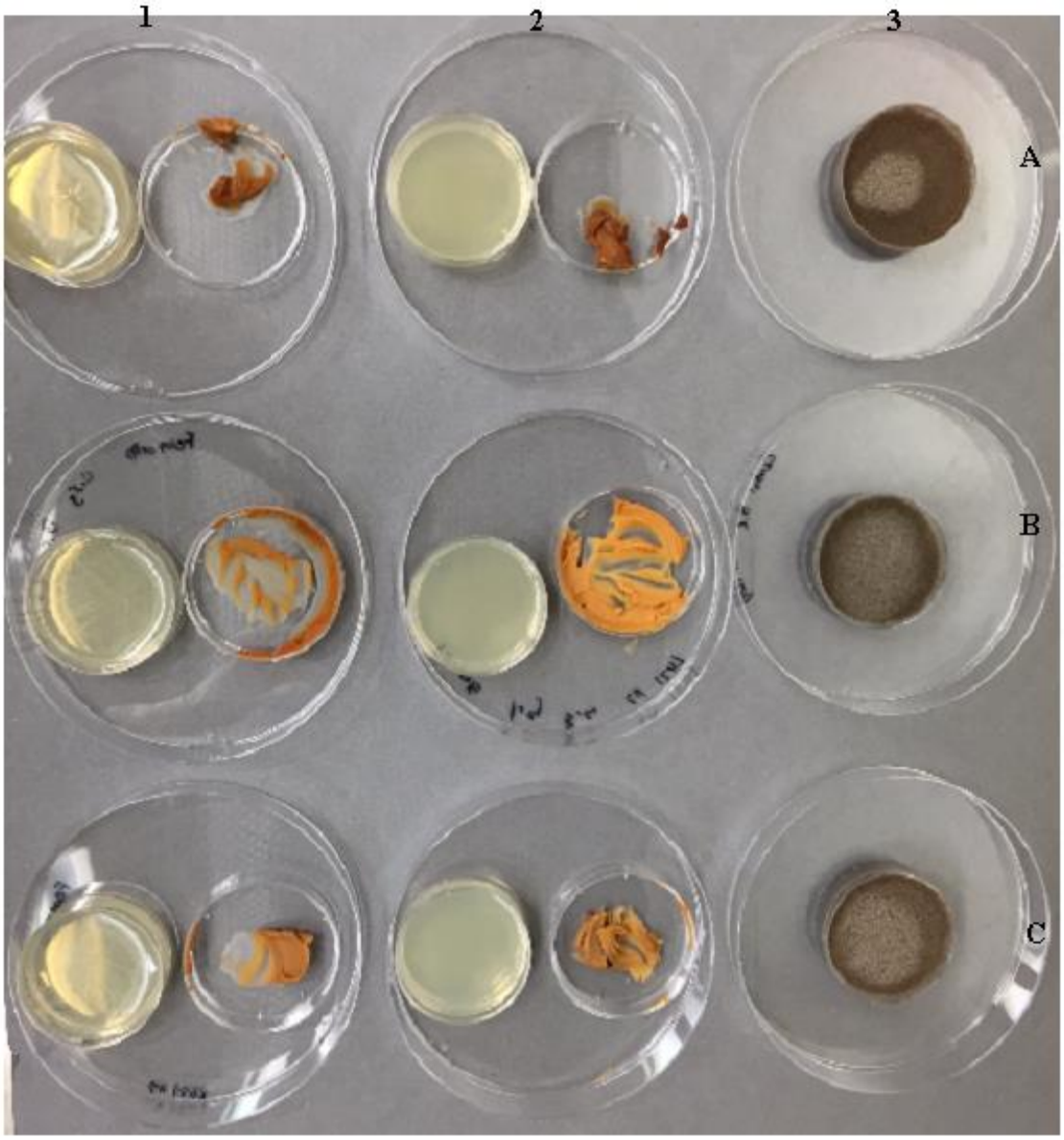

Figure 3.9 B. cinerea spores on SDA co-cultured with fermentation paste at multiple EFT
Horizontal rows: EFT 45.40 (A); EFT 68.70 (B); EFT 93.65 (C). Columns: 0.5g paste (1), 1g paste (2), 0g paste (3). Experiments were performed in triplicate with consistent results for all 3

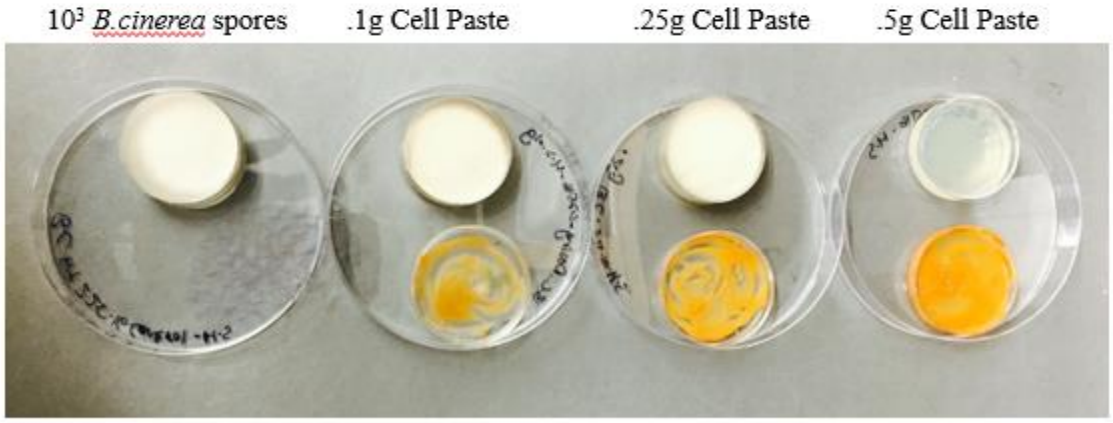

Figure 3.10 B. cinerea spores($10^{3)}$ minimal inhibitory mass assay
(0.1-0.5g tested and performed in triplicate with consistent results)

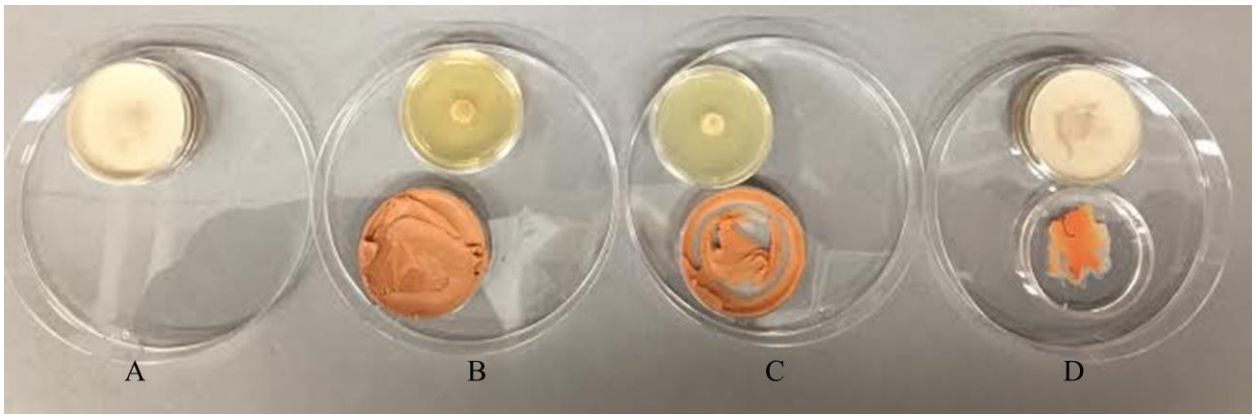

Figure 3.11 B. cinerea mycelia plug fungal antagonism
Shared airspace assay with 0g (A), 2g (B), 1g (C), and 0.1g fermentation paste (D)

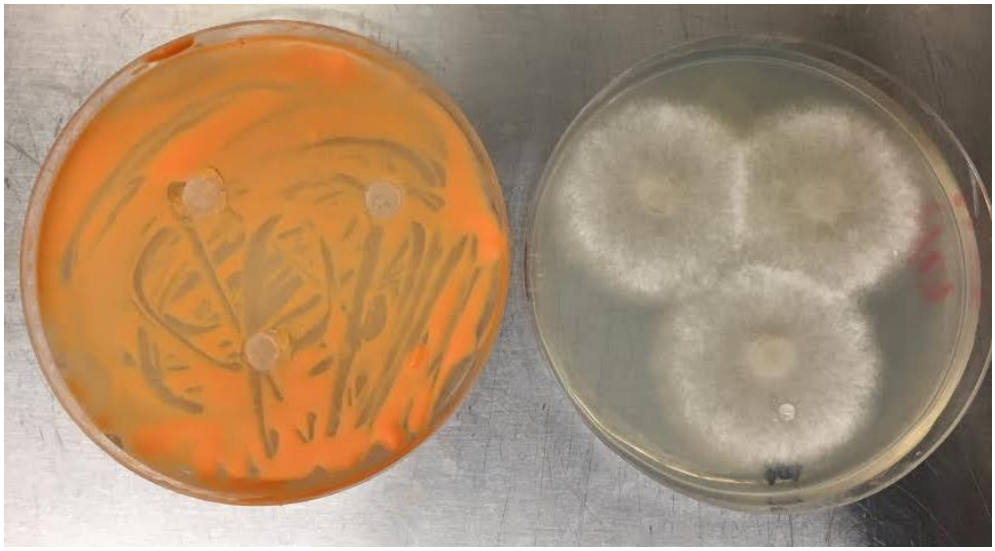

Figure 3.12 B. cinerea mycelia plug fungal antagonism
1g of fermentation paste (L) and control (R)

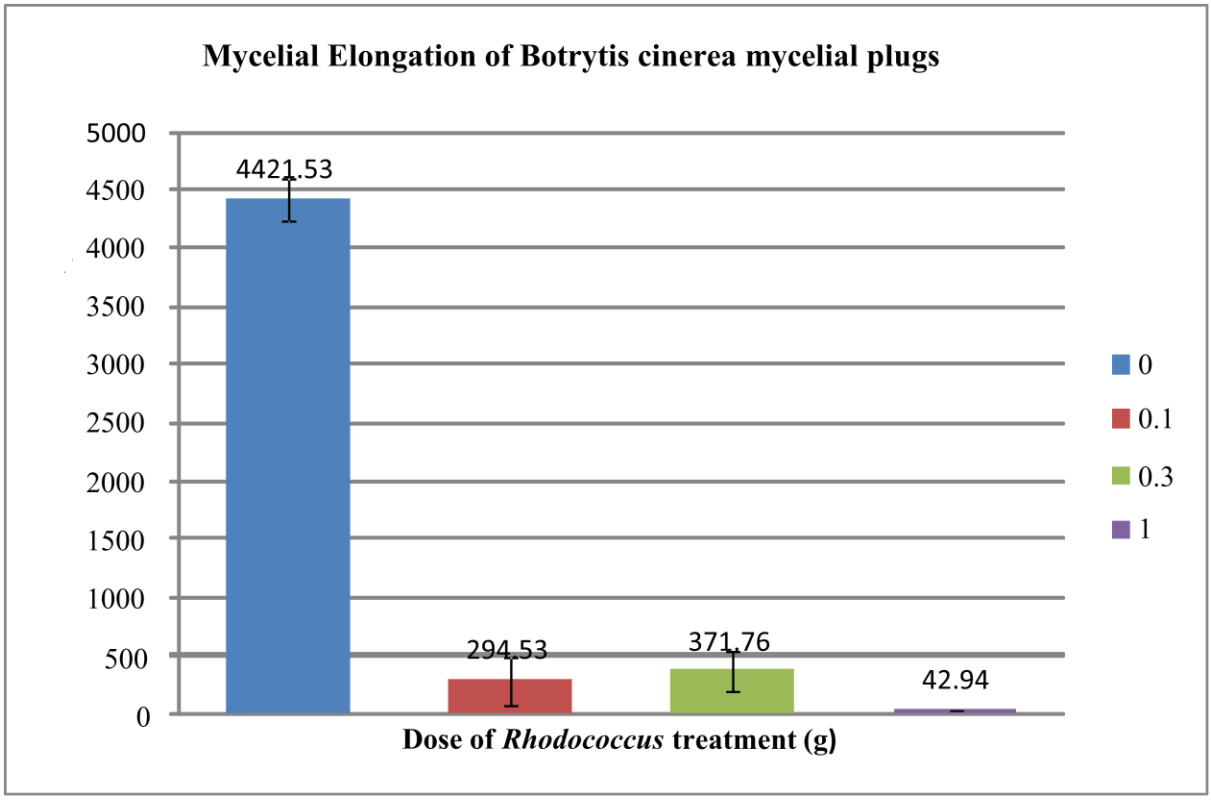

Figure 3.13 B. cinerea *mycelia elongation in response to multiple doses of fermentation paste (0,0.1,0.3, and 1g)*
Standard error bars of the mean. The mean is indicated above the bar

Figure 3.14 Grapes inoculated with 10^3 B. cinerea NRRL 1650 conidia (A-D)
Exposed to 5g R. rhodochrous DAP 96253 cells at 25 °C for 7 days (A,B). B and D are close-up pictures of A and D respectively

Figure 3.15 Botrytis cinerea mycelial plugs on Driscoll strawberries
Untreated strawberries on day 0 (A) and day 1 (B). Non-contact treated strawberries on day 0 (C) and day 1 (D)

3.9 Product assessment against *P. destructans*

In previous work completed at a smaller scale, 1g plate- generated cell paste demonstrated inhibition of *P. destructans* spore germination on SDA (Appendix A). In an experiment testing inhibitory effects of fermentation paste harvested from multiple time points (at 1 and 0.5g per shared airspace assay), 0.5g-1g of fermentation paste were inhibitory to *P. destructans* spores for *EFT 68.70 and EFT 93.65 harvests, while no inhibitory effects were seen for the EFT 45.40 test time (Fig. 3.16).* In further studies, fermentation paste (harvested after

48hrs) demonstrated *P. destructans* spore inhibition at a dose of 0.3- 0.5g per 10^3 spores on SDA, with extremely slight batch-to-batch variations (Fig. 3.17). Fermentation paste demonstrated slight batch-to-batch variations on contact-independent inhibition of 10^3 *P. destructans* mycelia plugs, but the dosage tested that resulted in the greatest inhibition of mycelia elongation was 3g paste per a 0.5cm plug (Fig. 3.18). In the direct fungal control treatment method, the mycelia plugs were inhibited proportionally as the treatment dosage increased, but elongation was inhibited with at least 0.25g paste (Fig 3.19)

In a trial utilizing white-nose positive bats, upon 72hour contact-independent exposure to fermentation paste all treated bats showed higher mean body masses at the conclusion of hibernation than their untreated counterparts. In the treated group, there was a 60% survivorship of bats while 0% in the untreated (Figures 3.20- 3.21). An additional bat study was completed to assess wing health of treated bats, and the treated bat showed healthier wings concluding the study. The untreated bat had extreme tissue damage and died, while lived through the study to be continually monitored for health and showed better wing health throughout the study (Fig. 3.22).

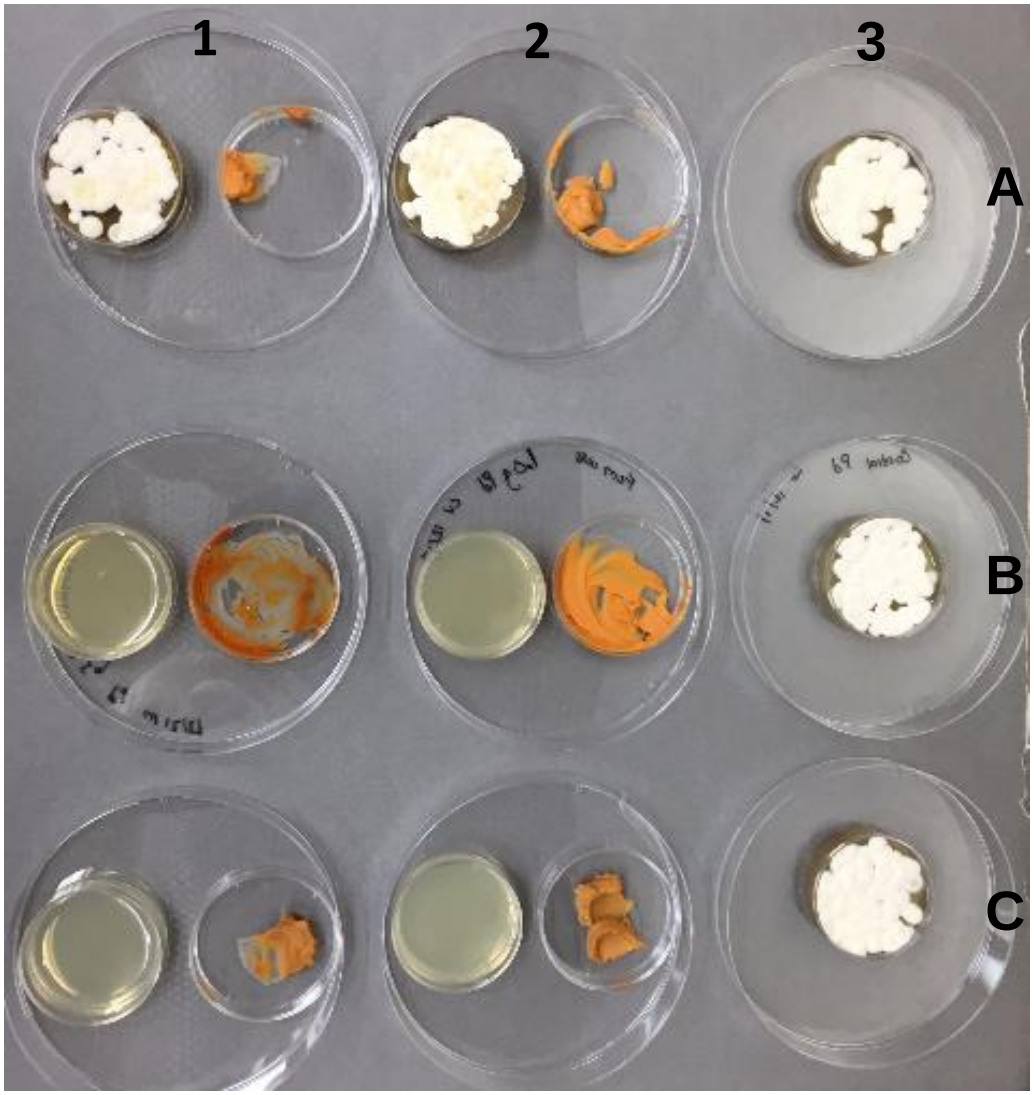

Figure 3.18 10^3 Pseudogymnoascus destructans spores inoculated on SDA co-cultured with fermentation paste harvested at various time points. Horizontal rows: EFT 45.40 (A); EFT 68.70 (B); EFT 93.65 (C). Columns: 0.5g paste (1), 1g paste (2), 0g paste (3). Experiments were performed in triplicate with consistent results for all 3.

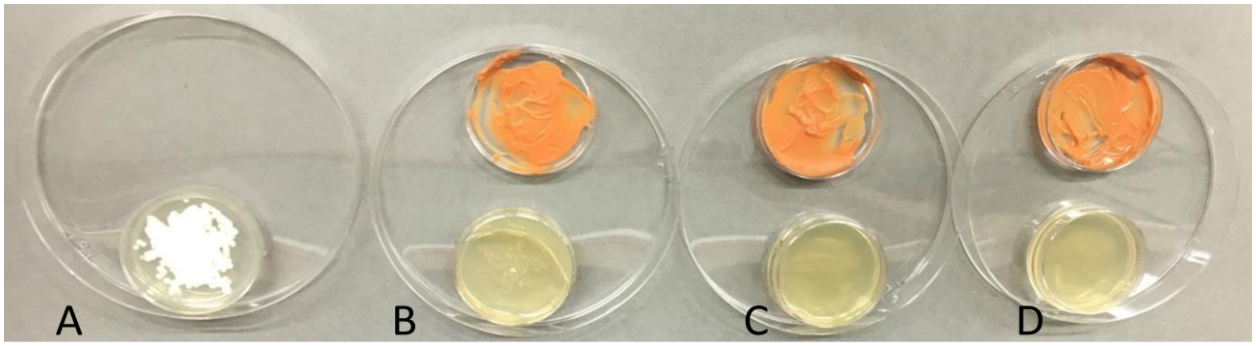

Figure 3.16 Dosing assays against10^3 Pseudogymnoascus destructans spores (15˚C)
Control (A); 0.4g paste (B); 0.5g paste (C); 0.6g paste (D)

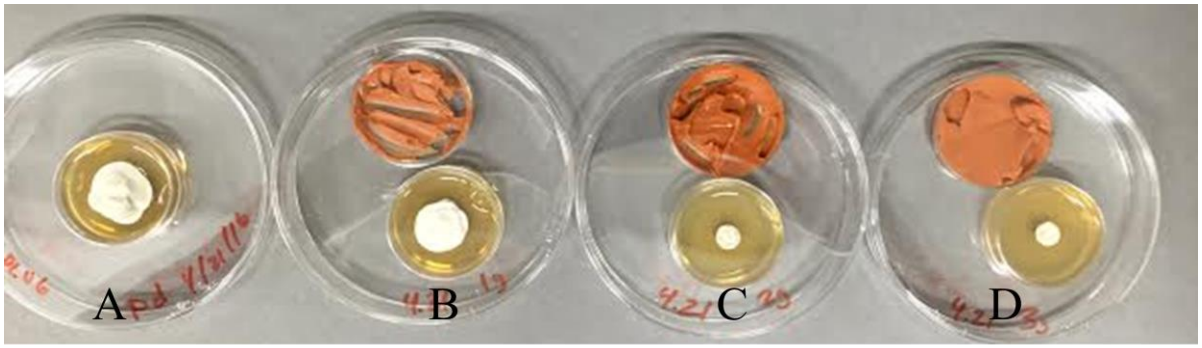

Figure 3.17 P. destructans mycelia plugs on plates with varying fermentation paste doses
0g (A), 1g (B), 2 (C), and 3g (D)

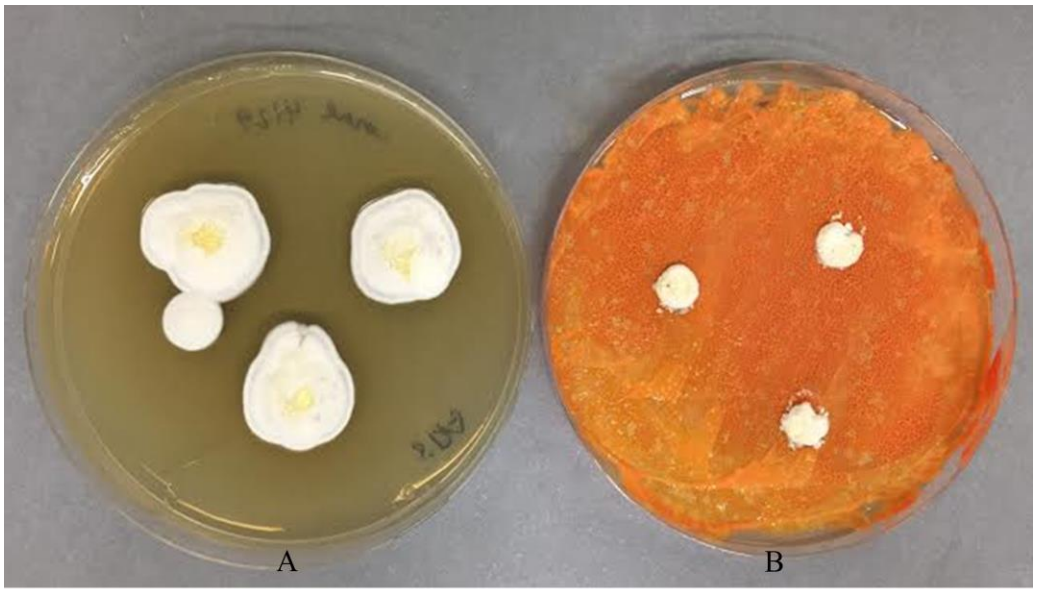

Figure 3.18 P. destructans plugs in contact- independent assays with 0g paste (A) and 0.25g fermentation paste (B)

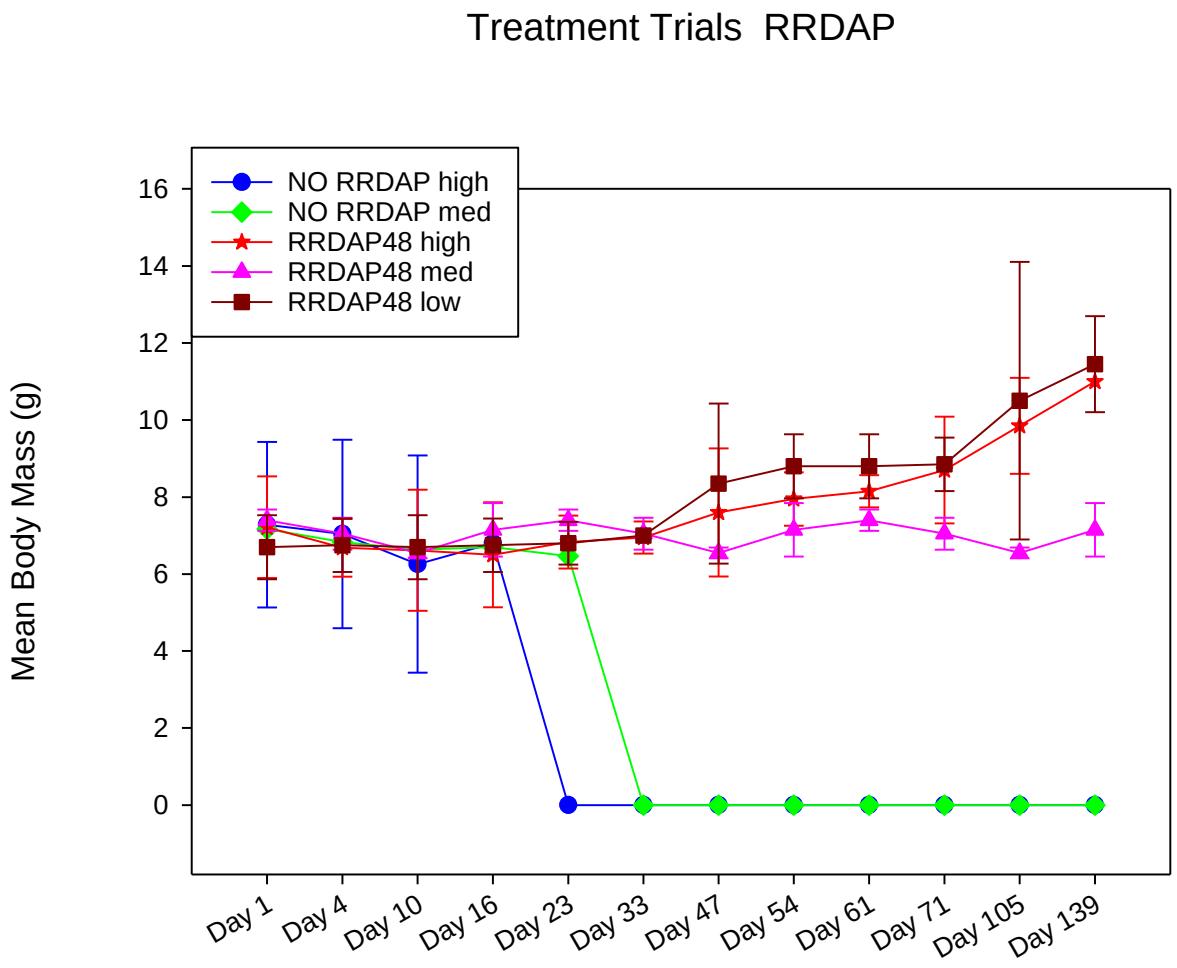

Figure 3.19 Treatment trial on bats. Mean body mass over time for untreated and treated bats.

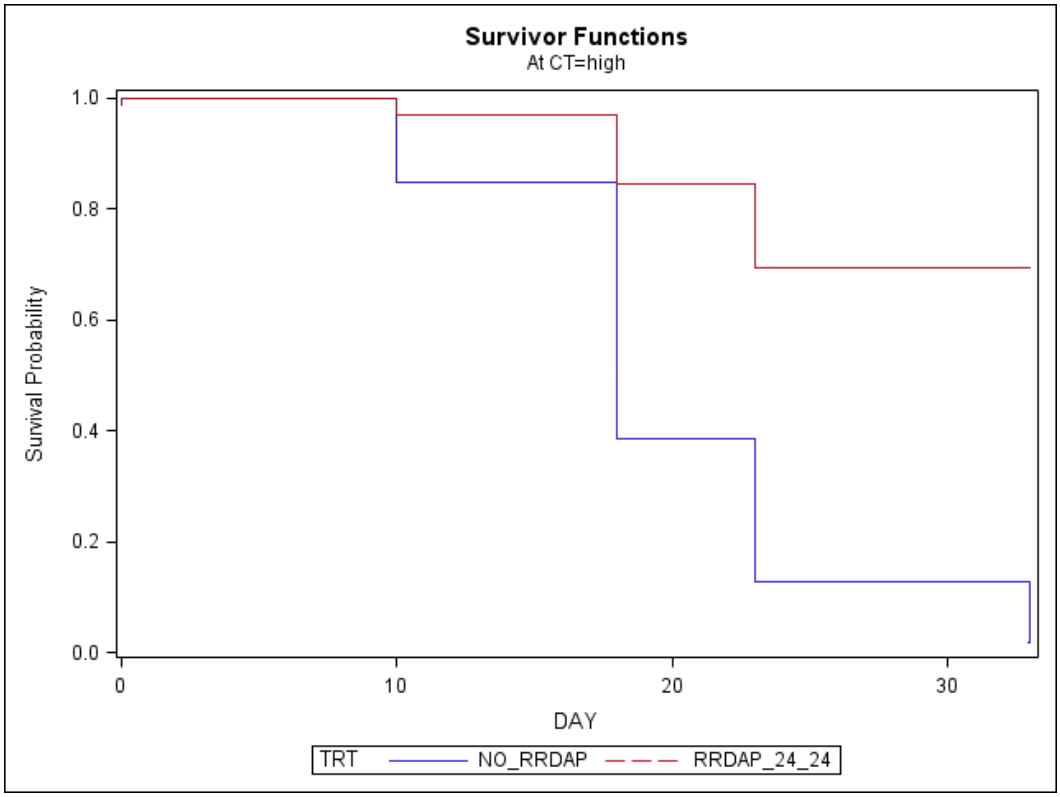

Figure 3.20 Survivorship of bats as a function of time. Treated (red); untreated (blue)
Treatment – ~60% survival, Untreated – 0% survival

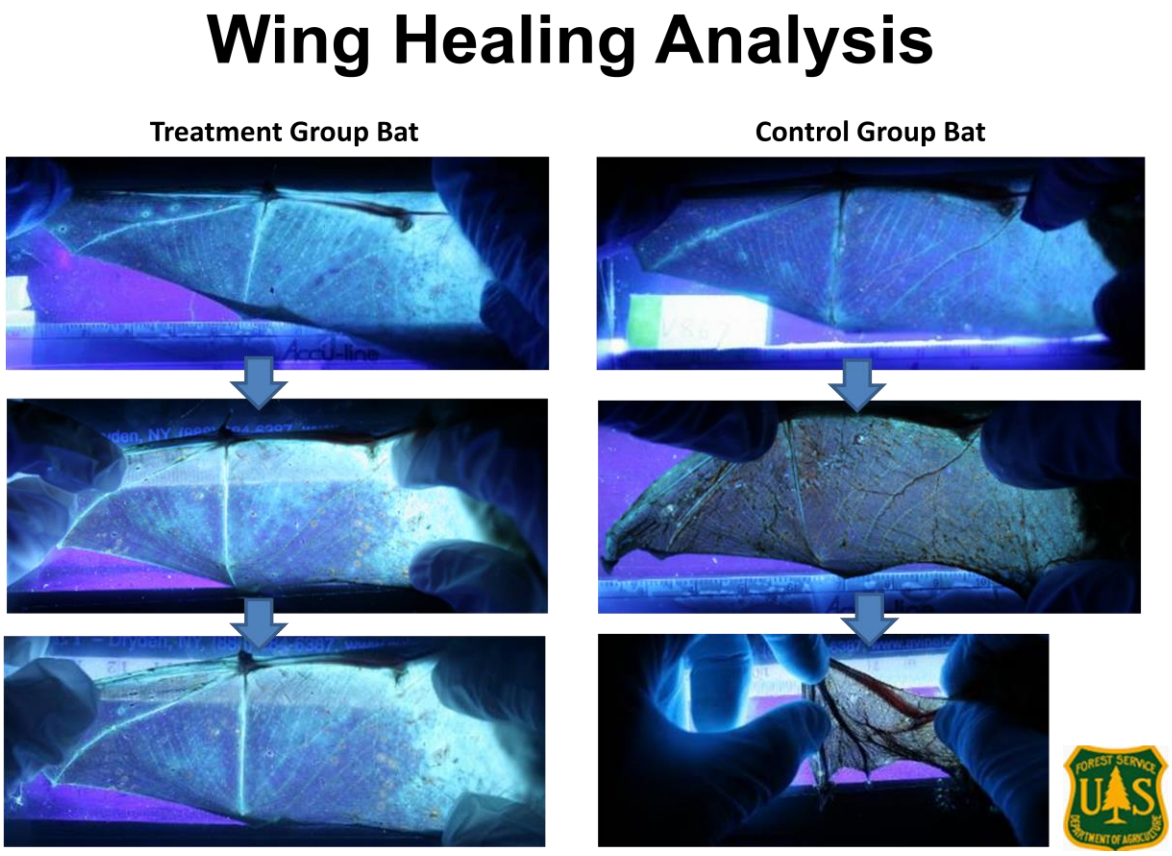

Figure 3.21 Trial 2 (bat wing healing analysis
Treatment group timecourse (L) and control group (R)

3.10 Stability of product and application techniques tested

Cells harvested from the bioreactor were shown to have stable activity for up to at least three months when stored at 4˚C (data not shown). This was defined as 1g showing complete inhibition of 10^3 *P. destructans* or *B. cinerea* spores on SDA in shared airspace assays. Cells frozen in liquid nitrogen and then moved to -80˚C for prolonged storage demonstrated complete inhibition when stored for at least 6 months (still in progress July, 2016).

Several application techniques for fermentation paste were tested against *B. cinerea* and *P. destructans:* contact-independent shared airspace assays, direct fungal control on a shared SDA plate, mini-plate membranes, catalyst dialysis membrane packs, calcium- alginate beads (Fig. 3.22), and catalyst-edible fruit coating boxes (Fig 3.26). Direct fungal control demonstrated the greatest activity against mycelia plugs on both fungal pathogens (see above).

Contact- independent shared airspace assays with fermentation paste demonstrated to be inhibitory to spores, for both *B. cinerea* and *P. destructans* (see above). Membranes were tested against tomatoes and showed to have contact-independent activity (figure 3.33). Calcium-alginate beads demonstrated no inhibition when tested against *B. cinerea* and *P. destructans* (3.23). Beads were imaged and the micrograph demonstrated that the surface area was reduced by this method of immobilization (Fig. 3.24). Dialysis membranes containing induced cells were utilized to control *P. destructans, B. cinerea* spores,and on native strawberry fungal pathogens (Fig. 3.36). Formulations of iduced cells and edible wax coated boxes were utilized for a strawberry and tomato trial to control pathogens present on the fruit and showed to be beneficial for fungal inhibition when utilized at room temperature or refrigeration (4^{o}C) (Figures 3.35).

Figure 3.22 R. rhodochrous DAP 96253 membranes designed for us in tomato trials

Figure 3.23 R. rhodochrous DAP 96253 immobilized in calcium-alginate beads

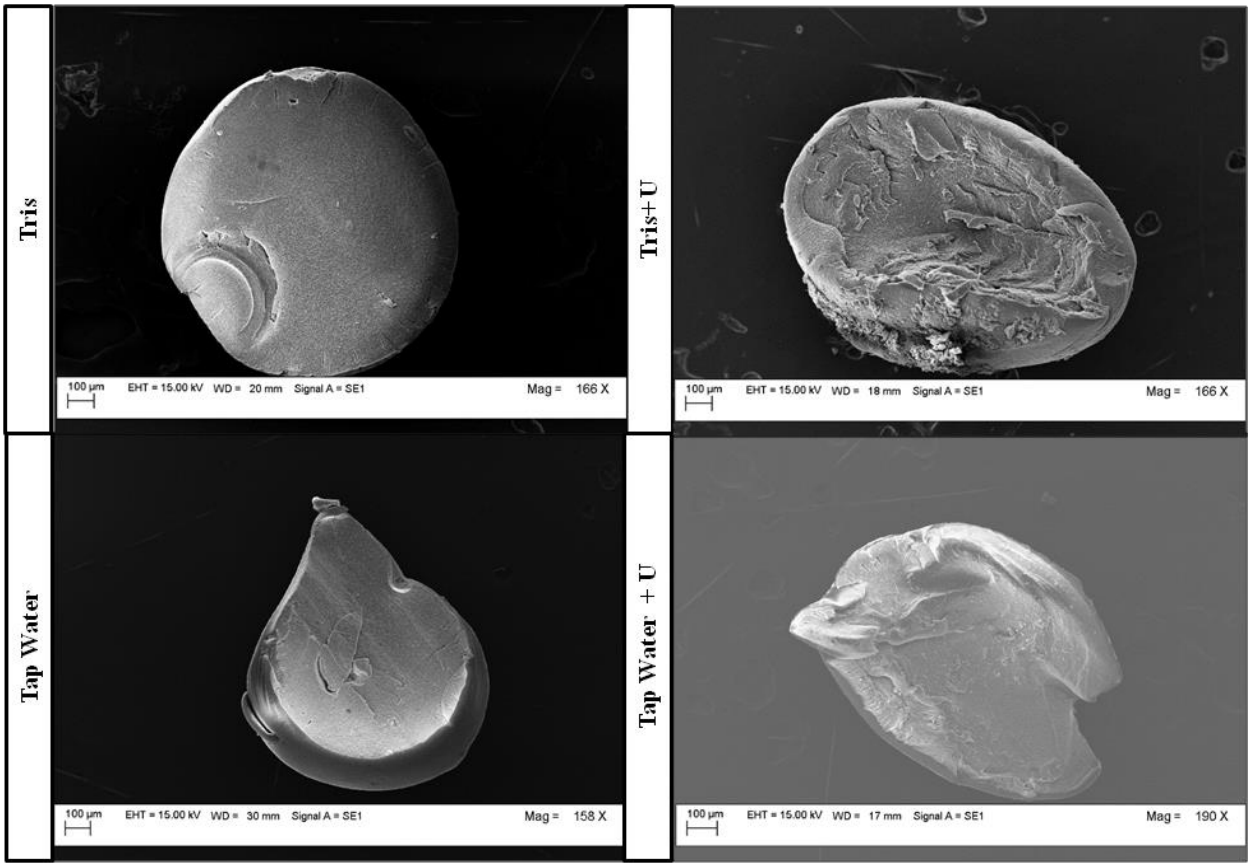

Figure 3.24 Formulation of induced cells calcium-alginate bead micrograph

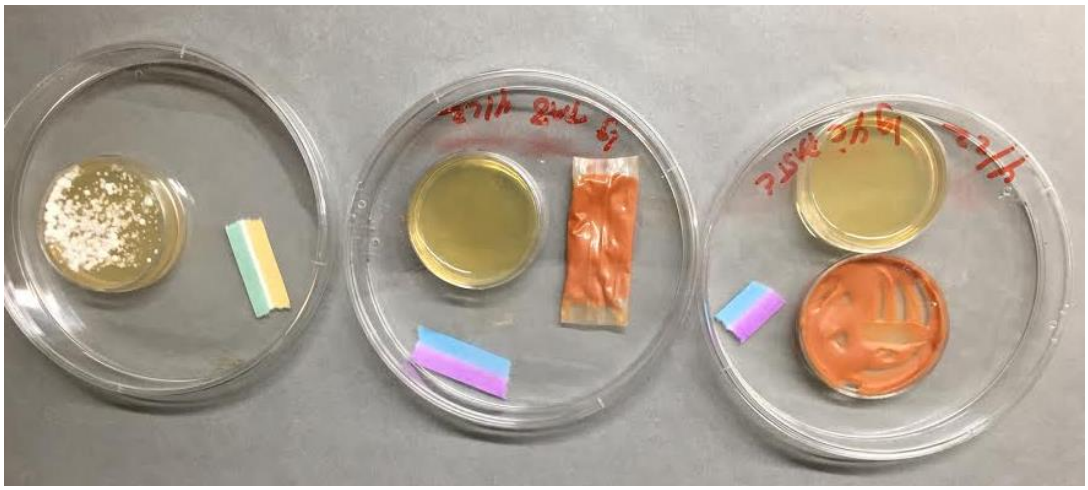

Figure 3.25 Dialysis membranes containing R. rhodochrous DAP 96253

Control (Left) and experimental 1g in dialysis membrane (middle) and 1g paste (Right)

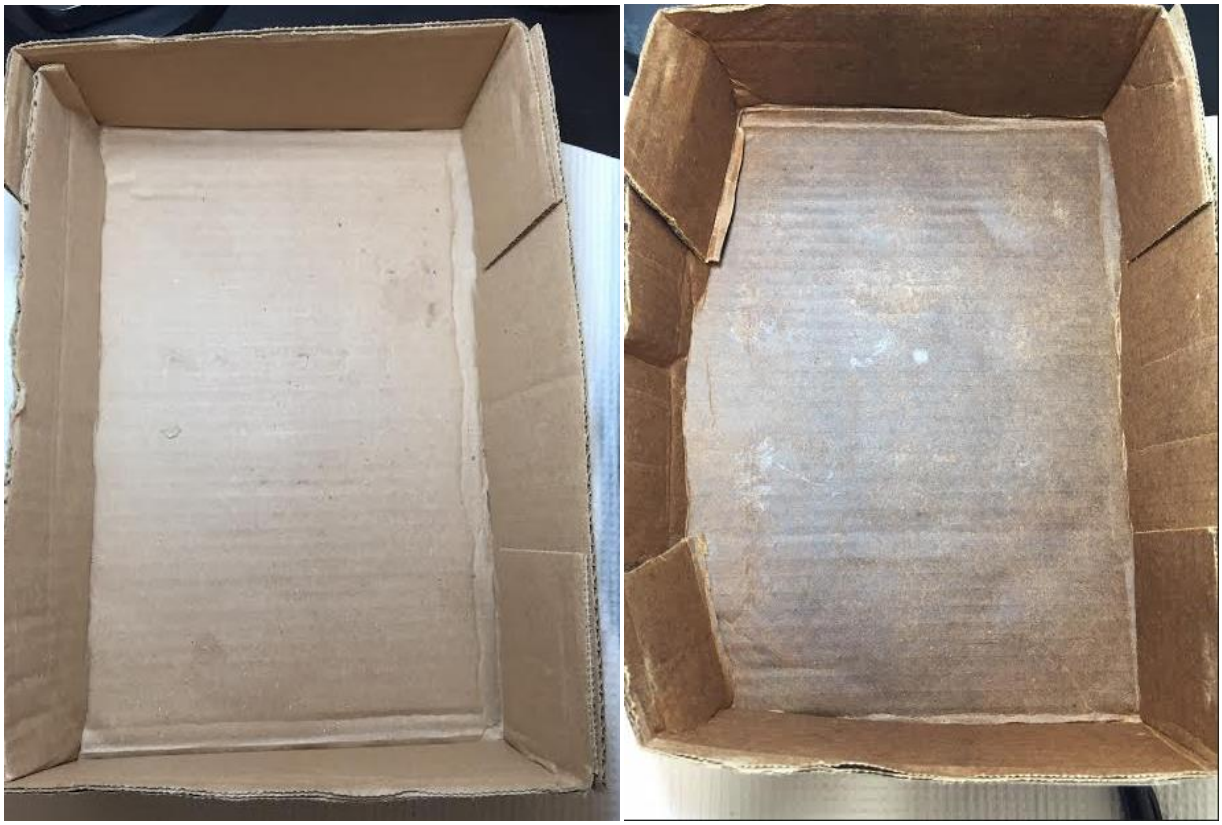

Figure 3.26 Formulation of induced cells of R. rhodochrous DAP 96253 in edible wax (LUSTR295®)
Treated (Right) and untreated box (Left).

3.11 Alkanilization of head space by bacterial volatiles

Two types of pH paper were utilized to demonstrate the alkalinization of the headspace upon contact- independent methods. The slanted cut paper demonstrated that the pH of multiple fermentation pastes were at least 10, while one was antifungal and the other was not (Fig. 3.27). This demonstrates the need for buffer in this product to handle the NH_3.

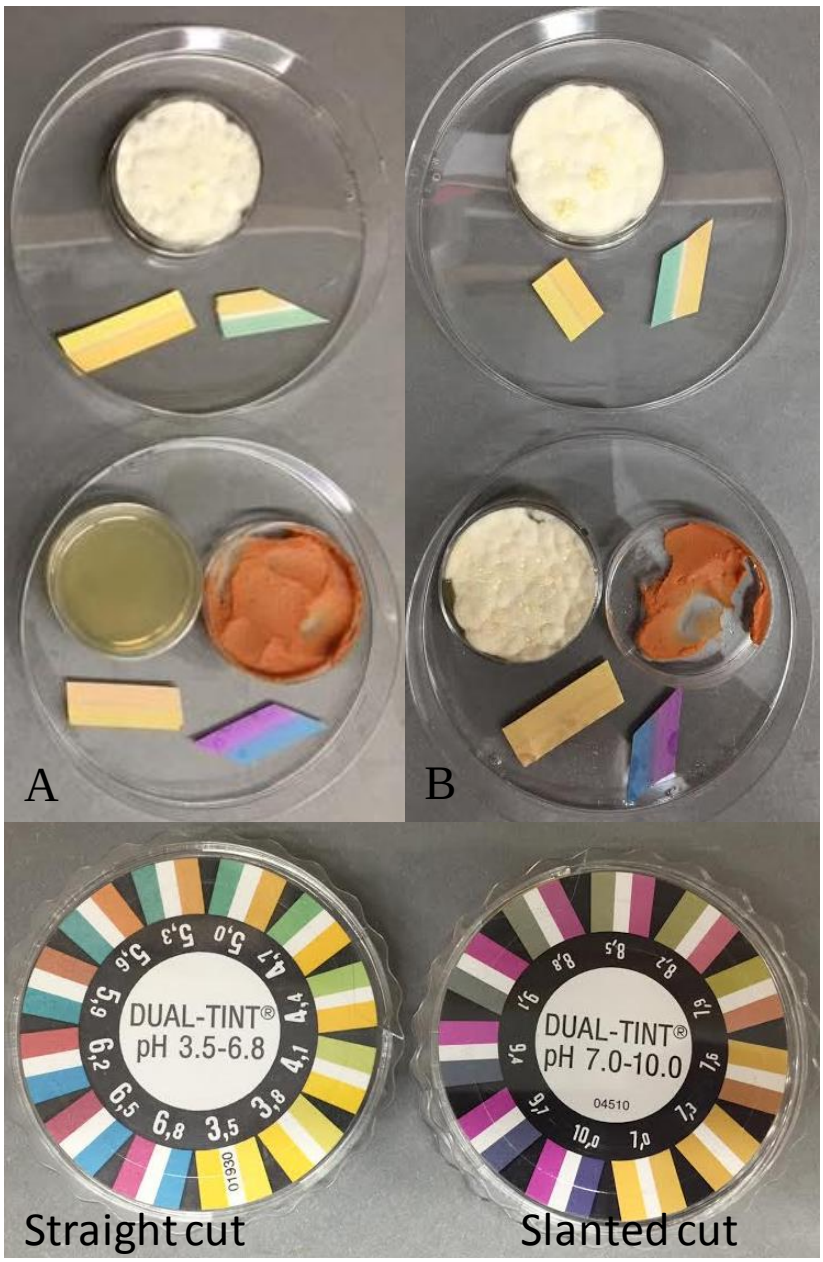

Figure 3.27 Alkalinization of headspace by fermentation cells that are antifungal (A) and not antifungal (B).

3.12 Control of fungal spoilage on peaches

Fresh peaches were used to conduct a fungal control trial of the fermentation paste. Concluding a week stored at room temperate (~25˚C), the peaches from treated and untreated boxes were separated into edible and moldy groups and images were captured (Figures 3.28-3.31). There were more edible peaches in the treated than the untreated boxes at the conclusion of the study (Table 3.3). The table shows the percentages of peaches lost to mold in each box. The internal and external peach tissues of the treated peaches were in better condition concluding the trial compared to the untreated (Fig. 3.32).

Table 3.3 Summary of images from peach trial

	Moldy	Total peach number	% Moldy
Control A	33	46	71.7
Control B	30	47	63.8
Treated C	14	45	31.1
Treated D	20	45	44.4

Figure 3.28 Peach Control (A)
At conclusion of experiment, the peaches were removed from the box and separated into edible (3 left columns) and moldy (6 right columns)

Figure 3.29 Peach Control (B)
At conclusion of experiment, the peaches were removed from the box and separated into edible (3 left columns) and moldy (5 right columns)

Figure 3.30 Treated peaches (C)
At conclusion of experiment, the peaches were removed from the catalyst-coated box and separated into edible (3 left columns) and moldy (5 right columns)

Figure 3.31 Treated peaches (D)
At conclusion of experiment, the peaches were removed from the catalyst-coated box and separated into edible (3 left columns) and moldy (5 right columns)

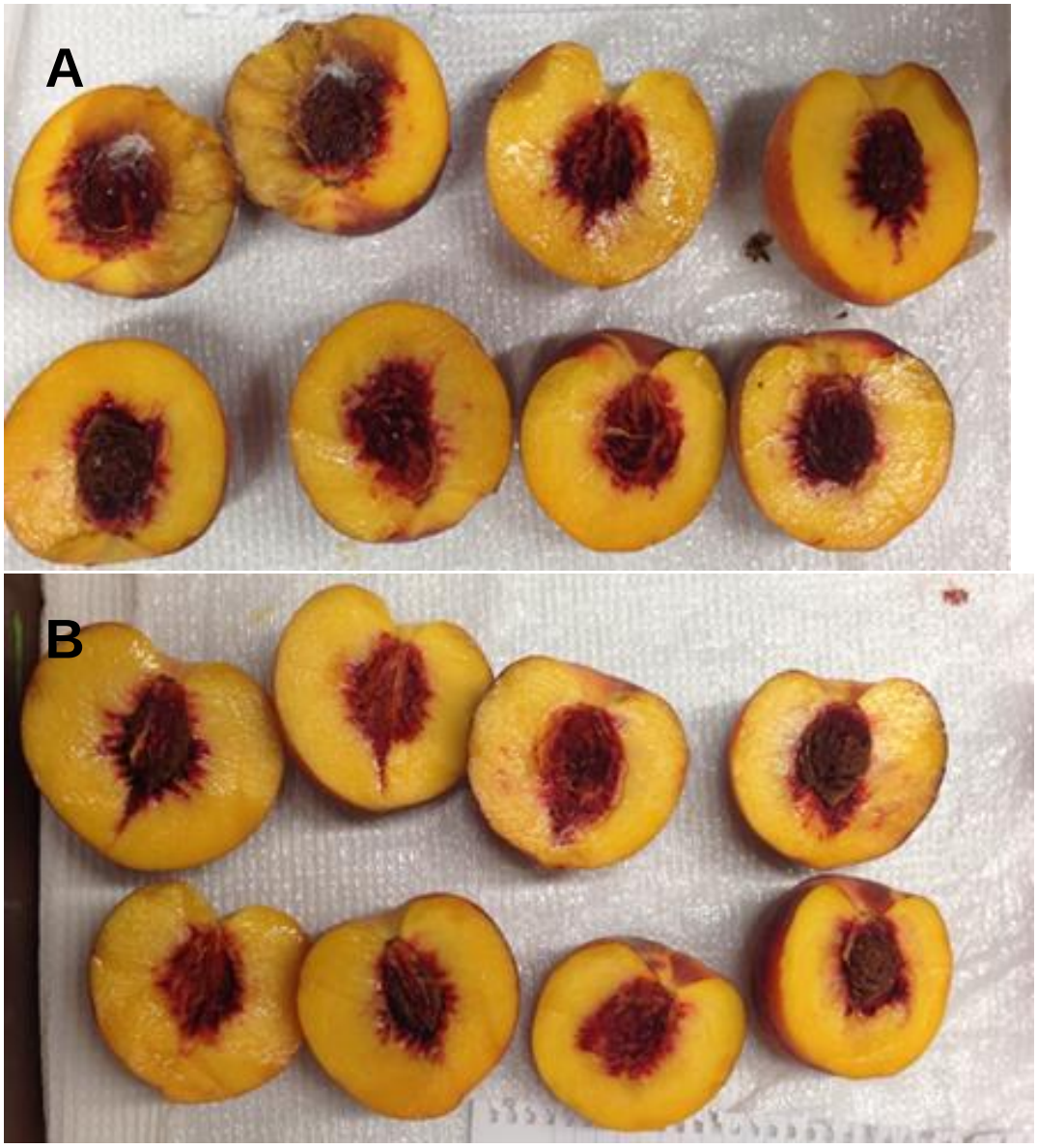

Figure 3.32 Images taken at conclusion of peach work
Control peaches (A) and treated peaches (B)

3.13 Control of fungal spoilage on brown grape tomatoes

A trial on brown grape tomatoes was conducted utilizing Rhodococcal fermentation paste membranes as the application technique (see above). The clamshell containers were stored at room temperature (~25˚C) for 21 days at which point the tomatoes were separated and rendered into edible, dehydrated, and moldy upon visual examination. For ambient phase 2 tomatoes,

there were less moldy in the treated group, while more moldy tomatoes in the treated group for the chilled subset. Both of the phase 4 ambient groups had more moldy tomatoes in the untreated than the treated groups (Fig. 3.10). Both of the chilled phase 4 untreated tomatoes had a higher number of moldy tomatoes than both of the treated groups (Fig 3.33).

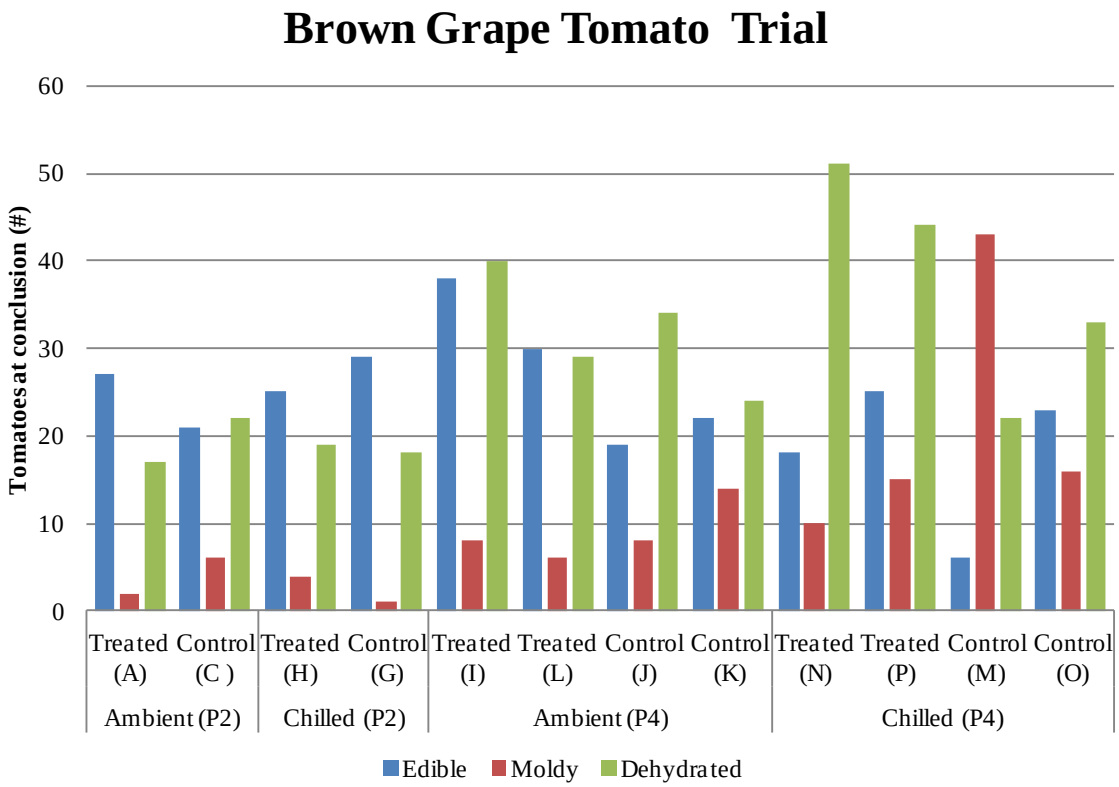

Figure 3.33 Brown grape tomato totals and conclusion of trial
Phase 2 (P2) and Phase (P4) experiments were conducted at ambient (25˚C for 21 days) and chilled (7 days at 14˚C and then subsequent storage at 25˚C for 14 days)

3.14 Control of fungal spoilage on strawberries

Rhodococcal-LUSTR295® coated boxes and Rhodococcal- dialysis membrane packs were utilized for a strawberry trial that extended for 3 days at 25^{O}C. Images were captured after 1,2, and 3 days and upon visual examination the untreated controls were the moldiest and in

the worst condition after 3 days. The treated boxes has more mold than the membrane pack containers, however the Rhodococcal-LUSTR295® coated boxes were in the best shape following a 3-day incubation on the benchtop at 25ºC (Figures 3.34-3.36).

In the refrigerated trial, the treated box was coated with LUSTR295® preparation and following two weeks refrigeration, the clamshells in the treated box showed les mold by visual examination the the untreated box (Figures 3.37, 3.38).

Figure 3.34 Untreated strawberries from day 1 (left) to day 2 (middle), and day 3 (right)

Figure 3.35 Treated-induced R. rhodochrous DAP 96253 edible wax (LUSTR295®) coated boxes
Strawberries from day 1 (left) to day 2 (middle), and day 3 (right)

Figure 3.36 Treated- (dialysis membranes containing R. rhodochrous DAP 9625)
Strawberries from day 1 (left) to day 2 (middle), and day 3 (right)

Figure 3.37 Refrigerated trial. Untreated
Strawberries from day 0 (left) to day7 (middle), and day 14 (right)

Figure 3.38 Refrigerated trial. Treated-induced R. rhodochrous DAP 96253 edible wax (LUSTR295®) coated boxes
Strawberries from day 0 (left) to day 7 (middle), and day 14 (right)

4 DISCUSSION

4.1 Inoculum

The first important feature in developing a scalable fermentation procedure is to produce a pure culture inoculum of appropriate mass, to inoculate the bioreactors, while taking into account the time it takes the cells to acclimate to the conditions in the bioreactor. In pilot- to –plant scale biomanufacturing systems, a large flask is sometimes used to inoculate the seed bioreactor, which in turn is used to inoculate a much larger production bioreactor. In previous work, methods using NB, NA, and plate- media containing glucose, yeast extract, malt extract, and urea (yemea plates) were used in the preparation of the seed inoculum. This represented a 10-day process, and on the final day these cells were manually scraped off of plate media, suspended in PBS, poured into a syringe and injected into the production vessel to initiate a fermentation run (Drago, 2006). With increased steps and the scraping of up to 100 petri plates to inoculate 1 bioreactor, contamination risks were abundant. Additionally, cells were not well acclimated to the conditions that represent those seen in the bioreactor, as the bioreactor is aqueous and the plates were extremely dry after a long incubation period.

In this study, the inoculum preparation procedures were minimized to include only two steps: NB and 1X R3A broth. This novel seed inoculum preparation method not only represented a step towards a more appropriate scale-up in process development efforts, but introduced the cells to an aqueous environment early on to shorten the acclimation time in the bioreactor. Also important to note, the media utilized in preparation of the seed was identical to that utilized in the bioreactor, and the preparation process was shortened to 6 days. By doing so, the risks of contamination were reduced by subtracting the amount of steps necessary from 5 to 3 steps. Additionally, the inoculum was pumped into the vessel through a previously un-used

“needle port line’, which reduced risks associated with injecting something directly into the vessel with a needle syringe like in previous work (Drago, 2006). In the case of scale-up to plant-scale operations in the future, the seed preparation method developed within this study will remain to have utility within the first steps of the plant-scale processes.

4.2 Process improvements within the bioreactor

When available, process development and optimization strategies should be involved when designing or improving a biomanufacturing process. This work does not represent a statistical approach or process optimization due to the restraint of having one 20L and one 30L bioreactor and limitations that a true optimization present such as needing identical bioreactors (kLa concerns (can only be calculated for a given bioreactor and will not account for different surface areas of vessels)) and possible concerns such as inconsistent yields, inconsistent product efficiency, and sheer number of runs that would have to be conducted (up to hundreds of runs to account for each variable of interest). Throughout this study, cells were consistently utilized for other projects in the laboratory and multiple batches were confidently provided to other research organizations for studies on fungal inhibition/ treatment on white-nose positive bats. For these reasons process improvements were the primary goal of this work in contrast to an optimization study. The results acquired within this preliminary fermentation study can be used as a resource for a future optimization study preceding a scale-up to plant-scale production.

In large scale biomanufacturing processes, it is common practice to use a seed bioreactor and larger production tanks. The seed bioreactor would initially get inoculated with a small volume, like that of a flask, followed by the seed bioreactor inoculating a much larger tank. By doing so, yields and profits can be exponentially increased. In this work, a similar system was

developed, but instead of scaling up to a much larger vessel from the seed bioreactor (20L), a 30L bioreactor was used as the production bioreactor. Even in transferring over 1L of inoculum from the seed tank to the production tank, both runs would yield at least 2-3kg of cells combined per the four days of the entire process (both served as production tanks). In a scaled-up version of the biomanufacturing scheme developed in this work, the entire 20L or 30L vessel could be transferred over to a much larger tank for even greater yields and profit efficiency. By utilizing a larger volume bioreactor as the production tank, bulk supplies could be ordered at extremely lower costs compared to the research-grade chemicals currently utilized.

In this study, the production process was further refined within the bioreactor utilizing multiple factors such as EFT, glucose/maltose concentrations, dissolved oxygen (DO) at time of harvest and inducer concentration as guiding parameters. The primary goal of this work was to produce catalyst with consistently improved antifungal capacity, coinciding with increased yields and greater consistency and predictability of the runs. Through this process multiple variables were measured and potential indicators of cells with antifungal activity were elucidated. Lastly, multiple variables were identified that could be linked to cells with no antifungal activity, and these could be utilized to elucidate the factor(s) involved/ not involved in the mechanism of fungal antagonism. Whether the variables highlighted in this study have a direct causal relationship with fungal antagonism is still unknown, however it is hypothesized that the data acquired within this study will undoubtedly lead to a greater insight into the process conditions required to produce *R. rhodochrous DAP 96253* as an industrial antifungal catalyst.

4.3 Dextrose/Maltose/ DO

R. rhodochrous DAP 96253 is known to utilize both glucose and maltose as sole carbon sources (Zopf, 1891). In previous work on *R. rhodochrous* fed-batch fermentation, for increased nitrile hydratase activity, it was demonstrated that the addition of 10g/L maltose to the fermentation feed medium increased cell yields and decreased nitrile hydratase activity in the resulting cells (Drago, 2006). While, *R. rhodochrous* grows relatively slow compared to other common contaminant-organisms, maltose was added and utilized to provide slow-release glucose, increasing cell yields.

Within this study, a negative correlation was seen between increasing concentrations of glucose/maltose and the resulting antifungal activity of fermentation paste. When maltose was considered as being 2 glucose (as this is how it is utilized by *R. rhodochrous*), and total glucose units was summed, this correlation was consistent. Higher carbohydrate concentrations tested in this study resulted in paste with little to no antifungal properties. Medium-low glucose concentrations tested resulted in less cell yields, than when using maltose in the feed medium, however the resulting cells displayed fungal antagonistic properties against the select fungal pathogens tested. It is important to note that low-medium glucose still resulted in improved cell yields than in previous work (>120g/L) and more importantly, the activity of the cells was at least doubled within this study. This was defined by comparing doses required for inhibition of *B. cinerea* and *P. destructans* with cells from previous work where conditions in the bioreactor were not refined to that of the current conditions defined within this study. When taking product costs and profits into account, this improvement represented a major milestone in the production development of this process.

When high carbohydrate concentrations were utilized in the fermentation medium and the cells were harvested between 3 and 4 days, it was noticed that the DO would remain low upon up to 1-2day(s) of the feed medium running out. Low (around 30%) DO demonstrated that the cells were probably still utilizing copious amounts of Oxygen as an electron acceptor in carbohydrate metabolism. It is important to note that at these time points, glucose was measured to be at a concentration of 0g/L suggesting that maltose was being consumed. Within the given restraints, only glucose concentrations could be measured using the YSI glucose analyzer, so it was unknown how long maltose concentrations remained high in the vessel. It is hypothesized that the DO remained low (around 30-50%) due to excess maltose in the feed medium and the need for an electron acceptor (as seen by the PO_2% graphs). In low-medium carbohydrate runs, there was a lag time after glucose concentration was 0g/L and the DO increased, however a marked difference in these runs was that the DO increased before the cells were harvested to at least 60% or higher.

In conclusion, glucose can be measured, so it is the ideal candidate for an affordable sole carbohydrate source in the fermentation media (not taking into account the minimal glucose concentrations in the other plant extracts present in the media). Glucose alone does not interfere with antifungal capabilities of the resulting cells, as long as it is fully utilized (0g/L after feed medium is exhausted), and the cells are left in the bioreactor to reach the stage where glucose utilization and high Oxygen demand have deceased for around 12 hours. It was seen that very high amounts of excess glucose were correlated with no antifungal properties of the cells, so this is a factor that could be optimized for in future work once the volatile profiles of fermentation cells are elucidated. Additionally, it is hypothesized that this is the process that should be

considered induction as cells that did not transition to this stage showed decreased antifungal properties.

4.4 Dissolved Oxygen in the bioreactor

Within the bioreactor, dissolved Oxygen (DO) concentrations are set to 30% utilizing a PID loop which uses stirrer to oxygenate the media (max 450; min 150RPM) and gasmix (air; O_2 pulse) . At initiation of all fermentation runs the initial DO is close to 100% as there is no mechanism that decreases the amount of O_2 present as a stir rate below 150RPM would be too slow to adequately agitate cells. At the time when the cells begin to enter exponential growth rate the speed of feed medium addition is increased to maintain glucose concentrations as close to 1.5g/L as possible. O_2 utilization spikes due to carbohydrate utilization and the stir rate begins to increase to aerate the media in response to the PID loop. The DO remains around 30% ±10 until the glucose is depleted. Following depletion, the DO has shown to remain below around 70% for 1-2 days. It is hypothesized that at this time the cells enter another metabolic stage. At this stage the DO remains low while the concentration of glucose in the bioreactor is 0g/L (as measured by the YSI glucose analyzer). It is hypothesized that once glucose is depleted the cells shift into an alternate metabolic pathway catabolizing proteins or other substrates that result in the production of ammonia, and other volatile compounds that could be involved in fungal antagonism. It has been demonstrated that the cells alkalinize the headspace once harvested, however a direct correlation between the increased pH and antifungal activity has not been demonstrated. It is hypothesized that the alkaline environment could be involved in fungal antagonism, and is most likely one of many factors in the multifaceted and complex interaction between the catalyst and fungal pathogens.

4.5 Amidase/ Urease/ Cyanidase/ Ammonia/ Alkalization of headspace

The enzymes involved in ammonia evolution evaluated in this study were amidase, cyanidase, and urease. Of these, all three were elevated in cells that were ranked as highly or slightly antifungal against the fungal pathogens tested in this study. The available ammonia was also higher in these two groups, than the cells displaying no fungal antagonism, but it is not clear as to whether these factors could be involved in fungal antagonism. Future work investigating the concentrations of *R. rhodochrous* ammonia and the linkage to fungal antagonism could decipher the statistical relevance of these enzymes. It is hypothesized that these enzymes are involved in alkalinizing the headspace of their immediate environment and could work synergistically with other enzymes or volatiles involved in the mechanism of fungal antagonism.

4.6 Fungal antagonism

Fermentation cells produced were grouped into antifungal (+), slightly antifungal (/), and non-antifungal (-). The cells that displayed fungal antagonism did so against the plant pathogen *B. cinerea* and the bat pathogen *P. destructans*. The cells grouped as slightly antifungal had activity against *B. cinerea* of the two species tested, however it is likely that there are a multitude of other sensitive species, as seen by the experiments on fruit. Fermentation runs were conducted in order to produce cells that were reliably antifungal. This biological control agent produced in this study were utilized in experiments exploring the control of post-harvest losses of fruits and in multiple preliminary experiments as a treatment tool for bats with WNS.

The trials on peaches, tomatoes, and strawberries completed within this study demonstrated promising results for the contact-independent control of post-harvest losses due to

'native' fungal species (those not inoculated, but naturally occurring). The application technique that showed to control the most fungal growth was the LUSTR295® cell formulation air-sprayed on boxes or fruit cartons. This application is extremely appropriate for larger-scale operations because the plastic clamshells holding the fruit can be placed within the treated cardboard box or carton, and the method of control is completely non- contact. In fact, it it hypothesized that shorter exposure times for clamshells of fruits to the treated boxes could be utilized. By doing so, the product could be "re-used" multiple times to increase the profitability of the process.

Over the span of this research the fermentation runs generated catalyst that was utilized for several preliminary trials on white-nose positive bats The results indicated no toxicity to bats, increased survivorship, and allowed greater wing healing (tissue regeneration) of bats in the treated groups. These small studies could warrant larger scale trials upon the approval of the appropriate agencies. Bat populations in the United States (US) are plummeting and the direct result of this could be an increase in insect populations and a decrease in the biodiversity of the US. This research supports justification for larger scale trials on bats with *R. rhodochrous DAP 96253* as a treatment tool for WNS.

4.7 Summary

In summary, this work clearly outlines the process conditions that will consistently produce 1-3kg of fungal antagonist, within nine days, with a decreased chance of contamination or inconsistency (including seed preparation). The cells are appropriate not only in the treatment trials for WNS, but in the control of post-harvest fruit losses. This study has pinpointed multiple parameters that can be monitored throughout the process, and multiple features that could be used as indicators for antifungal activity. Future work should be aimed

towards characterizing the volatile profiles of cells harvested from the bioreactor. In this work there were cells that were harvested at earlier time points (while still undergoing glucose metabolism and DO around 30-50%) and these were not antagonistic to *B. cinerea* and *P. destructans.* However, cells harvested at later time points (once glucose had been exhausted for longer periods of time and DO raised) were fungal antagonistic cells that remained active for several months when stored at refrigeration or freezer temperatures (data not shown). It is clear that the difference either lies in an enzyme 'induction' occurring in the second stage of the run, new volatile formation, increased volatile formation, or likely a combination of all of the above.

In future work, gas chromatography coupled with mass spectrometry will help to elucidate the differences between the types of paste that are produced when excess glucose is present and hence DO increased and when the exhaustion of glucose occurs and the DO increases in the vessel. Technically, all the cells used in this study were "induced'', however this research suggests that they did not all display the same activities, so more work defining the induction process will be important to further characterize the mechanism and efficiency of the catalyst.

Once the mechanism of fungal control is determined and the enzymes or volatile compounds involved are identified, than the fermentation can be further improved or optimized for the causal factor(s) that are statistically relevant in the mechanism of fungal antagonism.

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APPENDICES

Appendix A. Publication 1. Cornelison *et al*, 2014

Cornelison *et al. BMC Microbiology* 2014, 14:246
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BMC Microbiology

RESEARCH ARTICLE Open Access

A preliminary report on the contact-independent antagonism of *Pseudogymnoascus destructans* by *Rhodococcus rhodochrous* strain DAP96253

Christopher T Cornelison[1*†], M Kevin Keel[2†], Kyle T Gabriel[1†], Courtney K Barlament[1], Trudy A Tucker[1], George E Pierce[1] and Sidney A Crow Jr[1]

Abstract

Background: The recently-identified causative agent of White-Nose Syndrome (WNS), *Pseudogymnoascus destructans*, has been responsible for the mortality of an estimated 5.5 million North American bats since its emergence in 2006. A primary focus of the National Response Plan, established by multiple state, federal and tribal agencies in 2011, was the identification of biological control options for WNS. In an effort to identify potential biological control options for WNS, multiply induced cells of *Rhodococcus rhodochrous* strain DAP96253 was screened for anti-*P. destructans* activity.

Results: Conidia and mycelial plugs of *P. destructans* were exposed to induced *R. rhodochrous* in a closed air-space at 15°C, 7°C and 4°C and were evaluated for contact-independent inhibition of conidia germination and mycelial extension with positive results. Additionally, *in situ* application methods for induced *R. rhodochrous*, such as fixed-cell catalyst and fermentation cell-paste in non-growth conditions, were screened with positive results. *R. rhodochrous* was assayed for *ex vivo* activity via exposure to bat tissue explants inoculated with *P. destructans* conidia. Induced *R. rhodochrous* completely inhibited growth from conidia at 15°C and had a strong fungistatic effect at 4°C. Induced *R. rhodochrous* inhibited *P. destructans* growth from conidia when cultured in a shared air-space with bat tissue explants inoculated with *P. destructans* conidia.

Conclusion: The identification of inducible biological agents with contact-independent anti- *P. destructans* activity is a major milestone in the development of viable biological control options for *in situ* application and provides the first example of contact-independent antagonism of this devastating wildlife pathogen.

Keywords: *Pseudogymnoascus destructans*, Mycelia, Conidia, *Rhodococcus rhodochrous*, White-Nose Syndrome, Biocontrol

Background

The rapid spread and high mortality rates associated with white-nose syndrome (WNS) make the development of *in situ* treatment options for the causative agent, *Pseudogymnoascus destructans* [1,2], a significant objective for wildlife management agencies. Accordingly, the development of biologically-derived treatment options may have advantages over chemical or physical treatments, since classic examples of chemical and physical treatments in karst environments are now a cautionary tale [3]. To this end, "A National Plan for Assisting States, Federal Agencies, and Tribes in Managing White-Nose Syndrome in Bats" [4] was released in May, 2011. In this plan, significant focus was placed on the identification and development of biological control options for WNS.

Rhodococcus rhodochrous strain DAP 96253 is a ubiquitous, soil-associated, Gram-positive bacterium with tremendous metabolic and physiological diversity [5-9]. *Rhodococcus rhodochrous* has been used extensively in bioremediation as well as in the production of nitrile-containing compounds [5-7] and it has demonstrated delayed fruit ripening activity with climacteric fruits and

* Correspondence: ccornelison1@gsu.edu
†Equal contributors
[1]Applied and Environmental Microbiology, Georgia State University, 161 Jesse Hill Jr. Dr, Atlanta, GA, USA
Full list of author information is available at the end of the article

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vegetables [8]. Several enzymes have been shown to have increased activity and prevalence in bacteria induced to delay fruit ripening and these enzymes may play a role in the observed antifungal activity [8]. Initial investigation of the potential antagonism of *P. destructans* by *R. rhodochrous* indicated that, when induced under the protocol outlined in US patents 7,531,343, and 7,531,344 [10,11], *R. rhodochrous* strain DAP 96253 demonstrated significant contact-independent antagonism of *P. destructans in vitro*. As a result, the principal objective of this was evaluation of *R. rhodochrous* induced with urea for potential *in situ* application as a biological control agent for *P. destructans*.

In addition to the strong evidence established via *in vitro* analysis of the observed antagonism, the evaluation of the efficacy of induced *R. rhodochrous* was pursued in order to establish *in vivo* efficacy at preventing fungal invasion of bat tissue. This goal was accomplished using a bat-skin explant assay. The evaluation of induced *R. rhodochrous* to prevent or reduce the infective potential of *P. destructans* conidia was demonstrated by the inhibition of *P. destructans* growth on living bat tissue. This is the first example of antifungal efficacy on living bat skin for any biological control agent of WNS and represents a major milestone in this effort.

In order to optimize biocontrol efficacy and reduce potential cross-contamination of karst environments, various whole- and fixed-cell applications were investigated. The evaluation of various application methods of induced cells of *R. rhodochrous* for potential *in situ* application, including whole-cell application, non-growth fermentation cell-paste, and fixed-cell catalyst [8,12,13], were conducted. Non-growth fermentation cell-paste demonstrated persistent inhibitory activity and represents the most promising application method evaluated. The associated cell-paste activity is a significant development as it represents multiple hallmarks of ideal biocontrol agents.

Methods

Culture acquisition and maintenance

All *P. destructans* isolates used in the project were acquired from the WNS diagnostic lab at The University of Georgia Southeastern Cooperative Wildlife Disease Study (UGA SCWDS). Initial investigations have shown very low genetic and physiological variability amongst *P. destructans* isolates [14]. Accordingly, all assays were conducted with a small isolate sample size ($n \leq 3$). *P. destructans* cultures were maintained on Sabouraud Dextrose Agar (SDA, Difco) or in Sabouraud Dextrose Broth (SDB, Difco) at 4°C, 7°C, or 15°C depending on anticipated usage. *P. destructans* conidia were harvested from fungal lawns on SDA plates by adding 10 ml of conidia harvesting solution (CHS; 0.05% Tween 80, 0.9% NaCl) to the surface of the plate and gently scrapping with a sterile loop to dislodge conidia. The resulting solution was filtered through glass wool and centrifuged at 5000 rpm for 10 minutes. The resulting supernatant was removed and the spore pellet washed with 5 mL of sterile phosphate buffered saline (PBS, pH = 7), re-suspended, and filtered through glass wool. Conidia were stored in sterile PBS at −20°C. Conidia were stored no longer than six weeks prior to use based on in-house assessment of conidial viability under these conditions (unpublished data). *R. rhodochrous* strain DAP 96253 cells were maintained as glycerol stock aliquots (30% v/v) from 10 l fermentations carried out at GSU. Fresh glycerol stocks were used as the source of cells at the onset of each assay. The induction process was performed using the addition of urea or urea and cobalt as described in US patents 7,531,343 and 7,531,344 [8,10,11].

Co-culture assays with *R. rhodochrous*

A single-compartment Petri plate (150 mm × 15 mm) was used for a contained air-space to assess *P. destructans* growth characteristics in the presence of induced cells of *R. rhodochrous*. A 10 μl inoculum of *P. destructans* conidia solution (10^6 ml^{-1}) in a phosphate buffer solution was spread onto SDA in Petri plates (35 mm x 10 mm). Multiply induced cells of *R. rhodochrous* [10,11] were inoculated onto Petri plates (35 mm × 10 mm) containing Yeast Extract/Malt Extract agar (YEMEA) with or without urea (7.5 g/l) [8], and cultured in the contained air-space for up to 30 days. All assays were conducted in triplicate. The ability of induced *R. rhodochrous* to inhibit healthy established hyphae of *P. destructans* was assessed using mycelial plug assays. A lawn of *P. destructans* was allowed to grow for up to 20 days at which time a 5-mm-diameter transfer tube was used to remove a plug from the mat of fungus. The plugs were then inserted into a similarly sized core removed from an uninoculated culture plate. The plates were co-incubated in a shared air-space as described previously and radial growth from the plug was assessed over time.

Induced *R. rhodochrous* germule suppression assay

Thin layers (~750 μl) of 10% SDA were applied to standard microscope slides (24.5 × 76.2 mm) and 100 μl of *P. destructans* conidia solution (10^6 ml^{-1}) were spread across the agar surface. *R. rhodochrous*-inoculated Petri plates (35 mm × 10 mm) were placed in larger Petri plates (150 mm × 15 mm) and sealed with parafilm. Negative controls consisted of similarly-cultured conidia with no *R. rhodochrous* exposure. All trials were conducted in triplicate. At 4 and 7 days post-inoculation, conidia were observed in a light microscope at 200X magnification for the presence of germule formation. Germules were defined as single mycelial extensions emanating from conidia with a length equal to or greater

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than the intact conidia. Control and exposed slides were retained and examined daily for up to 21 days after germule formation was first observed on control slides. Recovery of conidia was determined by removing the *R. rhodochrous* after 24 hours, 72 hours, and 7 days. Slides were observed for 21 days after removal of control agent to assess recovery.

Preparation and evaluation of fixed-cell catalyst and fermentation cell-paste in non-growth conditions

Immobilization of whole bacteria was carried out based on the methods of DeFilippi [12] and Lopez-Gallego *et al.* [13]. Refinement of immobilized cells to produce active catalyst was carried out according to the methods of Pierce *et al.* [10,11]. Evaluation of anti-*P. destructans* activity of fixed-cell catalyst and fermentation cell-paste was determined in co-culture assays with *P. destructans* conidia and mycelial plugs with various amounts of control agent (<1.0 g), as described previously. Efficacy was determined by observation of germule formation as compared to unexposed controls for growth from conidia, and as percent reduction in radial growth of mycelial plugs.

Ex vivo anti-infectivity assay

The potential for induced *R. rhodochrous* to inhibit fungal growth on bat skin explants was evaluated using an *ex vivo* model of WNS. A 10-mm-diameter biopsy punch was used to collect full-thickness samples of skin (n = 40) from the patagium of bats (n = 2) immediately after euthanasia. The explants were adhered to a mesh support with tissue adhesive (TissueTek®) so that they would retain their shape and could be supported at the medium surface without allowing media to come in contact with the inoculated surface of the skin. The skin explants were then maintained on Eagle's modified minimal essential medium supplemented with antibiotics (kanamycin, 100 μg/ml: amikacin, 20 μg/ml; and vancomycin 50 μg/ml). A suspension of spores was placed onto the center of the explant and allowed to dry. The inoculated explants were incubated in a shared air-space with induced *R. rhodochrous*. Uninoculated control explants were incubated alone or with uninduced *R. rhodochrous*. Initial experiments were conducted at 7°C. Anti-infective efficacy was determined by visual and microscopic evaluation of bat wing membrane tissue cultures exposed to induced *R. rhodochrous* as compared to unexposed and uninduced controls.

Results

Anti-*P. destructans* activity of induced *R. rhodochrous*

Initial experiments with induced cells of *R. rhodochrous* demonstrated complete inhibition of growth from conidia of *P. destructans* when cultured with a shared air-space at 15°C (Figure 1a-c). Uninduced cells of *R. rhodochrous* showed no signs of inhibition, and were comparable to unexposed controls. Subsequent testing at 4°C demonstrated fungistatic activity of induced cells of *R. rhodochrous* and resulted in slower germination and reduced total mycelial growth as compared to uninduced cells of *R. rhodochrous* and unexposed controls (Figure 1d-f). Inclusion of activated carbon into the shared air-space abolished the anti-*P. destructans* activity of induced *R. rhodochrous* (Figure 1c). Mycelial plugs of *P. destructans* cultured in a shared air-space with induced *R. rhodochrous*

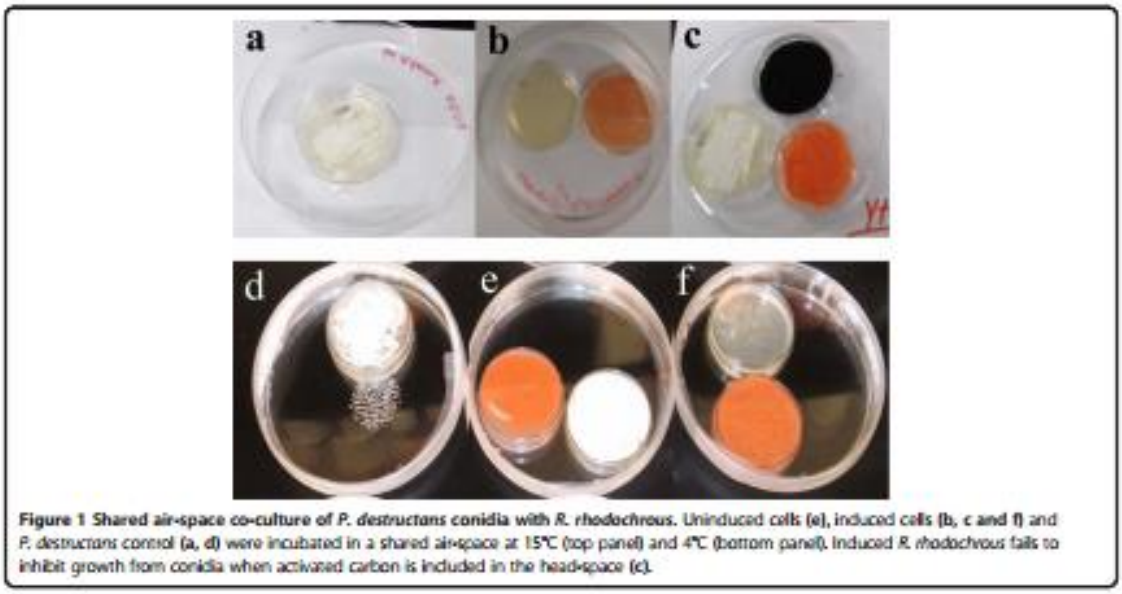

Figure 1 Shared air-space co-culture of *P. destructans* conidia with *R. rhodochrous*. Uninduced cells **(e)**, induced cells **(b, c and f)** and *P. destructans* control **(a, d)** were incubated in a shared air-space at 15°C (top panel) and 4°C (bottom panel). Induced *R. rhodochrous* fails to inhibit growth from conidia when activated carbon is included in the head-space **(c)**.

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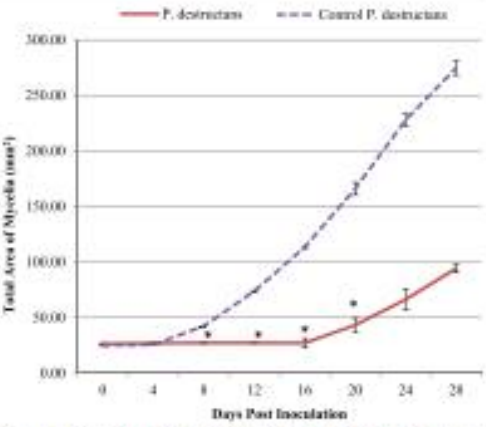

Figure 2 Induced *R. rhodochrous* inhibits radial mycelial growth of *P. destructans*. Growth areas of *P. destructans* plugs exposed to induced *R. rhodochrous* compared to *P. destructans* control plugs. All trials were conducted at 15°C. * indicates days post inoculation with statistically significant ($P \leq 0.05$) radial growth inhibition.

had a significant reduction in radial mycelial extension as compared to control plugs cultured in the absence of induced cells of *R. rhodochrous* (Figure 2). Radial growth of induced *R. rhodochrous*-exposed *P. destructans* at 28 days post inoculation indicated a 35% reduction in radial mycelial extension as compared to unexposed controls. This inhibitory activity was statistically significant ($p \leq 0.05$) on days 8, 12, 16, and 20 across all replicates (Figure 2).

Induced *R. rhodochrous* permanently and persistently inhibits conidia germination

Slide agar overlays inoculated with *P. destructans* conidia and exposed to induced *R. rhodochrous* failed to produce germules 21 days after removal of *R. rhodochrous* (Figure 3). Conidia exposed to induced cells of *R. rhodochrous* for only 24 hours revealed no signs of germule formation, whereas conidia exposed for 4 and 7 days exhibited early signs of germination but no obvious germules (Figure 3).

Ex vivo anti-infectivity activity of induced *R. rhodochrous*

Induced *R. rhodochrous* completely inhibited the colonization of bat wing explants by *P. destructans* conidia in all replicates ($n = 20$) when incubated in a shared air-space for up to 21 days at 7°C (Figure 4). Explants exposed to uninduced *R. rhodochrous* and unexposed explants were fully colonized at 14 days post inoculation. Histopathological assessments of explants were conducted. However, in this experiment no fungal growth was detected on any induced *Rhodococcus* exposed explants. Therefore the histopathology of otherwise "healthy" explants provided no additional data to this experiment. Histopathology of the control explants adheres to the histopathology of WNS in bats as described by Cryan *et al.* [15]. Spore germination assays, and the bat wing explant study relied upon qualitative visual and microscopic evaluation and

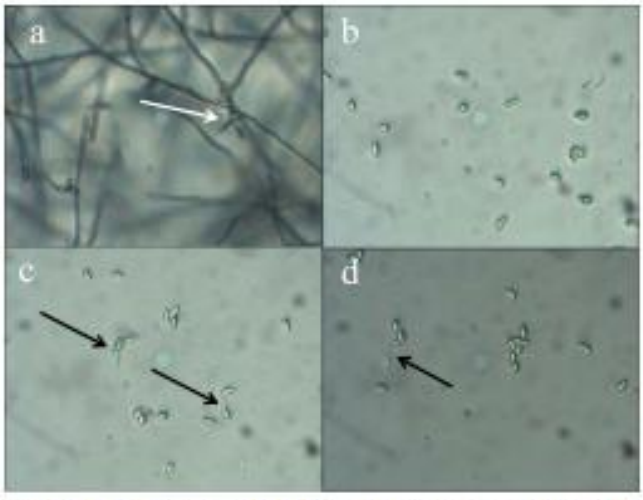

Figure 3 Persistent suppression of *P. destructans* germination by induced *R. rhodochrous*. *P. destructans* conidia are unable to recover after 24-hour exposure to induced *R. rhodochrous*. *P. destructans* control slide (a) produced significant mycelia growth and conidiation (white arrow) after 5 days. *P. destructans* conidia exposed to induced *Rhodococcus* for 24 hours (b), 72 hours (c) and 7 days (d) failed to form germules 21 days after removal of induced *R. rhodochrous*. Halted germination was observed in 72-hour and 7-day exposures (black arrows). All images were captured at 200X magnification.

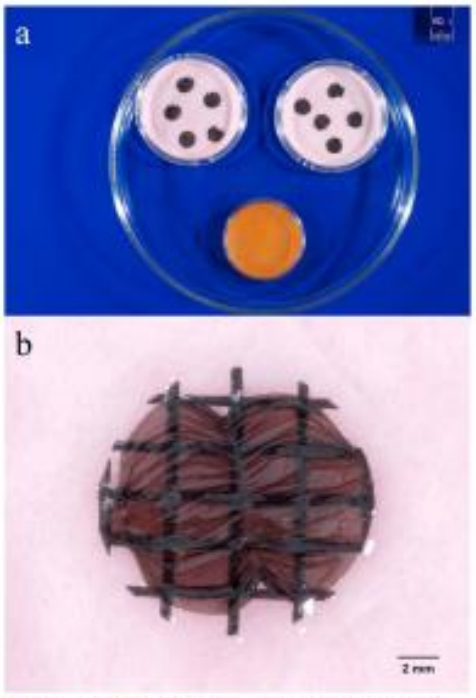

Figure 4 Induced *R. rhodochrous* prevents fungal colonization of bat tissue when contained in a shared air-space. Bat wing tissue explants in a shared air-space with induced *R. rhodochrous* 21 days post-inoculation with *P. destructans* conidia **(a)**. Magnified image of a control explant with visible fungal colonization 21 days post-inoculation **(b)**.

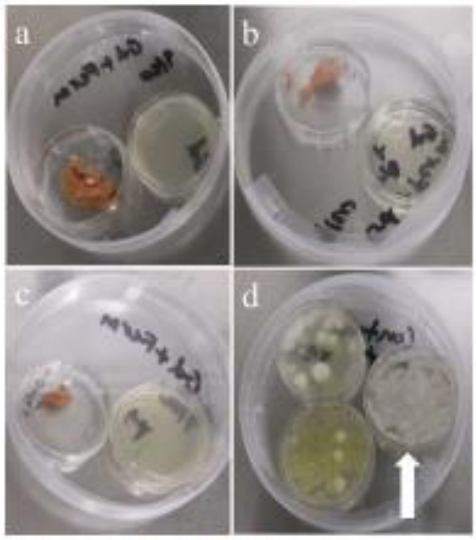

Figure 5 Non-growth cell-paste of *R. rhodochrous* inhibits growth from conidia of *P. destructans*. Non-growth fermentation cell-paste of induced *R. rhodochrous* was incubated in a shared air-space with *P. destructans* conidia inoculated plates. Quantities of 1.0 g, 0.5 g, and 0.25 g **(a, b, and c)** all demonstrated complete inhibition of growth from conidia of *P. destructans* as compared to unexposed controls **(d, white arrow)**. Image taken 21 days post-inoculation.

produced definitive results (i.e. no exposed explants developed fungal growth) therefore a statistical evaluation is unwarranted and omitted.

Evaluation of fixed-cell catalyst and fermentation cell-paste

Fixed-cell catalyst [8,10,11] failed to inhibit or slow growth from conidia of *P. destructans* when grown in a shared air-space. Fermentation cell-paste in quantities of 1.0 g, 0.5 g, and 0.25 g completely inhibited growth from conidia of *P. destructans* for greater than 80 days (Figure 5a-c).

Discussion and conclusion

Since its initial documentation in 2006, WNS has spread to twenty-four states and four provinces and has been implicated in the mortality of millions of North American bats [16-18] which may have a significant impact on North American agricultural practices [19]. WNS is characterized by invasive mycelial growth on the wings, muzzle and ears of hibernating bats that perturbs physiological functions of the host tissues leading to mortality [15]. Cave closures and culling of infected individuals appears to have little to no impact on the spread and mortality associated with this devastating disease [20]. Classic disease management practices applied in agriculture, such as vaccination and broad-spectrum dissemination of antibiotics, present many challenges in the management of disease in wild, highly disseminated, and migratory animal populations. Consequently, the development of novel treatment options are needed to avert the spread of WNS and reduce the mortality associated with currently infected hibernacula. To this end, the development of biologically-based control tools is the preferred option for application in karst environments.

Since the publication of the national response plan [4], several groups have initiated investigations to identify potential biological control agents for *P. destructans* [21-23]. Several of the investigations have relied on traditional sources of biocontrol agents or probiotics such as bacilli and lactobacilli, or competitive exclusion fungi such as *Trichoderma* sp., as well as attempts to isolate bat-skin-associated microbes with anti-*P. destructans* activity [21-23]. While these approaches have proven successful in agricultural and human health applications [24-27], their application in the attempted remediation

of WNS in bats has not been demonstrated. The requirement for contact with *P. destructans* and the bat hosts is a major hurdle for any agents reliant on competitive exclusion or non-volatile antimicrobial compound production. These potential control agents may prove to have limited efficacy against *P. destructans in situ* and potentially be harmful to the bat hosts. In contrast, the evaluation of induced *R. rhodochrous* strain DAP 96253 for application as a biological control agent of *P. destructans* aligns ideally with the needs of wildlife management agencies tasked with combatting WNS and is the first documented contact-independent microbial antagonism of *P. destructans*.

The evolutionary lineage of *R. rhodochrous* lends itself to VOC-based fungistasis due to its terrestrial ancestry [28-30]. The global prevalence of fungistatic soils is a measure of the natural antagonisms that exists in these complex environments [28-32]. Due to the ubiquity of *R. rhodochrous* in soils [5], it can be expected that *R. rhodochrous* as well as many other soil-dwelling bacteria have the potential to contribute to VOC-based fungistasis observed in these environments [29,30]. However, the development of induction methodologies is required to optimize this activity for biocontrol applications and is a decidedly advantageous quality of *R. rhodochrous* strain DAP 96253 as a potential biological control agent of WNS [33]. Leveraging this naturally evolved antagonism for control efforts has many benefits, particularly in the case of WNS. The complexity of soil ecology selects for antagonisms that are effective at low concentrations in diverse, compartmentalized environments where soluble diffusion may be limited [29]. Therefore, the production of antagonistic VOCs provides a viable means for soil-dwelling bacteria to compete with soil-dwelling fungi for resources and equates favorably with the environmental conditions of susceptible bat hibernacula. The ability of *R. rhodochrous* to detect and interfere with volatile signals has also been demonstrated in its delayed fruit-ripening activity [8] and is hypothesized to mediate the observed anti-*P. destructans* activity.

While the efficacy of urea-induced *R. rhodochrous* under growth conditions is promising for *in situ* management of WNS, the need for growth media supplementation poses problems for field application. The long term *in vitro* efficacy of non-growth-condition cell-paste at 4°C allows for increased confidence in forecasting the efficacy of this biocontrol agent in managing WNS in the field as this temperature is a sound approximation of average winter temperature of North American bat hibernacula [34]. The lack of growth media reduces the costs associated with application as well as reduces the likelihood of cross-contamination of control agent media with native cave microflora. In addition, the contact-independent basis of the non-growth antagonism will allow for *in situ* application methods that will reduce the potential for ecological impacts associated with introducing exogenous organisms to karst environments. The ecological impacts of any potential control agent are of significant concern for wildlife management agencies and the evaluation of potential ecological impacts must be assessed in order to circumvent ecological disasters associated with augmenting cave microflora (e.g. Lascaux cave) [4].

The evaluation of *R. rhodochrous* using *ex vivo* bat tissue explants as an indicator of anti-infective activity was paramount to establishing *R. rhodochrous* as a viable biocontrol agent of *P. destructans*. This was the first demonstration of inhibition of fungal colonization of bat tissue by a biological control agent. This *ex vivo* efficacy justifies further *in vivo* studies with live bats and should be pursued vigorously.

The ability of dormant conidia to remain viable in host-free environments increases long-term impacts of fungal pathogens and renders contaminated environments inhospitable to re-colonization [35]. The impact of WNS in locations such as New York has been tremendous, vastly reducing the populations of insectivorous bats over a broad geographic range. The permanent and persistent inhibition of conidia germination is a promising result and indicates that treatment of previously decimated hibernacula to inactivate resident conidia prior to re-colonization attempts may be feasible by applying induced *R. rhodochrous* in these environments. However further investigations are needed to confirm the applicability of this approach.

The evaluation of *R. rhodochrous* strain DAP 96253 has demonstrated the tremendous potential of this organism for application as a biological control agent of *P. destructans*. This is the first and only demonstration of contact-independent antagonism of *P. destructans* and represents a significant step toward the development of biologically-based treatment tools for WNS.

Competing interests
GEP and SAC are contributing authors on the seminal patents for the induction and application of *Rhodococcus rhodochrous* DAP 96253 cited in the text. These patents are held by Georgia State University Research Foundation.

Authors' contributions
CTC, SAC, and GEP conceived and designed the experiments conducted at GSU. KTG TAT and CKB developed and carried out the methodology to assess and produce induced *R. rhodochrous* as well as collected and analyzed data. MKK provided *P. destructans* isolates from his diagnostic work as well as designed and conducted the experiments with bat tissue explants at UGA. CTC, KTG, and MKK wrote the manuscript. All authors read and approved the final version of the manuscript.

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Author details
[1]Applied and Environmental Microbiology, Georgia State University, 161 Jesse Hill Jr. Dr, Atlanta, GA, USA. [2]Department of Pathology Microbiology & Immunology, University of California Davis, One Shields Avenue, Davis, CA, USA.

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Appendix B. Publication 2. Cornelison *et al*, 2014

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Inhibition of *Pseudogymnoascus destructans* Growth from Conidia and Mycelial Extension by Bacterially Produced Volatile Organic Compounds

Christopher T. Cornelison · Kyle T. Gabriel · Courtney Barlament · Sidney A. Crow Jr.

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Abstract The recently identified causative agent of white-nose syndrome (WNS), *Pseudogymnoascus destructans*, has been implicated in the mortality of an estimated 5.5 million North American bats since its initial documentation in 2006 (Frick et al. in Science 329:679–682, 2010). In an effort to identify potential biological and chemical control options for WNS, 6 previously described bacterially produced volatile organic compounds (VOCs) were screened for anti-*P. destructans* activity. The compounds include decanal; 2-ethyl-1-hexanol; nonanal; benzothiazole; benzaldehyde; and*N*,*N*-dimethyloctylamine. *P. destructans* conidia and mycelial plugs were exposed to the VOCs in a closed air space at 15 and 4 °C and then evaluated for growth inhibition. All VOCs inhibited growth from conidia as well as inhibiting radial mycelial extension, with the greatest effect at 4 °C. Studies of the ecology of fungistatic soils and the natural abundance of the fungistatic VOCs present in these environments suggest a synergistic activity of select VOCs may occur. The evaluation of formulations of two or three VOCs at equivalent concentrations was supportive of synergistic activity in several cases. The identification of bacterially produced VOCs with anti-*P. destructans* activity indicates disease-suppressive and fungistatic soils as a potentially significant reservoir of biological and chemical control options for WNS and provides wildlife management personnel with tools to combat this devastating disease.

Keywords *Pseudogymnoascus destructans* · Mycelia · VOC · White-nose syndrome · Fungistatsis

Electronic supplementary material The online version of this article (doi:10.1007/s11046-013-9716-2) contains supplementary material, which is available to authorized users.

C. T. Cornelison (✉) · K. T. Gabriel · C. Barlament · S. A. Crow Jr.
Applied and Environmental Microbiology, Georgia State University, 24 Peachtree Center Ave, Atlanta, GA, USA
e-mail: ctcornelison1@gsu.edu

Introduction

White-nose syndrome (WNS) was first documented near Albany, New York, in 2006 [2, 15]. Since its discovery, WNS has caused severe declines in bat populations in the Eastern United States and Canada [9, 12, 23]. Although the exact ecological and economic impact of this disease has yet to be determined, many researchers agree that continued declines in insectivorous bat populations will have a significant impact on forest management, agriculture and insect-borne disease [4]. The rapid spread of WNS and the high mortality rates associated with the disease [9, 13] necessitate the rapid development of disease management tools. In 2011, the fungus *Geomyces destructans* was shown to be the putative causative agent of WNS [18].

Recently, the fungus *Geomyces destructans* has been reclassified as *Pseudogymnoascus destructans* [19, 20]. *P. destructans* is a psychrophilic ascomycete with optimal growth at 12.5–15.8 °C [15, 23]. Its psychrophilic nature makes *P. destructans* ideally suited for colonization of bats in torpor, when body temperatures and immune function are greatly depressed [3, 5]. The clinical manifestation of *P. destructans* infection is characterized by fuzzy white growth on the muzzle and wings of hibernating bats and results in severe physical damage to bat wing membranes [8]. Due to the recent identification of *P. destructans*, many ecological and physiological traits and their influence on virulence are yet to be elucidated.

The rapid spread and high mortality rates associated with WNS make the development of in situ treatment options for *P. destructans* a significant objective for wildlife management agencies. Accordingly, the development of biological and chemical treatment options is a priority for State and Federal agencies as stated in the 2011 National WNS Management Plan established by the United States Fish and Wildlife Service (USFWS) [1]. With this goal in mind, 6 previously described bacterially produced antifungal VOCs [7, 11] were assayed for their in vitro potential to inhibit the growth and proliferation of *P. destructans*.

Previous investigations of fungistatic soils were able to identify bacteria that produced antifungal VOCs which were later identified via SPME/GC/MS of cultures and soils. The VOCs were produced by *Pseudomonas* and *Bacillus* spp. and demonstrated broad spectrum antifungal activity [7, 11]. Volatile-based fungistasis in soils has been observed in terrestrial environments around the globe [25]. Due to the biological and chemical complexity of these environments, the ultimate source of the active VOCs is often unknown but typically attributed to bacteria [16, 25]. The geology and ecology of soil make the presence of inhibitory volatiles of particular interest, as low levels of VOCs are able to inhibit fungal growth in a dense, compartmentalized, and diverse ecosystem [10, 11, 14, 16]. Using the soil ecosystem as an ideal example of naturally occurring biological control of fungal proliferation, we began to investigate biologically derived VOCs with known antifungal activity.

The influence of the VOCs on the growth from conidia and mycelial extension of *P. destructans* was evaluated using digital imaging techniques. In an effort to optimize the efficacy of the VOCs for potential in situ applications, formulations of VOCs were evaluated for potential synergistic effects. Combinations of two VOCs applied at equal quantities as individual VOCs revealed several potentially synergistic combinations. Accordingly, these synergistic blends were used to establish formulations of three VOCs ultimately yielding highly effective formulations with greatly increased anti-*Pseudogymnoascus* activity at relative concentrations of <1 ppm. The identification of biologically produced inhibitory volatiles expands the pool of potential biocontrol agents of *P. destructans*, and the development of chemical formulations with significant anti-*Pseudogymnoascus* activity at low concentrations provides promising chemical control options for in situ management of WNS.

Materials and Methods

Culture Acquisition and Maintenance

All *P. destructans* isolates used in the project were provided by Kevin Keel through his WNS diagnostic work at the University of Georgia's Southeastern Cooperative Wildlife Disease Study (SCWDS). *P. destructans* cultures were maintained on Sabauroud Dextrose Agar (SDA) or in Sabauroud Dextrose Broth (SDB) (BD, Maryland) at 4–15 °C. *P. destructans* spores were stored in phosphate-buffered saline (PBS) at −20 °C. Spores were stored no longer than 3 weeks prior to use.

VOC Exposure Assays and Evaluation of Bacterially Produced VOCs for Anti-*P. destructans* Activity

Volatile organic compounds previously shown to be produced by bacteria [7, 11] were screened for anti-*P. destructans* activity via VOC exposure to spores and mycelial plugs. The VOCs included: decanal; 2-ethyl-1-hexanol; nonanal; benzothiazole; benzaldehyde; and N,N-dimethyloctylamine (Sigma-Aldrich, Missouri). All VOCs were chosen based on their identification in fungistatic soils and their observed production in bacteria [7, 11]. All VOCs purchased as pure, liquid, research grade reagents and used directly, without

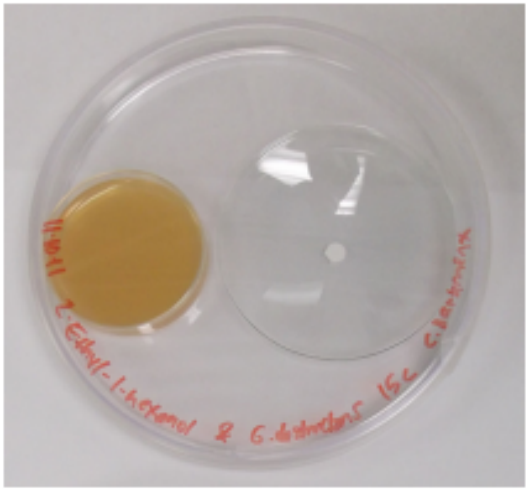


Fig. 1 Shared airspace assay with previously described bacterially produced VOCs

modification, in all subsequent assays. A single-compartment Petri plate (150 mm × 15 mm) was used for a contained airspace to assess *P. destructans* growth characteristics in the presence of fungistatic VOCs. Ten microliters of *P. destructans* conidia suspension (10^6 conidia ml^{-1} in PBS) was spread onto SDA plates (35 mm × 10 mm). Aliquots of 30, 3.0, or 0.3 µl of each VOC corresponding to maximum possible relative concentrations ranging from 113 ppm (v/v) to 1.13 ppm (v/v) (Table S1) were pipetted onto a sterile filter paper disk (12.7 mm) on a watch glass (75 mm). Each VOC containing disk and watch glass was placed inside a large Petri plate (150 mm × 15 mm) along with a *P. destructans*-inoculated SDA plate (35 mm × 10 mm) (Fig. 1). *P. destructans* mycelial plugs cut from the leading edge of actively growing colonies were inserted into fresh SDA plates (35 mm × 10 mm) and placed in large Petri plates (150 mm × 15 mm) with each formulation or pure VOC containing paper disk and sealed with parafilm M (Sigma-Aldrich, Missouri). Plates were then incubated at 15 °C for 21 days. Unexposed cultures and the addition of activated carbon to exposure assays served as negative controls for each trial. Anti-*P. destructans* activity was scored on a plus/minus scale for conidia-inoculated plates, and the radial growth from mycelial plugs was used to determine percent inhibition by comparing growth area of VOC exposed plugs to unexposed controls. All assays were performed in triplicate and averaged.

VOC Formulation Assay for Anti-*P. destructans* Activity

VOC formulations utilizing combinations of two pure VOCs were created with all fifteen possible combinations of the six VOCs by applying volumes corresponding to 2.0 µmol of each VOC to separate absorbent disks and arranging combinations of two disks of different VOCs on a single watch glass. Volumes corresponding to 4.0 µmol of each pure VOC were used as synergism controls to determine synergism. *P. destructans* mycelial plugs were harvested and inserted into fresh SDA plates (35 mm × 10 mm) and sealed with parafilm in large Petri plates (150 mm × 15 mm) with each formulation or pure VOC. Plates were then incubated at 15 °C for 21 days as described above. Each test was conducted in triplicate. Area measurements were conducted every 2 days post-inoculation with the use of digital photography and computer analysis as described below.

Area Measurement of Radial Growth with Digital Photography and Open-Source Software

Filamentous fungi grow by hyphae elongation and not by distinct cellular division. Accordingly, measuring the difference between the area growth of control agent-exposed mycelial plugs and control plugs has been a vetted method for assessing antimicrobial susceptibility [11, 17, 22]. The use of a ruler to measure the area of mycelial growth of filamentous fungi has its own challenges. Mycelial plugs will often grow asymmetrically, either naturally or because of exposure to the compound being tested. To provide more accurate measurement of mycelial growth, a digital photography and analysis technique was developed.

The GIMP (GNU Image Manipulation Program) is open-source, freely distributed software for image editing and authoring, compatible with GNU/Linux, Microsoft Windows, Mac OS X, Sun OpenSolaris, and FreeBSD operating systems. This software allows for the direct measurement of the number of pixels in a given selected area of a photograph. GIMP version 2.8.2 for Microsoft Windows was used at the time of this writing. A Nikon D3100 digital single lens reflex camera with an 18–55 mm lens was used to capture images. A standard three-leg tripod was used for support during capture.

The camera was attached to the tripod and aimed down to a surface to provide a consistent distance from the lens to the mycelial surface being photographed; ensuring the same pixel to millimeter ratio was retained for all acquired images. Images of mycelial plugs had their corresponding image numbers catalogued for later identification. All Petri plate agar heights were similar to ensure the focal point remained consistent as well as to retain the same pixel to distance ratio. Manual focus was activated to retain the same focal point throughout all image captures, and a remote shutter release device was used to assure stable, shake-free images were acquired.

Contrast between the growth medium and mycelium was required to obtain an accurate selection for measurement as well as to be able to discern the margin of the ruler graduation marks with GIMP. Therefore, the camera's white balance, exposure, f-stop, and ISO were adjusted to retain a consistent contrast between photograph acquisitions. A photograph of a ruler was used to set the focal point for the proceeding photographs as well as serving as a calibration device for determining the length of each pixel during image analysis.

The ruler tool was used to determine the number of pixels between two demarcations of a photographed ruler placed at the level of the agar surface in the Petri plates. The resulting pixel count was used to determine the millimeter-to-pixel ratio.

A different set of tools were necessary to measure the mycelial area. The selection tools were used to outline the margin of the mycelia. The Histogram tool was used to determine the number of pixels that comprised the selected area. The area of the selection was converted from the number of pixels to mm^2 with our previously derived number of pixels per mm and Eq. 1.

$$\left(\frac{\text{Number of pixels in area}}{\text{Number of pixels per mm}}\right)^2 = \text{Area of mycelia in } mm^2 \quad (1)$$

Tape Mount Preparation and Microscopic Evaluation

Pseudogymnoascus destructans cultures with aberrant phenotypes as compared to control cultures and published descriptions [14] were examined microscopically by tape mount. The adhesive side of standard transparent packaging tape was gently pressed against the surface of plate grown fungal colonies. The resulting tape-adhered sample was treated with 10 µl of 70 % ethanol and placed onto a microscope slide with lactophenol cotton blue dye. Slides were viewed on a light microscope (Nikon optiphot-2) at 200 × magnification and images captured using a scope mounted camera (QImaging micropublisher 3.3 RTV).

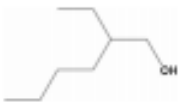
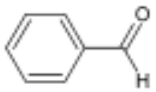
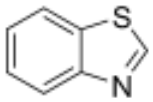
Results

Anti-*P. destructans* Activity of Bacterially Produced Volatiles

Initial investigation demonstrated inhibitory activity for most VOCs at relative concentrations less than 1 ppm. Decanal; 2-ethyl-1-hexanol; nonanal; benzothaizole; dimethyltrisulfide; benzaldehyde; and N,N-dimethyloctylamine all demonstrated anti-*P. destructans* activity when 30 µl of the respective compound were placed adjacent to SDA plates inoculated with *P. destructans* conidia in a closed system at 15 °C (Table 1). Control plates containing 1 g activated carbon showed no inhibition for decanal; 2-ethyl-1-hexanol; and benzaldehyde, while the remaining compounds inhibitory activity persisted in the presence of activated carbon (Table 1). Subsequent assays with 3 µl of each compound demonstrated similar results with only N,N-dimethyloctylamine unable to completely inhibit *P. destructans* growth from conidia at 7 days (Table 1). The addition of activated carbon abolished all inhibitory activity of the assayed compounds at 3 µl (Table 1). At 11 days of exposure to 3 µl of each respective compound, only 2-ethyl-1-hexanol, decanal, and nonanal demonstrated inhibitory activity, with all activated carbon controls abolishing the inhibitory activity (Table 1). Additionally, *P. destructans* cultures from conidia exposed to 3 µl benzothiazole without activated carbon revealed unique colony morphology characterized by increased pigmentation of the underside of the culture and diffusion of pigment into the growth media as compared to unexposed cultures and cultures exposed to benzothiazole in the presence of activated carbon (Fig. S1).

Assays using mycelial plugs cut from the leading edge of actively growing *P. destructans* colonies on SDA exposed to the previously described bacterially

Table 1 Evaluation of anti-*P. destructans* activity of bacterially produced antifungal VOCs with *P. destructans* conidia

VOC	Chemical structure	30 µl	30 µl[c]	3 µl[a]	3 µl[a,c]	3 µl[b]	3 µl[b,c]
2-ethyl-1-hexanol		−	+	−	+	−	+
Benzaldehyde		−	+	−	+	+	+
Benzothiazole		−	−	−	+	+	+
Decanal		−	+	−	+	−	+
Nonanal		−	−	−	+	−	+
N,N-dimethyloctylamine		−	−	+	+	+	+
Control		+	+	+	+	+	+

+, growth from spores; −, no visible growth

[a] 7 day exposure

[b] 10 day exposure

[c] Incubated with activated carbon

produced volatiles at 30, 3, and 0.3 µl of each respective compound and incubated in a contained air space at 15 °C gave varied results. At 30 µl, all compounds completely inhibited the growth of *P. destructans* mycelia for up to 9 days (Fig. 2a). At 14 days of exposure, only *P. destructans* plugs exposed to decanal showed any radial growth, with 83 % reduction in growth as compared to unexposed controls (Fig. 2a). At 3 µl of each compound, decanal and N,N-dimethyloctylamine yielded only minor reductions in radial growth, whereas the remaining compounds completely inhibited radial mycelial growth of *P. destructans* for up to 14 days (Fig. 2b). At 0.3 µl of each compound, only benzothiazole demonstrated significant inhibitory activity with a 60 % reduction in radial growth after 14 days of exposure (Fig. 2c). Interestingly, at 0.3 µl, N,N-dimethyloctylamine induced growth as compared to unexposed controls (Fig. 2c). This result may be due to hormesis [21].

In order to forecast the in situ efficacy of the VOCs additional in vitro evaluation was conducted at 4 °C to more accurately represent the environmental conditions of North American hibernacula. Exposure to 30 µl or 3.0 µl of each respective VOC completely inhibited radial growth of *P. destructans* for greater than 21 days (data not shown). Exposure to 0.3 µl of each respective VOC inhibited radial growth for all VOCs except benzaldehyde (Fig. 2d). The greatest degree of inhibition was observed with decanal which demonstrated a greater than 99 % reduction in growth area at 35 days post-inoculation (Fig. 2d). Based on these initial results, VOC exposure was standardized to 4.0 µmol per headspace for subsequent evaluations. In addition to evaluating individual VOCs, formulations were investigated for potential synergistic effects.

VOC Formulations Demonstrate Synergistic Anti-*P. destructans* Activity

Three VOC formulations comprised of two VOCs were observed to synergistically inhibit the growth of *P. destructans* mycelial plugs, more than the combined inhibition of each of the pure VOCs alone.

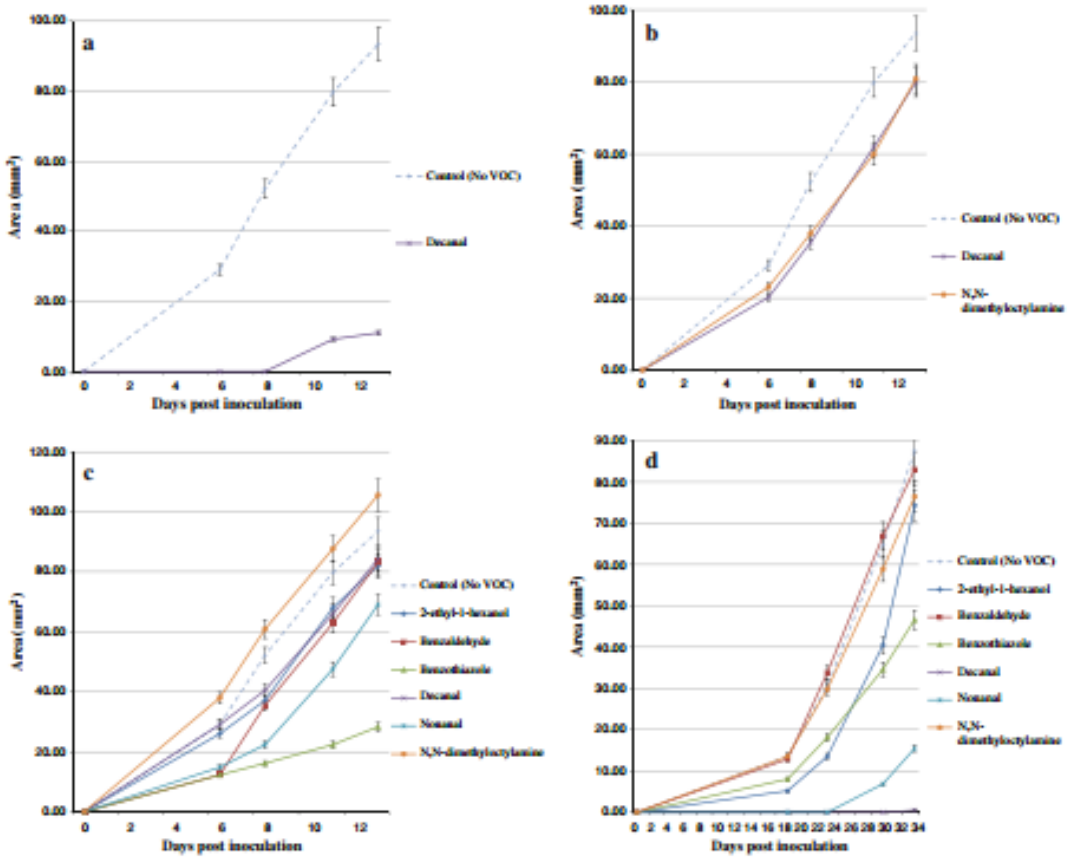


Fig. 2 Growth areas of *P. destructans* mycelial plugs exposed to bacterially produced VOCs at 15 °C at 30 µl (**a**), 3 µl (**b**), 0.3 µl (**c**), respectively. Growth area of mycelial plugs exposed at 4 °C to 0.3 µl (**d**) of bacterially produced VOCs. VOCs not shown in the legend completely inhibited radial growth for the duration of the experiment

Those include 2-ethyl-1-hexanol and benzaldehyde; 2-ethyl-1-hexanol and nonanal; 2-ethyl-1-hexanol and decanal; and 2-ethyl-1-hexanol and N,N-dimethyloctylamine (Fig. 3a, 3b, 3c, respectively). The greatest inhibition by the formulation occurred with 2-ethyl-1-hexanol and nonanal, which demonstrated greater than 95 % reduction in growth as compared to unexposed controls 14 days post-inoculation (Fig. 3c).

Two VOC formulations comprised of three VOCs at 1.33 µmol, respectively, were observed to synergistically inhibit the growth of *P. destructans* mycelial plugs, more than the combined inhibition of each of the pure VOCs alone at 4.0 µmol. Those include 2-ethyl-1-hexanol; benzaldehyde; and decanal; as well as 2-ethyl-1-hexanol; nonanal; and decanal (Fig. 4a, 4b).

Discussion

Since its initial documentation in 2006, *P. destructans* has spread to twenty-four states and four Canadian providences and is implicated in the mortality of 5.5 million bats [13]. Cave closures and culling of infected individuals appear to have little to no impact on the spread and mortality associated with this devastating disease. Classic disease management practices applied in agriculture such as broad spectrum dissemination of antibiotics are not realistic options for management of disease in wild, highly disseminated, and migratory animal populations. Accordingly, the development of novel treatment options is needed to avert the spread of

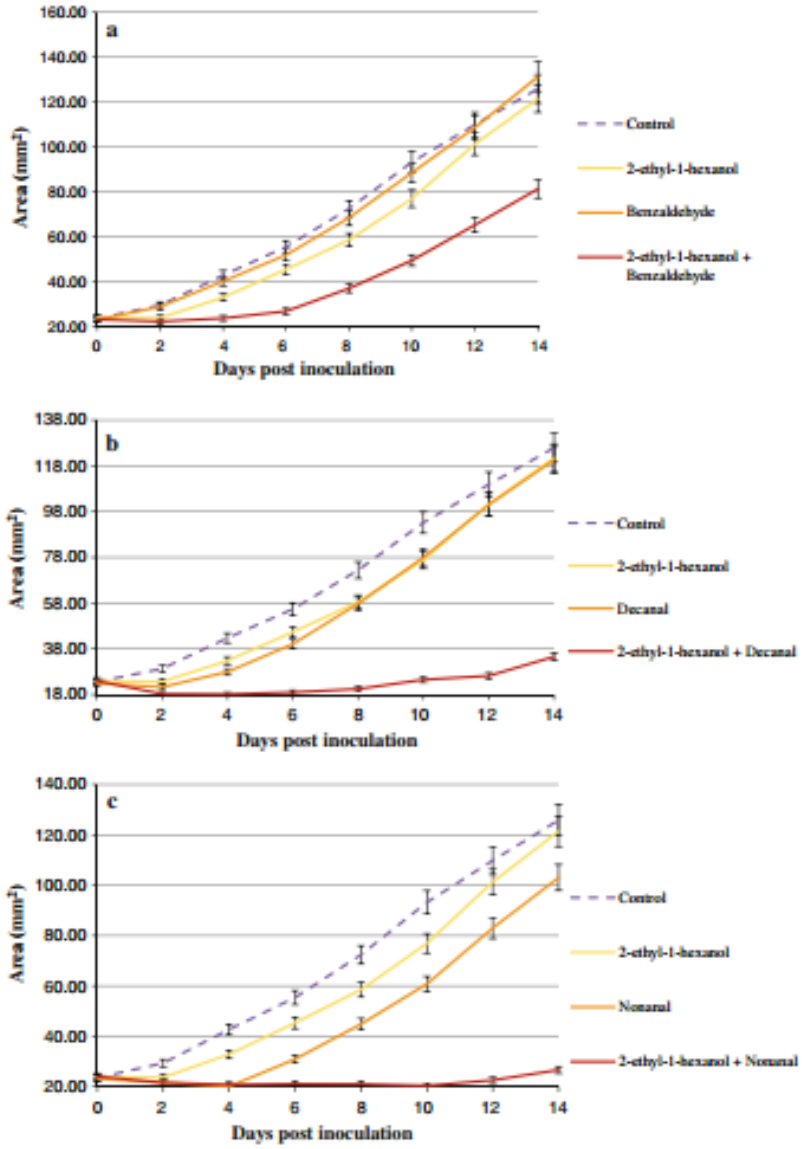


Fig. 3 Growth areas of *P. destructans* mycelial plugs exposed to each individual VOC as well as formulations at 15 °C. Measurements taken every 2 days for 14 days. a 2-ethyl-1-hexanol and benzaldehyde, b 2-ethyl-1-hexanol and decanal, and c 2-ethyl-1-hexanol and nonanal

this disease and reduce the mortality associated with currently infected hibernacula. To this end, the evaluation of previously described bacterially produced antifungal volatiles was conducted to identify potential chemical control agents as well as identify potential environmental reservoirs of anti-*P. destructans* activities and expand the pool of potential biological control agents.

Bacterially derived volatile fungistasis is a well-documented microbial antagonism and may be common in terrestrial ecosystems [7, 10, 11, 14, 16, 25]. Harnessing the potential of these natural antagonisms is already a powerful tool in the development of highly effective biological and chemical control options [7, 11, 17]. The biological origin of many fungistatic VOCs lends itself to obtainable inhibitory applications

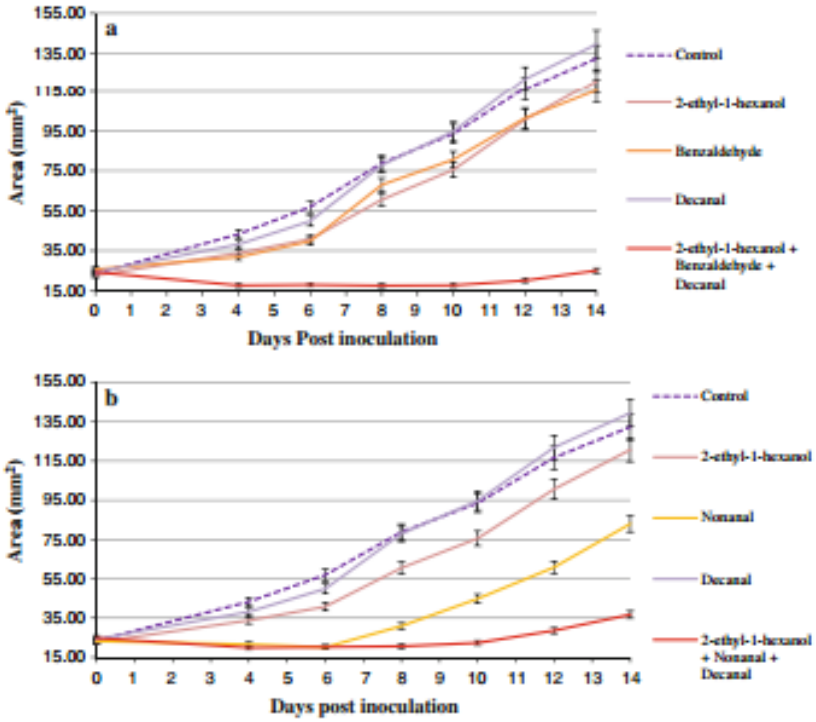


Fig. 4 Growth areas of *P. destructans* mycelial plugs exposed to each individual VOC as well as formulations at 15 °C. Measurements taken every 2 days for 14 days. **a** 2-ethyl-1-hexanol; benzaldehyde; and decanal. **b** 2-ethyl-1-hexanol; nonanal; and decanal

due to the typically low level of production in the natural hosts and the significant antagonistic activity observed at these low levels [7, 10, 14, 16, 21, 24]. The contact-independent activity of antagonistic VOC has several advantages over topical and oral, contact-dependent, treatment options that have been shown to be highly effective at inhibiting the growth of *P. destructans* in previous studies [6]. Contact-independent antagonisms allow for treatment of many individuals with a single application and ensure uniform exposure, avoiding the potential for microbial refugia on the host that may facilitate re-colonization of the host once the inhibitory compound has been removed or degraded. Accordingly, the evaluation of previously described bacterially produced VOCs reduces the processing required to identify viable treatment options, and the contact-independent activity of antagonistic VOCs has several advantages over topical and oral antifungal compounds and should be a focus of studies tasked with identifying novel treatments for newly emerging fungal diseases.

The coevolution of soil microbiota has produced antagonisms ideally suited for the complex ecology of soil. Harnessing these natural antagonisms can be a powerful tool in combating WNS as many of the traits of these antagonisms equate favorably with the ecology of hibernacula and the terrestrial heritage of the Geomycota and *Pseudogymnoascus* spp. warrants their susceptibility. The long-term efficacy of low quantities of VOCs illustrates the potential of these compounds for in situ application in the treatment of WNS. Additionally, the development of synergistic blends bolsters the appeal of soil-based fungistasis as a source of potential control agents as VOC mixtures are likely responsible for the observed fungistatic activity of repressive soils [14, 16, 24]. While several pure VOCs and blends produced significant growth inhibition, compounds and/or quantities unable to significantly inhibit growth caused noteworthy stress to *P. destructans* as determined by the abnormal phenotypes observed under these conditions (Fig. S1). The evaluation of bacterially derived VOCs has expanded the pool of potential biological control agents as well produced several VOC formulations with excellent anti-*Pseudogymnoascus* activity. The availability of volatile formulations for control of *P. destructans*

growth could prove to be a powerful tool for wildlife management agencies if appropriate application methods can be developed.

Current technology for dissemination of VOCs and essential oils for control of odors and pests in indoor environments is common with several companies, including TimeMist$_{tm}$, Aire-Master$_{tm}$, Prolitec$_{tm}$ and Air-Scent$_{tm}$ actively marketing these systems. Manufacturers claims vary significantly depending on product with treatment capacities varying from 6,000 to 50,000 ft^3 for a single unit and maintain 1–10 ppm concentrations in that area based on timed releases with various product lines. Although these claims are promising, appropriate scientific validation is lacking. Fragrance dispenser systems are compatible with a wide range of VOC and essential oils and their efficacy claims warrant further investigation as a potential application method for anti-*Pseudogymnoascus* VOCs in the treatment of WNS in hibernating bats.

The ecology of susceptible bat populations makes treatment of WNS difficult. The highly dispersed, difficult to access, and potentially hazardous nature of bat hibernacula require the development of control options with persistent inhibitory activity at low levels. Our results indicate a strong inhibitory activity for several compounds and have provided valuable leads for identification of potential biological control agents. The increased inhibitory effect observed in low temperature (4 °C) exposures is promising for field application as they represent a promising duration at the low temperatures associated with hibernacula during the time of infection and could have a significant impact on the mortality associated with infected hibernacula. Currently, the prognosis for susceptible North American bat populations is bleak at best. The development of biological and chemical treatment options must be investigated to provide wildlife management agencies with tools for control of *P. destructans* transmission and infectivity.

This project identified biologically derived chemical control agents that can potentially disrupt transmission by inhibiting growth from conidia as well as decrease infection/mortality rates in hibernacula currently infected with *P. destructans*. Additionally, several of the compounds evaluated in this study have the potential for application in captive-recovery programs purposed in the National Response Plan [1]. Cumulatively, this study has highlighted potential control options for further investigation for application in management of WNS as well as identified fungistatic soils as a potentially significant reservoir of biological control agents.

Acknowledgments This work was funded by the Georgia State University Department of Biology, as well as the Georgia State University Environmental Research Program. The authors would like to thank Kevin Keel for providing the fungal cultures used in this study. The authors would also like to thank Ian Sarad, Blake Cherney, and Ben Poodiak for their contributions to this effort.

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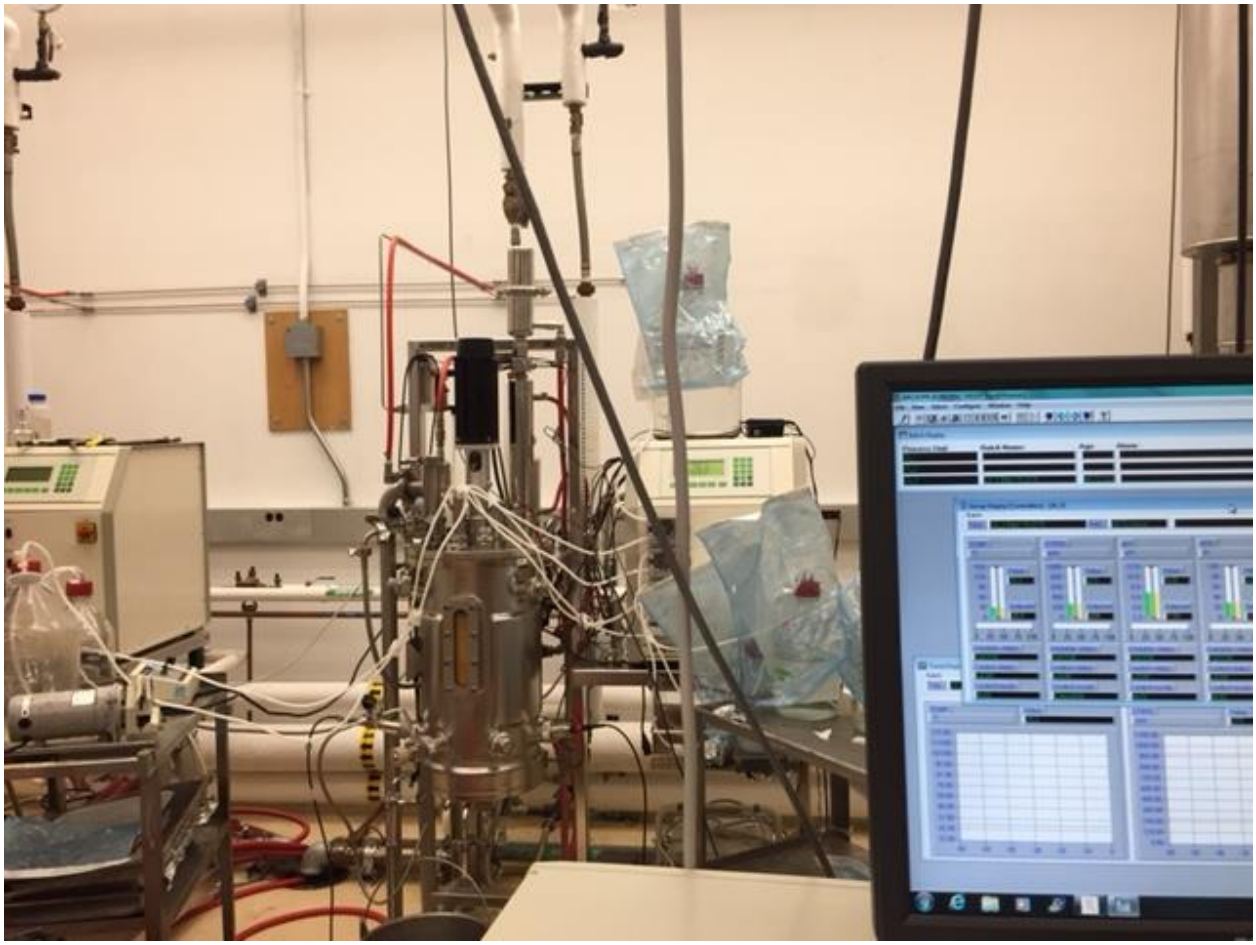
Appendix C. Fermentation Vessels

Sartorius Stedim Biostat C^+ 30L

(Image: Biostat C+ 30L; Kind permission of AEM Fermentation Group GSU)

Biostat C 20L



See page 52

Appendix D. Related equipment

YSI Glucose Analyzer

See page 53

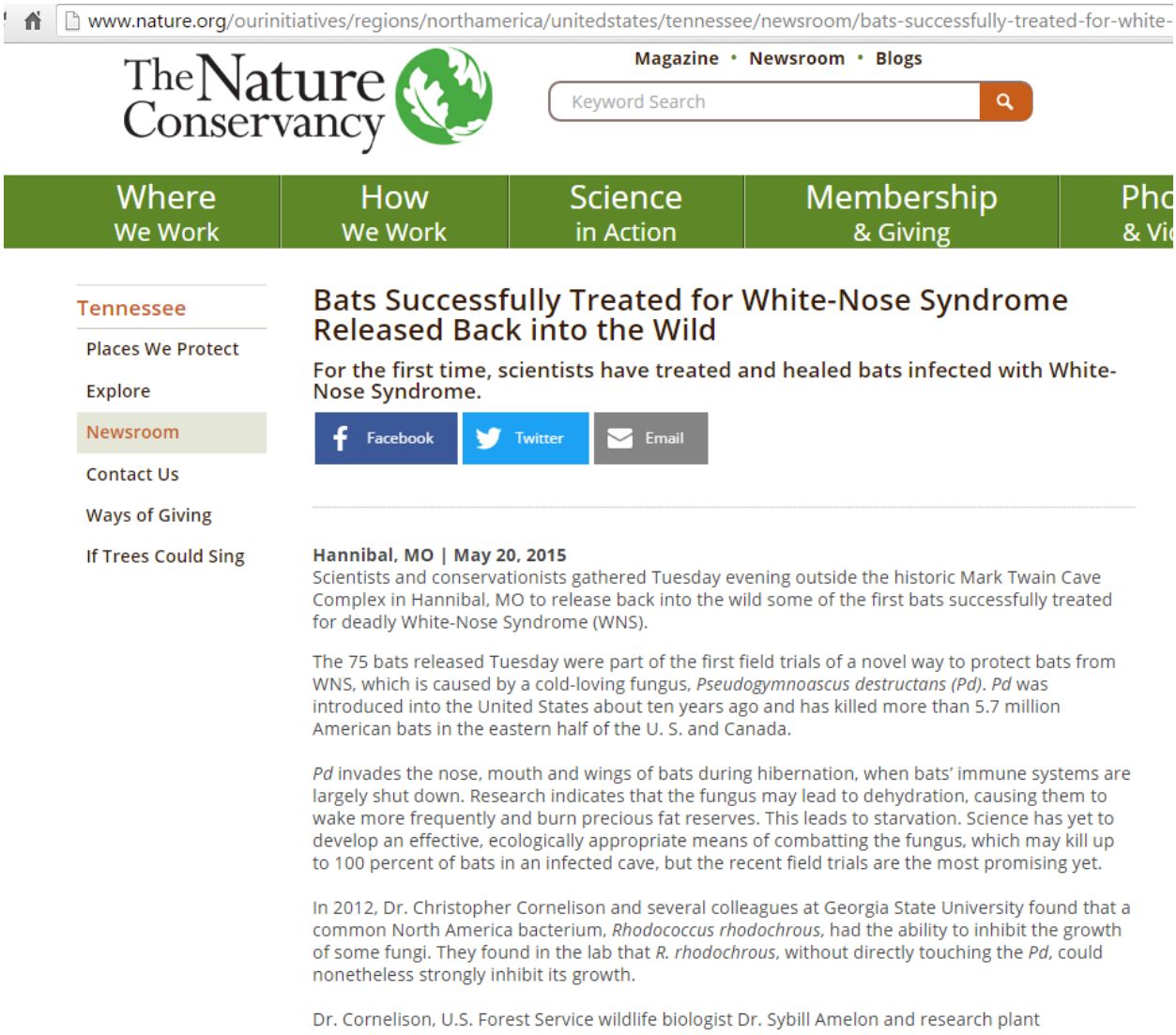
Carr Pilot Powerfuge

See page 54

Harvested Fermentation Paste

See page 54

Appendix E. Nature.org article on catalyst treated bats surviving winter for release back into the wild Missouri, 2015



www.nature.org/ourinitiatives/regions/northamerica/unitedstates/tennessee/newsroom/bats-successfully-treated-for-white-

The Nature Conservancy

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Ways of Giving

If Trees Could Sing

Bats Successfully Treated for White-Nose Syndrome Released Back into the Wild

For the first time, scientists have treated and healed bats infected with White-Nose Syndrome.

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Hannibal, MO | May 20, 2015
Scientists and conservationists gathered Tuesday evening outside the historic Mark Twain Cave Complex in Hannibal, MO to release back into the wild some of the first bats successfully treated for deadly White-Nose Syndrome (WNS).

The 75 bats released Tuesday were part of the first field trials of a novel way to protect bats from WNS, which is caused by a cold-loving fungus, *Pseudogymnoascus destructans (Pd)*. *Pd* was introduced into the United States about ten years ago and has killed more than 5.7 million American bats in the eastern half of the U. S. and Canada.

Pd invades the nose, mouth and wings of bats during hibernation, when bats' immune systems are largely shut down. Research indicates that the fungus may lead to dehydration, causing them to wake more frequently and burn precious fat reserves. This leads to starvation. Science has yet to develop an effective, ecologically appropriate means of combatting the fungus, which may kill up to 100 percent of bats in an infected cave, but the recent field trials are the most promising yet.

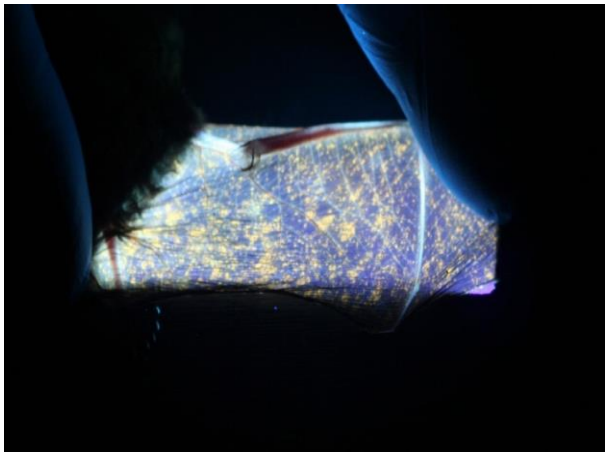
In 2012, Dr. Christopher Cornelison and several colleagues at Georgia State University found that a common North America bacterium, *Rhodococcus rhodochrous*, had the ability to inhibit the growth of some fungi. They found in the lab that *R. rhodochrous*, without directly touching the *Pd*, could nonetheless strongly inhibit its growth.

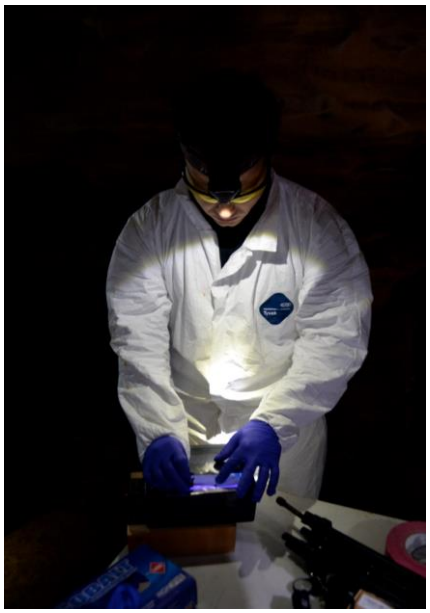
Dr. Cornelison, U.S. Forest Service wildlife biologist Dr. Sybill Amelon and research plant

(Reprinted with the kind permission of C. Cornelison).

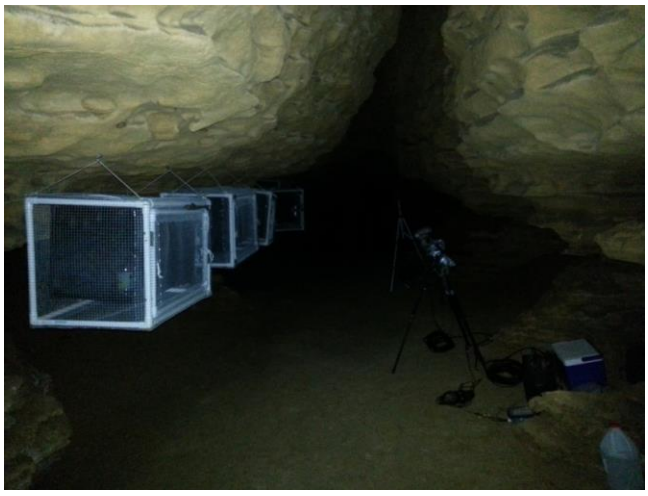
Appendix F. Evaluation of *P. destructans* infected bat wing tissue

(Turner *et al,* 2014)





Appendix G. Bat field trial images



Appendix H. Genetic Analysis of *R rhodochrous* DAP 96253.

(Sequence analysis determined by US Forest Service). Queries based on run sequence data- conducted by J. Neville. Tables prepared by G. Pierce.

Number in [ ] indicates number of gene copies identified.

Where ID of the MO is precisely identified the EC number is provided.

1. Flavin Family Monooxygenase [5]
2. 4-hydroxy-phenylacetate-3-Monooxygenase [3]
3. Ubiquinone biosynthesis Monooxygenase [2]
4. Luciferase-like Monooxygenase [1] (similar to alkane MO)
5. Putative Monooxygenase [11]
6. Nitrilotriacetic Acid Monoxygenase – Component- B (EC 1.14.13) [13]
7. Nitrilotriacetic Acid Monoxygenase – Component- A (EC 1.14.13) [2]
   (this is the reductase)
8. Alkane-1-Monooxygenase (1.14.15.13) [2]
9. Methane Monooxygenase Regulatory Protein B [1]
10. Methane Monooxygenase Component A: β-chain (EC 1.14.13.25) [2]
11. Methane Monooxygenase Component C (EC 1.14.13.25) [1]
12. Alkane Sulfonate Monooxygenase (EC 1.14.14.5) [1]
13. Pyrimidine catabolism Rut A Monooxygenase [1]

14. Peptide Monooxygenase [1]

15. Cyclohexanone Monooxygenase (EC 1.14.13.22) [14]

16. Rifampin Monooxygenase [1]

17. Antibiotic Synthesis Monooxygenase [1]

18. Coenzyme F420 dependent N5,N10 –methyltetrahydromethanopterin reductase [1]

19. FAD-Monooxygenase (PheA/TfdB) [1]

20. Monooxygenase-FAD binding Reductase (EC 1.14.13.20) [1]

Appendix I. Chemicals for Amidase, cyanidase, and urease

Cyanidase

Substrate: Potassium cyanide (0.05mg/mL). 10mL of the substrate would be 0.5mg of KCN in 10mL of ddH_2O

2mL sodium phenate (25g phenol, 78mL 2N NaOH in 800mL ddH_2O

3mL of 0.01% sodium nitroprusside

3mL 0.15% sodium hypochlorite

Amidase

Substrate: Acrylamide (1mg/mL). 10mL of solution would be 10mg of acrylamide in 10mL of ddH_2O

2mL sodium phenate (25g phenol, 78mL 2N NaOH in 800mL ddH_2O

3mL of 0.01% sodium nitroprusside

3mL 0.15% sodium hypochlorite

Urease

Substrate: Urea stock (1mg/mL). 10mL of solution would be 10mg of urea in 10mL of ddH_2O

2mL sodium phenate (25g phenol, 78mL 2N NaOH in 800mL ddH_2O

3mL of 0.01% sodium nitroprusside

3mL 0.15% sodium hypochlorite