INVESTIGATION OF BACILLUS SUBTILIS AS A BIOPESTICIDE
AGAINST BOTRYTIS CINEREA

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TITLE: INVESTIGATION OF BACILLUS SUBTILIS AS A
BIOPESTICIDE AGAINST BOTRYTIS CINEREA

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ABSTRACT

INVESTIGATION OF BACILLUS SUBTILIS AS A BIOPESTICIDE AGAINST

BOTRYTIS CINEREA

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The objective of this thesis was to investigate BiOWiSHTM-Aqua, a commercial dry solid formulation containing a consortium of bacteria and yeast, as a biopesticide for treatment of *Botrytis cinerea*, a gray mold that affects strawberries. BiOWiSHTM-Aqua was compared with another commercial product specifically used as a fungicide and bacteriocide, Serenade[®] Garden Disease Control Spray (concentrated *Bacillus subtilis* strain QST 713). Both laboratory tests as well as *in vivo* lab tests were conducted. BiOWiSHTM-Aqua results varied widely from plate to plate, regardless of experimental conditions. In some of these plates, inhibition zones were observed around colonies from BiOWiSHTM-Aqua, indicating efficacy. The organism responsible for the inhibition zones of *B. cinerea* growth was isolated from BiOWiSHTM-Aqua, and 16s rRNA analysis identified this culture as a strain of *B. subtilis*. This strain was designated as *B. subtilis* ssp. KLB. The *B. subtilis* KLB concentration required to completely inhibit *B. cinerea* was 9.1×10^4 CFU/mL when *B. subtilis* KLB was inoculated 48 hours before *B. cinerea*, 1.3×10^5 CFU/mL at 24 hours, and 3.2×10^6 CFU/mL when both were inoculated at the same time. Various preliminary experiments using *B. subtilis* KLB were also conducted to investigate its economic feasibility, to characterize the organism, and to test its post-harvest *in vivo* viability. *B. subtilis* KLB cell concentration was 1.6×10^9 CFU/mL in a bioreactor with LB at the end of the log growth phase. *B. subtilis* KLB achieved cell concentrations as high as 5×10^9 CFU/mL in shake flasks with food-grade tapioca as a carbon source. Inoculation of *B. subtilis* KLB on post-harvest strawberries did not have an effect on *Botrytis* infection rates compared to the negative control. These various experiments were the first step in research to potentially produce *B. subtilis* KLB on a commercial scale.

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CHAPTER 1: INTRODUCTION

Methyl bromide (MeBr) is a broad spectrum pesticide used in U.S. agriculture to control pest insects, weeds, and pathogens. In U.S. agriculture, MeBr is primarily used as a soil fumigant, the process of filling acreage with gaseous pesticides (EPA, 2011). However, MeBr causes the destruction of the ozone layer and the potential death of human beings. MeBr causes central nervous system and respiratory system failure in workers directly exposed to the gas (Thomas, 1996). MeBr also depletes the ozone layer by rising into the atmosphere and forming BrO, a powerful ozone-depleting substance (ODS). The result of depleting the ozone layer is UV radiation, which can genetically alter life forms at the cellular level, cause fatal skin cancer, and cause blindness in humans (Morrissey, 2007). In fact, the Montreal Protocol, which is designed to phase out substances that deplete the ozone layer, called for a 100 percent phase-out of MeBr from 2005 onward (EPA, 2011). Despite the consequences of using MeBr, its use as a fumigant is necessary to control agricultural pests for various crops. For example, in the Locascio, 1997 study, no MeBr alternatives were able to provide the consistent broad spectrum control provided by MeBr for polyethylene-mulched tomato growth in Florida. For many crops dependent on MeBr fumigation, there simply is no clear viable alternative.

A major California crop dependent on MeBr is strawberries. In fact, in 2004, California strawberry producers applied 3.2 million pounds of MeBr primarily for soil fumigation, accounting for 45% of the 7.1 million pounds of total MeBr used in California

(Fennimore, 2008). Organic fields, which do not use chemical pesticides, produce only 25 to 60 percent of conventional yields (USDA, 1999). As a result, parties to the Montreal Protocol and the rulemakings by EPA made an exception for usage of MeBr in California strawberry production (EPA, 2011).

Fumigation plays a large role in Integrated Pest Management (IPM), an effective and ecological approach to pest management that relies on a combination of common-sense practices including cultural, physical, biological, and chemical practices (Brennan, 2011). One of the longer-term goals of IPM programs is to reduce or even eliminate chemical pesticide usage by encouraging biologically and culturally based alternatives (Cooley, 1996). One of the ways of achieving this is by increasing the role of biological pesticides, or “biopesticides”. While strawberries are attacked by a large number of pests, IPM targets key pests within an agroecosystem (Cooley, 1996). The gray mold *Botrytis cinerea* is the most common and most serious disease of California strawberries (USDA, 1999). In the strawberry industry, IPM could greatly reduce or even potentially eliminate the use of conventional pesticides while minimizing the economic impact of using more natural means.

The purpose of my research is to investigate a biological means for controlling pest growth on strawberries. Specifically, a biopesticide, in this case an organism, will be tested against Botrytis gray mold. Firstly, biopesticide growth conditions for optimal inhibition of *Botrytis* will be determined *in vitro*. Secondly, economic feasibility of the

biopesticide will be explored by using less expensive growth media and by scaling up from flask growth to bioreactor growth. Lastly, *in vivo* experiments involving direct contact of the biopesticide on strawberry fruit bodies will test possible post-harvest inhibition of *Botrytis*.

CHAPTER 2: BACKGROUND

California produces more than 80 percent of all fresh market and processed strawberries grown in the United States and 20 percent grown in the world (USDA, 1999). Two-thirds of this acreage is located in the Santa Maria region alone, which encompasses the coastal regions from Santa Cruz to northern Santa Barbara (USDA, 1999). This amounts to over 1.5 billion pounds of strawberries annually, worth \$750 million (USDA, 1999). In fact, California is the only state that can grow strawberries at a high enough productivity per acre at 49,000 lbs/acre/year, over four times higher than the United States average (11,000 lbs/acre) (USDA, 1999).

To achieve this productivity, chloropicrin, a highly toxic chemical that affects the respiratory system, is used in conjunction with MeBr due to cost-effectiveness and efficacy of the combination (Fennimore, 2008). Several weeks before strawberry planting, a plastic tarp is placed over the target acreage in preparation for fumigation. The tarp is usually removed five days after fumigation unless bed fumigation is used, in which the tarp is then removed after the plants are removed. A mulch, typically made of clear polyethylene and used to separate strawberries and plant foliage from the ground, is placed right after planting (USDA, 1999).

A major reason for the necessity of MeBr is to control the growth of *B. cinerea*, which causes a major strawberry disease called Botrytis fruit rot, or commonly known as gray

mold. Gray mold grows not only on the fruit body, but also on petioles, stems, and flowers (Agrios, 1997). In California, *Botrytis* fruit rot is responsible for fruit losses of 30 to 40 percent where chemical pest management is not practiced (USDA, 1999). The disease cycle starts when *B. cinerea* spores (conidia) overwinter (the process of waiting out the winter to germinate in more favorable conditions) on decayed foliage and fruit as shown in Figure 2-1. In the next spring, during strawberry growing season, increasing temperatures and moisture promote fungal growth and production of conidia. The conidia are then spread through wind and rain onto developing strawberry plants (Burr, 1985).

Figure 2-1. *Botrytis* disease cycle on strawberry infection (Burr, 1985).

As a potential replacement for MeBr, the EPA approved MeI as a strawberry pesticide in the State of California in December 2010. Although MeI is not an ozone depleting substance, the United States Department of Agriculture (USDA) contends that there are concerns about the environmental fate of MeI after its fumigation. In addition, MeI is carcinogenic and exhibits moderate to high acute toxicity for inhalation and ingestion (Guo, 2009).

The dangers of using chemical pesticides have prompted the use of less toxic alternatives. One method to control pests such as *B. cinerea* is to limit or to replace the use of conventional pesticides with biopesticides in an integrated pest management program specific to strawberry growth. According to the EPA, biopesticides are derived from natural materials and fall into three major classes: microbial pesticides, Plant-Incorporated-Protectants (PIPs), and biochemical pesticides (EPA, 2000). In fact, even in 1990, biopesticide sales were estimated to be increasing 10 to 25 percent each year while the agrochemical market was static or even decreasing (Rodgers, 1993).

The focus of my thesis research is the use of microbial pesticides, which consist of a microorganism such as a bacterium as the active ingredient. Some of the advantages of using biopesticides compared to conventional pesticides include lower toxicity, specificity of pest targets, and effectiveness in small quantities.

Bacillus sp. are common antibiotic-producing microbial pesticides. In fact, an average of four to five percent of a *B. subtilis* genome is devoted to antibiotic production, including its potential to produce more than two dozen antibiotics (Stein, 2005). The most prominent biopesticide is *B. thuringiensis* (Bt), which accounts for 90 percent of all marketed biopesticides. Bt produces spores and endotoxins that target specific pests, a major one being lepidopteran larvae, or caterpillars (Nicholson, 2002). Besides Bt, many different species and strains of *Bacillus* successfully inhibit the growth of specific fungi such as *B. cinerea*.

One example is commercial product Serenade[®], consisting of *Bacillus subtilis* QST 713 and used on a variety of food crops to control various types of fungi and bacteria that cause diseases such as powdery mildew, sour rot, early blight, and late blight (Burr, 1985). The product is a powder mixed with water and sprayed on foliage. In addition, it poses no harmful health effects to humans and no adverse effects to non-target organisms (EPA Fact Sheet, 2000).

Another example is the use of EcoGuard[®] (*Bacillus licheniformis*), rotated with chlorothalonil, a synthetic fungicide, to control Dollar Spot (*Sclerotinia homoeocarpa*), a serious disease of turfgrass worldwide. This effective IPM practice, to rotate a biological component with a chemical component, outperformed a standard conventional fungicide program on Bermuda grass. In fact, EcoGuard[®] exhibited 95 percent control alone against Dollar Spot (Brennan, 2011).

In a case of *Bacillus* controlling the growth of a type of *Botrytis*, *Bacillus amyloliquefaciens* B190 has successfully been used to control mold on lily pads, or *Botrytis elliptica*. In fact, *B. amyloliquefaciens* B190 mixed with 0.025% calcium hydroxide and 0.05% sodium carbonate was able to completely inhibit *B. elliptica* germination and decreased lily pad disease severity (Chiou, 2003).

My thesis research consists of the investigation of BiOWiSH™-Aqua, which contains *B. subtilis*, as a biopesticide to inhibit *B. cinerea* growth (Figure 3-4). BiOWiSH™-Aqua is primarily used as a formulation used for wastewater treatment plants to biologically remove nutrients from wastewater. Some of the benefits include odor removal, reduction of sludge, and removal of ammonia and nitrates (BiOWiSH™-Technologies, 2012). However, BiOWiSH™-Aqua is also suitable for my research since it contains *B. subtilis*. A unique strain of *B. subtilis* was isolated from BiOWiSH™-Aqua and identified by NCBI BLAST as a very close relative to both *B. subtilis* and *B. amyloliquefaciens*, (Zhang, 2000). This isolate (named *B. subtilis* strain KLB) was used as the basis of my thesis to inhibit *Botrytis* growth.

CHAPTER 3: MATERIALS AND METHODS

3.1 Source and Culturing of Pathogen and Biopesticide

The pathogen was the gray mold *Botrytis* sp. (M. A. Yoshimura, personal communication, January 12, 2011) cultured from strawberries that came from Y Hayashi & Sons Farms based in Arroyo Grande, CA. The strawberries were placed in a beaker, covered with Parafilm[®] to prevent drying, and left at room temperature for five days in the lab at Building 13 Room 112 (13-112) in Cal Poly San Luis Obispo. After the five days the strawberry body, or the fruit tissue, contained the gray mold *Botrytis*, which had the appearance of a gray-brown and fluffy mass as shown in Figure 3-1. On a microscopic level under 1000x magnification, conidia were seen as shown in Figure 3-2. *Botrytis* conidia were collected using a sterile inoculum loop and inoculated into a test tube containing 10 mL of sterile deionized (DI) water. The test tube was vortexed and then 10 μ L of suspended conidia was inoculated in the middle of a Potato Dextrose Agar (PDA) plate as shown in Figure 3-3. The plate was placed in an incubator at 30°C for 48 hours, and then used as the source for *Botrytis* inoculum.

Figure 3-1. *Botrytis* growth on strawberry after five days.

Figure 3-2. A single *Botrytis* conidium at 1000x magnification.

Figure 3-3. *Botrytis* cultured on a PDA plate.

For *in vitro* testing of inhibition against *Botrytis*, a commercial product named BiOWiSHTM-Aqua, shown in Figure 3-4, was used as a biopesticide. BiOWiSHTM-Aqua is a dry solid formulation that contains a consortium of bacteria and yeast and is used for multiple applications including wastewater treatment, plant growth enhancement, pathogen inhibition, etc. BiOWiSHTM-Aqua consists of at least five known organisms, including *Pediococcus pentosaceus* Mees, *Bacillus subtilis*, *Pediococcus acidilactici*, *Dekkera anomala* and *Pichia farinosa*. Of particular interest is *Bacillus* spp., many of which produce antifungal peptide antibiotics (Rao, 2007).

In the initial testing, two controls were used. The first control was *B. subtilis* strain QST 713. The source was the commercial pesticide spray called Serenade[®] Garden Disease

Control Spray, consisting of concentrated spores in suspension, and used as a fungicide and bacteriocide. However, the *B. subtilis* QST 713 formulation is concentrated so much that cell concentration far exceeded any cell concentration in a normal liquid fermentation. The negative control consisted of sterile DI water.

Figure 3-4. BiOWiSH™-Aqua packaging.

3.2 In Vitro Experiments on PDA Plates

To determine which agar to use for plating, BiOWiSH™-Aqua and *Botrytis* were screened on different types of agar including Luria Broth (LB) agar, Plate Count Agar (PCA), and Potato Dextrose Agar (PDA). Although BiOWiSH™-Aqua grew on all types of agar, *Botrytis* was only able to grow on PDA.

3.2.1 BiOWiSHTM-Aqua Activation

Activation consisted of inoculating 10 g of BiOWiSHTM-Aqua into 1 L of tap water, equivalent to 10,000 parts per million (ppm). Prior to activation, a 1 L beaker was wiped with 70% ethanol and dried before addition of tap water. After inoculation, the top of the beaker was covered with Parafilm[®] to prevent contamination during incubation.

Incubation took place in a New Brunswick Series 25 Incubator Shaker at 60 rpm and 30°C for the three activation times in hours (T_{app}). After time T_{app} had elapsed, three different concentrations in ppm of BiOWiSHTM-Aqua (C_{bw}) were made in sterilized test tubes: 10,000 ppm (original concentration), 1,000 ppm, and 100 ppm. For the original concentration, the beaker was mixed well with a clean glass rod, and then the solids were allowed to settle before pipetting 10 mL of the BiOWiSHTM-Aqua suspension into the first test tube. The second and third test tubes were filled with 1,000 ppm and 100 ppm BiOWiSHTM-Aqua, respectively, by serially diluting 1 mL of BiOWiSHTM-Aqua from the previous test tube with 9 mL of tap water.

3.2.2 Microscope Enumeration of *Botrytis* conidia

A Olympus BX50 Microscope was used for both identifying cells and determining *Botrytis* conidia concentration (Figure 3). In order to determine conidia concentration, the area of the field of vision, the area of a cover slip, and the amount of inoculum were calculated using the steps below:

1. Adjusted microscope to 100x objective lens. The ocular lens was 10x. The total zoom of 1000x is the multiplication of the two lenses.

2. Calculated field of vision area by measuring radius with a calibration slide.
3. Calculated equivalent number of fields of vision in a cover slip area of 25 mm x 25 mm by using the formula: Area of cover slip (mm²) / area of field of vision (mm²) = Total # fields of vision / cover slip.

The following is the calculation of fields of vision / mL:

Radius = 0.12 mm

Field of vision area = $\pi r^2 = \pi (0.12)^2 = 0.0452 \text{ mm}^2$

Number of fields of vision / cover slip =

$(25 \text{ mm}^2 * 25 \text{ mm}^2) / \text{cover slip} * \text{field of vision} / 0.0452 \text{ mm}^2 =$

1.383×10^4 fields of vision / cover slip

Each cover slip completely contains 10 μL *Botrytis* inoculum, so:

1 cover slip =

$10 \mu\text{L} \ 1.383 \times 10^4$ fields of vision / cover slip * 1 cover slip / 10 μL

$1000 \mu\text{L} / 1 \text{ mL} =$

1.383×10^6 fields of vision / mL

The factor of 1.383×10^6 fields of vision / mL was used to calculate the concentration in the *Botrytis* inoculum. *Botrytis* conidia were suspended in DI water at a concentration of 1,000 conidia/mL. This suspension was prepared fresh on the days of inoculation.

Inoculating 10 μL provided an average of ten conidia on each PDA plate, more than

enough for strong radial growth of *Botrytis* mycelia. The following procedure was used to prepare the conidia suspension:

1. Flamed metal tweezers were used to take a pinch of *Botrytis* from the inoculum plate and suspended in a test tube with 10 mL DI water.
2. Pipetted 10 μ L of *Botrytis* suspension onto microscope slide. Placed a cover slip over the *Botrytis* solution.
3. Placed immersion oil on top of the cover slip, and used the coarse and fine adjustments to focus the field.
4. Twenty random fields were selected and the total number of conidia (Figure 3-2) were tallied. In order to avoid counting a field twice and to get a sample representation of the whole cover slip, the first field started at the top-left corner and the last field ended at the bottom-right corner of the cover slip.
5. The following calculations were performed to determine the amount of *Botrytis* solution and DI water to make a 10 mL *Botrytis* inoculum of 1,000 conidia/mL.
 - a. $\text{Counted number of } Botrytis \text{ conidia} / 20 \text{ fields of vision} * \text{fields of vision} / \text{one cover slip} = \text{Number of } Botrytis \text{ conidia} / \text{one cover slip} = \text{Number of } Botrytis \text{ conidia} / 10 \mu\text{L}.$

A sample calculation to dilute *Botrytis* solution to $C_{bot} = 1,000$ conidia/mL if 13 conidia are counted in 20 fields of vision:

$$13 \text{ conidia} / 20 \text{ fields} \times 1.383 \times 10^6 \text{ fields} / \text{mL} = 8.99 \times 10^5 \text{ conidia} / \text{mL}$$

- b. Dilute 11.1 μL *Botrytis* inoculum into 10 mL – 0.0111 mL = 9.989 mL DI water to obtain $C_{\text{bot}} = 1,000$ conidia/mL

6. The calculated amounts were pipetted into a sterile test tube and ready for use.

3.3 *In Vitro* Experiments of Biopesticide vs. Pathogen

A method was developed for screening BiOWiSHTM-Aqua (and later *Bacillus subtilis* KLB) against strawberry mold *Botrytis cinerea*. This *in vitro* method involved plating both BiOWiSHTM-Aqua and *Botrytis* on a PDA plate. To understand the interaction between the two, the experimental design was based on three important concerns. The first concern was BiOWiSHTM-Aqua's mode of action against *Botrytis*, that is, whether its organisms attack or inhibit *Botrytis* mycelial growth. The second was to determine which of the BiOWiSHTM-Aqua organism(s) would be effective against *Botrytis*. The last was to find the concentration (C_{bw}) of BiOWiSHTM-Aqua needed to be effective against 10 μL of $C_{\text{bot}} = 1,000$ CFU/mL of *Botrytis*. In order to address these concerns, three different variables in the experiment were defined, listed in section 3.3.1.

3.3.1 Experiment Variables

The following variables were tested to determine which parameter(s) had a significant effect on the inhibition of *Botrytis*:

3.3.1.1 Effect of *Botrytis* Incubation Period

T_{app} denotes time of BiOWiSHTM-Aqua incubation on agar plates before *Botrytis* inoculation – 0, 24, and 48 hours. For $T_{app} = 0$ hours, *Botrytis* is point-inoculated right after BiOWiSHTM-Aqua was streaked onto the plate. The reasoning behind this variable is to determine whether BiOWiSHTM-Aqua organisms can control or prevent *Botrytis* growth by inhibition. In this scenario, *Botrytis* would establish colonies around or overlapping the *Botrytis* in order to inhibit its growth.

3.3.1.2 Determination of Optimal BiOWiSHTM-Aqua Activation Time

T_{act} denotes time of BiOWiSHTM-Aqua activation – 48, 72, and 96 hours. Since BiOWiSHTM-Aqua contains organisms from the original inoculum (with the five types of organisms) as well as suspected unknown organisms from the raw material, the effectiveness of BiOWiSHTM-Aqua for *Botrytis* inhibition is dependent on which organisms are present. Different organisms may be predominant at different times of the activation, which may or may not affect *Botrytis* inhibition.

3.3.1.3 Determination of Optimal BiOWiSHTM-Aqua Concentration

C_{bw} denotes concentration of BiOWiSHTM-Aqua in ppm – 100, 1,000, and 10,000 ppm. Concentration is a critical variable to determine the amount of BiOWiSHTM-Aqua to use in *Botrytis* inhibition. A concentration too low may not inhibit *Botrytis* growth, but a concentration too high would be wasteful and more costly.

3.3.2 Fixed Experiment Parameters

To limit the number of variables, fixed variables were defined prior to the experiments. The following constants involved the concentration of *Botrytis* conidia onto each plate, temperatures for BiOWiSHTM-Aqua activation and incubation, shaking speed, and the agar plate medium used. In addition, tap water was used for all activation of BiOWiSHTM-Aqua.

C_{bot} = concentration of *Botrytis*, 1000 conidia/mL. Since 10 μ L was point-inoculated, this provided an average of 10 *Botrytis* conidia, enough for *Botrytis* to grow radially and to measure area of Petri dish occupied.

Temperature of activation = 30°C. This temperature was referenced from Rao, 2007, which was used to build up *B. amyloliquefaciens* B128 inoculum. At 30°C and a T_{act} of at least 48 hours, BiOWiSHTM-Aqua contained sufficient growth by observation of turbidity.

Temperature of incubation = 30°C. Plates were incubated at 30°C to keep the temperature consistent from activation to incubation. At this temperature, colonies grew to sufficient size for counting within 48 hours after plating.

RPM = activation of BiOWiSH™-Aqua, 60 rpm. Agitation at 60 rpm stirred

BiOWiSH™-Aqua material sufficiently to prevent settling/precipitation and to provide aeration without the liquid sloshing.

Agar plate medium = potato dextrose agar. Both BiOWiSH™-Aqua as well as *Botrytis* grew well on this medium as described in section 3.2.

3.3.3 Initial Inhibition Experiments Using BiOWiSH™-Aqua

A total of 27 combinations, every combination of the three variables, were plated in triplicate. The following conditions were tested, totaling 27 combinations:

$T_{app} = 0, 24, \text{ and } 48 \text{ hours}$

$T_{act} = 48, 72, \text{ and } 96 \text{ hours}$

$C_{bw} = 100, 1,000, \text{ and } 10,000 \text{ ppm}$

Plates were inoculated with 100 L of BiOWiSH™-Aqua at its specified concentrations and spread by using sterilized glass hockey sticks. $T_{app} = 0$ hour plates were inoculated with *Botrytis* immediately. $T_{app} = 24$ hours and $T_{app} = 48$ hour plates were inoculated with *Botrytis* 24 and 48 hours after BiOWiSH™-Aqua was spread plated, respectively.

All plates were incubated at 30°C for 48 hours.

Since not all *Botrytis* growth was radial, one of the following methods was used to determine the percentage of the plate overtaken by *Botrytis*:

- 1) Measured diameter of *Botrytis* to calculate area (Figure 3-5), then divided by total agar plate area to determine percentage:

$$\text{Percentage } Botrytis \text{ growth} = (\pi r_{bot}^2 / \pi r_{plate}^2) * 100$$

2) If *Botrytis* growth did not resemble a circle, growth was visually inspected and estimated.

Measured diameters were converted into a percentage of the total surface area of a Petri dish (diameter = 8.65 cm). Since agar was filled halfway to the top of the Petri dish, the area of was calculated based on the average of the bottom and top inner diameters, 8.5 cm and 8.8 cm, respectively.

Figure 3-5. Sample plate with *Botrytis* growth circled in red.

Figure 3-6. Process of plate inoculation.

The experiment schedule was as follows:

Day 1 – Activated 10,000 ppm BiOWiSHTM-Aqua for $T_{act} = 96$ hours plates (step 1 in Figure 3-6).

Day 2 – Activated 10,000 ppm BiOWiSHTM-Aqua for $T_{act} = 72$ hours plates (step 1 in Figure 3-6).

Day 3 – Activated 10,000 ppm BiOWiSHTM-Aqua for $T_{act} = 48$ hours plates (step 1 in Figure 3-6).

Day 5 – Prepared 81 spread plates with BiOWiSHTM-Aqua (27 combinations in triplicate) as well as three concentrations of *B. subtilis* strain QST713 as control in triplicate (steps 2-3 in Figure 3-6). Also, point inoculated $T_{app} = 0$ hours plates with *Botrytis* (steps 4-6 Figure 3-6).

Day 6 – Point inoculated $T_{app} = 24$ hours plates with *Botrytis* (steps 4-6 in Figure 3-6).

Day 7 – Point inoculated $T_{app} = 48$ hours plates with *Botrytis*. Measured diameter of *Botrytis* growth of $T_{app} = 0$ hours plates (steps 4-6 in Figure 3-6).

Day 8 – Measured diameter of *Botrytis* growth of $T_{app} = 24$ hours plates.

Day 9 – Measured diameter of *Botrytis* growth of $T_{app} = 48$ hours plates.

Botrytis application (T_{app}) and BiOWiSHTM-Aqua activation (T_{act}) times were adjusted as needed for every subsequent experiment based on results and observations of the previous experiment. For example, if most or all spread plates at application time of 0 hours contained a high percentage of *Botrytis* growth, the next experiment focused on application times of 24 and 48 hours. If activation time of 72 hours showed greater

inhibition against *Botrytis* compared to the other times, the three activation times tested were lessened to 12-hour intervals, with the middle at 72 hours.

3.3.4 *B. subtilis* KLB Isolation

B. subtilis KLB was isolated from a BiOWiSHTM-Aqua plate. To confirm the identity of the isolate taxonomically, its 16S rRNA sequence was determined (courtesy of Alice Hamrick and Dr. Christopher Kitts) and analyzed using NCBI BLAST searching tool (details in Section 4.1.2.1).

Initially, KLB inoculum was grown in a test tube containing Luria Broth (LB), a nutrient-rich medium containing NaCl, tryptone, and yeast extract for fast cell growth (Figure 3-7). A colony was isolated using a flamed inoculum loop, placed into the test tube, and incubated at 30°C and 120 rpm for 24 hours. However, KLB, an obligate aerobe, congregated at the liquid surface to form a biofilm (Figure 3-7).

Figure 3-7. *B. subtilis* KLB biofilm layer in a test tube with LB broth.

KLB inoculum was subsequently grown in a 250 mL Erlenmeyer flask containing 50 mL broth at the same conditions as for growth in a test tube. The broth in the Erlenmeyer flask was turbid as opposed to transparent as shown in Figure 9. The shaking speed was increased from 60 rpm, the speed used to activate BiOWiSHTM-Aqua, to 120 rpm because lower speeds resulted in flocculation and biofilm growth. Fresh inoculum approximately 24 hours old was used because the cells in the inoculum were in log phase. A growth curve was constructed correlating optical density at 600 nm (OD₆₀₀) and cell count. For OD₆₀₀, cuvettes with KLB and its dilutions of 1:2, 1:4, 1:8, 1:16, 1:32, and 1:64 were placed into a Hitachi U-3010 UV-Visible Spectrophotometer. In order to gather cell count data, a 10⁻⁶ dilution of KLB (diluted 1:2, OD₆₀₀ = 0.948) was made and 100 µL of the 10⁻⁶ dilution was spread on each agar plate in triplicate. The plates were

incubated at 30°C for 48 hours and colonies were counted. To determine KLB concentration, the following equation was used:

$$\text{Concentration of } B. \textit{subtilis} \text{ KLB (C}_{\text{KLB}}) = \text{colonies} / (\text{dilution} * 0.1 \text{ mL})$$

Using this formula, cell densities were determined by finding OD₆₀₀ using the spectrophotometer.

A quick visual screening study was conducted to confirm that KLB specifically inhibited *Botrytis* growth and to determine the effective concentrations. Two initial plates were sectioned into thirds. KLB was spread plated at the specified concentrations. The two plates were point inoculated (applied) in the center with *Botrytis* at application times of 0 and 24 hours (Figure 4-14 and Figure 4-15, respectively).

3.3.5 Inhibition Experiments Using *B. subtilis* KLB

In subsequent *in vitro* inhibition experiments, BiOWiSH™-Aqua was replaced by *B. subtilis* KLB. The inoculum was cultured as described in Section 3.3.4. Experiments were conducted in the same manner as the previous experiments with BiOWiSH™-Aqua. However, there were a few key differences in the experimental setups between using BiOWiSH™-Aqua and KLB. The first difference was that activation time (T_{act}) was not used because KLB inoculum was incubated for 24 hours in every experiment. The second difference was instead of using C_{bw} (ppm), C_{KLB} (CFU/mL) was used in order to determine CFU/mL vs. percent growth of *Botrytis* of the total Petri dish surface area.

The last difference was that in the BiOWiSHTM-Aqua inhibition experiments, the concentrations were the same for every experiment at 100, 1,000, and 10,000 ppm. In the KLB inhibition experiments, instead of inoculating a powder, KLB was grown as a liquid inoculum and therefore, initial concentrations varied each experiment or trial. To obtain the desired KLB concentrations for the experiments, KLB inoculum was diluted n times, where n was the number of ten-fold dilutions made, and expressed as 10^{-n} . For example, if $n = 5$, then the dilution was expressed as 10^{-5} of the undiluted concentration. For these experiments, n ranged from 0 (the concentration of the inoculum) to 7 (diluted ten-fold seven times).

3.4 Initial Carbon Source Screening Experiments

The purpose of the carbon source screening experiments was to determine whether *B. subtilis* KLB was able to grow on food-grade carbon sources such as table sugar since LB broth is not viable economically as a growth medium on a commercial scale. In order to minimize growth medium costs while keeping cell counts high, two food-grade carbon sources bought in the supermarket, liquid molasses and cooked tapioca, were tested as variables. The liquid molasses, Grandma's[®] Unsulphured Liquid Molasses, was weighed and diluted in DI water for ease of pipetting. Precooked tapioca balls, Kraft Minute[®] Tapioca, were ground up into a fine starch by a Krups coffee blender. Amounts of trace minerals, peptone (8.0 g/L), and ammonium sulfate (1.8 g/L) were kept constant at levels based on a spore production optimization study of *B. amyloliquefaciens* B128, an

organism that is very similar to *B. subtilis* (Rao, 2007). As in the Rao, 2007 study, each carbon source was tested at three different concentration levels in triplicate to determine the optimal concentration for viable cell density (CFU/mL). In addition to the trace minerals, peptone, ammonium sulfate, and carbon source (liquid molasses or precooked tapioca balls), 0.25 g/L (2% of 12.5 g/L) of LB powder was added to include trace amounts of tryptone and yeast extract, ingredients vital for successful KLB inoculum growth (Section 3.3.4).

Experiment 1 consisted of the initial experiments using 5, 15, and 25 g/L of carbon source, with the low level (5 g/L) lower than the optimum concentration and the high level (25 g/L) higher than the optimum concentration based on the Rao, 2007 study. Experiment 1 was conducted twice (trials 1 and 2) with identical conditions, but at different times, in order to measure variation of cell counts. The experiments were done in triplicate 250 mL shake flasks with 50 mL media in each flask. Each flask was inoculated with 0.5 g of inoculum from liquid media (one percent by volume). The shake flasks were placed in a shaker at 30°C and 120 rpm for 48 hours. After growth, cells were diluted in test tubes containing sterilized DI water. The dilutions were in increments of 1:10 to yield approximately 30-300 CFU/plate. Each triplicate flask, three flasks of each 5, 15, and 25 g/L of carbon source, was plated three times, for a total of 27 plates. The diluted cells were spread plated on PDA and incubated at 30°C. Cells were counted after 48 hours of growth.

Experiment 2 consisted of three changes: 1) tapioca was the only carbon source, 2) the concentrations of tapioca tested were 5, 15, and 20 g/L, and 3) four trials instead of two were run in order to determine repeatability of the experiment.

3.5 Growth Curves of *B. subtilis* KLB and BiOWiSHTM-Aqua

A growth curve of *B. subtilis* KLB and comparative OD₆₀₀ readings of BiOWiSHTM-Aqua were determined to evaluate how the growth of a single organism, KLB, compared with the growth of a product containing multiple organisms, BiOWiSHTM-Aqua. Since BiOWiSHTM-Aqua contains multiple organisms, inhibition or proliferation of certain organisms may affect the overall growth curve.

Figure 3-8. Setric Genie 2L laboratory-scale bioreactor.

To determine the growth rates, a 2 L bioreactor shown in Figure 3-8 was used as a laboratory-scale bioreactor to grow KLB and separately BiOWiSHTM-Aqua to compare growth rates. A laboratory-scale bioreactor such as this one provided more control of growth conditions, such as propeller speed and air sparge rate, as opposed to a laboratory-scale flask. Since the goal was to compare growth rates, LB was used because both KLB and separately BiOWiSHTM-Aqua grew well in it. At the specified LB concentration of 25 g/L, the OD₆₀₀ reading was out of range because the concentration of cells was too high. Therefore, 1.25 L of LB at a concentration of 12.5 g/L (50% of the specified 25

g/L) was used. The LB broth was poured into the bioreactor and autoclaved at 121°C at 15 psi for 20 minutes. After the bioreactor reached room temperature, 12.5 mL of inoculum (1% of total media volume) was pipetted into the bioreactor. Agitation was set at 180 rpm (close to the 200 rpm in an orbital shaker by Rao, 2007) and temperature at 30°C. To determine the initial OD₆₀₀, 1 mL of cells were sampled from the bioreactor immediately after inoculation. OD₆₀₀ readings were subsequently taken every 8-12 hours, more often during the initial lag and growth phases, and converted to CFU/mL using a calibration curve (Figure 4-13).

3.6 Effect of *B. subtilis* KLB *in vivo* on Strawberry Fruit Bodies

The purpose of this experiment was to determine whether mold growth on post-harvest strawberry fruit bodies could be slowed with *B. subtilis* KLB as a biopesticide. The controls for this experiment were *B. subtilis* strain QST713 and no biopesticide (just water). Just as for the culturing of *Botrytis*, the strawberries for the *in vivo* experiments were obtained from Y Hayashi & Sons Farms based in Arroyo Grande, CA. The strawberries were placed in a chamber as shown in Figure 3-9. Tap water was filled in five beakers and placed into the chamber in order to increase humidity to prevent the strawberries from drying. The strawberries were sprayed with the following biopesticides:

Undiluted KLB (approximately 5.0×10^8 CFU/mL)

1:10 concentration KLB

1:100 concentration KLB

Undiluted *B. subtilis* QST 713 (from Serenade[®])

No biopesticide (just water)

3.6.1 Initial Experimental Setup

Strawberries from each biopesticide treatment were placed together as shown in Figure 3-10. Six strawberries were used for each treatment in Trials 1-3 and nine were used in Trial 4. Approximately 2.5 mL of the biopesticide was sprayed on each group of strawberries outside the chamber. The biopesticide was rubbed on the whole surface of each strawberry and placed into the chamber. Then, 1 mL of 100 CFU/mL *Botrytis* was sprayed onto each group of strawberries. The strawberries were observed daily for eight days for any *Botrytis* growth. Humidity and temperature were recorded at the same time as observation. Pictures were taken daily as well to visually document the extent of molding over time. A strawberry was tallied as infected with *Botrytis* if any sign of *Botrytis* was seen on the fruit body.

Figure 3-9. Chamber for *in vivo* experiments using strawberry fruit bodies.

Figure 3-10. Initial strawberry placement in chamber.

Figure 3-11. Modified strawberry placement in chamber.

3.6.2 Modification to Initial Experimental Setup

The experiment was later modified based on possible cross-contamination and positioning bias in the experiment. Strawberries in certain areas within the chamber might have been more or less susceptible to *Botrytis* growth, so strawberry placement was randomized in a grid structure in the chamber as shown in the figure below. Rows were labeled A through E and columns were numbered 1 through 8 as shown in Figure 3-11. Using the same biopesticide groups and the same inoculating procedures as in the Initial Experiment Setup, eight strawberries were used for each of the five groups. Each strawberry was randomly placed in the chamber and its position was recorded. The strawberries were observed daily for eight days and those infected with mold were marked.

CHAPTER 4: RESULTS

4.1 Inhibition Experiments

This section presents results for initial *Botrytis* inhibition experiments with BiOWiSHTM-Aqua, followed by inhibition experiments with the *B. subtilis* KLB isolate.

4.1.1 *Botrytis* Inhibition Results Using BiOWiSHTM-Aqua

The initial goal was to determine how concentration of BiOWiSHTM-Aqua, time of BiOWiSHTM-Aqua activation, and time of BiOWiSHTM-Aqua growth prior to *Botrytis* inoculation affected percent growth of *Botrytis* on a Petri dish. Four trials were conducted using BiOWiSHTM-Aqua as the biopesticide. Data for the four trials begin at Table 4-1 and Figure 4-3. Some plates contained radial *Botrytis* growth (Figure 4-1) while other plates contained more random *Botrytis* growth patterns (Figure 4-2). The variations in *Botrytis* growth patterns were due to the different compositions of organisms in each plate, even within the triplicates.

Figure 4-1. Sample plate with typical radial *Botrytis* growth 48 hours after inoculation.

Figure 4-2. Sample plate with *Botrytis* inhibition seen around various areas of the plate.

Table 4-1. Trial 1 data with *Botrytis* application right after BiOWiSH™-Aqua spread plated ($T_{app} = 0$ hours).

Percent growth of <i>Botrytis</i> on plate			
BiOWiSH™-Aqua conc. (ppm)	BiOWiSH™-Aqua activation time (T_{act})		
	48 hr	72 hr	96 hr
10^2	51.4	61.8	100
	41.9	81.3	100
	48.1	69.3	100
Avg	47.1	70.8	100.0
10^3	41.9	51.4	100.0
	65.5	48.1	100.0
	51.4	41.9	100.0
Avg	52.9	47.1	100.0
10^4	54.7	n/a	100.0
	54.7	58.2	100.0
	51.4	54.7	100.0
Avg	53.6	56.5	100.0

Figure 4-3. Effect of BiOWiSH™-Aqua activation time on *Botrytis* growth at 0 hours application time in Trial 1 (Table 4-1).

Botrytis application time was 0 hours relative to BiOWiSH™-Aqua, shown in Figure 4-3 and Table 4-1. Lower average *Botrytis* growth (more inhibition) was seen in plates of BiOWiSH™-Aqua activation times of 48 and 72 hours compared to activation time of 96 hours. Growth was defined as percentage of the plate overtaken by *Botrytis*, measured either by diameter or by estimation.

At activation time of 48 hours, average *Botrytis* growth was nearly identical, from 47 to 54 percent for all BiOWiSH™-Aqua concentrations. At activation time of 72 hours, average growth ranged from 47 to 71 percent at 1,000 ppm and 100 ppm, respectively. At activation time of 96 hours, 100 percent growth (Petri dish full of *Botrytis*) was observed for all samples.

Table 4-2. BiOWiSH™-Aqua Trial 1 control data of the negative control (no biopesticide) and *B. subtilis* QST 713.

Percent growth of <i>Botrytis</i> on plate			
	Application time of <i>Botrytis</i> (T _{app})		
	0	24	48
<i>B. subtilis</i>	10.5	0.0	0.0
QST 713	17.3	23.6	23.6
(undiluted)	15.4	12.0	12.0
Avg	14.4	11.9	7.1
No biopesticide	100.0	100.0	100.0
Avg	100.0	100.0	100.0

Figure 4-4. Control data of Serenade (*B. subtilis* QST 713) at three *Botrytis* application times and no biopesticide.

As expected, all plates with no biopesticide contained 100 percent growth of *Botrytis*, comparable to the plate in Figure 3-3. Application times did not apply to the “no biopesticide” negative control since *Botrytis* was inoculated on empty PDA plates.

Regardless of *Botrytis* application time, *B. subtilis* QST 713 kept average *Botrytis* growth to a minimum, from 7 percent at 48 hours to 14 percent at 0 hours (Table 4-2 and Figure 4-4). These two controls showed extremes of *Botrytis* growth since one is a commercial biopesticide, which greatly inhibited growth, and the other did not contain any biopesticide, which allowed *Botrytis* to grow undisturbed.

Table 4-3. Trial 1 data with *Botrytis* application 24 hours after BiOWiSH™-Aqua spread-plated.

Percent growth of <i>Botrytis</i> on plate			
BiOWiSH™- Aqua conc. (ppm)	BiOWiSH™-Aqua activation time (T _{act})		
	48 hr	72 hr	96 hr
10 ²	36.1	0.0	0.0
	65.5	0.0	0.0
	41.9	23.6	65.5
Avg	47.8	7.9	21.8
10 ³	54.7	33.4	100.0
	58.2	73.2	100.0
	73.2	69.3	100.0
Avg	62.0	58.6	100.0
10 ⁴	73.2	0.0	100.0
	65.5	0.0	48.1
	98.8	0.0	85.5
Avg	79.2	0.0	77.9

Figure 4-5. Effect of BiOWiSH™-Aqua activation time on *Botrytis* growth at 24 hours application time in Trial 1 (Table 4-3).

Botrytis application time was 24 hours relative to BiOWiSHTM-Aqua, shown in Figure 4-5 and Table 4-3. Percent growth of *Botrytis* varied widely from plate to plate. The least average *Botrytis* growth was seen at activation time of 72 hours. In fact, complete inhibition of *Botrytis* was seen on all of the triplicate plates with 10,000 ppm of BiOWiSHTM-Aqua and on two of the plates with 100 ppm. In contrast, average *Botrytis* growth was close to 60 percent at 1,000 ppm.

At activation time of 48 hours, average *Botrytis* growth ranged from 48 to 79 percent, increasing from 100 to 10,000 ppm. At activation time of 96 hours, average growth ranged from 22 percent at 100 ppm to full growth at 1,000 ppm. Plates at 100 ppm contained fewer and larger BiOWiSHTM-Aqua colonies. Select colonies formed inhibition zones to limit the growth of *Botrytis*.

Table 4-4. Trial 1 data with *Botrytis* application 48 hours after BiOWiSH™-Aqua spread-plated.

Percent growth of <i>Botrytis</i> on plate			
BiOWiSH™-Aqua conc. (ppm)	BiOWiSH™-Aqua activation time (T _{act})		
	48 hours	72 hours	96 hours
10 ²	69.3	39.0	21.4
	69.3	30.8	36.1
	51.4	45.0	41.9
Avg	63.3	38.2	33.1
10 ³	28.3	41.9	33.4
	21.4	36.1	30.8
	23.6	36.1	28.3
Avg	24.4	38.1	30.8
10 ⁴	0.0	65.5	39.0
	45.0	41.9	36.1
	39.0	36.1	19.3
Avg	28.0	47.8	31.5

Figure 4-6. Effect of BiOWiSH™-Aqua activation time on *Botrytis* growth at 48 hours application time in Trial 1 (Table 4-4).

Botrytis application time was 48 hours relative to BiOWiSH™-Aqua, shown in Figure 4-6 and Table 4-4. *Botrytis* growth was relatively similar throughout all BiOWiSH™-Aqua activation times compared to growth at *Botrytis* application times of 0 and 24 hours.

At application time of 48 hours, 24 to 63 percent average *Botrytis* growth was observed. At application time of 72 hours, average growth was consistent at 38 to 48 percent. At application time of 96 hours, average growth was almost identical at 31 to 33 percent.

Table 4-5. Trial 2 data with *Botrytis* application right after BiOWiSH™-Aqua spread plated.

BiOWiSH™- Aqua conc. (ppm)	Percent growth of <i>Botrytis</i> on plate		
	BiOWiSH™-Aqua activation time (T _{act})		
	60 hours	73 hours	85 hours
10 ²	100.0	100.0	80.3
	100.0	100.0	80.3
	85.5	100.0	81.3
Avg	95.2	100.0	80.6
10 ³	91.0	80.3	85.5
	85.5	100.0	100.0
	85.5	100.0	85.5
Avg	87.3	93.4	90.4
10 ⁴	85.5	71.2	85.5
	100.0	48.1	85.5
	81.3	48.1	51.4
Avg	88.9	55.8	74.1

Figure 4-7. Effect of BiOWiSH™-Aqua activation time on *Botrytis* growth at 0 hours application time in Trial 2 (Table 4-5).

In Trial 2, *Botrytis* application times were at 0 and 24 hours and BiOWiSH™-Aqua activation times focused around 72 hours, at 60, 73 and 85 hours.

Botrytis application time was 0 hours relative to BiOWiSH™-Aqua, shown in Figure 4-7 and Table 4-5. *Botrytis* growth was particularly high for all plates regardless of BiOWiSH™-Aqua activation time or concentration. One explanation of greater *Botrytis* growth is that the batch of BiOWiSH™-Aqua used in Trial 1 was different than the batch used in Trial 2. Another possibility is that since application time was 0 hours, BiOWiSH™-Aqua did not have time to grow on the Petri dish before application of *Botrytis*.

The least average *Botrytis* growth was observed (56 percent) at activation time of 73 hours with 10,000 ppm of BiOWiSH™-Aqua. The second least (74 percent) was at activation time of 85 hours with 10,000 ppm. All other plates contained average growth of at least 87 percent and most were at greater than 90 percent.

Table 4-6. Trial 2 data with *Botrytis* application 24 hours after BiOWiSH™-Aqua spread-plated.

Percent growth of <i>Botrytis</i> on plate			
BiOWiSH™- Aqua conc. (ppm)	BiOWiSH™-Aqua activation time (T _{act})		
	60 hours	73 hours	85 hours
10 ²	100.0	48.1	0.0
	100.0	18.8	0.0
	100.0	65.5	12.0
Avg	100.0	44.1	4.0
10 ³	0.0	0.0	1.3
	18.8	56.5	0.0
	1.3	40.4	1.3
Avg	6.7	32.3	0.9
10 ⁴	85.5	0.0	3.0
	48.1	12.0	21.4
	33.4	0.0	56.5
Avg	55.7	4.0	27.0

Figure 4-8. Effect of BiOWiSH™-Aqua activation time on *Botrytis* growth at 24 hours application time in Trial 2 (Table 4-6).

Botrytis application time was 24 hours relative to BiOWiSH™-Aqua, shown in Figure 4-8 and Table 4-6. *Botrytis* growth was considerably lower compared to application time of 0 hours. One of the possible reasons is that BiOWiSH™-Aqua colonies were already established on plates before *Botrytis* application at 24 hours.

No patterns of *Botrytis* growth were seen regardless of BiOWiSH™-Aqua concentration and activation time. At 100 ppm and activation time of 60 hours, all of the plates contained 100 percent growth. In contrast, at 1,000 ppm, a ten-fold increase, average growth was almost non-existent at 7 percent. At 10,000 ppm, plates yielded considerably more growth at an average of 56 percent.

At activation time of 73 hours, there was considerable growth variability from plate to plate, shown by relatively large standard error bars. For example, although the average was 44 percent at activation time of 100 ppm, the triplicate plates varied from 19 to 66 percent. For 1,000 ppm, one plate did not contain any growth while the average was 32 percent. Average growth at 10,000 ppm (4 percent) was almost non-existent. At activation time of 85 hours, minimal average growth was observed at 100 and 1,000 ppm at 4.0 and 0.9 percent, respectively. At 10,000 ppm, average growth was 27 percent.

Table 4-7. Trial 3 data with *Botrytis* application 24 hours after BiOWiSH™-Aqua spread-plated.

Percent growth of <i>Botrytis</i> on plate			
BiOWiSH™- Aqua conc. (ppm)	BiOWiSH™-Aqua activation time (T _{act})		
	59 hours	71 hours	80 hours
10 ²	65.5	56.5	60.9
	65.5	65.5	60.9
	65.5	56.5	65.5
Avg	65.5	59.5	62.4
10 ³	56.5	65.5	70.2
	65.5	60.9	80.3
	56.5	60.9	65.5
Avg	59.5	62.4	72.0
10 ⁴	56.5	56.5	60.9
	56.5	56.5	65.5
	48.1	60.9	60.9
Avg	53.7	57.9	62.4

Figure 4-9. Effect of BiOWiSH™-Aqua activation time on *Botrytis* growth at 24 hours application time in Trial 3 (Table 4-7).

Table 4-8. Trial 4 data with *Botrytis* application 23 hours after BiOWiSH™-Aqua spread-plated.

Percent growth of <i>Botrytis</i> on plate			
BiOWiSH™-Aqua conc. (ppm)	BiOWiSH™-Aqua activation time (T _{act})		
	65 hours	77 hours	89 hours
10 ²	56.5	44.2	48.1
	48.1	33.4	40.4
	48.1	40.4	44.2
Avg	50.9	39.3	44.2
10 ³	48.1	27.1	40.4
	44.2	24.1	44.2
	48.1	24.1	44.2
Avg	46.8	25.1	42.9
10 ⁴	36.8	40.4	40.4
	48.1	44.2	48.1
	48.1	48.1	40.4
Avg	44.4	44.2	43.0

Figure 4-10. Effect of BiOWiSH™-Aqua activation time on *Botrytis* growth at 23 hours application time in Trial 4 (Table 4-8).

Trials 3 and 4 (Figure 4-9 and Figure 4-10) were repetitions of Trial 2 based on relatively low *Botrytis* growth at application time of 24 hours. However, average *Botrytis* growth in Trial 3 was greater at nearly all BiOWiSH™-Aqua concentrations and activation times, from 54 percent to 72 percent (Figure 4-9 and Table 4-7). Trial 4 contained lower average *Botrytis* growth, from 25 percent to 51 percent (Figure 4-10 and Table 4-8). Repeatability of Trial 2 was unsuccessful in Trials 3 and 4 possibly due to the variability of BiOWiSH™-Aqua organisms.

Overall, BiOWiSHTM-Aqua had no observable effect on *Botrytis* growth. *Botrytis* growth in these plates ranged from perfectly circular growth to completely random growth, as shown in Figure 4-1 and Figure 4-2.

Figure 4-2 shows inhibition zones around certain colonies that inhibited *Botrytis* growth while others did not. This suggested that only one or some of the organisms in BiOWiSHTM-Aqua inhibited *Botrytis* growth, although that/those organisms were not present in the same proportions on every plate. One organism responsible for *Botrytis* inhibition was later isolated and identified as *B. subtilis* KLB (more in Section 4.1.2.1).

4.1.2 *B. subtilis* KLB Isolate

4.1.2.1 Isolation and Characterization of *B. subtilis* KLB

The spread plates of the BiOWiSHTM-Aqua inhibition experiments yielded some plates with colonies of various species (Figure 4-2) as well as some plates with colonies of what appeared to be of a single species (Figure 4-11). The colonies on plates similar to Figure 7 inhibited *Botrytis* growth. The surrounding area of the colony was clear of any *Botrytis* growth, indicating that *B. subtilis* inhibited *Botrytis* growth in an *in vitro* environment. The *Botrytis*, inoculated in the middle of the plate, was never able to form a fuzzy conidial mass in contrast to the control with no biopesticide (Figure 4-11). A colony close to the *Botrytis* point of inoculation shown in Figure 4-11 was isolated using

a flamed inoculum loop and smeared onto a microscope glass slide. The cells were then Gram-stained as shown in Figure 4-12. They were identified as *Bacillus* because they were rod-shaped and gram positive. In addition, its 16S rRNA sequence was analyzed using NCBI BLAST searching tool, which closely matched (> 99% homology) various strains of both *B. subtilis* and *B. amyloliquefaciens* (Zhang, 2000). The closest sequence match was to *B. subtilis* BSn5 (100% Max ident), an endophytic bacterium with antimicrobial activity against the plant pathogen *Erwinia carotovora*, with GenBank number CP002468.1.

Complete inhibition of *Botrytis* growth, as shown by the large spot

Figure 4-11. PDA plate where *B. subtilis* KLB completely inhibited *Botrytis* (the large spot shown by the arrow).

Figure 4-12. 1000x magnification of a Gram-stained slide with *B. subtilis* KLB.

Concentration of KLB inoculum with OD₆₀₀ of 0.948 was plated at 10⁻⁶ dilution. The numbers of cells counted on the three plates were 63, 51, and 72, an average of 62. The concentration of KLB was used to construct a calibration curve, which was calculated as:

$$62 \text{ CFU} / (10^{-6} * 0.1 \text{ mL}) = 6.2 \times 10^8 \text{ CFU/mL}$$

Figure 4-13. Growth curve of *B. subtilis* KLB.

The following formula on Figure 4-13 was interpreted as follows:

$$y = 7.292 \times 10^8 x, \text{ where}$$

y = cell density in CFU/mL

x = optical density at 600 nm (OD₆₀₀)

Using this formula, cell densities were determined by finding OD₆₀₀ using the spectrophotometer.

To confirm that *B. subtilis* KLB specifically inhibited *Botrytis* growth and to determine the effective concentrations, a quick visual screening study was conducted. Regardless of an application time of 0 or 24 hours, *Botrytis* growth was completely inhibited in the 4.84

$\times 10^6$ CFU/mL section (Figure 4-14 and Figure 4-15). *Botrytis* grew well on the 4.84×10^4 CFU/mL and control sections at an application time of 0 hours (Figure 4-14). *Botrytis* growth was mostly inhibited at an application time of 24 hours except for minimal growth, shown by the white fuzz, on the control section (Figure 4-15).

Figure 4-14. Different *B. subtilis* KLB concentrations at application time of 0 hours.

Figure 4-15. Different *B. subtilis* KLB concentrations at application time of 24 hours.

4.1.2.2 *Botrytis* Inhibition Results Using *B. subtilis* KLB

B. subtilis KLB replaced BiOWiSH™-Aqua as the biopesticide in further *Botrytis* inhibition experiments. The variables evaluated were concentration of KLB (C_{KLB}) and time of KLB growth on a plate prior to *Botrytis* application (T_{app}). Nine trials were conducted in all. The conditions of each trial were based on evaluation of results from the previous trial. Afterward, all of the trial data were statistically evaluated using Minitab to determine 1) KLB concentration (C_{KLB}) threshold for complete inhibition of *Botrytis* at each application time (T_{app}) of 0, 24, and 48 hours, and 2) relationship between percent growth of *Botrytis* on plate and KLB concentration at each application time.

Table 4-9. Trial 1 data with *Botrytis* application 24 hours after *B. subtilis* KLB spread-plated ($T_{app} = 24$ hours).

<i>B. subtilis</i> KLB, dilution	<i>B. subtilis</i> KLB, CFU/mL	Percent growth of <i>Botrytis</i> on plate ($T_{app} = 24$ hrs)
10^{-2}	2.5×10^5	4.8
		7.1
		0.0
Avg		4.0
10^{-3}	2.5×10^4	0.0
		16.4
		56.5
Avg		24.3
10^{-4}	2.5×10^3	43.4
		41.9
		13.7
Avg		33.0

Figure 4-16. *B. subtilis* KLB Trial 1 data from Table 4-9.

At application time of 24 hours in Trial 1, the data (Figure 4-16 and Table 4-9) show that as KLB concentration (x-axis) increased, percent growth of *Botrytis* on plate (y-axis) decreased. At the highest KLB concentration of 2.5×10^5 CFU/mL, almost complete inhibition of *Botrytis* was seen with average *Botrytis* growth of 4 percent. At a concentration of ten times less, 2.5×10^4 CFU/mL, average *Botrytis* growth was much greater at 57 percent, with a range from 0 to 57 percent. At a concentration of two logs lower compared to the original, 2.5×10^3 CFU/mL, average *Botrytis* growth was 33 percent, with a range from 14 to 43 percent. The results were expected since a higher concentration of KLB should have a stronger effect against *Botrytis* growth.

Table 4-10. Trial 2 data with *Botrytis* application 24 and 48 hours after *B. subtilis* KLB spread-plated.

<i>B. subtilis</i> KLB, dilution	<i>B. subtilis</i> KLB, CFU/mL	Percent growth of <i>Botrytis</i> on plate (T _{app} = 24 hrs)	Percent growth of <i>Botrytis</i> on plate (T _{app} = 48 hrs)
10 ⁻²	8.6 x 10 ⁴	25.9	33.4
		21.4	27.1
		33.4	24.1
Avg		26.9	28.2
10 ⁻³	8.6 x 10 ³	33.4	30.2
		22.5	27.1
		40.4	30.2
Avg		32.1	29.1
10 ⁻⁴	8.6 x 10 ²	0.0	48.1
		48.1	44.2
		46.5	44.2
Avg		31.5	45.5
10 ⁻⁵	8.6 x 10 ¹	43.4	33.4
		46.5	36.8
		43.4	30.2
Avg		44.5	33.5
10 ⁻⁶	8.6 x 10 ⁰	n/a	100.0
		n/a	50.0
		n/a	20.0
Avg		n/a	56.7

Figure 4-17. *B. subtilis* KLB Trial 2 data from Table 4-10.

At application times of 24 and 48 hours in Trial 2, the data (Figure 4-17 and Table 4-10) also show that as KLB concentration increased, percent growth of *Botrytis* on plate decreased as in Trial 1. KLB concentrations were lowered to observe a larger spectrum of concentrations against percent growth of *Botrytis*. Average *Botrytis* growth ranged from 27 to 45 percent at application time of 24 hours. Average growth ranged from 28 to 57 percent, at application time of 48 hours. No significant differences were seen between the two application times. One observation was at very low KLB concentrations, *Botrytis* growth was not radial and varied greatly. An example is the inhibition of *Botrytis* growth on plates with just 8.6 CFU/mL of KLB with application time of 48 hours. These plates contained only one or a few colonies, but they grew much larger in size compared to those on more concentrated plates and greatly inhibited *Botrytis* growth. As shown in

Figure 4-17, the standard error at the lowest concentration was large, where *Botrytis* growth was 0, 20, and 100 percent in the triplicate plates.

Table 4-11. Trial 3 data with *Botrytis* application 0 and 24 hours after *B. subtilis* KLB spread-plated.

<i>B. subtilis</i> KLB, dilution	<i>B. subtilis</i> KLB, CFU/mL	Percent growth of <i>Botrytis</i> on plate (T _{app} = 0 hrs)	Percent growth of <i>Botrytis</i> on plate (T _{app} = 24 hrs)
10 ⁰	6.6 x 10 ⁶	0.0	0.0
		0.0	0.0
		0.0	0.0
Avg		0.0	0.0
10 ⁻¹	6.6 x 10 ⁵	2.6	0.0
		0.7	0.0
		0.0	0.0
Avg		1.1	0.0

Figure 4-18. *B. subtilis* KLB Trial 3 data from Table 4-11.

Figure 4-19. Minimal *Botrytis* growth (2.6 percent) at 6.6×10^5 CFU/mL of *B. subtilis* KLB in Trial 3.

At application times of 0 and 24 hours in Trial 3, the data (Figure 4-18 and Table 4-11) show nearly complete inhibition of *Botrytis* growth. Application time of 0 hours was tested in this trial since minimal differences were seen between application times of 24 and 48 hours in Trial 2. Another change was increasing KLB concentrations by 10 and 100 times compared to Trial 2, since the KLB concentration threshold to completely inhibit *Botrytis* growth was not reached in the previous trial.. Average *Botrytis* growth ranged from 0 to 1 percent at application time of 0 hours. Complete inhibition of *Botrytis* growth was seen at all KLB concentrations with application time of 24 hours. Figure 4-19 shows minimal *Botrytis* growth seen on plates with application time of 0 hours and 6.5×10^5 CFU/mL of KLB. The results were expected because this trial contained very large concentrations of KLB.

Table 4-12. Trial 4 data with *Botrytis* application right after *B. subtilis* KLB spread-plated.

<i>B. subtilis</i> KLB, dilution	<i>B. subtilis</i> KLB, CFU/mL	Percent growth of <i>Botrytis</i> on plate (T _{app} = 0 hrs)
10 ⁻²	4.3 x 10 ⁵	22.5
		27.1
		23.6
Avg		24.4
10 ⁻³	4.3 x 10 ⁴	70.0
		60.0
		80.0
Avg		70.0
10 ⁻⁴	4.3 x 10 ³	100.0
		100.0
		100.0
Avg		100.0
10 ⁻⁵	4.3 x 10 ²	100.0
		100.0
		100.0
Avg		100.0

Figure 4-20. *B. subtilis* KLB Trial 4 data from Table 4-12.

At application time of 0 hours in Trial 4, the data (Figure 4-20 and Table 4-12) show a very strong relationship between the increase in KLB concentration and the decrease in percent growth of *Botrytis* on plate. In Trial 4, the goal was to determine the KLB concentration threshold to completely inhibit *Botrytis* growth at application time of 0 hours. At 4.3×10^2 and 4.3×10^3 CFU/mL, all triplicate plates were completely filled with *Botrytis* (100 percent growth). Average *Botrytis* growth was 70 percent at 4.3×10^4 CFU/mL and 24 percent at 4.3×10^5 CFU/mL. By following the trend of the graph, complete inhibition of *Botrytis* growth would be predicted if KLB concentration was increased ten-fold to 4.3×10^6 CFU/mL.

Table 4-13. Trial 5 data with *Botrytis* application right after *B. subtilis* KLB spread-plated.

<i>B. subtilis</i> KLB, dilution	<i>B. subtilis</i> KLB, CFU/mL	Percent growth of <i>Botrytis</i> on plate (T _{app} = 0 hrs)
10 ⁰	5.6 x 10 ⁶	7.1
		10.5
		7.7
Avg		8.4
10 ⁻¹	5.6 x 10 ⁵	11.2
		21.4
		15.4
Avg		16.0
10 ⁻²	5.6 x 10 ⁴	33.4
		30.8
		25.9
Avg		30.0

Figure 4-21. *B. subtilis* KLB Trial 5 data from Table 4-13.

At application time of 0 hours in Trial 5 (Figure 4-21 and Table 4-13), the highest KLB concentration was approximately 10 times higher, at 5.6×10^6 CFU/mL, compared to in Trial 4 (4.3×10^5 CFU/mL). Average *Botrytis* growth was 16 percent at 5.6×10^5 CFU/mL of KLB and 8 percent at 5.6×10^6 CFU/mL. As a comparison, average growth was 24 percent at 4.3×10^5 CFU/mL in Trial 4. Although complete *Botrytis* inhibition did not occur at 5.6×10^6 CFU/mL of KLB, average growth was limited to 8 percent.

Table 4-14. Trial 6 data with *Botrytis* application 24 and 48 hours after *B. subtilis* KLB spread-plated.

<i>B. subtilis</i> KLB, dilution	<i>B. subtilis</i> KLB, CFU/mL	Percent growth of <i>Botrytis</i> on plate (T _{app} = 24 hrs)	Percent growth of <i>Botrytis</i> on plate (T _{app} = 48 hrs)
10 ⁻³	5.3 x 10 ⁴	0.0	0.0
		0.0	0.0
		0.0	0.0
Avg		0.0	0.0
10 ⁻⁴	5.3 x 10 ³	0.0	0.0
		0.0	0.0
		25.0	0.0
Avg		8.3	0.0
10 ⁻⁵	5.3 x 10 ²	10.0	0.0
		50.0	0.0
		30.0	0.0
Avg		30.0	0.0

Figure 4-22. *B. subtilis* KLB Trial 6 data from Table 4-14.

Trial 6 (Figure 4-22 and Table 4-14) was mainly a repeat of Trial 2, with application times of 24 and 48 hours and very similar KLB concentrations used. Trial 2 conditions were revisited because KLB concentration thresholds were determined at application time of 0 hours (Trial 5). Average *Botrytis* growth ranged from 0 to 30 percent at application time of 24 hours. Complete inhibition of growth was seen at 5.3×10^4 CFU/mL of KLB. At application time of 48 hours, complete inhibition of growth was seen at all KLB concentrations. This was in contrast to Trial 2, in which average growth was 27 to 28 percent at 8.6×10^4 CFU/mL for both application times of 24 and 48 hours. Although conditions were identical between Trial 2 and Trial 6, the difference may be attributed to different growth phases of certain KLB colonies. For example, select colonies may grow

larger than others and consume most of the nutrients in the agar, which inhibit the growth of smaller colonies. From observation of the plates, these larger colonies greatly inhibited *Botrytis* growth.

Table 4-15. Trial 7 data with *Botrytis* application 24 and 48 hours after *B. subtilis* KLB spread-plated.

<i>B. subtilis</i> KLB, dilution	<i>B. subtilis</i> KLB, CFU/mL	Percent growth of <i>Botrytis</i> on plate (T _{app} = 24 hrs)	Percent growth of <i>Botrytis</i> on plate (T _{app} = 48 hrs)
10 ⁻³	4.3 x 10 ⁴	0.0	0.0
		0.0	0.0
		0.0	2.0
Avg		0.0	0.7
10 ⁻⁴	4.3 x 10 ³	0.0	1.6
		3.9	0.0
		23.6	30.0
Avg		9.1	10.5
10 ⁻⁵	4.3 x 10 ²	80.0	0.0
		90.0	2.3
		95.0	35.0
Avg		88.3	12.4
10 ⁻⁶	4.3 x 10 ¹	55.0	90.0
		85.0	100.0
		65.0	85.0
Avg		68.3	91.7

Figure 4-23. *B. subtilis* KLB Trial 7 data from Table 4-15.

Trial 7 (Figure 4-23 and Table 4-15) was a repeat of Trial 6, with application times of 24 and 48 hours. For both application times, complete inhibition of *Botrytis* growth was seen at 4.3×10^4 CFU/mL of KLB and approximately 10 percent growth at 4.3×10^3 CFU/mL. For application time of 48 hours, complete inhibition of growth was seen at all KLB concentrations. The KLB concentration threshold to completely inhibit *Botrytis* growth, at 4.3×10^3 CFU/mL, was similar to the threshold in Trial 6, at 5.3×10^3 CFU/mL.

Table 4-16. Trial 8 data with *Botrytis* application 24 and 48 hours after *B. subtilis* KLB spread-plated.

<i>B. subtilis</i> KLB, dilution	<i>B. subtilis</i> KLB, CFU/mL	Percent growth of <i>Botrytis</i> on plate (T _{app} = 24 hrs)	Percent growth of <i>Botrytis</i> on plate (T _{app} = 48 hrs)
10 ⁻³	4.0 x 10 ⁴	0.0	9.0
		0.0	16.4
		0.0	12.0
Avg		0.0	12.5
10 ⁻⁴	4.0 x 10 ³	10.0	0.0
		75.0	0.0
		50.0	15.0
Avg		45.0	5.0
10 ⁻⁵	4.0 x 10 ²	90.0	50.0
		75.0	50.0
		75.0	65.0
Avg		80.0	55.0
10 ⁻⁶	4.0 x 10 ¹	90.0	90.0
		90.0	75.0
		85.0	15.0
Avg		88.3	60.0

Figure 4-24. *B. subtilis* KLB Trial 8 data from Table 4-16.

Trial 8 (Figure 4-24 and Table 4-16) was a repeat of Trial 6 and Trial 7, with application times of 24 and 48 hours. At application time of 24 hours, complete inhibition of *Botrytis* growth was seen at 4.0×10^4 CFU/mL of KLB. At application time of 48 hours, average growth was 13 percent at 4.0×10^4 CFU/mL and even lower at 5 percent when KLB concentration was reduced ten-fold, at 4.0×10^3 CFU/mL. *Botrytis* growth was minimal starting at a KLB concentration of 4.0×10^3 CFU/mL.

Table 4-17. Trial 9 data with *Botrytis* application 0, 24, and 48 hours after *B. subtilis* KLB spread-plated.

<i>B. subtilis</i> KLB, dilution	<i>B. subtilis</i> KLB, CFU/mL	Percent growth of <i>Botrytis</i> on plate (T _{app} = 0 hrs)	Percent growth of <i>Botrytis</i> on plate (T _{app} = 24 hrs)	Percent growth of <i>Botrytis</i> on plate (T _{app} = 48 hrs)
10 ⁻²	4.3 x 10 ⁵	10.5	0.0	0.0
		12.0	0.0	0.0
		13.7	0.0	0.0
Avg		12.1	0.0	0.0
10 ⁻³	4.3 x 10 ⁴	45.0	0.0	0.0
		45.0	0.0	0.0
		30.0	0.0	0.0
Avg		40.0	0.0	0.0
10 ⁻⁴	4.3 x 10 ³	75.0	0.0	0.0
		85.0	0.0	0.0
		70.0	2.6	0.0
Avg		76.7	0.9	0.0

Figure 4-25. *B. subtilis* KLB Trial 9 data from Table 4-17.

Trial 9 (Figure 4-25 and Table 4-17) was based on the culmination of all eight previous trials. Application times of 0, 24, and 48 hours were tested. KLB concentrations used were within the threshold range for complete inhibition of *Botrytis* at the respective application times. At application times of 24 and 48 hours, complete or nearly complete inhibition was seen at all KLB concentrations, from 4.3×10^3 CFU/mL to 4.3×10^5 CFU/mL. At application time of 0 hours, a strong inverse relationship between *Botrytis* growth and KLB concentration was evident, with growth ranging from 12 percent at 4.3×10^5 CFU/mL to 77 percent at 4.3×10^3 CFU/mL. This suggested that inoculating KLB prior to inoculating *Botrytis*, either at application time of 24 or 48 hours, contributed to inhibition of *Botrytis* growth.

4.1.2.3 Statistical Data of Trials 1-9 Combined

The two goals were to predict *B. subtilis* KLB concentration thresholds for complete *Botrytis* inhibition as well as to predict the relationship between *Botrytis* growth and KLB concentration at different application times. One possibility is that at a greater application time, *Botrytis* inhibition is less dependent on KLB concentration. At higher application times, KLB colonies will have matured and formed inhibition zones to inhibit *Botrytis* growth.

All data from the nine trials were transferred onto Minitab. An equation was predicted to estimate percent growth of *Botrytis* as a function of *Botrytis* application time and log

concentration of KLB. The data were fitted using linear regression on a semi-log plot (Figure 4-26).

Botrytis growth data of 6.6×10^5 and 6.6×10^6 CFU/mL of KLB (Trial 3, Figure 4-18) at application time of 24 hours were omitted because complete *Botrytis* inhibition was already seen at a lower KLB concentration of 4.3×10^5 CFU/mL (Trial 9, Figure 4-25). These outliers may have skewed the equation to predict higher KLB concentration thresholds.

Complete *Botrytis* inhibition was seen on many plates below the threshold KLB concentrations, but these data were not omitted because at least one of the plates in triplicate contained *Botrytis* growth. An example is in Trial 3 (Figure 4-18), in which *Botrytis* growth percentages were 0, 0.7, and 2.6 percent at application time of 0 hours and 6.6×10^5 CFU/mL of KLB.

Figure 4-26. Linear regression graphs of all three *Botrytis* application times of 0, 24, and 48 hours from data of all nine trials.

The equation to model Figure 4-26 data was as follows:

Predicted Percent Growth of *Botrytis* on plate =

$$127 - 0.928 T_{\text{app}} - 18.6 \log C_{\text{KLB}} \quad (R^2 = 0.55, \text{ from Appendix A})$$

The predicted KLB concentration thresholds are shown as y-intercepts in Figure 4-26, where percent growth of *Botrytis* on plate is 0 percent. At the three application times, the predicted threshold KLB concentrations are as follows:

$$T_{\text{app}} = 0 \text{ hours: } C_{\text{KLB}} = 3.2 \times 10^6 \text{ CFU/mL}$$

$$T_{\text{app}} = 24 \text{ hours: } C_{\text{KLB}} = 1.3 \times 10^5 \text{ CFU/mL}$$

$$T_{\text{app}} = 48 \text{ hours: } C_{\text{KLB}} = 9.1 \times 10^4 \text{ CFU/mL}$$

The predicted threshold KLB concentration at application time of 0 hours was more than one log greater compared to predicted thresholds at application times of 24 and 48 hours. KLB colonies were already established on the plates at 24 and 48 hours as opposed to at 0 hours, in which KLB was inoculated at the same time as *Botrytis*.

The model in Figure 4-27 predicts the relationship between *Botrytis* growth and log KLB concentration at different application times. Figure 4-27 is a truncated version of Figure 4-26, in which all data at less than 2.5×10^3 CFU/mL of KLB were removed. The reason for truncation was to focus on KLB concentrations closer to the predicted thresholds. In addition, future *in vivo* strawberry field trials will likely involve higher KLB concentrations to completely inhibit *Botrytis* growth.

Figure 4-27. Truncated version of Figure 4-26 with only data containing *B. subtilis* KLB concentrations greater than 2.5×10^3 CFU/mL.

The equation to model the truncated data in Figure 4-27 was as follows:

Predicted Percent Growth of *Botrytis* =

$$143 - 2.93 T_{\text{app}} - 22.1 \log C_{\text{KLB}} + 0.453 \text{ interaction } (R^2 = 0.50, \text{ from Appendix A})$$

The interaction term predicts whether at least one of the slopes is “different” from the others. The slope is explained as the reduction in the percent growth of *Botrytis* on plate per log increase in KLB concentration. Since the interaction term was significant (P-value < 0.05,) at least one of the three slopes is predicted to be “different” from the others (Appendix A). The interaction terms between 0 and 48 hours, and 0 and 24 hours were significant, which means that the slope at application time of 0 hours was “different” from the others. The interaction term between 24 and 48 hours was not significant, which means there was not enough evidence to conclude that the two slopes are “different” from each other. In other words, the dependence of KLB concentration on *Botrytis* inhibition was different at application time of 0 hours compared to at 24 and 48 hours.

Figure 4-28. Linear regression graph with truncated data from application time of 0 hours.

Figure 4-29. Linear regression graph with combined truncated data from application times of 24 and 48 hours.

Two regression equations were predicted, one at application time at 0 hours and the other with combined data at 24 and 48 hours. The two equations were as follows (from Appendix A):

$T_{\text{app}} = 0$ hours (Figure 4-28):

Predicted Percent Growth of *Botrytis* on plate = $181 - 28.0 \log C_{\text{KLB}}$ ($R^2 = 0.81$)

$T_{\text{app}} = 24$ and 48 hours (Figure 4-29):

Predicted Percent Growth of *Botrytis* on plate = $38.9 - 6.49 \log C_{\text{KLB}}$ ($R^2 = 0.07$)

At application time of 0 hours, a one-log increase in KLB concentration resulted in a 28 percent decrease in *Botrytis* growth. At application times of 24 and 48 hours, a one-log increase in concentration resulted only in a 6.5 percent decrease in growth. However, the R^2 value for the equation at application times of 24 and 48 hours is only 0.07 (Figure 4-29). One of the reasons for the low R^2 value is because at lower KLB concentrations, *Botrytis* growth widely varied from plate to plate. Some plates completely inhibited *Botrytis* growth while other plates contained substantial growth especially at lower KLB concentrations. KLB colonies in some plates varied in size, in which larger colonies typically contained a larger inhibition zone. Therefore, KLB concentration did not matter as long as KLB colonies had time to grow.

4.2 Initial Carbon Source Screening Results

Tapioca and molasses were screened as commercially available carbon source alternatives for growth of *B. subtilis* KLB. Experiment 1 was conducted twice (Trials 1.1 and 1.2) and consisted of the two carbon sources at 5, 15, and 25 g/L. Experiment 2 was conducted four times (Trials 2.1 to 2.4) and consisted of only tapioca at 5, 15, and 20 g/L. Only tapioca was used in Experiment 2 because cell counts were significantly higher compared to molasses. Each data point in Figure 4-30 and Figure 4-31 was the average of nine data points – three flasks for each carbon source concentration multiplied by three plates for each flask. The incubation temperature was 30°C, time of incubation was 48 hours, and agitation speed was 120 rpm.

Table 4-18. Cell counts in triplicate for Experiment 1, Trial 1.1.

<i>B. subtilis</i> KLB (x 10 ⁷ CFU/mL)						
Flask	Molasses			Tapioca		
	5 g/L	15 g/L	25 g/L	5 g/L	15 g/L	25 g/L
1	53	16	60	27	700	306
	60	14	26	29	450	386
	160	1	13	38	500	500
2	2	94	103	10	500	174
	3	47	150	35	600	164
	9	16	400	51	650	312
3	38	130	250	16	322	185
	12	49	130	19	356	135
	2	27	39	64	362	153
Avg 1	91	10	130	31	550	346
Avg 2	5	52	218	32	583	217
Avg 3	17	69	140	33	347	158
Avg total	38	44	162	32	493	240

Figure 4-30. Cell counts of KLB grown on molasses and tapioca from Table 4-18 (Experiment 1, Trial 1.1).

Table 4-19. Cell counts in triplicate for Experiment 1, Trial 1.2.

<i>B. subtilis</i> KLB (x 10 ⁷ CFU/mL)						
	Molasses			Tapioca		
Flask	5 g/L	15 g/L	25 g/L	5 g/L	15 g/L	25 g/L
1	n/a	1	220	37	296	380
	n/a	0	57	17	270	38
	n/a	0	19	356	140	171
2	107	62	21	77	119	38
	37	0	12	137	45	138
	53	0	3	185	215	192
3	2	5	99	26	50	16
	18	1	34	19	25	32
	12	1	18	8	20	3
Avg 1	107	0	54	137	235	112
Avg 2	66	21	12	133	126	123
Avg 3	11	2	50	18	32	17
Avg total	38	8	39	96	131	84

Figure 4-31. Cell counts of KLB grown on molasses and tapioca from Table 4-18 (Experiment 1, Trial 1.2).

Cell counts for Trial 1.1 (Table 4-18 and Figure 4-30) for both carbon sources were about five times higher compared to those for Trial 1.2 (Table 4-19 and Figure 4-31).

Conditions of Trial 1.1 and 1.2 were identical, with the exception that the starting concentrations of KLB inoculum varied since the trials were conducted on different days. Trial 1.1 tapioca averaged 493 colonies per plate at a 10^{-7} dilution, or 4.9×10^9 CFU/mL, while molasses averaged 4.4×10^8 CFU/mL. Trial 2 tapioca averaged 1.3×10^9 CFU/mL while molasses averaged 8×10^7 CFU/mL. Nonetheless, in Trial 1.1 at 15 g/L of carbon source, tapioca yielded a cell concentration of 11.1 times the concentration of molasses (Figure 4-30). In Trial 1.2 at 15 g/L, tapioca yielded 16.3 times the concentration of molasses (Figure 4-31).

Table 4-20. Cell counts in triplicate for tapioca, Experiment 2, Trials 2.1-2.4.

<i>B. subtilis</i> KLB (x 10 ⁷ CFU/mL)												
	Trial 2.1			Trial 2.2			Trial 2.3			Trial 2.4		
Flask	5 g/L	15 g/L	20 g/L	5 g/L	15 g/L	20 g/L	5 g/L	15 g/L	20 g/L	5 g/L	15 g/L	20 g/L
1	46	480	71	2	0	284	0	286	29	320	150	81
	75	159	14	2	0	136	0	123	73	30	32	10
	206	201	24	0	1	45	3	22	128	80	30	23
2	23	15	257	0	246	193	36	11	264	50	451	186
	3	26	79	0	249	45	80	25	90	40	250	81
	3	21	82	1	104	40	101	102	34	270	11	9
3	96	259	297	194	240	87	65	300	0	0	490	321
	26	500	72	48	93	36	50	62	0	0	137	181
	66	600	31	8	42	17	47	108	0	0	30	188
Avg 1	109	280	103	1	0	98	1	144	69	143	71	120
Avg 2	10	21	139	0	200	93	72	46	129	120	237	92
Avg 3	63	453	133	83	125	47	54	157	0	0	219	230
Avg total	60	251	125	28	108	79	42	115	66	88	176	147

Figure 4-32. Cell counts of KLB grown on tapioca from Table 4-20 (Experiment 2, Trials 2.1-2.4).

Figure 4-33. Average cell counts of KLB grown on tapioca from Table 4-20 (Experiment 2, Trials 2.1 to 2.4).

Conditions of Trials 2.1 to 2.4 (Table 4-20 and Figure 4-32) were identical, with the exception that the starting concentrations of KLB inoculum varied since the trials were conducted on different days. Experiment 2 consisted of tapioca at 5, 15, and 20 g/L based on Experiment 1 data as well as the optimal tapioca concentration of 16.7 g/L for production of *B. amyloliquefaciens* B128 spores (Rao, 2007). Although this experiment focused on determining an optimal tapioca range for viable cell production instead of spores, Trials 2.1 to 2.4 show that between the three tapioca levels, 15 g/L of tapioca produced the highest average cell concentration with 164 colonies per plate at a 10^{-7} dilution, or 1.6×10^9 CFU/mL. At 5 g/L of tapioca, the average cell concentration was 5.5×10^8 CFU/mL and at 20 g/L it was 1.1×10^9 CFU/mL (Figure 4-33).

4.3 *Botrytis* Growth Curve Experiment Results

Cell growth over time of both *B. subtilis* KLB and BiOWiSHTM-Aqua were compared using OD₆₀₀.

Figure 4-34. The OD₆₀₀ values of *B. subtilis* KLB and BiOWiSHTM-Aqua with corresponding KLB cell counts.

The primary y-axis (left) shows KLB cell counts and the secondary y-axis (right) shows OD₆₀₀ of both KLB and BiOWiSHTM-Aqua (Figure 4-34).

By using an enriched LB medium, the KLB lag phases were relatively short. The lag phases of the KLB trials lasted about 5-6 hours. The growth phase for both trials lasted until 50-55 hours. A slight dip in KLB concentration occurred in Trial 2 at 46 hours, but the next cell concentration at 53 hours increased, thus indicating the cells were still in growth phase. The maximum OD₆₀₀ readings of the KLB trials were reached at around 66 hours, but the beginning of the stationary phase began earlier, possibly around 53

hours. The two trials indicate that the stationary phases, where death of cells is approximately equal to growth of new cells, lasted between 24 and 36 hours. Cell concentrations of both trials at stationary phase were around 1.6×10^9 CFU/mL. The death phases, where the deaths of cells exceed the growth of new cells, began between 78 and 86 hours. However, although cell concentration was expected to decrease at a constant rate during the death phase, the cell concentrations unexpectedly stayed constant based on OD₆₀₀ readings after approximately 100 hours. OD₆₀₀ reading may be inaccurate in this case because both dead and live cells, as well as spores, may have been taken into account in the OD readings.

The BiOWiSHTM-Aqua grew rapidly starting at 10 hours, which resembled a lag phase. OD₆₀₀ peaked at 48 hours at 3.3. OD₆₀₀ was at a sharp and abrupt peak as it quickly dropped from about 3.3 to 2.7. In comparison to KLB, the maximum OD₆₀₀ of BiOWiSHTM-Aqua at 3.3 was significantly greater than that of KLB at 2.2, or 1.6×10^9 CFU/mL. OD₆₀₀ fluctuated from 60 to 160 hours, with an upward trend in OD₆₀₀ toward the end. No resemblance of a stationary or a death phase was present in the BiOWiSHTM-Aqua data. The proportion of KLB in BiOWiSHTM-Aqua was unknown. An explanation is that different organisms in BiOWiSHTM-Aqua were growing at different times, hence the high OD₆₀₀ values compared to KLB.

4.4 *B. subtilis* KLB as Biopesticide on Strawberry Fruit Bodies

Strawberries and several beakers of water were placed in a chamber as shown in Figure

3-9. Temperatures ranged from 18-22°C and humidity ranged from 90-99%.

Table 4-21. Average *Botrytis* infection rate (%) four days after biopesticide inoculation.

Trial	Percent of strawberries molded 4 days after biopesticide inoculation				
	<i>B. subtilis</i> KLB			Serenade [®] (undiluted)	Control
	No Dilution	1:10 Dilution	1:100 Dilution		
1	33.3	83.3	33.3	33.3	16.7
2	83.3	50.0	50.0	33.3	50.0
3	100.0	100.0	100.0	83.3	100.0
4	33.3	66.7	88.9	44.4	55.6
5	50.0	100.0	87.5	75.0	100.0
Avg infection rate (%)	60.0	80.0	71.9	53.9	64.4

Figure 4-35. Average *Botrytis* infection rate (%) four days after biopesticide inoculation from Table 4-21 data.

The original concentrations of *B. subtilis* KLB inoculum were approximately 5.0×10^8 CFU/mL. Commercial product Serenade® Garden Disease Control Spray (*B. subtilis* QST 713) was at the unknown original concentration. The control contained no biopesticide.

Table 4-21 and Figure 4-35 show the *Botrytis* infection rate (%) of gray mold-infected strawberries four days after biopesticide inoculation. Trials 1 to 4 contained identical conditions with strawberry placement as shown in Figure 3-10 and described in Section 3.6.1. Trial 5 was conducted using the modified scheme in Figure 3-11 and described in Section 3.6.2. Infection typically began three days (72 hours) after biopesticide

inoculation. Shortly after, the fruit body itself was broken down by the *Botrytis* (shown by a puddle of red juice).

The data in Table 4-21 were analyzed using Analysis of Variance (ANOVA F-test) to determine whether average *Botrytis* infection rates between the five biopesticide groups were statistically different (Appendix B). The null hypothesis was average infection rates between the five groups were equal, and the alternative hypothesis was at least one average infection rate was different from the others. There was insufficient evidence ($F = 0.64 < 2.87$) to reject the null hypothesis at the five percent significance level. In other words, there was no statistical evidence that the average *Botrytis* infection rates between the five biopesticide groups were different. Therefore, none of the biopesticides had an effect on *Botrytis* infection rates of strawberries at 18-22°C and 90-99% humidity in a chamber.

CHAPTER 5: CONCLUSIONS

5.1 Experimental Conclusions

The original goal of this research was to investigate the potential of BiOWiSH™-Aqua as a biopesticide against *Botrytis*. Results were measured as percent *Botrytis* growth on plate, which resulted in varied percentages of growth because every plate possibly contained a different cell composition. This led to results that were varied even within plates in triplicate. The lack of homogeneity of commercial product BiOWiSH™-Aqua was the probable cause of the vast differences. On average, BiOWiSH™-Aqua somewhat inhibited *Botrytis* growth, but was far from an optimal biopesticide.

The organism responsible for *Botrytis* inhibition was isolated from the BiOWiSH™-Aqua plates. Inhibition zones were seen around these specific colonies, which were identified as an isolate (*B. subtilis* KLB) courtesy of Alice Hamrick and Dr. Christopher Kitts. The closest sequence match was to *B. subtilis* BSn5 (100% Max ident), which contains antimicrobial activity against plant pathogen *Erwinia carotovora*. It was probable that KLB produced an antibiotic substance, such as the antifungal lipopeptide fengycin, to inhibit *Botrytis* growth (Vanittanakom, 1986). Hang (2005) had similar success with another strawberry biopesticide, *B. subtilis* S1-0210, which produces antibiotic substance that diffuses into the agar medium and inhibits the mycelial growth of *Botrytis*.

Once KLB was isolated, it was tested in place of BiOWiSH™-Aqua as the biopesticide used against *Botrytis*. KLB was much more successful and predictable in terms of *Botrytis* inhibition. In contrast to BiOWiSH™-Aqua, KLB showed a trend in which the longer KLB grew on plates before *Botrytis* inoculation, the less concentrated the KLB culture needed to be for complete *Botrytis* inhibition. The threshold was 3.2×10^6 CFU/mL of KLB at application time of 0 hours, as opposed to 1.3×10^5 CFU/mL and 9.1×10^4 CFU/mL at 24 and 48 hours, respectively. KLB concentration also mattered much less at higher application times. *Botrytis* growth was reduced by 28 percent for every log increase in KLB concentration at application time of 0 hours, as opposed to only 6.5 percent at 24 and 48 hours.

The probable cause of these trends was that KLB produced an antifungal agent during the application time before the inoculation of *Botrytis*. This means at application time 0 hours, *Botrytis* grew before the antifungal agent was produced because KLB started growing at the same time. Given that commercial product BiOWiSH™-Aqua is used for various applications due to the multitude of organisms, the potential of using KLB as a specialized product for strawberries was explored.

Subsequent experiments involved investigation of KLB for use as a commercial product directly on strawberries. First, commercially available carbon sources, liquid molasses and cooked tapioca balls, were tested to determine whether cell counts similar to those grown in LB could be achieved. While results varied between trials, every trial showed

15 g/L of tapioca was the optimal amount of carbon source. This makes sense because tapioca is a starch (a polysaccharide consisting of many glucose molecules) and KLB is very closely related to *B. amyloliquefaciens*, which can metabolize tapioca using amylases to break down the starch (Rao, 2007). However, the tapioca made the solution very turbid because residue was very apparent. Flocculation may have contributed to the disparity in cell counts of each trial as shown in Figure 4-32.

Another experiment to characterize both BiOWiSHTM-Aqua and the isolate was to compare their OD₆₀₀ values at different times. Seen in Figure 4-34, the KLB growth curve was a characteristic growth curve with clearly defined lag, log, stationary, and death phases. In contrast, BiOWiSHTM-Aqua growth was more random over time, with OD₆₀₀ increasing even when KLB was well into its death phase. This indicates that different organisms grew at different times. OD₆₀₀, which was used to calculate cell counts, did not identify particular cells. Rather, the BiOWiSHTM-Aqua growth curve just showed the aggregate of all of the organisms growing and dying. While one organism was in the death phase, another organism may have been in the log phase. The proportion of KLB in BiOWiSHTM-Aqua at any given time was not determined.

Using a biopesticide on post-harvest strawberries did not inhibit gray mold much. A similar result was described previously in which pre-application (on leaves and flowers) of a biopesticide was much more effective than post-application on ripe strawberries (Hang, 2005). Another problem was high humidity in the chamber at 90 to 99 percent,

which promoted fungal growth. The data in Table 4-21 as well as the ANOVA show that there was no significant difference between using a biopesticide and not using a biopesticide on post-harvest strawberries. In other words, KLB worked well to inhibit *Botrytis* in plate (*in vitro*) but not on strawberries (*in vivo*).

5.2 Future Research

The success of *in vitro* experiments using *B. subtilis* KLB as a biopesticide against *Botrytis* suggests that KLB should be successful in field trials. Hang (2005) showed *B. subtilis* S1-0210 could reduce infection rates from 70 percent in an untreated control to under five percent using a wettable powder formation of the biopesticide for pre-treatment. Field trials confirming these results should be conducted by comparing the following: using fumigation (chemical pesticide) only, using KLB only, and planning IPM by reducing chemical usage and increasing biological usage (biopesticides).

Post-harvest treatment testing by using KLB to increase shelf life of strawberry fruits can also be conducted. Lowering the temperature along with decreasing humidity may lead to more success in *Botrytis* inhibition.

Other tests related to the commercial feasibility of using KLB should be done. Some of the factors that determine whether KLB is an economic alternative are the ease of successfully growing the organism in large batches and the use of cheap commercial

ingredients in the media (molasses and tapioca). While my experiments aimed at these concerns, the results were preliminary. More bench-top scale fermentation in a bioreactor needs to be conducted to determine whether scaling up from flasks can be successful in terms of cell counts while minimizing contamination.

Once field trials confirm the effectiveness of KLB on *Botrytis* inhibition, the main priority will be to conduct research on its commercial feasibility as a biopesticide against *Botrytis* to address the increasing need of alternative pesticides needed in the California strawberry industry.

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Appendix A

Regression Analysis: % Growth of Botr versus T(app), hr, log C(KLB) - Figure 4-26

The regression equation is

% Growth of Botrytis on plate = 127 - 0.928 T(app) - 18.6 log C(KLB)

156 cases used, 117 cases contain missing values

Predictor	Coef	SE Coef	T	P
Constant	127.128	7.423	17.13	0.000
T(app)	-0.9281	0.1064	-8.73	0.000
log C(KLB)	-18.631	1.415	-13.16	0.000

S = 21.9864 R-Sq = 54.8% R-Sq(adj) = 54.2%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	2	89718	44859	92.80	0.000
Residual Error	153	73960	483		
Total	155	163679			

Source	DF	Seq SS
T(app)	1	5963
log C(KLB)	1	83755

Regression Analysis: % Growth of Botr versus T(app), hr, log C(KLB) - Figure 4-27

The regression equation is

% Growth of Botrytis on plate = 143 - 2.93 T(app) - 22.1 log C(KLB)
+ 0.453 interaction

108 cases used, 84 cases contain missing values

Predictor	Coef	SE Coef	T	P
Constant	143.24	13.98	10.24	0.000
T(app)	-2.9341	0.5297	-5.54	0.000
log C(KLB)	-22.077	2.702	-8.17	0.000
interaction	0.4525	0.1125	4.02	0.000

S = 17.9677 R-Sq = 50.3% R-Sq(adj) = 48.8%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	3	33916	11305	35.02	0.000
Residual Error	104	33575	323		
Total	107	67491			

Source	DF	Seq SS
T (app)	1	11679
log C (KLB)	1	17016
interaction	1	5221

Regression Analysis: % Growth of Botrytis on plate versus log C(KLB), Tapp = 0 hours - Figure 4-28

The regression equation is
 % Growth of Botrytis on plate = 181 - 28.0 log C (KLB)

33 cases used, 33 cases contain missing values

Predictor	Coef	SE Coef	T	P
Constant	181.38	13.18	13.76	0.000
log C (KLB)	-28.024	2.466	-11.36	0.000

S = 14.6829 R-Sq = 80.6% R-Sq(adj) = 80.0%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	27830	27830	129.09	0.000
Residual Error	31	6683	216		
Total	32	34513			

Regression Analysis: % Growth of Botrytis on plate versus log C(KLB) – Figure 4-29

The regression equation is
 % Growth of Botrytis on plate = 38.9 - 6.49 log C (KLB)

75 cases used, 51 cases contain missing values

Predictor	Coef	SE Coef	T	P
Constant	38.95	12.26	3.18	0.002
log C (KLB)	-6.485	2.791	-2.32	0.023

S = 16.0403 R-Sq = 6.9% R-Sq(adj) = 5.6%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	1389.7	1389.7	5.40	0.023
Residual Error	73	18782.2	257.3		
Total	74	20171.9			

Appendix B

One-way ANOVA: KLB Undiluted, KLB 1:10, KLB 1:100, Serenade, Control - Figure 4-35

Source	DF	SS	MS	F	P
Factor	4	2081	520	0.64	0.638
Error	20	16190	809		
Total	24	18271			

S = 28.45 R-Sq = 11.39% R-Sq(adj) = 0.00%