EFFECT OF CO-CULTURING *STREPTOMYCES GRISEUS* WITH SELECTED INDUSTRIAL MICROBES TO OPTIMIZE ANTIBIOTIC YIELDS

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Introduction

Co-cultures

Microorganisms often exhibit interspecies interactions in nature. This co-existence of different species can incur helpful, synergistic interactions in secondary metabolite production (9). Often, these interactions result in the production of secondary metabolites that help the organisms compete for limited space and resources. In the laboratory, secondary metabolite producing microorganisms can be co-cultured with other microorganisms in order to simulate these natural interactions. This technique has produced many benefits when compared to pure cultures. Some examples of the benefits include enhanced yields of biological molecules, higher growth rates, better utilization of mixed substrates, and protection from contamination because contaminants are less likely to flourish in a co-culture (11). Multiple studies have shown the many applications to the benefits of co-culturing; including the production of useful consumer goods, increased yields of industrial compounds, and the discovery of novel biological compounds.

Co-cultures used to Produce Fermented Foods

The technique of co-culturing microbes has been around for thousands of years and has been used to produce many of the fermented foods and beverages consumed around the world. The mixed species used for the production of these foods are responsible for the flavors and aromas that we have come to know from fermented dairy, vegetables, and beverages. Co-cultures are also being used in food fermentation to add to the nutritional value and safety of foods.

Co-culturing is a very important component of many popular dairy products; including yogurt, cheese, sour cream, and butter. Yogurt requires the use of multiple species of lactic acid bacteria grown in the same culture to produce the lactic acid and flavors associated with the ancient fermented milk product (38). The most common microbes found in yogurt fermentation include *Lactococcus salaivarius* subsp. *thermophilis*, *Lactobacillus delbrueckei* subsp. *bulgaricus*, *Lactobacillus acidophilus* and *Bifidobacterium bifidum* among others. It has been found that more diverse probiotics are found in yogurt when it is produced in a co-culture. Cheese is another fermented dairy product that owes its aromas and flavors to co-cultures of bacteria and molds. Most cheeses are produced from a starter culture that contains the various types of bacteria and molds needed to produce the desired variety of cheese (36). Although the list of different microbes used to make cheese is far too extensive to list here, some include *Penicillium roqueforti*, *Lactococcus cremoris*, *Lactococcus lactis*, and *Propionibacterium* species (16). It has been found that when *Enterococcus faecium* is added to the starter cultures for cheese, it produces bacteriocins capable of inhibiting *Listeria* and *Clostridium*, two potential contaminants (21). Sour cream fermentation utilizes the co-culture of *Lactococcus cremoris*, *Lactococcus lactis*, and *Leuconostoc cremoris* to produce lactic acid and give it its flavor (16). For butter, the sour cream is churned to allow oxygen for *Leuconostoc* to produce diacetyl from citric acid, which imparts characteristic flavor and aroma associated with butter.

Fermented vegetables also use co-culturing to produce many foods and condiments that we have become familiar with. Soybeans are a major contributor to fermented foods that require co-cultures. Soy sauce is a product of soybeans that are

co-cultured with *Aspergillus oryzae* and *Aspergillus soyae* in a solid fermentation (koji) before being co-cultured with multiple yeasts and lactic acid bacteria in a liquid (maromi) brine fermentation (11). Miso, another fermented soybean product, is a paste that uses the same co-culture as the solid fermentation of soy sauce with the addition of *Saccharomyces rouxii* and *Candida* species. Soybeans are also used in the production of tempeh, which uses co-cultures of *Bacillus subtilis* and *Rhizopus oligosporus* to ferment this legume. It has been found that when *Klebsiella* is added to the tempeh fermentation, vitamin B_{12} is produced (10). This indicates that co-culturing can increase the nutritional value of food products. Other vegetables using co-cultures for fermentation include cucumbers for pickles and cabbage for sauerkraut (16). To produce pickles, cucumbers are often fermented in a brine solution with a co-culture of *Lactobacillus plantarum* and *Pichia cerevisiae*. For sauerkraut, salted cabbage is co-cultured with the microbes that naturally grow on the cabbage. These include *Enterobacter cloacae*, *Leuconostoc mesenteroides*, and *Lactobacillus plantarum*, which grow in shifts as the fermentation cultures select for the different species.

Co-cultures are needed to produce some very popular alcoholic beverages; the most prevalent being wine. Grape juice (must) contains several types of yeast and bacteria that influence the flavor and aroma of the wine (1). *Saccharomyces cerevisiae* is the yeast used to produce the ethanol found in wine, but other yeasts, including non-*Saccharomyces* genera, add to the flavor and aroma complexity of wine (4). Aside from the co-cultures of yeast in the grape must, there are also co-cultures of lactic acid bacteria used for the malo-lactic fermentation to produce the final wine product (1). Another alcoholic beverage that requires the use of co-culturing is sake. This traditional

Japanese spirit uses *Aspergillus oryzae* and *Saccharomyces cerevisiae* to biodegrade the soybeans and ferment ethanol respectively (11).

Without co-culturing, the array of fermented foods and beverages to choose from would be far more limited. Many delicious and complex flavors would not exist without the benefit of utilizing more than one microorganism producing these fermented foods. Also, many nutritionally important molecules, such as probiotics and vitamins, would be absent or lacking in these foods without the synthesis from microbe species in these co-cultures.

Co-cultures used to Increase Industrial Production

Multiple studies have shown that microbes grown in a co-culture are capable of increasing the yields of their secondary metabolites. The secondary metabolites observed to be increased through the use of co-cultures include antimicrobial compounds, anticancer molecules, ethanol, and extracellular enzymes. Although it is still unclear exactly how co-cultures can increase yields of industrially important metabolites, it may be because the interspecies interactions turn on previously silent/cryptic genes (34) or the consortia more efficiently uses the nutrients metabolized from the media (3).

Researchers have found that antimicrobial compound production can be increased when an antimicrobial producing microorganism is co-cultured with other microbes. When 53 different species of marine bacteria were co-cultured in liquid media with *Streptomyces tenjimariensis*, a marine bacterium known for producing the antibiotic istamycin, researchers found that 12 of the co-cultures resulted in a 2-fold

increase of istamycin production over the amount of istamycin produced in pure cultures of *S. tenjimariensis* (37). They found that *S. tenjimariensis* needed to be added 1 day before the other microbes. If the other microbes were added first, a significant reduction in istamycin yields occurred. Another example of an increase in antimicrobial production comes from the co-culture of microbes found in tempeh production (10). It was found that when *Bacillus subtilis* was co-cultured with *Rhizopus peka* or *Rhizopus oligosporus*, antimicrobial activity in the fermentation culture increased. The *Rhizopus* species did not produce any antimicrobial activity on their own. The researchers did not identify which antimicrobial produced by *B. subtilis* was increased. The exposure of 16 different marine bacteria to heat-killed *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* in culture also resulted in increased antimicrobial activity when compared to pure cultures of the marine bacteria (27). This study was unable to determine whether the cell walls of these heat-killed bacteria or other compounds were driving the induction mechanism.

Co-culturing has also been found to increase the production of the anticancer and immunosuppressant molecule, undecylprodigiosin (25). Undecylprodigiosin is produced by the bacterium *Streptomyces coelicolor*. When this bacterium was grown with *E. coli* in the same bioreactor, the researchers found up to a 6-fold increase in undecylprodigiosin. They found that undecylprodigiosin was increased the most when *S. coelicolor* and *E. coli* were inoculated into the bioreactor at the same time. Interestingly, an increase of this molecule was also found when heat-killed *Bacillus subtilis* and *Staphylococcus aureus* were added to the bioreactor containing *S.*

coelicolor (24). The heat-killed cells were added to the bioreactor at the same time as *Streptomyces coelicolor*.

Along with antimicrobials, co-culturing has also been found to increase yields in ethanol and natural enzyme production. When looking for a cheaper and more efficient way to produce ethanol, a co-culture of *Saccharomyces cerevisiae* with the fungi *Trametes hirsute, Trametes versicolor* and *Chalara parvispora* produced 3 times more ethanol compared to *Saccharomyces cerevisiae* alone (12). This increase in ethanol production is a result from better utilization of carbohydrates in the culture medium by the consortium. Other ethanol research involving co-cultures are utilizing xylose-fermenting microbes with glucose-fermenting microbes to increase ethanol production using lignocellulose as the raw material. Multiple different combinations of microbes, including *Saccharomyces cerevisiae*, *Pichia stipitus*, *Candida tropicalis*, and *Zymomonas mobilis* have shown the ability increase ethanol production by fermenting xylose and glucose that make up the lignocellulose raw material (3). An increase in extracellular enzyme production has also been found when the fungi *Aspergillus niger* and *Aspergillus oryzae* were co-cultured together in liquid culture (15).

Co-cultures used for Novel Natural Product Discovery

Recently, microbial co-cultures have been found to produce previously undiscovered antimicrobial and anticancer compounds. Many of these compounds have shown effectiveness against microbes that have built a resistance to antibiotics already in use. With so many possibilities of combinations, co-culturing microbes could

be a valuable technique in the search for novel natural compounds to help fight emerging diseases.

A novel benzophenone antibiotic, pestalone, was discovered when a species of the fungus *Pestalotia* was grown with an unidentified marine bacterium (5). It was found that this antibiotic was effective against both methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium*. For the co-cultures, the researchers added the unidentified marine bacterium 1 day after *Pestalotia*. When they tried adding cell-free culture supernatants of the marine bacterium to a *Pestalotia* culture, the antibiotic was not formed. This indicates that pestalone production was induced by living cell-to-cell interactions or by recognizing components found on the cell of the marine bacterium.

The new aminoglycoside antibiotics, rhodostreptomycins A and B, were discovered when *Rhodococcus fascians* and *Streptomyces padanus* were co-cultured (20). These antibiotics were not found in either pure culture. In the co-cultures that produced these novel antibiotics, researchers found that *Streptomyces padanus* DNA was taken up by *Rhodococcus fascians*, indicating that horizontal gene transfer could produce novel antibiotic biosynthesis pathways.

Several new lipoaminopeptides, acremostatins A, B, and C, were produced when the fungi *Acremonium* sp. Tbp-5 and *Mycogene roseum* were co-cultured (6). These species produced their own antibiotics in pure cultures, but the acremostatins were only produced in co-cultures of the two microbes. For their co-cultures, *Acremonium* Tbp-5 was established for 7 days before *M. roseum* mycelia was added to the culture. The authors believe that *M. roseum* adds an acid to the nitrogen terminus of the

leucinostatins already produced by *Acremonium* Tbp-5 when the two species are grown in co-culture. This discovery appears to show that biosynthetic pathways can be combined to produce chimeric antibiotics using co-cultures.

The co-culture of a species of the marine fungus *Emericella* and the actinomycete *Salinispora arenicola* led to the detection of two new cyclic depsipeptides: emericellamides A and B (31). After finding these molecules in the co-culture, the researchers then discovered that they were being produced in the *Emericella* pure cultures, however at a 100-fold decrease when compared to a co-culture. Because the pure culture produced such a small amount of antibiotic, it would have gone undetected in normal analysis. The emericellamides were found to have some antimicrobial activity against methicillin-resistant *Staphylococcus aureus*.

A new xanthone derivative with signs of antifungal activity, 8-hydroxy-3-methyl-9-oxo-9H-xanthene-1-carboxylic acid methyl ether, was discovered when two mangrove fungi were co-cultured (22). The xanthone derivative was not detected in pure cultures of either fungus. The same two fungi were also found to produce an anticancer molecule, (-)-byssochlamic acid bisdiimide, in co-culture but not in their separate pure cultures (23). For their co-cultures, the two different fungi were added to the culture at the same time.

Other anticancer drugs, diterpenoids known as libertellenones A-D, were discovered when a *Libertella* species was co-cultured with the marine bacterium co-cultured with *Pestalotia* species mentioned above (30). These molecules were not produced in pure cultures. For the co-culture, *Libertella* was inoculated into the co-culture 3 days before the other bacterium. When the researchers tried adding heat-killed

bacterial cells or cell-free culture supernatants to the *Libertella* culture, there was no production of libertellenones, indicating that cell-to-cell interactions were needed to produce these molecules.

Streptomyces griseus

Streptomyces griseus is a Gram-positive saprophytic bacterium most often found in soil (2). Known for its contribution to the earthy odor of soil, *S. griseus* is also a major producer of secondary metabolites used in industry. This non-motile species of bacteria belongs to the Actinobacteria phylum and is unusual in the fact that its colonial morphology resembles filamentous fungi when grown on agar media (13).

In the laboratory, a single spore of *S. griseus* is all that is required to begin a colony (13). From the spore, substrate hyphae are formed that attaches to the agar medium and retrieves nutrients. Aerial hyphae then begin to form from the substrate hyphae that eventually lead to the production of more spores. As aerial hyphae are produced, *S. griseus* begins producing secondary metabolites in laboratory cultures (14). These secondary metabolites involve the production of multiple different types of antibiotics. The strain of *S. griseus* used in our lab is capable of producing both streptomycin and cycloheximide.

Streptomycin

Streptomycin is an antibacterial aminoglycoside antibiotic capable of inhibiting prokaryotic ribosomal function. Although there is much controversy surrounding the history of streptomycin, it was discovered by Dr. Selman Waksman and his graduate

student in 1943 while looking for a treatment for tuberculosis (17). They found that their strain of *S. griseus* was capable of producing an antibacterial compound when grown on an agar plate with tuberculosis bacteria. The antibiotic proved to be very effective in treating tuberculosis and the plague (8). Currently, streptomycin is used alongside other antibiotics to fight infections caused by the *Streptococcus* and *Mycobacterium* genera. The aminoglycoside is still capable of treating the plague without the help of other antibiotics.

Like all aminoglycoside antibiotics, streptomycin inhibits protein synthesis by binding irreversibly to 30S ribosomes found in bacteria (8). To reach the ribosomes and be effective, streptomycin needs to be taken up by the bacteria. This is why streptomycin is generally more successful in the presence of other antibiotics, especially β-lactams that can prevent cell wall synthesis and enable entry of streptomycin into the bacterial cells.

The biosynthesis of streptomycin by *S. griseus* is complex process. This bacterium does not immediately produce streptomycin at the beginning of fermentation, also known as the trophophase when cells are growing exponentially (26). Instead, the antibiotic is produced during the idiophase, the period when cell growth levels off and nutrients become limited. This occurs just after *S. griseus* begins to produce the molecule known as A-factor. A-factor (2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone) is a bacterial autoregulatory hormone that controls secondary metabolite and sporulation in *S. griseus* (14). The genes encoding the biosynthesis of streptomycin are repressed by the A-factor-binding protein while the *S. griseus* cells mature during trophophase (28). Once A-factor is produced, it binds to A-factor-binding

protein, which releases from the promoter of the streptomycin biosynthesis genes and allows for the production of streptomycin (14).

Cycloheximide

Cycloheximide is an antifungal antibiotic capable of inhibiting protein synthesis in eukaryotes. The history of cycloheximide is not as well documented as streptomycin, but it was first noticed by Dr. Selman Waksman shortly after the discovery of streptomycin (39). He referred to it as *Streptomyces griseus*' "second antibiotic". In 1946, scientists from the Upjohn company in Kalamazoo, Michigan found that this antibiotic possessed no obvious antibacterial activity, but was effective at inhibiting *Cryptococcus neorformans*, indicating that it was an antifungal agent (40). The antibiotic was originally named actidione (41) but eventually became known as cycloheximide. It has since become the most common reagent used in the laboratory grown microbe cultures to prevent protein synthesis (35).

Cycloheximide works by blocking the elongation phase of eukaryotic translation (35). This occurs by preventing the movement of transfer RNA into and out of the donor site of ribosomes (29). In sufficient concentrations, this blocking of translation results in complete inhibition of eukaryotic protein synthesis.

Although cycloheximide is a relatively old antibiotic, not much is known about its biosynthetic pathway (7). There is very little literature regarding how cycloheximide is synthesized, but many studies have been conducted to see how the antibiotic is controlled. Cycloheximide production has been found to be controlled by end-product repression (19). This was confirmed when cycloheximide was added to a culture of

cycloheximide-producing *Streptomyces griseus* and cycloheximide production decreased (33). It was also found that this feedback inhibition control could be relieved by physically removing the cycloheximide from the fermentation as it was produced (18). The physical removal was performed through the use of dialysis fermentation.

Research Objectives

The objective of this research was to test multiple co-cultures containing different combinations of *S. griseus* and challenge microbes to determine if streptomycin and/or cycloheximide production could be optimized. We believed that one combination would exceed the others in terms of antibiotic production. The conditions of this combination could then be optimized to further increase antibiotic production yields. The methods and results of these experiments could then be used as a model for the optimization of secondary metabolite production in industry.

Materials and Methods

Co-cultures

Streptomyces griseus (isolated by BSU undergraduate student John Jarosh from plant compost in 1997) and the challenge microbes were each cultured in a seed medium first. This medium was then used to inoculate the production (fermentation) medium. The seed medium consisted of (g/L): 10.0 glucose, 20.0 soluble starch, 5.0 yeast extract, 5.0 NZAmine A, and 1.0 $CaCO_3$. Fifty mL of this seed medium was first sterilized within an Erlenmeyer flask. Once the seed medium was prepared, each flask containing seed medium was inoculated with a challenge microbe or *S. griseus*. The challenge microbe flasks were aseptically inoculated with two separate colonies that were approximately the same size grown on plates containing seed medium that also contained 1.5% (w/v) agar. The *S. griseus* flasks were inoculated with 0.5 mL of thawed suspension of *S. griseus* stored at -80°C. The seed flasks inoculated with the challenge microbes were placed on an orbital shaker running at 210 rpm at 24°C for 7 days. The seed flasks inoculated with *S. griseus* were placed on an orbital shaker using the same settings but were only grown for 48 hours. After their respective times of growth, the seed cultures were removed from the shaker, blended with three 5-second bursts in a sterile stainless steel blender, and used to inoculate the antibiotic production medium.

The production medium consisted of (g/L): 60.0 glucose, 15.0 white bean flour, 2.5 yeast extract, 5.0 $(NH_4)_2SO_4$, 8.0 $CaCO_3$, 4.0 NaCl, and 0.2 KH_2PO_4. The production medium was first sterilized in an 250mL Erlenmeyer flask (5 flasks/treatment), allowed to cool to room temperature, inoculated with 0.5 mL of the blended *S. griseus* seed culture, and placed on an orbital shaker adjusted to 210 rpm at

24°C for 7 days. After the first 24 hours on the shaker, 0.5 mL of blended challenge microbe seed culture was aseptically added to the production medium to evaluate the effects of co-culturing on antibiotic yields. Following harvest of the production flasks, an aliquot was removed and stored at -80°C to perform future bioautograms. Positive control flasks of production medium contained pure cultures of *S. griseus* and negative control flasks contained pure cultures of the challenge microbe(s). A total of 15 different challenge microbes were evaluated at random: *Penicillium chrysogenum, Fusarium oxysporum, Saccharomyces cerevisiae, Pullularia pullulans, Rhizobium leguminosarum, Saccharopolyspora erythraea, Streptomyces fradiae, Streptomyces antibioticus,* and *Streptomyces* isolates (*Strepto*1, *Strepto*4, *Strepto*8, S2A, S4A, *Strepto*Blue1 and *Strepto*Blue2). Challenge microbes were previously identified by Dr. James Mitchell and obtained from Ball State University. These were chosen because they significantly increased antibiotic production in previous experiments (32).

Cycloheximide Bioassay

To observe cycloheximide (anti-fungal) production, the susceptible fungus *S. cerevisiae* NRRL Y-139 was cultured on potato dextrose agar (PDA) plates and incubated at 24°C for two days. After incubation, the *S. cerevisiae* cells were removed using a sterile swab and suspended in sterile 0.85% w/v NaCl solution. This *S. cerevisiae* solution was then adjusted to McFarland #10 turbidometric standard to maintain a consistent concentration. The turbid yeast solution was then added to 50°C sterile TGY agar (ATCC Medium #123) at 0.5% v/v, resulting in a yeast-seeded agar. The TGY agar was composed of (g/L): 3.0 glucose, 3.0 tryptone, 3.0 yeast extract, 1.0 K_2HPO_4, and 15.0 agar. The yeast-seeded agar suspension was mixed and 10 mL was

aseptically pipetted into each empty sterile plastic Petri dish (15x100mm). The seeded agar plates were then refrigerated for 24 hours, after which they were used to conduct the Kirby-Bauer assay for cycloheximide production.

Streptomycin Bioassay

To observe streptomycin (anti-bacterial) production, 5 mL of tryptic soy broth was inoculated with a loopful of the susceptible bacterium *Bacillus subtilis* NRRL B-765 and incubated for 20 hours at 37°C. Following incubation, the culture was vortexed and aseptically added to 50°C sterile TGY agar at 1% v/v, resulting in a bacteria-seeded agar. This suspension was mixed and 10 mL was aseptically pipetted into each empty sterile plastic Petri dish. The seeded agar plates were then refrigerated for 24 hours, after which they were used to conduct the Kirby-Bauer assay for streptomycin production.

Kirby-Bauer Protocol

To quantify the cycloheximide and streptomycin activity in the production medium cultures, the Kirby-Bauer bioassay technique was used. After harvest of the production flasks following 7 days incubation, a 1:20 dilution of the production suspension was performed for the cycloheximide bioassay and no dilution of samples was performed for the streptomycin bioassay. Twenty µL of sample was added per 6mm diameter paper disk (Whatman; Cat No. 2017-006) and each disk was placed onto their respective microbe-seeded agar plate to assay for antibiotic activity. Bioassay plates were cultured for 24 hours (incubated at 28°C for cycloheximide; 37°C for streptomycin) to

allow sufficient time for the antibiotics to diffuse radially from disks after which the diameter of the antibiotic inhibition zones were measured. The Kirby-Bauer bioassay technique was also performed from two-fold serial dilutions (2000 – 2 μg/mL) of purified cycloheximide and streptomycin sulfate using stock solutions prepared in deionized water. Results from known antibiotics were used to generate best-fit regression models (standard curves) using Minitab 16 Statistical Software. These models were employed to calculate the amounts of antibiotic activity (μg/ml) found in production media flasks and compared using One-Way ANOVA.

Optimization: Time of Challenge Microbe Addition

Seed cultures of *S. griseus* and the challenge microbes were inoculated and grown in seed medium as described above. However, for the treatments, challenge microbes were added to the production medium 2 days before inoculating *S. griseus*, 1 day before inoculating *S. griseus*, the same time as *S. griseus* (day 0), and 1, 2, 3, 4, 5, and 6 days after inoculating *S. griseus*. The production medium was then shaken as described above for 7 days following inoculation of *S. griseus* on day 0. The streptomycin and cycloheximide bioassays with the Kirby-Bauer protocol described above were used to quantify the amount of each antibiotic produced by each treatment.

Optimization: Effect of Heat-kill

Two colonies of *Strepto*8 were added to a seed flask prepared as described above and placed on an orbital shaker adjusted to 210 rpm at 24°C for 7 days. The seed culture was then removed from the shaker and blended with three 5 second bursts

in a sterile stainless steel blender. Ten mL of this blended culture was added to a #18 test tube that was submerged in a boiling water bath (100°C). Following 0, 5, 10, 15, 20, 30, 40, 50, and 60 minutes of boiling, 0.2 mL of cell suspension was removed and aseptically spread (using glass hockey stick) onto seed medium plates containing 15% agar. Plates were incubated for 4 days at 28°C, colonies counted on each plate and the results were recorded in cfu/mL. This experiment was only performed once (with one plate per time point) to determine amount of time required to heat kill *Strepto*8 cells that would be used in the experiment described below. The second time point that was found to result in <1 cfu/mL (15 minutes) was used for the living vs. dead vs. cell-free challenge microbe experiment.

Optimization: Living vs. Dead vs. Cell-Free Challenge Microbe

Seed cultures of *S. griseus* and the challenge microbe were inoculated and grown in seed medium as described above. Three of the seed cultures were removed from the shaker and pooled together into one flask. After mixing, 20 mL of seed culture was aliquoted into a sterile centrifuge tube on ice. This was used for Part A (live intact cells). The remaining seed culture (~130 mL) was blended with three 5-sec bursts in a stainless steel blender and aseptically poured into an empty, sterile 250 mL Erlenmeyer flask kept on ice. This portion was used for Parts B-E (described below). After inoculation, the production medium was then cultured as described above for 7 days following inoculation of *S. griseus*. The streptomycin and cycloheximide bioassays with the Kirby-Bauer protocol described above were used to quantify the amount of each antibiotic produced by each treatment. A flow diagram summarizing these treatments is shown in Figure 1.

Part A details the method used to inoculate the production flasks with unblended (intact), washed, live challenge microbe cells. The 20 mL aliquot taken from the pooled seed culture was centrifuged at 15,000Xg at 4°C for 10 minutes to separate the cells from the supernatant. The supernatant was then discarded and the pellet was resuspended in 20 mL sterile 0.85% NaCl before centrifuging at 15,000Xg at 4°C for 10 minutes again. Following centrifugation, the supernatant was discarded and the pellet was resuspended again in 20 mL sterile 0.85% NaCl before centrifuging at 15,000Xg at 4°C for 10 minutes again. After this centrifugation, the supernatant was discarded and the pellet was resuspended in 20 mL sterile 0.85% NaCl. Five hundred µL of this suspension was then placed into each of the production medium flasks.

Part B details the method used to inoculate the production flasks with live (blended) unwashed challenge microbe seed culture used as the control. Five hundred µL of blended, pooled seed culture was placed into each of the production medium flasks, as performed in the co-cultures method above.

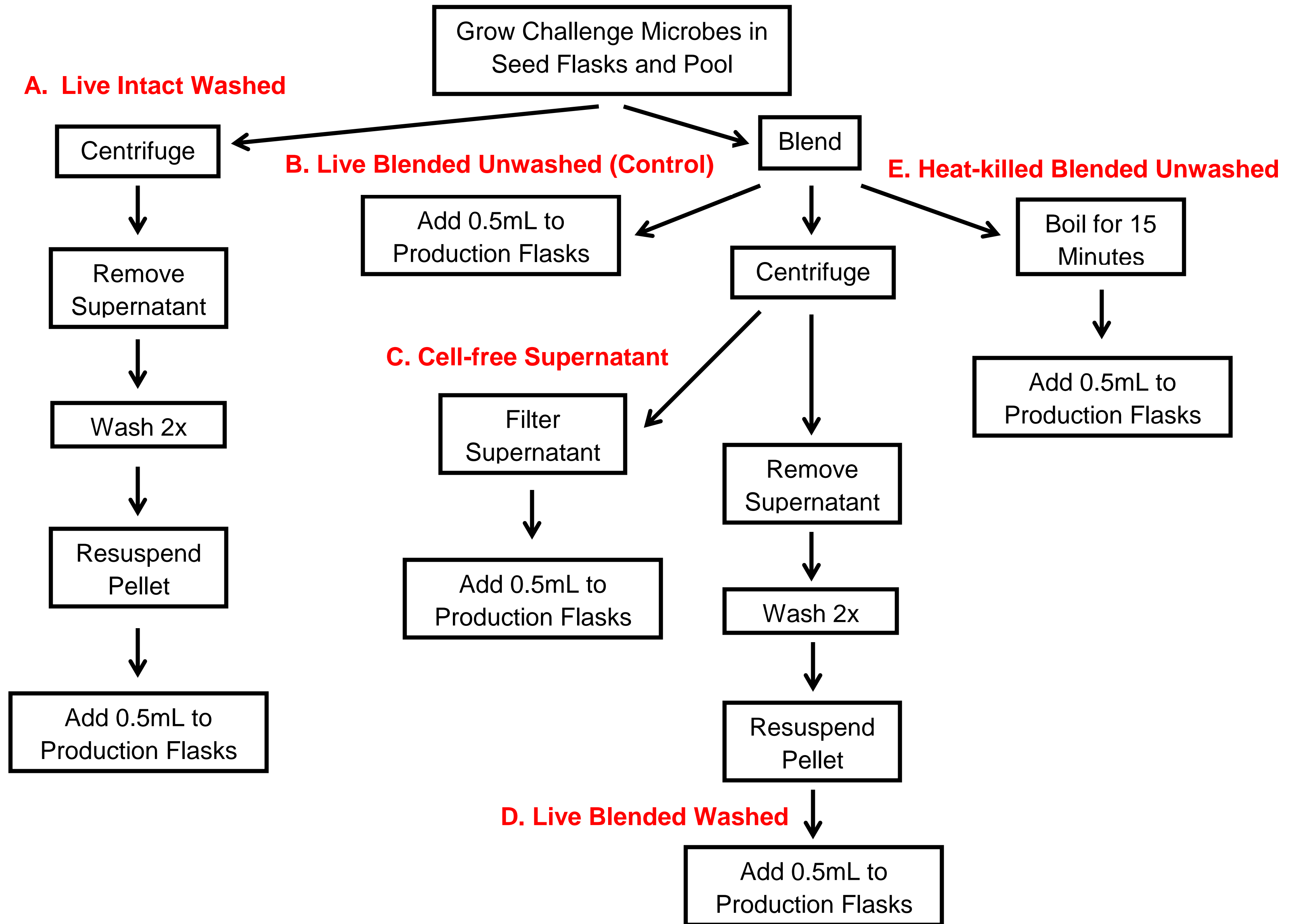
Part C details the method used to inoculate the production flasks with cell-free culture supernatant. Twenty mL of blended, pooled seed culture was centrifuged at 15,000Xg at 4°C for 10 minutes to separate the cells from the supernatant. The supernatant was aseptically poured into a sterile #18 test tube and placed on ice. This was then filter sterilized using a 0.45 µm Millipore syringe filter. Five hundred µL of the sterile supernatant was inoculated into each of the production medium flasks.

Part D details the method used to inoculate the production flasks with blended, washed, live challenge microbe cells. Twenty mL of blended, pooled seed culture was centrifuged at 15,000Xg at 4°C for 10 minutes to separate the cells from the

supernatant. The supernatant was then discarded and the pellet was resuspended in 20 mL sterile 0.85% NaCl before centrifuging at 15,000Xg at 4°C for 10 minutes again. Following centrifugation, the supernatant was discarded and the pellet was resuspended again in 20 mL sterile 0.85% NaCl before centrifuging at 15,000Xg at 4°C for 10 minutes again. After this centrifugation, the supernatant was discarded and the pellet was resuspended in 20 mL sterile 0.85% NaCl. Five hundred μL of this suspension was then inoculated into each of the production medium flasks.

Part E details the method used to inoculate the production flasks with heat-killed challenge microbes. Ten mL of blended, pooled seed culture was added to a sterile #18 test tube and submerged in a boiling water bath at 100°C. After 15 minutes, the test tube was removed from the water bath and placed on ice. Five hundred μL of this heat killed seed culture was then inoculated into each of the production medium flasks.

Figure 1. Flow diagram of Living vs. Dead vs. Cell-Free Challenge Microbe Experiment.



Bioautograms

Frozen co-culture production medium containing the microbes in question was removed from the -80°C freezer and allowed to thaw in water at room temperature. Ten mL of this thawed culture was centrifuged at 5000Xg for 10 minutes. The pellet was discarded and the supernatant was filtered through a 0.45 µm Millipore filter attached to a syringe and filtrate placed in a small sterile beaker (antibiotics are suspended in filtrate). Fifteen and 30 µL of filtered production medium culture were applied/spotted 2.5 cm from the bottom of a 25mm x 75 mm thin layer chromatography (TLC) sheet (Baker-flex, Silica gel IB2). Along with these sheets, a spot containing 15 µL of production medium + 5 µL of 1 mg/mL cycloheximide and a spot containing 15 µL of production medium + 5 µL of 1 mg/mL streptomycin sulfate were also added to a TLC sheet to compare migration of these known antibiotics to production flask samples. Five µL spots containing only 1 mg/mL cycloheximide or 1 mg/mL streptomycin sulfate were also added to the TLC sheets to compare migration of the pure antibiotics. After drying with blow-dryer, the chromatograms were run using an ethanol:acetone (1:2) solvent system for 75 minutes to separate antibiotics. Chromatograms were then air dried within a chemical fume hood and placed onto the surface of their respective TGY seeded agar plates, prepared as described above using 22x22 cm square Petri dishes containing 80 mL of medium, for 60 seconds to allow transfer antimicrobials. The chromatograms were then removed and the seeded plates were incubated for 24 hours (28°C for antifungal; 37°C for antibacterial), after which the inhibition zones were measured and the distance the compound traveled on the chromatogram (Rf values) were recorded.

Results

Phase I

For Phase I, 15 different microbes (11 bacteria and 4 fungi) demonstrated previously to improve antibiotic yields (when co-cultured individually) with *S. griseus* were evaluated in co-cultures containing up to 3 challenge microbes + *S. griseus* to simulate natural interactions. Five experiments, each containing 3 randomly assigned microbes and *S. griseus*, were used to assess all 15 challenge microbes using fractional-factorial design previously described. Phase I was performed to determine if >2 microbes could be co-cultured together and improve antibiotic yield.

Experiment A

The three different challenge microbes used in Experiment A were *Streptomyces antibioticus*, *Rhizobium leguminosarum*, and *Strepto*8. These challenge microbes were co-cultured with *S. griseus* in different combinations as well as grown in pure cultures. Results are summarized in Tables 1-3.

Table 1. Experiment A Cycloheximide Results using One-Way ANOVA.

Treatment	Mean (µg/mL)	Tukey	LSD
S. griseus	530.8	bc	c
S. griseus + *S. antibioticus**	618.1	ab	bc
S. griseus + *R. leguminosarum*	681.5	ab	bc
S. griseus + *S. antibioticus** + *R. leguminosarum*	647.3	ab	bc
S. griseus + *Strepto*8	725.0	ab	ab
S. griseus + *S. antibioticus** + *Strepto*8	889.0	a	a
S. griseus + *R. leguminosarum* + *Strepto*8	606.2	abc	bc
S. griseus + *S. antibioticus** + *R. leguminosarum* + *Strepto*8	693.1	ab	bc
*S. antibioticus**	331.0	c	d
R. leguminosarum	0.0	d	e
*Strepto*8	0.0	d	e

Means followed by different letters are significantly different ($P \leq 0.05$).
* Denotes that the challenge microbe independently exhibits antifungal activity.

Table 2. Experiment A Cycloheximide Results using T-test.

Treatment	Observed	Expected	P:Observed=Expected*
S. griseus + *S. antibiotica* + *Strepto*8	889	862	0.730
S. griseus + *S. antibiotica* + *R. leguminosarum* + *Strepto*8	693	862	0.126

*Two-sample T-test significantly different if p ≤ 0.05.

Table 1 provides the cycloheximide results for Experiment A. The *S. antibioticus* challenge microbe produced an antifungal on its own in a pure culture, so any combination containing this microorganism that appeared to increase cycloheximide yields had to be analyzed with a t-test (Table 2). According to the ANOVA (LSD) analysis (Table 1), the *S. griseus* + *S. antibioticus* + *Strepto*8 and *S. griseus* + *Strepto*8 combinations each significantly increased cycloheximide yields. The *S. griseus* + *S. antibioticus*, *S. griseus* + *R. leguminosarum*, *S. griseus* + *S. antibioticus* + *R. leguminosarum*, *S. griseus* + *R. leguminosarum* + *Strepto*8, and *S. griseus* + *S. antibioticus* + *R. leguminosarum* + *Strepto*8 combinations each exhibited no significant effect on cycloheximide production. None of the co-cultures significantly decreased cycloheximide production.

The Tukey analysis found that the *S. griseus* + *Strepto*8 combination had no significant effect on cycloheximide production while all other combinations were the same as the LSD analysis (Table 1). The t-test results demonstrated that the expected and observed cycloheximide values for the *S. antibioticus* combinations were not

significantly different, indicating that no synergy had occurred in cycloheximide production (Table 2).

Table 3 provides the streptomycin results for Experiment A. According to LSD analysis, the *S. griseus* + *R. leguminosarum* + *Strepto*8 combination significantly increased streptomycin production. All other combinations had no significant effect on streptomycin production. According to Tukey analysis, all combinations used for Experiment A exhibited no significant effect on streptomycin production (Tables 1 and 3).

Table 3. Phase I Experiment A Streptomycin Results using One-Way ANOVA.

Treatment	**Mean (µg/mL)**	**Tukey**	**LSD**
S. griseus	475.3	a	b
S. griseus + *S. antibioticus**	464.9	a	b
S. griseus + *R. leguminosarum*	482.8	a	b
S. griseus + *S. antibioticus** + *R. leguminosarum*	584.2	a	ab
S. griseus + *Strepto*8	524.0	a	b
S. griseus + *S. antibioticus** + *Strepto*8	590.2	a	ab
S. griseus + *R. leguminosarum* + *Strepto*8	698.2	a	a
S. griseus + *S. antibioticus** + *R. leguminosarum* + *Strepto*8	506.1	a	b
*S. antibioticus**	3.4	b	c
R. leguminosarum	0.0	b	c
*Strepto*8	0.0	b	c

Means followed by different letters are significantly different ($P \leq 0.05$).
* Denotes that the challenge microbe independently exhibits antibacterial activity.

Experiment B

The three different challenge microbes used in Experiment B were *Streptomyces* S4A, *Strepto*4, and *Streptomyces* S2A. These challenge microbes were co-cultured with *S. griseus* in different combinations as well as grown in pure cultures (Tables 4-7).

Table 4 provides the cycloheximide results for Experiment B. Isolates S4A and S2A challenge microbes produced antifungal activity on their own in a pure culture, so any combination containing these microorganisms that appeared to increase cycloheximide yields had to be analyzed using the t-test (Table 5). According to the LSD analysis (Table 4), the combination containing *S. griseus* + S4A significantly increased cycloheximide production; however, after analyzing t-test results, there was no significant difference between the observed and expected results, indicating that no synergism was occurring (Table 5). All other co-culture combinations had no significant effect on cycloheximide production. The Tukey analysis found the same results. Table 5 also shows significant t-test results for co-cultures containing S2A. However, these results show a negative significant difference, indicating that antagonism is occurring while S2A is in the co-culture.

Table 4. Experiment B Cycloheximide Results using One-Way ANOVA.

Treatment	**Mean (μg/mL)**	**Tukey**	**LSD**
S. griseus	522.8	c	cd
S. griseus + S4A*	907.5	ab	ab
S. griseus + *Strepto*4	677.1	abc	c
S. griseus + S4A* + *Strepto*4	713.0	abc	bc
S. griseus + S2A*	642.2	abc	c
S. griseus + S4A* + S2A*	578.1	bc	cd
S. griseus + *Strepto*4 + S2A*	623.1	bc	c
S. griseus + S4A* + *Strepto*4 + S2A*	665.1	abc	c
S4A*	413.0	c	d
*Strepto*4	0.0	d	e
S2A*	965.6	a	a

Means followed by different letters are significantly different ($P \leq 0.05$).
* Denotes that the challenge microbe independently exhibits antifungal activity.

Table 5. Experiment B Cycloheximide Results using T-test.

Treatment	Observed	Expected	P:Observed=Expected*
S. griseus + *S*4A	907	936	0.873
S. griseus + *S*2A	642	1488	0.004
S. griseus + S4A* + S2A*	578	1901	0.001
S. griseus + *Strepto*4 + S2A*	623	1488	0.002
S. griseus + S4A* + *Strepto*4 + S2A*	665	1901	0.001

*Two-sample T-test significantly different if P ≤ 0.05.

Table 6 provides the streptomycin results for Experiment B. Isolates S4A and S2A challenge microbes produced antibacterial activity on their own in a pure culture, so any combination containing these microorganisms that appeared to increase streptomycin yields had to be analyzed with a t-test (Table 7). According to LSD analysis, none of the combinations used for Experiment B significantly increased streptomycin production. The *S. griseus* + *Strepto*4 combination had no effect on streptomycin production. All other co-culture combinations significantly decreased streptomycin production.

According to Tukey analysis, the only difference was that the *S. griseus* + *S.* S4A + *Strepto*4 combination significantly decreased streptomycin instead of causing no effect (Table 6). All other combinations produced similar results. Table 7 demonstrates that all of the combinations analyzed using the t-test had a negative significant difference between the observed and expected values of streptomycin yields.

Table 6. Experiment B Streptomycin Results using One-Way ANOVA.

Treatment	Mean (µg/mL)	Tukey	LSD
S. griseus	1464.6	ab	a
S. griseus + S4A*	999.1	cd	b
S. griseus + *Strepto*4	1535.5	a	a
S. griseus + S4A* + *Strepto*4	1012.1	bcd	b
S. griseus + S2A*	852.6	d	bcd
S. griseus + S4A* + S2A*	874.7	d	bc
S. griseus + *Strepto*4 + S2A*	581.6	d	d
S. griseus + S4A* + *Strepto*4 + S2A*	611.6	d	cd
S4A*	1368.4	abc	a
*Strepto*4	0.0	e	e
S2A*	975.3	cd	b

Means followed by different letters are significantly different ($P \leq 0.05$).
* Denotes that the challenge microbe independently exhibits antibacterial activity.

Table 7. Experiment B Streptomycin Results using T-test.

Treatment	Observed	Expected	P:Observed=Expected*
S. griseus + *S*4A	999	2833	<0.001
S. griseus + *S*2A	853	2440	<0.001
S. griseus + S4A* + S2A*	875	3808	<0.001
S. griseus + *Strepto*4 + S2A*	582	2440	<0.001

*Two-sample T-test significantly different if P ≤ 0.05.

Experiment C

The three different challenge microbes used in Experiment C were *Pullularia pullulans*, *Saccharopolyspora erythraea*, and *Penicillium chrysogenum*. These challenge microbes were co-cultured with *S. griseus* in different combinations as well as grown in pure cultures (Tables 8-10).

Table 8 provides the cycloheximide results for Experiment C. None of the challenge microbes produced antifungal activity on their own in a pure culture. According to LSD analysis, the *S. griseus* + *P. chrysogenum*, *S. griseus* + *P. pullulans* + *P. chrysogenum*, and *S. griseus* + *P. pullulans* + *S. erythraea* + *P. chrysogenum* combinations each significantly increased cycloheximide production (Table 8). All other combinations had no significant effect on cycloheximide production. The Tukey analysis found that all of the co-culture combinations used in Experiment B had no significant effect on cycloheximide production.

Table 8. Experiment C Cycloheximide Results using One-Way ANOVA.

Treatment	Mean (µg/mL)	Tukey	LSD
S. griseus	1098.8	a	bc
S. griseus + *P. pullulans*	1047.0	a	c
S. griseus + *S. erythraea*	1115.0	a	bc
S. griseus + *P. pullulans* + *S. erythraea*	1241.3	a	abc
S. griseus + *P. chrysogenum*	1387.2	a	a
S. griseus + *P. pullulans* + *P. chrysogenum*	1458.7	a	a
S. griseus + *S. erythraea* + *P. chrysogenum*	1349.8	a	ab
S. griseus + *P. pullulans* + *S. erythraea* + *P. chrysogenum*	1405.4	a	a
P. pullulans	0.0	b	d
S. erythraea	0.0	b	d
P. chrysogenum	0.0	b	d

Means followed by different letters are significantly different ($P \leq 0.05$).

Table 9 shows the streptomycin results for Experiment C. The *S. erythraea* challenge microbe produced an antibacterial on its own in a pure culture, so any combination containing this microorganism that appeared to increase streptomycin yields had to be analyzed with a t-test (Table 10). According to the LSD results, the *S. griseus* + *S. erythraea* + *P. chrysogenum* and *S. griseus* + *P. pullulans* + *S. erythraea* + *P. chrysogenum* combinations each significantly increased streptomycin production. All

other combinations had no significant effect on streptomycin production. According to Tukey analysis, the *S. griseus* + *S. erythraea* + *P. chrysogenum* combination had no significant effect on streptomycin production, but all other combinations produced the same results as LSD analysis. Table 10 demonstrates that there was no significant difference between the observed and expected results for each combination; indicating that no synergism was occurring.

Table 9. Phase I Experiment C Streptomycin Results using One-Way ANOVA.

Treatment	**Mean (µg/mL)**	**Tukey**	**LSD**
S. griseus	471.2	b	c
S. griseus + *P. pullulans*	525.0	b	bc
S. griseus + *S. erythraea**	422.6	b	c
S. griseus + *P. pullulans* + *S. erythraea**	439.3	b	c
S. griseus + *P. chrysogenum*	564.9	b	bc
S. griseus + *P. pullulans* + *P. chrysogenum*	477.1	b	c
S. griseus + *S. erythraea** + *P. chrysogenum*	665.1	ab	b
S. griseus + *P. pullulans* + *S. erythraea** + *P. chrysogenum*	867.1	a	a
P. pullulans	0.0	c	d
*S. erythraea**	97.5	c	d
P. chrysogenum	0.0	c	d

Means followed by different letters are significantly different ($P \leq 0.05$).
* Denotes that the challenge microbe independently exhibits antibacterial activity.

Table 10. Experiment C Streptomycin Results using T-test.

Treatment	Observed	Expected	P:Observed=Expected*
S. griseus + *S. erythraea** + *P. chrysogenum*	665	569	0.214
S. griseus + *P. pullulans* + *S. erythraea** + *P. chrysogenum*	867	569	0.092

*Two-sample T-test significantly different if P ≤ 0.05.

Experiment D

The three different challenge microbes used in Experiment D were *Streptomyces fradiae*, *Strepto*1, and *Saccharomyces cerevisiae*. These challenge microbes were co-cultured with *S. griseus* in different combinations as well as grown in pure cultures (Tables 11-13).

Table 11 provides the cycloheximide results for Experiment D. None of the challenge microbes produced antifungal on their own in a pure culture. According to LSD analysis, none of the co-culture combinations significantly increased cycloheximide production (Table 11). The *S. griseus* + *S. fradiae*, *S. griseus* + *S. cerevisiae*, *S. griseus* + *S. fradiae* + *S. cerevisiae*, and *S. griseus* + *Strepto*1 + *S. cerevisiae* combinations had no significant effect on cycloheximide production. The other co-culture combinations each significantly decreased cycloheximide production. According to Tukey analysis, the only combination that significantly decreased cycloheximide production was *S. griseus* + *Streptomyces fradiae* + *Strepto1*. All other co-culture combinations had no significant effect on cycloheximide production.

Table 11. Experiment D Cycloheximide Results using One-Way ANOVA.

Treatment	Mean (µg/mL)	Tukey	LSD
S. griseus	1873.8	ab	abc
S. griseus + *Streptomyces fradiae*	1815.6	abc	bcd
S. griseus + *Strepto1*	1430.8	bc	def
S. griseus + *Streptomyces fradiae* + *Strepto1*	1087.9	c	f
S. griseus + *Saccharomyces cerevisiae*	2206.4	a	ab
S. griseus + *Streptomyces fradiae* + *Saccharomyces cerevisiae*	2285.0	a	a
S. griseus + *Strepto1* + *Saccharomyces cerevisiae*	1635.5	abc	cde
S. griseus + *Streptomyces fradiae* + *Strepto1* + *Saccharomyces cerevisiae*	1310.6	bc	ef
Streptomyces fradiae	0.0	d	g
Strepto1	0.0	d	g
Saccharomyces cerevisiae	0.0	d	g

Means followed by different letters are significantly different ($P \leq 0.05$).
* Denotes that the challenge microbe independently exhibits antibacterial activity.

Table 12 provides the streptomycin results for Experiment D. The *S. fradiae* and *S. cerevisiae* challenge microbes produced an antibacterial activity on their own in a pure culture, so any combination containing these microorganisms that appeared to increase streptomycin yields had to be analyzed with a t-test (Table 13). According to the LSD analysis, the *S. griseus* + *S. cerevisiae*, *S. griseus* + *S. fradiae* + *S. cerevisiae*, and *S. griseus* + *Strepto1* + *S. cerevisiae* combinations each significantly increased

streptomycin production. The *S. griseus* + *Strepto1*, *S. griseus* + *S. fradiae* + *Strepto1*, and *S. griseus* + *S. fradiae* + *Strepto1* + *S. cerevisiae* combinations each had no significant effect on cycloheximide production. The *S. griseus* + *S. fradiae* combination significantly decreased streptomycin production.

According to the Tukey results, the *S. griseus* + *S. fradiae* + *S. cerevisiae* combination significantly increased streptomycin production but all other combinations in Experiment D had no significant effect on streptomycin production. Table 13 demonstrates that the *S. griseus* + *S. fradiae* + *S. cerevisiae* combination had a positive significant difference between observed and expected streptomycin yields (P=0.044). This indicates that the increase in streptomycin yields was because of synergism. The other combinations had no significant difference between the observed and expected results for each combination, indicating that no synergism was occurring.

Table 12. Experiment D Streptomycin Results using One-Way ANOVA.

Treatment	Mean (µg/mL)	Tukey	LSD
S. griseus	917.3	bcd	de
S. griseus + *Streptomyces fradiae**	468.4	de	f
S. griseus + *Strepto1*	539.8	cde	ef
S. griseus + *Streptomyces fradiae** + *Strepto1*	1252.3	ab	bcd
S. griseus + *Saccharomyces cerevisiae**	1556.5	ab	ab
S. griseus + *Streptomyces fradiae** + *Saccharomyces cerevisiae**	1654.2	a	a
S. griseus + *Strepto1* + *Saccharomyces cerevisiae**	1499.7	ab	abc
S. griseus + *Streptomyces fradiae** + *Strepto1* + *Saccharomyces cerevisiae**	1159.6	abc	cd
*Streptomyces fradiae**	23.0	e	g
Strepto1	0.0	e	g
*Saccharomyces cerevisiae**	4.6	e	g

Means followed by different letters are significantly different ($P \leq 0.05$).
* Denotes that the challenge microbe independently exhibits antibacterial activity.

Table 13. Experiment D Streptomycin Results using T-test.

Treatment	Observed	Expected	P:Observed=Expected*
S. griseus + *S. cerevisiae**	1557	922	0.054
S. griseus + *S. fradiae** + *S. cerevisiae**	1654	945	0.044
S. griseus + *Strepto1* + *S. cerevisiae**	1500	922	0.065

*Two-sample T-test significantly different if P ≤ 0.05.

Experiment E

The three different challenge microbes used in Experiment E were *Fusarium oxysporum*, *Strepto*Blue2, and *Strepto*Blue1. These challenge microbes were co-cultured with *S. griseus* in different combinations as well as grown in pure cultures (Tables 14-16).

Table 14 provides the cycloheximide results from Experiment E. None of the challenge microbes produced antifungal on their own in a pure culture. According to LSD analysis, the *S. griseus* + *Strepto*Blue2, *S. griseus* + *Strepto*Blue1, *S. griseus* + *F. oxysporum* + *Strepto*Blue1 and *S. griseus* + *F. oxysporum* + *Strepto*Blue2 + *Strepto*Blue1 each significantly increased cycloheximide production. The *S. griseus* + *F. oxysporum*, *S. griseus* + *F. oxysporum* + *Strepto*Blue2, and *S. griseus* + *Strepto*Blue2 + *Strepto*Blue1 combinations each had no effect on cycloheximide production. None of the co-culture combinations significantly decreased cycloheximide production. According to Tukey analysis, *S. griseus* + *Strepto*Blue2 was the only combination that significantly increased cycloheximide production. The other co-culture combinations each had no significant effect on cycloheximide production.

Table 14. Experiment E Cycloheximide Results using One-Way ANOVA.

Treatment	**Mean (µg/mL)**	**Tukey**	**LSD**
S. griseus	888.2	b	d
S. griseus + *Fusarium oxysporum*	980.9	ab	cd
S. griseus + *Strepto*Blue2	1313.3	a	a
S. griseus + *Fusarium oxysporum* + *Strepto*Blue2	1055.1	ab	bcd
S. griseus + *Strepto*Blue1	1192.2	ab	ab
S. griseus + *Fusarium oxysporum* + *Strepto*Blue1	1152.8	ab	abc
S. griseus + *Strepto*Blue2 + *Strepto*Blue1	886.1	b	d
S. griseus + *Fusarium oxysporum* + *Strepto*Blue2 + *Strepto*Blue1	1104.7	ab	bc
Fusarium oxysporum	0.0	c	e
*Strepto*Blue2	0.0	c	e
*Strepto*Blue1	0.0	c	e

Means followed by different letters are significantly different ($P \leq 0.05$).
* Denotes that the challenge microbe independently exhibits antibacterial activity.

Table 15 provides the streptomycin results for Experiment E. The *F. oxysporum* challenge microbe produced an antibacterial on its own in a pure culture, so any combination containing this microorganism that appeared to increase streptomycin yields had to be analyzed with a t-test (Table 16). According to LSD analysis, the *S. griseus* + *F. oxysporum* + *Strepto*Blue2, *S. griseus* + *F. oxysporum* + *Strepto*Blue1, *S. griseus* + *Strepto*Blue2 + *Strepto*Blue1, and *S. griseus* + *F. oxysporum* + *Strepto*Blue2 +

*Strepto*Blue1 combinations each significantly increased streptomycin production. The *S. griseus* + *F. oxysporum*, *S. griseus* + *Strepto*Blue2, and *S. griseus* + *Strepto*Blue1 combinations each had no effect on streptomycin production (Table 15). None of the co-culture combinations significantly decreased streptomycin production.

Tukey analysis demonstrates that each co-culture combination in Experiment E had no significant effect on streptomycin production. Results suggest that there was no significant difference between the observed and expected results for each combination; indicating that no synergism was occurring (Table 16). Table 16 does show that the co-culture containing *S. griseus* + *F. oxysporum* + *Strepto*Blue1 produced a significant t-test results (P=0.011). However, these results show a negative significant difference, indicating that antagonism is occurring with these microbes in together in a co-culture.

Table 15. Experiment E Streptomycin Results using One-Way ANOVA.

Treatment	**Mean (µg/mL)**	**Tukey**	**LSD**
S. griseus	759.0	a	d
S. griseus + *Fusarium oxysporum**	782.9	a	cd
S. griseus + *Strepto*Blue2	807.2	a	bcd
S. griseus + *Fusarium oxysporum** + *Strepto*Blue2	899.2	a	ab
S. griseus + *Strepto*Blue1	874.4	a	abcd
S. griseus + *Fusarium oxysporum** + *Strepto*Blue1	947.8	a	a
S. griseus + *Strepto*Blue2 + *Strepto*Blue1	899.2	a	ab
S. griseus + *Fusarium oxysporum** + *Strepto*Blue2 + *Strepto*Blue1	880.2	a	abc
*Fusarium oxysporum**	11.9	b	e
*Strepto*Blue2	0.0	b	e
*Strepto*Blue1	0.0	b	e

Means followed by different letters are significantly different (P ≤ 0.05).
* Denotes that the challenge microbe independently exhibits antibacterial activity.

Table 16. Experiment E Streptomycin Results using T-test.

Treatment	Observed	Expected	P:Observed=Expected*
S. griseus + *F.oxysporum** + *Strepto*Blue2	899	771	0.078
S. griseus + *F. oxysporum** + *Strepto*Blue1	948	771	0.011
S. griseus + *F. oxysporum** + *Strepto*Blue2 + *Strepto*Blue1	880	771	0.174

*Two-sample T-test significantly different if P ≤ 0.05.

Table 17 is a summary of the different co-culture combinations that increased at least one antibiotic. The first portion of the graph highlights the co-culture combinations that significantly increased cycloheximide production and provides their respective fold increases over the *S. griseus* control. The second portion highlights the co-culture combinations that significantly increased streptomycin production and shows their respective fold increases over the *S. griseus* control. The third portion highlights those combinations that increased both cycloheximide and streptomycin and displays the fold increases over the *S. griseus* control for each antibiotic.

Table 17. Fold Increases of Combinations that Increased Antibiotic Production.

CYCLOHEXIMIDE

Organisms	Fold Increase
*Strepto*8	1.40
*Strepto*8 + *S. antibioticus**	1.10
S4A*	1.59
P. chrysogenum	1.28
P. pullulans + *S. erythraea** + *P. chrysogenum*	1.31
*Strepto*Blue2	1.55
P. pullulans + *P. chrysogenum*	1.37
*Strepto*Blue1	1.37
*F. oxysporum** + *Strepto*Blue1	1.37
*F. oxysporum** + *Strepto*Blue2 + *Strepto*Blue1	1.26

STREPTOMYCIN

Organisms	Fold Increase
P. pullulans + *S. erythraea** + *P. chrysogenum*	1.72
*S. cerevisiae**	2.04
*S. fradiae** + *S. cerevisiae**	2.17
*F. oxysporum** + *Strepto*Blue1	1.24
*Strepto*8 + *R. leguminosarum*	1.46
*Strepto*Blue2 + *Strepto*Blue1	1.19
*S. erythraea** + *P. chrysogenum*	1.26
*Strepto*1 + *S. cerevisiae**	2.04
*F. oxysporum** + *Strepto*Blue2	1.18
*F. oxysporum** + *Strepto*Blue2 + *Strepto*Blue1	1.16

CYCLOHEXIMIDE + STREPTOMYCIN

Organisms	Cyclo Fold Increase	Strepto Fold Increase
P. pullulans + *S. erythraea** + *P. chrysogenum*	1.31	1.72
*F. oxysporum** + *Strepto*Blue1	1.37	1.24
*F. oxysporum** + *Strepto*Blue2 + *Strepto*Blue1	1.26	1.16

Each of these co-cultures contains *Streptomyces griseus*.

* Denotes that the challenge microbe independently exhibits antibacterial activity. Fold Increase was determined by dividing the co-culture antibiotic yield by the *S. griseus* control antibiotic yield.

Table 18 summarizes how many microbes were combined in the co-cultures that increased, decreased, or had no effect on antibiotics yields. Having four microbes within the co-culture, 40% of the combinations resulted in increased yields, 20% decreased yields, and 40% of isolates exhibited no effect on yields for both cycloheximide and streptomycin antibiotics. When there were three microbes in the co-culture, 20% of the combinations increased cycloheximide while 47% increased streptomycin, 7% decreased cycloheximide while 20% decreased streptomycin, and 73% had no effect on cycloheximide yields while 33% had no effect on streptomycin yields. When there were two microbes in the co-culture, 33% of the combinations increased cycloheximide while 7% increased streptomycin, 7% decreased cycloheximide while 20% decreased streptomycin, and 60% had no effect on cycloheximide yields while 73% had no effect on streptomycin yields.

Table 18. Comparisons of the Amount of Different Microbes in the Co-cultures.

Cycloheximide			
# Microbes in Co-culture	Combinations that increased yields	Combinations that decreased yields	Combinations that had no effect
4	40% (2)	20% (1)	40% (2)
3	20% (3)	7% (1)	73% (11)
2	33% (5)	7% (1)	60% (9)
Streptomycin			
# Microbes in Co-culture	Combinations that increased yields	Combinations that decreased yields	Combinations that had no effect
4	40% (2)	20% (1)	40% (2)
3	47% (7)	20% (3)	33% (5)
2	7% (1)	20% (3)	73% (11)

Phase II

For Phase II, three of the co-culture combinations that increased cycloheximide production while not producing antibiotics of their own were investigated further. These co-culture combinations were *Strepto*8 + *S. griseus*, *Strepto*Blue2 + *S. griseus*, and *Penicillium chrysogenum* + *S. griseus*. Phase II experiments included a time of challenge microbe addition experiment, bioautograms to determine if there is novel antibiotic production, and a dead vs. living vs. cell-free supernatant challenge microbe experiment.

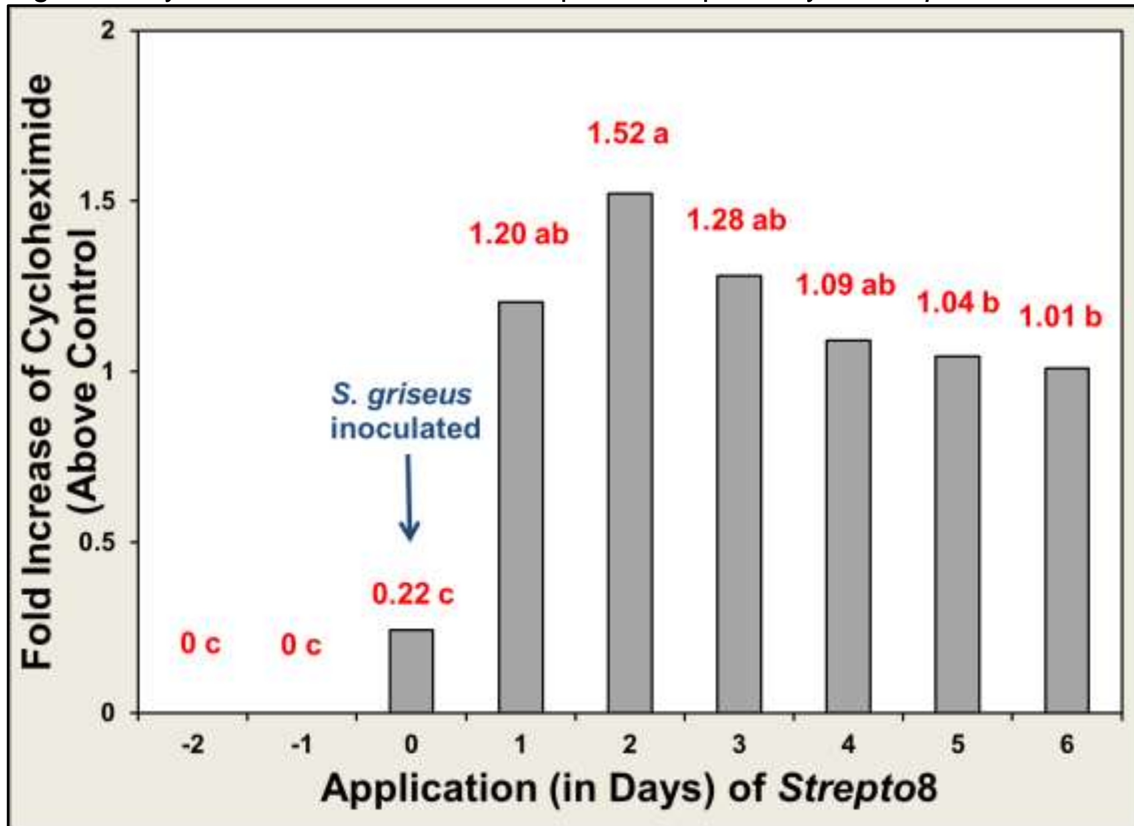
Time of Challenge Microbe Addition vs. Cycloheximide Synthesis

The first experiment in Phase II was the time of challenge microbe addition experiment. For this experiment, we wanted to determine when the optimal time was to add the challenge microbe(s) to the co-culture. Challenge microbes were added from two days before *S. griseus* to six days after *S. griseus*. Three different experiments, one each for the different combinations carried over to Phase II, were performed to determine when the optimal time to add challenge microbes to the co-culture was (Figures 2-5).

The first time of challenge microbe experiment was performed with *Strepto*8 as the challenge microbe (Figure 2). When *Strepto*8 was added to the production medium one or two days before *S. griseus*, cycloheximide production was completely inhibited. When *Strepto*8 was added on the same day as *S. griseus*, cycloheximide production was partially inhibited, producing only 0.22x the cycloheximide produced by *S. griseus* alone. When *Strepto*8 was added one day after *S. griseus*, cycloheximide production

was increased by 1.20x. When *Strepto*8 was added two days after *S. griseus*, cycloheximide production was increased by 1.52x. When *Strepto*8 was added three days after *S. griseus*, cycloheximide production was increased by 1.28x. When *Strepto*8 was added four days after *S. griseus*, cycloheximide production was increased by 1.09x. When *Strepto*8 was added five days after *S. griseus*, cycloheximide production was increased by 1.04x. When *Strepto*8 was added six days after *S. griseus*, cycloheximide production was increased by only 1.01x.

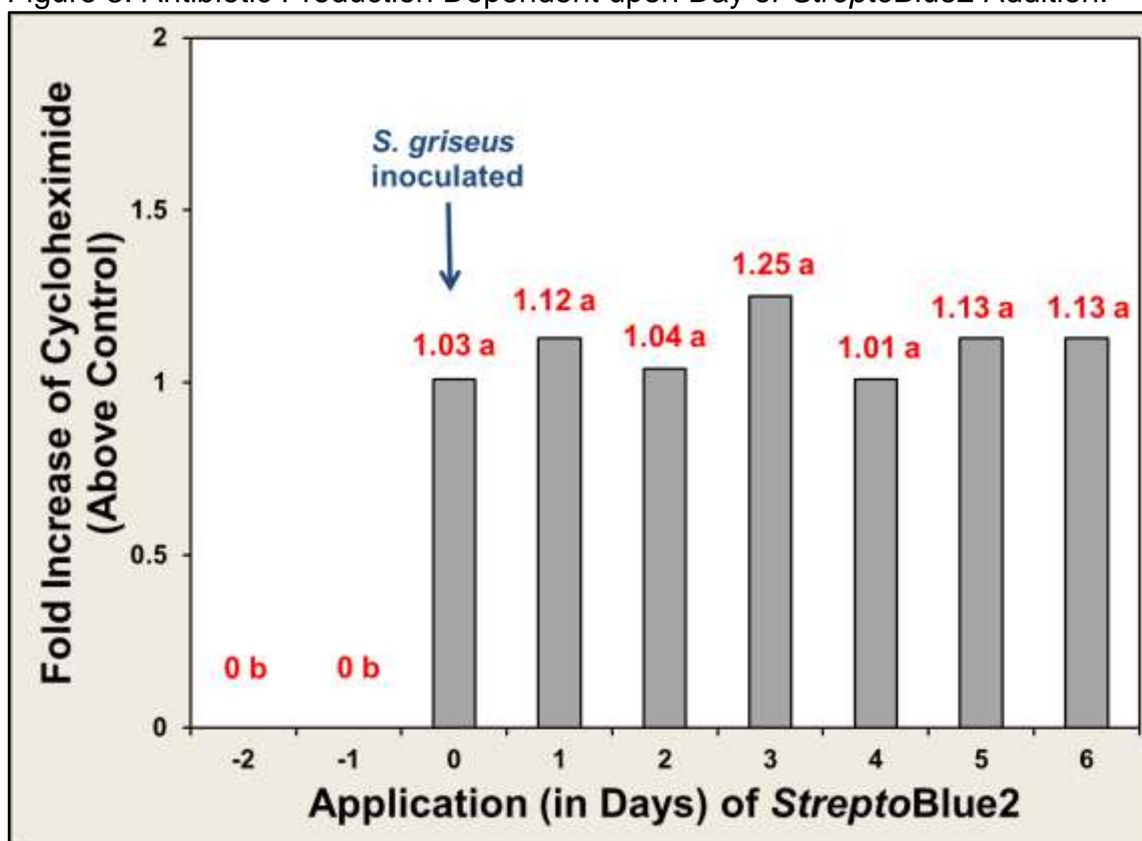
Figure 2. Cycloheximide Production Dependent upon Day of *Strepto*8 Addition.



Cycloheximide values were analyzed using One-way ANOVA. Means followed by different letters are significantly different ($P \leq 0.05$) using LSD analysis.

The second time of challenge microbe experiment was performed with *StreptoBlue*2 as the challenge microbe (Figure 3). When *StreptoBlue*2 was added to the production medium one or two days before *S. griseus*, cycloheximide production was completely inhibited. When *StreptoBlue*2 was added on the same day as *S. griseus*, cycloheximide production was increased by 1.03x over cycloheximide produced by *S. griseus* alone. When *StreptoBlue*2 was added one day after *S. griseus*, cycloheximide production was increased by 1.12x. When *StreptoBlue*2 was added two days after *S. griseus*, cycloheximide production was increased by 1.04x. When *StreptoBlue*2 was added three days after *S. griseus*, cycloheximide production was increased by 1.25x. When *StreptoBlue*2 was added four days after *S. griseus*, cycloheximide production was increased by only 1.01x. When *StreptoBlue*2 was added five days after *S. griseus*, cycloheximide production was increased by 1.13x. When *StreptoBlue*2 was added six days after *S. griseus*, cycloheximide production was increased by 1.13x.

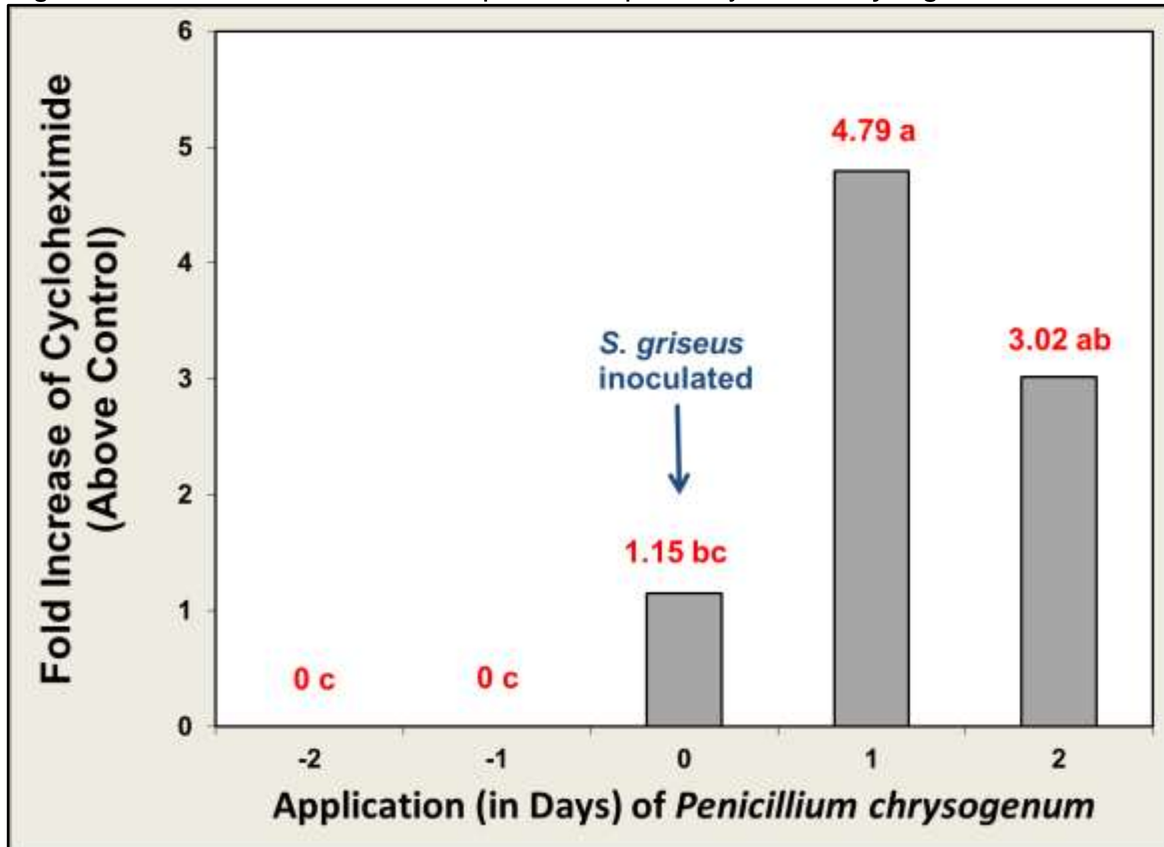
Figure 3. Antibiotic Production Dependent upon Day of *Strepto*Blue2 Addition.



Cycloheximide values were analyzed using One-way ANOVA. Means followed by different letters are significantly different ($P \leq 0.05$) using LSD analysis.

The final time of challenge microbe experiment was performed with *P. chrysogenum* as the challenge microbe (Figure 4). When *P. chrysogenum* was added to the production medium one or two days before *S. griseus*, cycloheximide production was completely inhibited. When *P. chrysogenum* was added on the same day as *S. griseus*, cycloheximide production was increased by 1.15x over cycloheximide produced by *S. griseus* alone. When *P. chrysogenum* was added one day after *S. griseus*, cycloheximide production was increased by 4.79x. When *P. chrysogenum* was added two days after *S. griseus*, cycloheximide production was increased by 3.02x.

Figure 4. Antibiotic Production Dependent upon Day of *P. chrysogenum* Addition.

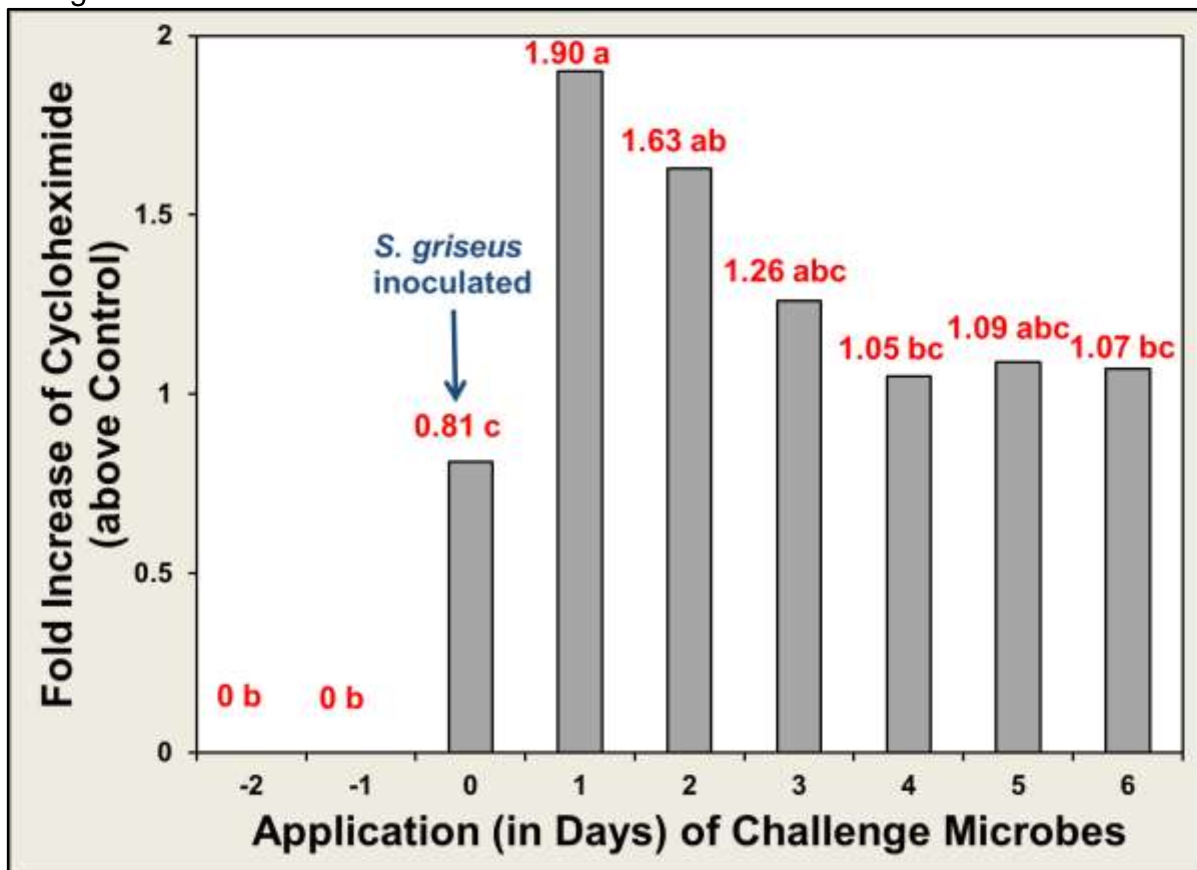


Cycloheximide values were analyzed using One-way ANOVA. Means followed by different letters are significantly different ($P \leq 0.05$) using LSD analysis.

Figure 5 provides the pooled data from all three challenge microbes tested. When the challenge microbes were added to the production medium one or two days before *S. griseus*, cycloheximide production was completely inhibited. When the challenge microbes were added on the same day as *S. griseus*, cycloheximide production was partially inhibited, producing only 0.81x the cycloheximide produced by *S. griseus* alone. When the challenge microbes were added one day after *S. griseus*, cycloheximide production was increased by 1.90x. When the challenge microbes were added two days after *S. griseus*, cycloheximide production was increased by 1.63x.

When the challenge microbes were added three days after *S. griseus*, cycloheximide production was increased by 1.26x. When the challenge microbes were added four days after *S. griseus*, cycloheximide production was increased by only 1.05x. When the challenge microbes were added five days after *S. griseus*, cycloheximide production was increased by 1.09x. When the challenge microbes were added six days after *S. griseus*, cycloheximide production was increased by 1.07x.

Figure 5. Antibiotic Production Dependent upon Day of Challenge Microbe Addition Using Pooled Data.



Pooled cycloheximide values were analyzed using One-way ANOVA. Means followed by different letters are significantly different ($P \leq 0.05$) using LSD analysis.

Bioautograms

The second experiment in Phase II involved performing bioautograms on the *Strepto*8 and *StreptoBlue*2 co-cultures to determine if any novel antibiotics were being produced. For each co-culture, a sample was chromatographed and bioassayed along with pure antibiotic controls and sample + pure antibiotic controls. Retardation factors (Rf)-values were obtained by dividing the distance the antimicrobial compound migrated by the distance the solvent front migrated. Results are summarized in Table 19.

Table 19. Rf values of Cycloheximide and Streptomycin for the *Strepto*8 Bioautogram.

Treatment	**Rf value**
Co-culture (Cycloheximide)	0.879
Co-culture + Cycloheximide	0.874
Cycloheximide Control	0.895
Co-culture (Streptomycin)	0.027
Co-culture + Streptomycin	0.027
Streptomycin Control	0.027

The cycloheximide in the *Strepto*8 co-culture migrated to produce an Rf value of 0.879, which compared/equaled to the co-culture sample + cycloheximide control value of 0.874 and the pure cycloheximide control value of 0.895 (Table 19). The streptomycin in the *Strepto*8 co-culture migrated to produce an Rf value of 0.027, which was the same as the co-culture sample + streptomycin control and the pure streptomycin control.

The cycloheximide in the *Strepto*Blue2 co-culture migrated to produce an Rf value of 0.871, which matched the co-culture sample + cycloheximide control value of 0.871 and the pure cycloheximide control value of 0.895 (Table 20). The streptomycin in the *StreptoBlue*2 co-culture migrated to produce an Rf value of 0.006, which compared well to the co-culture sample + streptomycin control value of 0.006 and the pure cycloheximide control value of 0.019.

Table 20. Rf values of Cycloheximide and Streptomycin for the *Strepto*Blue2 Bioautogram.

Treatment	**Rf value**
Co-culture (Cycloheximide)	0.871
Co-culture + Cycloheximide	0.871
Cycloheximide Control	0.895
Co-culture (Streptomycin)	0.006
Co-culture + Streptomycin	0.006
Streptomycin Control	0.019

Determining Time for Heat-killing *Strepto*8 Cells

Before using heat-killed *Strepto*8 in future experiments, an experiment was performed to determine how long *Strepto*8 seed culture needed to be boiled to devitalize all of the cells. Results are summarized in Table 21.

Before boiling the *Strepto*8 seed culture, the amount of *Strepto*8 colony forming units grown on an NZAmineA agar medium was too numerous to count (Table 21).

After boiling for 5 minutes, the amount of viable cells decreased to 10 cfu/mL. After boiling for 10 minutes or longer, <1 viable cfu/mL was detected.

Table 21. *Strepto*8 colony forming units remaining after boiling seed culture for different periods of time.

Time Boiled (min)	**CFU/mL**
0	TNTC
5	10
10	<1
15	<1
20	<1
30	<1
40	<1
50	<1
60	<1

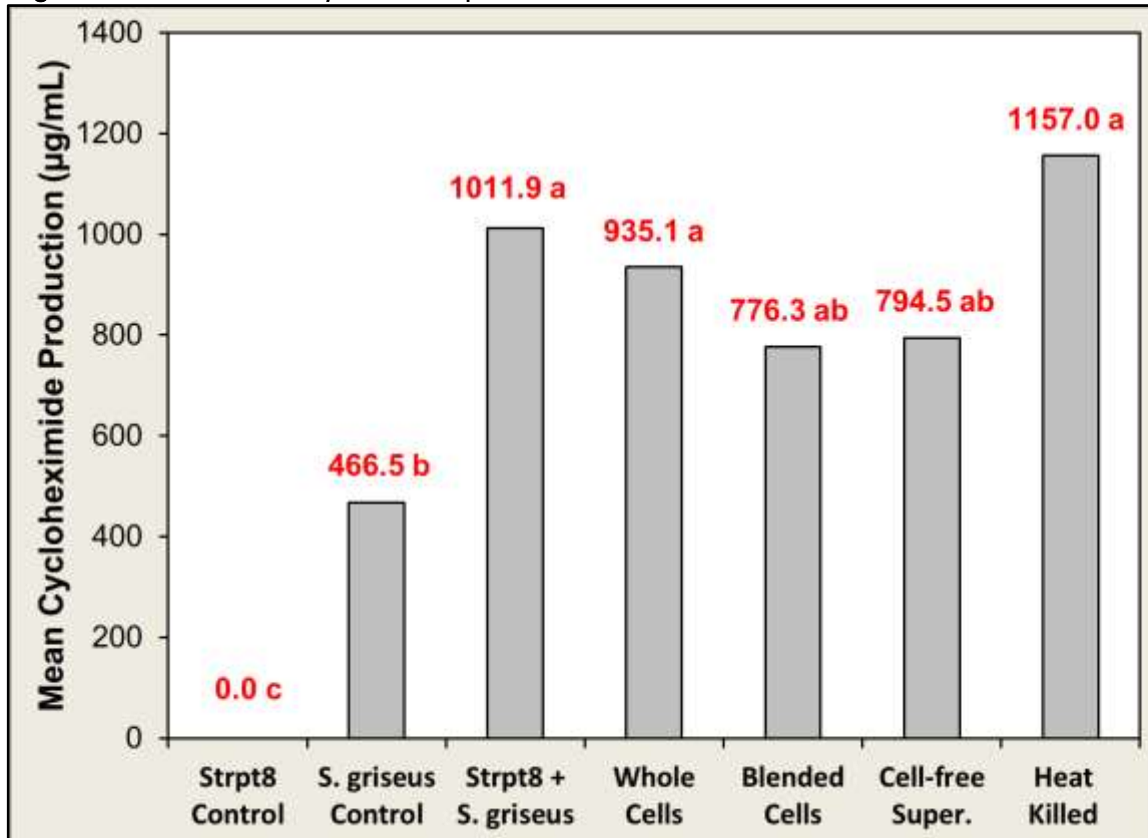
TNTC = Too numerous to count

Dead vs. Living vs. Cell-Free Challenge Microbe

The final experiment in Phase II was the dead vs. living vs. cell-free challenge microbe experiment. Live cells, dead cells, and various components of *Strepto*8 were co-cultured with *S. griseus* to learn more about which components may be causing an increase in cycloheximide production. Controls containing only *S. griseus* and only *Strepto*8 were run simultaneously with these treatments for comparison.

For this experiment, the pure culture of *Strepto*8 (Strpt8 control) did not produce any cycloheximide (Figure 6). The pure culture of *S. griseus* produced 466.5 μg/mL cycloheximide. The live co-culture of *Strepto*8 and *S. griseus* produced 1011.9 μg/mL cycloheximide, a significant increase of nearly 117% over the *S. griseus* control. The co-culture containing live washed Strepto8 cells and *S. griseus* produced 935.1 μg/mL cycloheximide, a significant increase of 100% over the *S. griseus* control. The co-culture containing blended, live washed Strepto8 cells and *S. griseus* produced 776.3 μg/mL cycloheximide, also a significant increase of 66% over the *S. griseus* control. The culture containing cell-free *Strepto*8 culture supernatant and *S. griseus* produced 794.5 μg/mL cycloheximide, a significant increase of 70% over the *S. griseus* control. The culture containing heat-killed *Strepto*8 cells and *S. griseus* produced 1157.0 μg/mL cycloheximide, the greatest amount of activity observed, which was significantly different from *S. griseus* control (an increase of 148%), but not significantly different from any of the other treatment combinations.

Figure 6. Effect of *Strepto*8 Components on Antibiotic Production in Co-culture.



Cycloheximide values were analyzed using One-way ANOVA. Means followed by different letters are significantly different ($p < 0.05$) using LSD analysis.

Discussion

After performing co-culture experiments on 35 different combinations of challenge microbes grown with *S. griseus* in Phase I, we found that 17 unique consortia increased the production of at least one antibiotic (Table 17). Of these 17 combinations, 10 increased cycloheximide, 10 increased streptomycin, and 3 increased both antibiotics. Based on these results, it appears that the co-culturing method used in this study may be a useful protocol in future industrial applications regarding large-scale production of streptomycin and cycloheximide.

As Table 18 demonstrates, there did not appear to be a relationship between an increase in antibiotic production and the number of microbes utilized in the co-culture. Of the 17 combinations found to increase antibiotic yields, 2 contained 4 different microbes in the co-culture, 9 contained 3 different microbes, and 6 contained 2 different microbes. In cycloheximide synthesis experiments containing 4 microbes in the co-culture, 40% of unique microbe mixtures increased yield, 20% decreased and 40% had no effect. When investigating a total of 3 microbes in the co-culture 20% increased, 7% decreased and 73% had no effect (Table 18). These results suggest when you go from adding 3 to 4 microbes in a co-culture you increase the number of combinations which improve cycloheximide, increase # combinations which decrease yield and decrease # combinations which have no effect on cycloheximide yield. If one compares testing a similar number of microbes (from 3 to 4) used in co-cultures on streptomycin synthesis, a different trend (compared to cycloheximide) is found: a decrease in # of combinations which improve streptomycin, no change in # decreased yields and increase # combinations which have no effect (Table 18). With the limited data (# co-cultures

tested) obtained in this study, no visible trend in yield improvement was determined with regard to the number of microbes grown within the co-culture, indicating that each co-culture behaves uniquely and is more dependent on the species of challenge microbes rather than the quantity. Testing a larger number/diversity of microorganisms may provide a better grasp as to whether any trends may occur when investigating yield optimization.

The main goal of Phase I was to produce a 'short list' of challenge microbes that significantly improve antibiotic yields so we could perform additional experiments to optimize antibiotic yield (Phase II). The 17 successful combinations found in Phase I were too numerous to carry over into Phase II, so we decided to reduce the number of potential combinations. While analyzing the antibiotic production of the different co-cultures and challenge microbes, it was determined that several of the challenge microbes produced antibiotics on their own in our production medium. Because results were more difficult to analyze for significant improvement when this occurred, we decided to move forward with the co-cultures containing challenge microbes that did not produce antibiotics on their own. This process cut the number of successful combinations down to 7. To further narrow our list, we decided to use co-cultures containing only 2 different microbes, with the thought that these co-cultures were less complex and would lead to more accurate bioassay and statistical analyses for Phase II. Additionally, results from Table 18 indicated no trend in yield improvement when testing co-cultures containing from 2-4 microbes; therefore, we decided to continue testing those combinations (co-cultures) that only increased cycloheximide production: the fungus *P. chrysogenum* and the bacteria *Strepto*8 and *StreptoBlue*2.

The first experiment performed in Phase II was to determine the optimal time to add the challenge microbe(s) to the production medium. Although each co-culture gave varying results, pooled data from testing all 3 challenge microbes demonstrated that it was best to inoculate challenge microbes 1-3 days following *S. griseus* (Figure 5). When the challenge microbe was added prior to or alongside *S. griseus*, cycloheximide production was inhibited, indicating that pre-establishment by *S. griseus* was essential to the production of cycloheximide. Similar results were observed when *S. tenjimariensis* was co-cultured with different marine bacteria to increase istamycin production (37). They found that pre-establishment of *S. tenjimariensis* one day before the challenge microbes was essential for an increase in antibiotic production but co-inoculation or pre-establishing the challenge microbes first decreased antibiotic production. However in, contrast, the co-cultures containing *S. coelicolor* increased undecylprodigiosin most when the microbes were added at the same time (25). This implies that each co-culture may be unique in terms of pre-establishment of the antibiotic producing organism and challenge microbes. For our study, when the challenge microbe was added after day 3, cycloheximide production was increased only slightly, perhaps because any interaction between the different microbes was introduced too late in the fermentation process. These observations indicate that significant increased yields of cycloheximide occur when the co-culture is added within a narrow window of time from 1-2 days following *S. griseus*.

The second experiment in Phase II was conducted to determine if any novel antibiotics were produced in the co-cultures which were assessed by performing bioautograms. Because of time constraints, only co-cultures containing *Strepto*8 and

*StreptoBlue*2 were analyzed with this method. The Rf values of the culture samples compared well with the Rf values of the controls; therefore, we determined that no novel antibiotics were produced in the co-cultures sampled (Tables 19 and 20). Although novel antibiotics were not found with these two co-cultures, we were able to confirm that cycloheximide and streptomycin were the only antibiotics synthesized in these samples. By screening more combinations, it is possible that novel combinatorial antibiotic(s) may be found in some of the non-tested co-cultures performed from Phase II. These cultures were frozen and can be thawed and tested in the future.

The final experiment in Phase II was to investigate whether living vs. dead challenge microbe and/or culture filtrate would improve antibiotic yield. If live cells are not necessary, we wanted to identify which component(s) may be invoking the increased production in this scenario. To help identify these components, whole washed cells, blended washed cells, and cell-free seed culture supernatant were added to the production flasks separately.

Results indicate that the addition of dead challenge microbes to the production medium increased cycloheximide yields comparable to the addition of live challenge microbes (Figure 6). Similar results were observed with undecylprodigiosin synthesis in which dead *E. coli* and *S. aureus* cells were added to a culture of *S. coelicolor* (24). This suggests that live challenge microbes are not required to significantly increase cycloheximide or other antimicrobial yields in co-culture. Various components of challenge microbes significantly improved cycloheximide yield more than with *S. griseus* cultured alone. Visibly, the best yield was observed when using heat-killed cells (but was not significantly different than *S. griseus*). These results suggest that a

component(s) of the challenge microbes added to the production medium contains the factor(s) that increase cycloheximide production. The most likely explanation for this is a molecule(s) produced by the challenge microbe that is produced intracellularly (e.g. intact washed cells 935.1 μg/ml) and capable of being excreted into the supernatant (e.g. cell-free supernatant 794.5 μg/ml) as shown in Figure 6. Because the heat-killed cells increased antibiotic yields, it is suspected that the molecule(s) is heat stable. More experimentation will be needed to confirm these results and further define other characteristics of the molecule(s).

Future Experiments

Continue Isolation of Cycloheximide-Enhancing Molecule(s)

After determining that a molecule(s) from the challenge microbe seed culture is most likely increasing the production of cycloheximide, we could further analyze the culture supernatant to characterize it. The next experiments could separate seed culture supernatant components by size/molecular weight to narrow down the range of possible biomolecules. One could centrifuge cell culture supernatants within tubes containing various-sized filters (e.g. 10,000 or 50,000 mw) to obtain different molecular weight fractions. One could also utilize column chromatography (e.g. molecular exclusion or ion exchange) to isolate fractions/components that could be tested to determine which may improve antibiotic yield.

Optimize Inoculum Rates of Challenge Microbes and *S. griseus*

In our study, the inoculum rate for each challenge microbe and *S. griseus* was kept constant at 1% v/v. Using response surface methodology (RSM), one could evaluate different inoculum rates (0.5-20% v/v) of both the challenge microbe(s) and *S. griseus* that result in optimizing antibiotic yields.

Optimize (Customize) Production Medium

The current production medium used in this study was based on the production medium developed by Kominek in 1975 to culture *S. griseus* (18, 19). The only modification made to the medium was to substitute white bean flour in place of soybean flour for the nitrogen source. Using response surface methodology, we could test

different concentrations of the ingredients already used in the formulation or test entirely different components to optimize antibiotic yields.

Optimize Fermentation Conditions

The current fermentation conditions used in this study were also based on the experiments of Kominek in 1975 (18, 19). We could alter/test the effects of varying the initial pH of the production medium, changing shaker speed, or reducing the amount of production medium in the flasks to increase dissolved oxygen. Response surface methodology could once again be employed to set these experiments up and analyze the data.

Co-culture More Than 4 Different Microbes

No co-cultures tested in this study contained more than 4 different microbes. Future experiments could have 4 or more challenge microbes added to the same production culture with *S. griseus*. This could help us determine a trend between the number of microbes added to a co-culture and an increase in antibiotic yields. Testing a larger number of different microbes may result in better identifying any possible trend of using two or more microbes in co-culture to improve antibiotic yield. I recommend using the fractional/partial factorial design to perform these types of screening as was used in the current experiment. Although a full-factorial design will potentially result in identifying more positive-interactions, the number of reps required for such would not be practical for rapid screening/downstream processing.

Scale-up to 2 L Bioreactor

Another future experiment could be scaling up the co-culture process into a 2 L bioreactor. Conditions of the bioreactor would be adjusted to closely mirror those of the shake flasks from our experiments to determine if similar results are obtained. This experiment could go a long way in shaping whether or not the technique of co-culturing could be used in much larger scale for industrial applications.

Assay More Co-cultures for Novel Antibiotics Using Bioautograms

Only two different co-cultures were analyzed for novel antibiotics using bioautograms in this study. Future experiments would analyze more co-culture combinations to determine if any novel antibiotics are being produced. During Phase I of this study, aliquots from each different co-culture combination tested were stored at -80°C for future novel antibiotic assessment.

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