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Development of Mixed Microbial Screening and Cultivation Methods for Novel Antibiotic Discovery

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Abstract

With antibiotic resistance on the rise, researchers are eager to maximize natural product discovery to find novel antibiotic compounds. One way to do this is through high-throughput liquid culture screening of bacterial libraries. Co-culture methods have proven successful in stimulating the expression of silent genes which can trigger the production of compounds that are not produced in monoculture. This expression is caused through the simulation of a competitive environment among the bacterial strains under the stress of minimal media conditions. Using this ecological theory, a more natural environment for bacterial cultivation can be created in the laboratory to maximize the production of novel compounds. We hypothesize that maintaining minimal media conditions and increasing the number of bacteria in culture together (tri-culture and multi-cultures) will further stimulate the production of novel natural products that would not be expressed in monoculture or in co-culture. Prior work has been done in the Wolfe laboratory to assess the antimicrobial activity of compounds produced by soil bacteria isolated from Western NC garden soil in both monoculture and co-culture using 96-well high throughput assay technique. This work expands the utility of this technology by developing a quantitative and robust method for screening microbial cultures encompassing three bacterial strains. Furthermore, during this screening study, it was found that the tri-culture of soil bacteria 540, 442, and 657 showed strong inhibition against *S. aureus*. To determine the nature of this antibiotic activity, a multi-liter liquid culture scale-up method was developed to maximize the antibiotic secondary metabolites produced by this culture. Antibiotic compounds isolated using this method are then purified using organic techniques and compound characterization can commence.

1. Introduction

Antibiotic resistance is a huge health care crisis, with nearly 3 million people in the US facing antibiotic-resistant infections every year. Because of this, there is a critical need for the discovery of novel antibiotic compounds that overcome resistance mechanisms in infectious bacteria.[1] To give some perspective, due to current resistance trends, it is predicted that multi-drug resistant bacteria will cause 10 million deaths worldwide by the year 2050. The continuous surge, and predicted surge, in antibiotic resistance is due to the overuse or misuse of antibiotics in medicine and agriculture causing a strong selective pressure promoting the evolution of resistant bacterial strains. Bacteria can become resistant to an antibiotic very quickly due to their ability to reproduce rapidly and the processes of horizontal gene transfer, which spreads advantageous mutations to other cells. Furthermore, these mutations that allow for antibiotic resistance can render an entire class of drugs ineffective because they all share the same mechanism of action.

Compounding the increase in resistance, there has also been a steady decline in novel antibiotic compound discovery since the 1960s.[3] Natural product discovery through high-throughput screening of bacteria libraries has shown to be a promising novel antibiotic compound source.[2] In fact, natural products account for two-thirds of new antibiotics approved between 1980 to 2010. Despite the decline in discovery efforts in the past few decades, naturally derived products are still a promising source for antibiotic compounds.[3] There are many different natural product sources

including microbiota isolated from soil environments. Due to high resource competition, range in conditions, and high microbial species diversity, soil environments, particularly the rhizosphere, are a good source for antibiotic producing bacteria species.[4]

Once potential antibiotic-producing bacterial libraries are assembled, biological screening assays are often used to determine the effectiveness of a discovered compound against a pathogen. Xie et al. developed a novel screening method for peptides that target bacterial membranes. They utilize a ribosome display system which begins with the construction or utilization of a preexisting DNA library. Peptide complexes are then formed from isolated peptide coding nucleotides, and these peptides are then tested against immobilized model bacterial membranes. The before mentioned screening assay, and ones like it, are essential to drug discovery because they are specific to membrane-targeting peptides and membrane targeting is often the mechanism of action utilized by antimicrobial compounds.[5] Taking a different approach, the Omura research group utilizes ultraviolet spectra, mass spectrometry, and color reaction in a physicochemical (PC) screening method. This method can be used on many individual compounds produced in bacterial culture broths and serves as a way to quantify the physicochemical properties of novel compounds. In doing this, the PC screening method achieves something biological screening methods cannot: test metabolites that are produced in very low amounts.[6]

One major problem researchers face is the rediscovery of already identified antibiotic compounds. New methods are needed to prevent this from happening and one way to do this is through an early mechanism of action (MOA) elucidation. Very recently, Wex et al developed an agar-based screening technique using biomarkers that will provide MOA and whole-cell bioactivity information for compounds produced by soil bacteria. This method is unique because it can be used with just bacterial strains grown on agar, culture extracts, supernatants, impure extracts, and pure compounds. Biomarkers indicative of bacterial metabolic pathway interference were used in this study, specifically bioreporters that respond to DNA stress, RNA stress, cell envelope stress, and difficulties in protein translation. Assay conditions were modified to achieve fast and reliable results along with sensitive detection. Actinomycetes from the Tubingen strain collection with unknown MOA were studied and they found 270 out of 600 to be antibiotic producers. The researchers were able to identify the expected MOA for the producers at various stages in the production and purification processes; furthermore, they isolated compounds that had not been previously found in this strain collection.[7]

Various studies have identified bacteria that produce antimicrobial metabolites in monoculture (cultivated individually) and many techniques have emerged to lessen the chances of rediscovering known antibiotics. One such technique, bacterial co-culture (growing multiple strains of bacteria together in low food environments) technique, has been utilized in a range of drug discovery applications and is shown to stimulate the production of compounds that are not produced in monoculture. Hoshino et al utilized co-culture technique to stimulate the production of secondary metabolites in actinomycete strains. Streptomyces are known for having secondary metabolite-biosynthetic gene clusters (SM-BGCs) that produce antimicrobial compounds and there is evidence that non-streptomyces actinomyces have a large number of SM-BGCs as well; however, they are poorly expressed by non-streptomyces in monoculture. Co-culturing mono-streptomyces with mycolic acid-containing bacteria and other actinomycetes mimic natural microbial interactions and activate the cryptic SM-BGCs that are not expressed in monoculture.[8] Co-culture technique can be used for bacteria strains from marine environments as well. Ibrahimi et al used co-cultivation techniques to stimulate the production of antimicrobial compounds by Actinobacteria. The research team isolated 142 predatory bacteria along the Moroccan coast and used a range of gram-negative and gram-positive natural prey as the sole nutrient source in the hopes to discover a link between predator-prey relationships and an increase in secondary metabolite production. They discovered that when co-cultivated on Bennet medium most of the Actinobacteria species produced antimicrobial molecules and the compounds that were produced could not be produced in monoculture. This study provides yet another example of co-culture application and how it may increase the production of antimicrobial natural products.[9]

Prior work has already been done in the Wolfe lab to determine co-culture candidates from soil bacteria. Murray et al worked to develop a high-throughput screening method to screen mono and co-culture bacteria mixtures *in vitro* against multidrug-resistant bacteria strains. Screening methods, such as the one discussed in this paper, are essential for research involving bacterial mono or co-culture techniques for drug discovery because any bacteria that show antimicrobial activity during the screening process are then candidates for mono or co-culture studies (Murray 2019)[10]. This work will expand upon Murray et al's method to screen mix microbial solutions (3-4 bacterial strains), with a focus on tri-cultures, for the production of antimicrobial natural products. Furthermore, this work will provide the methodology for stepwise liquid cultivation of tri-cultures to liter quantities, explain how to use organic methods to purify antibiotic compounds from the tri-culture secondary metabolites, and outline current efforts to characterize isolated antibiotic compounds.

2. Experimental

2.1 General Procedures

The careful and selective cultivation of specific bacteria strains is pertinent to this study. Because of this, sterile techniques are used in each step of this process. All liquid cultures and assays are created using single-use plates and test tubes, and the liquid media and pipet tips are sterilized in the autoclave at 121℃ for 60 minutes before use. In addition to this, test tube and plate inoculations are performed under a propane flame and the workbench is wiped down with a 10% bleach solution before and after inoculation. The Gen5 All-In-One Microplate Reader software with BioTek Synergy HTX Multimode Microplate Reader is used to analyze absorbance data. The Sorvall Legend XTR Centrifuge is used at 25℃ and speed 1000 g for 10 minutes when centrifuging. Any culture shaking for the soil bacteria occurred at 160rpm in an Excella E25 incubator set to 25℃. Bacteria in the library are in the form of 25% glycerol stocks and stored at -80℃. To be used in this experiment, the bacteria are quadrant-streaked onto 10% tryptic soy broth (TSB) agar plates and grown for 36-48 hours at 25℃. Liquid soil cultures are made by inoculating one bacteria colony isolated from an agar plate in 10 mL 10% TSB liquid media and incubating at 25 ℃. Similarly, liquid pathogen cultures are made the same way but using full-strength TSB and incubating at 37℃. All HPLC separation is acquired using the Reverse Phase HPLC BetaSil Phenyl column (Thermo Scientific 2.1 x 150 mm, 3µm) instrument running a 40% to 100% gradient mobile phase protocol that spans 30 minutes. Proton NMR data is acquired using a Varian Gemini 2000 and Oxford Instruments 400 Hz superconducting magnet which uses TMS as an internal standard.

2.2 Preliminary Growth Assessment

To begin, soil bacterial strains that do not express antibiotic compounds in monoculture or co-culture are isolated from previous monoculture and co-culture screening assay data.[10] Those bacterial strains (usually five or six total) are then screened in all possible combinations of three. The growth assay method is a five-day process beginning with the soil bacteria being inoculated in 10 mL of 10% TSB liquid media. These cultures are left to incubate for 24 hours at 25°C with constant shaking. The following day, the growth plate is made, using a 96-well round bottom plate, by combining 10 µL of each bacterial strain in a single well then diluting to 200 µL with 10% TSB. Each bacterial strain is grown in monoculture along with the tri-cultures to allow for comparison between monoculture growth trends and the tri-culture growth trends and all cultures were grown in triplicate (Figure 1). It's important to note that to prevent evaporation in the wells containing the bacterial cultures, the first and last row of the plate, as well as the far left and far right columns of the plate, were filled with 200 µL of 10% TSB to create an evaporative barrier. The growth plate is placed directly into the Biotek Plate reader where 590 nm absorbance readings are collected every 2 hours for a total of 96 hours.

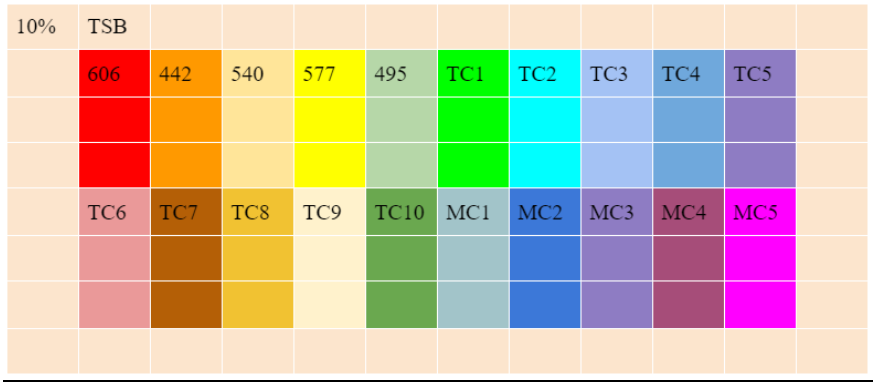

Figure 1. Example Growth Assay Plate Setup

To begin processing the absorbance data, the 10% TSB background is found using the absorbance average from the TSB containing border wells. The absorbances for each culture are averaged. Then the background absorbance is subtracted out. Following this, the absorbance is plotted over time for each monoculture and tri-culture independently.

Then the absorbance graphs for each strain in every tri-culture are overlaid with the respective tri-culture absorbance data.

2.3 Tri-Culture Bioassay Screen Design

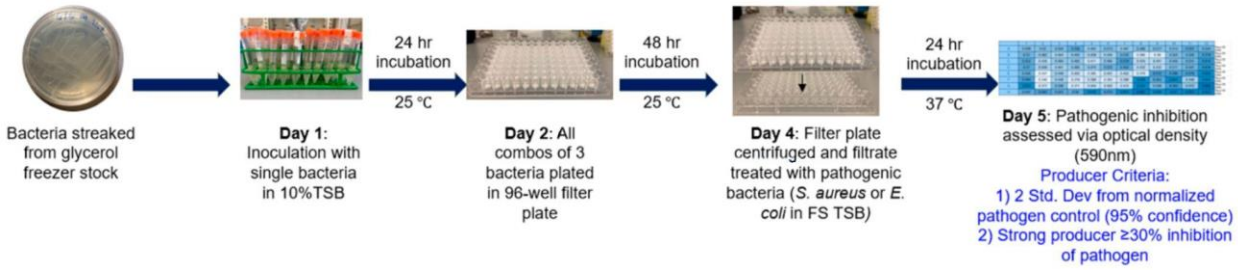

Figure 2. Bioassay 5-day screening process

Murray et al's design used in their mono and co-culture screens served as a guide for the development of a tri-culture bioassay screening method. This method uses a 96-well filter plate stacked with a secondary 96-well round bottom plate.[10] Similar to the growth assay, the screening assay is a 5-day process (Figure 2) that begins with the six selected soil bacteria inoculation in 10 mL of 10% allowing them to grow for 24 hours at 25 °C with constant shaking. After the 24-hour incubation, the filter plate(s) are created by combining 10 µL of each bacterial strain in a single well then diluting to 200 µL with 10% TSB. All possible tri-cultures (20 total) are cultivated in two sets of triplicate (6 wells total for each culture) to allow for screening for antibacterial activity against both *Staphylococcus aureu*s (S. *aureus*) and *Escherichia coli (E. coli)* (Figure 3). It's important to note a 10% TSB border is used in this plate setup and that the filter plate is placed on top of the secondary 96-well round-bottom plate during this process. The filter plate is left to grow with constant shaking for 48 hours at 25 ℃. On the third day, pathogen overnight cultures are made for both *S. aureus* and *E. coli* by inoculating 10mL of full-strength TSB. The pathogen cultures are left to incubate for 24 hours at 37°C and with gentle shaking. By the fourth day, both the pathogen cultures and filter plate(s) have finished incubation and it is time to make the pathogen plate. The filter plate(s) are centrifuged at 1000g for 10 minutes to allow for all secondary metabolites produced by the tri-cultures to pass through the filters and into the secondary plate(s) below. Following this step, the filter plates are discarded and 50µL of either *S. aureus* or *E. coli* are added to the round bottom plate. The new plate is then allowed to incubate for a final 24 hours at 37 ℃ and with gentle shaking before absorbance data is gathered at 590 nm.

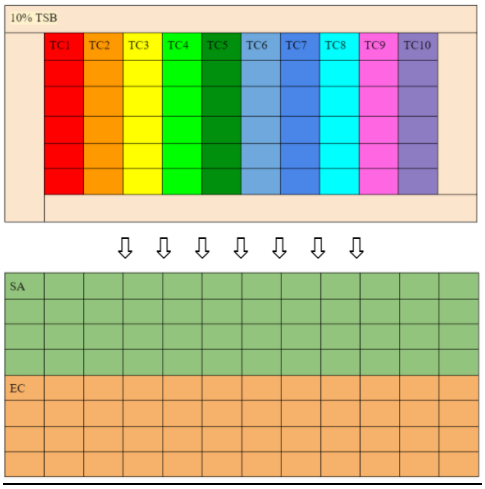

Figure 3. Example Filter Plate and Secondary Pathogen Plate Setup

2.4 Scale-Up Procedure and Secondary Metabolite Purification

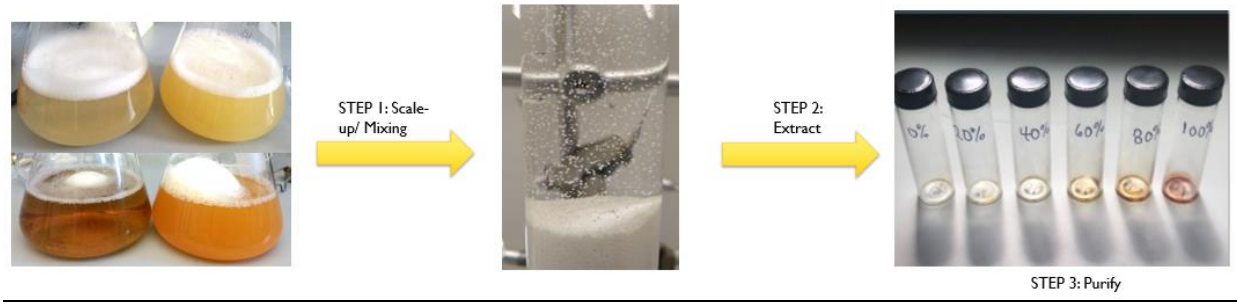

Figure 4. Scale-Up Procedure

For the 540, 442, 657 tri-culture scale-up, 10% TSB is used as the liquid media, and sterile conditions are maintained until the culture reaches the final 3L volume and the culture is mixed. First, 10 mL 10% TSB is inoculated with the bacteria colony using a pipet tip and cultures are left to grow for 24 hrs to make overnight cultures. The next day, the 10mL cultures are added to 20 mL of fresh media (30 mL total) and left to grow for another 24hrs. Then the 30 mL cultures are added to fresh media to give a total volume of ~300mL and are left to grow for 24hrs again. Finally, the 300mL cultures are independently added to the three remaining 2700 mL of fresh media to give 3L total and left to grow for a final 24hrs. The following day, the 540, 442, 657 3L cultures are mixed in equal portions and left to incubate for ~96hrs. The 9Ls of the tri-culture are centrifuged at 4000g for 20 minutes twice and the supernatant (containing the bacterial secondary metabolites) is collected both times (Figure 4).

Column chromatography is used for rough purification of the tri-cultures supernatant. 20g per liter Diaion HP-20 beads are prepared by being soaked in excess methanol and added to the supernatant. After ~6hrs, the liquid is discarded and the beads are poured into a 1L prepped (with cotton and sand) 1L open column, and any excess liquid is left to drain off. Gradient methanol to water mobile phases are flushed through the column in 1L quantities at the following percentages: 0%, 20%, 40%, 60%, 80%, and 100% CH_3OH:H_2O. HPLC was used for further purification of the active fraction (Figure 5).

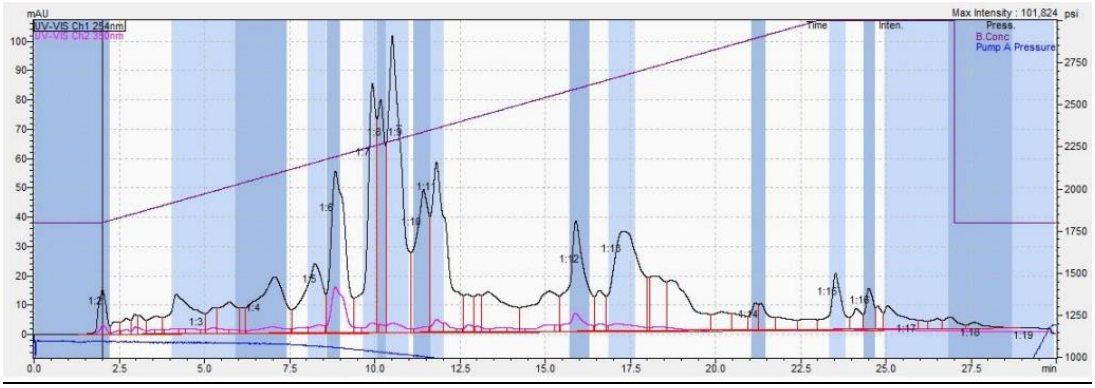

Figure 5. Representative HPLC Spectra

Cell death assays were used to determine the antibiotic activity of each fraction during the purification process. 96 well plate technique is utilized, first pathogens are pipetted into the plates along with the tri-culture fractions dissolved in DMSO (DMSO as a negative control and Chloramphenicol as a positive control) (Figure 6). Note that the compounds would have been diluted to a concentration of 100 mg/mL; however, due to extremely small amounts of product, 20 µL of DMSO were added to each sample. Absorbance readings were collected using the Biotek Plate reader at 590 nm. Generally, a lower normalized absorbance value compared to the negative control indicates cell death. For this assay, weak inhibition is characterized as less than 30% inhibition while strong inhibition is greater than 30% inhibition.

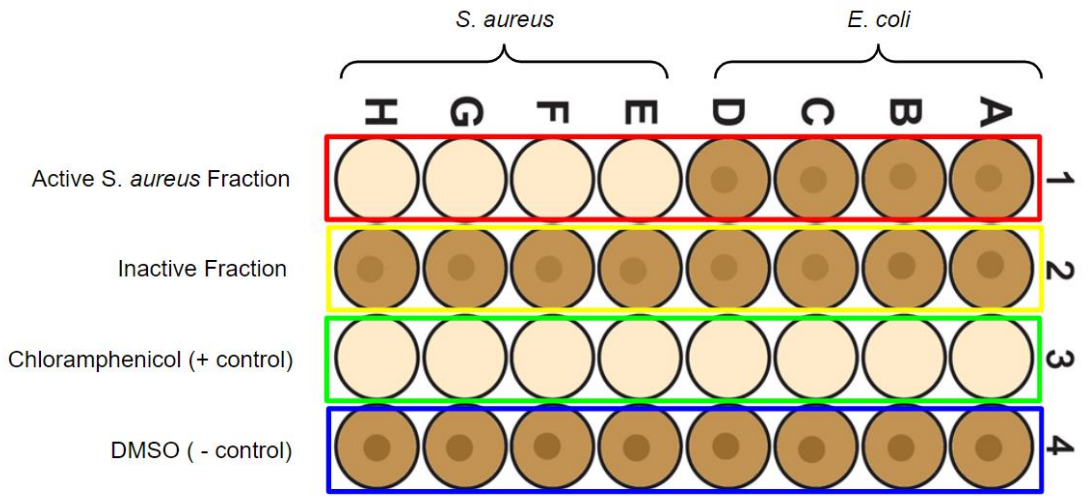

Figure 6. Cell Death Assay Design

3. Results and Discussion

3.1 Growth Assay Results

A total of eight growth assays were performed and soil bacteria were selected for screening based upon similar growth timelines and max absorbance values (Figure 6). A few multi-cultures comprising four bacterial strains were also tested out of curiosity in leftover wells in the growth assays and they showed some promising growth; however, tri-culture growth assessment and screening methodology is the main focus of this research.

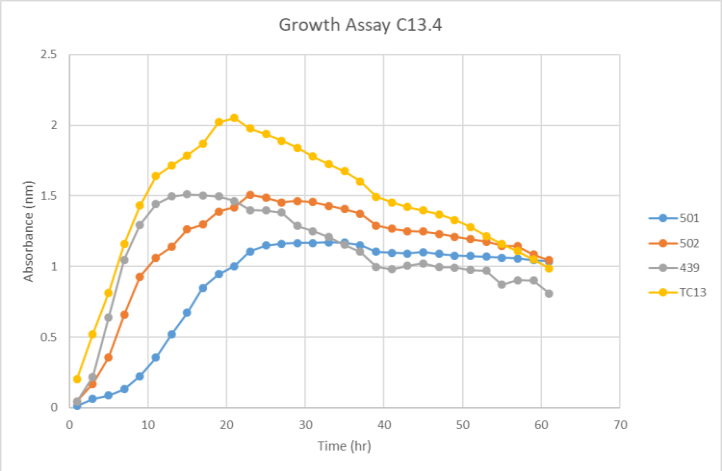

Figure 7. Representative Growth Assay Absorbance Data

3.2 Tri-culture Bioassay Results

There were some unexpected issues caused by plate evaporation in the first few trials of the screening bioassay. In the first trial assay, it was suspected that the plate itself was accidentally rotated too far to one side because the absorbance data showed an unusual absorbance gradient across the plate and the plate itself had a volume gradient across the wells. The assay was repeated using the same methods as before and yielded the same result indicating that there was

some other cause for the volume inconsistencies. It was determined that evaporation within the plate during the growth period in the incubator was causing these issues. During the first two screens, the plates were located too close to the ventilation source in the incubator itself thus, causing the side of the plate facing the vent to experience significantly more airflow and evaporation than the other side of the plate. To mitigate this, the position of the plate attachment within the incubator itself was moved to be on the opposite side of, and parallel with, the air vent. The plates themselves were sealed with two layers of parafilm and scrap cardboard was used to create a wind barrier around the plates.

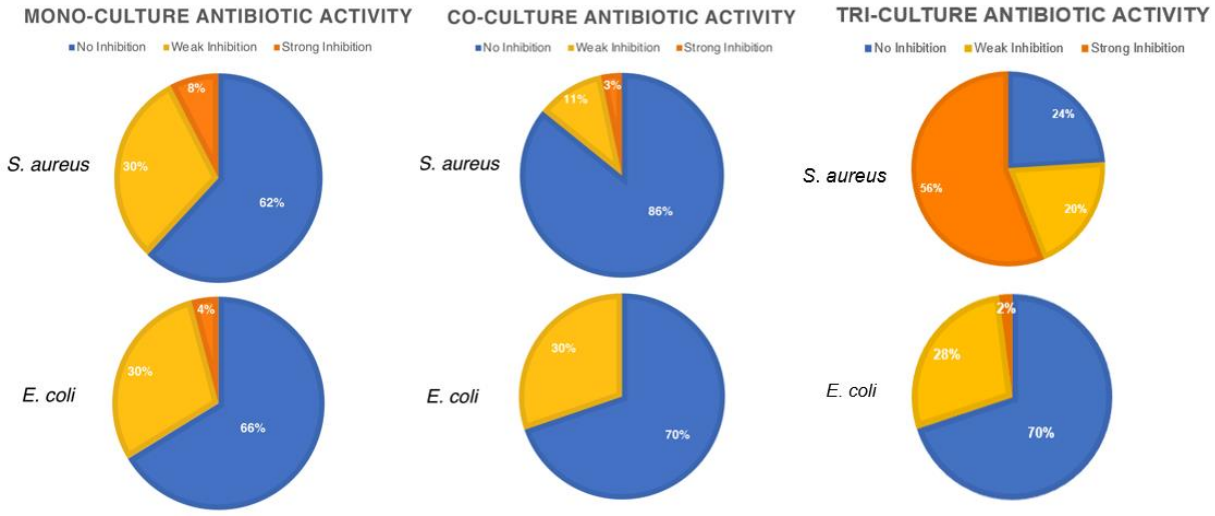

Figure 8. Collective Mono-culture, Co-culture, and Tri-culture Results for Bacteria Screened from the Wolfe Lab Bacteria Library

Four screening assays were performed, comprising 60 tri-cultures, and from those assays, it was determined that 48 of the tri-cultures showed some form of antibiotic activity against SA while only 22 showed activity against EC (Figure 8). As you can see, some renewed activity was observed, particularly for the inhibition of SA. Inhibitory data against SA for each screening assay is outlined in Figure 9, and no inhibition indicates no change in pathogen growth, weak inhibition indicates less than 30% inhibition of pathogen, and strong inhibition indicates greater than 30%inhibition.

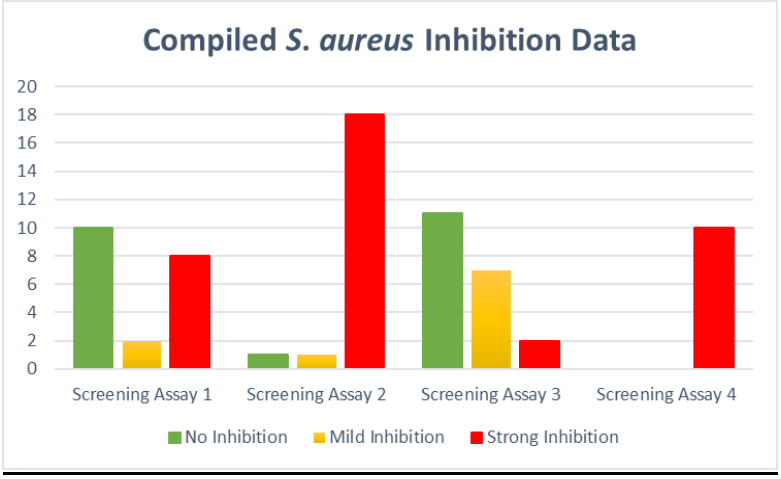

Figure 9. Compiled Screening Bioassay Results for Inhibition of *S. aureus.*

Table 1. Tri-culture Normalized Growth Values

Culture Name	Bacteria Combination	SA Normalized Growth Values	EC Normalized Growth Values
C11	442, 657, 569	----------	$7.813 \times 10^{-1} \pm 4.385 \times 10^{-2}$
C12	442, 657, 438	$5.853 \times 10^{-1} \pm 6.113 \times 10^{-2}$	$8.390 \times 10^{-1} \pm 3.202 \times 10^{-2}$
C13	442, 657, 540	$5.321 \times 10^{-1} \pm 5.220 \times 10^{-2}$	$7.872 \times 10^{-1} \pm 2.318 \times 10^{-2}$
C14	442, 569, 438	$6.023 \times 10^{-1} \pm 6.130 \times 10^{-2}$	$8.254 \times 10^{-1} \pm 9.801 \times 10^{-3}$
C15	442, 569, 540	$5.905 \times 10^{-1} \pm 4.824 \times 10^{-2}$	$7.578 \times 10^{-1} \pm 2.066 \times 10^{-2}$
C16	442, 438, 540	$5.826 \times 10^{-1} \pm 4.338 \times 10^{-2}$	$7.796 \times 10^{-1} \pm 1.721 \times 10^{-2}$
C17	657, 569, 438	$5.971 \times 10^{-1} \pm 5.583 \times 10^{-2}$	$8.395 \times 10^{-1} \pm 4.421 \times 10^{-2}$
C18	569, 438, 540	$6.069 \times 10^{-1} \pm 6.578 \times 10^{-2}$	$7.921 \times 10^{-1} \pm 5.284 \times 10^{-2}$
C19	657, 438, 540	$6.863 \times 10^{-1} \pm 6.442 \times 10^{-2}$	$9.947 \times 10^{-1} \pm 4.314 \times 10^{-1}$
C20	657, 438, 540	$7.461 \times 10^{-1} \pm 7.979 \times 10^{-2}$	$9.914 \times 10^{-1} \pm 6.559 \times 10^{-2}$

It is important to look for patterns between the bacteria present in the cultures and the level of inhibition seen to gain further insight into which bacteria is directly responsible for the production of the antibiotic compound. C13 (strongest SA inhibitor) and C15 (strongest EC inhibitor) comprise both 442 and 540. Surprisingly, C17 has neither 442 or 540 and showed strong inhibition against SA (Table 1). The trends regarding the importance of 657, 438, or 569 are unclear; however, when cultured together some antibiotic activity is seen. A clearer trend can be seen with the presence of both 442 and 540 in the tri-cultures and the resulting strong inhibition of SA as well as the weak inhibition of EC.

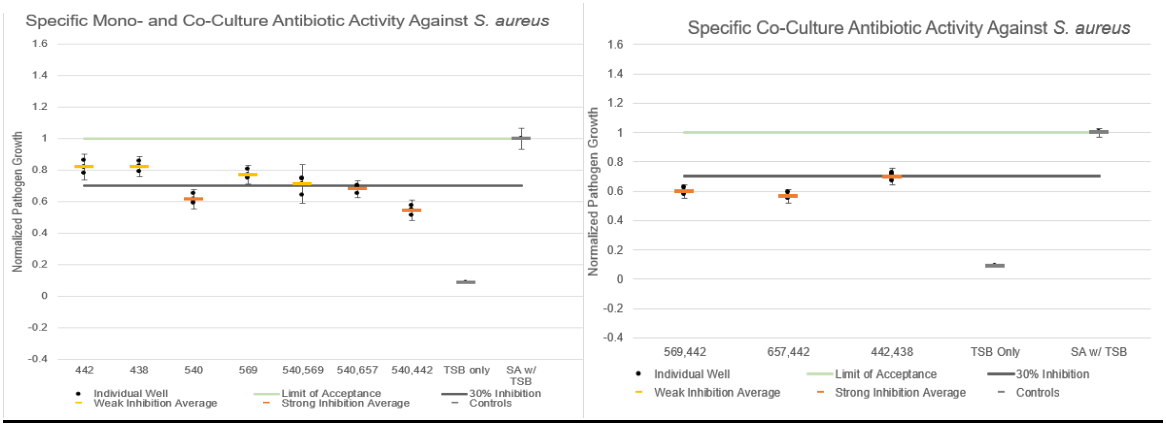

Figure 10. Mono- and Co-culture results for inhibition of SA

Even though these bacteria have been previously screened in monocultures and co-culture by a previous experiment, they were screened again in mono- and co-cultures to give a direct means for normalized growth value comparison. Contrary to previous reports, strong inhibition of SA is seen for 540 in mono-cultures and weak inhibition is seen for 442/438, and 569 in mono-cultures. Furthermore, co-cultures containing 442 all showed strong inhibition against SA and all co-cultures containing 540 showed strong inhibition against SA except for culture 540/569 (Figure 10). For EC, 442 and 540 were found to show weak inhibition in mono-cultures, while 438, 569 and 657 do not. All co-cultures screened against EC showed no inhibition. It's important to note that co-culture 442 and 540 were excluded from this

data set because the results do not fit in a 95% confidence interval. This indicates that 442 and 540 have some not previously reported antibiotic-producing capabilities, and these two strains may be responsible for the antibiotic activity seen in C13.

Table 2. Percent Pathogen Growth Inhibition by Mono-, Co-, and Tri-cultures of 540 and 442.

	Pathogen	
Soil Culture	SA (% Growth Inhibtion)	EC (% Growth Inhibition)
540	38.7 ± 0.0312%	12.6 ± 0.0343%
442	18.3 ± 0.0410%	23.2 ± 0.0392%
540, 442	45.7 ± 0.0317%	19.5 ± 0.0968%
540, 442, 657	46.8 ± 0.0522%	24.3 ± 0.0232%

When comparing mono-, co-, and tri-culture inhibition, a trend starts to appear for 540 and 442. 540 showed increasingly stronger inhibition of SA from mono- to co-culture than to tri-culture. Similarly, 442 only showed weak inhibition in monoculture but showed strong inhibition in co-culture and even stronger inhibition in tri-culture. For EC, both 540 and 442 showed weak inhibition in monoculture, no inhibition in co-culture (although this is without the consideration of a 442/540 co-culture), and showed the 'strongest' weak inhibition in tri-culture (Table 2)

3.3 Scale-Up and Cell Death Assay Results

Five scale-ups have been completed for the 540, 442, 657 tri-culture and antibiotic compounds were extracted from two of those scale-ups. There was some initial inconsistency in scale-up results and some purification error, but antibiotic compounds were successfully purified from the tri-culture scale-up after some trial and error. This indicates that this scale-up method developed for tri-cultures can potentially be used for other tri-cultures in the future. After an initial cell death assay using the rough column fractions, the 80% and 100% fractions from the first scale-up showed antibiotic activity against SA. The 18 HPLC fractions isolated from the 80% rough fraction were screened for antibiotic activity against EC and SA first due to a stronger antibiotic response than the 100%. No strong inhibition of EC was seen; however, fractions 5.1-12.1 showed strong inhibition of SA (Figure 11). TLC indicated the presence of one (possibly two) antibiotic compounds spread out among those fractions.

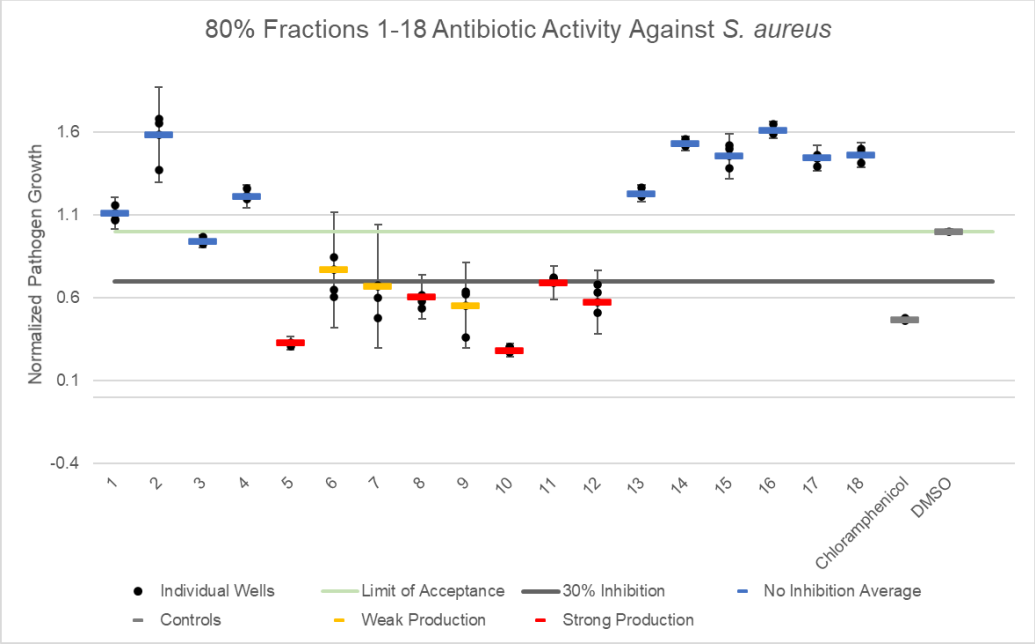

Figure 11. Cell Death Assay Results of 80% HPLC fractions 1-18 against *S. aureus*

In the fourth scale-up, an initial cell death assay showed that the 80% and 100% fractions showed antibiotic activity against SA. Of the 14 HPLC fractions, fractions 8.4 and 14.4 showed the strongest antibiotic activity, 14.4 being comparable to the positive control (Figure 12). The strong antibiotic activity of compound 14.4 was very surprising due to the inactivity of compound 14.1. However, because of this activity, compound 14.4 was further analyzed.

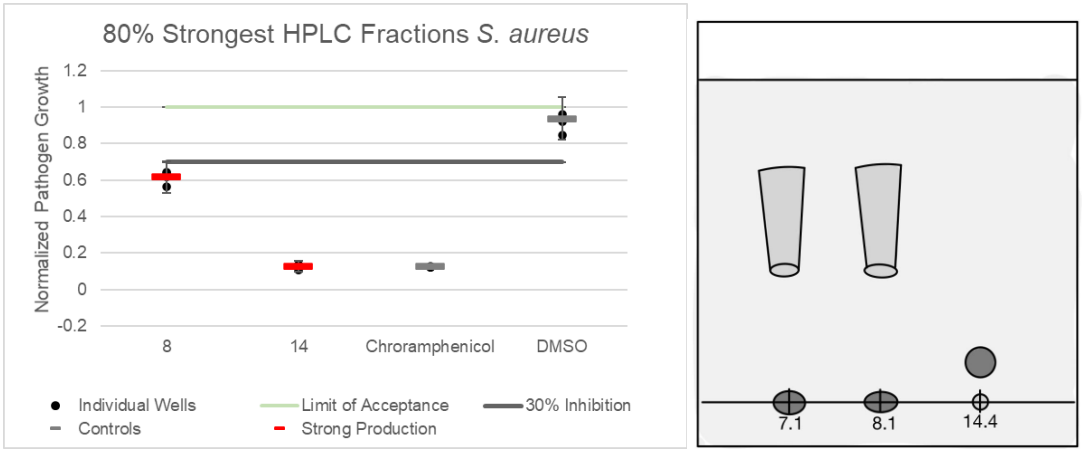

Figure 12. Cell Death Assay Results for 80% HPLC fractions 8 and 14 against *S. aureus* and TLC comparison

TLC confirmed that 14.4 is not the same as the antibiotic compounds 7.1 or 8.1 from the first scale-up. (Figure 12). Furthermore, through TLC it was determined that compound 14.4 was more polar than compounds 7.1 and 8.1 due to its location on the TLC plate. This aligns with the HPLC retention time as well since compound 14.4 eluded much later than the other compounds. 14.4 is also strongly long wave active while 7.1 and 8.1 are not indicating the presence of a conjugated double bond system. Following this confirmation, compounds 7.1 and 8.1 were combined and will be referred to as 7.8 while compound 14.4 will be referred to as compound 14 while reporting characterization data.

3.3 Spectroscopic Identification of Active Compounds.

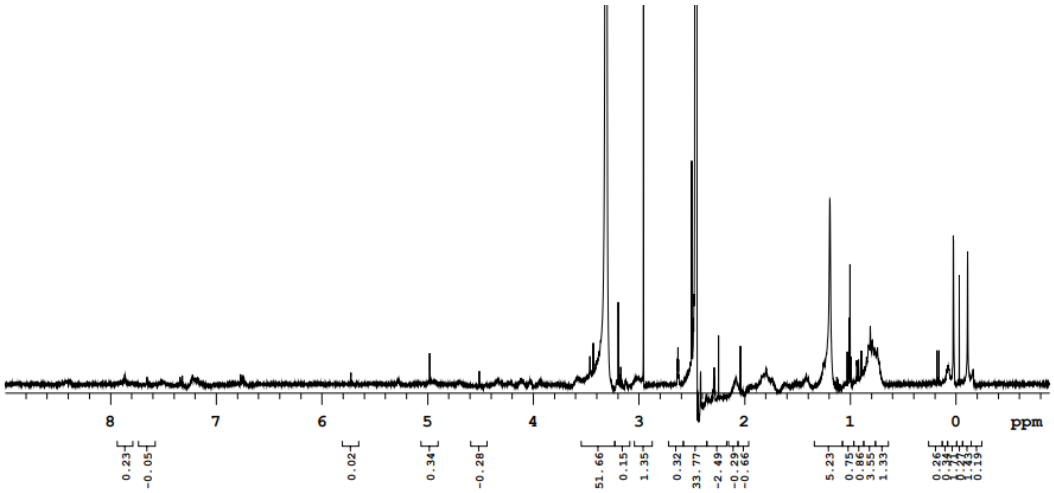

Figure 13. 7/8 H^1NMR, DMSO-d6

Proton NMR was collected for compound 7/8 and due to only having a small amount of the active compound, only a few small peaks were observed below 3ppm. The presence of these peaks indicates the possible presence of saturated alkanes, methyl groups, and either OH or NH functional groups (Figure 13).

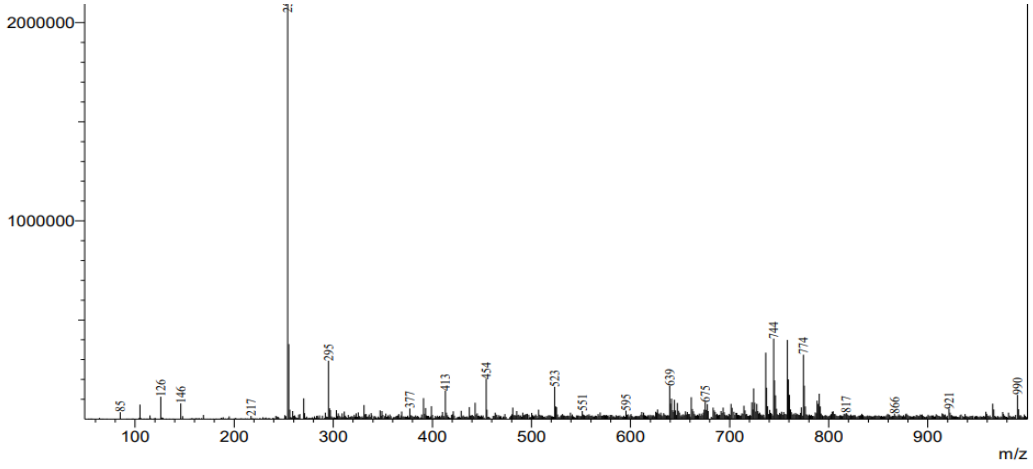

Figure 14. 7/8 LC-MS (+)

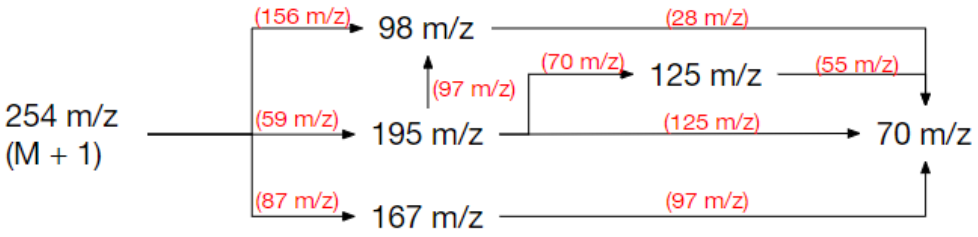

Figure 15. 7/8 SIM

Mass spectrometry was performed and following a full scan (detecting up to 1000 m/z) 254m/z was the most abundant mass for the positive ionization mode (Figure 14). Following this, selective ionization mode (SIM) was used for the 254 m/z and the fragmentation pattern is outlined in Figure 15. Masses lost between fragmentation events are also recorded and some patterns can be observed. The 195 m/z ion seems to decrease as both the 125 m/z and 70 m/z ions increase in abundance indicating that they originated from the 195 m/z. This is supported by the observation that 125 plus 70 equals 195. A mass of 97m/z was lost in two separate fragmentations, 195 m/z to 98 m/z and 167 m/z to 70 m/z. Furthermore, all larger masses seem to be fragmenting into 70 m/z as the smallest stable unit. The 70 m/z ion is most likely one of the following compounds: $C_2H_2N_2O$, $C_2H_4N_3$, $C_3H_2O_2$, C_3H_4NO, $C_3H_6N_2$, or C_4H_6O

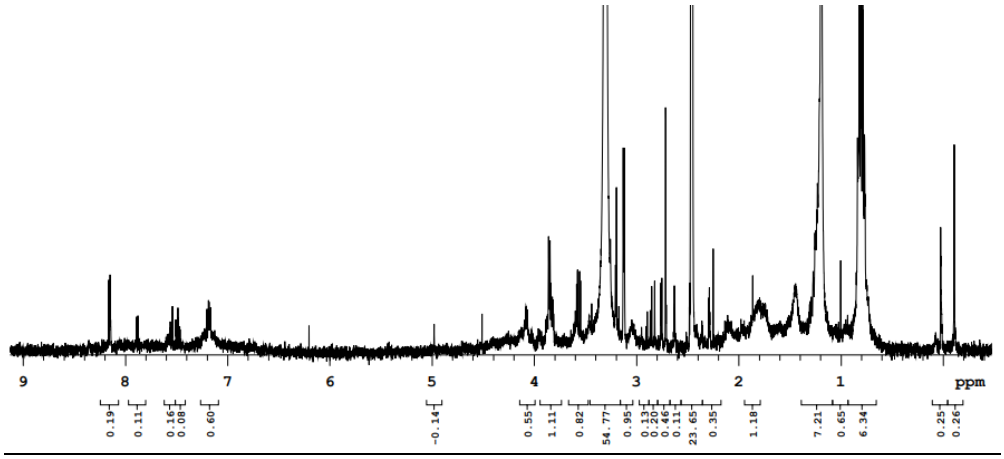

Figure 16. 14 H^1NMR, DMSO-d6

Proton NMR was acquired for compound 14 and peaks were observed in the aromatic region and, mostly, below 4 ppm. Peaks between 8.5 ppm and 7 ppm agree with previous data indicating that 14 is more nonpolar than the previously discovered antibiotic compounds and explains the long wave activity. The presence of peaks below 4 ppm indicates possible saturated alkanes, methyl groups, ether groups, and OH or NH functional groups.

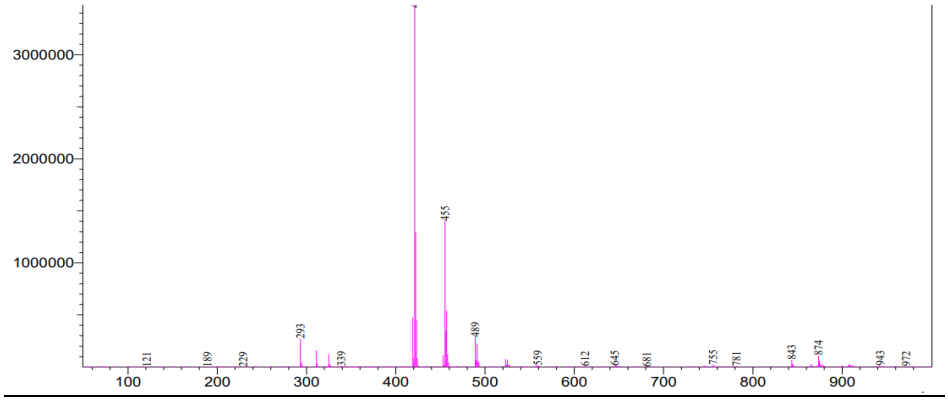

Figure 17.14 Full Scan (-)

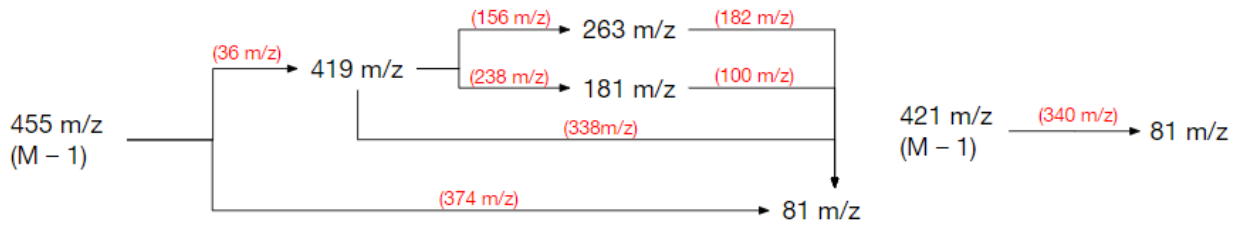

Figure 18. 14 SIMs

Mass spectrometry was performed and following a full scan (detecting up to 1000 m/z) 421 m/z was the most abundant mass, with 455 m/z as the second most abundant mass for the negative ionization mode (Figure 17). Following this, selective ionization mode (SIM) was used for both 421 m/z and 455 m/z, and the fragmentation pattern is outlined in Figure 18. Surprisingly, the most abundant 421 m/z mass only fragmented into one ion, a very stable 81m/z ion. This 81m/z ion is most likely one of the following compounds: C_3HN_2O, C_2HN_4, C_4H_3NO, $C_3H_3N_3$, C_5H_5O, $C_4H_5N_2$, or C_5H_7N. 340 m/z loss is observed for this mass and, due to its magnitude, could be many different compounds. 455 m/z fragmented into many more ions, thus providing slightly more information. The smallest mass lost, 36 m/z, is somewhat unusual, and can only be attributed to the loss of C_3H. Similar to the 421 m/z mass, all 455 m/z fragment ions seemed to become the very stable 81 m/z ion. 81 m/z could be many different compounds, including C_2N_3O or $C_4H_2O_2$.

4. Conclusions

To combat the increasing healthcare crisis caused by antibiotic resistance, new techniques are needed to bring the gap in antibiotic discovery to an end. Bacterial libraries can be a rich resource if mined thoroughly using robust screening techniques. Bacterial co-culture and minimal media methodology can be used to further stimulate antibiotic expression in bacterial strains. This research takes co-culture methodology a step further and provides a method to screen tri-cultures robustly and quantitatively. These screening studies resulted in multiple tri-cultures with inhibitory properties. A method to grow tri-culture 540, 443, 657 at liter quantities and purify the antibacterial compound was provided. 76% of the tri-cultures screened showed some form of antibiotic activity indicating the possible antibiotic mining potential for future tri-culture screening projects. The results of this research indicate a trend of increased inhibition for the same bacteria screened in mono-, co-, and tri-cultures. This research also has led to the discovery of two potential antibiotic compounds (**7/8** and **14**) that are strongly active against SA. In lieu of these results, tri-cultures may be used to increase antibiotic compound production in bacteria that show weak production in mono- and co-cultures. Researchers can also successfully extract these antibiotic compounds from the secondary metabolites using organic purification techniques. Characterization of antibiotic compounds **7/8** and **14** has begun with the successful acquisition of H^1NMR, Full Scan Mass Spectrometry, and SIM Mass Spectrometry data. Fragmentation patterns have been analyzed and work to identify fragment and ion loss molecular formulas is ongoing. To streamline this natural product isolation and identification workflow, I will continue to alter the scale-up and purification processes to increase the precision and reproducibility of this method. Finally, I believe this research will lay the foundation for the future development of mixed microbial cultivation methodology and screening methodology encompassing more than 3 bacteria species, and create a novel avenue for drug discovery.

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