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DETERMINATION OF THE CHEMICAL STRUCTURE OF THE ANTIMICROBIAL SECONDARY METABOLITES PRODUCED BY *STREPTOMYCES* SPP. ICC1 AND ICC4

2023 | NATALIE MARIE PIETRAMALA

B.Sc. HONOURS THESIS – CHEMICAL BIOLOGY

THOMPSON RIVERS UNIVERSITY

DETERMINATION OF THE CHEMICAL STRUCTURE OF THE ANTIMICROBIAL SECONDARY METABOLITES PRODUCED BY *STREPTOMYCES* SPP. ICC1 AND ICC4

by

NATALIE MARIE PIETRAMALA

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF

BACHELOR OF SCIENCE (HONS.)
in the
DEPARTMENTS OF PHYSICAL AND
BIOLOGICAL SCIENCES
(Chemical Biology)

This thesis has been accepted as conforming to the required standards by:
Dr. Heidi Huttunen-Hennelly (Ph.D.), Thesis Supervisor, Dept. Physical Sciences
Dr. Naowarat (Ann) Cheeptham (Ph.D.), Co-supervisor, Dept. Biological Sciences
Dr. Kingsley Donkor (Ph.D.), External examiner, Dept. Physical Sciences

Dated this 11th day of May, 2023, in Kamloops, British Columbia, Canada

ABSTRACT

Streptomyces spp. ICC1 and ICC4 are strains of bacteria isolated from the Iron Curtain Cave in Chilliwack, British Columbia. The secondary metabolites of these bacteria have antimicrobial properties that could be utilized in a variety of medicinal purposes. The goal of this project is to isolate and characterize the secondary metabolites of *Streptomyces* spp. ICC1 and ICC4. These two *Streptomyces* strains were first cultured and then tested for antimicrobial activity against a non-multidrug resistant target bacterium, *Escherichia coli* #59 and *Staphylococcus aureus*, and a strain of yeast, *Candida albicans*. Once antimicrobial activity was determined, the culture was extracted with diethyl ether to isolate the secondary metabolites. After extracting a greater volume of the secondary metabolites, they will be purified by reversed-phase high performance liquid chromatography (RP-HPLC). The bioactivity of the separated metabolites will be re-confirmed against *Escherichia coli* #59, *Staphylococcus aureus*, and *Candida albicans*. The metabolites will continue to be analyzed by mass spectrometry (MS) and proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy to determine a mass and structure of the compounds, respectively. Once the structure has been elucidated, further research into why those particular compounds exhibit antimicrobial properties will follow. This research will further the body of knowledge on *Streptomyces* spp. ICC1 and ICC4, and its pharmaceutical potential.

Thesis Supervisor: Dr. Heidi Huttunen-Hennelly

ACKNOWLEDGMENTS

I would like to extend my gratitude towards Dr. Heidi Huttunen-Hennelly and Dr. Naowarat Cheeptham for their guidance, resources, and support throughout this project. Heidi, thank you for all of the time and attention you provided during all of the chemical analyses. Ann, thank you for demonstrating biological assays and microbiology techniques. I would also like to acknowledge Dr. Kingsley Donkor for his positivity throughout the project and his role as external examiner. I would like to thank Kathy Baethke and the members in Dr. Cheeptham's research group for their advice and guidance in the lab. Additionally, thank you to Dr. Eric Bottos and Dr. Don Nelson for their advice on how to better showcase my work in a poster format.

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LIST OF ABBREVIATIONS

BGCs: Biosynthetic Gene Clusters

BSC: Biosafety Cabinet

^{1}H-NMR: Proton Nuclear Magnetic Resonance

HT: Hickey-Tressner Media

HT_N: Hickey-Tressner Normal Strength

ICC1 Flask A – F: ICC1 isolates of Flask C originating from Read's[16] cultures

ICC1 Flask C.3 and C.4: Additional flasks of ICC1 Flask C created to upscale the volume

LB: Modified Lysogeny Broth/Luria-Bertani with pharmamedia

MALDI: Matrix Assisted Laser Desorption/Ionization

MDR: Multidrug Resistant

MS: Mass Spectrometry

NA: Nutrient Agar

NB: Nutrient Broth

NRPS: Non-Ribosomal Peptide Synthetase

PDB: Potato Dextrose Broth

R2A: Reasoner's 2A broth

RiPPs: Ribosomally Synthesized and Post-Translationally Modified Peptides

RP-HPLC: Reverse Phase High Performance Liquid Chromatography

Rpm: Revolutions per Minute

INTRODUCTION

Antibiotic resistance in microorganisms is a continuously growing dilemma and a great threat to humans.[10,13] By studying *Streptomyces* ICC1 and ICC4 and their antimicrobial secondary metabolites, we may be able to make a significant contribution towards solving this ever-growing issue.[9] Bacteria that are capable of producing these secondary metabolites are extensively utilized in medicine as antifungal, antibacterial, and anticancer treatments.[4]

It was concluded that the *Streptomyces* ICC1 contains polar secondary metabolites that contain hydrophobic side groups from Milward[14] and Read[16]; however, the mode of action and structure of the metabolites are still unknown.

Iron Curtain Cave

The Iron Curtain Cave (ICC) is located in Chilliwack, British Columbia.[8] The temperature ranges from 4 to 12°C; the cave also inhabits six bodies of water which results in a humid environment.[8] It is primarily composed of carbonate and has iron and limestone deposits throughout.[8] Mineral deposits from the water sources in the cave have formed three speleothem types depicted in Figure 1: bacon, popcorn, and soda straws.[8]

Figure 1. Speleothem deposits throughout the ICC. (A, B) Bacon – calcium carbonate. (C) Popcorn. (D, E) Soda Straws – calcium carbonate.[8]

The cave is not open to the public, and any personnel that enter are restrained to a marked path, which is shown as the red line in Figure 2; this restricted entry ensures the cave habitats are preserved.[8]

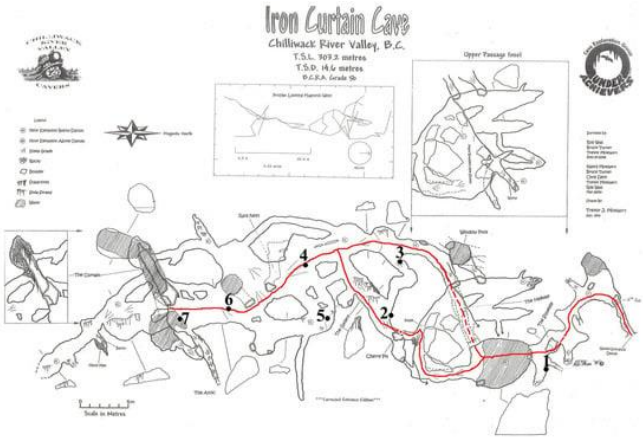

Figure 2. Map of the Iron Curtain Cave, Chilliwack, BC. Both *Streptomyces* ICC1 and ICC4 were collected from Point 1, the Connection Room.[8]

***Streptomyces* spp. ICC1 and ICC4**

The two strains of *Streptomyces* ICC1 and ICC4 were collected from Point 1 in Figure 2.[8,9] Both strains have macroscopic and microscopic similarities to *Streptomyces* spp. such as being filamentous and Gram-positive.[7] The ICCI and ICC4 isolates also have slight differences between them in their genome, which is discussed more in the following paragraph, and microscopic morphology as shown in Figure 3; ICC1 has a ridged surface and filamentous microscopic morphology, whereas ICC4 has a smooth surface on a mass of branching filaments.[8]

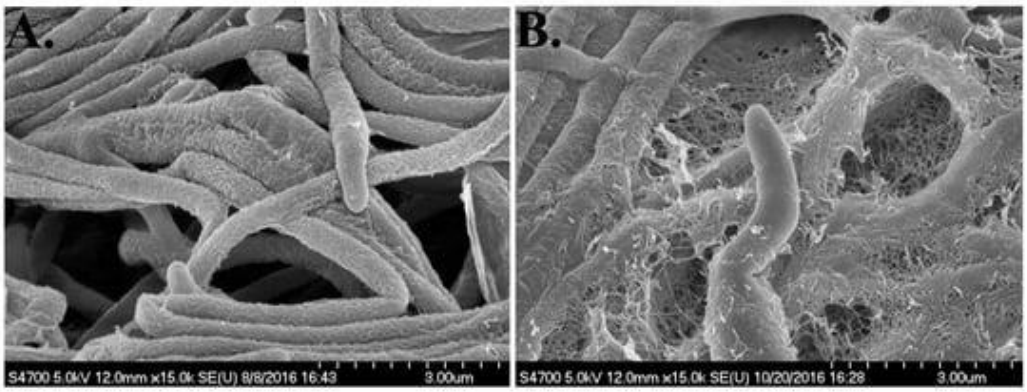

Figure 3. Microscopic morphologies of the two isolates imaged using Scanning Electron Microscopy. (A) *Streptomyces* ICC1, 5 μm scale bars. (B) *Streptomyces* ICC4, 3 μm scale bars.[8]

Streptomyces ICC1 and ICC4 were determined to have a total genome length of around 9.0 kbp and both had a 72% G+C content.[9] The isolates were genomically compared to *Streptomyces lavendulae* strain CCM 3239 and had a 54% alignment match.[9] ICC1 has previously demonstrated antimicrobial properties against the multidrug resistant (MDR) *Escherichia coli* 15-124 and 15-102, and ICC4 demonstrated antimicrobial properties against those same strains of *E. coli*, as well as *Pseudomonas aeruginosa* and *E. coli* 15-318 for MDR, and *Staphylococcus aureus* and *E. coli* non-MDR strains.[8]

Some strains of microorganisms can grow and survive in extreme temperatures ranging from boiling to freezing, including volcanic caves and glaciers.[3,5] Since the *Streptomyces* ICC1 and ICC4 are found in an extreme cave environment with minimal nutrients, light exposure, and below average temperatures, the bacteria are in competition with other organisms to survive; consequently, the isolates must provide the energy to produce these antimicrobial biomolecules to outcompete other bacteria that also have similar defence mechanisms to survive in a cave.[15]

Secondary Metabolites

Secondary metabolites are an integral part of the survival of bacteria.[12] Organisms that are part of the *Streptomyces* genus tend to produce abundant and diverse secondary metabolites; the diversification in the metabolites from ICC1 and ICC4 are likely a factor of the extreme cave environment they were grown and survived in.[9] These secondary metabolites are held in biosynthetic gene clusters (BGCs) which can be analyzed via bioinformatic technologies.[9] BGCs can include a multitude of biomolecules, including ribosomally synthesized and post-translationally modified peptides (RiPPs) and non-ribosomal peptides, and organic molecules, including terpenes and polyketides.[9] Additionally, *Streptomyces* produce melanoid pigments/melanins that give the solution a dark brown colour when producing their secondary metabolites.[2,6] These pigments are negatively charged, hydrophobic, classified as diverse molecular polymers, and are the products of oxidative polymerization of indolic and phenolic molecules.[2,6] The melanoid pigments assist the bacteria in their survival by absorbing some of the ultraviolet radiation from the sun that could damage the bacteria's DNA.[1,2]

The software AntiSMASH in the study by Gosse et al.[9] was able to identify and quantify the BGCs present in the metabolites of ICC1 and ICC4. It was found that most of the BGCs were identified as being terpenes, and there was a total of 35 BGCs in the ICC4 isolate and 37 BGCs in the ICC1 isolate.[9] This software was also utilized to identify the pathways the organism uses to synthesis the BGCs; some of the pathways identified led to the synthesis of SapB (morphogenic peptide), coelichelin, and lantipeptides.[9] There was also a molecule that had a peptide core which could not be confidently confirmed, so it was deemed as unidentified.[9] There was also a pathway in the ICC4 strain that synthesized a sulphur-containing thiopeptide.[9]

Another software called BAGEL was applied to try and identify the specific lantipeptides that the pathways led to.[9] From this analysis, a new RiPP cluster was noted, but the identity of the lantipeptides remained inconclusive.[9]

Lastly, a manual observation into the BGCs identified another two BGCs, in ICC1, including the non-ribosomal peptide synthetase (NRPS) enzyme and pyoverdine which increases bacterial virulence by taking in iron, similar to coelichelin.[9, 11]

Objectives

The goal of this research project was to identify the antimicrobial active molecules present in *Streptomyces* ICC1 and ICC4 bacteria. Broth optimization was necessary in trying to improve the laboratory growth conditions to decrease the duration of the secondary metabolite production. Obtaining enough sample to be purified on RP-HPLC was important for the further chemical analyses done on ^{1}H-NMR and MS help elucidate the metabolite secondary structure.

MATERIALS AND METHODS

Broth Optimization

A total of four different broths were tested to observe which would be most beneficial in optimizing the growth and secondary metabolite production of the *Streptomyces.* These broth medias were Hickey-Tressner (HT), Nutrient Broth (NB), Modified Lysogeny Broth/Luria-Bertani with pharmamedia (LB), and Reasoner's 2A (R2A); the formulas for these media are located in Table A1 of the Appendix. These broths were made in a normal strength batch, and a batch that was 1/10 the concentration. A 4 mL volume of each broth was sorted into labelled test tubes indicating their contents before the bacteria of interest was added (discussed further in the next paragraph). The broth that produced the darkest brown colour in the shortest amount of time was deemed as the most optimal broth for the bacterial growth.

Subculturing ICC1 Bacterial Colonies from Previous Cultures

The six broth culture flasks of *Stretomyces* ICC1 in NB from the previous research project conducted by Read[16] were used to subculture new volumes of the isolates. Those six flasks were labelled A – F, and a 1 mL aliquot of each flask was added to one test tube of each type of media broth mentioned previously.

The bacteria and broth solutions were incubated at 15°C, without a rotary shaker, and checked every 1 – 2 days for the dark brown pigment to appear. Since HT was the most optimal media, six Erlenmeyer flasks with 75 mL of HT_N and six Erlenmeyer flasks of 75 mL of HT 1/10 were made to increase the bacterial volume; the test tubes were then poured into their corresponding flask. The larger bacterial cultures were placed in a rotary shaker incubator set at

95 rpm and 15°C. These flasks were monitored for the brown pigment every few days for about a month before being tested for bioactivity via a Kirby-Bauer Disk Diffusion Assay.

Once bioactivity was observed, a portion of the bioactive culture was inoculated into five 500 mL Erlenmeyer flasks and placed into the same incubator. The bacteria were left to become confluent and were tested for bioactivity every 2 weeks – 1 month.

Subculturing ICC4 Bacterial Colonies from Ottawa Plates

Plates containing the ICC4 isolates were shipped from the University of Ottawa. Colonies that appeared to be free of contamination were inoculated into 250 mL of HT_N broth. The broth cultures were placed in the same incubator and conditions as the ICC1 cultures were in. The ICC4 isolates were monitored for a colour change and were tested for bioactivity every 2 weeks – 1 month.

Gram-stain

Due to potential contamination in some of the received plates from Ottawa, a Gram-stain was done on those samples, as well as some of the darkest flasks shown in Figure 5, to confirm if the bacteria growing was, in fact, *Streptomyces*. Utilizing proper aseptic techniques, a quarter sized amount of 85% saline and bacteria broth were added onto a clean microscope slide and left to dry. Once fully dried, the slides were briefly flamed to fix the bacteria in place. Next, Crystal Violet dye was applied to the flasks and left on for 30 s – 1 min before being washed with water gently. Following the wash, Gram's Iodine was added and left for 30 s – 1 min before being rinsed, as in the previous step. Acetone was applied until no colour was coming off; the slides were then

washed. Lastly, Pink Fuchsin was applied and left for 10 – 30 s before the final rinse. All slides were dried on bibulous paper and then viewed under a microscope.

Subculturing *Escherichia coli* #59*, Staphylococcus aureus*, and *Candida albicans*

A non-multidrug resistant strain of *E. coli*, #59, and *S. aureus*, as well as a strain of yeast, *C. albicans*, were used in the bioassay to test for antimicrobial activity. Each bacterium was inoculated into 1-2 mL of NB from a nutrient agar (NA) plate and placed in a 37°C shaking incubator overnight to allow for confluent growth. The following day, the broth cultures were analyzed on a spectrophotometer set at 600 nm; the target absorbance range was 0.600 – 1.000.

Extraction of the Secondary Metabolites

A liquid-phase organic extraction was completed to separate and extract the metabolites from the media and other biomolecules. In Milward's[14] and Read's[16] work, it was concluded that diethyl ether was the most suitable solvent in extracting the secondary metabolites from the broth due to the retention in bioactivity in the extracts; therefore, diethyl ether was used for this portion of the project as well.

A 2:1 ratio of broth culture to diethyl ether was combined in a separatory funnel. The funnel was shaken and frequently vented. The aqueous layer settled to the bottom since water has a greater density than diethyl ether; the aqueous layer was transferred into a tube and placed to the side. The organic layer was transferred into a sterile glass container. This process was completed two more times for a total of three extractions.

The extracts were left in the fume hood for about 1 – 3 hours, or until about 1 – 2 mL remained, to allow for the majority of diethyl ether to evaporate off to increase the concentration

of metabolites. The containers were then closed with a lid/cap and stored in the 15°C incubator with no rotary motion until a bioassay was conducted.

Kirby-Bauer Disk Diffusion Assay

The Kirby-Bauer disk assay was utilized to observe antimicrobial activity through the presence of zones of growth inhibition. To begin, NA was made, autoclaved, and placed in a water bath at 55 – 60°C for about an hour or until the media was the same temperature so that it would not kill the target bacteria when it is added.

While the NA was being autoclaved, the autoclaved paper susceptibility disks were prepared in the biosafety cabinet (BSC) following its UV light sterilization for 20 mins. The disks were prepared in two ways: 1) 8 mm disks were dunked in the organic layer extracts or the aqueous broth and left in solution for 30 mins before being taken out and dried for 1 hour, or, 2) disks with a 6 mm diameter were pipetted with 30 μL of supernatant and left to dry for 1 hour, and this method was repeated two more times for a total of 90 μL aliquots and 3 hours of drying time.

Once the NA had reached the desired temperature, a 0.02 % and a 1% aliquot of target bacteria to agar was added to each plate. For example, a plate containing 20 mL of agar would have a 0.02% and 1% volume of 0.004 mL or 0.20 mL of either *E. coli*, *S. aureus*, or *C. albicans*, respectively. After the target bacteria was combined with the NA, it was poured into plates and left to cool between 2 – 6 hours so that the contained bacteria would not become confluent.

After the agar was set, the dried paper disks were placed onto the gel with an even distance between disks. An antibiotic disk of 10 μg/mL gentamycin, that targets against Gram-negative bacteria, was used on the plates containing *E. coli*, and an antibiotic disk targeting both Gram-positive and Gram-negative bacteria, cephalothin in 30 μg/mL, was placed onto all to act as a

positive control. The assay plates were placed into an incubator set to 37°C, to allow for target bacterium growth, overnight. If zones of inhibition appeared, that indicated that the particular strain of *Streptomyces* tested was producing its antimicrobial secondary metabolites.

A flowchart outlining the methodology employed throughout this project is included in Figure 4 below.

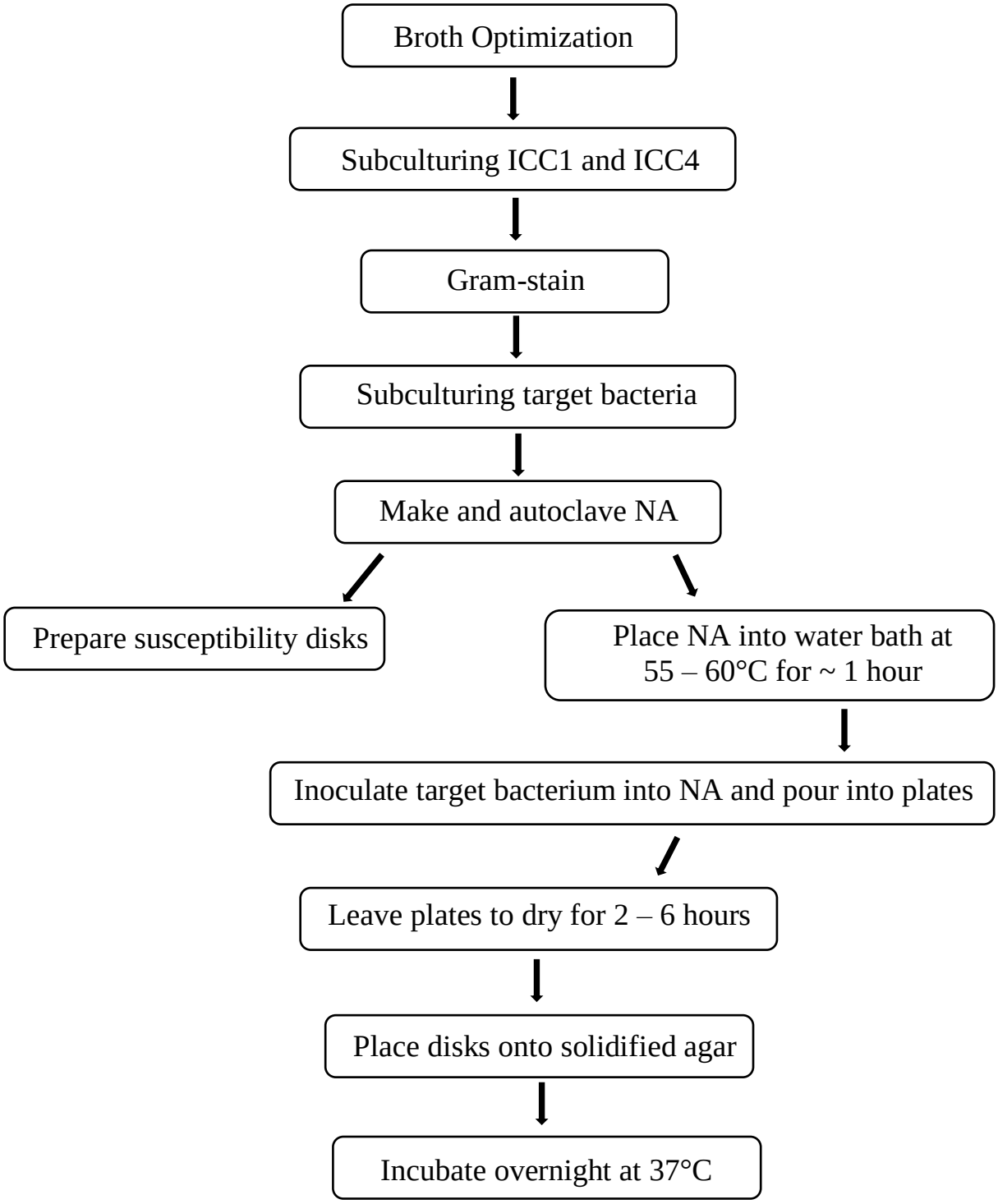

Figure 4. Flowchart of the methodologies and procedures followed in this experiment.

RESULTS AND DISCUSSION

Broth Culture Flasks

From the Broth Optimization, the media which was determined to be the most optimal was the normal strength of Hickey-Tressner (HT_N). The cultures in this broth produced the melanin pigment faster than the other broth medias tested. The larger flasks containing HT_N are shown in Figure 5 below.

Figure 5. Six flasks of ICC1 in HT_N broth after 22 days of incubation, with Flask C (third from the left) being the darkest.

The three flasks that contained the bioactive strains, are imaged below in Figure 6. Two of these three flasks depicted as B and C in Figure 6, were a dark brown and were made in HT_N; the other flask, labelled A in the Figure below, did not inhabit the dark brown pigmentation and was made in a blend of HT 1/10 strength and HT_N media.

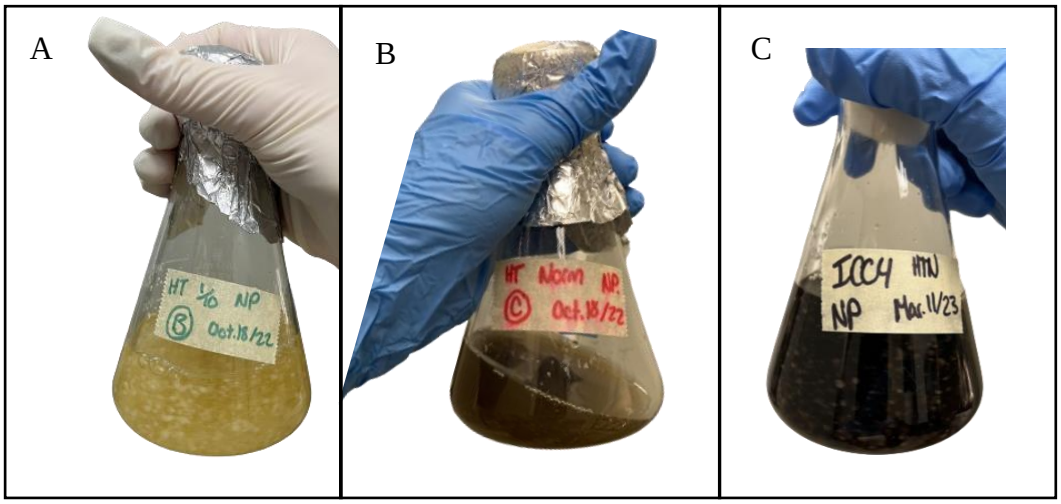

Figure 6. Flasks of the *Streptomyces* broth cultures in HT media. (A) ICC1 Flask B. (B) ICC1 Flask C. (C) ICC4 Flask.
Note: Flask B was originally grown in 1/10 HT media, but a 250 mL aliquot of HT_N was added about 5 months after.

Gram-stain

The results obtained during the Gram-stain indicated growth of *Streptomyces* due to the purple colour, indicating Gram-positive bacteria, and filamentous rods that are indicative of the *Streptomyces* genus.[7] The stains of the bioactive flasks are imaged below in Figure 7.

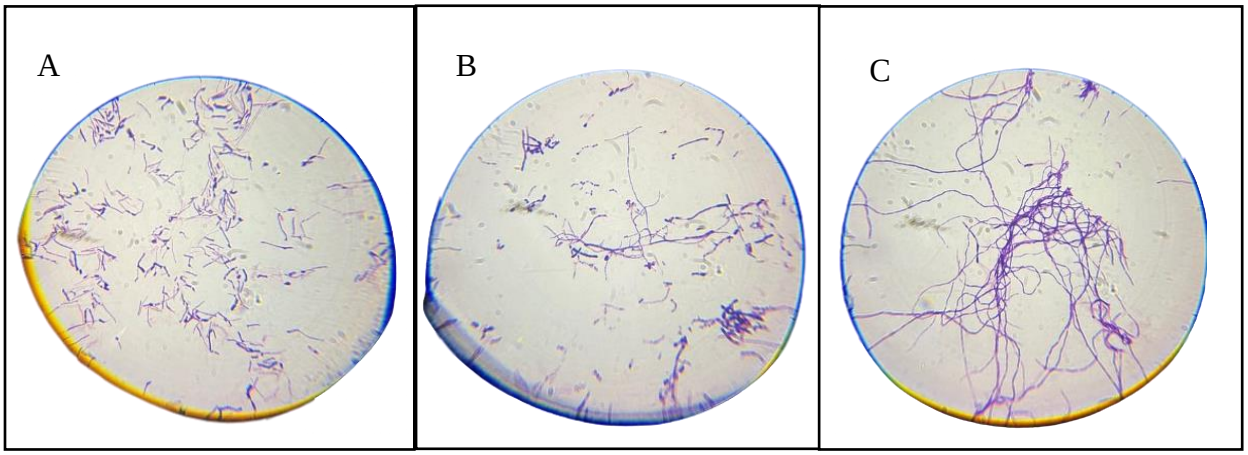

Figure 7. Gram-stains showing Gram-positive filamentous bacilli. (A) HT_N ICC1 Flask C. (B) HT_N ICC1 Flask B. (C) ICC4 isolate from Ottawa.

Kirby-Bauer Disk Diffusion Assay

Following the Kirby-Bauer Disk Diffusion Assay protocol, found in the Materials and Methods section, a multitude of Kirby-Bauer assays were completed to test for antimicrobial activity.

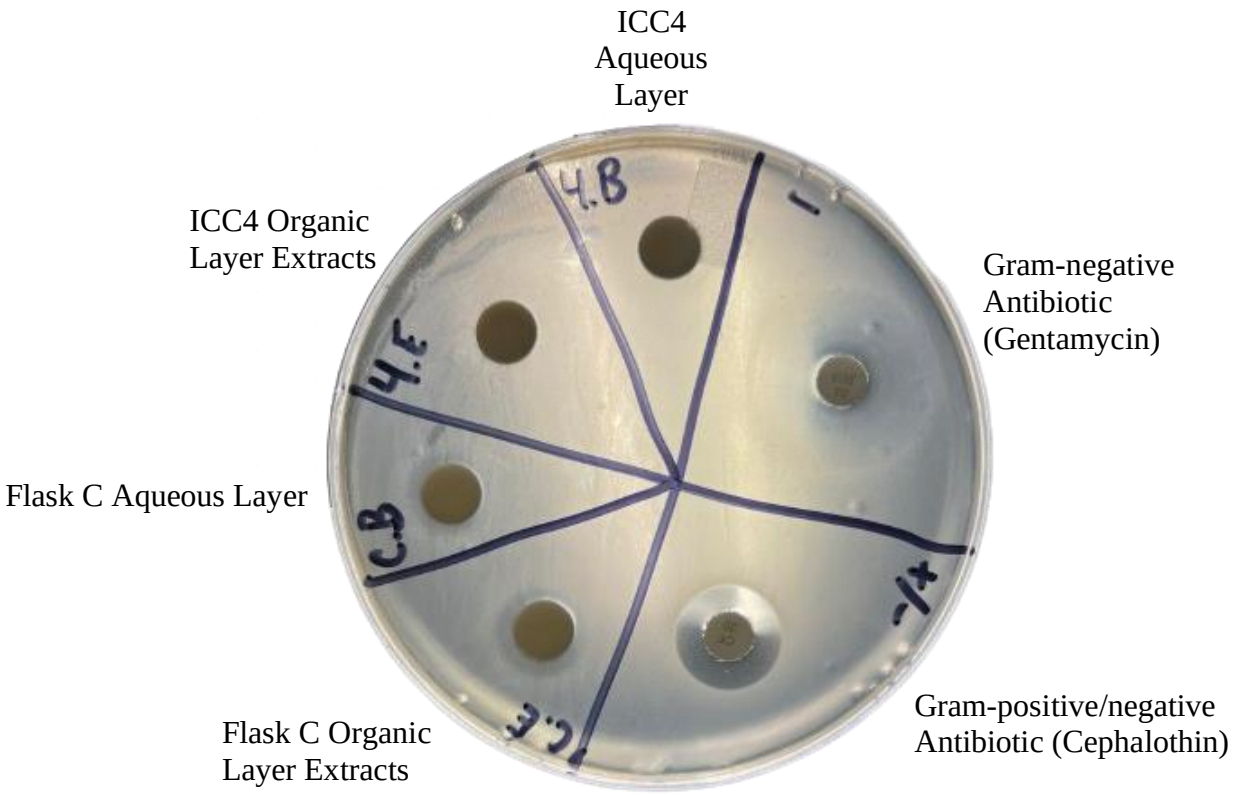

Figure 8. Kirby-Bauer assay with *E. coli* #59 as the target bacterium with blank susceptibility disks with an 8 mm diameter that were impregnated with the crude strains of interest. Zones of inhibition are present around the extracts of ICC1 Flask C and ICC4.

Table 1. Zone of inhibition measurements for the crude sample disks in Figure 8 with *E. coli* as the target bacterium.

Disk	**Distance from Centre of Disk (mm)**
Gram-negative Antibiotic (Gentamycin)	12
Gram-positive/negative Antibiotic (Cephalothin)	7
Flask C Organic Layer Extracts	7*
Flask C Aqueous Layer	7*
ICC4 Organic Layer Extracts	0
ICC4 Aqueous Layer	0

Note: * results were increased by 2 mm to adjust for the 8 mm impregnated disks compared to the 6 mm antibiotic disks.

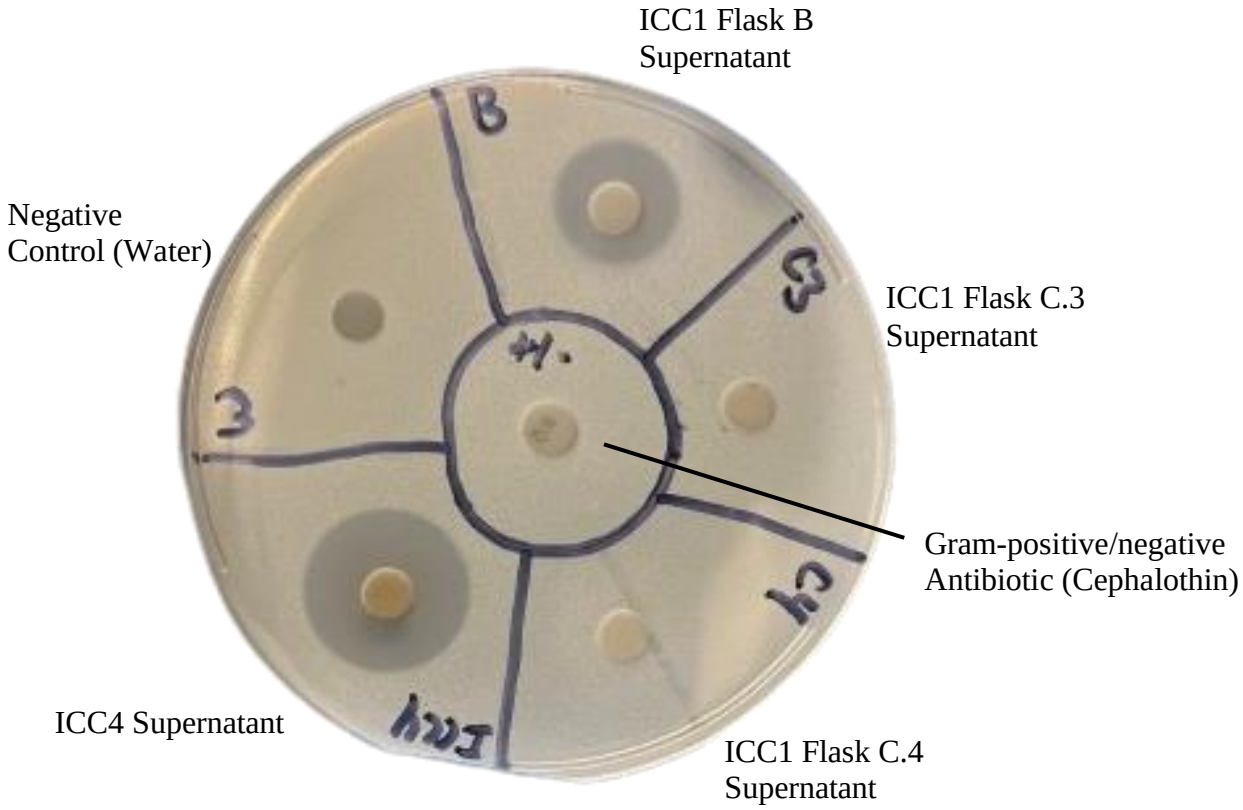

Figure 9. Kirby-Bauer assay *C. albicans* as the target bacterium with blank susceptibility disks with a 6 mm diameter that were impregnated with the crude strains of interest. Zones of inhibition are present around the supernatants of ICC4 and ICC1 Flask B.

Table 2. Zone of inhibition measurements for the crude sample disks in Figure 9 with *C. albicans* as the target bacterium.

Disk	**Distance from Centre of Disk (mm)**
Negative Control (Water)	0
Gram-positive/negative Antibiotic (Cephalothin)	0
ICC1 Flask C.4 Supernatant	0
ICC4 Supernatant	10
ICC1 Flask B Supernatant	7.5
ICC1 Flask C.3 Supernatant	0

Discussion

After the initial subculturing, brown pigmentation began to appear approximately 3 – 5 days post inoculation, and the entire broth had a dark brown colour after 1 month of incubation. Pellet formation in the broth was visible since the beginning since pellets were inoculated into the new broth media from the prior six flasks.

When upscaling broth volume via subculturing, the newly subcultured flasks would have an appearance similar to its parent flask; for example, if a dark brown flask was used to subculturing a new flask, that new flask would also be dark brown in colour. Observations regarding pigmentation and pellet formation was difficult to be made due to the immediate appearance of colouration and/or pellets.

All of the ICC1 C Flasks and ICC4 broth had the dark brown hue indicative of melanin production, but ICC1 Flask B did not; as seen in Figure 7. Since Flask B had antimicrobial activity even without the melanoid pigmentation, it suggests that perhaps the brown pigmentation of the broth is not a reliable indication of antimicrobial secondary metabolite production with the HT

media. Additionally, the broth in ICC1 Flask B was fairly transparent and there were white pellets that contained some filamentous nature, which is indicative of *Streptomyces*, and that was the reason it was tested for bioactivity even without a brown hue. This type of transparent broth and pellet morphology was also clearly seen in the ICC4 flask; the pellet appearance in ICC1 Flask C was difficult to observe since the broth itself was more cloudy. Additionally, the original ICC1 flasks left by Read[16] had differences in broth morphology as well; some contained a brown cloudy colour, and others were more of a yellow/beige colour that was transparent with visible white pellets inside. More studies are needed to confidently determine the role of melanin in relation to antimicrobial metabolite activity.

The lengthy duration of time needed for the broth cultures to exhibit bioactivity was substantially greater than the times found by Milward[14] and Read[16] which was around 5 – 40 days of incubation. A potential reason for this is that both of the prior researchers had grown their culture in NB instead of HT media. Even though HT_N media produced the brown pigmentation faster, NB may have nutrients that foster the bacterial growth and metabolite production more efficiently. Furthermore, Milward[14] and Read[16] kept their broths in a shaking incubator set at 150 rpm whereas the incubator for this project was kept at 95 rpm. The faster rotation could add more aeration into the broth cultures which could increase the bacteria growth rates.

The results of this project do indicate that the two isolates from the ICC have antimicrobial activity against a strain of non-MDR bacteria and a strain of yeast. From the results shown in Figures 7 and 8, bioactivity was present in organic layer extracts, aqueous layer extracts, and the culture supernatant. Prior results from Milward[14] and Read[16] found that the antimicrobial metabolites ended up in the organic extract layer only, namely when using diethyl ether. Therefore, Milward[14] and Read[16] concluded that the metabolites were polar and possess some hydrophobic

substituents since they partition preferentially into diethyl ether instead of water. Conversely, it was found in this research that there was activity in both the diethyl ether organic layer and in the water layer; this could be due to some unwanted mixing between the organic layer with the aqueous layer during the extractions or from an incomplete extraction of the metabolites from the aqueous broth.

Additionally, the supernatant disks were subjected to a total of 90 μL of broth, whereas the organic and aqueous layer disks were only subjected to about 30 μL (close to the maximum holding amount of the disks). This could lead to the greater zones of inhibition in Figure 9, or as would be expected, the concentration of metabolites in the supernatant to be higher than in the extracts.

In the comparison between Tables 1 and 2, it is evident that the zones of inhibition of the samples in Table 2 were all larger than the experimental samples in Table 1, even with the adjusted disk sizes. Also, the zones themselves are much clearer in Figure 9 than Figure 8. This could be an indication that the isolates were better able to inhibit the growth of *C. albicans* than *E. coli* #59; however, this is not conclusive since the metabolite concentrations were unknown and the samples applied to the disks were crude.

CONCLUSIONS AND FUTURE WORK

In conclusion of this project, it was determined that HT_N media was the best broth for the growth of *Streptomyces* ICC1 and ICC4 due to the dark brown colour that formed in the least amount of time; this is currently the best estimate for antimicrobial metabolite production. Additionally, it was found that there were prominent zones of inhibition around the disks of the ICC1 Flask B Supernatant and the ICC4 Supernatant against *C. albicans*, and around the ICC1 Flask C Organic Layer Extracts and Broth against *E. coli*, shown in Figures 8 and 7, respectively

However, due to lack of time, the broth cultures are not scaled up yet, so structural analysis could not be conducted.

In terms of the future work relating to this project, crude bacteria samples will be analyzed on Matrix Assisted Laser Desorption/Ionization (MALDI); this will provide the metabolite mass(es) present in the extracts so that the identity of the metabolites can be narrowed down.

Once a large volume of bioactive broth cultures is achieved, the samples will be purified and separated via reverse phase-high performance liquid chromatography (RP-HPLC) to separate compounds based on their polarities. Once this separation is complete, the samples will be analyzed on proton nuclear magnetic resonance (^{1}H-NMR) to elucidate the chemical structure of the compound(s).

Additionally, the results against *C. albicans* are promising for further research into this yeast. Since *Candida albicans* is a strain of yeast that is prevalent in and on the human body, if this yeast begins to grow uncontrollably, it can lead to infections in vital organs and body parts, such as the heart, vagina, brain, and mouth.[4] Consequently, if the *Streptomyces* isolates have reproducible activity against this yeast, then it can potentially be used as an antibiotic to fight those aforementioned infections.

In the future, the *C. albicans* will be grown in an overnight culture of Potato Dextrose Broth (PDB) and placed in a shaker incubator at 25°C to optimize its growth conditions. When conducting the Kirby-Bauer disk diffusion assay, the preparation steps for the *Streptomyces* susceptibility disks will be the same, except a Potato Dextrose Agar will be used, and Nystatin will be used as the positive control. The assay will also be placed in an incubator set at 25°C.

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APPENDIX

Table A1. Formulas for the types of media used in the Broth Optimization portion of the methodology. All masses are for a 500 mL volume of deionized water.

Media Type	Normal	1/10
Hickey-Tressner (adjusted to pH 7.3)	- 10 g **dextrin** - 1 g yeast extract - 1 g **enriched meat media** - 2 g N-Z amine	- 1 g **dextrin** - 0.1 g yeast extract - 0.1 g **enriched meat media** - 0.2 g N-Z amine
Nutrient Broth	- 1 g D(+)-glucose - 15 g peptone - 6 g sodium chloride - 3 g yeast extract	- 0.1 g D(+)-glucose - 1.5 g peptone - 0.6 g sodium chloride - 0.3 g yeast extract
Modified Lysogeny Broth/Luria-Bertani	- 5 g peptone - 5 g pharmamedia - 5 g yeast extract - 5 g sodium chloride	- 0.5 g peptone - 0.5 g pharmamedia - 0.5 g yeast extract - 0.5 g sodium chloride
Reasoner's 2A	- 3.15 g R2A powder	- 0.315 g R2A powder

Note: Dextrin from Potato Starch was used.
Note: Beef Extract Powder was used for the Enriched Meat Extract.