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Research Article

Isolation, Identification and Assessment of the Anti-bacterial, Anti-Fungal and Anti-biofilm Activity of a Novel Temperature Resistant Thermophilic *Actinomycetes* Gram Positive Bacteria Extracted from the Soil

Suvekshya Shreshta, Juhi Desai, Mariam Tinawi and Kokila Kota*

Ramapo College of New Jersey, Mahwah, NJ, USA

***Corresponding Author:** Kokila Kota, Principle Investigator, Ramapo College of New Jersey, Mahwah, NJ, USA.

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Abstract

Biofilms are a hallmark feature of various opportunistic pathogenic bacteria that allow them to communicate with each other. *Pseudomonas aeruginosa* is an opportunistic bacterium that results in serious infections in health-care settings, associating with over 70% of all clinical infections. *Pseudomonas* growing in biofilms exhibit adaptive resistance to essentially all antibiotics and there is an urgent need to work on new methods to supplement our current antibiotics. Therefore, the long-term goal of our research aims to develop anti-biofilm agents targeting polymicrobial communities rather than the planktonic bacteria.

An important group of soil bacteria, recognized as a source of commercially important enzymes and antimicrobials, is the largely underestimated *Actinomycetes.* Considering the potential of discovering *Actinomycetes* that can produce enzymes and antimicrobials, this study is aimed to isolate *Actinomycetes* samples collected from sites across Ramapo College campus soils and test the antimicrobials with industrial and medical applications. Several strains were isolated and screened using standard microbiology and biochemical diagnostic tests. One strain was identified as a thermophile and based on the colony morphology, gram staining and diagnostic tests had identified it to be a gram positive bacteria belonging to the genus *Actinomycetes*.

The isolated strain of *Actinomycetes* was further tested and the extracellular crude extract from the supernatant exhibited anti-bacterial and anti-fungal activity assessed by agar disk diffusion. It also exhibited anti-biofilm activity against the *Pseudomonas aeruginosa* biofilm. Our research addresses if pre-treatment of the bacterial biofilms with the purified supernatant increases their antibiotic susceptibility. A combined treatment of antibiotics with antibiofilm peptides may offer a very potent treatment of both biofilm and planktonic infections resulting in novel adjuvant therapies.

Keywords: *Pseudomonas aeruginosa; Actinomycetes;* temperature

Introduction

Pseudomonas aeruginosa is an opportunistic bacterium that often results in serious infections in health-care settings especially in immunocompromised patients. *Pseudomonas* growing in biofilms exhibit adaptive resistance to essentially all antibiotics and there is an urgent need to work on new methods and ways to supplement our current antibiotics. Therefore much of our current research is aimed at a long term goal of developing anti-biofilm agents targeting biofilm forming and the planktonic forms of the bacteria *Pseudomonas aeruginosa*.

One of the most important groups of soil bacteria, recognized as a source of commercially important enzymes and antimicrobials, is the *Actinomycetes* and the diversity of *Actinomycetes* is largely underestimated. Considering the potential of discovering *Actinomycetes* that can produce enzymes and antimicrobials with

industrial and medical applications, this study is aimed to isolate *Actinomycetes* from soil samples collected from selected sites of Ramapo campus soils. Several strains were isolated and screened using the standard microbiology and biochemical diagnostic tests. One of the several strains isolated was identified as a thermophile and based on the colony morphology, gram staining and the diagnostic tests it was identified to be a gram positive spore forming bacteria belonging to the genus *Actinomycetes.*

The isolated strain of *Actinomycetes* has the ability to withstand and thrive at a high temperature and based on the rate of growth at higher temperatures, it is identified as a thermophilic gram positive spore-forming bacteria. The extracellular crude extract from the cell free supernatant of the thermophile exhibited anti-bacterial and anti-fungal activity assessed by agar disk diffusion method. The thermophilic extract also showed anti-biofilm activity against the bacterial biofilm formed by the bacteria *Pseudomonas aeruginosa*. Addition of Proteinase-K to the extract showed a reduction in the antimicrobial activity of the crude extract which indicated that the cell-free supernatant has antimicrobial peptides (AMP) inhibiting the test microbes. Ammonium sulfate at a 60% saturation (w/v) was used to precipitate the extracellular AMP from the crude cell-free extract. To further purify and remove the ammonium sulfate, ultra-filtration technique (using Amicon filters) was used. The concentration of the partially purified AMP was measured using the Bradford Assay. Using the disk diffusion method, the antimicrobial activity was measured using the zones of inhibition against the gram positive bacteria *Staphylococcus aureus* and the gram negative bacteria *Pseudomonas aeruginosa* and *Escherichia coli*. It was also used to estimate the antifungal activity against *Candida albicans*. The results showed that the extract had both antibacterial, antifungal activities and also antibiofilm activity against the *Pseudomonas aeruginosa* biofilms. The novel thermophilic shows a promising antimicrobial activity and in the future our goal is to further purify the AMPs from the strain and examine the synergistic effects of the peptides with the commercial antibiotics on *Pseudomonas aeruginosa* biofilm inhibition. A combined treatment of antibiotics with antimicrobial peptides may offer a very potent treatment of both biofilm and planktonic infections resulting in novel adjuvant therapies.

Materials and Methods

Isolation of the thermophilic strain-materials

- Soil sample
- Test tubes
- Bunsen burner
- Distilled water
- Tryptic soy agar (TSA) plates
- Incubator.

Isolation of the thermophilic strain-method

- Collect a soil sample from various sites on Ramapo College campus.
- Heat shock and serially dilute the sample. The purpose of serial dilution is to perform a stepwise dilution to logarithmically decrease the concentration to an experimentally reasonable level.
- Inoculate the bacteria on tryptic soy agar (TSA) plates and place the plate in an incubator to be grown at various temperatures.
- One strain from the soil sample withstood and thrived in high temperatures. Therefore concluding it is a thermophilic strain.

Biochemical testing

Starch hydrolysis materials

- Isolated thermophilic strain
- Starch agar plate
- Sterile sticks
- Incubator
- Iodine solution.

Starch hydrolysis method

- Using a sterile stick, pick up the thermophilic strain and streak it on a starch plate in the form of an ‘S’ across the entire plate.
- Incubate the plate at 37°C for 48 hours.
- Add 2-3 drops of iodine solution directly onto the bacteria and allow it to interact with the bacteria for 10-15 minutes.
- Read the plates to determine if it was positive or negative. Adopted from Microbe Online [1].

Methyl Red/Voges-Proskauer test-materials

- Thermophilic strain
- MR-VP broth
- Inoculating wire
- Bunsen burner
- Incubator

- Methyl red indicator
- 5% alpha-naphthol
- 40% Potassium Hydroxide.

Methyl Red/Voges-Proskauer test-method

Methyl red test

- In a test tube containing the MR-VP broth, inoculate the thermophilic strain with a sterile inoculating loop and incubate at 35°C for 48 hours.
- Add 3-6 drops of methyl red indicator to the tube.
- Observe for a red color immediately and record.

VP test

- In a test tube containing the MR-VP broth, inoculate the thermophilic strain with a sterile inoculating loop and incubate at 35°C for 18 to 24 hours.
- Add 6 drops of 5% alpha-naphthol and mix.
- Add 2 drops of 40% potassium hydroxide and mix.
- Observe the color after 30 minutes and record. Adopted from Universe 84a [2].

Gelatinase test-materials

- Thermophilic strain
- Gelatinase test tube
- Inoculating loop
- Bunsen burner
- Incubator.

Gelatinase test-method

- Inoculate the thermophilic strain with a sterile inoculating loop into the gelatin and incubate at 35-37°C for 24-48 hours.
- Remove the gelatin tube from the incubator and place at 4°C to check for dissolution of the gelatin. Adopted from Microbiology Info [3].

Catalase test-materials

- Thermophilic strain
- Test tube
- Hydrogen peroxide solution
- Sterile swab

Catalase test-method

- Pour 1-2 mL of hydrogen peroxide into a sterile test tube.
- Using a sterile swab, add the thermophilic strain into the test tube.
- Observe for immediate results. Adopted from Microbiology Info [4].

Gram staining-materials

- Thermophilic strain
- Microscope slide
- Bunsen burner
- Inoculating loop
- Distilled water
- Crystal violet
- Iodine solution
- Decolorizer
- Safranin
- Bilirubin paper
- Microscope
- Immersion oil

Gram staining-method

- Place a small drop of water onto a slide and with a sterile inoculating loop, place a small drop of bacterial sample on the slide. Heat fix the bacteria by allowing it to air dry and passing it through the bunsen burner 3-4 times.
- Add enough crystal violet to cover the slide and allow it to sit for 1 minute. Rinse with water.
- Add enough iodine solution to cover the slide and allow it to sit for 1 minute. Rinse with water.
- Add enough iodine to cover the slide and allow it to sit for 8 seconds. Rinse with water.
- Add enough safranin to cover the slide and allow it to sit for 45 seconds. Rinse with water.
- Place the slide in bilirubin paper to allow it to dry completely.
- View the slide under a microscope to observe the shape to determine if it is gram-positive or negative. Adopted from ThoughCo [5].

Endospore staining-materials

- Thermophilic strain
- Microscope slide
- Distilled water
- Inoculating loop
- Bunsen burner
- Heat plate
- Beaker
- Blotting paper
- Malachite green solution
- Safranin
- Bilirubin paper
- Microscope
- Immersion oil

Endospore staining-method

- Fill a beaker with water and allow it to boil.
- Place a small drop of water onto a slide and with a sterile inoculating loop, place a small drop of bacterial sample on the slide. Heat fix the bacteria by allowing it to air dry and passing it through the bunsen burner 3-4 times. Add a blotting paper on top of the slide.
- Once the water is boiling and producing steam, place the microscope slide on top of the beaker.
- Saturate the blotting paper with malachite green solution and keep saturating it so the paper doesn't go dry. Allow the process to go for 5 minutes.
- Rinse the slide completely with distilled water.
- Add enough safranin to cover the slide and allow it to sit for 30 seconds. Rinse with water and dry with bilirubin paper.
- View the slide under a microscope to observe if endospores are present or not. Adopted from Microbiology Info [6].

Extraction and purification of antimicrobial peptides-materials

- Thermophilic strain
- Incubator
- Centrifuge
- Centrifuge tubes
- Pipette
- Ammonium sulfate
- Scale
- Weigh boats
- Stir plate/stir bar
- 20°C Fridge.

Extraction and purification of antimicrobial peptides-method

- Grow cells at 55°C for 48-72 hours.
- Centrifuge cells and pipette the cell free supernatant.
- Transfer supernatant to a beaker containing a stir bar.
- Weigh ammonium sulfate to bring the final concentration to 50% saturation.
- As the sample is stirring, slowly add ammonium sulfate and allow the solution to mix.
- Move the beaker to a 20°C fridge for future use. To determine its antimicrobial activity, perform agar disk diffusion. Adopted from Exalpha [7].

Agar disk diffusion method-materials

- *Escherichia coli*
- *Pseudomonas aeruginosa*
- *Staphylococcus aureus*
- *Candida albicans*
- Sterile swabs
- TSA Plates
- Isolated extract
- Forceps
- Bunsen burner
- Antibiotic discs
- Incubator

Agar disk diffusion method-methods

- Using a sterile swab, swab each bacteria into a lawn (*Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli, Candida albicans)* onto its own TSA plate.
- Using sterile forceps, dip an antibiotic disc into the extract from the previous experiment and place it in the middle of the TSA plate.
- Incubate the plates at 37°C for 24 to 42 hours. Adopted from Southeast Asian Fisheries Development Center [8].

Biofilm assay-materials

- Extracted thermophilic strain
- *Pseudomonas aeruginosa*
- LB Medium
- Microtiter plate
- Incubator
- Water
- 0.1% solution of crystal violet
- Acetic acid
- Spectrophotometer.

Biofilm assay-methods

- To grow the biofilm, grow a culture of *Pseudomonas aeruginosa* in LB medium. Add 100 uL of dilution per microtiter plate well and incubate at 37°C.
- After incubation, dump out the cells remaining in the plate and rinse with water.
- Add 125 uL of 0.1% solution of crystal violet into each well.
- Incubate the plate at room temperature for 10-15 minutes.
- Rinse the plates 3-4 times with water and leave the plate to dry for a few hours.
- Add 125 uL of 30% acetic acid to each well.
- Incubate the plate at room temperature for 10-15 minutes.
- Add 125 uL of solubilized CV to a new microtiter dish.
- Measure the absorbance at 595 nm and include a control. Adopted from JoVE [9].

Results

Figure 1: Starch hydrolysis test results indicate the thermophile is amylase-positive.

Figure 2: MR-VP test results indicate the thermophile is MR positive and VP negative.

Figure 3: Gelatinase test results indicate a positive result for the breakdown of gelatin.

Figure 4: Catalase test results indicate a positive result for this test.

Starch Hydrolysis	MR-VP	Gelatinase Testing	Catalase
+	+	+	+

Table 1: Overall findings from the four diagnostic tests performed.

Figure 5: Gram staining results indicated a violet pigment hence indicating a gram positive strain.

Figure 6: Endospore staining results. The above image shows that endospores are present hence the bacterial strain can produce endospores.

Figure 7: Agar Well Diffusion Using *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus,* and *Candida albicans*. Zones of inhibition were measured and recorded in table 2 and graphed in figure 8.

The thermophilic extract exhibited anti-bacterial and anti-fungal activity	
Bacteria	Zones of Inhibition (mm)
Escherichia coli	20
Pseudomonas aeruginosa	22.5
Staphylococcus aureus	16
Candida albicans	21

Table 2: After performing agar well diffusion, the zones of inhibition were measured to determine if the thermophilic extract can exhibit antimicrobial activity.

The thermophilic extract exhibited anti-bacterial and anti-fungal activity

Figure 8: The above figure shows the zones of inhibition that the thermophilic extract can inhibit growth with the four bacteria's used.

The thermophilic extract also exhibited anti-biofilm activity against *Pseudomonas aeruginosa*	
Thermophilic Extract Added (mg/mL)	**Absorbance at 595 nM**
Control	0.85
0.2	0.78
0.4	0.65
0.6	0.62
0.8	0.57
1	0.45
1.2	0.3

Table 3: Our next experiment looked at determining if the amount of extract added affected the inhibition of growth of *Pseudomonas aeruginosa.*

The thermophilic extract also exhibited anti-biofilm activity against *Pseudomonas aeruginosa*

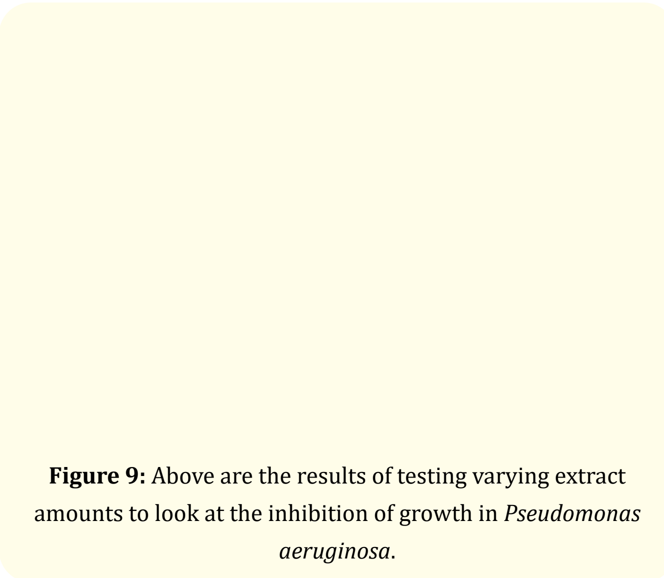

Figure 9: Above are the results of testing varying extract amounts to look at the inhibition of growth in *Pseudomonas aeruginosa*.

Discussion

To determine initial characterization of the strain we isolated, biochemical testing was necessary. We were confident that the strain we isolated was a thermophile as it was able to withstand high temperatures but to be sure, four tests were performed. Starch hydrolysis was performed to determine if our thermophilic strain can secrete exoenzymes such as amylase. After flooding the plate with iodine, we saw a zone of inhibition around the streaked bacteria indicating that the thermophilic strain can hydrolyze the starch indicating that it is amylase-positive. The next test performed was the MR-VP test. In figure 2a, the methyl red test is used to determine if a bacterial strain can ferment glucose. After the methyl red indicator was added, the color immediately turned red indicating a positive test and that the bacteria can produce sufficient acid. In figure 2b, the VP test is used to see if a bacterial strain can produce acetyl methyl carbinol from glucose fermentation. In the image, a pink-red color was not achieved, hence concluding the thermophile is negative and cannot produce acetyl methyl carbinol from glucose fermentation. Following, a gelatinase test was used to determine if the bacterial strain can produce the enzyme, gelatinase that specifically liquefies gelatin. The tube in figure 3 was liquefied indicating a positive result for the breakdown of gelatin. Lastly, the catalase test was used to determine if the bacterial strain contains the enzyme catalase. Catalase is responsible for the breakdown of hydrogen peroxide into its byproducts, oxygen and water. The plate in figure 4 bubbled after hydrogen peroxide was added, indicating a positive result for this test. Two types of staining, gram staining and endospore staining were also conducted to further identify characteristics that would help us identify the strain. Gram staining was used to determine whether the thermophilic strain was gram positive or negative. After performing the needed steps, the image in figure 5 produced a violet pigment. This indicates that the strain is gram positive. Endospore staining is a good indicator of if the strain is able to produce endospores or not. In figure 6, under the microscope green dots appeared hence indicating that the species is able to produce spores. The results of the above tests help indicate that the isolated bacterial strain we extracted from the soil was part of the *Actinomycetes* species. To determine the specific genus, the strain would be sent for 16srRNA sequencing.

Actinomycetes can produce enzymes and antibacterials that can be isolated and used in industrial and medical applications. After extracting the antimicrobial peptides from *Actinomycetes,* we were able to perform various experiments to determine its antibacterial and antifungal activity. Agar well diffusion is a common method to evaluate antimicrobial activity of microbial extracts. With the four bacterias used, *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus,* and *Candida albicans,* we added a soaked antibiotic disk with the extract to the plate. To no surprise, the extract was able to inhibit growth in all four plates. However, to our surprise, the extract inhibited most in *Pseudomonas*

aeruginosa. This is beneficial because *Pseudomonas aeruginosa* is an opportunistic bacterium that results in serious infections in health-care settings. Due to its ability to form biofilms, there is an urgent need to work on new methods to supplement our current antibiotics. If the extract inhibits *Pseudomonas aeruginosa,* this can be beneficial to produce a new treatment for both biofilm and planktonic infections resulting in novel adjuvant therapies. The next experiment we conducted was a biofilm assay to determine if *Pseudomonas aeruginosa* ability to form biofilms is affected by the thermophilic extract. Various amounts of extract were added to the biofilm as seen in table 3 and figure 9. Looking at the control, the absorbance is around 0.85 nm, concluding that *Pseudomonas aeruginosa* still has a high affinity to form biofilms. As the thermophilic extract is added, the absorbance decreases hence concluding that the formation of biofilm is also decreasing. The experiment can conclude that with the addition of more extract, *Pseudomonas aeruginosa* cells are unable to stick and are then unable to form biofilms.

Conclusion

Pseudomonas aeruginosa's ability to form biofilms with other opportunistic bacteria causes over 70% of all clinical infections. *Pseudomonas aeruginosa* is immune to all commercially ready antibiotics so the need to supplement our old methods for new methods that will target this rapidly growing bacteria. One group of soil bacteria known as the *Actinomycetes* species could be the answer researchers are looking for. This species can produce enzymes and antimicrobials that limit the growth of bacteria such as *Pseudomonas aeruginosa*. A soil sample was collected across Ramapo College campus and through the use of colony morphology, gram staining and the diagnostic tests, we were able to conclude that the sample contained the *Actinomycetes* species. From there, we performed experiments to isolate and purify the species' extracellular crude extract to obtain its antibacterial and antifungal activity. Its activity was assessed using agar disk diffusion which helped conclude that the extract has properties to limit growth of bacteria, especially in *Pseudomonas aeruginosa.* In addition, we also looked at the formation of biofilms after thermophilic extract was added. The extract was able to inhibit the production of biofilms as more extract was added as seen by the decrease in absorbance. This is beneficial to our research as *Pseudomonas aeruginosa's* ability to form biofilms exhibits resistance to all antibiotics. Seeing results that can lead to limitation of biofilm production in one of the most known hospital bacterial infections could lead to new treatments to limit serious infection in healthcare settings. Further research includes further purification using various protein purification techniques and assessing more antimicrobial activities of the *Actinomycetes* species. The species will be further identified by sending it for 16srRNA sequencing. Combining a unique treatment of antibiotics using the *Actinomycetes* species with antibiofilm peptides may be the answer to alleviating biofilm production especially from *Pseudomonas aeruginosa*.

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