IJNRD.ORG ISSN : 2456-4184
INTERNATIONAL JOURNAL OF NOVEL RESEARCH AND DEVELOPMENT (IJNRD) | IJNRD.ORG
An International Open Access, Peer-reviewed, Refereed Journal

ISOLATION AND EXTRACTION OF GEOSMIN FROM SOIL

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Abstract : Geosmin is a member of the largest family of natural compounds, the terpenoids and is a natural bicyclic terpene with an earthy odour that is produced by a group of soil dwelling bacteria- Streptomyces. Geosmin is the main constituent of Petrichor, the smell of Earth, is produced by the soil dwelling bacterial species *Streptomyces avermitilis*. This protein is released from the pores of the soil in the form of an aerosol due to rain. This study was conducted to isolate and characterise *Streptomyces avermitilis* for geosmin. Soil samples were collected from the Aare Forest and the Sanjay Gandhi National Park in Mumbai and isolation with a selective media was performed to isolate the streptomyces colonies. Various tests were carried out to identify and isolate the species from a soil sample and then GC MS was run on the isolated sample to confirm the presence of geosmin. The paper discusses the preparation of the soil samples, the isolation media and growth time, the ideal characteristics of a *Streptomyces avermitilis* colony and analyses the results from the chromatogram produced from running the GC MS. The study also identifies possible applications of the research in the real world.

IndexTerms **- Geosmin, *Streptomyces avermitilis*, Petrichor, characteristics.**

INTRODUCTION

The refreshing and pleasant smell that accompanies the first rain after a long period of dry weather, known as petrichor, has long been chased by scientists and even perfumers for its enduring appeal [1]. The fragrance was first thought of as the distinctive smell of rocks that originated from surface deposits and reactions of rock surfaces. The term evolved eventually and is now referred to as the smell of rain.

Petrichor is a big soup of various compounds such as ozone, terpenes, and geosmin which is a major component of petrichor. Geosmin is a member of the largest family of natural compounds, the terpenoids and is a natural bicyclic terpene with an earthy odour that is produced by a group of soil dwelling bacteria- Streptomyces. These bacteria are present in healthy soils and are often used to create antibiotics. These bacteria produce geosmin which is deposited into the tiny pores of the soil, rain droplets bounce on these pores and open them up due to pressure differences and release the substances in the pores as aerosoles which are formed like a mist and are carried by the wind, spreading the scent.

Geosmin has a much larger role in the environment, although it is perceived as a sensory delight. Geosmin was first discovered in 1965 and is mainly produced by actinobacteria and myxobacteria in soil, and by cyanobacteria in aquatic systems. Geosmin also occurs in ascomycete fungi, in basidiomycota in amoebae, in maize where it serves as a possible attractant of the corn earthwork Heliothis zea. It is also present in foods such as beetroot, red beans and rainbow trout. The broad distribution of geosmin among bacteria in both terrestrial and aquatic environments suggests that this compound has an important ecological function, for example, as a signal or as a protective specialised metabolite against biotic and abiotic stresses. Geosmin also plays an important role in biotechnology, as the smell creates an off flavour in water, wine and freshwater fish products that producers struggle to eliminate

[2]. However, it is still unclear what the exact function of geosmin is and the pathways and mechanisms involved in the production of geosmin in *Streptomyces avermitilis* remain an area of interest for research.

In this study, *Streptomyces avermitilis* was used due to various reasons such as a well characterised genome. This bacterium was the first of its genus to have its genome sequenced and has comprehensive genetic information available which provides a strong foundation for the study. It is also a model organism for secondary metabolite productions, and it is most well known for producing avermectins which are widely used as antiparasitic agents. The research on its secondary metabolite pathways gives valuable information on methods of identification of the bacteria. Most importantly, S*treptomyces avermitilis* is known for its high geosmin production compared to some other species.

This study aims to isolate and characterise *Streptomyces avermitilis* to better understand the production of geosmin. Soil samples were collected from a national park and a forest to identify where the bacteria are best. Isolation of the bacteria from different soil samples and characterization of the bacteria can provide better insights into the production of geosmin and this opens avenues for various biotechnology applications. It can enhance production of geosmin and provides an opportunity to further understand the role of geosmin in the lifecycle of the bacteria and the environment it is present in. It can contribute to the field of biofragrences and help understand its complex role in the environment.

METHODOLOGY

3.1. Collection and preparation of soil samples

Soil samples were taken from two different locations in mumbai. One sample was taken from the Aare Forest and another was taken from the Sanjiv Gandhi national park using gloves and the samples were stored in a clean polythene bag and put at XYZ temp until prepared. For each soil sample 1g of soil was suspended in 100mL of sterile saline and incubated at 28°C in an orbital shaker for 30 minutes at 150 rpm. The soil samples were then allowed to stand for a few minutes. For separation of spores from vegetative cells, the dilutions were placed in a 45°C-water bath for 16 hours [3].

3.2. Isolation of Streptomyces colonies from the soil samples

The media used for the isolation of the streptomyces colonies was the kusters agar media with the following components. 10g/L glycerol, 0.3g/L casein, 3g/L KNO_3, 2g/L K_2HPO_4, 2g/L NaCl, 0.05g/L $MgSO_4.7H_2O$, 0.02g/L $CaCO_3$, 0.01g/L $FeSO_4.7H_2O$, and 30g/L agar.The media was autoclaved to remove any biological contamination and then was melted using a hotplate and was poured into two petri plates. The Petri plates were left to harden. Later, the soil dilutions were spread on the two prepared plates and incubated at 28o celsius. For 4-5 days.

3.3. Subculturing of the grown colonies

Subculturing of the colonies was done in two different media. The first media was 2 flasks containing 100 ml of the Kusters Agar media without the agar, it had the following components: 10g/L glycerol, 0.3g/L casein, 3g/L KNO_3, 2g/L K_2HPO_4, 2g/L NaCl, 0.05g/L $MgSO_4.7H_2O$, 0.02g/L $CaCO_3$, and 0.01g/L $FeSO_4.7H_2O$. A colony was picked up from the original petri plate using a sterilised nichrome loop and spun around in the flask with the media. This was labelled as KB 1. The same was repeated with the second flask and it was labelled KB 2.

The second sub-culturing was done in a synthetic media which was most suitable for the GC-MS analysis according to research cited at [4]. The media consisted of: 60g/L glucose, 2g/L $(NH_4)_2SO_4$, 0.1g/L of $MgSO_4.7H_2O$, 0.5g/L of K_2HPO_4, 2g/L of NaCl, 0.05g/L of $FeSO_4.7H_2O$, 0.05g/L of $ZnSO_4.7H_2O$, 0.05g/L of $MnSO_4.4H_2O$, 5g/L of $CaCO_3$, and 2g/L of yeast extract. 100ml of the synthetic broth media was prepared and 5 test tubes were prepared with 10 mL of the synthetic broth media. Colonies from the original plate were picked up and transferred into each of the five test tubes using a sterilised nichrome loop and spun around in the tube to allow the colony to dissolve. The test tubes were left to incubate for 4 days.

3.4. Characterization of the streptomyces colonies

3.4.1. Gram staining

4 colonies from the petri plate with the forest sample were identified and picked up into separate microscope slides using a sterilised nichrome loop and 1 drop of millique was added to the sample and the sample was spread around using the look and heat sealed. Then, gram staining was carried out in the standard gram staining process.

3.4.2 Catalase testing

This experiment was done after subculturing the colonies into the kusters media and synthetic broth media. Colonies which were used for gram testing were identified and a few drops of hydrogen peroxide were dropped on top of them and then was observed to identify the formation of bubbles indicating catalase positivity.

3.4.3 Antibacterial testing

Well diffusion method was used for studying antimicrobial activities of the isolates. Two plates of nutrient agar were prepared with 13g/L nutrient broth and 30g/L agar. The media was autoclaved upto 15kPa and then allowed to melt on a hotplate before being poured onto two petri plates which were left for a few hours to harden.

Nutrient broth was prepared with the following composition: 13g/L nutrient broth. 10 ml of the nutrient broth (NB) was added to two different test tubes and placed in a water bath to digest. Later, *Escherichia coli was* streaked from a yeast extract slant using a sterile nichrome loop and dissolved in the NB tube which was labelled as E.coli. The same process was repeated with the other NB test tube with *Staphylococcus. aureus* which was labelled S. aureus.

A few drops of the E.coli induced NB was added onto one plate and was swabbed using a sterile earbud. The NB was spread throughout the plate. The plate was labelled as E.coli. The second plate was swabbed with a sample from the S. aureus NB test tube and labelled S. aureus. Four wells were created on each plate using a sterilised borer. The wells were labelled below each plate as -control, SB, KB1, KB2. Using a p20 pipette and a new pipet each time,15 µl of millique was added to the -control well, 15 µL of sample from one of the five SB test tubes was added to the SB well, 15µL of the sample from KB1 was added to the KB1 well ans 15µL from the KB2 flask was added to the KB 2 flask. The same was done for both the plates. The plates were placed in an incubator set at 37o celsius for a day.

3.5. GC MS analysis of Volatile Organic Products of S. avermitilis

One of the 5 test tubes containing SB was taken out 2 days after incubation and transferred to a falcon tube and centrifuged at 3000 rpm for 2 minutes to isolate all the cells and debris from the organic products, the supernatant was pulled up a syringe which was attached to a 0.2 µL filter and collected in a test tube. 10 mL of n-hexane was added to 10 mL of the extracted sample and was passed through a filter paper containing $MgSO_4$. 15 µL of the sample was loaded into a GC MS tube and GC MS was run using a temperature program of 50-280°C, temperature gradient of 20°C/minute. The same process was repeated with the SB test tube sample 4 days after incubation. The chromatogram was compared to the preloaded database in the MS attached to the GC, to confirm the presence of geosmin.

RESULTS & DISCUSSION

Figure 1: picture of the prepared soil samples, the right side being the forest sample and the left being the national park sample

Figure 2: picture of the isolation media after being frozen

Figure 3: picture of the petri plate with kusters agar media and the Aare forest sample 4 days after incubation

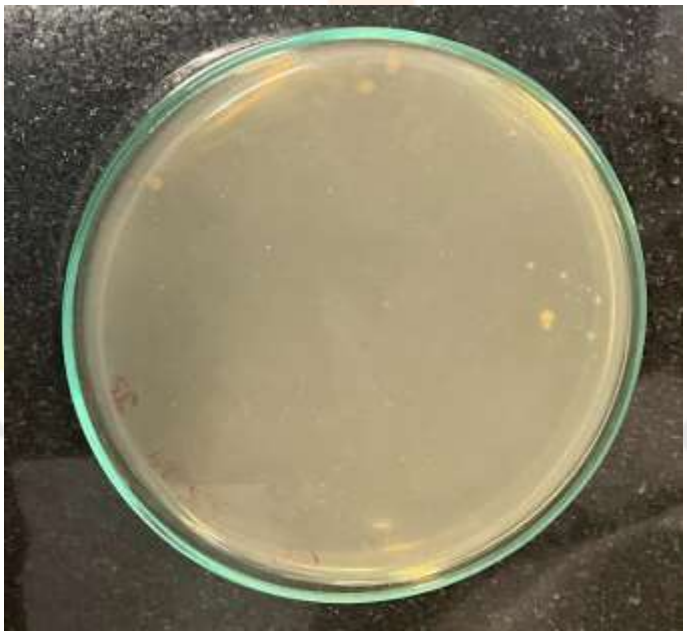


Figure 4: picture of the petri plate with kusters agar media and the national park sample 4 days after incubation

The forest and national park were picked for soil collection as they are far away from anthropogenic activities and have a deeper smell of petrichor during rains. These deeper smells indicate the higher presence of streptomyces species in the soil. Thus, the soil was collected from a forest and from a national park in Mumbai.

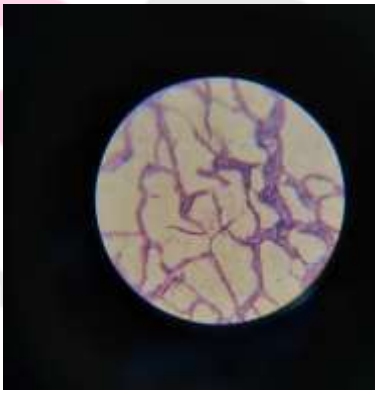
Kusters agar was used as the media of growth as it is an isolation media for the streptomyces species and only promotes growth of the streptomyces colonies on the plate. The isolative media was helpful in filtering all the bacteria in the soil to obtain only the streptomyces species as all the colonies that grew on the forest plate were streptomyces but the colony on the national park forest was not streptomyces as discussed later.

There was more growth in the forest sample. This was expected as forests are less exposed to pollution and deep forests are subjected to almost no anthropogenic activity. National parks, on the other hand, are subjected to little anthropogenic activity from tourists and are in a much-controlled environment. This could have been the reason for more grown with the forest sample.

Table 1: table showing colony characteristics of selected colonies

Colony characteristics							
Colony number	Size	Colour	Surface	Elevation	Shape	Margin	opacity
A1	3mm	Cream white	Glistening	Umbonate	Irregular	Undulate	Opaque
A2	5mm	Cream white	Glistening	Umbonate	Irregular	Undulate	Opaque
A3	6mm	Cream white	Glistening	Raised	Irregular	Undulate	Opaque
A4	2.5mm	White	Glistening	Raised	Irregular	Undulate	Opaque
B1	3mm	Cream white	Glistening	Flat	Circular	Even	Opaque

Table 2: table showing the results of gram staining

Colony number	Gram staining result	Image under microscope
A1	Gram Positive Rod	

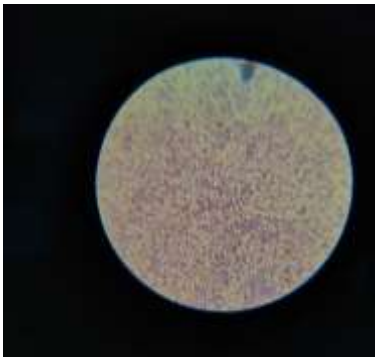
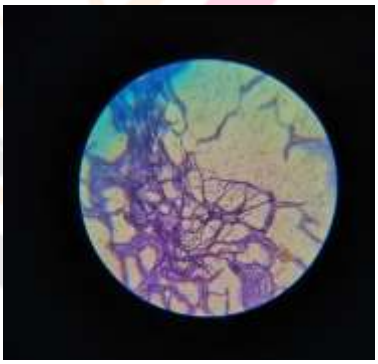
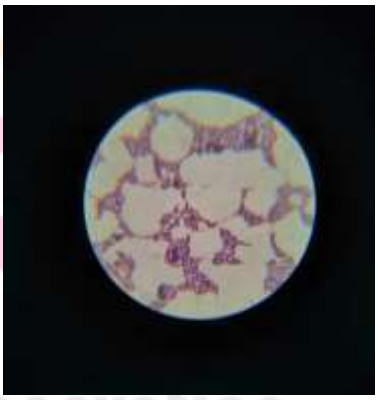
A2	Gram Negative Cocci	
A3	Gram Negative Vibrio	
A4	Gram Positive cocci in chain	
B1	Gram Positive Bacilli	

Table 3: table showcasing the results of the catalase test

Colony number	Catalase test result
A1	Positive
A2	Negative
A3	Negative
A4	Negative
B1	Negative

Streptomyces colonies often appear dull with a chalky creamy colour. Their surface is often glistening as they have a leathery surface and their elevation is usually raised, convex, or umbonate. Their shape is usually irregular or rhizoid. Based on these characteristics, colonies A1, A2, A3, and A4 can qualify as streptomyces colonies.

Streptomyces avermitilis is a gram positive and catalase positive bacteria. A1, A4, and B1 are gram positive bacteria. However, *S.avermitilis* grows in thread like, filamentous manner, forming a complex network of vegetative hyphae. Thus only A1 or A4 can be *S.avermitilis*. However, only A1 can be categorised as *S.avermitilis* as it is also catalase positive and thus it was used for subculturing.

Figure 5: picture of the results of the antimicrobial test against *E. coli*

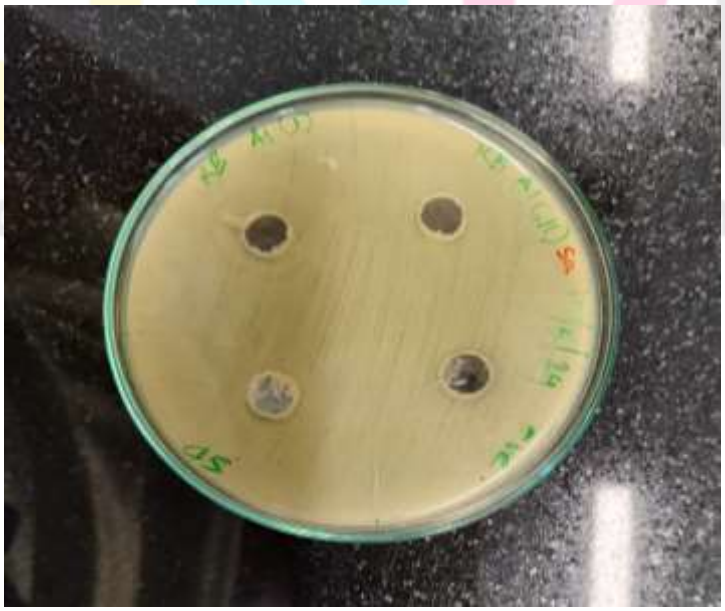


Figure 6: picture of the results of antimicrobial activity against *S. aureus*

Agar well diffusion method was used to determine the antimicrobial activities and minimum inhibitory concentrations because the size of inhibition zone provides a clear indication of the antimicrobial activity, making it easy to interpret. It also allows a wide range of antimicrobial agents to be tested simultaneously, which was used in the case of this experiment.

The zone of inhibition is the clear area surrounding an antimicrobial agent on an agar plate where bacterial growth was prevented. This zone is used to measure the effectiveness of the antimicrobial agent against the bacteria being tested.

Partial inhibition was observed against *E.coli*, as seen in figure 3, but there was no inhibition on the *S.aureus* plate at all. There was mad growth of *E.coli* and *S.aureus* on the plate as expected as they were grown in a nutrient agar with suitable conditions overnight. *Streptomyces avermitilis* has inhibition to *E.coli* primarily due to the production of avermectins. It also has partial inhibition to *S. aureus* which is primarily due to Bacteriocins and Lytic Enzymes. It can be expected that these enzymes were damaged or that they were not in optimal condition to work.

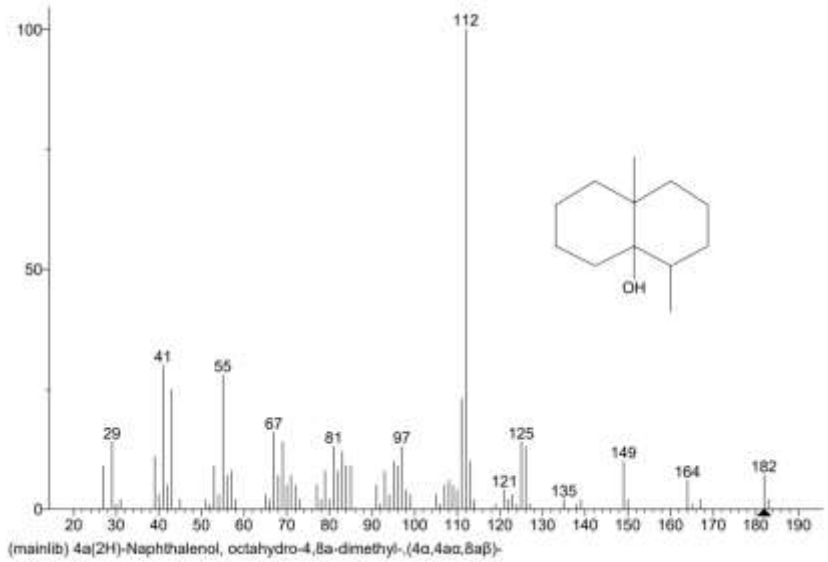


Figure 7: chromatogram showing the results of the KB sample in the GC MS.

Figure 8: samples filtered on day 2

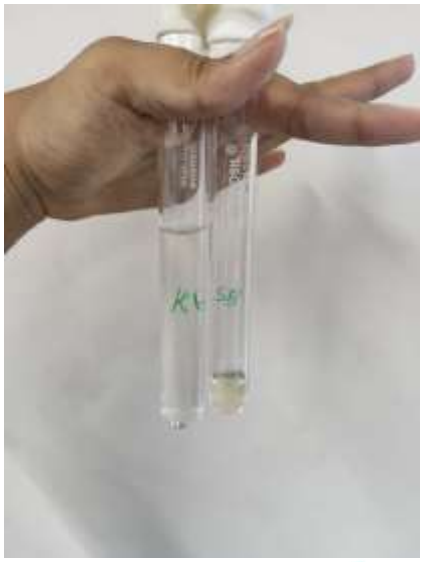


Figure 9: samples filtered on day 4 (both the isolations from the kusters broth were mixed)

The filtered samples had a strong odour. The ones filtered on day 2 had a more pleasant odour compared to the odour of the samples filtered after 4 days. The samples which were filtered after 4 days had an unpleasant strong smell, this could be due to fermentation of several proteins that were produced and had time to accumulate. The filtration process was also much easier on day 2 compared to day 4 and this is because of the increased growth on day 4 which would have had many more cellular components and bacteria. GC-MS was conducted to confirm the presence of geosmin in the extraction produced.

Figure 7 shows the chromatogram produced after running the KB sample in the GC MS and geosmin's presence is indicated at the peak at 182 indicated with an arrow. The graph was analysed with the NIST library to identify geosmin. The sample also shows peaks at several other positions and hence the sample requires purification so only geosmin is present and the peaks also explain the unpleasant smell. The other proteins in the sample would have added other fragrances to the sample.

CONCLUSION

The study was successful as we were able to identify the presence of geosmin in the samples. We were able to determine that forest soil samples can be the prime producers of this smell since they consist of more bacterial colonies compared to other soils which are in contact with pollution. We were able to characterise the *Streptomyces avermitilis* colonies as well and determined their likely characteristics. The project can be further enhanced by conducting the same experiments multiple times to confirm that geosmin was really produced by the bacteria. The process can be optimised by repeating it further on and the GC MS process could have been optimised to give out more accurate results. The method could have been changed slightly to make the process quicker and to make the bacteria grow faster, since they take a lot of time to grow.

The project can be used for the mass production of geosmin or to produce avermectins which are widely used in antibiotics. Geosmin production can contribute to the field of biofragrences which would greatly reduce the amount of chemical usage in perfumes and can increase use in natural products. Geosmin production can also contribute to our understanding of the environmental pathways geosmin, and rain are involved in. Studies can be carried out by exposing several soil species to the smell of geosmin to observe their responses.

ACKNOWLEDGEMENT

I would like to acknowledge all the individuals and organisations whose support have been fundamental to the completion of this research project.
Special thanks to Mrs. Bhavna Pandya, my dedicated advisor for her unwavering guidance and insightful feedback throughout.
I acknowledge the help of Ms. Apeksha Kumari Patel in all the lab work throughout the project.
I acknowledge Bioriidl for allowing me to use their lab instruments.
I acknowledge Athena Education for their pivotal role in connecting me with Bioriidl.

REFERENCES

[1] Sam Macer. “Recreating the Smell of Rain (Petrichor) With Geosmin.” *YouTube*, 5 Sept. 2022, www.youtube.com/watch?v=B5JHJ8k1geM.

[2] Garbeva, Paolina, et al. "Volatile Sensation: The Chemical Ecology of the Earthy Odorant Geosmin." *Environmental Microbiology*, vol. 25, no. 9, Mar. 2023, pp. 1565–74. https://doi.org/10.1111/1462-2920.16381.

[3] "Geosmin - American Chemical Society." *American Chemical Society*, www.acs.org/molecule-of-the-week/archive/g/geosmin.html.

[4] Cane, David E., et al. "Geosmin Biosynthesis in Streptomyces Avermitilis. Molecular Cloning, Expression, and Mechanistic Study of the Germacradienol/Geosmin Synthase." *The Journal of Antibiotics*, vol. 59, no. 8, Aug. 2006, pp. 471–79. https://doi.org/10.1038/ja.2006.66.

[5] Kitani, Shigeru, et al. "Avenolide, a Streptomyces Hormone Controlling Antibiotic Production in Streptomyces Avermitilis." *Proceedings of the National Academy of Sciences*, vol. 108, no. 39, Sept. 2011, pp. 16410–15. https://doi.org/10.1073/pnas.1113908108.

[6] Halton, By Mary. *Petrichor: Why Does Rain Smell so Good?* 27 July 2018, www.bbc.com/news/science-environment-44904298.

[7]Tankeshwar, Acharya, and Acharya Tankeshwar. "Shapes of Bacteria: Cocci, Bacilli, and Spirochetes." *Microbe Online*, 27 Dec. 2023, microbeonline.com/characteristics-shape-of-pathogenic-bacteria.

[8]"Fig. 5 Mass Spectra of Natural Geosmin (a, B) [2 H 4]-geosmin at M/Z..." *ResearchGate*, www.researchgate.net/figure/Mass-spectra-of-natural-geosmin-a-b-2-H-4-geosmin-at-m-z-186-along-with-the-two_fig4_346533610.

[9] PubChem. "Geosmin." *PubChem*, pubchem.ncbi.nlm.nih.gov/compound/Geosmin#section=IUPAC-Name.

[10] Bristow, R. L., et al. "An Extensive Review of the Extraction Techniques and Detection Methods for the Taste and Odour Compound Geosmin (Trans-1, 10-dimethyl-trans-9-decalol) in Water." *TrAC Trends in Analytical Chemistry*, vol. 110, Jan. 2019, pp. 233–48. https://doi.org/10.1016/j.trac.2018.10.032.

[11] *The Chemical Ecology of Geosmin - Spectrum: Concordia University Research Repository*. 2021, spectrum.library.concordia.ca/id/eprint/988023.

[12] Whitbourne, Kathryn, and Desiree Bowie. "Petrichor: What Causes the Earthy Smell After Rain?" *HowStuffWorks*, 14 Sept. 2023, science.howstuffworks.com/nature/climate-weather/atmospheric/question479.htm.