THE EFFECT OF GEOSMIN ON THE GROWTH OF BACILLUS CEREUS

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The purpose of this study was to determine the effect of varying concentrations of geosmin on the growth of Bacillus cereus. In aquatic environments where geosmin is a major odor compound, it would be advantageous to have information concerning its effect upon various indigenous microorganisms in water supplies.

Viable cell count and optical density determinations showed that geosmin, an odor metabolite of certain aquatic actinomycetes and blue-green algae, stimulates the growth of Bacillus cereus. The stimulation was observed as a reduction in the generation time during logarithmic growth.
THE EFFECT OF GEOSMIN ON THE GROWTH OF BACILLUS CEREUS

THESIS

Presented to the Graduate Council of the North Texas State University in Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

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A vast amount of research has been conducted in the area of tastes and odor problems in water supplies. Tastes and odors in an aquatic environment are contributed by a variety of microorganisms. Both actinomycetes and certain species of algae have been shown to be sources of odor problems in various water supplies. Adams (1933) was apparently the first to attribute the problem of earthy odors in raw water to actinomycetes. He examined the Nile River and suggested that the earthy odors which occurred from time to time were due to volatile compounds formed by the actinomycetes. Thaysen (1936) described the isolation of ether-soluble metabolites of actinomycetes. He observed that the odor was manure-like in high concentration but earthy when the metabolites were diluted.

Silvey et al. (1950) noted that most of the earthy tastes and odors in rivers, streams, natural lakes, and reservoirs throughout the Southwestern part of the United States were due to actinomycetes of the genus Streptomyces. The organisms were cultured under laboratory conditions and
found to produce characteristic earthy-odors similar to the ones associated with aquatic systems. This was the first conclusive account of the role of actinomycetes as the primary odor producers. Silvey and Roach (1956) redirected attention to the importance of actinomycetes as a major contributor of taste and odor problems in water. Following this work, other publications implicated taste and odor problems in water to actinomycetes (Hoak, 1957; Morris, 1962; Erdei, 1963).

Hoehn (1963) concluded that *Bacillus cereus* reduced typical actinomycetic tastes and odors in aquatic systems and also noted that aquatic actinomycetes produced metabolites which were stimulatory to *B. cereus*. He also hypothesized that one might possibly minimize taste and odor problems in water supplies by maintaining populations of *Bacillus* organisms during the period of odor occurrences. Under the direction of Dr. J. K. G. Silvey, application of quantities of *B. cereus* cultures to Lake Hefner, Oklahoma has proven effective in controlling the malodorous problem.

In a comprehensive report on Lake Hefner, Oklahoma, Silvey and Roach (1964) published work concerning the microbiotic cycles of surface waters. The work included a study of population densities and interrelationships of
microorganisms in an aquatic habitat. A close relationship was observed between blue-green algae, actinomycetes, and Gram-positive spore-forming bacilli of the genus *Bacillus*. According to the data, the blue-green algae attained peak growth in late summer. Through the stationary and declining phases of the algal growth, the actinomycete concentration increased dramatically until it reached a maximum in September. During the actinomycetes' stationary and declining phase, a definite increase in the Gram-positive spore-forming bacilli was observed. As the actinomycetes decreased, the data reflected a concomitant increase in *Bacillus* and a reduction of threshold odor. This and similar works prompted further research in microbiotic related taste and odor problems in water supplies.

Romano and Safferman (1963) stimulated progress in the study of earthy-odor compounds by preparing an actinomycetic concentrate of *Streptomyces griseoluteus* and finding that a $10^9$ dilution of the compound gave a fetid smell. The authors concluded that the odors of *Streptomyces* cultures were particularly more offensive than those of other actinomycete genera.

The increased development and application of gas chromatographic techniques stimulated extensive research in
isolation and identification of odor compounds. Gerber and Lechevalier (1965) isolated an earthy-smelling compound from *Streptomyces griseus* and other *Streptomyces* which they named geosmin. Geosmin was found to be a neutral oil with a boiling point of 270°C. Reaction with acid yielded the odorless compound argosmin, a neutral oil with a boiling point of 230°C. Wasserman (1966) related that geosmin was produced by *S. fradiae*, *S. griseus*, *S. odorifer*, and *S. antibioticus* and that it was responsible for the typical smell of soil. High resolution mass spectroscopic studies by Gerber (1967) indicated an empirical formula of $\text{C}_{12}\text{H}_{22}\text{O}$ for geosmin and $\text{C}_{12}\text{H}_{20}$ for argosmin. Medsker et al. (1968) suggested structures for geosmin using chemical and spectroscopic evidence. The workers verified the $\text{C}_{12}\text{H}_{22}\text{O}$ empirical formula proposed by Gerber and described the compound as a dimethyl substituted, saturated, bicyclic tertiary alcohol with the hydroxyl group sterically hindered. In high concentrations, geosmin had a strong camphoric odor which was altered to an earthy smell upon dilution. Acid treatment destroyed the odor as was shown by Gerber. Contrary to Gerber's findings, Medsker revealed the following results: (a) presence of an $-\text{OH}$ group determined by spectroscopy, (b) two rather than three methyl groups present,
(c) acid treatment of geosmin yielded several isomeric hydrocarbons rather than a single compound.

Gerber (1968) concluded that geosmin is trans-1,10-dimethyl-trans-9-decalol with the structural formula as shown in Figure 1. She proposed that, biochemically, geosmin appears to be a sesquiterpene which has lost an isopropyl group.

![Figure 1](image_url)

Figure 1--Proposed structure for geosmin, trans-1,10-dimethyl-trans-9-decalol.

In conjunction with this, Rosen et al. (1968) conducted gas chromatographic analyses of odor compounds extracted from S. griseoluteus. The extracted material and a sample of pure geosmin were compared and they exhibited identical retention times, identical odors, and identical responses to HCl. The data coincided with findings of Medsker (1968) that acid treatment of geosmin yielded more than a single compound.
The workers concluded that the odor compound which they isolated was identical to geosmin.

For many years, algae have been implicated with taste and odor problems of water supplies, although evidence was lacking to substantiate the extent of its malodorous effect. The odors due to algae in aquatic habitats are of three distinct types and origins: (a) odors produced during active growth stages of algae, (b) odors resulting from common intermediates or metabolites released upon cell lysis, (c) odors resulting from decomposition of the organisms after death. Preliminary studies by Maloney (1963) using Chlorococcum, a unicellular green alga in pure culture, indicated that the fetid material is retained within the cells until released through autolysis or induced disintegration. This is in agreement with the work of Krough and Lange (1930) who found that the odorous material synthesized by algae is quantitatively stored in the algal cells.

Safferman et al. (1967) presented the first evidence which suggested the possible biosynthesis of geosmin by microorganisms other than actinomycetes. These workers isolated an earthy-smelling compound produced by the blue-green alga Symploca muscorum which was identical to geosmin isolated by Gerber and Lechevalier (1965). A study by
Medsker et al. (1968) reinforced the findings of Safferman (1967) suggesting the synthesis of geosmin by organisms other than actinomycetes. The work was concerned with the isolation and characterization of geosmin from aquatic actinomycetes and from two blue-green algae, <i>S. muscorum</i> and <i>Oscillatoria tenuis</i>. Henley (1970a) isolated a planktonic blue-green alga, <i>Anabaena circinalis</i> from samples of bloom-laden water during a taste and odor problem in Garza-Little Elm Reservoir. The cultured organism was observed to produce odors of the same variety as that occurring during the fetid bloom. Upon subsequent analysis, geosmin was isolated and identified as the odor compound from <i>A. circinalis</i>. These works therefore establish geosmin as an important odorous metabolite of various aquatic microorganisms.

Current research on geosmin and related odor compounds has been primarily concerned with laboratory cultures. The presence of geosmin in the environment had not been demonstrated until Rosen et al. (1970) isolated geosmin from Grand Lake, Ohio during an intense odor problem. Geosmin was isolated in pure form and identified by mass spectroscopy. This was the first account demonstrating geosmin's presence and its capacity as an odor compound in natural water systems.
Geosmin-related experiments have been conducted recently in an attempt to specifically explain some of the conjectured biological effects in aquatic systems. Henley (1970b) showed a degradative effect of B. cereus on geosmin. Using gas-chromatographic analyses, he found a marked reduction of the geosmin peak after a 48-hour incubation period. This could be an intuitive explanation of the reduction of taste and odor problems where adequate concentrations of the Bacillus organism are present. Camp (1971) indicated that geosmin acted as a positive effector in stimulating the metabolic activity and the subsequent growth of one strain of an actinomycete. This formation reveals that geosmin may have a definite biological effect on various indigenous microorganisms in an aquatic environment.

Statement of Problem

In aquatic environments where geosmin is a major odor compound, it would be advantageous to have information concerning its effect upon various indigenous microorganisms in water supplies. The purpose of this research was to determine the effect of varying concentrations of geosmin on the growth of Bacillus cereus.
CHAPTER II

MATERIALS AND METHODS

Mass Culturing of *Anabaena Circinalis*

The laboratory procedure outlined by Henley (1970a) was applied in the mass culturing of the blue-green alga, *Anabaena circinalis* as the source of geosmin. Organisms were first grown for purposes of inoculation into mass culture. The stock cultures were grown by inoculating a 1.0 ml aliquot of the organism (O.D. of 0.05 at 750 nm) into 250 ml Erlenmeyer flasks containing 100 ml of the ASM-1 salts medium of Gorham (1964). The inoculated cultures were placed on a rotary shaker at approximately 140 rpm. Room temperature and light intensity of 60-80 foot-candles were attained throughout the culturing process. After a period of 16-18 days, the cultures had obtained an O.D. in the range of 0.4. Five 5 gallon glass carboys containing 18 liters of ASM-1 medium plus 100 mg/L of HCO₃⁻ were inoculated with 400 ml of the pooled stock culture inoculum. The carboys were incubated at room temperature (approximately 26 °C) with a light intensity of 60-80 foot-candles and continuous aeration. The cultures attained an optical density of approximately 0.5
after a six-week growth period. The organisms were then harvested by use of a continuous-flow Sharpel's Super Centrifuge. The packed cells were then stored at -20 °C.

Geosmin Extraction and Concentration

The protocol used for the extraction and concentration of geosmin was a modification of methods utilized by Henley (1970a). A diagram of the extraction and concentration procedure is shown in Figure 2. Twenty grams of algal cells (wet wt.) were suspended in 500 ml of distilled water in a 2 liter distilling flask for each distillation. A twenty ml aliquot of petroleum ether was added to the collection flask before subsequent distillation and a plastic tube was attached to the condenser to form a direct contact of the distillate and the petroleum ether in the collecting flask to insure that geosmin was not lost to the atmosphere. After 50% of the total volume had been distilled, the residue was discarded since only trace amounts of geosmin remained. The distillate was extracted two times with 30% (v/v) nannograde petroleum ether. Prior to the final extraction, the distillate was saturated with NaCl. The petroleum ether extracts were combined and dried with 5% (w/v) NaCl. The combined extracts were concentrated by distillation at 40 °C.
20 gm cells + distilled H₂O  
(total volume of 500 ml)

→ distillation  
50%

→ 250 ml geosmin distillate

→ distillate saturated ← extract w/ petroleum ether  
with NaCl  
(30% v/v)

→ extract w/ petroleum ether  
(30% v/v)

→ discard aqueous phase

→ combine extracts

→ dried w/ NaCl  
(5% w/v)

→ concentrate

→ distillation @ 40 °C

→ 1-2 ml petroleum ether concentrate

→ geosmin collection w/ gas-chromatography

Figure 2--Extraction and concentration protocol for geosmin preparation.
to approximately 1-2 ml concentrate. The petroleum ether concentrate containing geosmin was then stored at -20 C to await gas-chromatographic analyses.

Gas-chromatographic Collection of Geosmin

Assay of the odor compound geosmin was ascertained with the Varian Aerograph dual column gas chromatograph. The column used in the collection procedure was an aluminum, 10' x 3/8" packed with 10% SE-30 on Chromasorb G, 60/80 mesh.

Fifty µl samples of the petroleum ether concentrate were injected into the gas chromatograph for separation of organic compounds. Confirmation of geosmin was determined by injecting petroleum ether-geosmin sample which was obtained from Dr. David Jenkins, University of California at Richmond.

Collection of geosmin was achieved by inserting a 2.0 mm U-shaped glass collecting tube into the exit port of the gas chromatograph at the time of peak response on the recorder. Prior to geosmin collection, the collection tube was washed with analytical grade acetone, dried, and rinsed with deionized double distilled water to insure that additional organic compounds were not present. The collection tube was placed in an acetone-dry ice bath during collecting periods. The ends of the collecting tube were sealed and the collection tube was stored at -20 C.
Procedure for Preparing Geosmin Concentrations

L-Salts medium, Leadbetter and Foster (1958), was flushed through the collecting tube using a 50-microliter syringe into a 25 ml volumetric flask. The total volume was increased to 25 ml volume with L-Salts medium.

Geosmin concentrations were calculated from total organic carbon values measured by the Beckman Model 915 Total Organic Carbon Analyzer. Since geosmin is 79.12% carbon, a relationship is noted where 1.0 mole of geosmin carbon represents 1.264 mole of geosmin. From this, calculations were made to quantitate the geosmin concentration. The organic carbon content of the L-Salts medium-geosmin solution was observed by injecting a 25 μl sample into the analytical gas chromatograph.

Culture Flask Preparation

The initial phase of this investigation was concerned with establishing a minimal medium for Bacillus cereus that would produce an exponential growth rate. After a series of trial and error studies, L-Salts medium plus 0.75% glucose and 0.075% yeast extract were chosen and employed throughout the study. Culture flasks were prepared by dispensing 75 ml aliquots of L-Salts medium into 500 ml side-arm Erlenmeyer flasks. Yeast extract and glucose were added in desired
quantities to attain 0.075% and 0.75% respectively to a total volume of 100 ml. The medium was then autoclaved and found that approximately 4.0 ml of the medium were lost due to autoclaving.

The desired concentrations of geosmin were achieved by adding the appropriate amount of sterile L-Salts medium-geosmin solution to the Erlenmeyer culture flasks that gave the desired geosmin concentration in 100 ml. The total volume of the culture medium was increased to 100 ml with sterile L-Salts medium.

Experimental Approach

Geosmin concentrations of approximately 0.1, 1.0, and 3.0 mg/L were chosen for the study. A single experimental set-up included (a) duplicate control flasks of Bacillus cereus in 100 ml L-Salts medium plus 0.075% yeast extract and 0.75% glucose, and (b) duplicate test flasks of L-Salts medium plus 0.075% yeast extract and 0.75% glucose with B. cereus and the desired quantity of geosmin. A 0.1 ml inoculum of an 18-hour logarithmic culture of B. cereus (O.D. of 0.3 at 420 nm) was added to the desired culture flasks. The experimental and control flasks were placed in a walk-in environmental chamber and agitated on a rotary
shaker at approximately 100 rpms in a temperature of 25 °C ±1. An experimental run was conducted for each geosmin concentration.

The effect of geosmin on the growth of B. cereus was measured by concomitant optical density (Bausch and Lomb Spectronic-20) at 420 nm and viable cell count (total plate count) determinations. Samples were taken at 0, 3, 6, 9, 12, 18, 24, and 36 hours to determine the subsequent effect of geosmin. Optical density measurements were performed by adjusting the spectrophotometer with L-Salts medium plus 0.075% yeast extract and 0.75% glucose in a tube similar in refractility to the side-arm tubes. A "dark box" was fitted over the Erlenmeyer flask to minimize the amount of light entering the phototube. Optical density readings were taken at the designated intervals.

The serial dilution technique was employed in the viable cell count determination. One ml aliquots were taken at specified intervals and appropriate serial dilutions were performed using 9.0 ml Tris-buffered water blanks. A range of the Bacillus concentration was chosen at each interval followed with the serial dilution method and streaked on
Plate Count Agar in triplicate plates. The plates were incubated at 37 °C ± 1 for 12 hours followed by visible quantitation.
CHAPTER III

RESULTS

Gas-chromatographic Collection of Geosmin

The pure sample of geosmin which was obtained from Jenkins reflected a single peak at approximately 16 minutes under the gas chromatographic parameters utilized during the collecting period. A chromatogram as shown in Figure 3 exhibits the retention time of a pure sample of geosmin dissolved in petroleum ether. Figure 4 reflects the different peaks of a petroleum ether concentrate of Anabaena circinalis. Comparison of Figures 3 and 4 confirms the capacity of A. circinalis to synthesize geosmin.

Procedure for Preparing Geosmin Concentrations

Approximately 59.4 mg/L geosmin were collected from combined A. circinalis extracts. The total quantity of geosmin dissolved in the 25 ml L-Salts medium was approximately 1.50 mg. Table I explains the procedure employed in determining geosmin concentration. The chromatogram as seen in Figure 5 represents the organic carbon content of the L-Salts medium-geosmin solution.
Figure 3—Gas chromatographic analysis of a pure sample of geosmin.
Figure 4—Gas chromatographic analysis of an *Anabaena circinalis* concentrate.
TABLE I

DETERMINATION OF GEOSMIN CONCENTRATION USING TOTAL AND INORGANIC CARBON STANDARDS*

<table>
<thead>
<tr>
<th>Total Carbon Standards</th>
<th>mg/L</th>
<th>Units</th>
<th>Inorganic Carbon Standards</th>
<th>mg/L</th>
<th>Units</th>
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<tr>
<td></td>
<td>20</td>
<td>25</td>
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<td></td>
<td>50</td>
<td>58.5</td>
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<tr>
<th></th>
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<th>mg/L</th>
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<tr>
<td>Total Carbon Geosmin Sample</td>
<td>93±1</td>
<td>80</td>
</tr>
<tr>
<td>Inorganic Carbon Geosmin Sample</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td>Geosmin Organic Carbon</td>
<td></td>
<td>65 mg/L</td>
</tr>
<tr>
<td>Total Carbon L-Salts (Blank)</td>
<td>41.5±.5</td>
<td>33</td>
</tr>
<tr>
<td>Inorganic Carbon L-Salts (Blank)</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td>Organic Carbon L-Salts (Blank)</td>
<td></td>
<td>18 mg/L</td>
</tr>
<tr>
<td>Pure Geosmin Organic Carbon</td>
<td></td>
<td>65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>47 mg/L</td>
</tr>
</tbody>
</table>

\[(47 \text{ mg/L})\cdot(0.25\text{L})\cdot(1.264) = 1.5 \text{ mg Geosmin}\]

*Duplicate injections were performed
Figure 5—Analytical gas chromatographic analysis of the L-Salts medium-geosmin standard.
Experimental Results

Figures 6, 8, and 10 exhibit the effect of geosmin on the growth of *Bacillus cereus* as determined by viable cell count determinations. The generation time ($G_t$) was reduced from approximately 38 minutes to approximately 26 minutes during the exponential growth phase. Optical density readings as shown in Figure 7, 9, and 11 reveal similar results showing that geosmin enhances the growth of *B. cereus*. 
Figure 6—The effect of geosmin (3 mg/L) on the growth of *Bacillus cereus*.
Figure 7—The effect of geosmin (3 mg/L) on the growth of *Bacillus cereus*.
Figure 8—The effect of geosmin (1 mg/L) on the growth of Bacillus cereus.
Figure 9—The effect of geosmin (1 mg/L) on the growth of *Bacillus cereus*.
Figure 10—The effect of geosmin (0.1 mg/L) on the growth of Bacillus cereus.
Figure 11—The effect of geosmin (0.1 mg/L) on the growth of *Bacillus cereus*.
CHAPTER IV

DISCUSSION

Viable cell count and optical density determinations have shown that geosmin, an odor metabolite of certain aquatic actinomycetes and blue-green algae, stimulates the growth of *Bacillus cereus*. The stimulation is manifested by a reduction in the generation time during logarithmic growth.

The microbiotic cycle studies of Silvey and Roach (1964) reveals a significant increase in *Bacillus* organisms during the actinomycetes' stationary and declining phase. The increased *Bacillus* population could possibly be explained as a growth enhancement effect of geosmin. As the actinomycete reach stationary and declining phase of its growth, cell lysis occurs. Upon cell lysis, geosmin is liberated into the aqueous environment making it available to the *Bacillus* organisms. At that point, geosmin may possibly stimulate the growth of the Gram-positive bacilli resulting in an increased population.

Geosmin has been isolated from several species of *Streptomyces* and from various blue-green algae including *Symploca*, *Oscillatoria*, and *Anabaena* genera, thus implying
a biochemical link between these two groups of procaryotes. Included with this, geosmin has been found to stimulate the growth of a geosmin-producing actinomycete of the genus Streptomyces and now a bacterium Bacillus cereus. Other studies appear to be necessary to determine the exact mechanism involved in the geosmin enhancement. The actual biochemical pathway(s) that are involved in geosmin biosynthesis in both actinomycetes and blue-green algae are not known. The elucidation of these pathways or mechanisms would further increase the understanding and possible control of taste and odor problems in aquatic environments.
LITERATURE CITED


