# A Study on Fractal Morphogenesis in Bacteria as a Response to Environmental Stress

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## Abstract

Bacteria respond to the biochemical and physiochemical stimuli within their environment by formation of fractal colonies. This phenomenon has been studied and is thought to be the result of chemical signaling between single cells, as well as activation or deactivation of genes and even conjugation. This study was performed to demonstrate fractal morphogenesis in *Escherichia coli*, a gram negative bacterium, and *Micrococcus luteus*, a gram positive bacterium, as a response to herbal mixtures and diffusion-limited agar. Actively growing *Micrococcus luteus* and *Escherichia coli* were surface plated on standard 12% LB agar, 8% LB agar, 17% LB agar, standard agar + garlic, standard agar + golden seal, standard agar + sage, and differential media eosin methylene blue agar, selective for *E. coli*, and mannitol salt agar, selective for *M. luteus*, for 24 to 48 hours at 37°C and 25°C, respectively.

Non-fractal colony growth ranged in size from 0.1-1.0mm and fractal colony growth ranged from 3.0-7.5mm in size. All herbs tested: goldenseal, garlic, and sage, proved to encourage fractal growth in hard and soft agar with hard agar being the more promising fractal growth medium. Therefore, hard agar limits the amount of nutrient diffused throughout the agar, promoting fractal growth and development. On all media tested the death rate for bacterium with garlic administered was 50% for *M. luteus* and 100% for *E. coli* at 24 hrs. Sage and goldenseal had far less of an effect, with bacterial death rate on all media not overcoming 50% for either *E. coli* or *M. luteus* using sage and goldenseal at 24 hrs and 48 hrs, respectively. Colonies surviving displayed fractal growth as well as non-fractal growth. The fractal colonies formed were tested for their viability which showed them remaining viable up to 30 days without refrigeration.

These results indicate that bacterial fractal growth and development was promoted as an adaptive response to the use of retardant nutrient dispersal agar and the presence of herbal tinctures as unusual and irregular environment situations.

# Introduction

Microbiology is the study of microorganisms that cannot be seen with the naked eye. Microbiology is a vast area that includes various subjects, many viruses, parasites, and largely bacteria. A few of these bacteria, and the way they evolve and react to the environment and its abrupt or subtle changes, are the area of interest within this study.

Bacteria exist in few shapes and all are relatively the same size. Bacteria can be round-shaped, termed cocci, or rod-shaped. They can also be gram-positive, meaning they retain crystal-violet dye during gram staining, or gram-negative, meaning that they do not retain crystal-violet dye during gram staining. For any study to be performed validly, both types of bacteria should be taken into consideration within the study. The bacteria that will be used in this proposed study will be harmless lab strains of *Escherichia coli (E. coli),* a rod-shaped gram-negative bacterium, and *Micrococcus luteus (M. luteus),* a gram-positive cocci.

The amount of knowledge the world possesses about bacteria, relative to the amount we know about other organisms, is meager in comparison. However, this small bank of knowledge giants over the even tinier amount of knowledge our forefathers held. Not so long ago, people believed that several illnesses that are known today to be caused by bacterial infections (tuberculosis, tetanus, and other infectious diseases) were caused by something wrong, an evil act that they had committed in their lives. They believed that sickness was a type of punishment for your wrongdoings, whether it affected you, or someone you cared about, such as your child or a sibling. Today, we know that killing the infectious organism via antibiotics can cure many diseases. We have developed these antibiotics after strenuous study of these microorganisms and how they interact with their outside world.

However, not all bacteria interact harmfully with their surroundings. Out of all the bacterial strains that we have knowledge of today, only 5% are harmful or pathogenic. The other 95% are nonpathogenic, and many are bacteria whose existence is necessary for human viability. For example,

there is a strain of *Escherichia coli* that lives within our intestines and produces vitamins needed for intestinal health, as well as aiding our intestines fight off other infectious diseases of the gut.

The study to be performed will analyze how different bacterial colonies form and whether or not their formation can be altered by their surroundings. In a previous study (Woriax and Woriax, 2007), it was determined that the addition of herbal tinctures composed of garlic, sage, or goldenseal caused serious deterioration of bacterial colonies and hindered bacterial growth under otherwise optimal conditions. However, the growth and morphology of the bacterial colonies without environmental stressors has never been studied in detail and is the key area of interest within this study.

# Review of Literature

Arouh and Levine (2000) analyzed the effect of nutrients in agar to the colony formation of bacterial colonies. Branching of colonies occurs due to limited nutrients, with the branching becoming a mechanism for diffusion of the limited nutrient. When the bacteria is assisted chemotactically, the effect is quite different and results in suppression of colony branching. Calculations for this experiment were done via model systems, but will presumably remain the same in a more realistic situation.

Ben-Jacob et al (1995) modeling demonstrates how distinct growth patterns of bacteria can be due to cooperative behavior, occurring since environmental conditions are unfavorable. This model incorporates random walkers, which demonstrate bacterial aggregates, which move in response to environmental deviations such as nutrient gradients. Due to these deviations, the walkers will communicate with each other chemotoxically, and the colony will respond efficiently to the situation presented.

A great morphological change in *Bacillus subtilis* colonies was observed after growing under ultraviolet radiation, in comparison to growth under normal conditions (Delprato et al, 2001). Around the central area of the colonies, the bacteria migrate towards the colony's edge and form a ring during uniform spatial radiation exposure. When the exposure ceases, the colonies grow in a regressive form back towards the center as well as towards the exterior region. This phenomenon indicates that the pattern is not formed due to lack of nutrients in the central area.

Ginovart and co-workers (2002) developed a model to demonstrate and simulate the behavior and growth of bacteria. This individual-based model was named INDISM, an acronym for Individual Discrete Simulations. This model's results have been used in the study of the relationship between rate of growth and nutrient material, biomass distributions, and metabolic changes and variations in

bacterial colonies. These results prove to be useful and correlate with available experimental data, providing great insight into the mechanisms and characteristics involved in the colony growth.

Another model was developed by Lacasta et al (1999), which presented a diffusion-reaction model to demonstrate bacterial colony growth. What is often observed in colony growth as a reaction to environmental stresses is represented by a nonlinear diffusion term. A response that can present hysteresis activates the presence of this mechanism. Changing agar concentrations as well as initial nutrients will allow the numerical integration of the model to demonstrate the various growth patterns of *Bacillus subtilis* OG-01.

A model was made regarding the hydrodynamics and evolution of bacterial colonies growing on soft agar plates (Laga and Passot (2003)). This model is comprised of reaction-diffusion equations coupled with a hydrodynamics equation and captures the dynamics within the colony and along its boundaries. This is beneficial when related to the chemotaxic strategies of many bacteria in colonial growth.

The previous work done by Lega and Passot (2004) to model the hydrodynamics of a bacterial colony are represented in a phase diagram. As did the model, the diagram represents how hydrodynamics are related to colony growth and chemical signaling within a bacterial colony.

Matsuyama et al (1993) analyzed the fractal morphogenesis of bacterial colonies based on environmental factors. They concluded that bacterial colony growth depended on the agar medium's nutrient concentration. When nutrients were low, bacteria grew slowly and in a more isolated form. However, in agar-rich medium bacterial colonies grew quite the opposite. Other factors found to have been involved in colony growth were wetting agents that yielded more movement of colonies.

In another paper, fractal growth of *Serratia marcescens* was described (Matsuyama et al (1989)).The growth was irregular and lake-like and contained fractal colonies. For this growth to be analyzed, mutants defective in production of surface-active exolipids, as well as mutants lacking

flagella, were isolated. The fractal spreading growth occurred in correlation with the exolipid (namely serrawettin W1 and W3) mutants. When these exolipids were added exogenously, the bacteria grew in normal fashion. Thus, the irregular growth was due to the serrawettin defect.

Mendelson et al (1999) analyzed the projection motions of *Bacillus subtilis* colonies as well as the correlated fluid flows. In wet dense populations, individual cells projected at rates around 76-116μm/s. Swimming cells were classified into patterns of whirls and jets. These patterns were relatively short-lived, lasting around 0.25s. Whirls and jets in motion never appeared to get very close to each other, but, instead, would turn to go in the other direction upon approaching another whirl or jet. Jets were faster than whirls, moving at a speed of 27μm/s, whereas whirls moved at a speed of 19μm/s. The addition of water into the colonies resulted in an immediate increase in the rate of swimming. The results of this analysis showed that cell motility within colonies is highly organized.

Mitchell and Wimpenny (1997) tested the effects of the concentration of agar medium on the growth and morphology of several types of bacteria. They found that growth is dependent on medium concentration. Shapiro's work (1995) is an analysis of bacterial colony patterns and their meanings. The way colonies of bacteria grow can indicate several features and indications of the bacteria, including DNA rearrangement systems. Chemotactic aggregation, group motility, and long-range chemical signaling can also be related to colony growth. All of these mechanisms were explored and explained on a molecular level.

Wakano et al (2004) measured sporulation rate and demonstrated it using reaction diffusion equations to model spatial growth patterns. One-dimensional simulation was used to prove the existence of traveling wave solution. The wave form was studied as a function of the initial nutrient concentration. The velocity of the traveling wave showed sharp transition in the nonlinear diffusion model, consistent with experimental results. Results of two-dimensional simulation are presented and analyzed as well.

In another work led by Wakano (2003), researchers modeled the processes by which *Bacillus circulans* grow. *Bacillus circulans* develop in a knotted-branching pattern, which consists of aggregate trajectories that move, mature, and reproduce together. This bacterial growth was modeled by combining a nutrient dynamic reaction diffusion system, the nucleation theory for aggregate generation, and individual dynamics of growth and motion of aggregates. Computer simulation produces branching growth and nutrient response not unlike that of *Bacillus circulans*.

A recent exposition (Zhang et al, 2007) described development of a technique using ultraviolet (UV) irradiation for the nanolubrication of hard disks. The UV irradiation is used to induce bonding of a perfluoropolyether on CN disks for further application of magnetic hard disks. The use of UV irradiation allows for the control of the degree of bonding on the CN coatings. The effect of this wavelength efficiency in bonding was compared using 100% mobile PFPE, 100% bonded PFPE, and a collection of bonded and mobile combinations in several laboratory tests. Using several different microscopes, the wear and friction characteristics of these different cases were analyzed. It was concluded that mixed PFPE shows the highest rupture strength.

Researchers Zhang et al (2005) modified the pre-existing bacterial growth model proposed by Delprato. The modifications were performed to reflect the morphological transition of bacterial colonies under ultraviolet radiation exposure. This modified model considers four factors, which include a chemotaxis initiated by radiation, the bacteria's two-stage destruction rate, the lubricant fluid generated by colonies, and the ultraviolet intensity. There were several results found by the experimenters, including the fact that rate of growth and death is dependent on the intensity of radiation, and that once exposure I stopped, growth initiates not only on the outer region, but also on the inner region of the colony. Lubricant fluid and the two-stage destruction rate were also found to be critical in colony growth dynamics when exposed to ultraviolet radiation.

# Methodology

## Experiment 1

In the first experiment, the manner in which bacterial colonies form given the involvement of herbal components within its growth environment was determined. This experiment was conducted by preparing 10mL Luria-Bertani (LB) broth, a growth medium used in microbiology that is composed of several nutrients needed for optimal microbial growth in test tubes. These test tubes were then inoculated with swabbed loops of each bacterium, and then 0.5g of each herb used was added to the test tube. Each culture was then diluted to a concentration of $10^{-8}$. These dilutions were then streaked onto previously prepared agar plates of soft (8%), standard (12%), and hard (17%) concentrations of agar. After the plates are streaked, they were then incubated at 37°C *(E. coli*) and 25°C (*M. luteus*), the optimal temperature for both bacteria, for 24 hours. After 24 hours, the colonial growth was recorded. The plates were then incubated for another 24 hours, and the growth recorded again.

## Experiment 2

The four points of analysis in Experiment 2 were viability, point inoculation, Herbs in agar plates, and Herbs streaked from liquid culture. The "viability" experiment is an analysis on each bacterium's ability to grow again after having been previously streaked and grown on an agar plate from a liquid medium. This technique was done using bacteria from recently streaked agar plates, and transferring a small portion onto a freshly made agar plate. The plate dilutions in the previously stated Experiment 1, once after the 24 hours and once after the 48 hours, were swabbed, and the bacteria were streaked onto fresh plates. These plates were then incubated at 37°C (*E. coli***)** and 25°C (*M. luteus*) for 24 hours, and the growth was recorded.

"Point inoculation" was performed by using the tip end of sterile pipettes. The bacterial strains from previously grown bacteria on agar plates were transferred onto sterile agar plates using the pipet

tip end. The plates were then incubated at 37°C for 24 hours, and the growth was recorded.

"Herbs in agar plates" was an experiment intended to harness a different approach on observing the interactions between herbal environments and bacteria. The experiment was conducted by preparing 125 mL of LB agar for the addition of each herb. The agar was then sterilized. Then 5.0 g of each herb was added to the sterilized 125 mL of agar once it cooled to 55°C, and the agar plates were poured. The plates were then allowed to harden and streaked with both *M. luteus* and *E. coli*. The plates were incubated at 37°C (*E. coli*) and 25°C (*M. luteus*) for 24 hours, and the growth was recorded.

"Herbs streaked from liquid culture" was performed as follows. 10 mL LB test tubes were prepared by swabbing loops of each bacterium, then adding 0.5 g of each herb to each allotted test tube as well. Bacteria were then streaked onto previously prepared soft (8%), hard (17%), and standard (12%) agar. The bacteria were incubated at 37°C (*E. coli*) and at 25°C (*M. luteus*). The plates were observed, and the growth recorded after 24 and 48 hours.

## Results

### *E.coli*

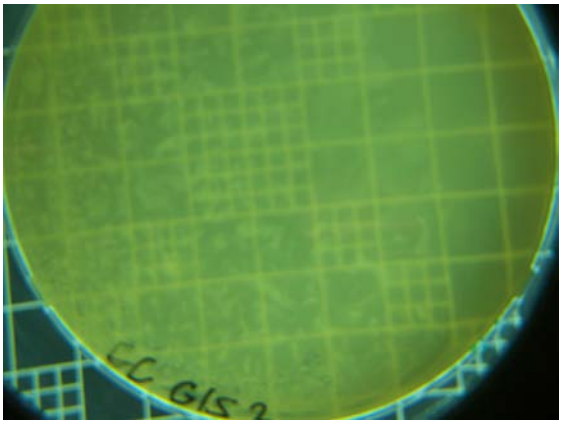

Figure 1:  Non-fractal colonies on standard media (no magnification)

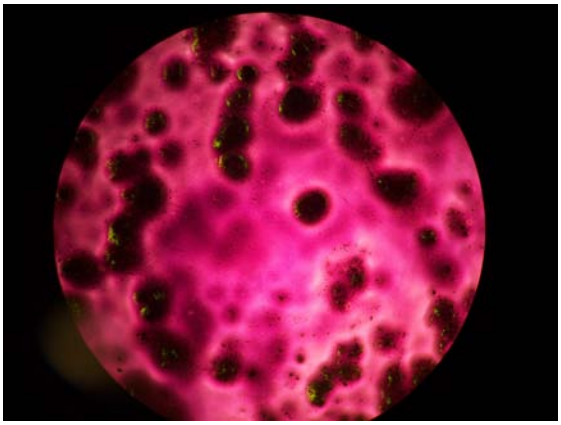

Figure 2: Non-fractal and fractal colonies on EMB (no magnification)

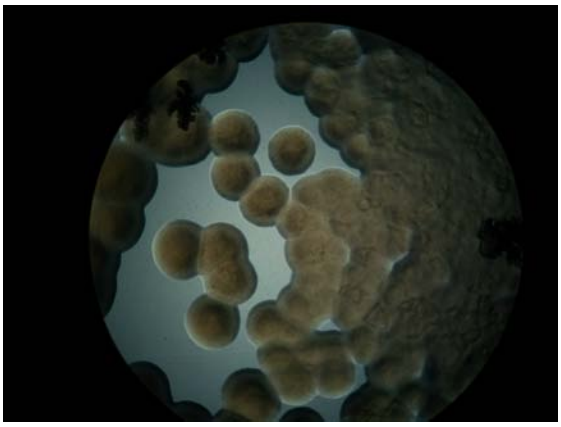

Figure 3: Fractal colonies at 100x

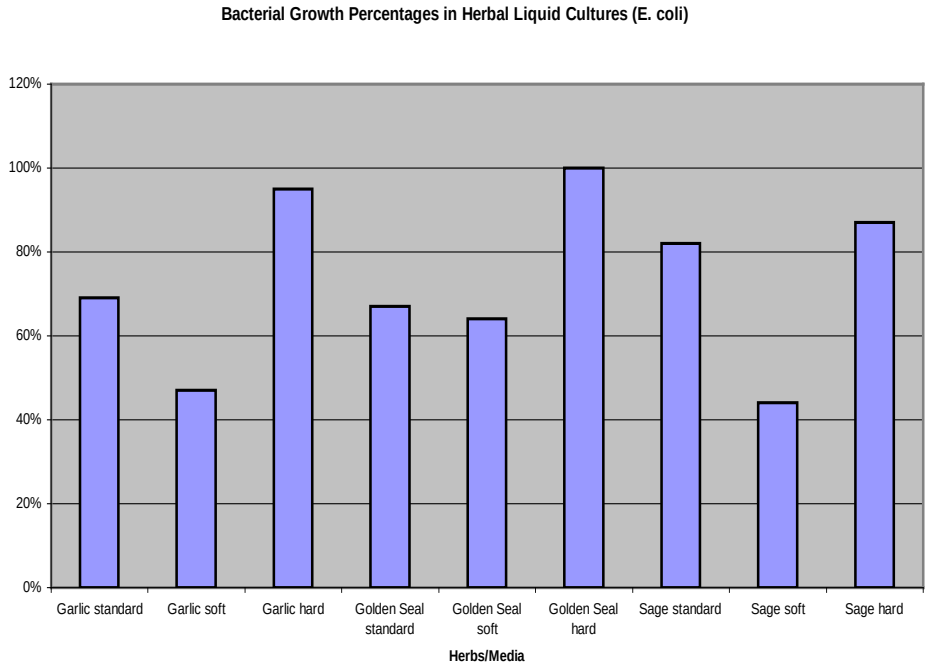


Figure 4: Graph of colonial growth percentages for fractal colonies on herbal media

*M. luteus*

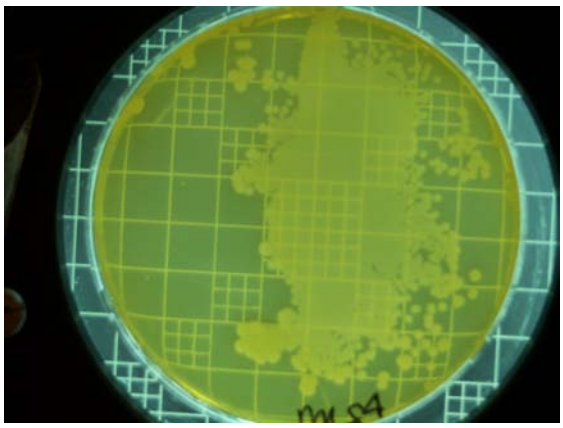

Figure 1: Non-fractal colonies on standard media (no magnification)

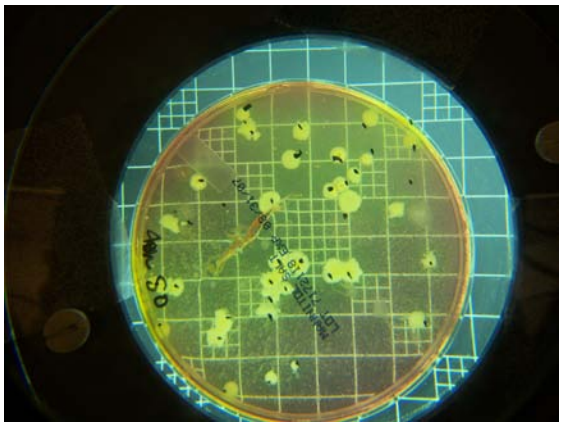

Figure 2: Non-fractal and fractal colonies on MSA (no magnification)

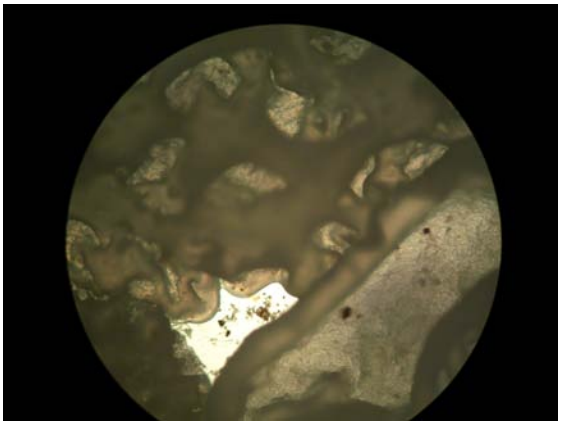

Figure 3: Fractal colonies at 100x

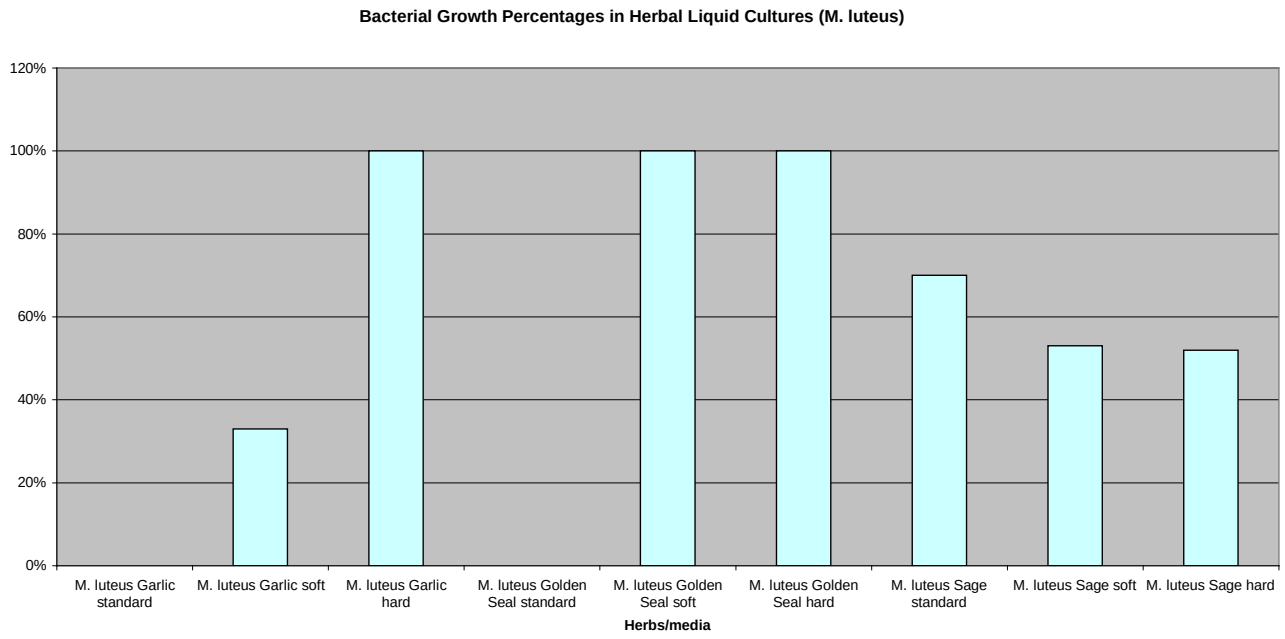


Figure 4: Graph of colonial growth percentages for fractal colonies on herbal media

# Discussion

## Experiment 1

Initially, the 18 hr. broth cultures made were at a standard plate count of 1.0-2.0x$10^8$. The herbs helped the formation of fractal colonies in soft and hard agar, with hard agar producing more prominent results. It was concluded from this experiment that the hardness of the agar decreased the nutrient dispersion, thus promoting the growth and development of fractal colonies. Fractal colonies emerged in an array of sizes, specifically from 3.0-7.5mm. These fractal colonies were present with distinct branching growth. The non-fractal colonies that grew were from 0.1-1.0mm in size, without branching growth, displaying only compressed growth. On all media, garlic eradicated 50% of *M. luteus* and 100% of *E. coli* within 24 hrs, with the kill on hard, soft, and standard media at 48 hrs being 98%, 98%, and 100%, respectively. The small amount of surviving colonies showed only fractal growth and development on soft agar. Therefore, it was concluded that garlic eradicated gram negative bacteria at a rate superior to that which gram positive bacteria were eradicated. However, results for sage and goldenseal were far less drastic, with the bacterial death rate on all media not overcoming 50% for either *E. coli or M. luteus*, using sage and goldenseal at 24 hrs and 48 hrs, respectively. Also, the colonies surviving displayed fractal growth as well as non-fractal growth. Thus, it was concluded that garlic proved a better herb than sage or goldenseal for annihilating bacteria.

## Experiment 2

Conclusions reached by altering certain variables confirmed the conclusions reached in Experiment 1. Also established were that decreasing garlic concentration and increasing goldenseal and sage concentration show more valuable and practical results. The implantation of herbs into the agar proved to be a viable manner to inhibit the growth of both bacterium, although more so with *M. luteus* inhibition.

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