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Article

Analyzing honey's ability to inhibit the growth of *Rhizopus stolonifer*

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SUMMARY

***Rhizopus stolonifer* is a mold commonly found growing on bread that can cause many negative health effects when consumed. Preservatives are the well-known answer to this problem; however, many preservatives are not naturally found in food, and some have negative health effects of their own. We focused on honey as a possible solution because of its natural origin and self-preservation ability. We hypothesized that honey would decrease the growth rate of *R. stolonifer*. We evaluated the honey with a zone of inhibition (ZOI) test on agar plates. Sabouraud dextrose agar was mixed with differing volumes of honey to generate concentrations between 10.0% and 30.0%. These plates were then inoculated with a solution of spores collected from the mold. The ZOI was measured to determine antifungal effectiveness. A statistically significant difference was found between the means of all concentrations except for 20.0% and 22.5%. Our findings support the hypothesis as we showed a positive correlation between the honey concentration and growth rate of mold. By using this data, progress could be made on an all-natural, honey-based preservative.**

INTRODUCTION

Food preservation is vital in order to prevent food waste and allow for as much storage as possible. Preservatives allow for food to be available for longer than their usual shelf life. This is largely beneficial because it results in less production and waste. It is estimated that roughly 30% of all food produced goes unused (1). Natural preservatives are becoming increasingly popular as people are searching for organic alternatives to their chemical counterparts (1). Many chemical and artificial preservatives, such as calcium propionate, are effective, but have also been shown to lead to negative health effects such as respiratory issues and even some cancers (1).

Natural preservatives popularity has recently been boosted because of the assurance that you are consuming real organic ingredients rather than something created in a lab (1). Honey specifically has been shown to contain antioxidants and nutrients and has even been used to treat wounds (2). We believe that honey would be able to meet the effectiveness levels of the other preservatives while also providing health benefits and tasting good. Our experiments with honey aim to expand the field of natural preservatives and their possible incorporation into the food industry. Making this switch on a large scale could provide and promote overall healthier lifestyles.

The goal was to study the interactions between honey and *Rhizopus stolonifer* to determine honey's antifungal properties. Using zone of inhibition (ZOI) measurements, we aimed to determine how the growth rate of a common bread mold, *R. stolonifer*, was affected by the various honey concentrations. *R. stolonifer* is most commonly found growing on foods, which provide it the nutrients needed to grow (3). We hypothesized that honey would decrease the growth rate of *R. stolonifer.* Specifically, we predicted that higher concentrations of honey would be more effective than lower concentrations at reducing the growth of the mold. After conducting our experiment and finding a statistically significant effect of honey on ZOI size, we concluded that honey is an effective inhibiting agent of *R. stolonifer*.

RESULTS

Our initial testing consisted first of a well diffusion method, where honey was poured into a well in the center of an agar plate, allowing it to spread through the agar on its own. Throughout this original testing we also used large pieces of mold coated agar rather than the later smaller concentrations. Both of these methods were ineffective and unrealistic as the mold grew to cover the entirety of the plate after just 24 hours. These findings resulted in method changes.

To simulate mold growth on food, we mixed raw honey with sabouraud dextrose agar at concentrations of 10%, 20%, 22.5%, and 30% with a control of 0% (pure agar) as a substitute for bread. We then inoculated *R. stolonifer* spores around the outer edge of the plate and incubated for 48 hours.

The mean ZOI for each concentration of honey were as follows: 0% - 0.11 inches, 10% - 1.57 inches, 20% - 2.71 inches, 22.5% - 2.63 inches, and 30% - 3.35 inches (**Figure 1**). The positive control group of 10% calcium propionate showed a mean ZOI of 3.35 inches. The largest achievable value for this measurement was 3.35 inches which was the diameter of the petri dish. A one-way ANOVA test was used to determine that differing percentages of honey and calcium propionate did have an effect on the growth of *R. stolonifer* ($p << 0.001$). This was followed by Tukey's test to ascertain which concentrations varied significantly from each other. The corresponding tests all showed statistical significance ($p < 0.05$) except for the test between 20% and 22.5% ($p = 0.93$).

There was also a relationship between concentration of honey and growth rate of the mold (**Figure 2**). Using the data we collected from various times after inoculation, we created a logistic model to visualize the rate at which the mold grew. The control group had the steepest slope meaning the fastest growth of the mold. The 22.5% concentration of honey had the flattest slope and, therefore, the slowest growth. As the concentrations of honey increased, the growth rate of *R.*

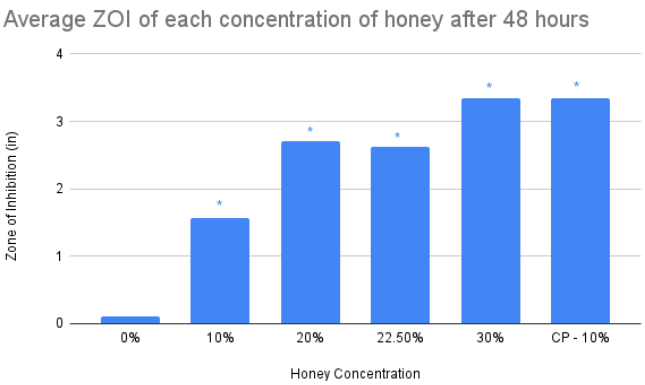

Figure 1: Average ZOI of each concentration of honey after 48 hours. Tests were conducted with honey and agar combined. A one-way ANOVA was conducted for significance from 0% honey: *$p < 0.001$. Data shown as mean ± SD. n(0%)=17, n(10%)=24, n(20%)=19, n(22.5%)=13, n(30%)=19, n(10% calcium propionate)=10.

stolonifer decreased.

We completed additional testing to determine the minimum inhibitory concentration (MIC) of honey against *R. stolonifer*. The lowest concentration of honey that fully inhibited mold growth was 30%, with 25% resulting in mold growth with roughly half of the trials.

DISCUSSION

The aim of this study was to determine the effects varying honey concentrations had on the growth of *R. stolonifer*. Initial tests consisted of diffusing honey from a well in the center into premade agar plates. All concentrations of honey failed to inhibit any mold growth. There are two likely reasons for this. First, the process of mold inoculation was unrealistic as mold starts growing from a single spore, not a large group of healthy mold (3). Also, fully grown mold contains mycelium, which absorb nutrients causing the mold to grow faster than if in spore form (3). Upon discovering this, we filtered spores and switched to using a concentration of 500,000 spores per mL. Secondly, we did not distribute the honey evenly throughout the plate, but rather soaked into the agar from the well in the center. This led to an inconsistent concentration of honey throughout the plate, with less honey as the distance from the well increased. Because of this, the mold, which was inoculated where the least amount of honey was contained, may have been able to grow into a healthy form and overpower the higher concentrations of honey near the center.

Later, minimum inhibitory concentration (MIC) tests using honey mixed directly with the agar produced results that matched our hypothesis. This is a test that determines the smallest amount of honey needed to effectively inhibit mold growth. The negative control saw the most growth as expected, yet still less than the initial testing methods. Since the mold was starting from a spore, we obtained more realistic results. The mold did not completely overgrow the plates after 24 hours, which allowed the growth rate to be better monitored. The data collected and presented in our results clearly shows that as the concentration of honey incorporated into the agar increases, so does the ZOI.

We used the one-way ANOVA and Tukey's Test to compare all of the recorded ZOI for each concentration of honey against one another. All comparisons were found to be significant except the 20% and 22.5% trials. We believe this to be because the honey concentrations were too close in proximity to produce marked differences.

Our results are comparable to other published research. An experiment on the antifungal activity of different Nigerian honeys on various molds is one of these examples (4). Anyanwu completed these experiments with mold, however not *R. stolonifer* specifically. An MIC was determined for the five molds just as we did in the latter experiments. The MIC varied between 12.5% and 40% depending on the honey being used and the mold being grown (4). *Aspergillus niger,* a mold that also grows on food (primarily fruits and vegetables), displayed an MIC of 20-25%, which is similar to the 30% MIC of *R. stolonifer* in our experiment (4). At a 25% honey concentration, mold only grew on the plates half of the time, causing us to conclude that it is right around the MIC. The 30%

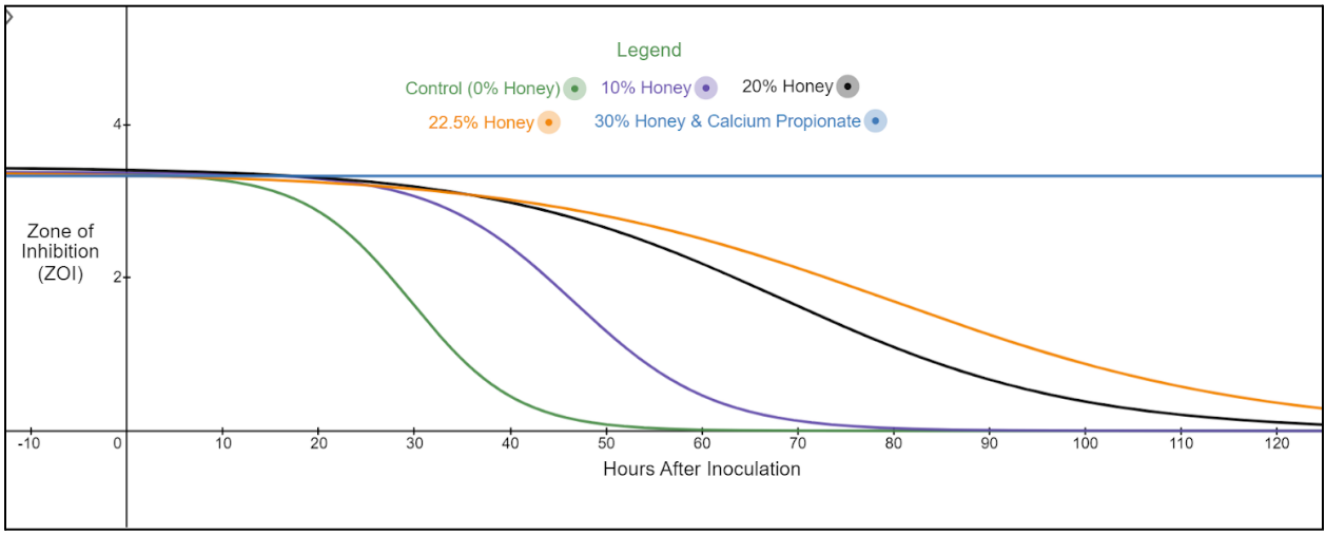

Figure 2: Zone of inhibition vs. hours after inoculation. Best fit curve modeling the average change in zone of inhibition of *R. stolonifer* as time passes. The model was made for each concentration from the average of 10-24 measurements taken at 24 hour intervals, whose averages are displayed on the graph. This data came from the honey mixed with agar tests.

honey results fell into the MIC range of other molds (4). These experiments did use honey from a different source, which as previously mentioned, can have differing antifungal strength. To have better compared our results with this past work, MIC tests on the same molds could have been completed.

Our study's results are intended to be applied to food preservation, specifically in bread, where *R. stolonifer* is commonly found (5). We suggest that honey could be used as a natural food preservative by applying a glaze of raw honey to the outside of bread. The suggested dilution of honey in this glaze would be 30% because that is the concentration that fully inhibited growth in this experiment.

A limitation of this research is that almost all honey displays differing anti-fungal properties (4). Therefore, the ideal concentration of a different kind of honey than what we tested may not be 30%. Future researchers should start by inoculating *R. stolonifer* at a concentration of 500,000 spores per milliliter onto a plate of 30% honey-sabouraud agar. If full inhibition of mold occurs, the tested honey could be used at a 30% concentration for the glaze.

Additional research should also be completed to ensure that the increase in ZOI is in fact due to the increase in honey concentration and not the decrease of available nutrients from the agar. To complete this, a mixture of 70% sabouraud dextrose agar should be poured into Petri dishes and R. *stolonifer* spores should be evenly distributed around the edge of the plate. If growth occurs, then it is reasonable to credit honey for the inhibition of R. *stolonifer* growth in our experiment.

MATERIALS AND METHODS

Initial Testing

The initial concentrations of the first set of tests were created by mixing the appropriate amounts of honey and distilled water and later mixed straight into the agar. Each of these tests had its own control of 100% agar with no honey. Finally, calcium propionate, a known inhibitor of *R. stolonifer*, was used as a positive control (6).

In order to ensure consistency and sterility of the experiments, sterile sabouraud dextrose agar (Carolina Biological, #776560) and syringes (Amazon, B08HM4T3GX) were used. These were removed from the packaging only minutes before use, while the agar itself was boiled before plates were poured. The raw, natural honey was purchased from a local honey supplier in Michigan, U.S.A., and stayed in a sealed jar. All beakers and stirring devices were cleaned and sterilized prior to and following each test. The colonies of *R. stolonifer* (Carolina Biological, #156223) were kept in a closed environment away from all testing. In order to keep a constant supply of mold, new colonies were grown rather than purchased. Throughout the process, old colonies were recycled to create fresh ones by inoculating blank agar plates with leftover mold.

Adjusted Testing

Our method of operation was adjusted due to a failure to produce results from our original methods. Uniform testing was achieved using a predetermined concentration of spores through the process of spore suspension. Here, the spores from a colony were scraped into a beaker and mixed with distilled water. The larger mycelial pieces of the mold were filtered out with the use of a Grade 4 Whatman filter (Amazon, B01MU70YUB). Using a pipette, the resulting mixture was then plated onto a hemocytometer (Amazon, #8906121590470). This is a device with a grid that allows for the counting of small particles, such as mold spores, under a microscope (Meiji Techno Co, MT-51). The concentration of spores was adjusted by adding additional water or spores to reach the desired concentration of 500,000 spores per mL. This concentration was selected to model previous MIC tests done on mold, as well as to eliminate the confounding variable of differing spore counts, which leads to an inconsistent rate of mold growth (4).

The adjusted tests, used for final data collection, were conducted using this inoculation method, and the raw honey was mixed directly into the agar. Sabouraud agar jars (Amazon) were melted using a Bunsen burner before pouring 100 mL into a beaker. The agar was mixed with raw honey to achieve the correct dilution for the current test (10%, 20%, 22.5%, or 30%). Next, 15 mL of this mixture was carefully poured into Petri dishes and cooled for 24 hours, with all contaminated plates thrown away. From here, a pipette was used to evenly distribute 60 µL of the spore suspension solution around the outer edge of the plate. The inoculated plates were then placed into the isolated chamber, kept at room temperature (21± 2°C), and measured every 24 hours for 5 days. This process was repeated for each honey concentration. All ZOIs were measured by recording the mean width of the inhibition zone, using a digital caliper (**Figure 3**). Because the ZOI is not a perfect circle, this was achieved by recording the diameter in evenly spaced intervals then taking the average.

In the analysis of our statistical data we used a one-way ANOVA test paired with a Tukey's HSD test. We determined any results with a p value less than 0.05 to be statistically significant. These tests were run in an online software listed

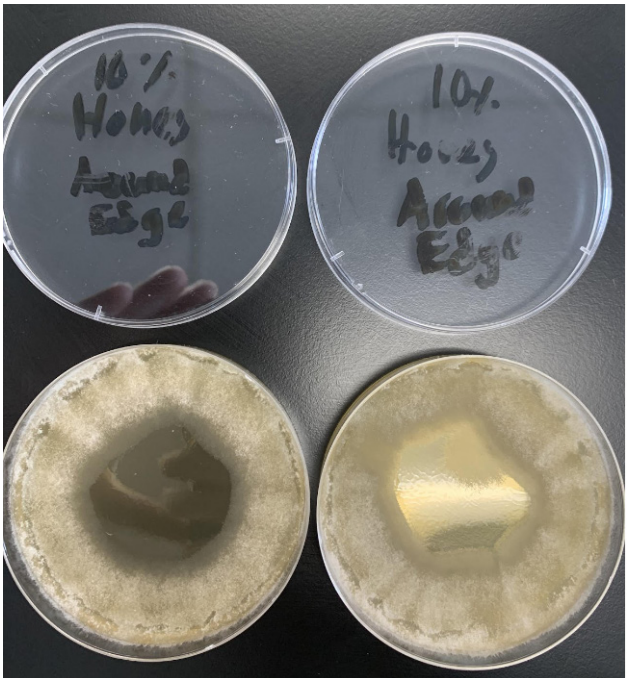

Figure 3: Zone of inhibition (ZOI) for 10% honey agar mix 24 hours after inoculation. This figure shows our honey mixed with agar tests experimental setup, which displayed variable ZOI. We took 2 measurements of each plate and recorded the average ZOI.

in the references below (7).

ACKNOWLEDGEMENTS

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REFERENCES

1. Schwab, Clarissa. "New Research Will Unveil the Future for Natural Preservation of Food Products." *EurekAlert!*, EurekAlert!, 2021, www.eurekalert.org/news-releases/499500. Accessed 20 Nov. 2022.
2. Nolan, Victoria C., *et al*. "Dissecting the Antimicrobial Composition of Honey." *Antibiotics*, vol. 8, no. 4, 2019, p. 251. doi.org/10.3390/antibiotics8040251.
3. Kaiser, Gary. "8.3: Molds." *Biology LibreTexts*, Libretexts, 9 Apr. 2022, www.bio.libretexts.org/Bookshelves/Microbiology/Book%3A_Microbiology_(Kaiser)/Unit_4%3A_Eukaryotic_Microorganisms_and_Viruses/08%3A_Fungi/8.3%3A_Molds. Accessed 28 Nov. 2022
4. Anyanwu, CU. "Investigation of in Vitro Antifungal Activity of Honey." *Journal of Medicinal Plants Research*, 1 Jan. 1970, doi:10.5897/JMPR12.577
5. Gabler, Milkota. "Survival of Spores of *Rhizopus Stolonifer*, *Aspergillus niger*, *Botrytis cinerea* and *Alternaria alternata* after exposure to ethanol solutions at various temperatures", *Journal of Applied Microbiology,* 8 Apr. 2004, doi:10.1111/j.1365-2672.2004.02280.x.
6. Vazhacharickal, Prem Jose. "Effect of Concentration and pH on the Preservative Action of Calcium Propionate Against Black Bread Mold (Rhizopus stolonifer) in Kerla", *CIBTech Journal of Biotechnology*, Apr. 2015.
7. One-Way Repeated Measures ANOVA Calculator. Social Science Statistics, Jeremy Stangroom, www.socscistatistics.com/tests/anovarepeated/default.aspx.