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An Alternative to Conventional Antibiotics - The Antimicrobial Properties of Deacetylated Chitin Extracted from *Gongronella Butleri*: A Research Protocol

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Abstract
Antimicrobial resistance (AMR) is a growing threat to global public health and development. It is estimated that AMR was directly responsible for 1.27 million deaths worldwide in 2019. Due to the superfluous use of antibiotics, AMR is increasingly widespread, and bacterial infections are becoming more difficult to treat. Chitin is a potential alternative that has demonstrated antimicrobial properties. Most commonly extracted from insects and crustaceans, chitin can also be found in the cell walls of fungi. In previously performed experiments, the results supported the hypothesis that chitin derived from crustaceans and insects displayed antimicrobial properties. Due to the different chemical composition and attributes of fungal chitin, this study will explore whether fungal chitin also displays antimicrobial properties. In this experiment, chitin will be extracted from *Gongronella butleri* fungus and will be deacetylated into two degrees of deacetylation (70% and 90%) to form chitosan in liquid form. Chitosan easily interacts with the bacterial cell wall and inhibits the formation of the cell wall. The chitosan will then be added to two bacterial cultures, *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative), and the diameter of inhibition will be assessed to determine the efficacy of chitosan as an antimicrobial agent. The control Petri dishes will contain the most common antibiotic used for each bacterial culture (penicillin for *S. aureus* and streptomycin for *E. coli*). The results will be analyzed using a two-way Analysis of Variance (ANOVA) test. It is anticipated that chitosan will inhibit the growth of both bacterial cultures. It is expected that the low-molecular-weight chitosan will be more effective against *E. coli* and the high-molecular-weight chitosan will be more effective against *S. aureus*. If the diameter of inhibition of the bacterial cultures with chitosan is equal to or greater than the diameter of inhibition of the control, then it can be concluded that chitosan is an effective antimicrobial agent. The results will indicate whether fungal chitin has antimicrobial properties and can be used as an alternative to antibiotics. This experiment can be expanded, to test how metal salts, temperature, pH, and varying degrees of deacetylation influence the antimicrobial properties of chitin.

Keywords: Gram-positive bacteria; Gram-negative bacteria; medicine; degree of deacetylation (DDA); chitin; chitosan; antibacterial properties; antimicrobial resistance (AMR); zygomycetes

Introduction

Antimicrobial resistance (AMR) is a growing concern worldwide, with devastating effects on health and the economy. It is estimated that AMR was directly responsible for 1.27 million deaths worldwide in 2019 and contributed to 4.95 million deaths globally [1]. The use of antibiotics eliminates bacterial infections, but their overuse results in the development of AMR [1].

As antibiotics are becoming less effective, research is being done on the antimicrobial properties of chitin. Chitin is a biopolymer abundant in the cell walls of fungi [2]. The deacetylation of chitin in sodium hydroxide (NaOH) solution forms chitosan, a cation with proven bacterial inhibitory properties [3]. The degree of deacetylation (DDA) is the amount of surface amino group left after removing the acetyl group [3].

Chitin can be extracted from a variety of sources, including shrimp, crab shells [4], insects, and crustaceans [2]. Previous research has also demonstrated the antimicrobial properties of chitin [2]. However, if chitin is to be used against bacteria commercially, then an abundant source must be available. As such, chitin produced from fungi is a more sustainable option, allowing large amounts of chitin to be produced [5]. Although different sources of chitin have been found to have antimicrobial properties, fungal chitin has not been thoroughly explored [5]. Therefore, this experiment looks at the antimicrobial properties of different degree of deacetylation (DDA) of fungal chitin on bacteria.

When chitin is deacetylated, the number of acetyl groups is reduced, causing the overall charge of the newly formed chitosan to be positive [3]. The higher the DDA, the larger the positive charge of the chitosan. The cell walls of bacteria tend to have a net negative charge [3]. When a bacterial cell

wall comes into contact with deacetylated chitin (chitosan), the difference in charges (the positive chitosan and the negative bacterial cell wall) causes the chitosan to attach to the bacterial cell wall, resulting in bacterial lysis [3, 6].

Chitosan is classified into high-molecular-weight (HMW) and low-molecular-weight (LMW) chitosan based on the number of acetyl groups present [7]. HMW chitosan has a higher number of acetyl groups present compared to LMW chitosan, and forms a polymeric film with the bacterial cell wall, which prevents the delivery of nutrients and causes the death of bacterial cells [7]. This is because HMW chitosan forms an impermeable layer around the cell due to hydrophilic and ionic interactions, which changes the cell's permeability and blocks transport into the cell [7]. LMW chitosan can penetrate bacterial cells and bind with the negatively charged intracellular components, such as phosphate residues of deoxyribonucleic acid (DNA) molecules. This blocks transcriptional reactions and messenger RNA (mRNA) synthesis [7]. Since chitosan is positively charged, it is attracted to the negatively charged bacterial membrane and disrupts the membrane, which eventually leads to bacterial death [8].

In this experiment, *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative), will be used. HMW chitosan has higher antibacterial effects on Gram-positive bacteria due to their thicker cell walls, as the HMW chitosan binds to the cell membrane. LMW chitosan is more effective against Gram-negative bacteria with thinner cell walls, as it can penetrate the bacterial cell and bind to intracellular components [2, 7].

Objective

The objective of this study is to determine whether the degree of deacetylation (DDA) of chitin to chitosan, extracted from *Gongronella butleri* fungus, affects chitosan's antibacterial properties against *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative).

Rationale

Up until now, chitin has been extracted from shrimp, crab shells [4], insects, and crustaceans [2]. The goal of this study is to determine if chitin found in the cell walls of fungi also display antibacterial properties. Since fungi can be cultured in the lab, large amounts of chitin can be extracted [9]. This would reduce the ethics associated with animal use and may be a more long-term solution as fungal sources are easier and less costly to grow compared to harvesting crustaceans [9].

Methods

The experiment will first require the extraction of fungal chitin to form chitosan. Chitin will be extracted from the *G. butleri* fungus, belonging to the class Zygomycetes, which contains higher amounts of chitin compared to other fungi [9]. It will be extracted through the deacetylation process [10].

Strong basic (alkali) solutions are used, such as 40 wt% sodium hydroxide (NaOH), to break down the fungal cell walls, at 100 °C for one hour [10]. The alkali insoluble material (AIM) product is derived [11]. Next, AIM needs to be treated with a strong acid like hydrochloric acid (HCl), to remove insoluble components from the extract. The remaining material, the cell wall, will then be precipitated and further processed [12].

The deacetylation process is repeated twice with varying concentrations of alkali to produce chitosan with 2 different DDA (70% and 90%), as a higher DDA results in a lower molecular weight of chitosan [12]. Once these stages are completed, the chitosan will be in powder form, therefore, it must be liquified so it can be easily added to the Petri dishes. The extracted chitosan will be dissolved in an aqueous acidic solution like acetic acid or lactic acid, which will liquify it [13]. A disk diffusion susceptibility test can then be performed, by dipping the disks into the liquified form of the chitosan [14].

The most common antibiotic for each bacterial culture will be used as a control. In this case, penicillin will act as the control for *S. aureus* (Gram-positive) and streptomycin will act as the control for *E. coli* (Gram-negative) [15]. The antibiotics will then be dissolved in distilled water; the disks will be dipped into the liquified form and added to the bacterial cultures [14]. The experiment will be conducted in triplicates.

Each day, after 4-6 days of incubation, the diameter of inhibition around the disks in the bacterial cultures will be measured and analyzed with statistical software [14]. Specifically, a two-way analysis of variance (ANOVA) test will be used, as there are two independent variables (degree of deacetylation and type of bacteria), which are being measured against the dependent variable (diameter of inhibition) [16].

Results

The experiment should take 2-3 weeks to complete. It is expected that the chitosan will inhibit the growth of both bacterial cultures on the Petri dishes. It is expected that the LMW chitosan will be more effective against *E. coli* and the HMW chitosan will be more effective against *S. aureus* [2].

Discussion

The larger the diameter of inhibition is, the greater the antimicrobial properties of the chitosan against the bacterial culture. The diameter of inhibition of the bacterial cultures with the chitosan added will be compared to the control Petri dishes with the common antibiotics added. If the diameter of inhibition with chitosan is similar to or greater than that of the control, it indicates that the chitosan is effective against the bacterial culture.

The diameters of inhibition will be analyzed using a two-way ANOVA [16], to determine whether there is a

difference between the experimental and control conditions. The results will also be used to determine if there is a difference in the antimicrobial properties of chitosan against Gram-positive and Gram-negative bacteria, by comparing the p-value of the two-way ANOVA test.

The results from this experiment can be compared to previous experiments that tested the antimicrobial effects of chitin on bacteria. The aim is to determine whether chitin from fungi has equal or greater antimicrobial properties compared to chitin extracted from sources such as shrimp and insects. These results will help determine whether fungal chitin is a viable source of chitin for antimicrobial properties, allowing chitin to be produced in large quantities.

Limitations

Despite the strengths of this research protocol, there are some limitations. Since fungi have not normally been used to extract chitin, the deacetylation process has been simplified to ensure some chitin can be obtained. A large amount of chitin may not be obtained, however, since this is preliminary research, the procedure can be expanded in the future to obtain more chitin. An example of this is exploring technologies that could help increase the amount of chitin obtained from fungi, such as by using enzymes and ionic liquids [17]. Furthermore, this research protocol only tested the antimicrobial properties of fungal chitin on two bacterial cultures, namely *E. coli* and *S. aureus*. Since bacterial infections can be caused by various bacterial cultures [18], the antimicrobial properties of fungal chitin cannot be determined after testing only two bacterial cultures. In the future, various types of bacteria should be tested against fungal chitin.

Conclusions

In conclusion, this study addresses a major issue that is plaguing modern medicine – the lack of effective antibiotics due to growing antimicrobial resistance (AMR). By providing an alternative method for treating bacterial infections, individuals can still be treated despite antibiotics not being effective. The research will be beneficial in determining whether chitin, extracted from fungi, can have antimicrobial properties. This could uncover a new strategy of targeting bacterial infections, providing direction for future research on AMR.

In the future, the scope of this research can be expanded to test how the presence of sodium, calcium, magnesium, and other metal salts influences the antibacterial properties of chitin. The effect of temperature, pH [3], and varying DDA can also be explored in further studies. Additionally, various bacterial cultures can be tested against fungal chitin and chitin can be extracted from various fungal cultures, to determine whether different fungi have varying antimicrobial properties.

List of Abbreviations

AMR: antimicrobial resistance
ANOVA: analysis of variance
DDA: degree of deacetylation
HMW: high-molecular-weight
LMW: low-molecular-weight
DNA: deoxyribonucleic acid
mRNA: messenger RNA
AIM: alkali insoluble material
HCl: hydrochloric acid
pH: potential of hydrogen

Conflicts of Interest

All authors declare that they have no conflicts of interest.

Ethics Approval and/or Participant Consent

Ethics approval and participant consent were not required as no humans or animals were used in the study.

Authors' Contributions

MP: Contributed to the introduction, results, discussion and conclusion sections; made substantial contributions to study design, drafted/revised the manuscript, and approved final publication.
ATA: Contributed to the methodology; made substantial contributions to study design, drafted/revised the manuscript, and approved final publication.
FL: Contributed to the introduction, made substantial contributions to study design, drafted/revised the manuscript, and approved final publication.

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