Abstract: Fungal spoilage contributes to the food losses which is a massive problem globally. The objective of the study is to determine the optimal and Minimum Inhibitory Concentration of the solution consisting of waste oyster shell powder with bio-silica from rice husk ash enhanced by Akapulko crude extract against Aspergillus niger and Penicillium chrysogenum, slow growing fungi that caused food spoilage. Waste oyster shells were pulverized while bio-silica is extracted using sol-gel method. The preliminary results showed that the solution containing a concentration of 90 mg/mL, 10 mg/mL and 580 mg/mL of oyster shell powder, Bio-silica and Akapulko crude extract, respectively, is the optimal concentration ratio against molds. The inhibition of the solution against the molds was further evaluated by utilizing Minimum Inhibitory Concentration Test and Agar Well Diffusion Test. Based from the Minimum Inhibitory Concentration (MIC) Test, the results showed that 680 mg/mL of the solution is capable of inhibiting both fungi. Basing from Agar Well Diffusion Test, the antifungal solution exhibited 74.10% of the efficacy of the standard control against both fungi. The outcome of the study entailed that the antifungal solution is a potential fungicide to minimize food spoilage.

Keywords: Minimum Inhibitory Concentration; Agar Well Diffusion Test, bio-silica; sol-gel method; fungal spoilage

I. INTRODUCTION

Food waste is a massive problem in most countries. The cost of food losses related to fungal spoilage is estimated at $10,000,000 annually [38]. Bread is a major culprit, with 32% of loaves purchased thrown out as a waste, according to figures from the Department for Environment, Food and Rural Affairs (DEFRA). In addition, half of the fruits and vegetables harvested in the tropics are lost due to fungal spoilage [38]. The appearance of molds is the main reason why food is thrown away by consumers. As a response to this, fungal spoilage must be taken into account by deeming it a potential spoilage agent. According to The Food Safety and Inspection Service, some common foodborne molds are often found on meat and poultry includes Alternaria, Aspergillus, Botrytis, Cladosporium, Fusarium, Geotrichum, Monilia, Manosascus, Mortierella, Mucor, Neuspora, Oldium, Oospora, Penicillium, Rhizopus, and Thamnidium. Aspergillus niger is a type of mold and the most common fungus in the Aspergillus genus. The word Aspergillus is derived from the Latin word “aspergillum” which basically means “holy water sprinkler”. When viewed under a microscope, these fungi resemble the shape of these sprinklers. Its molds are black or dark brown in color. They often have a white layer beneath the surface. This fungus is highly thermotolerant; it can survive in extreme conditions such as freezing weather or heat waves. In addition, this asexual saprophyte is not picky when deciding what to contaminate, so it can be found almost anywhere. Aspergillus niger is frequently pinpointed as the source of black mold and is the most common mold concern in homes. It is a food spoilage organism and can be found on practically any stored food, particularly in warmer climates. This includes fruits and vegetables, nuts, cereal grains, and dried, smoked and or fish and meat products [38]. Moreover, its strains produce mycotoxins, including ochratoxin A (OTA). OTA is a chemically stable compound; hence, ordinary food processing measures fail to substantially reduce its presence in foods and beverages. OTA has been shown to be toxic and carcinogenic in animals. The kidney is the main target organ for OTA; OTA is a potent renal carcinogen in several animal species [9, 19, 22, 23, 47]. Additionally, individuals who have severe mold allergies could also be affected by the fungus, with reactions such as asthma attacks and allergic alveolitis. It can even cause fungal balls to develop in the lungs of individuals exposed to it in large quantities. Another food borne mold is Penicillium, it is well known and one of the most common fungi occurring in a diverse range of habitats, from soil to vegetation to air, indoor environments and various food products. It has a worldwide distribution and a large economic impact on human life. Its main function in nature is the decomposition of organic materials, where species cause devastating rots as pre- and postharvest pathogens on food crops [12, 38, 40], as well as producing a diverse range of mycotoxins [12]. Penicillium chrysogenum is frequently identified as a food spoilage agent [40]. P. chrysogenum is well-known both as an allergen and as a pathogen, it has been known to cause a variety of opportunistic infections [17], mostly in people whose immune systems are weakened.
due to already suffering from another disease. Aside from relatively benign conditions such as skin rashes and ear infections (otomycosis), this fungus has been known to have caused sinusitis, posttraumatic endophthalmitis, necrotizing esophagitis in an AIDS patient [17], necrotizing pneumonia [7], intestinal invasion and disseminated disease [1]. The main objective of the study is to utilize an antifungal product composed of bio-silica from rice husk ash combined with waste oyster shell powder and enhanced with Akapulko extract that will inhibit Penicillium chrysogenum and Aspergillus niger that is responsible for the molding of breads. The study aims to answer the following specific problems:

- What is the optimal concentration ratio between waste oyster shell and bio-silica from rice husk ash that will inhibit the growth of Penicillium chrysogenum and Aspergillus niger?
- What is the Minimum Inhibitory Concentration of the antifungal product against Penicillium chrysogenum and Aspergillus niger?
- What is the percentage inhibition ratio based on the optimal concentrations?

II. METHODOLOGY

A. Research Design

This study used an experimental method to test the antifungal activity of pulverized Oyster shell and bio-silica from rice husk ash. The researchers conducted experiments to know the potential of pulverized Oyster shell with bio-silica as it inhibits the growth of Penicillium chrysogenum and Aspergillus niger. Different proportions of the combined raw materials were tested to get the optimal concentration in relation to the Minimum Inhibitory Concentration (MIC) and Agar Well - Diffusion Method for the percent inhibition ratio.

B. Collection and Preparation of Raw Materials

The oyster shells were collected from the market area in Ilocos region and the rice husk was collected from the rice mill in Guiset Norte, San Manuel, Pangasinan. The oyster shell was boiled for two hours and air dried to lessen the amount of moisture. It was then crushed into smaller pieces using mortar and pestle before oven drying at 100 °C for 1 hour. The crushed oyster shell was sent to the Mining Department for further pulverization. For the rice husk ashing, the collected rice husk was washed to remove impurities. Then, it was treated with hydrochloric acid at 60°C with at a concentration of 0.5 M for 30 min with constant stirring. The acidic solution was drained off and the rice husk was rinsed with distilled water until free from acids and was filtered and air-dried. The acid leached rice husk was placed in a muffle furnace heated at 650°C until all rice husk turned into ashes. For the preparation of the adjuvant, Akapulko leaves were washed with water for the removal of dirt. After cleaning, the leaves were pounded using mortar and pestle and then squeezed to collect its juice.

C. Extraction of Silica from Rice Husk Ash

25 grams of rice husk ash sample was dispersed in 175mL of 12% (caustic soda) NaOH solution by dissolving 24 grams of NaOH in 200 mL of distilled water. The NaOH solution and the ash were placed on a round bottom flask and heated for 1 hour. The solution obtained was filtered from any unburned, carbon impurities by the use of a filter press. The clear filtrate is the aqueous sodium silicate solution which was further concentrated in the oven at a temperature range of 100°C – 150°C for 60 – 90 mins.

Growth of Penicillium Chrysogenum and Aspergillus niger in Bread

Penicillium chrysogenum and Aspergillus niger were cultured in moistened leftover loaf breads by placing them in dark place until growth of molds were visible.

D. Inoculation of the Molds

All the apparatus used were sterilized by autoclaving at 180°C for 1 hour. The petri dishes were placed in an oven to remove excess moisture. Enough melted agar were poured into each sterile petri dish and were cooled at room temperature. The agar medium was left to set like stiff gelatin at room temperature and is ready for storage or use. The inoculation was done by letting a sterile cotton swab touch the collection area and then uncovering the petri dishes long enough to make a pattern of zigzag inoculation lines in the agar. This was done by gliding the cotton swab over the surface of the agar and the cover was placed back in the plate immediately. The plates were turned upside down and were stored in a dark place for a week until fungal growth is visible.

E. Preparation of the Slurry for Assay

0.02 grams of pulverized oyster shell, 0.02 grams of bio-silica and 2 drops of adjuvant (Akapulko extract) were mixed and stirred thoroughly. The proposed antifungal agent were mixed with 10 mL of Sabouraud Dextrose Broth. The total amount of antifungal agent prepared was 0.04 grams. The mixture was mixed until a homogenous solution between the broth and the proposed antifungal agent was achieved. The solution was transferred into a sterile test tube and was mixed for 15 seconds. The test tube was rolled further in between the palms for 30 seconds. In a 1.0 ml of this mixture, it contains 4mg or 4000µg of the proposed antifungal agent.

F. Determination of Minimum Inhibitory Concentration (MIC)

A two-fold dilution process was used in this section. Two-fold dilution refers to the reduction of the concentration of a solution by a factor of two, reducing the original concentration by one half. This method was used for inhibition tests to establish titers of the test samples. 1 mL of the proposed antifungal agent was added into a test tube containing 1 mL of the broth that resulted in a 2 mL volume. By doing this, the concentration of the antifungal agent in the
tube was diluted by half of its previous concentration. 13 test tubes were sterilized and labeled accordingly. Using a pipette, 1 mL of Sabouraud dextrose broth was placed into test tube #2 to #11 while 2 mL of Sabouraud dextrose broth was introduced to test tube #12.1 mL of the antifungal agent was introduced to test tube #1 and #2. The test tubes were covered with aluminum foil and the content of test tube #2 was shaken gently. 1 mL of the contents of test tube #2 was withdrawn and then transferred to test tube #3 and then was covered after. The content of test tube #3 was shaken. This process was continued until 1 mL from test tube #9 was added to test tube #10. 1mL content from test tube #10 was withdrawn using a pipette and was disregarded. Since originally the antifungal agent has a concentration of 4 mg/mL in test tube #1, the concentration was reduced to half in test tube #2 as 2 mg/mL, test tube #3 as 1 mg/mL, test tube #4 as 0.5 mg/mL and so on until test tube #10. One milliliter of the prepared fungi that was diluted was introduced to test tubes 1 to 11 and test tube number 13. 1 mL of the original antibiotic agent was added to test tube #13. The test tubes were incubated at 25-30°C for 3 to 5 days. The test tubes were examined for fungal growth using turbidimeter after the incubation period to compare the turbidity in the tubes. The tube that has the lowest concentration of proposed antifungal agent at which no growth or turbidity observed was stated as the MIC of the antifungal agent against Penicillium chrysogenum and Aspergillus niger.

G. Antifungal Activity Test by Agar Well Diffusion Method
Petri dishes containing 20 mL of Potato Dextrose Agar medium with the fungi samples were used for antifungal activity assay performed by the solid media using agar well diffusion method. An imaginary partition of four quadrants was drawn at the bottom of the petri dishes. The agar medium was punched with a hole of 6-8 mm at the center of each quadrant and equidistant to each other. The proposed antifungal agent with the adjuvant was placed inside the four holes using the optimal concentration based from the MIC. The agar plates were sealed tightly and were incubated at 25°C for 5 to 7 days until the growth of Penicillium chrysogenum and Aspergillus niger along with the antifungal takes effect.

H. Treatment of Data
Antifungal activity is the measure of the amount of inhibited fungi. It is a function of growth treatment with respect to growth inhibition and control. Calculation of the growth inhibitions of each of the fungal strains will be by the percentage of inhibition of radial growth relative to the control along with antifungal effect on the fungi.
To determine the antifungal activity of Pulverized Oyster Shell and Bio-silica from Rice Husk Ash, the following formula was used:

\[
\text{% inhibition ratio} = \frac{\text{treatment}}{\text{control}} \times 100\%
\]  
(1)

Where:
control = mycelium growth of control, mm
treatment = mycelium growth of treatment, mm

III. RESULTS AND DISCUSSION
A. Optimal Concentration Ratio of the Antifungal agent against *Penicillium chrysogenum* and *Aspergillus niger*
The antifungal agent exhibited a potential in the inhibition of the growth of the fungi, *Penicillium chrysogenum* and *Aspergillus niger*. It was tested and showed the optimal concentration ratio of the mixture as an antifungal agent against the fungi. The Table 1 and Table 2 show the response of the fungi to the antifungal solution at different concentration with corresponding presence of inhibition.

**TABLE I. INHIBITION TEST FOR OPTIMAL CONCENTRATION RATIO OF THE ANTIFUNGAL AGENT AGAINST *PENICILLIUM CHRYSOGENUM***

<table>
<thead>
<tr>
<th>Variation of Concentrations, mg/mL</th>
<th>Presence of Zone of Inhibition</th>
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</thead>
<tbody>
<tr>
<td><strong>Oyster shell powder</strong></td>
<td><strong>Bio-silica</strong></td>
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<tr>
<td>70</td>
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<td>80</td>
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<td>90</td>
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From Table 1, it is observed that fungi, *Penicillium chrysogenum* responded negatively to the first, second and fourth variations of the concentration of the antifungal solution. However, it can be seen that on the third variant of concentration, apparent inhibitions were exhibited by the fungi. Thus, the solution containing a concentration of 90 mg/mL, 10 mg/mL and 580 mg/mL of oyster shell powder, Bio-silica and Akapulko crude extract, respectively, is the optimal concentration ratio of the antifungal agent against the fungi.

**TABLE II. INHIBITION TEST FOR OPTIMAL CONCENTRATION RATIO OF THE ANTIFUNGAL AGENT AGAINST *ASPERGILLUS NIGER***

<table>
<thead>
<tr>
<th>Variation of Concentrations, mg/mL</th>
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Data shown in Table 2 is the result of an experimental test conducted to determine the optimal concentration ratio of the three components for the antifungal solution against *Aspergillus niger*. It was noticed that from the 4 trials executed with the varying compositions of the antifungal solution, the mixture with 90 mg/mL oyster shell powder, 10 mg/mL Bio-silica, and 580 mg/mL of Akapulko extract exhibited a visible zone of inhibition for *A. niger*. Thus, these components consisted the optimal concentration ratio of the antifungal agent against *A. niger*. The antifungal property of the oyster shell powder and bio-silica could be attributed to the presence of the CaCO$_3$ and Silica. The 3rd variation confirms that a cross linking in between the layers of silica and calcium oxide obtained from the oyster shell is achieved and a substantial decrease in the chain length of silica proves the decrease of concentration (Yongja He, et al, 2013).
B. Minimum Inhibition Concentration of the Antifungal solution against Penicillium chrysogenum and Aspergillus niger

Figure 1 shows that test tube no. 1 to 10 represents the varying concentration of the solution with test tube no. 1 having the highest concentration decreasing respectively until test tube no. 10 having the lowest concentration. Test tubes no. 11, 12, and 13 are the controls. The volume of inoculum and the antifungal standard were added accordingly in specified test tubes.

The test tube no. 11 which is the negative control tube did show a visible growth of the fungi while test tube no. 12 and 13 did not show any visible growth. The negative control contains both the broth and the inoculum, a growth of the fungi was observed. The positive control with the antibiotic standard (potassium sorbate) shows no visible growth of Penicillium chrysogenum and Aspergillus niger.

TABLE III. INHIBITION TEST FOR MINIMUM INHIBITION CONCENTRATION OF THE ANTIFUNGAL AGENT AGAINST PENICILLIUM CHRYSOGENUM

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<th>TRAIL</th>
<th>Tube # contents</th>
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<tbody>
<tr>
<td>I</td>
<td>- + + + + + + + CONTROL</td>
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<td>2</td>
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The response conveyed as positive (+) growth for the presence of mycelia growth or high turbidity and negative (-) for no apparent growth of fungi or low turbidity. After the incubation period, the tubes were examined. Based from Table 3, it can be seen that at test tube no. 1, there is no visible presence of fungi, P. chrysogenum. However, as the solution was diluted, a turbid solution indicating the existence of the fungi was observed. Thus, the prepared original concentration of the antifungal agent can be considered as the minimum inhibitory concentration against the fungi.

TABLE IV. INHIBITION TEST FOR MINIMUM INHIBITION CONCENTRATION OF THE ANTIFUNGAL AGENT AGAINST ASPERGILLUS NIGER

<table>
<thead>
<tr>
<th>TRAIL</th>
<th>Tube # contents</th>
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Similar with the serial dilution executed for the determination of minimum inhibition concentration of the antifungal solution for P. chrysogenum, the solution is only capable of inhibiting A. niger at 100% of the optimal concentration ratio as shown in Table 4. Thus, low turbidity was observed from the 1st tube while the succeeding tubes with diluted concentration were highly turbid. The results gathered in the MIC assay is considered valid because the extract contains active compounds that has antifungal properties as it inhibits the growth of the fungi which confirms that not all tubes exhibit growth. The result shows the lowest concentration of the antifungal agent that inhibits the growth P. chrysogenum and A. niger as stated in the Manual of Antimicrobial Susceptibility Testing (Coyle M., 2005). The solution requires a higher amount of Oyster shell powder but minimal Bio-silica.

C. Determination of Percent Inhibition of the Antifungal Agent

The optimal concentration acquired from the MIC was used for the agar well diffusion method to establish the percent inhibition ratio. Table 5 shows that the optimal concentration of the concentration, 680 mg/mL, an average of 1.23 mm zone inhibited P. Chrysogenum. Comparing to the 1.66 mm inhibition of the antifungal control, potassium sorbate, the antifungal solution exhibited 74.10% of the efficacy of the standard control against the fungi. Thus, as the diameter increases, the percentage of inhibition also increases.

Table 6 shows the data gathered in the determination of percent inhibition of the antifungal agent against A. niger using potassium sorbate as the standard fungicide. With a zone of inhibition with a mean of 1.23 mm diameter, the solution exhibited 74.10% of the efficacy of potassium...
sorbate whose mean diameter is 1.66 mm.

The similarity of the results obtained for the determination of antifungal activity of the prepared solution against Penicillium chrysogenum and Aspergillus niger is due to the fact that both fungi are both slow growing and easily developed resistance to environmental changes. This true to the fact that P. chrysogenum and A. niger are examples of dimorphic fungi which grew as a filamentous fungi and yeast (McDonald W, 2002). In addition, this is the reason why a highly concentrated antifungal agent was utilized aside from unidentified exact value of active agents in Akapulko extract and Oyster shell powder.

IV. CONCLUSIONS AND RECOMMENDATIONS

The study of the antifungal activity of waste oyster shell powder with bio-silica from rice husk ash enhanced with Akapulko extract against Aspergillus niger and Penicillium chrysogenum was performed in the microbiology laboratory of BSU (Benguet State University) and the results obtained upon performing the experiments could be concluded as follows. [1] The antifungal activity of waste oyster shell powder with bio-silica from rice husk ash enhanced with Akapulko extract can potentially inhibit the growth of Aspergillus niger and Penicillium chrysogenum because of the presence of the zone of inhibition against the fungi. [2] Based from the two-fold dilution process for Minimum Inhibitory Concentration test performed, 100% of the concentration of solution shows no growth of fungi organisms. [3] An optimal concentration of ratio 90 mg/mL oyster shell, 10 mg/mL bio-silica, and 580 mg/mL of Akapulko extract can inhibit the growth of Aspergillus niger and Penicillium chrysogenum producing an average of 1.23 mm diameter inhibition.

From the results obtained, the researchers could therefore conclude that the waste oyster shell powder with bio-silica from rice husk ash enhanced with Akapulko extract could be used as an antifungal agent against Aspergillus niger and Penicillium chrysogenum. With this research, a biologically-based, environmentally friendly natural fungicide can be an alternative.

From the research study, the researchers recommend that further study can be made to expand the scope of this paper. Thereby the following were recommended: [1] Analysis of phytochemical content: The researchers recommend performing an analysis of the phytochemical content of Akapulko and composition of the solution by Scanning Electron Microscopy that specifically inhibit the growth of the fungi. [2] Analysis of compatibility: The determination of the factors that made the three raw materials (Oyster shell, Bio-silica and Akapulko juice) compatible with each other should be considered by the next researchers. [3] The researchers recommend further research on the shelf life of the antifungal agent and its effectiveness at varying days. [4] Different variations between Oyster shell and Akapulko Juice could be checked and if the absence of the Bio-silica in the solution could give the same or better inhibition. [5] Use of other techniques: Further research should also be rendered to come up with other techniques in extracting the raw materials as well as other tests in inhibiting the growth of the fungi. [6] Application to other fungi species: The inhibition potential of the solution should also be attempted that may widen the scope of fungi that the solution can inhibit. [7] Use of different plant extract: Other plant extracts that exhibit antifungal properties could be attempted to replace the Akapulko extract that may enhance the concentration. [8] Other application: The possibility of the solution being an antimicrobial agent could also be attempted that may widen the purpose of the solution. [9] Industrial application: A bioplastic could be made from the solution to be able to serve its original purpose—to inhibit the growth of such molds in foods such as bread, fruits and vegetables.

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