Comparison of antifungal activities of scallop shell, oyster shell and their pyrolyzed products

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Abstract Waste shells create several serious problems, however, only parts of them are being utilized now. Therefore, the ideal solution would be to convert the waste shells into a product that is both environmentally beneficial and economically viable. Scallop and oyster shells were exposed to heat treatment at 1050 °C. SEM and XRD analysis results showed that the resultant powder turned completely into CaO after the treatment. The antifungal activities of non-treated and heat-treated scallop and oyster shell powder slurry were investigated. Non-treated oyster shell powder exhibited a significant antifungal activity at 25,000 ppm. Its antifungal activities against Physalospora piricola Nose (P. piricola) and Rhizoctonia solani Kühn (R. solany) were even up to 100%. Moreover, increasing culture time did not alter the antifungal activities. Heat-treated scallop and oyster shell powder exhibited obvious antifungal activity at 500 ppm, at which concentration 100% inhibition of R. solany was observed. The possible antifungal mechanism of the oyster shell and its heat-treated counterpart was studied using R. solani Kuhn as model. The results illustrated that oyster shell is able to affect the membrane permeability of the fungus. The above-mentioned results showed that it is possible for oyster shell to be an agriculture fungicide.

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Introduction

Marine aquaculture is a major industrial sector in coastal cities in China. The farming of different types of seafood species brings a great deal of marine products, such as fresh consumption, canned and precooked products. In the case of mollusks, the processing installations generate significant amounts of shell waste that accounts for hundreds of thousands of tons a year (Kwon et al., 2004). Until recently, the industry recognizes that it must take account of environmental factors, including wise stewardship of ecosystem resources and environmentally benign recycling of nature waste products, to retain sustainable development in the future. Therefore, recent
regulations and strategies on waste opened up new opportunities for sustainable development through the management and treatment of aquaculture materials. They encourage not only the application of green technologies, but also the recovery of the waste and the use of recovered materials as raw materials. In such a way, the sources of pollution can be minimized and the waste exploited as a resource.

In scallop and oyster, the total shell weight is around 60%, based on different collection seasons. As shell by-products, finding a use has become an important study subject around the world. Japanese researchers investigated the use of oyster shell waste as a substitute for aggregates in construction materials (Yoon et al., 2003, 2004; Yang et al., 2005) or cement clinker (Cheon and Song, 2003). The Korean Public finance was also increased in order to establish a fertilizer plant for oyster recycling (Yoon et al., 2003) and to solve problems of water eutrophication by means of transforming this material into a sustainable reagent for an efficient removal of phosphates from waste waters (Kwon et al., 2004). There are also reports that oyster shell powder was applied as additives of noodles, fried chicken, sardine ball (Suhara, 1995) and Kimchi (Choi et al., 2006) for quality improvement of extension of shelf life. Shell powder heated to over 700 °C exhibited a bactericidal activity (Sawai et al., 2001; Shiga et al., 1999). Likewise, scallop shells are reused in forestry road construction, as absorbent for phosphate (Yeom and Jung, 2009). Shell products were also used in e shellfish farming. For example, the cultch of certain shells was used as a substrate on which shellfish spat could form, grow and develop (Scottish Environment Protection Agency, 2005).

However, only parts of waste shells are being utilized now, leaving large amounts of shells piled up near the seaside, which creates several serious problems such as emission of offensive odors and soil pollution of heavy metals. Therefore, alternative approaches for recycling waste shells must be found. The ideal solution would be to convert the waste shells into a product that is both environmentally beneficial and economically viable.

In recent years, inorganic antimicrobial agents have attracted great interests for controlling of micro-organisms (Nakashima et al., 2001; Wilczynski, 2000). Sawai and Yoshikawa (2004) studied the antifungal activity of metallic oxide powders (MgO, CaO and ZnO) against Candida albicans NBRC1060. The results showed that MgO and CaO powders exhibited antimicrobial activities against all fungi examined in their study and showed minus difference between types of fungi. Oyster and scallop shells are mainly composed of calcium carbonate. This gives us a clue that oyster and scallop shells may have antifungal activities. The aim of our study is to investigate the antifungal activities of non-treated scallop shell and oyster shell powder and their heat-treated counterparts against four common crop-threatening pathogenic fungi (Physalospora piricola Nose (P. piricola), Rhizoctonia solani Kühn (R. solany), Alternaria solani (A. solany), and Phomopsis asparagi (Sacc.) (P. asparagi)). Moreover, Comparison of the effect of the antifungal activities of these samples was investigated. The initial mechanism of the oyster shell and heat-treated oyster shell against fungi was also studied in R. solani Kuhn model.

Materials and methods

Materials

The scallop shell and oyster shell were obtained from Dalian Zhangzidao Fishery Group Limited Company. The shell was washed several times and dried in an oven at 60 °C for 24 h. The shell was then ground and passed through 100 mesh, respectively.

Two kinds of shell were exposed to heat treatment at 1050 °C in air for 2 h, and ground using a ball mill and passed through 100 mesh, respectively. A suspension was prepared by suspending the ground shell powder in distilled water to yield the slurry at the powder concentrations of 0.5–25 mg/mL. Other chemical reagents were all of analytical grade. Four crop-threatening pathogenic fungi (P. piricola Nose (P. piricola), R. solani Kühn (R. solany), A. solani (A. solany), and P. asparagi (Sacc.) (P. asparagi)) were obtained from Qingdao Academy of Agricultural Sciences.

Methods

X-ray diffraction (XRD)

XRD measurement of the powder samples was performed with a D8 Advance diffractometer (Bruker) with Cu target (λ = 0.154 nm) at 40 kV. The scanning rate was 1.2°/min and the scanning scope of 2θ was 5–50°.

Scanning electron microscope (SEM)

The surface morphology of the sample concentration was analyzed by scanning electron microscopy by using KYKY-2800B SEM.

Antifungal assay

Antifungal assays were evaluated in vitro by mycelium growth rate test (Hermández-Lauzardo et al., 2008). The shell powder was suspended in distilled water to yield the suspension at the powder concentrations of 10–50 mg/mL. The solutions were autoclaved at 121 °C for 20 min and mixed with sterile molten potato dextrose agar (PDA) to obtain final concentrations of 0.5 and 25 mg/mL. When the agar was cooled, the mycelium of fungi was transferred to the plates and then incubated at 27 °C. The mixed medium without sample was used as the blank control. When the mycelium of fungi reached the edges of the blank control plate, the antifungal index was calculated with the following equation:

\[
\text{Antifungal index}(\%) = \frac{D_b - D_i}{D_b} \times 100
\]

Here, \( D_i \) is hyphal diameter in the test plate and \( D_b \) is hyphal diameter in the blank control.

Three replicates of each test were carried out and the results were averaged. The Scheffe method was used to evaluate the differences in antifungal index in the tests. Results with \( P < 0.05 \) were considered statistically significant.
The effect of oyster shell and their pyrolyzed products on the mycelial ultrastructure

One hundred micrograms per milliliter polyoxin, 12.5 mg/mL (prior to calcining) and 250 μg/mL (calcined) oyster powder culture medium were prepared. After cooling, the diameter of 5 mm bacteria block inoculated into medium, at the same time, 1 cm of the coverslip was inserted into the culture medium, which was cultured for 7 days at dark conditions for 29°C. Double distilled water served as the control. The coverslip was removed when the coverslip was attached with hyphae, then rapidly fixed in 2% glutaraldehyde solution at room temperature for 4 h, followed by 0.1 mol/L pH 7.2 phosphate buffer solution (PBS) washing, 1% osmic acid immobilizing 2 h, double distilled water rinsing, then respectively 30%, 50%, 70%, 80%, 90%, 95%, and 100% ethanol gradient dehydration, isoamyl acetate replacement 30 min overnight, drying, glue, coating, and observing under the scanning electron microscope.

The effect of oyster shell and their pyrolyzed products on medium electrical conductivity

The mycelial of R. solani Kuhn which was incubated in potato dextrose (PD) for a week. Then polyoxin, oyster shell and their pyrolyzed products were added to the PD to obtain the sample concentrations of 12.5 mg/mL (prior to calcining), 250 μg/mL (calcined) oyster powder and 100 μg/mL polyoxin, respectively. Double distilled water served as the control and polyoxin as positive control. The conductivities at 0 (\(J_0\)), 10, 20, 30, 60, and 120 min (\(J\)) were measured. In the end, the relative conductivity value was calculated: \(\Delta J = J - J_0\).

The effect of oyster shell and their pyrolyzed products on pyruvic acid content

Mycelial pyruvic acid content was determined according to the method described by Liu and Huang (2011). The upper clear layer (0.2 mL) of mycelial extract and distilled water (2.8 mL) were mixed with 2,4-dinitrophenylhydrazine (1.0 mL, 1 mg/mL), and then the mixture was swayed evenly and incubated for 10 min (37°C). Next, sodium hydroxide (5.0 mL, 1.5 mol/L) was added and swayed uniformly. The absorbance value of the mixture measured at 520 nm was converted into the value of pyruvate content (mg/L) by the standard curve of pyruvate. Distilled water served as the control.

Results

Morphology change of shell powder before and after treatment

Fig. 1 exhibited the surface morphology of non-treated and heat-treated shell powder. Fig. 1 showed that before the treatment of scallop and oyster shell powder, they all possess similar schistous structure. After heat-treatment, the schistous structure disappeared, but the surface morphology of two kinds of shell powder kept similar.

After the heat-treatment, the two kinds of shell powder exhibited similar XRD pattern, as shown in Fig. 2, in which these peaks were characteristic of absorption of CaO. Therefore, two kinds of shell powder became calcium oxide after heat-treatment. This result corresponds with the SEM result.

Antifungal activities of the non-treatment shell powder against four pathogenic fungi

Fig. 3 shows the effect of antifungal activities of non-treated scallop and oyster shell powder against four pathogenic fungi at different concentration and culture time. After culturing for 48 and 72 h, scallop and oyster shell powder barely had any antifungal activity against four pathogenic fungi at 500 ppm, for P. piricola and A. solany, while scallop and oyster shell powder exhibited slight antifungal activity after 48 h. However, after 72 h, scallop shell powder exhibited no antifungal activity. When the concentration of shell powder was increased to 25,000 ppm, scallop shell powder almost had no antifungal activity against the other three pathogenic fungi after 48 and 72 h, except for P. asparagi pathogenic fungi. However, it is noteworthy that the oyster shell powder exhibited significantly strong antifungal activity for four pathogenic fungi at 25,000 ppm at 48 and 72 h, and the antifungal activity of the oyster shell powder against P. piricola and R. solany even reached 100%. Moreover, the antifungal activity of oyster shell powder did not change with the increase of culture time.

Antifungal activities of the heat-treated shell powder against four pathogenic fungi

Fig. 4 shows the antifungal activities of heat-treated scallop and oyster shell powder against four pathogenic fungi at 500 ppm under different culture time. As shown in Fig. 4, heat-treated scallop and oyster shell powder all had significantly strong antifungal activity against four pathogenic fungi, especially against R. solany. Antifungal activity of the two kinds of shell powder had no evident difference among the four pathogenic fungi. However, after 72 h, except for R. solany pathogenic fungi, antifungal activity of two kinds of shell powder against the other three pathogenic fungi decreased with the increase of culture time.

As shown in Figs. 3 and 4, at 500 ppm, heat-treated shell powder had much stronger antifungal activity compared with non-treated shell powder. Heat-treated shell powder is CaO, while the composition of non-treated shell powder is mainly CaCO₃. Sawai and Yoshikawa’s (2004) research results showed that CaO powder exhibited good antimicrobial activities. Our research results are consistent with Sawai and Yoshikawa’s. However, at high concentration, non-treated oyster shell powder exhibited obvious antifungal activity, which needs further investigation.

Ultrastructural changes

To visualize the membrane disrupting process of oyster shell and heat-treated oyster shell, SEM study of the impact of oyster shell and heat-treated oyster shell on R. solani Kuhn cell membrane was conducted and cell morphology change and membrane damage were observed. SEM images (Fig. 5) showed that the control fungi remained intact, displaying smooth and bright surfaces and integrated envelopes, while the treatment mycelium lost its original structure. After being...
Figure 1  SEM of non-treatment and heat-treatment of shell powder: (a) scallop shell, (b) oyster shell, (c) heat-treated scallop shell, and (d) heat-treated oyster shell.

Figure 2  XRD patterns of heat-treated shell powder.
processed by 12.5 mg/mL oyster powder (prior to calcining), mycelium were much thinner and more bending, gathered into strands; after being processed at 250 μg/mL of oyster powder (calcined), hyphae distorted, bifurcation increased and hyphae swelled.

Effect of oyster shell and heat-treated oyster shell on change of medium electrical conductivity of R. solani Kuhn

The effects of oyster shell and heat-treated oyster shell on membrane permeability were detected in R. solani Kuhn, respectively. As shown in Fig. 6, after treated by the sample, the relative conductivity with oyster shell (before the calcination) treatment was found to be higher than that treated with polyoxin through all the time. However, the relative conductivity with heat-treated oyster shell was found to be lower than that treated with oyster shell and polyoxin.

Discussion

P. asparagi can cause severe stem blight of asparagus, and the disease has been discovered on the leaves and in any part of the stem of asparagus. When asparagus is affected by this pathogen, lesions form on the stems. At first the lesions appear light brown and later turn dark reddish brown. Asparagus will die in areas where the lesions have been formed around. A. solani is the causal agent of early blight disease of tomato. This pathogen colonizes various plant tissues including stems, leaves and fruit, and subsequently derives nutrients from host cells killed by the deleterious action of non-host specific, toxic secondary metabolites such as alternaric acid and zinniol. Epidemics caused by this economically important pathogen can result in severe tomato crop defoliation in areas with high humidity.
and frequent nightly dew. *R. solani*, is a pathogen which causes seed decay, seedling damping off, and root rot of bean and soybean. The disease causes death of crops with great economic losses (Elad et al., 1982). Therefore, the study on
antifungal agents is significant. As shown in the results, the heat-treated shell powders have much stronger antifungal activity than that of non-treated shell powder. At high concentration, the antifungal activity of oyster shell powder almost got to 100% against *P. piricola* and *R. solany*. For *A. solany* and *P. asparagi*, its antifungal index also exceeded 60%. Moreover, its antifungal activity was stable with the increase of action time.

In order to explore action mechanism of oyster shell, oyster shell was tested for its capacity of affecting membrane permeability of *R. solani* Kuhn, such as cell membrane ultrastructure changes, the relative permeability rate of cell membrane and mycelial pyruvate content. For cell membrane ultrastructure changes, oyster shell can significantly change the structural morphology of *R. solani* Kuhn cell. Oyster shell and heat-treated oyster shell were made of CaCO₃ and CaO, and had a lot calcium ions. They may first be attracted to fungi surfaces by electrostatic bonding between calcium cationic and anionic structures on the fungi surface. When the membrane was destabilized by calcium cation via ion exchange and formation of transmembrane pores (Kugler et al., 2005; Brogden, 2005), disturbing and lysis of fungi cells occurred. Secondly, because calcium ion is small molecule, it may permeate into cell membrane and change the concentration of sodium and kalium cations and affect the function of cell membrane.

The cytoplasm and membrane of micro-organisms are the targets for many inhibitive agents (Hwang et al., 2010). When membranes become compromised by antimicrobial agents, low molecular mass species such as K⁺ and PO₄³⁻ leached out, followed by DNA, RNA and other materials. In this study, to gain insight into the mode of action of oyster shell and heat-treated oyster shell, they were tested for their capacity of affecting membrane permeability of *R. solani* Kuhn. Their effect on the relative permeability rate of cell membrane and mycelial pyruvate content were determined. The relative conductivity with oyster shell and polyoxin treatment was obvious changed. Before the calcination oyster shell has better effect compared with after calcined oyster shell. The results may be attributed to the fact that polyoxin as a small molecule destroyed the cell membrane more quickly. For oyster powder (prior to calcining), it could be due to a greater concentration causing the increase of osmotic pressure, which then led to cell membrane rupture and electrolyte leakage. It may be also inferred that this kind of sample may interact with the membrane and cause the leach out of low molecular nucleic acid, proteins, and so on. However, the results of the lower effect of heat-treated oyster shell need to be further researched. Mycelial pyruvate plays an important role in energy metabolism of carbon source pathway. In this way, it was indicated that the oyster shell interfered with the construction of cell architecture and caused hypha growth inhibition. Through the mechanism analysis, it was concluded that the oyster shell executed antifungal properties may be associated with energy metabolism and cell wall-degrading.

Oyster shell is easy to access and non-toxic, and the major element in oyster shell, that is calcium, can improve crop growth. Therefore, if oyster shell was used in agriculture, it shall achieve environment protection and economizing resources effects. This will also be an ideal solution to convert the waste shells to a product that is not only environmentally beneficial, but economically viable.

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References

Elad, Y., Hadar, Y., Chet, I., Henis, Y., 1982. Prevention, with *Trichoderma harzianum* Rifai aggr., of reinestation by *Sclerotium rolfsii* Sacc. and *Rhizoctonia solani* Kühn of soil fumigated with...
methyl bromide, and improvement of disease control in tomatoes and peanuts. Crop Prot. 1, 199–211.


