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Antifungal activity of nano and micro charcoal particle polymers against *Paecilomyces variotii*, *Trichoderma virens* and *Chaetomium globosum*

Research Paper

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This study investigates the antifungal activity of a polymer integrated with nano-porous charcoal particles against *Paecilomyces variotii*, *Chaetomium globosum*, *Trichoderma virens*, which are all filamentous fungi. The charcoal polymers were prepared by combining charcoal powders with plastic resin under a vacuum to form charcoal particle protrusions on the polymer surface. The mycelial growth of *P. variotii* and *T. virens* exhibited a reduction of 10 and 30%, respectively, after the conidia were pre-treated with charcoal polymers, and in particular, no mycelial growth was found in *C. globosum* during 5 days of culture. The adsorption of Ca^{2+} into charcoal was suggested to inhibit growth due to the reduction in the flux of calcium ions (Ca^{2+}) into the hyphae. In 5 h, about 15 mM of Ca^{2+} were removed from $CaCl_2$ solution with 0.2 g/mL of polymers, and the nano-sized pores of the charcoals on the polymer were responsible for the Ca^{2+} adsorption.

Introduction

Molds are filamentous fungi that can be used to prepare a number of fermented foods, but some of these fungi can also cause human disease when food and agricultural products are contaminated [1,2]. Toxins produced in pathogenic fungi can induce constant irritation or even a fatal response [3]. Furthermore, fungal contamination of agricultural products is an aggravating factor of the food crisis [4]. *Paecilomyces* species are pathogenic fungi that are commonly found in soil, food products and cosmetics [5], and these can cause food spoilage in addition to various human illnesses, including sinusitis, endophthalmitis, wound infections following tissue transplants, cutaneous hyalohyphomycosis, onychomycosis, osteomyelitis, otitis media and dialysis-related peritonitis [5,6]. *Trichoderma* species are found in soil, wood and vegetables, and these pathogens aggravate immunocompromised host diseases [7]. *Chaetomium* species grow in soil, air and plant debris and are considered to be an allergen and a causative agent for onychomycosis (nail infection), peritonitis, and cutaneous lesions [8].

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It is more difficult to successfully treat fungal contamination or disease in humans and plants than it is to treat bacterial disease [9] because both fungi and host organisms are eukaryotic, so chemical treatments for pathogenic fungi are often toxic to humans and plants [10]. Recently, new antifungal reagents, such as chitosan, flavonoids and essential oils have attracted a great amount of attention due to their non-toxicity to humans and plants [11–14].

As their surface-to-volume ratio increases, a greater quantity of antifungal agent comes in contact with the fungi and penetrates the cells [15–17]. So the efficiency of the antifungal activity can be maximized by fabricating novel antifungal agents with materials that have micro- and nano-scale features. Silver nanoparticles are a representative fungicide that inhibits a broad range of biological processes in fungi [18]. Numerous studies have been carried out on the antifungal activity of nanomaterials by using nanoparticulated zinc oxide, graphene oxide and copper [16,19,20]. Charcoals obtained from wood have been used as detoxifying substances due to their physical adsorption resulting from their porous structure [21–23]. Previous studies have shown that charcoal could reduce aflatoxins in fungal-contaminated milk feed [24,25].

http://dx.doi.org/10.1016/j.nbt.2015.08.001

To date, few studies have investigated the antifungal activity of charcoal and its related molecular mechanisms. Thus, the objective of this study is to demonstrate the antifungal activity of charcoal polymers against three pathogenic fungi, *Paecilomyces variotii*, *Trichoderma virens* and *Chaetomium globosum*. The present study also elucidates an inhibitory biochemical mechanism of mycelium growth, which is expected to be helpful in using charcoal polymer as a functional packaging material for food, cosmetics and agricultural products.

Materials and methods

Materials

Charcoal polymers were produced as plastic chips with a constant size and surface area (3.0 mm × 2.0 mm × 1.5 mm, 10.8 mg per each). These were prepared by combining charcoal powder with a plastic resin under vacuum, as described in our previous reports [26]. Briefly, oak wood charcoals were milled with balls at 14,000°C for 6 hours to produce micro meter sized powders. Charcoal plastic chips were produced by mixing fine charcoal powders with plastic resin (HDPE, KCT, Seoul, Republic of Korea) in a vacuum. Potato Dextrose Agar (PDA) was purchased from Difco (Detroit, MI, USA), and L-(+)-Tartaric acid and trypan blue were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Fungi

Three filamentous fungi, *P. variotii* (ATCC 18502), *C. globosum* (ATCC 6205), and *T. virens* (ATCC 9845) were kindly provided by NGV, our collaborative partner.

Fungi culture

To harvest conidia, distilled water was dropped into a PDA plate containing fully grown fungi. The conidia suspension was harvested by aspirating the dropped water after rocking the PDA plates. The concentration of conidia was adjusted to 1.0×10^2 cells/mL by scoring the conidia in a haemocytometer (Superior Marienfeld, Lauda-Koenigshofen, Germany) under a microscope, Axio Imager 2 (Carl-Zeiss, Jena, Germany). After 10 mL of conidia suspension were incubated with or without charcoal polymers (50, 100, 200 mg/mL) in a shaking incubator (Vision scientific, Daejeon, Republic of Korea) at 26°C for 24 h, 50 μL of each conidia suspension were inoculated on a PDA plate in a 90 mm petri dish and were cultured in a BOD incubator (DongSung, Seoul, Republic of Korea) at 26°C. The mycelial growth was observed by taking photographs of each petri dish with a digital camera (Olympus, Tokyo, Japan) and conducting an image analysis.

Measurement of mycelial growth

The antifungal activity of the charcoal polymer was assessed by measuring the area of mycelial growth and comparing it to that of the control cultures (without charcoal). The area of mycelial growth was calculated from the photographs of the fungi culture by using an image analysis computer program (Image J, NIH, Bethesda, MD, USA). The mycelial growth was calculated as follows:

Percentage of mycelial growth

$$= \left[\frac{A_{\text{mycelium w/charcoal}}/A_{\text{whole plate}}}{A_{\text{mycelium w/o/charcoal}}/A_{\text{whole plate}}}\right] \times 100$$

where $A_{\text{mycelium w/charcoal}}$ is the average area of the mycelium grown from conidia previously incubated with charcoal polymers, and $A_{\text{whole plate}}$ is the entire area of the medium plate. $A_{\text{mycelium w/o charcoal}}$ is the average area of the mycelium grown from conidia previously incubated without charcoal polymer.

Scanning electron microscopy (SEM)

The morphology of the charcoal particles in plastic and the porous structure of the charcoal particles were observed using a scanning electron microscope (SEM, Hitachi S-4800, Tokyo, Japan) with an SE2 detector for secondary electrons. High density poly ethylene (HDPE) plastic containing 0.5% (w/w) charcoal particles was attached onto a carbon tape (Ted Pella Inc., Redding, CA, USA) and was observed under SEM with an accelerating voltage of 5.0 kV and a working distance of 8.3–8.4 mm. Charcoal particles filtered using a 325-mesh nylon membrane were dropped on carbon tape and were observed under an SEM with an accelerating voltage of 15.0 kV and a working distance of 8.0 to visualize the porous structure of the charcoal surface.

Measurement of calcium ion concentration

The changes in the calcium ion concentration due to the presence of charcoal polymers were monitored by employing an EDTA titration process in the presence of a Patton-Reeder indicator, calconcarboxylic acid. The original color of the free Patton-Reeder indicator is blue, and this color changes to pink as a result of the formation of a complex between the indicator and Ca^{2+}. However, the color can change back into the original blue by adding EDTA to the complex solution because the binding affinity between EDTA and Ca^{2+} is stronger than that between the Patton-Reeder indicator and Ca^{2+}. Since the stoichiometric ratio between Ca^{2+} and EDTA at the point at which the color changes is 1:1, the calcium ion moles can be calculated by measuring the volume of EDTA used during titration. The molar concentration of Ca^{2+} adsorbed into the charcoal particles was evaluated using the Ca^{2+} moles calculated from the EDTA titration. For the EDTA titration, 0.1 M of calcium chloride (CaCl2) solution was prepared, and the residual magnesium ions (Mg^{2+}) were removed by adding 0.1 M NaOH (pH 12). The CaCl2 solutions were incubated with or without charcoal polymers in a shaking incubator at 26°C for 2 h. The Ca^{2+} content in the CaCl2 solutions was evaluated by measuring the volume of 0.01 M EDTA that was added to the Patton-Reeder indicator−Ca^{2+} complex.

Statistical analysis

The experimental data were gathered from at least three independent experiments and are represented as the mean values and standard deviations (±S.D.). The statistical significance was calculated using Student *t-test* (Sigma Plot 10.0, SPSS Inc., Chicago, IL, USA), and p values less than 0.05 or 0.01 indicate a significant difference in the data.

Results and discussion

Nano and micro charcoal particles on plastics

Scanning electron microscopy (SEM) was employed to investigate whether charcoal particles exist on the surface of the plastic (Fig. 1). The size of the charcoal particles was determined from SEM images to range from 100 nm to 10 μm, and about 60% of the