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Free chlorine and phytic acid synergistically inactivated conidia of *Aspergillus* spp.

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Abstract−Chlorination has been widely used to disinfect various microbials in the environment, but its fungicidal activity is known to be limited. Here, we demonstrate that a combinatorial treatment with free chlorine and phytic acid exerted high fungicidal activities against selected species of *Aspergillus*. Treatment with either chlorine (7 mg/*l*) or phytic acid (~400 mg/*l*) without pH adjustment caused marginal inactivation of *Aspergillus niger* conidia within 5 min. However, the combinatorial treatment with free chlorine and phytic acid inactivated 98% of *A. niger* conidia within 5 min ($\overline{CT}$=25.7 mg/*l*·min). Overall fungicidal efficiency of combinatorial application was higher (~256%) than the sum of inactivation levels by individual treatment, suggesting a synergistic effect between free chlorine and phytic acid. Transmission electron microscopy observation showed that free chlorine primarily disrupted nucleo-cytosolic organs, whereas phytic acid preferentially disintegrated the cell wall and plasma membrane. The combination of both agents demolished the conidial structure of *A. niger*. The effects of these chemicals on the cell membrane were verified with propidium iodide staining, lipid peroxidation, and extracellular ATP secretion. Fungicidal activities of chlorine and phytic acid were further confirmed against *A. parasiticus* and *A. flavus*. Our data suggest that the mixture of free chlorine and phytic acid without any additional preparation may efficiently disinfect *Aspergillus* spp. through the synergistic activities of individual components.

Keywords: Free Chlorine, Phytic Acid, Combinatorial Treatment, *Aspergillus* spp., Inactivation

INTRODUCTION

Aspergillus is a genus of conidial fungi that is almost ubiquitously present in indoor and outdoor environments [1,2]. The genus has been taxonomically divided into eight subgenera containing more than 200 species. Several *Aspergillus* spp. are associated with mycotoxin excretion and infectious diseases of humans and animals. Aspergillosis, a representative infectious disease mostly caused by *Aspergillus fumigatus*, is a common infection of the respiratory tissues of mammals [3]. In addition, species such as *A. parasiticus* and *A. flavus* produce aflatoxin, a notorious mycotoxin and carcinogen [4]. Aflatoxin increases the risk of metabolic aberration and DNA mutations, thereby having adverse effects on various organs. *A. niger* also generates ochratoxin A (OTA), which is considered harmful to the kidneys and possibly carcinogenic to humans [5]. Regardless of the high pathogenic potential and broad distribution of *Aspergillus*, the efficiency and safety of disinfection methods against these fungi have been investigated under limited conditions and are not well established.

Various sanitizers such as chlorine derivatives [6], UV irradiation [7], photocatalytic nanoparticles [8], ozone [9], and organic acids [10] have been used to disinfect fungal contamination. Among them, due to easy accessibility and cost-effectiveness, chlorine derivatives have been widely used to sanitize water and surface fungi in practical application [11]. Recently, free chlorine inactivated spores and conidia of fungal species belonging to *Cladosporium*, *Phoma*, *Aspergillus*, and *Penicillium*, have been studied with different levels of sensitivity [12]. Similarly, chlorine dioxide reduced the viability of fungi (e.g., *Cladosporium* spp., *Trichoderma* spp., *Penicillium* spp.), depending on pH, temperature, and concentration [13]. However, chlorination alone was reported to be insufficient to completely inactivate fungi from drinking water [14,15]. Alternatively, the combinatorial and/or sequential effects of multiple treatments have been also suggested. UV irradiation in combination with chlorination was shown to effectively inactivate *A. flavus* [16]. Integrated treatment with hypochlorite, UV, and cold atmospheric plasma (CAP) also reduced conidial viability in three *Penicillium* species on a 1-3 log scale [17,18]. In addition, treatment with chlorine derivatives along with organic acids such as peracetic acid and humic acid was found to increase the inactivation of target fungal species [13,14]. However, the synergistic effects of chlorine and organic acid were found to be limited. Nevertheless, due to natural existence, low toxicity, and high germicidal activities, organic acids could be versatile disinfectants to inactivate fungi and need to be investigated in broader spectrum.

To date, several organic acids have been evaluated for their potential applications as disinfectants against fungi [10]. The fungicidal activity of most organic acids is pH dependent. However, several derivatives of carboxylic acids, fatty acids, and/or amino acids exerted antifungal activities that were associated with polyvalent functionalities such high oxidation potentials, hydrophobicity, and amphipathic properties, thereby causing oxidative stress, membrane disintegration, and inhibition of fungal cell metabolism [19]. Propionic acid,

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a naturally existing carboxylic acid, was shown to efficiently induce death of yeast cells by mediating oxidative stress [20]. Fatty acids such as undecylenic acid and acetylenic acid exerted potent antifungal activities against *Candida*, *Aspergillus*, and *Trichoderma* [21,22]. 2-Hydroxyisocaproic acid, an amino acid derivative, displayed antifungal activities against *Candida* and *Aspergillus* [23]. Poacic acid was also found to inactivate various plant pathogenic fungi through the inhibition of glucan synthesis in fungal cell walls [18].

Phytic acid, a polyvalent organic acid used as a food additive and metal chelator, was reported as a safe germicide against various bacteria [24]. Phytic acid alone and in combination with sodium hypochlorite was used to eradicate *Enterococcus faecalis* [25] and *Bacillus subtilis* [26], the most resistant bacteria in endodontic dental treatment. Phytic acid was also efficient in inactivating *Escherichia coli* O157:H7 [27,28]. However, regardless of its high germicidal activity and polyvalent functionality, phytic acid has never been investigated for its antifungal activity. Therefore, evaluation of the effects of phytic acid alone and/or with other agents on fungal growth will be of interest to facilitate the development of advanced disinfectants against fungi.

In the present study, we investigated the effects of free chlorine and phytic acid alone and in combination against conidia produced by *A. niger*. We monitored the ultrastructural changes of conidia treated under various conditions and explored the potential mode of action of free chlorine and phytic acid. In addition, we compared the disinfectant efficiency of the combination of free chlorine and phytic acid with three representative species of *Aspergillus* (*i.e. A. niger*, *A. flavus*, and *A. parasiticus*).

EXPERIMENTAL DETAILS

1. Chemical Agents and Fungal Strains

Sodium hypochlorite solution (5%, Junsei Co., Japan) and phytic acid solution (50%, Sigma-Aldrich Co., USA) were used as sources of free chlorine and phytic acid, respectively. All stock solutions were freshly prepared before experiment. Mycelial plates of *A. niger* (KACC 43547), *A. flavus* (KACC 41730), and *A. parasiticus* (KACC 46037) strains were obtained from the Korean Agricultural Culture Collection (KACC).

2. Preparation of Fungus Culture and Conidium Suspension

Mycelia of *Aspergillus* spp. were initially inoculated in 2 ml of MP media (malt extract 10 g/*l*, Bacto-peptone 10 g/*l*, pH 7.4 adjusted with potassium hydroxide [KOH]) and cultured with gentle shaking at 25 °C for 24 h. Mycelial suspensions were obtained and re-inoculated onto potato dextrose agar (PDA, Difco Co., USA) plates, followed by incubation at 25 °C. After seven days, the conidia produced were collected by washing with sterilized 0.1% Tween-80 solution and filtering with sterile Miracloth (Millipore, Billerica, MA). Conidia were harvested by centrifugation at 4,000 rpm for 10 min and subjected to three rounds of washing with phosphate-buffered saline (PBS, pH 7.2, Sigma-Aldrich Co., USA). The number of viable conidia in the stock was determined by accessing colony-forming units (CFUs) per milliliter on PDA plates and adjusted to 3×10^6 CFU/ml for conidia inactivation tests.

3. Investigation of Fungal Conidia

To investigate the efficiency of free chlorine and/or phytic acid to inactivate *Aspergillus* spp. conidia, 30 ml of a reaction mixture containing conidia (9×10^5) and chemicals was stirred in Pyrex reactor at room temperature (20±1 °C) for designated time course. The reaction was terminated with sodium thiosulfate (Sigma-Aldrich Co., USA), which was used to quench the residual free chlorine. Concentration of free chlorine in the reaction mixture was determined with *N,N*-diethyl-*p*-phenylene diamine (DPD, Hach Co., USA) using colorimetric method with a Pocket Colorimeter II (Hach Co., USA). After treatment, 1 ml of the reaction mixture was inoculated onto PDA plates at various dilutions. After two days of incubation at 25 °C, the number of fungal colonies was counted.

The delayed Chick-Watson model with a time-averaged free chlorine concentration was chosen to explain the kinetics of *Aspergillus* spp. conidia in the presence of free chlorine, as described by Eq. (1) [29-31].

$$\mathrm{Log}\frac{N}{N_0}=\begin{pmatrix} 0 & \text{if } \ \overline{C}T\le\overline{C}T_{lag}=\frac{1}{k}\mathrm{Log}\left(\frac{N}{N_0}\right) \\ -k(\overline{C}T-\overline{C}T_{lag}) & \text{if } \ \overline{C}T\ge\overline{C}T_{lag}=\frac{1}{k}\mathrm{Log}\left(\frac{N}{N_0}\right) \end{pmatrix} \quad (1)$$

where N=concentration of viable *Aspergillus* conidia at time t (CFU/ml), N_0 = initial concentration of viable *Aspergillus* conidia (CFU/ml), $\overline{C}=\int_0^t C/t\ dt$=time-averaged free chlorine concentration (mg/*l*), C=free chlorine concentration at time t (mg/*l*), k=inactivation rate constant (*l*/mg·min), $\overline{C}T_{lag}$=x-axis intercept of the inactivation curve.

4. Transmission Electron Microscopy (TEM)

To examine their ultrastructure after treatment with chemical agents, conidia were subjected to TEM observation. Conidia were washed with 10 mM PBS (pH 7.2) and fixed with Karnovsky's fixative (2% glutaraldehyde and 2% paraformaldehyde mix [Sigma-Aldrich Co., USA]) [32] at 4 °C for 24 h. The samples were washed with 0.05 M sodium cacodylate buffer (pH 7.2) and post-fixed with 1% osmium tetroxide (OsO_4, Sigma-Aldrich Co., USA) at 4 °C for 2 h. After washing twice with distilled water, the conidia were subjected to electro-conductive staining at 4 °C for overnight using 0.5% uranyl acetate (VWR™, USA). The sample solution was subsequently dehydrated with sequential changes of solvent to 30%, 40%, 50%, 70%, 80%, 90%, and 100% ethanol. The solvent was substituted with 100% propylene oxide, and conidia were embedded in Embed 812 resin mixture (VWR™, USA). Thin specimens of approximately 70 nm thickness were sectioned using an ultramicrotome (LC7, LEICA, Germany) and placed on copper grids of 200 mesh (Sigma-Aldrich Co., USA). The sectioned specimens were stained with 2% uranyl acetate for 7 min and incubated in Reynold's lead citrate (LC, Agar Scientific Co., UK) for 7 min. Finally, the prepared specimens were observed with TEM (Hitachi H-7650, UK).

5. Propidium Iodide (PI) Staining of Conidia

A. niger conidia were stained with PI [33]. After chemical treatment, 1 ml of the reaction mixture was mixed with 10 µl PI stock (Sigma-Aldrich Co., USA) and incubated in dark conditions for 30 min. The fluorescence intensity emitted at 617 nm was meas-

ured with an absorbance/fluorescence microplate reader (Tecan Co., Austria).

6. Lipid Peroxidation Assay of Conidia

To investigate the lipid peroxidation in conidia membrane, we determined the production of total malondialdehyde (MDA) by modifying a previously described procedure (EZ-TBARS kit, DoGenBio, Korea). About 2×10^6 conidia were exposed to each chemical treatment and harvested by centrifugation at 6,000 rpm for 5 min. The harvested conidia were suspended in 200 µl of 10 mM PBS (pH 7.2) and subjected to cell lysis at 95 °C for 30 min. MDA standards and conidia samples were mixed with thiobarbituric acid (TBA) at equal volumes and incubated at 65 °C for 45 min. The total amount of MDA was determined by monitoring the absorbance at 540 nm using a spectrophotometer.

7. Determination of ATP Released from Conidia

Conidia leakage induced by chemical treatment was accessed by evaluating the content of extracellular ATP released from conidia. A total of 2×10^6 conidia per treatment were collected by gentle centrifugation at 6,000 rpm for 5 min and suspended in 200 µl of 10 mM PBS (pH 7.2). Conidial suspensions were vigorously vortexed for 30 min, followed by high-speed centrifugation at 14,200 rpm for 15 min. The amounts of ATP in standards and sample conidia were determined using Sigma ATP bioluminescent assay kit (Sigma-Aldrich Co., USA) and tube luminometer (Lumat LB 9508, Berthold, Germany).

8. Statistical Analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) in R software (https://www.r-project.org/). Multiple comparisons of means were analyzed by Duncan's multiple range tests at $p<0.05$.

RESULTS AND DISCUSSION

1. Inactivation of *A. niger* Conidia by Free Chlorine and Phytic Acid

Free chlorine was previously shown to efficiently inactivate conidial spores of several *Aspergillus* spp. [6,12]. In addition, the combinatorial treatment of free chlorine and organic acids was found to exhibit antifungal effects, but the synergistic effects of thee agents were limited [13,14]. In the present study, we investigated the fungicidal action of the combination of free chlorine and phytic acid, another polyvalent organic acid. The number of colonies formed from the conidia treated with free chlorine and phytic acid alone and in combination was counted for different time points (Fig. 1). The treatment with free chlorine (7 mg/*l*) alone without pH adjustment resulted in a gradual conidial inactivation in a time-dependent manner. According to 5 min of treatment, the germinated CFU/ml of *A. niger* conidia was reduced in a 0.5 log scale (approximately 70% inactivation). Inactivation rate as the function of $\overline{CT}$ value was estimated as 53.23 mg/*l*·min. Even though the parameters were not equivalent to be compared in parallel, previous study reported that *A. niger* was resistant up to 10 mg/*l* of free chlorine for 5 min [14], which was similarly observed in our study. On the other hand, the measured pH of the chlorine suspension was pH 8.8. When pH of free chlorine was adjusted to 7 prior to experiment [6,12], the $\overline{CT}$ values required to inactivate various *Aspergillus* spp. to 0.5 log scale were estimated to be less than 20 mg/*l*·min. This inactivation rate was more efficient than the $\overline{CT}$ value determined in this study (i.e., 53.23 mg/*l*·min). In general, disinfection activity of free chlorine was dependent on the amount of hypochlorous acid (HOCl, pKa=7.4) and was high in the pH range of 4 to 8 [34,35]. At pH above 8, hypochlorite ions (OCl^-) undergo dissociation as major species and their germicidal activity is 80% less than that of HOCl. Thus, the low fungicidal activity of free chlorine observed herein was likely due to the high pH (i.e., 8.8).

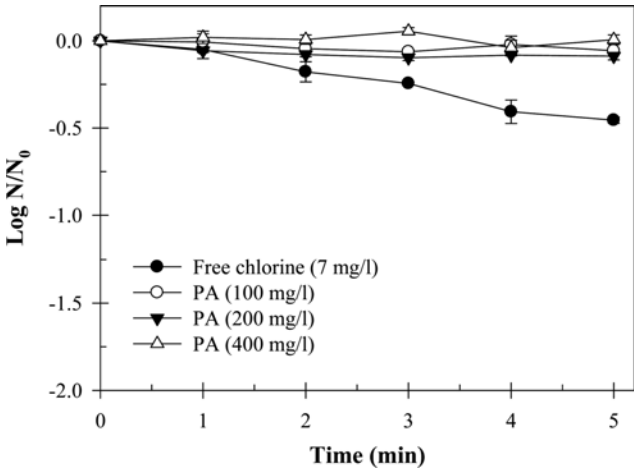

Fig. 1. Effect of individual treatment with free chlorine and phytic acid on the inactivation of *A. niger* conidia. Free chlorine and phytic acid were used to treat *A. niger* conidia (3×10^4 CFU/ml) without any pH adjustment. The inactivation of *A. niger* conidia was presented as the log N/N_0 scale. N denotes the CFU/ml at a designated time and N_0 is the CFU/ml at initial time point.

The treatment with phytic acid alone had no significant inactivation effects on *A. niger* conidia in the range of 100 to 400 mg/*l* within designated time courses (~5 min of treatment) (Fig. 1). Up to the treatment with 400 mg/*l* of phytic acid for 5 h resulted in a decrease in the viability of *A. niger* conidia to only 0.6 log scale (i.e., 60% inactivation) (Supplementary Information Fig. S1). Taken together, the individual treatment with free chlorine without pH adjustment or phytic acid was insufficient to completely inactivate *A. niger* conidia.

Next, we examined the combined effect of free chlorine and phytic acid on the inactivation of *A. niger* (Fig. 2). In comparison with the condition treated with free chlorine only, combinatorial treatment of agents showed a strong inactivation of conidia, proportionate to the concentration of phytic acid. Treatment with free chlorine (7 mg/*l*) and 400 mg/*l* of phytic acid for 5 min resulted in a reduced viability of *A. niger* conidia to about 1.78 log scale (approximately 98% inactivation). In terms of inactivation kinetics, $\overline{CT}$ value at 1.78 log scale was estimated to be 25.2 mg/*l*·min (data not shown). This inactivation efficiency was higher than that previously determined (37.55 to 57.35 mg/*l*·min for 2 log scale) with free chlorine at around pH 7 for *A. fumigatus* and *A. versicolor* [6].

Our data indicated that combinatorial application of free chlorine and phytic acid efficiently inactivated *A. niger* conidia. As the function of 'percent synergistic effect' in inactivation efficiency in

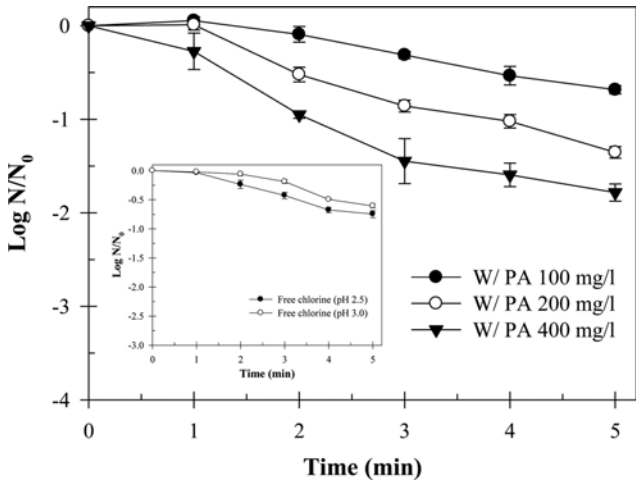

Fig. 2. Effect of the combinatorial treatment with free chlorine and phytic acid on the inactivation of *A. niger* conidia. Free chlorine and phytic acid were mixed to obtain $[HOCl]_0$=7 mg/*l* and $[phytic\ acid]_0$=100-400 mg/*l* and used to treat *A. niger* conidia (3×10^4 CFU/ml) without pH adjustment. The inactivation of *A. niger* conidia was presented as the log N/N_0 scale (N, CFU/ml at designated time; N_0, CFU/ml at initial time point. Inset, inactivation efficiency of free chlorine under low pH condition.

Table 1. Synergistic effect in combinatorial treatment with free chlorine and phytic acid on the inactivation of *A. niger* conidia

Disinfection agent	HOCl PA	7 mg/*l* 100 mg/*l*	7 mg/*l* 200 mg/*l*	7 mg/*l* 400 mg/*l*
Predicted inactivation[a]		0.56 log	0.59 log	0.50 log
Observed inactivation		0.68 log	1.35 log	1.78 log
Percentage increase		21.4%	128.8%	256.0%

[a]Arithmetic sum of inactivation level ($logN/N_0$) via individual treatment of free chlorine and phytic acid (ex., 0.56 log was the sum of 0.50 log by HOCl 7 mg/*l* and 0.06 log by PA 100 mg/*l*)

log N/N_0 scale [36], combination of free chlorine and phytic acid increased fungal inactivation 21.4-256%, compared to the arithmetic sums of individual treatments (Table 1). This strongly suggests that free chlorine and phytic acid inactivated the *A. niger* conidia via the synergistic effect rather than additive effect.

The pH might be an important factor for the fungicidal activities of chlorination and/or acid treatment. In our study, pH values of conidia suspensions treated with free chlorine in presence of 100, 200, and 400 mg/*l* of phytic acid were 2.83, 2.68, and 2.58, respectively. In comparison with the condition treated with free chlorine only (Fig. 1), combined treatment with free chlorine and

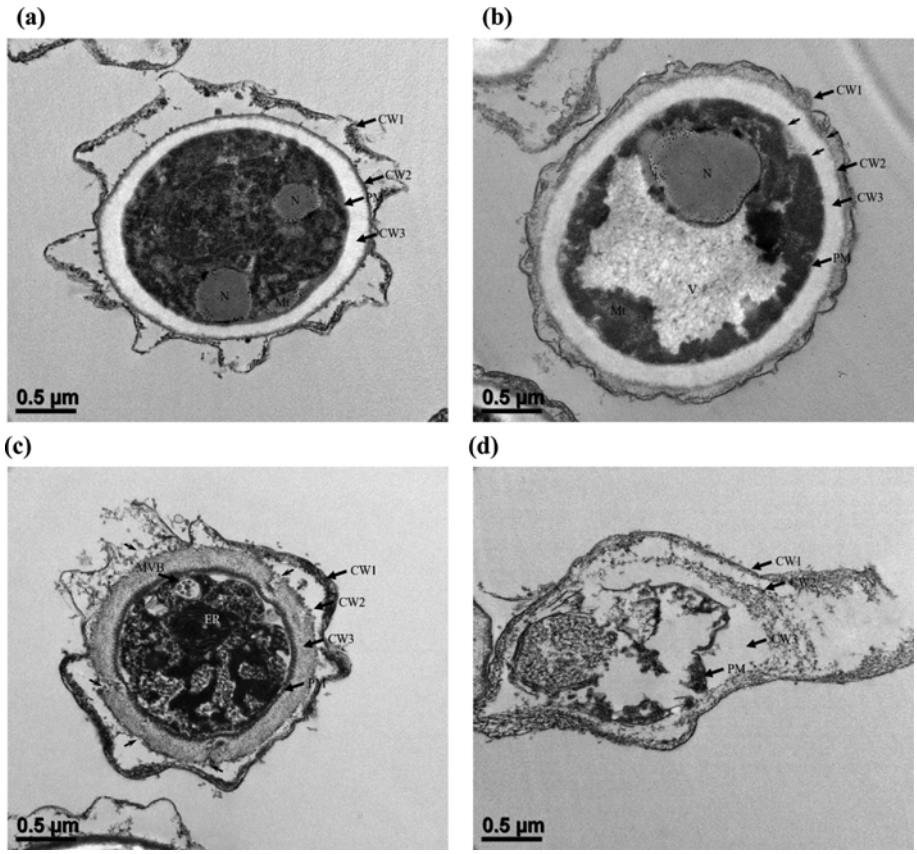

Fig. 3. Ultrastructure of *A. niger* conidia treated with free chlorine and phytic acid. Transmission electron micrographs of *A. niger* conidia untreated (a), treated with free chlorine (b), phytic acid (c), and mixture of free chlorine and phytic acid (d). The pH of the medium was not controlled. $[HOCl]_0$=7 mg/*l*, $[phytic\ acid]_0$=400 mg/*l*, treatment time=5 min, CW, cell wall; V, vacuole; N, nucleus; Mt, mitochondria; MVB, multivesicular body; ER, endoplasmic reticulum.

phytic acid showed a significant reduction in the pH of conidia suspension. Therefore, the high fungicidal activity with the combination of free chlorine with phytic acid could be attributed to the pH-lowering effect. To clarify this mechanism, we adjusted the pH of the conidia suspension treated with free chlorine alone to 2.5 and 3.0 with hydrochloric acid (HCl), resulting in the reduction in the viability of conidia to less than 0.75 log scale within 5 min (Fig. 2 subset). Noticeably, such inactivation level was not enough to account for the inactivation scale (i.e., 1.78 log; Fig. 2) observed by combinatorial treatments without adjusting pH. This implied that the high fungicidal activity with the combinatorial treatment could be associated with the pH-lowering effect along with an additional but yet to be known modes of action of phytic acid.

2. Ultrastructural Changes of *A. niger* Conidia Treated with Free Chlorine and Phytic Acid

To investigate the mode of action underlying the fungicidal effects of free chlorine and phytic acid against *A. niger*, we monitored the changes in the ultrastructure of conidia following treatment with the two chemicals using TEM (Fig. 3). Untreated *A. niger* conidia (Fig. 3(a)) exhibited intact cell walls and plasma membranes. The nucleus, mitochondria, and other organelles also displayed intactness and definite shapes. The conidia treated with free chlorine (Fig. 3(b)) also maintained the morphology of a normal cell wall and plasma membrane, but their nuclei and cell peripheries were less confined and appeared disrupted. HOCl is known to penetrate across the plasma membrane and cause damage to proteins and lipids, leading to the inhibition of cell metabolism in the cytosol and disruption of DNA integrity in the nucleus [14,37]. In general, sodium hypochlorite dominantly formed HOCl in the pH range of 4 to 8. In our study, the pH of the conidia suspension treated with free chlorine alone was 8.8. Under these circumstances, OCl^- functions more favorably than HOCl [34,35]. Regardless of the low amount, however, HOCl produced at pH 8.8 may have disrupted the ultrastructure of the cytosol. On the other hand, the conidia treated with phytic acid only (Fig. 3(c)) revealed intact cytosolic organelles but partially impaired cell walls and plasma membranes. The intactness of the conidia subcellular organelles supported the low fungicidal activities of phytic acid alone (Fig. 1). The combinatorial treatment of free chlorine and phytic acid induced the complete disruption and burst of *A. niger* conidia (Fig. 3(d)). Taken together, although the individual treatment with free chlorine and phytic acid was insufficient to cause any damage to conidia, each chemical partially disrupted *A. niger* conidia with a different potential mode of action. The combinatorial treatment with the two chemical agents caused death of *A. niger* conidia in a synergistic manner.

3. Membrane Disintegrity of *A. niger* Conidia Treated with Free Chlorine and Phytic Acid

To investigate how free chlorine and phytic acid inactivated *A. niger* conidia, we examined several parameters reflecting the membrane integrity of conidia. PI is a fluorescent dye that stains the nucleus by binding to the DNA but is often known to indicate membrane disintegration, owing to its inability to across the intact lipid bilayer [38,39]. In our study, the conidia treated with free chlorine and/or phytic acid showed higher fluorescence intensity than those from untreated condition (Fig. 4(a)). In particular, the com-

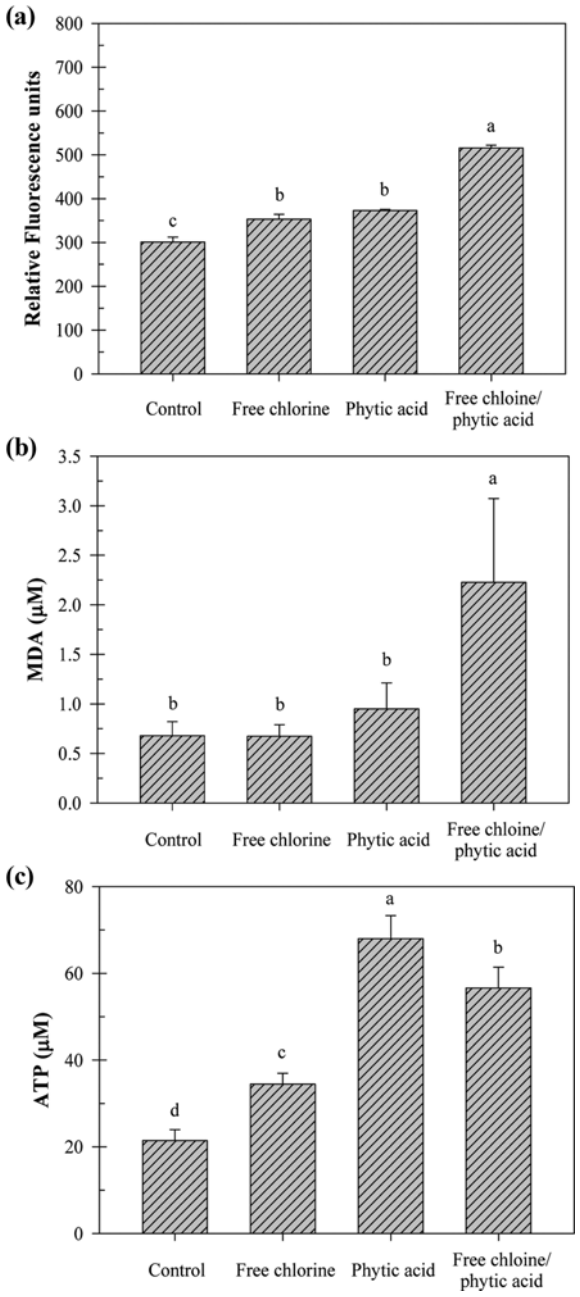

Fig. 4. Influence of free chlorine and phytic acid on the membrane integrity of *A. niger* conidia. (a) The comparison of fluorescence intensities of conidia stained with PI after treatment with free chlorine and phytic acid. (pH not controlled, $[HOCl]_0$= 7 mg/*l*, $[phytic\ acid]_0$=400 mg/*l*, treatment time=5 min, excitation wavelength=535 nm, emission wavelength=617 nm). (b) Level of MDA produced from the conidia treated with free chlorine and phytic acid. The concentration of total MDA produced from conidia was determined by monitoring adduct with TBA (pH not controlled, $[HOCl]_0$=7 mg/*l*, $[phytic\ acid]_0$= 400 mg/*l*, treatment time=5 min, absorbance evaluated at 532 nm). (c) Quantitation of extracellular ATP excreted from the conidia. The total concentration of ATP excreted from conidia was determined by measuring luminescence from the luciferase reaction requiring ATP as a cofactor. $^{a\text{-}d}$Mean values with the different letters are significantly different per Duncan's multiple comparison test ($p<0.05$).

binatorial treatment with free chlorine and phytic acid resulted in the highest fluorescence of PI.

Free chlorine and several acids are known to cause lipid peroxidation and disruption of proton gradient in the plasma membrane. To investigate the effects of free chlorine and phytic acid on the oxidation state of the plasma membrane of *A. niger* conidia, we examined the concentration of MDA produced from the conidia treated with the disinfectants (Fig. 4(b)). MDA level was similar between untreated and free chorine-treated samples, implying that free chlorine may not be effective enough under these conditions to cause lipid peroxidation. Treatment with phytic acid treated alone (400 mg/*l*) caused a slight increase in the level of MDA as compared with that reported for non-treated control and free chlorine-treated samples. However, we observed almost a twofold increase in the MDA level for the combinatorial treatment group as compared with other treatment groups. Although the impact on the redox state of the membrane relies on the dosage and time of chemical treatment, the individual treatment with free chlorine (7 mg/*l*) and phytic acid (400 mg/*l*) caused no detectable changes in lipid oxidation state. However, the combinatorial treatment with same dosages caused a significant level of lipid peroxidation.

The conidial leakage induced by free chlorine and phytic acid was examined by monitoring the concentration of ATP that potentially diffused from the organelles of *A. niger* conidia. In comparison with the untreated conidia, those treated with phytic acid alone and together with free chlorine showed a significant leakage of ATP (Fig. 4(c)). However, as shown in Fig. 1, phytic acid alone (~400 mg/*l*) had no effect on the inactivation of *A. niger* conidia within 5 min. Thus, our data suggest that phytic acid facilitated the leakage of conidia but was insufficient to kill the fungi. In previous reports, phytic acid similarly disrupted the bacterial membrane, although the cells remained viable [28,40]. Phytic acid, often called as myo-inositol hexakisphosphate (IP6), carries negative charges and acts as an efficient chelator. Phytic acid was bound to various divalent cations (e.g., Mg^{2+} and Ca^{2+}), which played a role in the maintenance of the stable structure of the fungal cell wall [28]. In addition, membrane integrity in bacteria was affected by ethylenediaminetetraacetic acid (EDTA) or citric acids [41-43], suggesting that chelating or binding of cations may interrupt with the process of maintenance or synthesis of cell wall and membrane integrity.

4. Combination Treatment of *A. niger*, *A. parasiticus*, and *A. flavus* Conidia with Free Chlorine and Phytic Acid

The fungicidal activity of free chlorine and phytic acid was further tested against other *Aspergillus* spp. such as *A. parasiticus* and *A. flavus* (Fig. 5). Within 5 min of treatment with free chlorine (7 mg/*l*) and phytic acid (400 mg/*l*), almost 99.9% (3 log scale) of *A. parasiticus* and *A. flavus* conidia were inactivated. Under same conditions, free chlorine and phytic acid reduced the conidial viability by up to 1 log scale. In terms of kinetics, $\overline{CT}$ values required to achieve 90% reduction (1 log scale) in conidial viability for *A. niger*, *A. parasiticus* and *A. flavus* were 22.1, 17.1, and 17.8 mg/*l*·min (Supplementary Information Fig. S2). Taken together, *A. parasiticus* and *A. flavus* were more sensitive to the combinatorial treatment of free chlorine and phytic acid than *A. niger*. These differences in sensitivities among *Aspergillus* spp. have been previously reported, and *A. niger* was recalcitrant to disinfection with free chlorine [14].

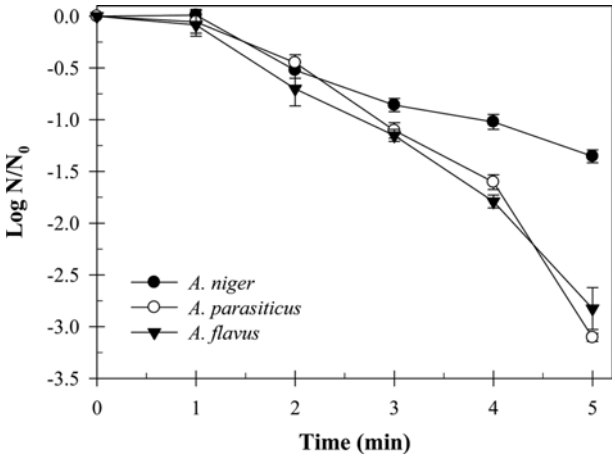

Fig. 5. Comparison of inactivation kinetics for conidia from *Aspergillus* spp. with free chlorine/phytic acid system (pH not controlled, $[HOCl]_0$=7 mg/*l*, $[\text{phytic acid}]_0$=200 mg/*l*).

Nonetheless, the combination of free chlorine and phytic acid efficiently inactivated the three major species of *Aspergillus*.

CONCLUSION

The establishment of efficient and safe disinfection agents and methods is critical to controlling fungal contamination in the environment. So far, chlorination has been widely used to disinfect water and surface, but toxicity, pH dependence, and by-products are the major disadvantages that restrict the practical application of this technique. Therefore, reducing usage or developing alternative disinfectants with chlorine species would be desirable to establish a good sanitization technology. In the present work, we exploited the safe nature of phytic acid as a food additive and its efficient germicidal activity against *Aspergillus* spp. The combination treatment with free chlorine and phytic acid without pH adjustment served as a rapid and an efficient inactivation method against selected *Aspergillus* spp. Therefore, the simple mixing of free chlorine and phytic acid could be a promising alternative as a disinfectant to conventional chemicals. Our study provides an insight into the mechanism underlying the effects of these chemicals on the inactivation of fungal species.

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SUPPORTING INFORMATION

Additional information as noted in the text. This information is available via the Internet at http://www.springer.com/chemistry/journal/11814.

REFERENCES

1. J. Guinea, T. Pelaez, L. Alcala and E. Bouza, *Med. Mycol.*, **44**, 349 (2006).
2. B. Mousavi, M. T. Hedayati, N. Hedayati, M. Ilkit and S. Syedmousavi, *Curr. Med. Mycol.*, **2**, 36 (2016).
3. H. Brahm and M. D. Segal, *N. Engl. J. Med.*, **360**, 1870 (2009).
4. P. Kumar, D. K. Mahato, M. Kamle, T. K. Mohanta and S. G. Kang, *Front. Microbiol.*, **7**, 2170 (2016).
5. L. Zhu, B. Zhang, Y. Dai, H. Li and W. Xu, *Toxins (Basel)*, **9**, 113 (2017).
6. X. Ma and K. Bibby, *Water Res.*, **120**, 265 (2017).
7. H. Nourmoradi, M. Nikaeen, C. R. Stensvold and H. Mirhendi, *Water Res.*, **46**, 5935 (2012).
8. M. Sokmen, I. Tatlidil, C. Breen, F. Clegg, C. K. Buruk, T. Sivlim and S. Akkan, *J. Hazard. Mater.*, **187**, 199 (2011).
9. K. C. Piacentini, G. D. Savi and V. M. Scussel, *Qual. Assur. Saf. Crop.*, **9**, 383 (2017).
10. R. Hassan, S. El-Kadi and M. Sand, *J. Adv. Biol.*, **2**, 1 (2015).
11. S. Fukuzaki, *Biocontrol. Sci.*, **11**, 147 (2006).
12. V. J. Pereira, R. Marques, M. Marques, M. J. Benoliel and M. T. Barreto Crespo, *Water Res.*, **47**, 517 (2013).
13. G. Wen, X. Xu, T. Huang, H. Zhu and J. Ma, *Water Res.*, **125**, 132 (2017).
14. M. Sisti, G. Brandi, M. De Santi, L. Rinaldi and G. F. Schiavano, *J. Water Health*, **10**, 11 (2012).
15. D. Kanzler, W. Buzina, A. Paulitsch, D. Haas, S. Platzer, E. Marth and F. Mascher, *Mycoses*, **51**, 165 (2008).
16. H. M. Al-Gabr, T. Zheng and X. Yu, *Sci. Total. Environ.*, **463-464**, 525 (2013).
17. M. N. Groot, T. Abee and H. van Bokhorst-van de Veen, *Food Microbiol.*, **81**, 108 (2019).
18. J. S. Piotrowski, H. Okada, F. Lu, S. C. Li, L. Hinchman, A. Ranjan, D. L. Smith, A. J. Higbee, A. Ulbrich, J. J. Coon, R. Deshpande, Y. V. Bukhman, S. McIlwain, I. M. Ong, C. L. Myers, C. Boone, R. Landick, J. Ralph, M. Kabbage and Y. Ohya, *Proc. Natl. Acad. Sci. USA*, **112**, E1490 (2015).
19. C. H. Pohl, J. L. F. Kock and V. S. Thibane, Antifungal free fatty acids: A review, Formatex, Badajoz (2011).
20. J. Yun and D. G. Lee, *FEMS Yeast Res.*, **16**, 1 (2016).
21. M. Petrovic, D. Bonvin, H. Hofmann and M. Mionic Ebersold, *Int. J. Mol. Sci.*, **19**, 1 (2018).
22. X. C. Li, M. R. Jacob, S. I. Khan, M. K. Ashfaq, K. S. Babu, A. K. Agarwal, H. N. Elsohly, S. P. Manly and A. M. Clark, *Antimicrob. Agents. Chemother.*, **52**, 2442 (2008).
23. M. Sakko, C. Moore, L. Novak-Frazer, V. Rautemaa, T. Sorsa, P. Hietala, A. Jarvinen, P. Bowyer, L. Tjaderhane and R. Rautemaa, *Mycoses*, **57**, 214 (2014).
24. S. Puvvada, P. Latha, K. B. Jayalakshmi and S. K. Arul, *Int. J. Appl. Dent. Sci.*, **3**, 19 (2017).
25. R. Nassar and M. Nassar, *Int. Arab. J. Antimicrob. Agents*, **6**, 1 (2016).
26. A. K. Yadav, P. Sirohi, S. Saraswat, M. Rani, M. P. Singh, S. Srivastava and N. K. Singh, *Curr. Microbiol.*, **75**, 849 (2018).
27. J. J. Hue, L. Li, Y. E. Lee, K. N. Lee, S. Y. Nam, Y. W. Yun, J. H. Jeong, S. H. Lee, H. S. Yoo and B. J. Lee, *J. Food Hyg. Saf.*, **22**, 37 (2007).
28. N. H. Kim and M. S. Rhee, *Appl. Environ. Microbiol.*, **82**, 1040 (2016).
29. M. Cho, H. Chung and J. Yoon, *Environ. Sci. Technol.*, **37**, 2134 (2003).
30. M. Cho, H. Kim, S. H. Cho and J. Yoon, *Ozone Sci. Eng.*, **25**, 251 (2003).
31. M. Cho, J. H. Kim and J. Yoon, *Water Res.*, **40**, 2911 (2006).
32. M. J. Karnovsky, *J. Cell Biol.*, **27**, A137 (1965).
33. L. K. Dhandole, Y. S. Seo, S. G. Kim, A. Kim, M. Cho and J. S. Jang, *Photochem. Photobiol. Sci.*, **18**, 1092 (2019).
34. M. D. Sobsey, T. Fuji and P. A. Shields, *Water Sci. Technol.*, **20**, 385 (1988).
35. A. M. Driedger, J. L. Rennecker and B. J. Mariñas, *Water Res.*, **34**, 3591 (2000).
36. M. Cho, V. Gandhi, T. M. Hwang, S. Lee and J. H. Kim, *Water Res.*, **45**, 1063 (2011).
37. C. L. Hawkins, D. I. Pattison and M. J. Davies, *Amino Acids*, **25**, 259 (2003).
38. P. Breeuwer and T. Abee, *Int. J. Food Microbiol.*, **55**, 193 (2000).
39. M. Berney, H. U. Weilenmann and T. Egli, *Microbiology*, **152**, 1719 (2006).
40. Q. Zhou, Y. Zhao, H. Dang, Y. Tang and B. Zhang, *J. Food Prot.*, **82**, 826 (2019).
41. L. Leive, *Biochem. Biophys. Res. Commun.*, **21**, 290 (1965).
42. M. Vaara, *Microbiol. Rev.*, **56**, 395 (1992).
43. I. M. Helander and T. Mattila-Sandholm, *J. Appl. Microbiol.*, **88**, 213 (2000).