J Food Sci Technol (July–August 2011) 48(4):515–519
DOI 10.1007/s13197-011-0268-5

SHORT COMMUNICATION

Inhibition effect of belzalkonium chloride treatment on growth of common food contaminating fungal species

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Revised: 11 May 2010 / Accepted: 12 May 2010 / Published online: 23 February 2011
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Abstract The improvement of disinfection applications for hard contact surfaces in food processing is critical for the control and prevention of disease-causing and food spoilage microorganisms. The objective in this study was to determine the efficiency of the antifungal agent benzalkonium chloride on growth and/or spore germination of postharvest fruit pathogenic fungi (*Aspergillus* spp., *Penicillum* spp., and *Alternae alternate*) in vitro. The benzalkonium chloride was found to be active against all fungal species but to a different extent. Addition of ethylenediamine-tetraacetic acid and its sodium salt increased the sensitivity of fungi to benzalkonium chloride. Thus, integrated washing and sanitizing with benzalkonium chloride or homologous surface active compounds combined with ethylenediamine-tetraacetic acid and its sodium salt is promising fungicide candidates for reducing fungal contamination of storage.

Keywords Fungi · Surfactant · Inhibition · Fungicide · Antimicrobial packaging

Introduction

Microorganisms (e.g., bacteria and molds) can form invisible biofilms on hard food contact surfaces. The need for an effective, persistent, and durable surface disinfectant sterilization mixture is felt in food industry for all food-product contact surfaces as well as for non-product surfaces, including food collection (e.g. equipment and utensils, refrigeration units), food processing (e.g. walls and ceilings in slaughterhouses), food packaging (e.g. ventilation, air conditioning, packaging apparatus), and food distribution (e.g. preparation surfaces in restaurants and food stores) (Chapman 2003; Velanquez 2009; Tanner 1989; Fu et al. 2007). The most commonly used chemicals approved by Food and Drug Administration (FDA) for use as food-contact surface sanitizers are chlorine, quaternary ammonium compounds, and hydrogen peroxide (Velanquez et al. 2009; Fu et al. 2007). In food-handling operations, they are used as rinses, sprayed onto surfaces, or circulated through equipment with Cleaning-In-Place operations (Chapman 2003). Chlorine, in its various forms, is the most commonly used sanitizer in food processing and handling applications, however, the major disadvantage for chlorine compounds is corrosiveness to many metal surfaces (especially at higher temperatures) and increasing health, safety concerns about the formation of carcinogenic chlorinated organic compounds and the emergence of new resistant pathogens (Singh et al. 2002). Hydrogen peroxide, whereas, has found only limited application in the food industry, while commonly used in the medical field. Therefore, it makes necessary to seek new surface sanitizers for reducing the microbial load.

Postharvest diseases caused by in particular acid-tolerant fungi result in tremendous losses in quantity and quality of fresh produce during storage. Fungi cause spoilage, produce off flavors and odors, discoloration of the product, and sometimes even cause human diseases (Basaran 2010). Food industry continuously seeks new surface sanitizers for

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effective removal of storage fungi. An ideal surface sanitizer should have a sufficient level of antimicrobial activity, no or a negligible effect on the organoleptic quality of the product, and there should be no or little health threating residue left on the product. Benzalkonium chloride (BC) (quaternary ammonium compound) is an odorless and colorless disinfectant commonly used by the food industry for surface disinfect against a number of microorganisms (Cabo et al. 2009; Sutterlin et al. 2008). The BC is active at low concentrations, considered as fast-acting disinfectants, it is slightly toxic to mammals (LD50= 430 mg/kg-bw), and under recommended usage and precautions, BC pose little toxicity or safety risks (Tanner 1989).

In recent studies, among a number of antifungal disinfectants tested, quaternary ammonium compounds showed the highest efficacy against Fusarium infection on banana plants at an exposure time of 30 s; and they were more effective as compared to chlorine bleach and thus recommended to replace the sterilants currently being used (Moore et al. 1999; Nel et al. 2007). Furthermore, more recently a number of anionic, cationic and non-ionic surfactants were tested for antifungal activities in surfactant-coated poly ethylcyanoacrylate nanoparticles (McCarron et al. 2007).

Aspergillus and *Penicillium* can cause postharvest decay in fruits and vegetables from surface-borne contamination that infects the fruit through injuries or micro wounds. These fungi are also able to infect stored fruits by mycelial spread from infected fruit to adjacent healthy fruit, causing sections of decay in bulk.

The main objective of this study was to examine BC with different ethylenediamine-tetraacetic acid (EDTA) and Na_2EDTA formulations for its ability to inhibit germination of conidia and mycelial growth of common food contaminating fungal species (*Penicillium* spp., *Aspergillus* spp. and *Alternaria alternate)*.

Materials methods

Fungal strains and growth conditions

Seven pathogenic fungal species were isolated from naturally infected rotting fruits in the Department of Food Engineering Laboratory (Isparta). To isolate pure culture, suspensions were spread on Potato Dextrose Agar (PDA) medium and individual single-spore colonies were picked, and the isolates were identified based on their respective morphological characteristics conidia, as well as their growth features as described earlier (Ozcelik 2010). All seven isolates (*Penicillium expansum*, *P. italicum*, *P. piceum*, *P. digitatum, P. allii*, *Aspergillus niger* and *Alternaria alternata*) were maintained on PDA at 4 °C. Fungi culturing conditions and spore preparation were performed as described earlier (Basaran 2010). Five ml of 0.05% (w/v) sterile peptone water was added onto agar plates and spores were rubbed from the agar surface with a sterile glass rod. The high-density spore suspension was passed through two layers of cheese cloth and spores were used for fungal studies.

Bilayer agar-well antifungal activity assays

BC was purchased from Sandoz (Istanbul, Turkey), EDTA, and Na_2EDTA were obtained from Merck (Germany). The antifungal activity of the compounds was determined using the bilayer agar-well diffusion method as described earlier (Basaran 2010). A solution of an anionic dye Commassie Brilliant Blue (CBB) (0.06%) was thoroughly mixed with agar (6.25%) and the mixture poured on 90 mm diameter glass Petri dishes in 2 mm thickness. After solidification, the dyed agar was punched in diameter of 3 mm, various concentrations of each test compound and mixtures (BC, EDTA and Na_2EDTA) were dissolved in water and loaded into 3 mm diameter hole and left for adsorption. Fungal spores of seven species (10^4 CFU ml^{-1}) were suspended in sterile dH_20 and this suspension was mixed with 10 ml of sterile PDA at 45 °C and poured onto the surface of the chemically loaded–colored agar. The inoculated plates were then incubated at 23–25 °C and 70% (RH) controlled incubator for 72 h in order to allow uniform and sufficient fungal growth. The susceptibility of the fungi to the test compounds was determined by the formation of an inhibitory zone (diameters in mm) surrounding the chemical loaded wells (Basaran 2010). Six replicates of three plates were used for each concentration, and the results are presented as mean values. A well containing only dH_2O was used as negative control.

Statistical analysis

Analysis of variance and Duncan's multiple range tests were performed to analyze the data using the SAS program (SAS Institute, Inc., NC, USA).

Results and discussion

Inhibition effect of BC on *Aspergillus* spp., *Penicillium* ssp. and *Alternaria alternata* in vitro plate screen

Black spot caused by *A. alternata* and spoilage caused by *P. digitatum, P. italicum*, *P. expansum* and *Aspergillus* are

economically important postharvest diseases of a variety of fruits. *A. alternata* penetrates during fruit growth, followed by quiescence until the fruit is harvested and develops during long storage periods (Prusky et al. 1999; Liu et al. 2007a, b). Furthermore, patulin is a very toxic mycotoxin associated with a wide range of *Penicillium* spp. (e.g., *P. expansum*) and has strict limits in international regulations (Paterson 2007). Therefore, prevention of development of these fungal species is of critical importance for extending storage and the final quality of the fresh produce.

The sensitivity of these presently investigated fungi was species dependent, and therefore identical mixtures had different results on fungal species. Among the seven pathogens examined, *A. niger* was the most sensitive to BC, whereas *Penicillium* spp. (*P. expansum, P. italicum*, *P. piceum*, *P. digitatum, P. allii*) exhibited the least sensitivity (Fig. 1). The obtained result was also in agreement with the earlier study that BC with a concentration of 0.005 mg ml^{-1} showed direct fungi toxicity and significantly inhibited mycelial growth of *A. parasiticus strain* 2929 in vitro (Basaran 2010). One−way ANOVAs indicated that in general, BC+ EDTA or BC+ Na_2EDTA was superior to BC alone, showing the benefit of surfactant effect except against *A. alternate*. BC combined with EDTA or Na_2EDTA mixture was more effective against conidial germination of *A. niger* and *A. alternata* than against that of *Penicillium* spp. EDTA, a chelator, demonstrates antimicrobial effect by limiting availability of cations and destabilize the cellular membrane (Economou et al. 2009). In vitro susceptibility of selected fungal species to various BC mixtures is summarized in Table 1. For the same fungal pathogen, the spore germination initiation was more sensitive than hyphal extension. There have been several previous effectiveness assessments undertaken on this substance by several studies. Romanova et al. (2007) reported disinfectant effect of BC against *Listeria monocytogenes.* When the effectiveness of BC, and ozonized

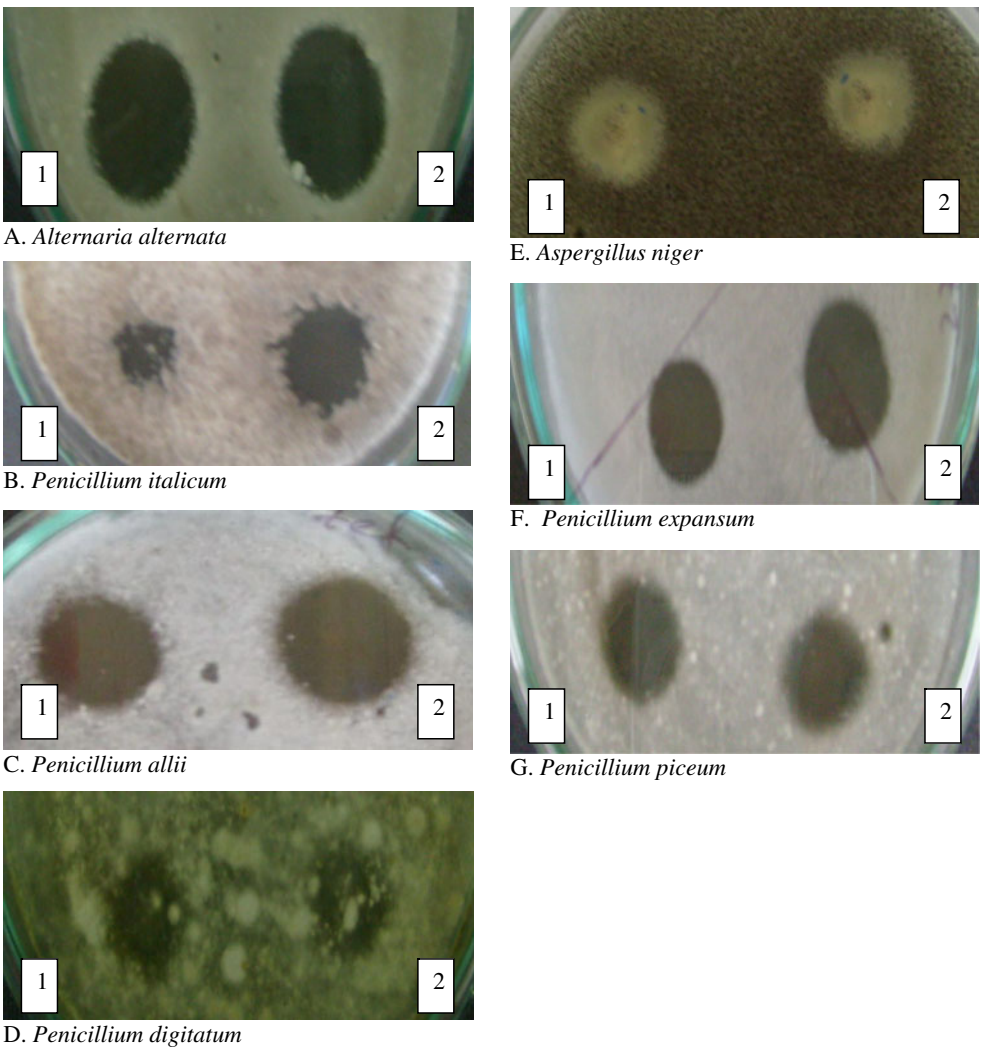

Fig. 1 Chemical inhibition zones of Benzalkonium chloride +EDTA (0.5 mg+0.05 mg/ml) (1) and Benzalkonium chloride +Na_2EDTA (0.5+0.05) treatment (2) (**a**. *Alternaria alternata*; **b**. *Penicillium italicum;* **c**. *Penicillium allii*; **d**. *Penicillium digitatum;* **e**. *Aspergillus niger*; **f**. *Penicillium expansum;* **g**. *Penicillium piceum*)

Table 1 In vitro activity of combined treatment of Benzalkonium chloride, EDTA and Na_2EDTA combinations against various pathogenic fungi

Fungal species	Treatment and inhibition zone (mm±SD) on PDA								
	EDTA 0.05 (g/ml)	Na_2EDTA 0.05 (g/ml)	BC 1 (mg/ml)	BC 0.50 (mg/ml)	BC 0.10 (mg/ml)	BC+EDTA 0.50+0.05 (mg +g/ml)	BC+EDTA 0.10+0.01 (mg +g/ml)	BC+Na_2EDTA 0.50+0.05 (mg +g/ml)	BC+Na_2EDTA 0.10+0.01 (mg +g/ml)
P.expansum	5.0±0.73[a]	35.0±0.51a	18.5±0.60[a]	14.3±0.30[a]	11.0±0.33[a]	17.5±0.19[a]	12.5±0.71[a]	17.0±0.15[a]	13.0±0.76[a]
P. italicum	5.0±0.59[a]	34.5±0.82a	16.0±0.54[b]	13.0±0.24[a]	10.0±0.41[a]	15.3±0.45[b]	13.0±0.82[a]	17.8±0.31[a]	12.0±0.54[a]
P. piceum	nd	40.5±0.73b	17.0±0.74[b]	10.0±0.29[b]	11.8±0.50[a]	15.0±0.54[b]	12.0±0.94[a]	15.3±0.23[a]	12.0±0.59[a]
P. digitatum	nd	37.0±0.95a	14.0±0.60[c]	14.0±0.54[a]	12.2±0.30[a]	15.3±0.34[b]	11.5±0.54[a]	14.0±0.24[b]	11.0±0.67[a]
P. allii	7.0±0.70[b]	30.0±0.97c	17.0±0.54[b]	13.0±0.23[a]	nd	15.0±0.67[b]	nd	15.0±0.14[b]	nd
Aspergillus niger	nd	30.0±0.65c	12.0±0.67[d]	6.0±0.36[c]	nd	10.0±0.54[c]	nd	10.0±0.11[c]	nd
Alternaria alternata	nd	25.0±0.76d	14.0±0.54[c]	14.0±0.45[a]	nd	14.0±0.52[b]	nd	14.5±0.6[b]	nd

(IZ:Inhibition zone was loaded with 50 µl compounds, nd:no zone formation. Each value represents the mean value of five replicate S.D. Means within treatments in a column not sharing a common superscript letter are significantly ($p \leq 0.05$) different)

water against *Staphylococcus aureus* was compared, BC was more effective and in less than 5 min at a concentration for <50 mg l^{-1} (Cabo et al. 2009).

BC exerted its antifungal effect by influencing various aspects of fungal development that included: (1) retarding conidium' germination initiation and reducing spore germination rate and (2) suppressing/slowing hyphial elongation of mycelia and colony growth. All these effects would be enormously functional in controlling fungal development. For instance, both conidia germination of fungi on plates and in liquid culture were greatly suppressed by BC+ Na_2EDTA or EDTA even at the lowest concentration of 0.05 mg ml^{-1}. These observations were in accordance with Liu et al. (2007b) who observed similar results with *P. digitatum*, *P. italicum*, *P. expansum*, *Botrytis cinerea* and *Monilinia fructicola*, when Aureobasidin A, an antifungal cyclic depsipeptide was applied (Liu et al. 2007b). To test whether BC could kill conidia or just delay the germination initiation, the time courses of inhibitory effect of BC at various concentrations were incubated up to 10 days and then plated on PDA. No germination occurred after 10 days of incubation, this concluded that BC effective for extended period of time. BC at ≤ 0.01 mg ml^{-1} killed these spores rather than delaying the conidial germination initiation, especially in the cases of the fungi of *P. marneffei*, *P. piceum*, *P. expansum* and *P. italicum*. In some cases, sub-inhibitory preservative concentrations could enhance fungal growth as seen in earlier studies. However, no stimulating effect on fungal growth was observed at any of the conditions tested when BC was added at the lowest concentration (0.005 mg ml^{-1}) (data not shown).

The effect of BC on the morphology of these pathogens was examined by comparison the microscopic morphologies of mycelia on BC-free PDA with those on BC containing PDA. To assess the effect of BC on the fungal morphology, fungi were exposed for different exposure times to microbial inhibition concentrations and examined them under a light microscope and scanning electron microscopes. Significant structural alterations in fungal hyphae were observed when the BC concentrations were higher than 0.05 mg ml^{-1} for *A. parasiticus* contaminated on hazelnuts as viewed under electron microscope (Basaran 2010). The mode of action of BC against fungal cells involves a general perturbation of lipid bilayer membranes. In fungi, specifically, surfactants form micelles with ergosterol molecules present in the phospholipids of plasma bilayer membrane and this bonding causes structural damage, the higher membrane permeability and loss of electrolytes and other cytoplasmatic components and as a consequence of which, the fungal cells die (Zhou et al. 2009). While, there is no evidence to support whether BC interferes with intracellular functions yet, additional experimentation will be needed to further elucidate the mode of BC activities.

Conclusion

In summary, storage infections caused by fungal pathogens are responsible for substantial postharvest loses, and therefore, to extend the storage life, sanitizing treatments should be integrated to current applications of modified atmospheres and storage at proper temperature (Liu et al. 2007a, b). The present study reveals that BC sanitizing treatment effective in reducing/suppressing fungal growth and delaying decay, however the effect depends on the fungal species.

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