Curvularia Haloperoxidase: Antimicrobial Activity and Potential Application as a Surface Disinfectant

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A presumed antimicrobial enzyme system, the *Curvularia* haloperoxidase system, was examined with the aim of evaluating its potential as a sanitizing agent. In the presence of hydrogen peroxide, *Curvularia* haloperoxidase facilitates the oxidation of halides, such as chloride, bromide, and iodide, to antimicrobial compounds. The *Curvularia* haloperoxidase system caused several-log-unit reductions in counts of bacteria (*Pseudomonas* spp., *Escherichia coli*, *Serratia marcescens*, *Aeromonas salmonicida*, *Shewanella putrefaciens*, *Staphylococcus epidermidis*, and *Listeria monocytogenes*), yeasts (*Candida* sp. and *Rhodotorula* sp.), and filamentous fungi (*Aspergillus niger*, *Aspergillus tubigensis*, *Aspergillus versicolor*, *Fusarium oxysporum*, *Penicillium chrysogenum*, and *Penicillium paxilli*) cultured in suspension. Also, bacteria adhering to the surfaces of contact lenses were killed. The numbers of *S. marcescens* and *S. epidermidis* cells adhering to contact lenses were reduced from 4.0 and 4.9 log CFU to 1.2 and 2.7 log CFU, respectively, after treatment with the *Curvularia* haloperoxidase system. The killing effect of the *Curvularia* haloperoxidase system was rapid, and 10^6 CFU of *E. coli* cells/ml were eliminated within 10 min of treatment. Furthermore, the antimicrobial effect was short lived, causing no antibacterial effect against *E. coli* 10 min after the system was mixed. Bovine serum albumin (1%) and alginate (1%) inhibited the antimicrobial activity of the *Curvularia* haloperoxidase system, whereas glucose and Tween 20 did not affect its activity. In conclusion, the *Curvularia* haloperoxidase system is an effective sanitizing system and has the potential for a vast range of applications, for instance, for disinfection of contact lenses or medical devices.

Bacteria and other microorganisms attach readily to surfaces in both natural and man-made ecosystems. If they are left undisturbed, and if nutrients are supplied, biofilms may form (9, 10). Microorganisms attached as single cells or in monolayers and more mature microbial biofilms are the cause of problems in several areas, for example, in the food industry, where bacteria colonize processing equipment and subsequently contaminate (or recontaminate) the products (22). There are reports of clones of pathogenic bacteria, such as *Listeria monocytogenes*, which have persisted in food-processing environments for several years (12, 27). Even more severe problems are seen in the medical area, where surface-adhered microorganisms cause infections from contaminated contact lenses (42) or medical devices, such as catheters or ear tubing (4, 28, 34). Effective cleaning and surface disinfection is essential to control these hazards.

A vast range of agents or compounds that can inactivate microorganisms are therefore used across many sectors, from the pharmaceutical industry to the food industry to water distribution lines. Such sanitizers are used both for surface disinfection and for water purification (15, 21, 25). Due to the environmental impact, there is a growing concern about the disposal of sanitizers and chemically synthesized disinfectants.

This has led to increased research efforts to identify and evaluate natural antimicrobial compounds as novel sanitizers (14, 30). Natural antimicrobial systems are widespread, occurring in mammals, cold-blooded animals, plants, and microorganisms (3). Examples of such compounds are iron-chelating substances, such as lactoferrin (41), small basic peptides (16, 17, 20), and enzymes with antimicrobial activity. Examples of the last are lactoperoxidase, secreted from various mammalian glands (14, 43), and lysozyme, found in body fluids, such as tears, saliva, and human milk (26).

Peroxidases other than lactoperoxidase may also exhibit antimicrobial activity. Thus, preliminary studies have demonstrated that a haloperoxidase isolated from the filamentous fungus *Curvularia verruculosa* has antimicrobial activity (18). *Curvularia* haloperoxidase (Novozymes A/S, Bagsværd, Denmark), examined in this study, is not commercially available but is produced by recombinant technology that allows production on an industrial scale. The enzyme oxidizes halides, such as bromide, chloride, and iodide, in the presence of hydrogen peroxide, and it is believed, albeit not experimentally verified, that reactive oxygen species with antimicrobial effects are produced. Commercial sanitizers based on, for example, hydrogen peroxide alone have several side effects, such as corrosion. The *Curvularia* haloperoxidase system uses 100-fold-lower concentrations of H_2O_2 and would therefore be expected to be less corrosive. The present study was undertaken with the aim of evaluating the *Curvularia* haloperoxidase system as a sanitizing agent. The stability of the system, as well as effects of potential interfering agents, were studied to evaluate potential areas of application. Finally, the system was tested on bacteria

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TABLE 1. Log CFU of selected bacteria, yeasts, and filamentous fungi ml^{-1} before and after treatment with
Curvularia haloperoxidase system (enzyme, halide, and H_2O_2)

Organism	Species	Strain no./origin or reference	Treatment			Log CFU $ml^{-1} \pm$ SD	
			Enzyme (mg liter^{-1})	H_2O_2 (mM)	Halide (mM)	Before treatment	After treatment[e]
Gram-negative bacteria	P. aeruginosa	PaO1	0.125	0.250	0.667[c]	6.1 ± 0.0	4.4 ± 0.1***
	P. putida	2442	0.125	0.250	0.667[c]	5.1 ± 0.0	3.1 ± 0.2***
	P. putida	2442	1.000	1.000	5.000[c]	5.2 ± 0.1	<1.0***
	P. putida	DOT-T1E (39)	0.050	0.100	0.250[c]	5.8 ± 0.0	2.6 ± 0.3**
	P. putida	DOT-T1E-PS30 (39)	0.050	0.100	0.250[c]	6.0 ± 0.0	3.7 ± 0.0***
	P. putida	DOT-T1E-PS34 (39)	0.050	0.100	0.250[c]	6.1 ± 0.2	3.8 ± 0.0**
	P. putida	DOT-T1E-82 (39)	0.050	0.100	0.250[c]	5.8 ± 0.2	3.0 ± 0.5**
	E. coli	ATCC 23282	0.125	0.250	0.667[c]	5.7 ± 0.0	1.2 ± 0.2***
	S. marcescens	ATCC 14041	0.125	0.250	0.667[c]	6.2 ± 0.0	<1.0***
	A. salmonicida[a]	Jno 3175/88	0.125	0.250	0.667[c]	5.7 ± 0.0	<1.0***
	S. putrefaciens[a]	A6/isolated from whole, gutted cod; 0°C	0.125	0.250	0.667[c]	5.5 ± 0.0	<1.0***
Gram-positive bacteria	S. epidermidis	DSM 20042	0.125	0.250	0.667[c]	5.6 ± 0.0	<1.0***
	L. monocytogenes	O32 (Campden Food and Drink Research Association)/Scott A; human case	0.125	0.250	0.667[c]	6.2 ± 0.0	1.9 ± 1.2*
Yeasts	Rhodotorula sp.[a]	B33-1/fish-processing factory	1.000	1.000	5.000[c]	4.4 ± 0.0	4.4 ± 0.0[NS]
	Rhodotorula sp.[a]	B33-1/fish-processing factory	0.100	0.100	0.100[c]	4.4 ± 0.1	<1.0***
	Candida sp.[a]	B114-3/fish-processing factory	1.000	1.000	5.000[c]	4.3 ± 0.0	4.2 ± 0.0*
	Candida sp.[a]	B114-3/fish-processing factory	0.100	0.100	0.100[d]	4.4 ± 0.0	<1.0***
Filamentous fungi	A. niger[b]	IBT 14491/Lechuigillia cave in New Mexico	1.000	1.000	5.000[c]	5.0 ± 0.1	4.9 ± 0.0[NS]
	A. niger[b]	IBT 14491/Lechuigillia cave in New Mexico	0.050	0.050	0.050[d]	5.4 ± 0.1	4.9 ± 0.1**
	A. niger[b]	IBT 3250/fermentation plant	0.050	0.050	0.050[d]	5.1 ± 0.1	2.5 ± 0.1***
	A. tubigensis[b]	IBT 22444/grape	0.050	0.050	0.050[d]	5.0 ± 0.1	1.9 ± 0.1***
	A. tubigensis[b]	IBT 4579	0.050	0.050	0.050[d]	5.5 ± 0.1	3.1 ± 0.0***
	A. versicolor[b]	IBT 23325/salt-evaporating plant	0.050	0.050	0.050[d]	5.1 ± 0.0	5.2 ± 0.0[NS]
	F. oxysporum[b]	IBT 2478/37°C hot water	1.000	1.000	5.000[c]	5.3 ± 0.1	5.3 ± 0.1[NS]
	F. oxysporum[b]	IBT 2478/37°C hot water	0.050	0.050	0.050[d]	5.3 ± 0.0	<1.0***
	P. chrysogenum[b]	IBT 23469/soil	0.050	0.050	0.050[d]	5.0 ± 0.1	<1.0***
	P. paxilli[b]	IBT 16202/optical instrument in Panama	0.050	0.050	0.050[d]	5.2 ± 0.1	<1.0***

[a] From culture collection at Danish Institute for Fisheries Research, Department of Seafood Research, DK-2800 Kgs. Lyngby, Denmark.
[b] From culture collection at BioCentrum, Section of Food Biotechnology and Mycology, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark.
[c] Bromide used as halide.
[d] Iodide used as halide.
[e] *, $P < 0.05$ comparing levels before and after treatment using Student's t test; **, $P < 0.01$ comparing levels before and after treatment using Student's t test; ***, $P < 0.001$ comparing levels before and after treatment using Student's t test; NS, nonsignificant comparing levels before and after treatment using Student's t test.

adhering to contact lenses to determine its potential as a surface disinfectant.

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MATERIALS AND METHODS

Microorganisms and growth conditions. The microorganisms (with strain numbers and origins) used in this study are listed in Table 1. Among the tested strains were three *Pseudomonas putida* efflux pump mutants (DOT-T1E-PS30, DOT-T1E-PS34, and DOT-T1E-82) (39). DOT-T1E-PS30 was a double mutant and lacked functional TtgDEF and TtgGHI pumps, while DOT-T1E-PS34 and DOT-T1E-82 were triple mutants lacking functional TtgABC, TtgDEF, and TtgGHI pumps. The sensitivities of these mutants to the *Curvularia* haloperoxidase system were compared to that of the wild-type parent strain, DOT-T1E. All microorganisms were cultured at 25°C. Gram-negative bacteria were cultured in tryptone soy broth (Oxoid CM129), while gram-positive bacteria were cultured in brain heart infusion (BHI) (Oxoid CM225) for 24 h. As an exception, *Listeria monocytogenes* was cultured at 37°C in BHI supplemented with 3 g of glucose liter^{-1}. Yeasts were grown for 48 h in yeast extract-peptone-dextrose (YPD) broth (containing [per liter] 5.0 g of yeast extract, 10.0 g of peptone, and 10.0 g of glucose, pH 5.5). Different media were used for sporulation of filamentous fungi. *Aspergillus* spp. and *Penicillium* spp. were grown for 5 to 10 days on Czapek yeast extract agar (35) and thereafter for 4 to 7 days on malt extract agar (MEA) modified after the method of Blakeslee (5). MEA was modified by adding (per liter of MEA) 1 ml of trace metal solution consisting of 10 g of $ZnSO_4 \cdot 7H_2O$ (Merck 8883) liter^{-1} and 5 g of $CuSO_4 \cdot 5H_2O$ (Merck 2790) liter^{-1}. *Fusarium*

sp. was grown for 7 and then for 8 days on Specieller Nährstoffarmer agar (31) under UV light.

Harvesting of cells and conidia. Bacteria and yeast cells were harvested (3,360 × g; 10 min), washed once in 0.05 M HEPES (Sigma H-3375; pH 7) or 0.05 M MES (morpholineethanesulfonic acid) (Sigma M-8258; pH 6) buffer and resuspended in HEPES or MES buffer. HEPES and MES buffer were used for evaluating the microbiocidal effect of *Curvularia* haloperoxidase with bromide and iodide, respectively, as the halide. Conidia from filamentous fungi on MEA and Specieller Nährstoffarmer agar plates were suspended in MES buffer, and hyphae were removed by filtering the suspension through sterile glass wool.

Enumeration of microorganisms. Plate counting was used to determine cell numbers following exposure to the *Curvularia* haloperoxidase system. Tenfold serial dilutions were prepared in sterile physiological saline with 0.1% peptone. To inhibit any carryover of the enzyme antimicrobial effect, the first 10-fold dilution was prepared in sterile physiological saline with 0.1% peptone and 0.5% bovine serum albumin (BSA) (Sigma A-7906), since BSA abolished the antimicrobial effect (see data below). Appropriate dilutions were surface plated, and all plates were incubated at 25°C. Gram-negative bacteria, gram-positive bacteria, and yeasts were enumerated on tryptone soy agar (Oxoid CM131), BHI agar (BHI broth with 15 g of agar liter^{-1}), and YPD agar (containing [per liter] 5.0 g of yeast extract, 10.0 g of peptone, 10.0 g of glucose, and 20 g of agar, pH 5.5), respectively. BHI agar supplemented with glucose (3 g liter^{-1}) was used for enumeration of *L. monocytogenes* cells, and plates were incubated at 37°C. Tryptone soy agar, BHI agar, and YPD agar plates were incubated for 2 to 3 days. Conidia of filamentous fungi were plated on MEA and incubated for 4 to 5 days.

Curvularia haloperoxidase antimicrobial effect. Harvested bacteria and yeast cells were diluted to an optical density of 0.4 at 450 nm and diluted 100 times in HEPES or MES buffer, yielding bacterial counts of ~10^6 CFU ml^{-1} and yeast

counts of 10^4 CFU ml^{-1}. The conidium solution was diluted to ~5 × 10^5 conidia ml^{-1} in HEPES or MES buffer. Separate solutions of *Curvularia* haloperoxidase, hydrogen peroxide (Merck 1.07209), and halide in the form of KBr (Sigma P-9881) or KI (Merck 1.05043) were made six times stronger than the final concentration used in the experiment. Stock solutions of *Curvularia* haloperoxidase and halide were prepared in HEPES when bromide was used as the halide or in MES buffer when iodide was used. The hydrogen peroxide solution was mixed in Milli-Q water. Volumes of 200 µl of cell or conidium suspension, 100 µl of *Curvularia* haloperoxidase, 100 µl of hydrogen peroxide, 100 µl of halide, and 100 µl of buffer were mixed in a sterile Eppendorf tube. The final concentrations varied between 0.125 and 1.000 mg of enzyme liter^{-1}, 0.250 and 1.000 mM hydrogen peroxide, 0.667 and 5 mM bromide, and 0.050 and 0.100 mM iodide. All enzyme experiments were performed at 25°C, because disinfection of contact lenses is used in this study as an example of surface disinfection, and contact lenses are disinfected at room temperature. Experiments were performed in duplicate, counts of bacteria were log transformed, and levels before and after treatment were compared using Student's *t* test.

Four different kinds of experiments were performed with *Curvularia* haloperoxidase to investigate (i) the antimicrobial spectrum, (ii) killing kinetics, (iii) the stability of the antimicrobial effect, and (iv) the effects of interfering compounds. These experiments were performed as follows.

(i) **Antimicrobial spectrum.** Suspensions of bacteria, yeasts, and filamentous fungi were incubated with the *Curvularia* haloperoxidase system for 20 min at 25°C under agitation (270 rpm), and cell numbers were determined by plate counting.

(ii) **Killing kinetics.** The total volume of enzyme, hydrogen peroxide, halide, and cell suspension was increased to 10 ml by mixing 3.34 ml of cell suspension, 1.66 ml of hydrogen peroxide solution, and 5.0 ml of combined enzyme and bromide solution. The hydrogen peroxide solution was prepared in Milli-Q water at six times the concentration used in the final mixture, and the combined enzyme and bromide solution was prepared in HEPES buffer at two times the concentration used in the final mixture. Suspensions of cells, hydrogen peroxide, and bromide plus enzyme were mixed in a 25-ml glass flask and incubated at 25°C under agitation (200 rpm). Samples were withdrawn every 2 to 5 min to determine cell numbers.

(iii) **Stability of antimicrobial effect.** *Curvularia* haloperoxidase, bromide, and hydrogen peroxide were mixed and left at room temperature. At intervals between 0 and 45 min after the components were mixed, cells of *Escherichia coli* suspended in HEPES were added, and the mixture was incubated for 20 min at 25°C under agitation (270 rpm). Cell numbers were determined by plate counting.

(iv) **Interfering compounds.** The following suspensions were prepared in Milli-Q water: 6% BSA (Sigma A-7906), 6% alginic acid (Fluka 05550), 0.6% gum xanthan (Sigma G-1253), 6% Tween 20 (Merck 8.22184), and 6% glucose (Merck 1.08337). A volume of 100 µl of one of these suspensions or dilutions thereof was added to 200 µl of *E. coli* cell suspension, 100 µl of *Curvularia* haloperoxidase solution, 100 µl of hydrogen peroxide solution, and 100 µl of bromide solution. Cell numbers were measured by plate counting 20 min after mixing the ingredients.

Disinfection of contact lenses. Cells of *Serratia marcescens* or *Staphylococcus epidermidis* were harvested as described above and suspended in 10 ml of HEPES buffer to ~10^6 CFU ml^{-1}. The bacteria were allowed to attach by immersing contact lenses (73% water, group II, nonionic; sterile UV multifocal; Rythmic, Essilor, France) in the bacterial suspension for 5 h at 25°C with agitation (125 rpm). The lenses were washed for 1 min in 10 ml of HEPES after exposure to the cell suspension to remove loosely attached cells. Thereafter, the lenses were incubated with the *Curvularia* haloperoxidase system or the constituents of the *Curvularia* haloperoxidase system for 20 min at 25°C without agitation. The proportions of enzyme-H$_2$O$_2$-bromide-HEPES were 1:1:1:7 ml. Solutions of enzyme, hydrogen peroxide, and bromide were therefore prepared 10 times stronger than the desired final concentration in the mixture. The final concentrations used were 0.125 mg of enzyme liter^{-1}, 0.667 mM bromide, and 0.250 mM hydrogen peroxide. For control experiments, the volume of the omitted constituents was replaced with HEPES buffer. The numbers of attached live cells on the lenses were determined after treatment by using indirect conductance measurements (2, 6, 11, 19, 33) with the Flexi 2000 instrument (Malthus Instruments Ltd., West Sussex, United Kingdom). In brief, the lenses were placed in Malthus glass tubes containing 3 ml of tryptone soy broth, and an insert with 0.5 ml of 0.1 N KOH was placed in each tube. Electrodes were immersed in the KOH solution, and the glass tubes were incubated in a water bath at 25°C. The conductance of the KOH solution was measured continuously, and detection times, e.g., the time from the start of the experiment until a significant change occurred in the conductance, were registered. Multiplication of microbial cells on

the contact lenses results in CO$_2$ development, and as CO$_2$ is absorbed in the KOH, conductance decreases. The time from the start of the measurement until a significant change occurs in conductance is inversely linearly proportional to the initial number of cells. A standard curve relating detection times to cell numbers was prepared for each of the two bacteria from a 10-fold dilution series of the bacterial cell suspension used for the attaching experiment.

RESULTS

Antimicrobial spectrum of *Curvularia* haloperoxidase system. The antimicrobial activity of the *Curvularia* haloperoxidase system was tested against selected bacteria, yeasts, and filamentous fungi (Table 1).

(i) Bacteria. The *Curvularia* haloperoxidase system clearly had a bactericidal effect against both gram-negative and gram-positive bacteria, and the numbers of all tested bacteria were considerably reduced after 20 min of treatment using 0.125 mg of enzyme liter^{-1}, 0.667 mM bromide, and 0.250 mM hydrogen peroxide. Using Student's *t* test, the reduction in numbers of *Pseudomonas aeruginosa*, *P. putida* 2442, *E. coli*, *S. marcescens*, *Aeromonas salmonicida*, *Shewanella putrefaciens*, and *S. epidermidis* cells were significant, with a *P* value of <0.001, and the reduction in *L. monocytogenes* was significant with a *P* value of <0.05.

The extent of the bactericidal effect was dependent on the concentration of the *Curvularia* haloperoxidase system, resulting in increased activity with increasing concentrations. *P. putida* 2442 was reduced from 5.1 ± 0.0 to 3.1 ± 0.2 log CFU ml^{-1} when exposed to 0.125 mg of enzyme liter^{-1}, 0.667 mM bromide, and 0.250 mM hydrogen peroxide. No *P. putida* cells could be detected (<1 CFU ml^{-1}) after 20 min when the doses were increased to 1 mg of enzyme liter^{-1}, 5 mM bromide, and 1 mM hydrogen peroxide.

P. putida 2442 appeared more resistant to the *Curvularia* haloperoxidase system than *E. coli*, *S. marcescens*, *A. salmonicida*, *S. putrefaciens*, *L. monocytogenes*, and *S. epidermidis* when the same concentrations of the enzyme system were used. Approximately 10^5 cells ml^{-1} of *P. putida* 2442 were reduced to 10^3 cells ml^{-1} by a treatment which caused reductions of the other bacteria to 10 cells ml^{-1} or below. To determine if the widespread occurrence of multidrug efflux pump systems in *Pseudomonas* spp. were the cause of the relative resistance, we evaluated the effects of the *Curvularia* haloperoxidase system on three *P. putida* efflux pump mutants, DOT-T1E-PS30, DOT-T1E-PS34, and DOT-T1E-82 (39). The sensitivities of these mutants to the *Curvularia* haloperoxidase system were compared to that of the wild-type parent strain, DOT-T1E. During treatment, the wild-type strain was reduced from 5.8 ± 0.0 to 2.6 ± 0.3 log CFU ml^{-1} (Table 1). The pump mutants were as sensitive as the wild-type parent strain; for example, DOT-T1E-82 was reduced from 5.8 ± 0.2 to 3.0 ± 0.5 log CFU ml^{-1}.

(ii) Yeasts. The number of cells of *Rhodotorula* sp. was unaffected by treatment with 1 mg of enzyme liter^{-1}, 5 mM bromide, and 1 mM hydrogen peroxide. A slight decrease (*P* < 0.05) was seen when *Candida* sp. was treated with this combination. When bromide was replaced with iodide, there was a marked effect against these strains (*P* < 0.001). *Rhodotorula* sp. was reduced from 4.4 ± 0.1 to <1.0 log CFU ml^{-1}, and *Candida* sp. was reduced from 4.4 ± 0.0 to <1.0 log CFU ml^{-1}
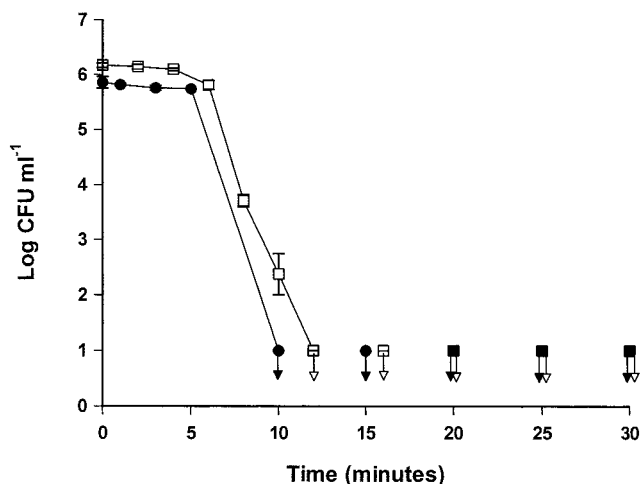
FIG. 1. Numbers of *E. coli* (●) and *S. marcescens* (□) cells during treatment with the *Curvularia* haloperoxidase system at 25°C. The arrows indicate samples below the detection level. The error bars represent standard deviations of duplicate determinations.

during treatment with 0.1 mg of enzyme liter^{-1}, 0.1 mM iodide, and 0.1 mM hydrogen peroxide for 20 min.

(iii) Filamentous fungi. Filamentous fungi, like yeasts, were not affected by the *Curvularia* haloperoxidase system when bromide was used as the halide. The numbers of conidia of *Aspergillus niger* and *Fusarium oxysporum* were unaffected by treatment with 1 mg of enzyme liter^{-1}, 5 mM bromide, and 1 mM hydrogen peroxide. When bromide was replaced with iodide, there was a marked effect against these strains. Except for the reduction in numbers of *A. niger* (IBT 14491) conidia, which was significant with a *P* value of <0.01, the reductions of the numbers of conidia of all other tested strains were significant with a *P* value of <0.001 (Table 1). Treatment with 0.05 mg of enzyme liter^{-1}, 0.05 mM iodide, and 0.05 mM hydrogen peroxide for 20 min reduced the number of *F. oxysporum* conidia from 5.3 ± 0.0 to <1.0 log CFU ml^{-1}. Using the same concentration of the *Curvularia* haloperoxidase system, *A. niger* (IBT 14491) conidia were reduced from 5.4 ± 0.1 to 4.9 ± 0.1 log CFU ml^{-1}.

Conidia from other strains of filamentous fungi were also treated with 0.05 mg of enzyme liter^{-1}, 0.05 mM iodide, and 0.05 mM hydrogen peroxide. *Aspergillus tubigensis* IBT 22444 and *A. tubigensis* IBT 4579 were reduced by this treatment from 5.0 ± 0.1 and 5.5 ± 0.1 to 1.9 ± 0.1 and 3.1 ± 0.0 log CFU ml^{-1}, respectively. By use of the same doses, *Penicillium chrysogenum* and *Penicillium paxilli* were reduced from 5.0 ± 0.1 and 5.2 ± 0.1, respectively, to <1.0 log CFU/ml. In contrast, treatment of *Aspergillus versicolor* conidia with 0.05 mg of enzyme liter^{-1}, 0.05 mM iodide, and 0.05 mM hydrogen peroxide for 20 min did not affect the number of conidia.

Killing kinetics. In order to assess the killing kinetics of the *Curvularia* haloperoxidase system, cells of *E. coli* and *S. marcescens* were treated in separate experiments with 0.125 mg of enzyme liter^{-1}, 0.667 mM bromide, and 0.250 mM hydrogen peroxide. The initial numbers of *E. coli* and *S. marcescens* cells were 5.9 ± 0.1 and 6.2 ± 0.0 log CFU ml^{-1}, respectively (Fig. 1). During the first 4 to 5 min of treatment, the numbers of both *E. coli* and *S. marcescens* cells remained constant, but

thereafter the numbers declined. Cells of *E. coli* or *S. marcescens* could not be detected after 10 to 12 min of treatment.

Stability of antimicrobial effect. The *Curvularia* haloperoxidase system, consisting of 0.125 mg of enzyme liter^{-1}, 0.667 mM bromide, and 0.250 mM hydrogen peroxide, was mixed, and the stability of the system was investigated. The bactericidal effect of the *Curvularia* haloperoxidase system was not stable. Suspending *E. coli* cells immediately after mixing the *Curvularia* haloperoxidase system caused complete killing (Fig. 2), and *E. coli* cells could not be detected after treatment. Five minutes after the *Curvularia* haloperoxidase system was mixed, the bactericidal effect against *E. coli* was reduced compared to the bactericidal effect exerted immediately after mixing. Only 3.5 ± 0.3 out of 5.8 ± 0.0 log CFU ml^{-1} survived treatment with the *Curvularia* haloperoxidase system when the system was left for 5 min before the addition of the cells. The bactericidal effect against *E. coli* was lost when the cells were suspended 10 min after the *Curvularia* haloperoxidase system was mixed, and all 5.8 ± 0.0 log CFU ml^{-1} survived.

Interfering compounds. The interferences of different compounds with the bactericidal effect of the *Curvularia* haloperoxidase system were evaluated. BSA interfered with the antimicrobial effect, and the degree of interference depended on the concentration of BSA, as well as the concentration of the *Curvularia* haloperoxidase system. A concentration of 0.005% BSA did not interfere with the bactericidal effect of the haloperoxidase system (Fig. 3). Using a low concentration of the *Curvularia* haloperoxidase system, a BSA concentration of 0.01% increased the number of surviving *E. coli* cells from <1.0 to 1.2 ± 0.2 log CFU ml^{-1}. Increasing the BSA concentration from 0.01 to 0.1% increased the number of surviving *E. coli* cells further, and all *E. coli* cells survived at a BSA concentration of 0.5% with a low enzyme concentration. Using a higher enzyme concentration, a smaller fraction of *E. coli* cells survived than in samples with similar BSA contents treated with a low enzyme concentration. Nevertheless, a BSA
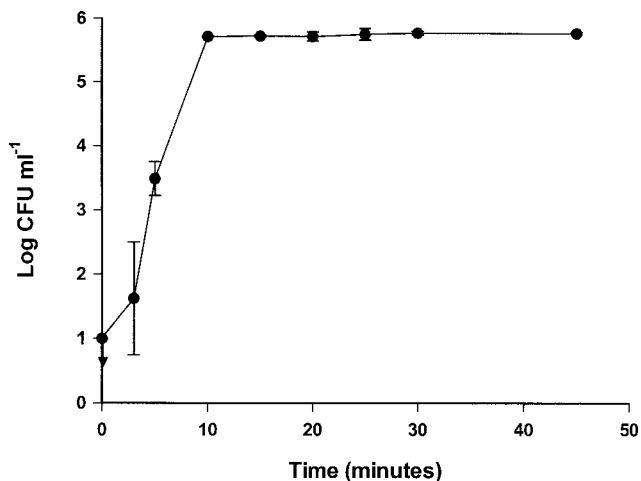

FIG. 2. Stability of *Curvularia* haloperoxidase system. The *Curvularia* haloperoxidase system was mixed and left for 0 to 45 min. Cells of *E. coli* were added, and the cell numbers were measured after 20 min. The arrow indicates a sample below the detection level. The error bars represent standard deviations of duplicate determinations.
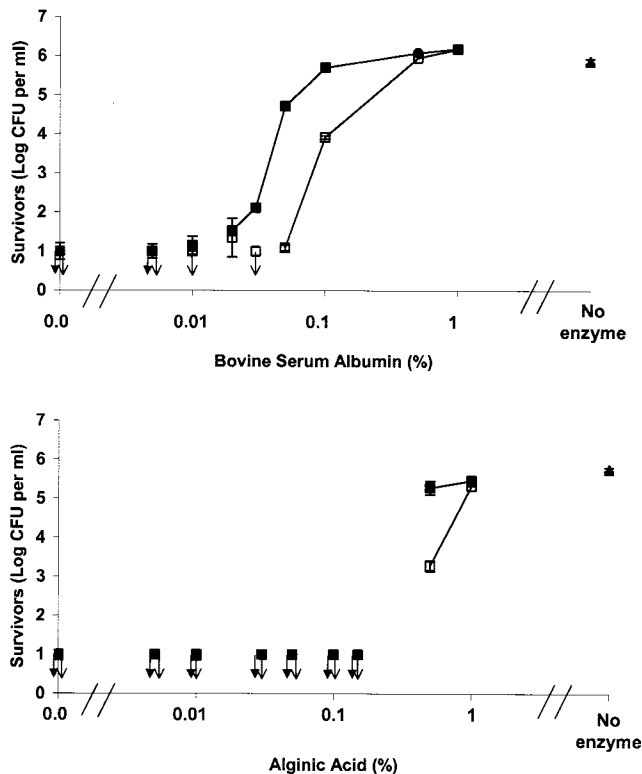
FIG. 3. Interference of protein and alginic acid with the bactericidal effect of the *Curvularia* haloperoxidase system against *E. coli*. □, low enzyme concentration (treatment with 0.125 mg of haloperoxidase liter^{-1}, 0.667 mM KBr, and 0.25 mM H$_2$O$_2$); ■, high enzyme concentration (1.0 mg of haloperoxidase liter^{-1}, 5.0 mM KBr, and 1.0 mM H$_2$O$_2$). The arrows indicate samples below the detection level. The error bars represent standard deviations of duplicate determinations.

content of 1% caused all *E. coli* cells to survive even when a high enzyme concentration was used.

Similarly to BSA, alginic acid interfered with the bactericidal effect of the *Curvularia* haloperoxidase system against *E. coli* (Fig. 3). Alginic acid in concentrations from 0 to 1% was used in killing experiments with *E. coli*, and cells were treated with the same concentrations of the *Curvularia* haloperoxidase system used to investigate protein interference. Alginic acid in concentrations up to 0.15% did not interfere with the bactericidal effect of the *Curvularia* haloperoxidase system. Adding 0.5% alginic acid caused a substantial number of *E. coli* cells to survive the treatment with both low and high enzyme concentrations. Similar to the protein interference, the interference of alginic acid was also dependent on the concentration of the *Curvularia* haloperoxidase system. Using a low enzyme concentration, 5.2 ± 0.2 log CFU ml^{-1} survived the treatment when 0.5% alginic acid was added compared to 3.3 ± 0.1 log CFU ml^{-1} with a high enzyme concentration and the same concentration of alginic acid; 1% alginic acid caused the survival of 5.5 ± 0.0 and 5.3 ± 0.0 out of 5.8 ± 0.0 log CFU ml^{-1} with low and high enzyme concentrations, respectively.

Glucose and Tween 20 in concentrations up to 1% did not affect the bactericidal effect of the *Curvularia* haloperoxidase system against *E. coli*.

Disinfection of contact lenses with the *Curvularia* haloperoxidase system. *S. marcescens* and *S. epidermidis* adhered readily to sterile contact lenses immersed for 5 h in a suspension of *S. marcescens* or *S. epidermidis* of ~6 log CFU ml^{-1}. After loosely attached cells were removed by washing the lenses in HEPES buffer, the log CFU counts per lens were between 4 and 5. The lenses were treated for 20 min with 0.125 mg of enzyme liter^{-1}, 0.250 mM hydrogen peroxide, and 0.667 mM bromide, reducing the numbers of both *S. marcescens* and *S. epidermidis* cells on the lens surfaces. After treatment with the *Curvularia* haloperoxidase system, the numbers of *S. marcescens* cells per lens were 1.2 ± 0.9 compared to 4.0 ± 0.4 log CFU on lenses placed in HEPES buffer for 20 min. The numbers of *S. epidermidis* cells were reduced by the treatment from 4.9 ± 0.1 log CFU on lenses placed in HEPES buffer to 2.7 ± 0.7 log CFU on the treated lenses. For both strains, control treatments with enzyme or a combination of bromide and hydrogen peroxide did not affect cell numbers on the lenses, showing that the bactericidal effect was attributable only to the three-component system of enzyme, bromide, and hydrogen peroxide.

DISCUSSION

The present study demonstrated that the *Curvularia* haloperoxidase system has a rapid antimicrobial effect against a broad spectrum of bacteria, yeasts, and filamentous fungi. Compared to other, monocomponent sanitizers, such as hydrogen peroxide (1), much lower concentrations are used and the exposure time is shorter. The concentration and contact time of hydrogen peroxide is important because of its corroding effect. Disinfection with 3% hydrogen peroxide caused corrosion of equipment used in ocular science, and after 144 disinfection soakings, the equipment was inaccurate because of the corrosion (7). When hydrogen peroxide is used as the sole antibacterial principle, exposure for 15 to 60 min using 0.1 to 0.6% concentrations has been required to kill urinary tract pathogens, such as *E. coli*, *S. epidermidis*, and *S. marcescens* (40). In comparison, using the *Curvularia* haloperoxidase system, a concentration of 0.25 mM (~0.0009%) hydrogen peroxide was used to kill these three species (Table 1). The concentration of hydrogen peroxide used in our study is, depending on species and strain, 110 to 670 times lower than the concentration used by Schaeffer et al. (40). It should be mentioned that the initial number of cells in our study (~10^6 CFU ml^{-1}) is lower than that in the study by Schaeffer et al. (40) (~10^8 CFU ml^{-1}), and the *Curvularia* haloperoxidase system may require higher concentrations of components if aimed at higher cell concentrations. Alasri et al. (1) showed that 0.31% hydrogen peroxide reduced *E. coli* (ATCC 8739) by 5 log reductions during 30 min of treatment, whereas 4.5 log reductions were obtained by treating *E. coli* with the *Curvularia* haloperoxidase system using 0.25 mM (~0.0009%) hydrogen peroxide. Since exposure time is also an important parameter when considering side effects of chemical sanitizers, it is advantageous that the killing effect of the *Curvularia* system is extremely rapid (Fig. 1). Also, the short-lived effect is beneficial, as the bioactive compounds disappear rapidly (Fig. 2) and thereby limit the introduction of potentially harmful

chemical agents, for example, into the eye after disinfection of contact lenses.

The *Curvularia* haloperoxidase system has a broad spectrum and exerts its killing effect against bacteria, yeasts, or filamentous fungi. It is likely, albeit not experimentally verified, that *Curvularia* haloperoxidase oxidizes halides, such as bromide, chloride, and iodide, in the presence of hydrogen peroxide and produces reactive oxygen species with an antimicrobial effect. Other peroxidase systems, such as the lactoperoxidase system, are well described (38, 43). The principal electron donor for lactoperoxidase is the pseudohalide thiocyanate (SCN^-). In the presence of hydrogen peroxide, lactoperoxidase oxidizes SCN^- to thiocyanogen $(SCN)_2$, which hydrolyzes rapidly to hypothiocyanous acid (HOSCN) or hypothiocyanate ($OSCN^-$). $(SCN)_2$ and HOSCN can oxidize protein sulfhydryl groups to sulfenyl thiocyanate derivates. It is believed that *Curvularia* haloperoxidase, like lactoperoxidase, oxidizes halides, for example, bromide, to its hypohalide hypobromite. These hypohalides have an oxidizing effect against proteins in the microorganism, causing the antimicrobial effect. The fact that protein, such as BSA, interferes with the antimicrobial effect of the system supports this hypothesis about the mode of action of the *Curvularia* haloperoxidase system.

The interfering effect of protein clearly limits the application of the *Curvularia* haloperoxidase system, which would not be suitable as an antimicrobial agent in environments rich in protein. Other compounds were also investigated for their potential interference because of the relevance to assessing applicability. Tween 20 was investigated instead of lipid because of its solubility and showed no interference with the antimicrobial effect. No interference was observed when the carbohydrate glucose was present during treatment. Alginate protected *E. coli* from the antimicrobial effects of the *Curvularia* haloperoxidase system. Exopolysaccharides are present in the matrix of a microbial biofilm (10), and their potential interference is therefore relevant when an antimicrobial system is considered as a surface sanitizer. Alginate is an extracellular polysaccharide produced by a variety of gram-negative bacteria, including *Azotobacter vinelandii*, *Pseudomonas fluorescens*, and *P. aeruginosa* (32). Structurally, alginate is a linear glycoronan composed of $(1 \rightarrow 4)$-linked residues of β-D-mannuronic acid and its C-(5)-epimer α-L-guluronic acid, and physically it has the property of forming viscous solutions and gels (8). Learn et al. (24) have investigated hypochlorite scavenging by *P. aeruginosa* alginate, and apparently the uronic acid core and the *O*-acetyl groups of pseudomonal alginate have the ability to scavenge hypochlorite. Furthermore, they demonstrated that a mucoid *P. aeruginosa* was more resistant to killing by hypochlorite than a nonmucoid mutant, and the addition of purified alginate to the nonmucoid mutant protected the organism by a greater resistance to killing by hypochlorite. As mentioned, we hypothesize that hypohalides are produced by the *Curvularia* haloperoxidase system, and the interference of alginate with the antimicrobial effect is thus to be anticipated based on the study by Learn et al. (24). Therefore, it is unlikely that the *Curvularia* haloperoxidase system can be used for the inactivation of mature biofilms.

In contrast, the *Curvularia* haloperoxidase system proved excellent as a surface sanitizer when used on bacteria adhering to contact lenses (Table 2). The U.S. Food and Drug Admin-

TABLE 2. *Curvularia* haloperoxidase killing of *S. marcescens* and *S. epidermidis* cells attached to the surfaces of contact lenses

Treatment	Log CFU/lens[a]	
	S. marcescens	*S. epidermidis*
Control		
HEPES	4.0 ± 0.4	4.9 ± 0.1
KBr + H$_2$O$_2$	4.3 ± 0.5	4.6 ± 0.1
Curvularia haloperoxidase	3.9 ± 0.3	4.7^b
Treatment		
Curvularia haloperoxidase system	$1.2 \pm 0.9^*$	$2.7 \pm 0.7^*$

[a] *, $p < 0.05$ comparing log CFU/lens on lenses treated with the *Curvularia* haloperoxidase system and on lenses treated with control treatments, using Student's t test.
[b] Only a single test.

istration requires that contact lens care products not only be antibacterial but also eliminate yeasts and some filamentous fungi (13). Since the *Curvularia* haloperoxidase system also has pronounced microbiocidal effect against yeasts and filamentous fungi (Table 1), it qualifies as a potential contact lens disinfectant. Miller et al. (29) have assessed the antimicrobial activity of lens care products and found that two out of five products examined did not meet the Food and Drug Administration's requirements for antimicrobial effect against yeasts and filamentous fungi. Although it is rare, eye infections can also be caused by protozoa, such as *Acanthamoeba*, and occurrences are often associated with contact lens use (23, 36, 37). The effect of the *Curvularia* haloperoxidase system against protozoa has not been tested. The antibacterial effect on thin layers of adhered bacteria and the rapid and unstable effect, as well as the overall low concentration of compounds, point to a vast range of other applications, for instance, as a sanitizer of equipment used in ocular science or medical devices, such as pacemakers and catheters. The exact mechanism of action of the *Curvularia* haloperoxidase system is unknown, and such knowledge will be crucial for predicting any potential side effects and for evaluating other areas of application.

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