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Susceptibility of *Penicillium expansum* Spores to Sodium Hypochlorite, Electrolyzed Oxidizing Water, and Chlorine Dioxide Solutions Modified with Nonionic Surfactants

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ABSTRACT

The use of water flotation tanks during apple packing increases the risk of contamination of apples by spores of *Penicillium expansum,* which may accumulate in the recirculating water. Routine addition of sanitizers to the water may prevent such contamination. Sodium hypochlorite (NaOCl), chlorine dioxide (ClO_2), and electrolyzed oxidizing (EO) water have varied activity against spores of *P. expansum*, and their effectiveness could be enhanced using surfactants. The objective of this study was to determine the ability of three nonionic surfactants, polyoxyethylene sorbitan monooleate (Tween 80), polyoxyethylene sorbitan monolaurate (Tween 20), and sorbitan monolaurate (Span 20), to enhance the efficacy of NaOCl, ClO_2, and EO water against spores of *P. expansum* in aqueous suspension at various temperatures and pH conditions. The efficacy of NaOCl solutions was enhanced by the addition of surfactants at both pH 6.3 and pH 8 (up to 5 log CFU reduction). EO water and ClO_2 were effective against *P. expansum* spores (up to 5 log CFU and 4 log CFU reduction, respectively), but addition of surfactants was not beneficial. All solutions were less effective at 4°C compared to 24°C irrespective of the presence of surfactants. Nonionic surfactants could potentially be used with NaOCl to improve control of *P. expansum* in flotation tanks, but the efficacy of such formulations should be validated under apple packing conditions.

Penicillium expansum is a concern in postharvest handling of apples, because it causes significant spoilage losses during longterm storage. Apples that are infected with *P. expansum* typically accumulate high levels of patulin, a potential human carcinogen with demonstrated cytotoxic effects in laboratory animals *(2, 11, 20, 21).* Manual culling and sorting of infected apples is a standard practice that significantly reduces the potential for patulin contamination of processed apple products by *P. expansum (7)*. However, these steps are difficult to monitor under the stringent protocols required in a hazard analysis and critical control point plan. Regulations to limit residual levels of patulin in apple products *(23)* have increased the need for other successful prevention strategies, many of which correctly target *P. expansum* in apple packinghouses.

Treatment of flotation tank water with sanitizers has long been used to inactivate fungal spores on apples and prevent cross contamination of sound fruit by spores present in the water *(14, 18).* Sodium hypochlorite (chlorine), the most commonly used sanitizer, usually has to be maintained above optimal concentrations and within a narrow pH range to be effective, but hypochlorite concentrations often drop below the minimum effective level due to the introduction of organic matter *(6, 12)* and lack of close monitoring. Furthermore, several strains of *P. expansum* can survive typical levels of chlorine or other sanitizers normally applied in the water if the optimal concentrations are not vigilantly monitored. Besides chlorine, other sanitizers such as chlorine dioxide, peracetic acid, ozone, and acetic acid among others, have been applied with varied levels of success against *P. expansum* in packinghouses *(8, 13, 15, 17).* Chlorine, however, remains the sanitizer of choice in most apple packinghouse operations *(11)*.

The insensitivity of certain fungi to some sanitizers might result from the hydrophobic nature of the spore *(9)* which may potentially reduce sanitizer contact and penetration. The addition of surfactants to the sanitizer solutions might therefore increase efficacy against spores of *P. expansum* and thereby allow lower concentrations of sanitizers to provide effective control of *P. expansum* in water flumes. Concerns about worker safety, environmental contamination, and accumulation of chlorinated byproducts such as chloramines related to the use of chlorine sanitizers could be minimized through reduced chlorine use *(1).*

The objective of this study was to determine the effect of three nonionic surfactants on the efficacies of sodium hypochlorite (NaOCl), electrolyzed oxidizing (EO) water, and chlorine dioxide (ClO_2) against spores of *P. expansum* in suspension. The surfactants were polyoxyethylene sorbitan monooleate (Tween 80), polyoxyethylene sorbitan monolaurate (Tween 20), and sorbitan monolaurate (Span 20). Additional experiments were conducted to determine the effect of pH and temperature on the interaction between the Tween 20 and NaOCl.

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MATERIALS AND METHODS

Microorganisms. Three apple isolates of *P. expansum* were used in this study, which were P99 and P301 (New York State Agricultural Experiment Station, Cornell University, N.Y.), and ATCC 28877 (American Type Culture Collection, Baltimore, Md.). Cultures were maintained on potato dextrose agar (PDA; Difco, Sparks, Md.) slants. To produce inoculum, each strain was grown on PDA plates for approximately 8 days at 25°C. The surface of each culture was then washed gently using 10 ml of sterile water containing 0.01% Tween 80, and the resulting suspensions were filtered through eight layers of cheesecloth to remove mycelial debris. A Bright-Line hemacytometer (Hausser Scientific, Horsham, Pa.) was used to estimate the spore concentrations. Each suspension was also serially diluted and spread plated on PDA to confirm the spore concentrations. Equal volumes of resulting suspensions were combined into a cocktail that was used as the inoculum for all experiments. The stock spore suspensions contained approximately 10^7 CFU/ml.

Treatment solutions and surfactants. The non-ionic surfactants, Span 20 (S20), Tween 80 (T80), and Tween 20 (T20) (Sigma-Aldrich, St. Louis, Mo.), previously autoclaved at 121°C for 21 min, were used to prepare treatment solutions. Sodium-hypochlorite (NaOCl; VWR International, West Chester, Pa.) solution was adjusted to pH 6.3 ± 0.1 using hydrochloric acid (HCl; Sigma-Aldrich) where applicable. The NaOCl solutions were modified with each surfactant to make up 0.01%, 0.05%, and 0.1% (vol/vol) of the surfactant in the mixture for the treatments in aqueous suspensions. Appropriate controls were prepared with NaOCl, the nonionic surfactants, as well as distilled deionized water. EO water was generated by an EO water generator (model ROX 20TA, Hoshizaki Electric Co., Ltd., Sakae, Toyoake, Aichi, Japan) and only the acidic portion was used. ClO_2 was generated by a chlorine dioxide generator (Halox Technologies Inc., Bridgeport, Conn.). Both EO water and ClO_2 solution were further diluted with distilled deionized water where necessary. S20, T20, and T80, previously autoclaved at 121°C for 21 min, were used to modify treatment solutions where indicated. Free chlorine in the solutions was determined by a colorimetric titration kit (Hach Co., Loveland, Colo.), while the oxidation-reduction potential (ORP) and pH were measured using an Orion meter (Thermo Electron Corp., Waltham, Mass.) with dual probes. Unless otherwise stated, all treatments were carried out at ambient temperatures (ca. 24°C).

Treatments. The cocktail spore suspensions of *P. expansum* prepared as before were exposed to the surfactant-sanitizer combinations for up to 5 min. T80, T20, and S20 at concentrations of 0.01, 0.05, and 0.1% (vol/vol) were each combined with NaOCl solutions containing approximately 50 ppm free chlorine. This concentration is below the recommended concentration *(12)* for NaOCl applications for sanitation in packing houses (100 to 200 ppm). Two sets of NaOCl solutions that were either alkaline (pH 8.5 ± 0.2) or slightly acidic (pH 6.3 ± 0.2) were prepared for each 100-ml combination. One milliliter of the inoculum, prepared as previously described, was then added to each solution to produce an initial spore density of approximately 10^5 CFU/ml. While gently agitating the solutions, 1-ml samples withdrawn at intervals of 30 s, 1 min, 2 min, and 5 min were serially diluted in deionized water, and viable spores were enumerated on PDA plates after 48-h incubation at 25°C. To remove any residual activity at the chosen time intervals, 0.1 ml of 0.1 M sodium thiosulfate was added into the first dilution tube before each experiment. A 50:50 dilution of EO water *(10)* and ClO_2 adjusted to a concentration of 3 ppm (maximum recommended concentration for water flumes) were similarly evaluated using the surfactants at 0.1%, vol/vol (the highest concentration of surfactant previously combined with NaOCl). The effect of temperature on the efficacy of NaOCl was determined at 4, 10, and 15°C, using combinations with T20 at 0.1%, vol/vol; EO water and ClO_2 were similarly evaluated at 4°C, but without any surfactants at this temperature. Both EO water and ClO_2 were further reformulated in phosphate buffer at pH 6.3 to determine the influence of pH on their efficacy when combined with T20.

TABLE 1. *Chemical properties (pH and ORP) and free chlorine concentration of sanitizer formulations*[a]

Treatment	pH	ORP (mV)	Free chlorine concn (ppm)
NaOCl	6.1	874	50
NaOCl+T20	6.1	877	51
NaOCl+T80	6.1	871	50
NaOCl+S20	6.1	881	52
NaOCl	8.5	644	50
NaOCl+T20	8.5	648	49
NaOCl+T80	8.5	638	49
NaOCl+S20	8.5	647	51
EO water	3.5	1,027	18
EO water+T20	3.4	1,022	17
EO water+T80	3.6	754	7
EO water+S20	3.5	1,019	21
ClO_2	3.7	888	3
ClO_2+T20	3.7	879	3
ClO_2+T80	3.6	872	3
ClO_2+S20	3.8	884	3

[a] Values for solutions containing surfactants represent those obtained at the highest surfactant concentrations used (0.1%, vol/vol).

Statistical analysis. Treatments performed in triplicate were analyzed for significant differences using ANOVA procedures with SPSS statistical software (SPSS, Inc., Chicago, Ill.). Significant differences were determined at 95% confidence intervals using Tukey's test *(24).*

RESULTS

The chemical properties (pH and ORP) of the treatment solutions used in this study are shown in Table 1. Addition of T20 and S20 (up to 0.1%, vol/vol) to the sanitizers did not affect their chemical properties. T80, however, reduced both the ORP and concentration of free chlorine in EO water without affecting the pH. Table 2 shows the activity of alkaline NaOCl (50 ppm free chlorine) modified with each of the surfactants on spores of *P. expansum,* while similar results from acidified NaOCl solutions are shown in Table 3. Except for 5 min of exposure in the presence of T20 and S20 at levels of 0.05% and 0.1%, alkaline NaOCl solutions did not significantly reduce the number of recovered viable spores. Alkaline NaOCl solutions modified with 0.05% and 0.1% T20 and S20 were more effective at reducing the viable spores recovered after a 5-min exposure, compared to alkaline NaOCl that had not been modified

TABLE 2. *Residual viable spores of* P. expansum *exposed to alkaline (pH 8.5 ± 0.2) NaOCl solutions modified with various surfactants for up to 5 min in aqueous suspension*[a]

Treatment	Viable spores recovered over time (log CFU/ml)[b]			
	30 s	60 s	120 s	300 s
NaOCl (control)	4.6 A a	4.7 A a	4.8 A a	4.5 AB a
0.01% T80+NaOCl	4.7 A a	4.6 A a	4.6 A a	4.7 A a
0.01% T20+NaOCl	4.6 A a	4.8 A a	4.8 A a	4.1 B b
0.01% S20+NaOCl	4.5 A a	4.8 A a	4.9 A a	4.8 A a
0.05% T80+NaOCl	4.4 A a	4.5 A a	4.5 A a	4.6 A a
0.05% T20+NaOCl	4.8 A a	4.6 A a	4.6 A a	3.7 C b
0.05% S20+NaOCl	4.7 A a	4.6 A a	4.8 A a	3.4 C b
0.1% T80+NaOCl	4.9 A a	4.9 A a	4.9 A a	5.0 A a
0.1% T20+NaOCl	4.4 A a	4.8 A a	4.5 AB a	2.6 D b
0.1% S20+NaOCl	4.7 A a	4.8 A a	4.3 B a	2.5 D b

[a] Numbers in the same column followed by different uppercase letters and numbers in the same row followed by different lowercase letters are significantly different ($P \leq 0.05$).

[b] The initial spore concentration for all treatments was approximately 10^5 CFU/ml.

TABLE 4. *Residual viable spores of* P. expansum *exposed to EO water and ClO_2 solutions modified with various surfactants (0.1%, vol/vol) for up to 5 min in aqueous suspension*[a]

Treatment	Viable spores recovered over time (log CFU/ml)[b]			
	30 s	60 s	120 s	300 s
Control	5.0 A a	4.7 A a	4.9 A a	4.9 A a
EO water	4.7 A a	3.4 B b	<1.0[c] E c	<1.0 E c
EO+T20	4.7 A a	4.0 A a	1.5 C b	<1.0 E c
EO+T80	4.8 A a	4.9 A a	5.0 A a	4.8 B a
EO+S20	4.3 A a	4.4 A a	3.3 A b	2.1 B c
ClO_2	3.6 B a	1.5 C b	1.0 D c	1.2 D c
ClO_2+T20	3.0 B a	1.9 C b	1.0 D c	1.0 D c
ClO_2+T80	4.3 A a	2.8 B b	2.3 B c	2.0 C c
ClO_2+S20	4.7 A a	4.5 A a	4.2 A a	3.4 C b

[a] Numbers in the same column followed by different uppercase letters and numbers in the same row followed by different lowercase letters are significantly different ($P \leq 0.05$).

[b] The initial spore concentration for all treatments was approximately 10^5 CFU/ml.

[c] <1.0 means that no viable spores were detected at the dilutions used.

(Table 2). The activity of acidified NaOCl was significantly higher than that of the alkaline solutions (Table 3). When the surfactants were added, no viable spores were detected except in solutions containing T80 and NaOCl. No viable spores were detected beyond 30 s of exposure to any of the pH-adjusted solutions.

Table 4 shows the viable spores recovered after treatments in EO water and ClO_2. Without surfactants, ClO_2 solutions caused a significant reduction (1.4 log CFU/ml) in viable spores within 30 s of exposure. A longer exposure time of 5 min in these solutions resulted in a reduction of 4 log CFU/ml. EO water by itself was also effective within 1 min, reducing viable spores by 1.6 log CFU/ml. When each solution was modified with surfactants, no improvement in their activity was realized. All the treatments with these two sanitizers that included surfactants were either as effective as the treatments without surfactants or less active when the surfactants were included. T80, for example, reduced the activity of EO water to insignificant levels, while both T20 and T80 delayed the destruction of the spores by this sanitizer; significant inactivation by the EO water only occurred after 1 min in the presence of these surfactants. The efficacy of ClO_2 modified with T20 was not significantly different from ClO_2 by itself, while both T80 and S20 delayed the inactivation of spores by this sanitizer; significant activity only occurred after 1 and 2 min, respec-

TABLE 3. *Residual viable spores of* P. expansum *exposed to slightly acidic (pH 6.3 ± 0.2) NaOCl solutions modified with various surfactants for up to 5 min in aqueous suspension*[a]

Treatment	Viable spores recovered over time (log CFU/ml)[b]			
	30 s	60 s	120 s	300 s
NaOCl (control)	3.9 B a	3.6 A a	4.0 B a	3.1 C a
0.01% T80+NaOCl	1.2 C a	<1.0[c] C b	<1.0 C b	<1.0 C b
0.01% T20+NaOCl	<1.0 C b	<1.0 C b	<1.0 C b	<1.0 C b
0.01% S20+NaOCl	<1.0 C b	<1.0 C b	<1.0 C b	<1.0 C b
0.05% T80+NaOCl	<1.0 C b	<1.0 C b	<1.0 C b	<1.0 C b
0.05% T20+NaOCl	<1.0 C b	<1.0 C b	<1.0 C b	<1.0 C b
0.05% S20+NaOCl	<1.0 C b	<1.0 C b	<1.0 C b	<1.0 C b
0.1% T80+NaOCl	<1.0 C b	<1.0 C b	<1.0 C b	<1.0 C b
0.1% T20+NaOCl	<1.0 C b	<1.0 C b	<1.0 C b	<1.0 C b
0.1% S20+NaOCl	<1.0 C b	<1.0 C b	<1.0 C b	<1.0 C b

[a] Numbers in the same column followed by different uppercase letters and numbers in the same row followed by different lowercase letters are significantly different ($P \leq 0.05$).

[b] The initial spore concentration for all treatments was approximately 10^5 CFU/ml.

[c] <1.0 means that no viable spores were detected at the dilutions used.

TABLE 5. *Residual viable spores of* P. expansum *exposed to EO water and ClO_2 solutions modified with T20 (0.1%, vol/vol) for up to 5 min in aqueous suspension*[a]

Treatment	ORP (mV)	Viable spores recovered over time (log CFU/ml)[b] 30 s	60 s	120 s	300 s
Control	366	4.9 A a	4.9 A a	4.8 A a	4.8 A a
EO water	787	4.5 A a	4.4 A a	4.0 B a	1.0 C b
EO+T20	769	4.6 A a	4.6 A a	4.2 B a	2.3 B b
ClO_2	814	4.3 A a	3.8 B b	2.5 C c	1.8 B d
ClO_2+T20	829	4.3 A a	3.7 B b	2.4 C c	<1.0[c] D d

[a] Solutions were prepared in phosphate buffer (pH = 6.3). Numbers in the same column followed by different uppercase letters and numbers in the same row followed by different lowercase letters are significantly different ($P \leq 0.05$).

[b] The initial spore concentration for all treatments was approximately 10^5 CFU/ml.

[c] No viable spores were detected at the dilutions used.

TABLE 6. *Residual viable spores of* P. expansum *exposed to NaOCl solutions and held at 4°C, 10°C, and 15°C for up to 5 min in aqueous suspension*[a]

Treatment	Viable spores recovered over time (log CFU/ml)[b] 30 s	60 s	120 s	300 s
4°C				
Control	5.0 A a	4.9 A a	5.1 A a	5.0 A a
NaOCl	5.0 A a	4.7 A a	3.2 C b	3.0 B b
NaOCl+T20	4.9 A a	4.7 A a	4.1 B b	3.0 B c
10°C				
Control	5.2 A a	5.1 A a	4.8 A a	4.8 A a
NaOCl	4.7 A a	4.3 B b	3.4 C c	3.3 B c
NaOCl+T20	4.5 AB a	3.7 C b	1.3 D e	<1.0 C d
15°C				
Control	5.0 A a	4.7 A a	4.9 A a	4.7 A a
NaOCl	4.3 B a	3.7 C b	3.3 C a	3.2 B c
NaOCl+T20	4.4 B a	2.5 D b	<1.0	<1.0[c]

[a] Numbers in the same column followed by different uppercase letters and numbers in the same row followed by different lowercase letters are significantly different ($P \leq 0.05$).

[b] The initial spore concentration for all treatments was approximately 10^5 CFU/ml.

[c] No viable spores were detected at the dilutions used.

tively, when the ClO_2 was modified using the two surfactants.

Experiments were conducted to assess the role of pH in the interaction between the T20 and both ClO_2 and EO water (Table 5). Because the NaOCl solutions were enhanced by surfactants (especially with T20 at pH 6.3), EO water and ClO_2 solutions originally formulated to a ''natural'' pH of 3.5 and 3.7, respectively, were prepared in phosphate buffer adjusted to a pH of 6.3. The results from these experiments determined that the low pH of EO water and ClO_2 that were combined with T20 did not contribute to the lack of improved efficacy in these solutions (Table 5). When the treatments were formulated in phosphate buffer (pH 6.3), the ORP of these solutions was less than that of the solutions at natural pH (Table 1). Consequently, the efficacy of the solutions was also lower than that at the natural pH. The addition of T20 to these sanitizers did not restore their effectiveness to levels achieved at the natural pH levels. NaOCl (50 ppm) solutions adjusted to pH 3.5 similar to EO water were included among the treatments to confirm that pH did not play a significant role in the synergism between hypochlorite and T20. At this pH, no viable spores were recovered from solutions containing hypochlorite, and T20 did not have deleterious effects on its activity (data not shown).

At 4°C, T20 did not improve the efficacy of NaOCl, but at 10°C, the NaOCl+T20 combination was more effective than NaOCl alone at 60 s and nearly all spores were killed after 5 min at 10°C (Table 6).

DISCUSSION

Alkaline NaOCl solutions did not show any significant activity after 5 min exposures except when combined with T20 and S20. At pH above 8.0, less than 25% of the NaOCl would be present as hypochlorous acid, the active form of this compound *(3).* The pH sensitivity of NaOCl as well as the intrinsic characteristics of *P. expansum* spores such as hydrophobicity that limit the activity of NaOCl may explain the lack of significant activity at this pH in the absence of surfactants. T20 and S20 may have lowered the surface tension in these solutions; hence, facilitating greater contact between the spores and hypochlorous acid molecules, and thereby improving activity. Hypochlorous acid in chlorine solutions peaks at pH levels between 5.0 and 7.0 *(3)*; therefore, the increased inactivation of *P. expansum* spores in the pH-adjusted solutions was not unexpected. Modification of the solutions with surfactants increased NaOCl efficacy further at this pH. Again, a reduction in surface tension in the solutions may have increased contact and penetration of NaOCl into the fungal spores, thus increasing its efficacy. A positive correlation between ORP and the efficacy of various sanitizers against *P. expansum* has previously been demonstrated *(10).* Since the ORP and pH of the NaOCl solutions did not change when surfactants were added (Table 1), the increase in activity of NaOCl where surfactants were present may be the result of a physical mechanism (e.g., reduced surface tension countering the effects of spore surface hydrophobicity) rather than some unknown chemical mechanism.

Roberts and Reymond *(13)* demonstrated that ClO_2 in aqueous suspension resulted in more than 99% spore mortality of *P. expansum* at 3 μg/ml and above, but levels of 1 μg/ml were not as effective. Previous experiments with EO water diluted to 25% strength with deionized water against *P. expansum* in aqueous suspension also resulted in more than 99% reduction in viable spores after 1 min *(10).* We expected that adding nonionic surfactants to both ClO_2 and EO water would enhance their activity against spores of *P. expansum* because we expected that the surfactants would increase wetting of the hydrophobic spores and therefore enhance contact *(4)* between the sanitizing agents

and the spore surface. Our results did not support this hypothesis. Unlike NaOCl, the addition of surfactants to either EO water or ClO_2 was not beneficial in enhancing efficacy against *P. expansum* spores. This finding may be a result of the thermodynamics of the active molecules of EO water and ClO_2 in solution. ClO_2, for example, exists in water as the dissolved gas and does not interact closely with water molecules *(22).* Because NaOCl molecules interact closely with water molecules, the presence of surfactants likely favors its close proximity to spores and, therefore, contact and inactivation. This scenario is unlikely in a ClO_2-surfactant-water system. In fact, the presence of surfactants may create a barrier to ClO_2 gaining efficient contact with spores; as the hydrophobic tails of the surfactants align with the spore surfaces, contact between the ClO_2 and these surfaces may be reduced. Although the active molecule in EO water is largely thought to be HOCl, other molecules also contribute to its overall efficacy. Among these are gaseous chlorine *(5),* and other free radicals, the thermodynamics of which may be negatively affected by surfactants, thus lowering the efficacy of EO water when combined with surfactants. The lower efficacy of the solutions at reduced temperatures was also not unexpected. As temperatures drop, molecular activity is reduced, thus generally reducing activity.

The combination of NaOCl with at least 0.05% vol/vol of surfactants such as T20 and S20 could potentially expand the control limits for pH and free chlorine concentration without causing sanitation failures, since activity could be retained at a higher pH and lower concentrations of NaOCl. Studies in our laboratory are underway to validate the findings in this study under simulated packinghouse conditions. Although ClO_2 and EO water could be applied to control *P. expansum* in lieu of hypochlorite solutions, the use of T20, S20, or T80 to enhance the efficacy of these two sanitizers does not seem advisable based on these results. Enhanced control of decay by *P. expansum* in the packinghouse using surfactants has been demonstrated *(16, 19),* but packinghouse experiments with the sanitizer-surfactant combinations applied in this study are warranted. Results can then be used to understand the factors to consider when choosing sanitizer-surfactant combinations that are practical for flotation tank conditions. While the addition of surfactants may, for example, prevent the contamination of apples in a flotation tank by *P. expansum* spores in the water, further research is needed to determine if modification with surfactants would improve the prevention of decay caused by spores already on the apples when treatments are applied.

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