

Efficacy of Reflex against Various Planktonic Bacteria, Yeasts, and Mold

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I. BACKGROUND

Reflex is an EPA approved formulated sanitizer/disinfectant produced at Enviro Tech Chemical Services, Inc. The active ingredient in Reflex is peroxyacetic acid (PAA). What makes Reflex unique is the introduction of nitric acid to lower the pH of the diluted solution more than PAA alone. Reflex was originally developed for dairy production facilities, which require antimicrobials to remain effective in the presence of protein. While many antimicrobials are hindered in these environments Reflex, with its additional acid component, remains efficacious. Recent studies, however, have shown Reflex to excel as a sanitizer in areas outside of the dairy industry. Oxidizers such as PAA function by chemically oxidizing the outer membrane of bacteria causing the cells to lyse, spilling the contents of the cell. By decreasing the pH of the solution the bacteria can be made more susceptible to this mode of action.

The nitric acid component of Reflex is also usefully employed in brewery and dairy operations for beerstone and milkstone removal and prevention. Thus, its use allows sanitation and beerstone/milkstone removal to be accomplished in one step.

The purpose of this study is to evaluate the efficacy of Reflex against two different bacteria, yeasts, and mold in solution. **Table 1** describes each organism challenged, as well as the concentration of PAA, and the respective contact times.

Table 1. The organisms, organism type, concentration, and contact time for the challenge study.

Organism	Type	Conc. PAA	Contact Time
<i>Listeria monocytogenes</i>	Bacteria	100 ppm	1 min.
<i>Salmonella typhimurium</i>	Bacteria	100 ppm	1 min.
<i>Candida albicans</i>	Yeast	100 ppm	1 min.
<i>Saccharomyces cerevisiae</i>	Yeast	100 ppm	1 min.
<i>Aspergillus niger</i>	Mold	100 ppm	2 min.
<i>Byssoschlamys fulva</i>	Mold	100 ppm	2 min.

The species used in the experiment are explained in detail below.

Bacterial Organisms

Listeria monocytogenes

Listeria species are gram-positive, bacillus-shaped, soil dwelling bacteria. The major species that pertains to humans is *Listeria monocytogenes* which is a very adaptable and rugged bacterium and is the pathogen that is the causative agent for the disease listeriosis. Listeriosis is usually contracted by ingesting contaminated foods such as meat or dairy products. Prevention of lister-

iosis as a food illness is important and requires reliable and effective sanitation practices.

Salmonella typhimurium

Salmonella typhimurium is a pathogenic, gram-negative bacteria predominately found in the intestinal lumen of mammals. *Salmonella typhimurium* is a serovar of *Salmonella enterica* which has over 2500 different serovars. Six of the serovars are the most common human pathogens with *Salmonella typhimurium* being one of the six. The most common foods that can harbor *S. typhimurium* are poultry and raw eggs. Refrigerating or freezing does not destroy the bacteria but simply arrests growth. The hazardous nature of *S. typhimurium* is the presence of an outer membrane consisting largely of lipopolysaccharides (LPS) which protects the bacteria from the environment. Strong oxidizers such as PAA are able to penetrate the membrane and destroy *S. typhimurium* with great efficiency.

Yeast Organisms

Candida albicans

Candida albicans is present in 80% of the population's normal flora without causing sickness but an overgrowth of *C. albicans* can lead to oral infection or even systemic infections. *C. albicans* is an opportunistic pathogen that usually affects immune-compromised individuals. *C. albicans* is very unique in that it is classified as a dimorphic organism meaning that it can grow either as yeast or mold. Since *C. albicans* can grow as yeast or mold, it has the ability to grow on many different substrates.

Saccharomyces cerevisiae

Saccharomyces cerevisiae is one the most common yeast. *S. cerevisiae* is commonly used in wine and beer production, baking, and as a model organism in science. *S. cerevisiae* can be isolated from the skin of grapes as well as other dark skinned fruits such as plums, and thus fruit juices are more susceptible to *S. cerevisiae* contamination. Overgrowth of *S. cerevisiae* can lead to a severe fungal infection. Prevention of *S. cerevisiae* is important to in the preservation of food from spoilage as well as protecting consumers from foodborne illness. *S. cerevisiae* can be resistant to heat processing.

Mold Organisms

Aspergillus niger

Aspergillus niger is a very common fungus that can be found virtually anywhere. *A. niger* is the causative agent in a fruit and vegetable disease called "black mold". The most common fruits and vegetables affected are grapes, onions, and peanuts. *A. niger* is ubiquitous to the soil but can also be found in the environment. Fungal infections caused by *A. niger* in humans are not

common but immune compromised individuals are susceptible to *A. niger* fungal infections. *A. niger* is more heat resistant to high temperatures and low water activity compared to other fungi which makes it dominate in warmer climates. Prevention of *Aspergillus niger* is important in preventing the spoilage of food.

Byssoschlamys fulva

Byssoschlamys fulva is a common spore producing fungus that is not affected greatly by elevated temperatures and low pH. *Byssoschlamys fulva*, being heat resistant, survives pasteurization. Canned and bottled fruits are the most susceptible to *B. fulva* contamination. Also, *B. fulva* is the pathogen that causes "Byssoschlamys rot" on strawberries. Since *Byssoschlamys fulva* can withstand high temperatures chemical sanitation is the best way for preventing contamination.

II. EXPERIMENTAL METHODS

In order to perform the study, the organisms had to be cultured in the lab.

Bacterial Organisms

Listeria monocytogenes (Hardy Diagnostic Cat# 0254FPC) was grown in Listeria Enrichment Broth (Criterion Cat. No.: C6030) and incubated at 35°C for 36 hours. The bacteria were separated from the broth by centrifugation. The liquid was decanted and the bacterial pellet was reconstituted in 1.0 L sterile phosphate buffer. The 1.0 L solution was then split into two 500 mL samples. One sample was used as the control and the other served the testing sample.

Salmonella typhimurium (ATCC® 14028) was grown in Sigma Nutrient broth at 35°C for 48 hours. The bacteria were separated from broth by centrifugation. The liquid was decanted and the bacterial pellet was reconstituted in 1.0 L sterile phosphate buffer. The 1.0 L solution was then split into two 500 mL samples. One sample was used as the control and the other served as the testing sample.

Yeast Organisms

Candida albicans (ATCC® 10231) pellet (5.1 x 10³ CFU/pellet) was reconstituted in 5 mL of Brain Heart Infusion Broth (Criterion Cat. No.: C5140). After the pellet was completely dissolved, 1.0 mL of the solution was plated on five Hardy Diagnostic Sabdex Agar (Cat no.: W70) and the plates were incubated at 25°C for 72 hours. After 72 hours of incubation the Sabdex Agar plates had several 3-4 mm round, off-white color colonies which is indicative for *Candida albicans* growth. The yeast was aseptically transferred to 1.0 L of sterile Butterfield's Buffer which was then thoroughly mixed to ensure homogeneity. The 1.0 L solution was then split into two, 500mL samples. One sample served as the control and the other served as the test sample.

Saccharomyces cerevisiae (ATCC® 18824) pellet was reconstituted in 5 mL of Criterion WL Nutrient Medium (Cat. No.: C7301). After pellet was completely dissolved, 1.0 mL of the solution was plated on five Hardy Diagnostic Sabdex Agar (Cat no.: W70) after which the plates were incubated at 25 °C for 72 hours. After 72 hours of incubation the Sabdex Agar plates had many 2-3 mm round, white color colonies which is indicative of *Saccharomyces cerevisiae* growth. The yeast was aseptically transferred to 1.0 L

of sterile Butterfield's Buffer which was then thoroughly mixed to ensure homogeneity. The 1.0 L solution was then split into two, 500mL samples. One sample served as the control and the other served as the test sample.

Mold Organisms

Aspergillus niger (ATCC® 16888) pellet was reconstituted in 5 mL of Brain Heart Infusion Broth (Criterion Cat. No.: C5140). After the pellet was completely dissolved, 1.0 mL of the solution was plated on five Hardy Diagnostic Sabdex Agar (Cat no.: W70) and the plates were incubated at 25 °C for 72 hours. After 72 hours of incubation the Sabdex Agar plates had several 4-5 mm filamentous white and black color colonies which is indicative of *Aspergillus niger* growth. The mold was aseptically transferred to 1.0 L of sterile Butterfield's Buffer which was then thoroughly mixed to ensure homogeneity. The 1.0 L solution was then split into two, 500mL samples. One sample served as the control and the other served as the test sample.

Byssoschlamys fulva (ATCC® 24474) powder was reconstituted in 5 mL of Brain Heart Infusion Broth (Criterion Cat. No.: C5140). After the powder was completely dissolved, 1.0 mL of the solution was plated on five Hardy Diagnostic Sabdex Agar (Cat no.: W70) after which the plates were incubated at 25°C for 72 hours. After 72 hours of incubation the Sabdex Agar plates were covered with filamentous white color, growth characteristic of *Byssoschlamys fulva* growth. The mold was aseptically transferred to 1.0 L of sterile Butterfield's Buffer which was then thoroughly mixed to ensure homogeneity. The 1.0 L solution was then split into two, 500 mL samples.

Control Sample Procedure

The control samples for each organism had the same testing protocol. An aliquot was removed from each control solution, serially diluted and plated on their respective media (see **Table 2**).

Test Solution Procedure

Each test sample solution was dosed with a nominal 100 ppm PAA from Reflex (0.79g). The lot used (#37-12-0723-1) was analyzed by iodometric titration and found to have a PAA concentration of 6.35% and a hydrogen peroxide concentration of 24.40%. The contact time for the bacterial and yeast organisms was one minute and the contact time for the molds was two minutes (See **Table 1**). The concentration of PAA was tracked using the Palin DPD methodology over the 1 or 2 minutes contact time. After the respective contact times, remaining peroxygens in each solution were neutralized with a calculated amount of erythorbic acid. After neutralization the test solution was analyzed again to verify that all active PAA and H₂O₂ was neutralized. Aliquots were then removed from each test solution, serially diluted, plated the appropriate media.

III. RESULTS AND DISCUSSION

In **Table 2** below the initial pH, final pH, the medium used for each organism, and concentration PAA (ppm) for each solution tested are described.

Table 2. Description of bacteria solutions used in the challenge study.

Organism	Init. pH	Final pH	Organism Medium	Conc. PAA (ppm)
<i>L. monocytogenes</i>	6.36	2.33	3M Environ. Listeria Petrifilms™*	94.3
<i>S. typhimurium</i>	7.77	2.83	3M Enterobacteriaceae Petrifilms™*	109.1
<i>C. albicans</i>	5.69	2.56	Hardy Diagnostic Sabdex Agar*	87.7
<i>S. cerevisiae</i>	7.23	2.88	Hardy Diagnostic Sabdex Agar*	104.9
<i>A. niger</i>	7.84	2.91	Hardy Diagnostic Sabdex Agar*	114.5
<i>B. fulva</i>	8.03	2.91	Hardy Diagnostic Sabdex Agar*	112.4

*3M Environmental Listeria Petrifilms™ were incubated at 35°C for 30 hours. 3M Enterobacteriaceae Petrifilms™ were incubated at 35°C for 24 hours. Sabdex Agar was incubated at 25°C for 72 hours.

Bacterial Organisms

The *L. monocytogenes* control sample had an average log₁₀ of 6.86 CFU/mL. There were zero detectable colonies remaining after treatment with 100 ppm PAA (from Reflex) for 1 minute. Therefore, treatment of 100 ppm PAA from Reflex on *Listeria monocytogenes* in solution had a log₁₀ reduction >6.86 CFU/mL which equates to a >99.9999%.

The *S. typhimurium* control had an average log₁₀ of 7.03 CFU/mL. After treatment with 100 ppm PAA from Reflex at a 1 minute contact time there was zero viable colonies remaining. Therefore, 100 ppm PAA from Reflex at a 1 minute contact time had a log₁₀ reduction >7.03 CFU/mL on *Salmonella typhimurium* in solution which equates to >99.9999% reduction.

Table 3. The average number of bacterial colonies present on the control Petrifilms® and the test sample Petrifilms® after a 1 minute contact time with 100 ppm PAA from Reflex.

Organism	Avg. log ₁₀	log ₁₀ Reduction	% Reduction
<i>Listeria monocytogenes</i> control	6.86	NA	NA
<i>Listeria monocytogenes</i> test	0	>6.86	>99.9999
<i>Salmonella typhimurium</i> control	7.03	NA	NA
<i>Salmonella typhimurium</i> test	0	>7.03	>99.9999

Yeast Organisms

The *Candida albicans* control had an average log₁₀ of 6.84 CFU/mL. Treatment with 100 ppm PAA from Reflex for 1 minute

reduced the *C. albicans* log₁₀ to 4.22 CFU/mL. This is log₁₀ reduction of 2.62 CFU/mL, which equates to 99.7%.

The *Saccharomyces cerevisiae* control had an average log₁₀ of 6.24 CFU/mL. Treatment with 100 ppm PAA from Reflex for 1 minute reduced the *S. cerevisiae* log₁₀ to 3.50 CFU/mL. This is a log₁₀ reduction of 2.74 CFU/mL, which equates to 99.8%.

Table 4. The average number of yeast colonies present on the control and test sample plates after a 1 minute contact time with 100 ppm PAA from Reflex.

Organism	Avg. log ₁₀	log ₁₀ Reduction	% Reduction
<i>Candida albicans</i> control	6.84	NA	NA
<i>Candida albicans</i> test	4.22	2.62	99.7
<i>Saccharomyces cerevisiae</i> control	6.24	NA	NA
<i>Saccharomyces cerevisiae</i> test	3.50	2.74	99.8

Mold Organisms

The *Aspergillus niger* control had an average log₁₀ of 5.83 CFU/mL. Treatment with 100 ppm PAA from Reflex for 2 minutes reduced the *A. niger* log₁₀ to 4.00 CFU/mL. This is a log₁₀ reduction of 1.83 CFU/mL, which equates to 98.5%.

The *Byssoschlamys fulva* control had an average log₁₀ of 7.04 CFU/mL. Treatment with 100 ppm PAA from Reflex for 1 minute reduced the *B. fulva* log₁₀ to 1.93 CFU/mL. This is a log₁₀ reduction of 5.11 CFU/mL, which equates to 99.999%.

Table 5. The average number of mold colonies present on the control and test sample plates after a 2 minute contact time with 100 ppm PAA from Reflex.

Organism	Avg. log ₁₀	log ₁₀ Reduction	% Reduction
<i>Aspergillus niger</i> control	5.83	NA	NA
<i>Aspergillus niger</i> test	4.00	1.83	98.5
<i>Byssoschlamys fulva</i> control	7.04	NA	NA
<i>Byssoschlamys fulva</i> test	1.93	5.11	99.999

IV. CONCLUSION

- Reflex demonstrates broad spectrum efficacy against a variety of microorganisms including bacteria, yeasts, and molds.
- As expected, *Salmonella typhimurium* and *Listeria monocytogenes* bacteria, being the lowest life forms, were totally eradicated using 100 ppm PAA from Reflex within one minute following the treatment.
- The higher life form yeast, represented here by *Candida albicans* and *Saccharomyces cerevisiae*, was much hardi-

er than the bacteria. The same PAA treatment effected close to a 3 log₁₀ reduction.

- *Aspergillus niger* mold proved to be the most robust organism with the highest resistance to 100 ppm of PAA from Reflex. A reduction of about 2 log₁₀ was elicited at a contact time of two minutes. However, *Byssochlamys fulva* mold was quite susceptible and was reduced in population by log₁₀ of 5.11 over two minutes.