JOSEPH DONABED, B.S.
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Efficacy of Reflex against Various Planktonic Bacteria, Yeasts, and Mold

BACKGROUND

Reflex is an EPA approved formulated sanitizer/disinfectant pro-duced at Enviro Tech Chemical Services, Inc. The active ingredi-ent 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 de-veloped for dairy production facilities, which require antimicro-bials 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 stud-ies, 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 con-centration 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
Listeria monocytogenes	Bacteria	100 ppm	1 min.
Salmonella typhimurium	Bacteria	100 ppm	1 min
Candida albicans	Yeast	100 ppm	1 min
Saccharomyces cerevisiae	Yeast	100 ppm	1 min
Aspergillus niger	Mold	100 ppm	2 min
Byssochlamys fulva	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 contami-nated foods such as meat or dairy products. Prevention of lister-iosis as a food illness is important and requires reliable and ef-fective sanitation practices.

Salmonella typhimurium

Salmonella typhimurium is a pathogenic, gram negative bacteria predominately found in the intestinal lumen of mammals. Sal-monella 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 ei-ther 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, bak-ing, and as a model organism in science. S. cerevisiae can be iso-lated 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 i*s 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. ni-ger is ubiquitous to the soil but can also be found in the envi-ronment. 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.

Byssochlamys fulva

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

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 sam-ples. One sample was used as the control and the other served the testing sample.

Salmonella typhimurium (ATCC® 14028) was grown in Sigma Nu-trient 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 buff-er. 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 indica-tive for Candida albicans growth. The yeast was aseptically trans-ferred to 1.0 L of sterile Butterfield's Buffer which was then thor-oughly 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 reconstitut-ed 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 fila-mentous 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.

Byssochlamys 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 solu-tion 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 cov-ered with filamentous white color, growth characteristic of *Bys-sochlamys 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 ana-lyzed by iodometric titration and found to have a PAA concentra-tion 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 solu-tion were neutralized with a calculated amount of erythorbic acid. After neutralization the test solution was analyzed again to verify that all active PAA and H_2O_2 was neutralized. Aliquots were then removed from each test solution, serially diluted, plated the appropriate media.

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 chal-lenge study.

Organism	Init. pH	Final pH	Organism Medium	Conc. PAA (ppm)
L. monocytogenes	6.36	2.33	3M Environ. Listeria Petrifilms™*	94.3
S. typhimurium	7.77	2.83	3M Enterobacteriaceae Petrifilms™*	109.1
C. albicans	5.69	2.56	Hardy Diagnostic Sabdex Agar*	87.7
S. cerevisiae	7.23	2.88	Hardy Diagnostic Sabdex Agar*	104.9
A. niger	7.84	2.91	Hardy Diagnostic Sabdex Agar*	114.5
B. fulva	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. There-fore, 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 samp le Petrifilms® after a 1 minute contact time with 100 ppm PAA from Reflex.

Organism	Avg. $\log_{10}$	$\log_{10}$ Reduction	% Reduction
Listeria monocytogenes control	6.86	NA	NA
Listeria monocytogenes test	0	>	6.86 > 99.9999
Salmonella typhimurium control	7.03	NA	NA
Salmonella typhimurium test	0	>	7.03 > 99.99999

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 reduc-tion 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
Candida albicans control	6.84	NA	NA
Candida albicans test	4.22	2.62	99.7
Saccharomyces cerevisiae control	6.24	NA	NA
Saccharomyces cerevisiae 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 reduc-tion of 1.83 CFU/mL, which equates to 98.5%.

The Byssochlamys 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 reduc-tion 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
Aspergillus niger control	5.83	NA	NA
Aspergillus niger test	4.00	1.83	98.5
Byssochlamys fulva control	7.04	NA	NA
Byssochlamys fulva test	1.93	5.11	99.999

CONCLUSION

- Reflex demonstrates broad spectrum efficacy against a variety of microorganisms including bacteria, yeasts, and molds.
- As expected, Salmonella typhimurium and Listeria mono-cytogenes bacteria, being the lowest life forms, were to-tally 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 hardier than the bacteria. The same PAA treatment effected close to a 3 log10 reduction.
- Aspergillus niger mold proved to be the most robust or-ganism 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.