Study Title

Efficacy of Perasan MP-2
Against *Salmonella* Heidelberg Inoculated Poultry Marinade

Efficacy of Perasan MP-2
Against *Campylobacter jejuni* Inoculated Poultry Marinade

Data Requirements

Efficacy Data for FSIS, USDA, FDA

Author/ Performing Laboratory

Joseph Donabed, B.S.

Enviro Tech Chemical Services, Inc.

Sponsor

Michael Harvey

Enviro Tech Chemical Services, Inc.
500 Winmoore Way
Modesto, CA 95358

Study Completion Date

26th June, 2015

Total pages: 9

Efficacy of Perasan MP-2 against *Salmonella* Heidelberg and *Campylobacter jejuni* Inoculated Poultry Marinade

Background

Marinated poultry products are very popular in the restaurant industry, primarily fast food restaurants. They can also be purchased at local grocery stores for consumer consumption. Currently, pathogen control in the marinade is virtually non-existent. Typically marinade is applied during secondary poultry processing. Although the poultry products are treated with a food contact substance (FCS) prior to being marinated, there is a high probability that the poultry products could be re-contaminated with potentially harmful pathogens after being treated with the FCS. In order to eliminate this risk, Enviro Tech Chemical Services proposes dosing peracetic acid (PAA) into the marinade. This application will have multiple benefits. First, the PAA will aid in reducing pathogens present in the marinade. Additionally, the PAA present in the marinade will eliminate any pathogens present on the poultry. This proposed application is a new microbial intervention that will increase food safety in the poultry industry.

Enviro Tech Chemical Services proposes a maximum concentration of 50 mg/L (ppm) PAA in the marinade with a hydrogen peroxide and 1-hydroxyethane 1,1-diphosphonic acid (HEDP) concentration equal to 18.4 ppm and 2.42 ppm respectively. Due to the accumulation, HEDP may build up to 7.2 ppm. The purpose of this study is to determine if 40 ppm PAA from Perasan MP-2 is sufficient at eliminating *Salmonella* Heidelberg and *Campylobacter jejuni* inoculated poultry marinade.

Materials and Methods

The Food Contact Substances:

Using iodometric titration, Perasan MP-2 (lot#825-061615-1) yielded a PAA concentration of 15.22% and a hydrogen peroxide (H_2O_2) concentration of 5.85%. A 10,000 mg/L of peracetic acid (PAA) was made by diluting 5.87 mL of the Perasan MP-2 to 100 mL using reverse osmosis water in a Class A 100 mL volumetric flask. This solution would serve as the PAA stock solution. The concentration of PAA in the stock solution was analyzed using the Palin Modified DPD methodology (Enviro Tech's Patent No. US 7,651,860 B2) and yielded a concentration of 9844 mg/L (ppm) PAA.

Part 1: Efficacy of Perasan MP-2 against *Salmonella* Heidelberg Inoculated Poultry Marinade

Test Solution:
Poultry Marinade

Two, 1-gallon samples of fresh marinade from a large poultry processing facility in the U.S. was packaged on dry ice and mailed overnight to Enviro Tech Chemical Services. Inc. (Modesto,

CA) on June 19, 2015. The large poultry processing facility did not wish to disclose the exact recipe of the marinade but a general composition of the ingredients was detailed:

- Onion and Garlic powder
- Monosodium glutamate (MSG)
- Yeast
- Citric acid
- Garlic powder
- Hydrolyzed corn protein
- Phosphates
- Sugar and Salt
- Soy Sauce

The temperature of the marinade on arrival was 1.2°C at a pH of 7.62. A total of 650 mL of marinade was measured and aseptically transferred to a sterile beaker. A magnetic stir bar was added to the marinade and the solution was mixed at 600 rpm on a magnetic stir plate.

Test System:
Salmonella Heidelberg bacteria (ATCC 8326)

Salmonella Heidelberg bacteria were cultured on 5% Sheep's Blood agar plates (Hardy Diagnostics, Cat # A600) by incubation for two days at 35^{o} C. The bacteria were scraped from the plates using an L-shaped spreader and transferred to the 650 mL marinade sample. The *S.* Heidelberg inoculated marinade was mixed for 15 minutes to ensure homogeneity of the bacteria. The pH of the inoculated marinade was 7.65. Next, three aliquots were taken from the stock solution, serially diluted, and plated on 3M Enterobacteriaceae Petrifilms™. These three sets would serve as the control for this study.

Treatment Solutions:

The remaining *S.* Heidelberg inoculated marinade was divided into three 200 mL samples. Using the 9844 ppm PAA stock solution, each of the 200 mL was dosed with a nominal 40 ppm PAA by adding 813 µL of the PAA stock solution to each 200 mL marinade sample. The solutions were mixed at 300 rpm and the concentration of PAA was measured over a 60 minute time interval using the Palin modified DPD methodology. After 30 and 60 minutes of contact with the nominal 40 ppm PAA, 10 mL aliquots were taken from each of the 200 mL samples and transferred to a sterile beaker. A total of 360 µL of a 10% erythorbic acid solution was added to each 10 mL aliquot to neutralize any active peroxygen. The neutralized aliquots were then serially diluted and plated on 3M Enterobacteriaceae Petrifilms™. The Petrifilms™ were incubated at 37°C for 24 hours then enumerated.

Part 2: Efficacy of Perasan MP-2Against *Campylobacter jejuni* Inoculated Poultry Marinade

Test Solution:
Poultry Marinade

Using the same marinade source used in Part 1, 650 mL of the marinade was measured out and aseptically transferred to a sterile beaker. A magnetic stir bar was added to the marinade and the solution was mixed at 600 rpm on a magnetic stir plate.

Test System:
Campylobacter jejuni bacteria (ATCC 33291)

Campylobacter jejuni (ATCC 33291) was cultured in Bolton Broth (Sigma Aldrich, lot number BCBB7257) containing 5% defibrinated sheep blood (Hardy Diagnostics) by anaerobic incubation at 40.2° C for 48 hours. The bacteria were separated from the nutrient broth by centrifugation. One mL of the concentrated bacteria mixture was removed and plated on Campy Cefex Agar (Hardy Diagnostics). This was repeated four times to achieve a total of four *Campylobacter*-inoculated Campy Cefex Agar plates. The plates were kept under anaerobic atmosphere and incubated for 48 hours at 40.2°C. After two days, the surfaces of the four *Campylobacter*-inoculated Campy Cefex Agar plates were aseptically scraped using a sterile L-shaped spreader and transferred to the 650 mL of poultry marinade. The *C. jejuni*-inoculated marinade was mixed for 15 minutes to ensure homogeneity of the bacteria. The pH of the inoculated marinade was 7.68. Next, three aliquots were taken from the stock solution, serially diluted, and plated on Campy Cefex Agar. These three sets would serve as the control for this study.

Treatment Solutions:

The remaining *C. jejuni* inoculated marinade was divided into three, 200 mL samples. Using the 9844 ppm PAA stock solution, each of the 200 mL was dosed with a nominal 40 ppm PAA by adding 813 µL of the PAA stock solution to each 200 mL marinade sample the solutions were mixed at 300 rpm. The concentration of PAA was measured over a 60 minute time interval using the Palin modified DPD methodology. After 30 and 60 minutes of contact with the nominal 40 ppm PAA 10 mL aliquots were taken from each of the 200 mL samples and transferred to a sterile beaker. A total of 360 µL of a 10% erythrobic acid solution was added to each 10 mL aliquot to neutralize any active peroxygen. The neutralized aliquots were then serially diluted and plated on Campy Cefex Agar plates. The agar plates were incubated under anaerobic conditions at 40.5°C for 48 hours then enumerated.

Results and Discussion

Part 1: Efficacy of Perasan MP-2 against *Salmonella* Heidelberg Inoculated Poultry Marinade

Table 1: PAA concentrations from Perasan MP-2 in the *S.* Heidelberg inoculated poultry marinade over the 60 minute time interval

Time (minutes)	Conc. PAA (mg/L)
0.5	39.59
10	36.81
20	28.03
30	26.75
45	22.47
60	20.97

Table 2: Average efficacy over three trials of a nominal 40 ppm PAA against *S.* Heidelberg in poultry marinade

Description	Avg. $\log_{10}$ (CFU/mL)	STD Dev.(CFU/mL)	Avg. $\log_{10}$ Reduction	% Reduction
Control	6.25	0.11	NA	NA
40 ppm PAA 30 minutes	2.76	0.06	3.49	99.9674
40 ppm PAA 60 minutes	0	0	>6.34	>99.9999

The control marinade sample (untreated) had an average $\log_{10}$ of 6.25 ± 0.11 CFU/mL of *Salmonella* Heidelberg in the three trials. After 30 minutes of treatment with a nominal 40 ppm PAA from Perasan MP-2, the average $\log_{10}$ of *S.* Heidelberg decreased to 2.76 ± 0.06 CFU/mL. After 60 minutes of contact with a nominal 40 ppm PAA there were no viable *S.* Heidelberg colonies remaining.

Part 2: Efficacy of Perasan MP-2 against *Campylobacter jejuni* Inoculated Poultry Marinade

Table 3: PAA concentration from Perasan MP-2 in the *Campylobacter jejuni* inoculated poultry marinade over the 60 minute time interval

Time (minutes)	Conc. PAA (mg/L)
0.5	39.16
10	37.45
20	32.31
30	28.25
45	23.33
60	21.40

Table 4: Average efficacy over three trials of a nominal 40 ppm PAA against *Campylobacter jejuni* in poultry marinade

Description	Avg. log_{10} (CFU/mL)	STD Dev.(CFU/mL)	Avg. log_{10} Reduction	% Reduction
Control	4.38	0.14	NA	NA
40 ppm PAA 30 minutes	1.12	0.09	3.26	99.95
40 ppm PAA 60 minutes	0	0	>4.38	>99.99

The control marinade sample (untreated) had an average log_{10} of 4.38 ± 0.14 CFU/mL of *Campylobacter jejuni* in the three trials. After 30 minutes of treatment with a nominal 40 ppm PAA from Perasan MP-2, the average log_{10} of *C. jejuni* decreased to 1.12 ± 0.09 CFU/mL. After 60 minutes of contact with a nominal 40 ppm PAA there were no viable *C. jejuni* colonies remaining.

Statistical Analysis

The Students' t-test was used to assess whether the means of the microbiological results obtained in this study are significantly different from each other when comparing the control (untreated) and the poultry marinade treated with PAA. In doing so, the "t-test: Two Sample Assuming Un-Equal Variances" (which is part of the Analysis ToolPak of Excel 2013) was used to perform the statistical analysis. This involved statistically comparing the means of the control and the means of the PAA treated marinade using the number of degrees of freedom and the t-table. Therefore, if the t stat > t value, the means of differences are significantly different. A probability of p = 0.05 means that the level of confidence that the data are correct is equal to 95%. The data obtained in this study and seen in Addendums 1and 2 show statistically significant ($p<0.05$) differences in the microbiological results obtained from the untreated poultry marinade (control) compared to the poultry marinade treated with 40 ppm PAA from Perasan MP-2

Conclusions

Part 1: Efficacy of Perasan MP-2 against *Salmonella* Heidelberg Inoculated Poultry Marinade

- The *Salmonella* Heidelberg inoculated marinade control contained a log_{10} of 6.25 CFU/mL. After 60 minutes of contact with a nominal 40 ppm PAA from Perasan MP-2, there were no viable S. Heidelberg colonies remaining.
- The results of this suggest that Perasan MP-2 at a concentration of 40 ppm as PAA is very effective at eradicating *Salmonella* Heidelberg in poultry marinade with a contact time of 60 minutes.

Part 2: Efficacy of Perasan MP-2 against *Campylobacter jejuni* Inoculated Poultry Marinade

- The *Campylobacter jejuni* inoculated marinade control contained a $\log_{10}$ of 4.38 CFU/mL. After 60 minutes of contact with a nominal 40 ppm PAA from Perasan MP-2, there were no viable *C. jejuni* colonies remaining.
- The results of this suggest that Perasan MP-2 at a concentration of 40 ppm as PAA is very effective at eradicating *Campylobacter jejuni* in poultry marinade with a contact time of 60 minutes.

ADDENDUM 1: Salmonella Heidelberg Inoculated Poultry Marinade

log_{10} (CFU/mL) Control (untreated) vs. 40 ppm PAA treated poultry marinade against *Salmonella* Heidelberg		
	Test Solutions	
Sample Number	log_{10} Remaining (CFU/mL) Control (Untreated)	log_{10} Remaining (CFU/mL) PAA (Treated)
Marinade #1 (10 min Contact)	6.34	2.71
Marinade #2 (10 min Contact)	6.12	2.82
Marinade #3 (10 min Contact)	6.28	2.75

t-Test: Two-Sample Assuming Un-Equal Variances			
		"Control vs. Treated" Samples	
		Variable 1	*Variable 2*
Mean		*6.247	*2.760
Variance		0.0129	0.0031
Observations		3	3
Hypothesized Mean Difference		0	
df		3	
t Stat[1]		47.6935	
P(T<=t) one-tail		1.01479E-05	
t Critical one-tail		2.353363435	
P(T<=t) two-tail		2.02958E-05	
t value		3.182446305	

1 Means within a grouping with an asterisk are significantly different (P<0.05) as determined by the t-test which assesses whether the means of two groups are statistically different from each other. Therefore if t Stat > t value the means of differences are significantly different. A probability of p = 0.05 (95% probability of making a correct statement).

Using a t-table, the confidence level was approximated. The t-stat (47.6935) exceeded the 99.9% confidence levels with a t-value of 3.1824. To determine the exact confidence level, the P- value was used in the determination (100% - 0.0000002%), thus, the confidence level is established at >99.999% that the average *Salmonella* Heidelberg treated marinade samples are statistically different when comparing the control (untreated marinade).

ADDENDUM 2: *Campylobacter jejuni* Inoculated Poultry Marinade

log_{10} (CFU/mL) Control (untreated) vs. 40 ppm PAA treated poultry marinade against *Campylobacter jejuni*		
	Test Solutions	
Sample Number	log_{10} Remaining (CFU/mL) Control (Untreated)	log_{10} Remaining (CFU/mL) PAA (Treated)
Marinade #1 (10 min Contact)	4.36	1.04
Marinade #2 (10 min Contact)	4.25	1.11
Marinade #3 (10 min Contact)	4.53	1.21

t-Test: Two-Sample Assuming Un-Equal Variances			
		"Control vs. Treated" Samples	
		Variable 1	*Variable 2*
Mean		*4.380	*1.120
Variance		0.0199	0.0073
Observations		3	3
Hypothesized Mean Difference		0	
df		3	
t Stat[1]		34.23684805	
P(T<=t) one-tail		2.73922E-05	
t Critical one-tail		2.353363435	
P(T<=t) two-tail		5.47844E-05	
t value		3.182446305	

1 Means within a grouping with an asterisk are significantly different (P<0.05) as determined by the t-test which assesses whether the means of two groups are statistically different from each other. Therefore if t Stat > t value the means of differences are significantly different. A probability of p = 0.05 (95% probability of making a correct statement).

Using a t-table, the confidence level was approximated. The t-stat (34.2368) exceeded the 99.9% confidence levels with a t-value of 3.1824. To determine the exact confidence level, the P- value was used in the determination (100% - 0.0000002%), thus, the confidence level is established at >99.999% that the average *Campylobacter jejuni* inoculated marinade treated marinade samples are statistically different when comparing the control (untreated marinade).