NUCRO TECHNICS

FINAL REPORT

ANTI-MICROBIAL EFFICACY EVALUATION OF PURO CANADA INC. OZONE DEVICE (NUTROZ) AGAINST GRAM NEGATIVE VEGETATIVE BACTERIA ON GLASS SURFACES

REPORT NO. 377096

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SIGNATORY PAGE

Prepared by:

Scott Chen
Supervisor, Microbiology Laboratories
Nucro-Technics

Aug. 3/21
Date

Reviewed and Approved by:

for John Fanaras
President
Nucro-Technics

Aug 13/2021
Date

1.0 PURPOSE

To demonstrate the antimicrobial efficacy of the NUTROZ device by determining the log reduction of vegetative bacterial populations on glass substrate coupons.

2.0 TEST ORGANISM

The following vegetative bacteria was used in testing:

Escherichia coli ATCC 8739 (Gram-negative)

3.0 TEST DEVICE

The NUTROZ device uses ozone (a form of oxygen comprised of three oxygen atoms: O_3). Ozone is generated and circulated within the NUTROZ device. The ozonation process is to be evaluated for its capability to kill vegetative bacteria on the surface of a representative hard, non-porous surface (glass coupons).

For testing, the NUTROZ device (S/N 19020013) was used with software version 6.2.10.

4.0 MATERIALS AND EQUIPMENT

- Centrifuge
- Filter funnels (sterile)
- Filtration manifold
- Glass slides (25mm x 75mm)
- Incubators (maintained at 30 – 35°C)
- Laminar Flow Hood
- Loops (10 µL volume, sterile)
- Membrane filters (0.45-micron mixed cellulose ester (MCE))
- Micropipettes (with appropriate sterile tips)
- Petri dishes (sterile)
- NUTROZ device (S/N 19020013)
- Serological pipets (sterile, various sizes)
- Syringe filters (0.2 micron, low-protein binding)
- Tubes (various sizes, sterile)
- Tweezers (sterile)
- Vortex mixer

5.0 MEDIA AND DILUENTS

Recovery/Growth medium:

- Tryptic Soy Agar with Lecithin and Polysorbate 80 (TS)
- Tryptic Soy Broth (TSB)

Diluents:

- 0.9% saline

6.0 PROCEDURE

Preparation of glass substrates:

Glass slides (25 mm x 75 mm) were used as the glass substrate coupon. Prior to testing, the glass slides were cleaned by slowly immersing into 95 – 100% ethanol three times, then slowly immersing in purified water three times. The cleaned slides were placed into glass Petri dishes and autoclaved to sterilize.

Preparation of culture suspension:

The vegetative test organism was transferred from a stock culture into TSB. The test organism was incubated aerobically for 18 – 24 hours at 30 – 35 °C. The test organism was then transferred into 3 x 15 mL volumes of TSB and incubated as above.

After incubation, the contents of the 3 culture suspensions were pooled and then dispensed, in 10 mL aliquots, into 4 centrifuge tubes. The culture suspensions were centrifuged at 2000 rpm for 30 minutes. The resulting supernatant was carefully removed from each tube, then the remaining pellets were each resuspended with 1 mL of sterile 0.9% saline. The resuspended culture was pooled and mixed (total volume = 4 mL), to be used as the inoculum suspension.

Enumeration of the inoculum suspension:

From the inoculum suspension, 10-fold serial dilutions were prepared up to 10^{-7}. From the last three dilutions (10^{-5} to 10^{-7}), 0.1 mL aliquots were plated in duplicate with TS, then incubated aerobically at 30 – 35°C for NLT 24 hours. After incubation, colonies were enumerated on the TS plates between acceptable limits of 25 – 250 CFU.

Test Procedure:

Seven (7) glass substrates were inoculated with 10 µL of inoculum suspension (approximately 10^7 CFU). The inoculate was evenly distributed over the surface of the coupons, taking care not to touch the edges. The inoculated substrates were placed into sterile Petri dishes in an incubator (30 – 35°C) until visibly dry (for 30 minutes).

Once dry, five (5) inoculated glass substrates were aseptically placed directly onto the middle shelf of the NUTR0Z device (inoculated side up). One cycle was run
using the 5 inoculated glass substrates.

The remaining two (2) inoculated glass substrates were used as viability controls and were not treated with the device. One viability control was assessed immediately after drying at the initial time (pre-cycle) while the other viability control was held at ambient conditions for the duration of the NUTR0Z device cycle and assessed after the cycle has completed (post-cycle).

To assess recovery from sample/viability controls, the glass substrates were transferred into separate sterile tubes containing 60 mL 0.9% saline. The glass substrate + saline suspensions (1 0^0) were mixed by vortex for 30 seconds, followed by 10-fold serial dilutions in 0.9% saline (up to 10^{-6}). For samples, the entire contents of each dilution (up to 1 0^{-6} and including 1 0^0) were filtered aseptically through a 0.45-micron mixed cellulose ester (MCE) membrane filter. For viability controls, the entire contents of the final 3 dilutions (1 0^{-4} to 1 0^{-6}) were filtered aseptically through a 0.45-micron MCE membrane filter. Each filter was rinsed with 50 mL of 0.9% saline, then aseptically transferred onto TS plates. The plates were incubated aerobically at 30 –35°C for NLT 24 hours.

After incubation, colonies were enumerated on the membrane filters between acceptable limits of 20 –200 CFU (or closest to this range where needed).

Negative controls:

Saline:
For each daycode/lot, 50 mL 0.9% saline was filtered through a 0.45 µm MCE membrane filter.
Filter was transferred onto a TS plate then incubated aerobically at 30 –35°C for NLT24 hours

TS:
One TS plate per media daycode was incubated aerobically at 30 –35°C for NL T 24 hours.

7.0 RESULTS

Table 1. Antimicrobial efficacy of NUTROZ device against *Staphylococcus aureus* on glass substrates

Cycle	Coupon	Recovered CFU/ coupon	Log value	Log reduction		Average log reduction	
				Pre	Post	Pre	Post
1	Treated Sample (Replicate 1)	84	1.9	3.7	2.8	2.8	2.0
	Treated Sample (Replicate 2)	3.6×10^3	3.5	2.0	1.2		
	Treated Sample (Replicate 3)	76	1.9	3.6	2.8		
	Treated Sample (Replicate 4)	3.4×10^4	4.5	1.0	0.2		
	Treated Sample (Replicate 5)	89	1.9	3.6	2.8		
	Viability Control (Pre-cycle)	3.4×10^5	5.5	N/A	N/A		
	Viability Control (Post-cycle)	5.3×10^4	4.7	N/A	N/A		

Inoculum control: 9.0×10^9 CFU/mL [9.0×10^7 CFU/0.01 mL]

From 5 replicates across 1 cycle run, the NUTROZ device (S/N 19020013, Software V6.2.10) demonstrated an average log reduction of *Escherichia coli* (ATCC 8739) on glass substrates of 2.8 from pre-cycle viability counts and 2.0 from post-cycle viability counts.

No growth was observed for either of the negative controls described in Section 6.0.

8.0 CONCLUSION

Based on the above-mentioned results from 5 replicates in 1 run, it can be concluded that treatment of glass substrate coupons through one full cycle of the NUTROZ device (S/N 19020013, Software V6.2.10, middle shelf) will reduce the presence of *Escherichia coli* (ATCC 8739) from the glass surface by an average 2.8-log value (based on pre-cycle viability counts).

DATE: ~~Jul 29/21~~ Jul 26/21 (*) ~~Jul 27/21 (*)~~ CUSTOMER: Magna Closures Inc. PROJECT NO.: 377096

Anti-microbial efficacy evaluation of Puro Ozone device against gram negative ~~vegetatit~~ vegetative bacteria on glass surfaces

1. Preparation of glass substrates:

Glass slides (25 mm x 75 mm) ~~will be~~ was used as the glass substrate coupon. Glass slides were cleaned by slowly immersing into 100% ethanol 3 times, then slowly immersing in purified water 3 times. Slides were then placed into a glass Petri dish and autoclaved to sterilize.

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2. Preparation of culture suspension:

Escherichia coli ATCC# 8739 (T4 - Vial 24) was transferred into 3x15mL TSB (day code: 2031 exp: Aug 05/21) Inc. @ 30-35°C (inc #1) for 18-24 hrs.

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3. After inubation, the contents of the 3 TSB culture suspensions were pooled, then dispensed in 10mL aliquots into 4 centrifuge tubes. The culture suspensions were centrifuged @ 2000 x rpm for 30 mins (centrifuge NI# 00953 cal due: Dec 04/21). The supernatant from each tube was removed, then the remaining pallets were resuspended with 1mL sterile saline 0.9%. All resuspended cultures were pooled and mixed (total volume = 4mL), this is the inoculum suspension.

4. Enumeration of the inoculum suspension:

Diluted the inoculum suspension up to 10^{-7} using 0.9% saline From the last three dilutions (10^{-5}, 10^{-6} & 10^{-7}), 0.1mL aliquots were plated in duplicate with TS (day code: 2041, exp: Aug 06/21). Inc. @ 30-35°C (inc#1) for NLT 24hrs. Time in: 1:30pm

* Spelling error, DL, ~~Jul 29/21~~ (*) Doumentation error, DL, Jul 26/21 wrong date, Jul 26/21, DL

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5. Test procedure:

7 glass substrates were inoculated with 10 µL of inoculum suspension under the biohazard hood* The inoculated substrates were placed into a sterile Petri dish in an incubator @ 30-35°C for 30 mins (Timer M-TM-22 cal due: Oct 13/21) to allow the inoculum suspension to dry.

Once drying was completed, 5 inoculated glass substrates were aseptically placed onto the middle shelf of the PURO device (inoculated side up). The remaining 2 inoculated glass substrates were used as viability controls.

After placing the 5 inoculated substrates inside the PURO device (S/N: 190.20013 Version 6.2.10 "40 min" generation, "20 min" neutralization), the cycle was initiated. Immediately, one viability control (pre-cycle) was assessed while the other one (post-cycle) was held at ambient conditions for the duration of the PURO device cycle.

To assess the pre-cycle viability control, the glass substrate was transferred into a sterile tube containing 60mL 0.9% saline. The tube was vortexed ~~by~~ for* 30 seconds, followed by 10-fold dilutions in 0.9% saline up to 10^{-6}. The entire contents of dilutions 10^{-4}, 10^{-5} & 10^{-6} were filtered through a 0.45µm NCE MF (LOT # 9090.5202 exp: Sept 30/22). The MF was rinsed with 50mL 0.9% saline, then transferred onto TS plates.

Once the cycle was completed, the post-cycle viability control was assessed in the same way as the pre-cycle control mentioned above.

Then, ~~samples~~* the recovery of from the 5 samples was tested. Each glass substrate was transferred into separate sterile tubes containing 60mL 0.9% saline. Each tube was vortexed for 30 seconds, followed by 10-fold dilutions up to 10^{-6} (using 0.9% saline). For each sample,

* Documentation error, DL, Jul 27/21

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5. Test procedure continues:

the entire contents of each dilution* (10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 10^{0}) was filtered through a 0.45µm MCE MF. The MF was rinsed with 50mL 0.9% saline, then transferred onto TS plates. (*Note: for sample 2, only 30mL were filtered for dilution 10^{0} due to spillage.)

TS plate were incubated @ 30-35°C (inc #1) for MLT 24hrs.

Time in: 1:30pm Time out: Jul 28/21 @ 2:00pm

6. Negative controls:

° Substrate: one uninoculated sterile glass substrate was transferred into a sterile tube containing 60mL 0.9% saline. The tube was vortexed for 30 seconds, then filtered through a 0.45µm MCE MF. The MF was rinsed with 50mL 0.9% saline, then transferred onto a TS plate.

° Saline: each separate day code of saline used was tested. 50mL 0.9% saline was filtered through a 0.45µm MCE MF. MF was transferred onto a TS plate.

° TS: one TS plate was incubated.

Inc. all neg. controls @ 30-35°C (inc #1) for MLT 24hrs.

Time in: 1:30pm Time out: Jul 28/21 @2:00 pm

Media & equipment	day code	exp:
Saline	1751	Dec 24/21
Saline	2011	Jan 20/22
Saline	2071	Jan 26/22
TS	2041	Aug 06/21
TS	1971	Jul 30/21

Vortex #07; Micropipetter NT# #328088 cal due: Jul 29/21; Micropipette NT#3990619 cal due: Aug 03/21; Biohazard hood NT# 002462; glass slides Corning L) 32220009

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Results:

Date/Time out/By: Jul 28/21 @ 2:00 pm DL

Calculations by: DL

Sample #/ Control	Observed dilution	Recovered CFU	CFU/substrate	log value	log reduction Pre-cycle	log reduction Post-cycle
#1	10^{0}	83	84	1.9	3.7	2.8
#2	10^{-1}	54	3.6×10^{3}	3.5	2.0	1.2
#3	10^{0}	75	76	1.9	3.6	2.8
#4	*~~10^{-2}~~	51	3.4×10^{4}	4.5	1.0	0.2
#5	10^{0}	88	89	1.9	3.6	2.8
Pre-cycle viability control	10^{-4}	51	3.4×10^{5}	5.5		
Post-cycle viability control	10^{-4}	8	5.3×10^{4} ~~10^{5}~~*	4.7 ~~5.7~~*		
				Average	2.8	2.0

*Documentation error, DL, Jul 28/21

Example calculation:

$$\text{CFU/substrate} = \frac{\text{CFU/plate}}{\text{Vol. filtered}} \times \text{dilution factor} \times \frac{\text{volume suspended}}{\text{substrate}}$$

$$\#1 \quad \text{CFU/substrate} = \frac{83 \text{ CFU}}{59 \text{ mL}} \times 10^{0} \times \frac{60 \text{ mL}}{\text{substrate}} = 84 \text{ CFU/substrate}$$

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Results:

Example log reduction calculation:

log reduction (pre-cycle / post-cycle) = log value from (pre-cycle viability control / post-cycle viability control) =

= log value of treated sample

Neg controls results:

- substrate: 0
- saline: 0 / 0 / 0
- TS: 0

Inoculum control results:

Recovered CFU	Observed dilution	Vol. plated
88 / 92	10^{-7}	0.1ml

CFU/ml: $\left(\frac{88+92}{2}\right)$ CFU $\times \frac{10^{7}}{0.1\text{mL}} = 9.0\times10^{9}$ CFU/mL

$(9.0\times10^{7}$ CFU $(0.01$ mL$)^{*}$

* information added by SC Jul 29/21

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