Campden Technology Limited
Station Road
Chipping Campden
Gloucestershire
GL55 6LD, UK

Tel: +44 (0)1386 842000
Fax: +44 (0)1386 842100
www.campden.co.uk

Campden BRI
food and drink innovation

EFFICACY OF OZONATED WATER PRODUCED BY BIOTEK EQUIPMENT AGAINST A RANGE OF BACTERIA AND FUNGI OVER A FIVE MINUTE CONTACT PERIOD

CONFIDENTIAL TO:

Colin McArthur
Biotek-ozone (UK)
4 Bowling Street
Coatbridge
ML5 1PP
&
Manufacturer: BioSure (a division of Biotek Environmental Science Ltd)
5F 98 Xingde Road
Sanchung District
New Taipei City
Taiwan

REPORT NUMBER: MB/REP/122919/1

21st February 2012

Lawrence Staniforth, Matthew Bennington

Report Authorised By:		Report Checked by:	
Name:	Mrs. J. Gaze		Mr. S. Ridgway
Function:	Deputy Head of Department, Microbiology		Technical Officer

This report shall not be reproduced except in full without written approval from Campden BRI

Campden Technology Limited, Registered no. 3836922, Incorporated in England & Wales
Registered Office: Station Road, Chipping Campden, Gloucestershire. GL55 6LD
Part of Campden BRI Group

Information emanating from Campden BRI is given after the exercise of all reasonable care and skill in its compilation, preparation and issue, but is provided without liability in its application and use.

ISO 9001 CERTIFIED

SUMMARY

The ozonated water was shown to be bactericidal and yeasticidal under both clean and dirty conditions within five minutes.

Based upon the results, ozonated water (3.7 ppm ozone), when used at a flow rate of 420 mL/minute, successfully produces a greater than 5 log reduction in all bacterial challenges and greater than 4 log reduction against yeast challenges within 5 minutes under both clean and dirty conditions.

The ozonated water was shown not to be fungicidal within 5 minutes against *A. niger* spores under either condition.

1. INTRODUCTION

Campden BRI was approached by Biotek Ozone (UK) and asked to undertake disinfection tests using ozonated water produced by Biotek Ozone (UK) system as part of the M&S approval scheme disinfectants.

The M&S approval scheme requires that disinfectants be tested to European standards EN 1276[1] (+ additional organisms at 10°C) and EN 1650[2] (+ additional organism at 20°C), these tests were designed for disinfectants that are produced as concentrates and then diluted prior to use and thus stable. Ozonated water is generated at point of use and cannot be generated at high active (ozone) levels. Thus and is given the nature of ozonated water alternative testing regimes was agreed between Dr. J. Holah (Campden BRI) and Mr. Ian Abbotts (M&S).

"Ozone (O_3) is an oxidizing agent which reacts readily with most types of organic matter and which is active against a wide range of microorganisms. It is more efficient at lower temperatures and requires a minimum humidity of ca. 60% for the disinfection of surfaces. It is only active against surface contaminants and is not suitable for use with fats meat etc. which are likely to be oxidized[3]." As ozonated water and its means of application are not suitable for aqueous suspension tests such as the EN 1276 and EN1650, the filtration method used was designed to maintain the principles described below:

- Organisms and level of challenge
- Use of "clean" and "dirty" conditions
- Requirement for a 5 log reduction against bacteria and 4 log reduction against fungi within 5 minutes
- Validation of the recovery method

In situ the factory machines would have a flow rate of 10L/minute, this cannot be emulated within the laboratory with a flow rate through the filter membranes of approximately 0.42L/minute or 1/25th that achieved by the factory machines.

2. MATERIALS AND EQUIPMENT

2.1 Products tested

Ozonated water generated from Biotek system.

2.2 Media and equipment

Sterile Petri dishes 90mm
Sterile Pall 0.45 µm filter units
WL3 safety cabinet
Vortex
pH meter
Volumetric flasks (100mL, 1000mL)
Calibrated pipettes of nominal capacities 10mL, 1mL, 0.1mL and calibrated automatic pipette with capability of 10µL
Water baths capable to being controlled at 45°C ± 1°C
Sterile inoculating loops
Incubator set at 37°C ± 1°C
Stopwatch
Sterile forceps
Propanol-2-ol, 70% (v/v)
TSA BS - Tryptone Soya Agar (Oxoid CM131)
MEA BS – Malt Extract Agar
SDW - Sterile Distilled Water.

2.3 Interfering substance

BSA - Bovine Serum Albumin, Cohn fraction V for Dubos medium (BDH) preparation for "Clean" conditions:
0.03g (± 0.01g) of BSA was dissolved in 100mL (± 1.0mL) of sterile distilled water and filter sterilised using a syringe and a membrane filter with a maximum effective pore size 0.45µm. The final concentration of bovine albumin in the test procedure was 0.3g L^{-1}.
BSA - Bovine Serum Albumin, Cohn fraction V for Dubos medium (BDH) preparation for "Dirty" conditions:
0.3g (± 0.01g) of BSA was dissolved in 100mL (± 1.0mL) of sterile distilled water and filter sterilised using a syringe and a membrane filter with a maximum effective pore size 0.45µm. The final concentration of bovine albumin in the test procedure was 3g L^{-1}.

2.4 Diluent

Diluent BS ingredients:
Tryptone pancreatic digest of casein (LABM MC5) - 1g
Sodium chloride - 8.5g
Made up to 1000mL with deionised water
pH 7.0 ± 0.2 when measured at 20°C after sterilisation

2.5 Rinse liquid Neutraliser

Sterile distilled water.

2.6 Challenge organisms

Pseudomonas aeruginosa FH72h
Staphylococcus aureus FH73h
Escherichia coli FH64h
Enterococcus hirae FH 65f
Listeria monocytogenes FH66d
Salmonella Typhimurium FH 68d
Yersinia enterocolitica FH67b
Escherichia coli O157:H7 (non toxigenic strain due to filtration being used).
Aspergillus niger
Candida albicans
Saccharomyces cerevisiae

2.7 Membrane filtration apparatus

A filter holder of at least 100 ml volume was set up for use with filters of diameter 47 mm to 50 mm and 0,45 µm pore size. The vacuum source used gave an even filtration flow rate (approx 420 mL per minute).

3. METHOD

3.1 Preparation of bacterial suspension

For each challenge organism, a bacterial suspension was prepared from an 18 - 24h, 2nd passage culture on TSA, incubated at 37°C.

The bacterial suspension was prepared by suspending the bacterial growth from the 2nd passage subculture. 5g of sterile glass beads and 9mL diluent (BS) were added to each slope. The slope was then shaken gently to remove the culture from the agar surface. The resultant suspension was then filtered through a funnel containing sterile glass wool and eluted with further diluent (BS) to maximise recovery. It was vortexed for 30 seconds. The concentration of the bacterial suspension was determined by spectrophotometer, using a calibration graph of optical density at 420nm against viable count. The suspension was diluted as necessary, using diluent (BS), to give a cell concentration of approximately $5.0x10^8$ CFU mL^{-1} and then serially diluted with diluent (BS) down to 10^3 CFU mL^{-1}

3.2 Preparation of bacterial suspension

For each challenge organism, a fungal suspension was prepared from an 18 - 24h, 2nd passage culture on MEA, incubated at 30°C.

The fungal suspension was prepared by suspending the yeast and mould spores grown from the 2nd passage subculture. 5g of sterile glass beads and 9mL diluent (BS) were added to each slope. The slopes/plates were then shaken gently to remove the culture from the agar surface. The resultant suspension was then filtered through a funnel containing sterile glass wool and eluted with further diluent (BS) to maximise recovery. It was vortexed for 30 seconds. The concentration of the yeast cells in suspension was determined by spectrophotometer, using a calibration graph of optical density at 420nm against viable count. The suspension was diluted as necessary, using diluent (BS), to give a cell concentration of approximately $5.0x10^8$ CFU mL^{-1} and then serially diluted with diluent (BS) down to 10^3 CFU mL^{-1}.

The concentration of mould spores was determined using microscopy and a cell chamber. The suspension was diluted as necessary, using diluent (BS), to give a cell concentration of approximately $5.0x10^8$ CFU mL^{-1} and then serially diluted with diluent (BS) down to 10^3 CFU mL^{-1}.

3.2 Microbial test suspension

Once the working culture was prepared and diluted to the required concentration of approx. 5.0E+08 the test clean and dirty inocula was prepared by mixing even (1:1) volumes of BSA (0.3g/100mL for clean or 3g/100mL for dirty) and the relevant organisms suspension at approx. 5.0E+08. The test inoculum was plated out with the appropriate recovery media to enumerate the microbial suspensions.

3.3 Product preparation

The Biotek Ozonated water generator was installed by Biotek personnel and was used on the hand wash setting to generate ozonated water. The measured ppm ozone within the water was 3.7 ppm.

3.6 Test procedure

Initial screening tests demonstrated that the ozonated water directly applied to the filter forced the inocula into the sides of the filter unit limiting the access to the bacterial challenge. This was demonstrated by rings of bacterial growth on the filter paper. Thus sterile bottles were continually filled with ozonated water and then poured over the inoculated filters.

The tests were performed in duplicate at an ambient temperature (18°C - 25°C).

1. 0.2mL 1:1 mixture was pipetted into 50mL of BP diluents held within a filter holder.
2. This was then filtered, and the filtered membrane was washed with ozonated water for the allotted time (5 minutes) whilst the vacuum was running (flow rate of approximately 450mL per minute total volume ozonated water approximately 2.25L).
3. After 5 minutes washing with ozonated water the membrane was washed with 100 mL of BP diluent.
4. The filter was aseptically removed from the filter unit and placed upon a prepoured plate of the recovery media (TSA BS for bacteria and MEA BS for the fungi)
5. The TSA BS plates were incubated at 37°C ± 1°C for 48h and the MEA BS plates were incubated at 30°C ± 1°C for 4 days.

3.7 Validation of recovery (for each challenge organism)

1 Filter membranes (Validation Test) were washed with ozonated water for the allotted time (5 minutes) whilst the vacuum was running (flow rate of approximately 450mL per minute total volume ozonated water 2.25L).
2 Filter membranes (Validation Control) were washed with sterile distilled water for the allotted time (5 minutes) whilst the vacuum was running (flow rate of approximately 450mL per minute total volume ozonated water 2.25L).
3 After washing the membranes with ozonated or non ozonated water they were washed with 100 mL of BP diluent.
4 0.2mL 1:1 of an approximately 5.0E+03 CFU/mL mixture was pipetted into 50mL of BP diluents held within a filter holder and then filtered.
5 The filter was washed with a further 100mL BP diluent.
6 The filter was aseptically removed from the filter unit and placed upon a prepoured plate of the recovery media (TSA BS for bacteria and MEA BS for the fungi).

7 The TSA BS plates were incubated at 37°C ± 1°C for 48h and the MEA BS plates were incubated at 30°C ± 1°C for 4 days.

During testing the ozone content was monitored using an ozone meter at 3.7ppm.

4. RESULTS

4.1 Microbial test suspension (see section 3.2)

All inoculum concentrations were within or greater than required specifications.

4.2 Efficacy of Ozonated water results (see section 3.6)

Tables 4.1 and 4.2 summarise the results as $\log_{10}$ reduction achieved by the treated surface compared to the WSH control.

Table 4.1:

Log_{10} reduction of challenge organisms achieved after 5 minutes washing with ozonated water under dirty conditions

Challenge organism Dirty conditions 5 minute contact	cfu/mL 1:1 inoculum N	cfu/mL No	Log No	CFU/filter recovered	Log CFU recovered	Log reduction	Validity control Mean CFU/plate	Validity check Mean CFU/plate	Results valid +/- 50% difference to control
P. aeruginosa FH72h	6.50E+07	1.30E+07	7.11	<15	<1.18	>5.93	126	137	Valid
S. aureus FH73h	3.55E+08	7.10E+07	7.85	<15	<1.18	>6.67	98	109	Valid
E. coli FH64h	5.60E+08	1.12E+08	8.05	<15	<1.18	>6.87	145	136	Valid
E. hirae FH 65f	2.80E+08	5.60E+07	7.75	<15	<1.18	>6.57	120	126	Valid
L. monocytogenes FH66d	3.20E+08	6.40E+07	7.81	<15	<1.18	>6.63	13	2	NOT valid
S. Typhimurium *FH 68d*	2.15E+08	4.30E+07	7.63	<15	<1.18	>6.45	186	181	Valid
Y. enterocolitica FH67b	4.30E+08	8.60E+07	7.93	<15	<1.18	>6.75	134	139	Valid
E. coli O157:H7	2.80E+08	5.60E+07	7.75	<15	<1.18	>6.57	78	63	Valid
C. albicans	4.05E+07	8.1E+06	6.91	<15	<1.18	>5.73	157	101	Valid
S. cerevisiae	4.1E+07	8.2E+06	6.91	<15	<1.18	>5.73	139	132	Valid
A. niger	1.05E+08	2.1E+07	7.32	>300	>2.47	<4.85	7	3	Valid

N inoculum
No test challenge

The results above demonstrate that when ozonated water was applied onto organisms on a surface (0.20um filter membrane) under dirty conditions a greater than 5 log reduction was achieved against bacteria and a greater than 4 log reduction against yeast.
Ozonated water was not shown to be fungicidal at the concentrations and volumes tested against *A. niger*.
The validation of the recovery method against *L. monocytogenes* failed, however, in the context of the whole test it was clear that the ozonated water was bactericidal within 5 minutes and had an effect upon *L. monocytogenes*.

Table 4.2:

Log_{10} reduction of challenge organisms achieved after 5 minutes washing with ozonated water in clean conditions

Challenge organism 5 min contact Clean conditions	cfu/mL 1:1 inoculum	cfu/mL No	Log No	CFU/filter recovered	Log CFU recovered	Log reduction	Validity control Mean CFU/plate	Validity check Mean CFU/plate	Results valid +/- 50% difference to control
P. aeruginosa FH72h	2.40E+08	4.80E+07	7.68	<15	<1.18	>6.5	126	137	Valid
S. aureus FH73h	3.60E+08	7.19E+07	7.86	<15	<1.18	>6.68	98	109	Valid
E. coli FH64h	1.28E+10	2.55E+09	9.41	<15	<1.18	>8.23	145	136	Valid
E. hirae FH 65f	2.30E+08	4.60E+07	7.66	<15	<1.18	>6.48	120	126	Valid
L. monocytogenes FH66d	3.68E+08	7.36E+07	7.81	<15	<1.18	>6.63	13	2	NOT valid
S. Typhimurium *FH 68d*	3.60E+08	7.19E+07	7.86	<15	<1.18	>6.68	186	181	Valid
Y. enterocolitica FH67b	2.30E+08	4.60E+07	7.66	<15	<1.18	>6.48	134	139	Valid
E. coli O157:H7	1.60E+08	3.20E+07	7.51	<15	<1.18	>6.33	78	63	Valid
C. albicans	4.05E+07	8.1E+06	6.91	<15	<1.18	>5.73	157	101	Valid
S. cerevisiae	4.1E+07	8.2E+06	6.91	<15	<1.18	>5.73	139	132	Valid
A. niger	1.05E+07	2.1E+06	6.32	>300	>2.47	<3.85	7	3	Valid

N inoculum
No test challenge

The results above demonstrate that when ozonated water was applied onto organisms on a surface (0.20um filter membrane) under dirty conditions a greater than 5 log reduction was achieved against bacteria and a greater than 4 log reduction against yeast.
Ozonated water was not shown to be fungicidal at the concentrations and volumes tested against *A. niger*.
The validation of the recovery method against *L. monocytogenes* failed, however, in the context of the whole test it was clear that the ozonated water was bactericidal within 5 minutes and had an effect upon *L. monocytogenes*.

5. CONCLUSION

Based upon the results, ozonated water (3.7 ppm ozone), when used at a flow rate of 420 mL/minute, successfully produces a greater than 5 log reduction in all bacterial challenges and greater than 4 log reduction against yeast challenges within 5 minutes under both clean and dirty conditions.

The ozonated water was shown not to be fungicidal within 5 minutes against *A. niger* spores under either condition.

The ozonated water was shown to be bactericidal and yeasticidal under both clean and dirty conditions within five minutes.

6 REFERENCES

1. EN1276:2009 Chemical disinfectants and antiseptics. Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas. Test method and requirements (Phase 2, step 1).
2. EN1650:1997 Chemical disinfectants and antiseptics. Quantitative suspension test for the evaluation of fungicidal activity of chemical disinfectants and antiseptics used in food, industrial, domestic and institutional areas. Test method and requirements (Phase 2, step 1).
3. Ozone; Dictionary Microbiology & Molecular Biology 3rd edition 2001 Wiley.