Antibiofilm Efficacy of Advanced Formula Zymox® Otic Plus with 1% Hydrocortisone

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ABSTRACT

The goal of this study was to evaluate the efficacy of Advanced Formula Zymox® Otic Plus on biofilms of *P. aeruginosa*, *S. aureus*, *Methicillin-resistant S. aureus* (MRSA) and *C. albicans* based on biofilm clearance and eradication using the MBEC assay. In addition, the goal was to also determine the effects of Advanced Formula Zymox® Otic Plus on MBC (minimum bactericidal concentration) and MBEC (minimum biofilm eradication concentration) confirmed by Log Reduction. The product was tested at 100% - 0.78% dilutions on the biofilm of these four microorganisms in Organism Specific Broth and evaluated at 10 and 30 minute exposure intervals. The product showed a significant increase in antimicrobial activity at both time points and effectiveness in biofilm eradication in as few as 10 minutes when the organism was exposed to the product at full strength.

INTRODUCTION

The expertise of Innovotech, Inc. is microbial biofilms. Biofilms are a cohesive matrix of microorganisms, mucopolysaccharides (slime), and extracellular constituents that exist in virtually every natural environment. Biofilms form in an environment in response to the presence of a solid surface as well as other factors such as shear force (flow) as a mechanism to avert being removed from that environment. Biofilm formation is a developmental process moving from attachment, to microcolony formation, and then to mature biofilm development under the control of specific biofilm genes. The production of a mucopolysaccharide (slime) on the surface that further protects the biofilm and can often be seen with the naked eye.

Once formed, biofilms are difficult to remove as they show an increased resistance to biocides and antibiotics when compared to planktonic (free-floating) microorganisms. Studies have shown a greater than hundred-fold resistance to antibiotics of biofilms when compared to the 2 same bacteria in a planktonic (free floating) state. This resistance is due to the mucopolysaccharide

coating that is developed and a physiological alteration in the microorganism.

Until recently it has been difficult to study biofilms due to the difficulties in repeatable culturing biofilm organisms in the lab. Various devices have been developed to produce biofilms. However, most have been cumbersome and prone to various technical problems. In 1996, microbiologists working at the University of Calgary developed a simple assay to reliably culture 96 identical biofilms at a time. The assay allows microorganisms to grow on 96 identical pins protruding down from a plate lid. By placing the biofilms on the pins into the wells of a micro titer plate, a matrix of compounds, concentrations and synergistic effects can easily be assessed. This allows rapid testing of compounds for anti-biofilm activity.

MATERIALS AND METHODS

Test Compound: Advanced Formula Zymox® Otic Plus with 1% Hydrocortisone

Organism	Source	Dilution Factor	Organism Specific Media (OSM)
Pseudomonas aeruginosa	ATCC 27853	1,000 X	Tryptic Soy Broth/Agar (TSB/TSA)
Staphylococcus aureus	ATCC 29213	100 X	Tryptic Soy Broth/Agar (TSB/TSA)
Staphylococcus aureus (MRSA)	USA 400	100 X	Tryptic Soy Broth/Agar (TSB/TSA)
Candida albicans	ATCC 18804	No Dilution	Sabourad Dextrose Broth/Agar (SDB/SDA

**Special Precautions: Standard BSL-2 safety precautions were in effect*

at 100% - 0.78% dilutions (two-fold dilutions performed in Organism Specific Broth (OSB).

Neutralizer

1.0 g L-Histidine

1.0 g L-Cysteine

2.0 g Reduced glutathione

• Prepared up to 20 mL in double distilled water.

• Passed through a syringe with a 0.20 µm filter to sterilize.

• This solution was stored at -20°C.

• Prepared 1 liter of the appropriate growth medium OSB. This medium was supplemented with 20.0 g per liter of saponin and 10.0 g per liter of Tween-80 and adjusted with dilute NaOH to the correct pH (7.0 ± 0.2 at 20º C).

• 500 µL of the universal neutralizer was added to each 20 mL of the surfactant supplemented growth medium used for recovery plates.

Organism Specific Media

• Tryptic Soy Agar

• Sabouraud Dextrose Broth supplemented with 500 mM Galactose

• 30g of Sabouraud Dextrose Broth was dissolved in 700 mL of distilled water and autoclaved at 121 ºC. This solution was allowed to cool.

• 300mL of Galactose (0.3g/mL) was added to the solution.

Galactose Stock Solution

• Galactose = 180.156g/mol, Solubility ~683g/L in water.

• (180.156*0.5M) (0.5 M = 500mM) so 90.027g per litre of SDA to make 500 mM final concentration of Galactose.

• 90g of galactose was dissolved in 300 mL water to make 0.3g/mL solution and this solution was filter sterilized.

Test Method

Utilized standard protocol for the experimental process of high-throughput antimicrobial susceptibility testing using the MBEC™ P&G assay, outlined below:

Culture/Inoculum Preparation

• Using a cryogenic stock (at -70ºC), a first sub-culture of the bacterial organ-

isms listed above were streaked out on Tryptic Soy Agar (TSA) or Sabouraud Dextrose Agar (SDA).

• The plates were incubated at 37ºC for 24 hours and stored wrapped in parafilm at 4ºC.

• From the first sub-culture, a second sub-culture was streaked out on TSA/SDA and incubated at 37ºC for 24 hours. The second sub-culture was used within 24 hours starting from the time it was first removed from incubation.

• From the fresh streak plate, each bacterial organism was inoculated in 200 mL of sterile Tryptic Soy Broth (TSB) for bacteria and Sabourad Dextrose Broth (SDB) for yeast strains.

• Organisms were grown in TSB or SDB at 35±2ºC on a rotary shaker (at approximately 150 rpm) for 12-18 hours. This achieved an inoculum density of approximately 109 CFU/mL.

• The inoculum was adjusted to an approximate cell density of 10^6CFU/mL by diluting the organisms (as described in the chart of microorganisms) in sterile TSB (except the C albicans strain which was diluted according to steps below for the yeast strain). The cell density was confirmed by serially diluting and spot plating triplicate samples of the inoculum.

Growing the Yeast Biofilm Strains

• 50 mL of the flask culture was moved to a 50 mL centrifuge tube and centrifuged at 3000x for 10 minutes.

• Following centrifugation, the supernatant was decanted and the pellet re-suspended in 50 mL PBS (pH 7.2-7.5). The cell density was confirmed by serial dilution and spot plating.

• 150uL of the yeast suspension in PBS was then added to the appropriate wells of a 96 well plate except for the SC wells.
NOTE: Those wells served as sterility controls (SC) and were not filled with inoculum.

• To sterility control wells sterile SDB was added.

• The Hydroxyapatite (HA) coated MBEC™ P&G lid was then inserted into the 96 well bottom and incubated at 37±1ºC for 90 minutes.

• Following incubation the inoculated device was transferred to a bottom plate containing 150uL of growth media.

• The device was placed on a shaker in a humidified incubator at 37ºC for 24 hours set at 110 revolutions per minute.

• A sample of the inoculum (3 replicates) was serially diluted (ten-fold). These were controls used to verify the starting cell number in the inoculum.

• The serial 10 fold dilutions of the inoculum from 10^{-7} to 10^0 were spot plated on an appropriately labelled series of agar plates and plates were incubated for an appropriate period of time and scored for growth.

Growing the Bacterial Biofilm Strains

• Using a micro pipette, 150 µL of the diluted organisms were added to the appropriate wells of a 96 well NUNC bottom according to the diagram in the chart regarding Preparation of Challenge Plates.

NOTE: SC wells served as sterility controls and were NOT filled with inoculum.

• To sterility control wells sterile TSB was added.

• The HA MBEC™ P&G lid was then inserted into the 96 well bottom.

NOTE: The volume of inoculum used in this step was calibrated such that the biofilm covered a surface area that was immersed, entirely, by the volume of antimicrobials used in the challenge plate set up (below). Using a larger volume of inoculum may lead to biofilm formation high on the peg that physically escapes exposure in this challenge step.

• The device was placed on a shaker in a humidified incubator at 37ºC for 24

Preparation of Challenge Plates

Plate 1. *Bacterial Strains (MBEC P & G)*

	Pseudomanas aeruginosa			*Staphylococcus aureus*			*Staphylococcus aureus (MRSA)*					
A	100%	100%	100%	100%	100%	100%	100%	100%	100%	GC	GC	GC
B	50%	50%	50%	50%	50%	50%	50%	50%	50%	GC	GC	GC
C	25%	25%	25%	25%	25%	25%	25%	25%	25%	GC	GC	GC
D	12.5%	12.5%	12.5%	12.5%	12.5%	12.5%	12.5%	12.5%	12.5%	SC	SC	SC
E	6.25%	6.25%	6.25%	6.25%	6.25%	6.25%	6.25%	6.25%	6.25%			
F	3.125%	3.125%	3.125%	3.125%	3.125%	3.125%	3.125%	3.125%	3.125%	BGC	BGC	BGC
G	1.56%	1.56%	1.56%	1.56%	1.56%	1.56%	1.56%	1.56%	1.56%	BGC	BGC	BGC
H	0.78%	0.78%	0.78%	0.78%	0.78%	0.78%	0.78%	0.78%	0.78%	BGC	BGC	BGC

Plate 2. *Yeast Strains (Hydroxyapatite(HA) Coated MBEC P & G))*

	Yeast					
A	100%	100%	100%	GC	GC	GC
B	50%	50%	50%	SC	SC	SC
C	25%	25%	25%			
D	12.5%	12.5%	12.5%			
E	6.25%	6.25%	6.25%			
F	3.125%	3.125%	3.125%			
G	1.56%	1.56%	1.56%			
H	0.78%	0.78%	0.78%	BGC	BGC	BGC

Note: Bacterial and Yeast organisms were tested using exposure time and points of 10 minutes and 30 minutes (4 challenge plates per time point). Three replicate samples of each dilution of the test compound were used.

hours set at 110 revolutions per minute

Preparation of Challenge Plates

- BGC wells were biofilm growth checks
- SC wells were sterility controls
- GC wells were the growth controls for each organism
- Percentages within the wells indicate test article concentration used
- Colors correspond with organism

Using a sterile 96-well microtiter plate the following was done aseptically to set up the above challenge plate:

- 200 µL of sterile OSM was added to wells labelled GC and SC of the challenge plate. These served as sterility control (SC) and growth control (GC) for each trial of each organism.
- 200 µL of the working solutions, diluted in OSM, were added to the appropriate wells of the challenge plates (see section, Preparation of Challenge Plates).

Antimicrobial Challenge of Biofilm

- Rinse plate(s) of 0.9% saline (200µL per well) were prepared.
- Planktonic cells were rinsed from biofilm that have formed on the lid of the MBEC™ device by dipping the lid into the saline for 1-2 minutes.

Biofilm Growth Check was performed as follows:

- BGC pegs were broken with flamed pliers.
- Each peg was placed into 200 µL of neutralizer (described in section 4.1) in a 96 well plate.
- The pegs were sonicated for 30 minutes.
- The plate was serially diluted and spot plated on Organism specific agar (OSA). This served as a biofilm

growth check.

• The lid was transferred to the challenge plate and exposed at room temperature for the time points listed in section 3.2.

• Recovery plate(s) of neutralizer were prepared as described in section 4.1 (200 µL per well) in another 96 well micro titre plate.

• The peg lid was transferred to recovery media and sonicated on high for 30 minutes to dislodge surviving biofilm. The plate(s) were placed in a dry stainless steel insert tray which sits in the water of the sonicator. The vibrations created in the water by the sonicator transferred through the insert tray to actively sonicate the contents of the 96 well recovery plate(s).

Qualitative Determination of the MBC (planktonic efficacy test)

• A new sterile 96 well plate was prepared containing 180 µL per well of neutralizer.

• After the specified contact time, 20 µL from each well of the last challenge plate used was removed, and placed into the corresponding wells of a fresh 96 well Nunc plate containing 180 µL neutralizer per well as prepared above. This was incubated at 35 ± 2°C as appropriate for 24 hours. MBC results were determined following the incubation by +/- growth.

Quantitative Determination of the MBEC (Biofilm removal efficacy test)

• Following sonication, 100 µL from each well of the MBEC™ plate was placed into the first 12 empty wells of the first row of a 96 well-micro titer plate. 180 µL of 0.9% sterile saline was placed in the remaining rows.

• A serial dilution (10^0-10^{-7}) was prepared by moving 20 µL down each of the 8 rows.

• 10 µL was removed from each well and spot plated on prepared TSA or SDA plates.

• Plates were incubated at 35 ± 2°C and counted after approximately 24 hours of incubation.

• Data was evaluated as Log10 CFU/peg.

• Log Reduction values were calculated by subtracting the Log10 CFU/peg recovered from the treated pegs from the Log10 CFU/peg recovered from the growth control pegs.

Qualitative Determination of the MBEC:

• 100 µL of sterile OSB was added to each well of the neutralizer plate.

• The neutralizer plate was covered with a new, sterile non-pegged lid and incubated at 35±2ºC for 24 hours. MBEC results were determined following the incubation by +/- growth.

DATA COLLECTION

• MBC value represented the lowest concentration which killed 99.9% of the population. To determine the minimum bactericidal concentration (MBC) values following the 24 hour incubation, checked for turbidity (visually) in the wells of the challenge plate. Clear wells were evidence of bactericidal activity following a suitable period of incubation.

• MBEC results were determined following the 24 hour incubation from the MBEC panels with Log10 reduction data. To determine the minimum biofilm eradication concentration (MBEC) values, checked for turbidity (visually) in the wells of the recovery plate. Clear wells were evidence of biofilm eradication. The MBEC is defined as the minimum concentration of antibiotic that inhibits growth of the biofilm. This would be the lowest concentration in which there was no growth observed in the majority of the three wells.

DATA ANALYSIS

• Appropriate data sheets and/or graphs were prepared to facilitate evaluation of data and comparison of

the different catheters and bacteria.

• Appropriate statistical techniques were applied to effect data comparison and evaluation as deemed appropriate. The supporting data (raw data sheets, notebook references, and spreadsheets calculating adhesion and comparative statistics) were signed and dated by the technician and verifier/reviewer as completed.

RESULTS

Calculations

CFU/mL = (CFU/10µL)/0.01mL

CFU/peg = (Raw Data/0.01mL)*0.2

Log10 CFU/peg = Log10(CFU/Peg+1)

Log Reduction= Average Log10 (Growth Control) - Average Log10 (Test Compound)

% Kill = ((Average CFU/peg Growth Control - Average CFU/peg Test)/(Average CFU/peg

Growth Control))*100

Statistics - Non-pairwise, two-tailed Student's T-test (for statistical significance, $p \leq 0.05$)

GC = Growth Control

SC = Sterility Control

BGC = Biofilm Growth Check

NOTE: A negative Log Reduction value indicates that there was more growth in the treated wells following exposure to the test compounds than in the growth control (untreated) wells. This led to a negative Log reduction value and percent kill was represented as < 0%.

MBC and MBEC values for each sample and organism tested based on visual reading of +/-growth. MBC value represents the lowest concentration which killed 99.9% of the population. The MBEC is defined as the minimum concentration of antimicrobial that inhibits growth of the biofilm.

Advanced Formula Zymox® Otic Plus showed a significant increase in antimi-

Ten Minute Data

	Advanced Formula Zymox Otic Plus											
	P. aeruginasa ATCC 27853				*S. aureus* ATCC 29213				*S. aureus* USA 400			
Dilution	Log Reduction	Percent Kill	P Value	S/NS	Log Reduction	Percent Kill	P Value	S/NS	Log Reduction	Percent Kill	P Values	S/NS
100%	0.90	88.57%	0.02	S	6.18	100.00%	0.00	S	4.97	100.00%	0.00	S
50%	-0.25	<0%	0.30	NS	0.61	78.80%	0.03	S	0.19	23.53%	0.61	NS
25%	0.00	<0%	1.00	NS	0.23	20.00%	0.50	NS	-0.46	<0%	0.16	NS
12.50%	0.06	14.29%	0.80	NS	0.37	48.00%	0.30	NS	-0.34	<0%	0.34	NS
6.25%	-0.24	<0%	0.26	NS	0.52	64.00%	0.14	NS	-0.28	<0%	0.36	NS
3.125%	0.17	30.00%	0.49	NS	0.42	59.20%	0.16	NS	-0.39	<0%	0.38	NS
1.56%	-0.01	<0%	0.97	NS	0.59	75.20%	0.03	S	-0.29	<0%	0.29	NS
0.78%	0.21	32.86%	0.44	NS	0.47	66.40%	0.07	NS	-0.57	<0%	0.35	NS

	Advanced Formula Zymox Otic Plus			
	C. albicans ATCC 18804			
Dilution	Log Reduction	Percent Kill	P Values	S/NS
100%	3.55	100.00%	0.01	S
50%	1.43	87.29%	0.31	NS
25%	1.75	99.15%	0.04	S
12.50%	2.67	99.83%	0.01	S
6.25%	3.11	99.92%	0.01	S
3.125%	1.72	99.07%	0.04	S
1.56%	1.63	98.90%	0.05	NS
0.78%	1.05	94.49%	0.11	NS

10 Minute		Advanced Formula Zymox Octic Plus
P. aeruginasa ATCC 27853	MBC	100%
	MBEC	>100%
S. aureus ATCC 29213	MBC	100%
	MBEC	>100%
S. aureus USA 400	MBC	100%
	MBEC	>100%
C. albicans ATCC 18804	MBC	50%
	MBEC	100%

crobial activity at both time points and the greatest at 30 minute time point as compared to the 10 minute time point when the test solution was used at full strength (100%) against the selected strains, as can be seen from the increase in Log reduction values.At the 10 minute time point Advanced Formula Zymox® Otic Plus was significantly effective at full strength against S. aureus ATCC 29213 and S. aureus USA 400 (Log reduction values >4.7)and also showed effective eradication at 10 minutes against C. albicans ATCC 18804 with Log reduction values >3 at full strength.

Thirty Minute Data

	Advanced Formula Zymox Otic Plus											
	P. aeruginasa ATCC 27853				*S. aureus* ATCC 29213				*S. aureus* USA 400			
Dilution	Log Reduction	Percent Kill	P Value	S/NS	Log Reduction	Percent Kill	P Value	S/NS	Log Reduction	Percent Kill	P Values	S/NS
100%	6.52	100.00%	0.00	S	5.49	100.00%	0.00	S	5.24	100.00%	0.00	S
50%	-0.01	<0%	0.97	NS	0.09	20.41%	0.58	NS	0.13	25.93%	0.38	NS
25%	0.31	54.55%	0.12	NS	0.49	10.20%	0.41	NS	-0.02	<0%	0.90	NS
12.50%	0.30	52.73%	0.13	NS	0.36	53.06%	0.17	NS	0.02	0.00%	0.89	NS
6.25%	0.01	1.82%	0.95	NS	0.31	51.02%	0.12	NS	-0.02	<0%	0.88	NS
3.125%	-0.24	<0%	0.48	NS	0.51	53.06%	0.28	NS	-0.06	<0%	0.60	NS
1.56%	-0.48	<0%	0.50	S	0.61	67.35%	0.16	NS	-0.15	<0%	0.22	NS
0.78%	-0.40	<0%	0.07	NS	0.96	87.35%	0.02	S	-0.51	<0%	0.16	NS

	Advanced Formula Zymox Otic Plus			
	C. albicans ATCC 18804			
Dilution	Log Reduction	Percent Kill	P Values	S/NS
100%	4.78	100.00%	0.00	S
50%	1.73	98.61%	0.02	S
25%	1.71	98.61%	0.02	S
12.50%	2.45	99.62%	0.01	S
6.25%	2.55	99.69%	0.01	S
3.125%	2.29	99.64%	0.01	S
1.56%	1.90	99.00%	0.01	S
0.75%	1.53	94.38%	0.06	NS

30 Minute		Advanced Formula Zymox Octic Plus
P. aeruginasa ATCC 27853	MBC	100%
	MBEC	100%
S. aureus ATCC 29213	MBC	100%
	MBEC	100%
S. aureus USA 400	MBC	100%
	MBEC	100%
C. albicans ATCC 18804	MBC	100%
	MBEC	100%