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Microbac Laboratories, Inc.

Venice Division

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Research Project for Zimek Technologies, LLC

MODULAR CLASSROOM TEST #01

95 minute treatment time – one Zimek machine

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Initiation of Project: April 21, 2006

Report Date: May 1, 2006

Background Information:

Mr. David Sparks, Executive Vice President of Research and Development for Zimek Technologies Management Company, contacted Microbac Laboratories, with an interest in determining, scientifically, the effectiveness of the micro environmental sanitation machine produced by Zimek Technologies, LLC ("Zimek") which produces negatively charged micro particles in delivering the microbicidal Zimek QD disinfectant wherever air flows. Zimek Technologies Management Company is the Manager of Zimek Technologies, LLC. The initial study was done in the Microbac Laboratory February 21, 2006 and was reported March 1, 2006.

Conditions of Field Test:

The purpose of the following test is to demonstrate the effectiveness of the Zimek micro particle treatment system under actual building treatment conditions. The site was the Chain of Lakes Middle School; 8700 Conroy Road; Orlando, Florida 32835. A double wide modular classroom (building P5) was used for the treatment site. The room measured 24' wide by 36' long by 8' tall for a total area of 864 square feet and volume of 6,912 cubic feet. Students had the day off and the classroom room remained unchanged with desks, bookshelves, books, student papers, teacher's desk, overhead projector, dry eraser board, telephone and student workbench. The only change made was the taping of a shower cap over the ceiling smoke detector to prevent the charged micro particles from setting off the smoke alarm.

Summary of Test Procedures:

Test A) Microbac Laboratories, Inc. took seventeen swab samples in representative areas using swabs containing 1 mL of Letheen Broth¹. A dry marker was used to make an oval test area two inches tall by five inches across with a line down the middle at most of the sites. Swab samples taken in the left hand area represent the bacterial count in colony forming units (CFU) per swab. A second set of seventeen swab samples was taken in the right hand area of each oval following the Zimek treatment and dwell time. Test areas such as the door handle, cabinet handle and telephone mouthpiece had smaller, yet adjacent, before and after test surfaces. The swab samples were placed in a cooler and returned to the Microbac Laboratories in Venice, Florida for analysis. The laboratory analysis will compare the levels of pre-existing bacterial contaminants to the levels after the Zimek treatment.

Test B) Microbac Laboratories, Inc. placed individual living cultures of four bacteria and three molds on each of seven 2" by 3" glass microscope slides and allowed the cultures to dry. Three sets were made: One set of seven culture slides was placed, culture side up, on the overhead projector. A second set of seven culture slides was taped vertically onto the bookcase. The third set of seven culture slides was taped onto the overhead ceiling light fixture with the culture surface facing downward. One set of controls was kept in the laboratory while a second set of controls was transported to the test site but not exposed to the Zimek treatment. The controls were used to determine the bacterial count or mold spore count on each slide before exposure. After the Zimek treatment, the slides were carefully removed from the overhead projector, the bookshelf and the light fixture to avoid any bacteria being added to the microscope slides. The culture slides with living and dead bacteria and mold spores were boxed under sterile conditions and placed in the cooler which also contained the swabs samples.

Zimek Treatment:

One Zimek machines was operating exposing the room to Zimek QD micro particles for 95 minutes. Upon peering in the door 15 minutes into the test, it was noted that the Zimek micro particle treatment penetrated everywhere from floor to ceiling. It was difficult to see the other end of the room. Following the 95 minute exposure period, the machine was turned off and the Zimek micro particles continued to treat the room for an additional 40 minute dwell time. As many of the micro particles remained suspended, a fan was used to assist evacuation of the micro particles from the class room.

Procedure for analyzing swab samples:

Seventeen swab samples were taken before treatment and seventeen swab samples were taken after treatment. These thirty-four swabs were transported back to the Microbac Laboratory in Venice, Florida in a cooler. The temperature of the swabs upon return to the laboratory April

21, 2006 was 10° C. Each swab was placed on the vortexor for seven seconds to thoroughly remove the bacteria from the swab into the 1 mL of Lethen Broth contained in the swab tube. Half of the Lethen Broth was removed under sterile conditions and pipetted into a zero dilution petri dish. The other 0.5 mL of Lethen Broth was added to a test tube containing 9.5 mL of sterile deionized water. The tube was shaken for seven seconds in a one foot arc to thoroughly disburse any bacteria present from the swab test. One mL of this mixture was pipetted into a second petri dish representing the 10⁻¹ dilution. A 0.1 mL aliquot was pipetted into a third petri dish representing the 10⁻² dilution. 12 to 15 mL of PCA, Plate Count Agar, at 46.2° C was poured into each petri dish. Each petri dish was then swirled eight times in a clockwise direction, eight times in a counter-clockwise direction and eight times in a figure eight fashion to thoroughly distribute any bacteria from the swab in the PCA. After cooling for thirty minutes, to form a gel, each petri dish was inverted and placed into the 35° C incubator for a twenty four hour incubation period. Counts of the bacterial colonies, each stemming from one colony forming unit, CFU, were recorded in the laboratory notebook. As each sample stems from one-half of the Lethen Broth, the laboratory counts are doubled in the report to represent the true count per swab.

Preparation of the bacteria samples on the glass slides:

The bacteria cultures were obtained from the Microbac Laboratory - Venice Division stock cultures.

Listeria innocua ATCC #33090
Salmonella enteritidis ATCC #13076
Escherichia coli ATCC #25922
Staphylococcus aureus ATTC #6538

A 1 mL aliquot of a 24 hour working *Listeria* culture was pipetted into a 99 mL Butterfield dilution blank. This dilution was shaken for seven seconds in a one foot arc to thoroughly disburse the bacteria. A 1 mL aliquot of this dilution was pipetted into a 99 mL blank of 10% strength TSB (Tryptic Soy Broth). This mixture was shaken for seven seconds in a one foot arc to thoroughly disburse the bacteria. A 0.1 mL aliquot of this bacterial dilution was pipetted under sterile conditions onto each of five clean 2" by 3" glass microscope slides. The water was allowed to evaporate at room temperature leaving the bacteria on the slide with a small amount of Tryptic Soy Broth. Bacteria that do not produce spores would dehydrate and become non-viable on a smooth clean surface with no nutrient. The test controls would have a zero count and nothing could be learned from the test.

One of the slides with the viable *Listeria* bacteria was kept in the laboratory while the other four were transported to the test site in a cooler. The first of the four glass slides was carefully taped to the bottom of the ceiling light fixture facing downward. The second slide was carefully taped to the side of a book case facing into the room. The third slide was placed flat on the top of the overhead projector facing upward. The fourth slide was used as a second control (one kept in the lab) and kept in the slide box with no treatment exposure.

A second, identical, procedure was used to prepare five glass microscope slides with the referenced level of viable *Salmonella* bacteria on them.

A third, identical, procedure was employed to prepare five glass microscope slides with the referenced level of viable *Escherichia coli* bacteria on them.

A fourth, identical procedure was employed to prepare five glass microscope slides with the referenced level of *Staphylococcus* bacteria on them.

Procedure for analyzing bacteria on glass slides:

- 1) The viable and non-viable bacteria were washed off each slide: One mL of Butterfield buffer water was pipetted onto a slide and allowed to run into a sterile petri dish. A sterile loop was run over the smeared area of the slide to assist in removal of bacteria. The 1 mL sample was pipetted several times and run over the slide into the sterile petri dish to remove all bacteria.
- 2) Half of the bacteria wash was removed under sterile conditions and pipetted into a zero dilution petri dish. The other 0.5 mL of bacteria wash was added to a test tube containing 9.5 mL of Butterfield buffer. The tube was shaken for seven seconds in a one foot arc to thoroughly disburse any bacteria present from the microscope slide test. One mL of this mixture was pipetted into a second petri dish representing the 10^{-1} dilution. A 0.1 mL aliquot was pipetted into a third petri dish representing the 10^{-2} dilution. 12 to 15 mL of PCA, Plate Count Agar, at 46.2° C was poured into each petri dish. Each petri dish was then swirled eight times in a clockwise direction, eight times in a counter-clockwise direction and eight times in a figure eight fashion to thoroughly distribute any bacteria from the microscope slide in the PCA.
- 3) After cooling for thirty minutes, to form a gel, each petri dish was inverted and placed into the 35° C incubator for a twenty four hour incubation period. Counts of the bacterial colonies, each stemming from one colony forming unit, CFU, were recorded in the laboratory notebook. As each sample stems from one-half of the Butterfield buffer bacteria wash, the laboratory counts are doubled in the report to represent the true count per swab. Standard approved procedures established by AOAC and FDA/BAM were used in the collection of bacteria and in their dilution into petri dishes with appropriate media and cultivation.^{2 & 3}

Preparation of the mold samples on the glass slides:

Concentrated spores in vials were obtained from the Microbac Laboratory - Knoxville Division:

Aspergillus niger ATCC 16888 - 5.0×10^8 spores per mL.

Penicillium citrinum FRR 1841 - 1.34×10^9 spores per mL.

Stachybotrys chartarum VAMH 6417 - 2.15×10^8 spores per mL.

A one mL aliquot of each spore concentrate was diluted into a vial containing 99 mL of sterilized deionized water. A 0.5 mL aliquot was transferred to each of four petri dishes, with each petri dish containing Malt Extract Agar. A sterile, plastic spreader "hockey stick" was used to spread the spores evenly across each petri dish. The petri dishes were incubated for five

days in the environmental chamber at 30° C and 97% relative humidity. Using sterile techniques, the spores were collected with sterile deionized water into a sterile 125 mL flask. Glass beads inside the flask were used to break up the clumped spores on the vortexing machine. The spores were washed with sterile deionized water and centrifuged three times. Following the last centrifugation (10 minutes at 2,000 rpm) and removal of wash water, 10 mL of sterile deionized water was pipetted into each centrifuge tube.

Microscope slides containing the referenced levels of viable fungal spores were prepared for this test to closely simulate normal conditions.

A one mL aliquot of *Aspergillus* fungal spore suspension was pipetted into a 99 ml deionized water dilution blank. The blank was shaken for 7 seconds in a one foot arc to thoroughly distribute the spore suspension. A one mL aliquot of this suspension was pipetted into a second deionized water dilution blank. This second dilution blank was shaken for 7 seconds in a one foot arc to thoroughly distribute the spore suspension. A 0.1 ml sample from this second dilution blank was placed onto the middle of each of five cleaned 2" by 3" glass microscope slides. These *Aspergillus* spore samples were allowed to air dry on the microscope slides. Four of these microscope slides containing *Aspergillus* were transported to the test site and the fifth remained in the laboratory as a control for the spore count before testing.

Of the four *Aspergillus* spore slides brought to the testing site, one was carefully taped to the bottom of the ceiling light fixture facing downward. The second slide was carefully taped to the side of a book case facing into the room. The third slide was placed flat on the top of the overhead projector facing upward. The fourth slide was used as a second control (one kept in the lab) and kept in the slide box with no treatment exposure.

A second, identical, procedure was used to prepare five glass microscope slides of *Penicillium* spores on them.

A third, identical, procedure was employed to prepare five glass microscope slides of *Stachybotrys* spores on them.

Procedure for analyzing molds on glass slides:

- 1) The viable and non-viable mold spores were washed off each slide: One mL of Butterfield buffer water was pipetted onto a slide and allowed to run into a sterile petri dish. A sterile loop was run over the smeared area of the slide to assist in removal of mold spores. The 1 mL sample was pipetted several times and run over the slide into the sterile petri dish to remove all mold spores.
- 2) Half of the mold spore wash was removed under sterile conditions and pipetted into a zero dilution petri dish. The other 0.5 mL of mold spore wash was added to a test tube containing 9.5 mL of Butterfield buffer. The tube was shaken for seven seconds in a one foot arc to thoroughly disburse any bacteria present from the microscope slide test. One mL of this mixture was pipetted into a second petri dish representing the 10^{-1} dilution. A 0.1 mL aliquot was pipetted into a third petri dish representing the 10^{-2} dilution. 12 to 15 mL of Malt Extract Agar, MEA, at 46.2° C was poured into each petri dish. Each petri dish was then swirled eight times in a clockwise direction, eight times in a counter-clockwise

direction and eight times in a figure eight fashion to thoroughly distribute any bacteria from the microscope slide in the PCA.

- 3) After cooling for thirty minutes, to form a gel, each petri dish was placed into the environmental chamber at 29° C and 97% relative humidity for a five day incubation period. Counts of the mold colonies, each stemming from one colony forming unit, CFU, were recorded in the laboratory notebook. As each sample stems from one-half of the Butterfield buffer mold spore wash, the laboratory counts are doubled in the report to represent the true count per swab. Standard approved procedures established by AOAC and FDA/BAM were used in the collection of mold spores and in their dilution into petri dishes with appropriate media and cultivation.^{2 & 3}

Results of swab tests in descending order of initial testing (CFU/swab):

	<u>Before Treatment</u>	<u>After Treatment</u>
1) Teacher's desk -left corner.	5,600	2
2) Desk #3 - Seat.	520	10
3) Teacher's Desk - work area.	200	6
4) Desk #1 - Seat.	1,000	12
5) Student Common Work Table.	13,200	180
6) Map Holder -Right Side.	660	8
7) Desk #4 - Top.	34	<2
8) Desk #2 - Top.	6	<2
9) Pencil Sharpener – Top Surface.	1,500	44
10) Door Handle – Upper Surface.	<2	<2
11) Dry Eraser Board.	2	<2
12) Ceiling Light - Bottom surface.	<2	<2
13) Phone - Mouth Piece.	14	<2
14) Ceiling - Round Metal Plate.	<2	<2
15) Cabinet Handle.	5,800	26
16) Light Switch Plate.	<2	<2
17) Desk # 1 - Bottom Surface.	<2	<2



Results of Bacteria Tests on Microscope Slides (CFU/Slide):

	<u>Before Treatment</u>	<u>After Treatment</u>
<i>Listeria</i>		
Flat Surface	60	<2
Vertical Surface	60	<2
Ceiling Surface	60	<2
<i>Salmonella</i>		
Flat Surface	110	<2
Vertical Surface	110	<2
Ceiling Surface	110	<2
<i>Escherichia coli</i>		
Flat Surface	140	<2
Vertical Surface	140	<2
Ceiling Surface	140	<2
<i>Staphylococcus</i>		
Flat Surface	3,320	<2
Vertical Surface	3,320	<2
Ceiling Surface	3,320	<2

Results of Mold Tests on Microscope Slides (CFU/Slide):

	<u>Before Treatment</u>	<u>After Treatment</u>
<i>Aspergillus</i>		
Flat Surface	120	<2
Vertical Surface	120	<2
Ceiling Surface	120	<2
<i>Penicillium</i>		
Flat Surface	100	<2
Vertical Surface	100	<2
Ceiling Surface	100	<2
<i>Stachybotrys</i>		
Flat Surface	290	<2
Vertical Surface	290	<2
Ceiling Surface	290	2

Conclusion:

- 1) The swab tests indicated where the highest levels of bacteria occur in a class room. The Zimek micro particle treatment was very effective in reducing the naturally occurring bacterial level.
- 2) The Zimek micro particle treatment was completely effective in killing the referenced levels of *Listeria*, *Salmonella*, *Escherichia coli* and *Staphylococcus* from the microscope slides in horizontal, vertical and ceiling positions.
- 3) The Zimek micro particle treatment was completely effective in killing the referenced levels of *Aspergillus* mold spores and *Penicillium* mold spores from the microscope slides in horizontal, vertical and ceiling positions. *Stachybotrys* is one of the most difficult mold spores to kill. The Zimek micro particle treatment was effective in killing the referenced levels of *Stachybotrys* in horizontal and vertical positions and 99.3% effective in killing the *Stachybotrys* spores on the ceiling microscope slide.

Reference Material:

1. Rediswabs were obtained from Biotrace International. Each contained 1 mL of Letheen Broth. Lot #RS06002, Expiration date 1/11/2007, ISO 9001-2000 Certified.
2. American Association of Analytical Chemists (AOAC) Official Method 966.23.
3. Federal Department of Agriculture/Bacteriological Analytical Manual (FDA/BAM) Chapter 3.

Respectfully submitted by:

A handwritten signature in cursive script, appearing to read "Kayse Hasiak".

Kayse Hasiak
Laboratory Director