PERMANON
CANADA

Report on Permanon Surface Modification Nanotech Products

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Project requested by Stewart Berry, MBA – Co-Owner and CEO - Permanon Canada Limited
Dr. Keith Warriner, University of Guelph –Food Sciences Faculty oversaw the experiments and tests.

Executive Summary

Mr. Berry viewed the telecast of CBC's Marketplace on Dirty Hotels (www.cbc.ca/news/canada/hotel-room-tests-...) and immediately contacted Dr. Warriner who was with the CBC investigative team and provided the assessment of the types of bacteria and levels of bacteria they found in multiple Hotel and Motel properties (http://www.cbc.ca/news/canada/hotel-ice-air-hold-potential-hazards-cbc-test-finds-1.1209109 across Canada.

During the past nine months Dr. Warriner and his staff have carried out a number of tests and experiments on surface modification with the objective being, "the assessment of multiple Permanon products on varying hard surfaces and their ability to reduce the long term growth of differing bacteria."

In addition they performed a number of experiments to assess the ability of Permanon Surface products to reduce or eliminate the ability of mould to multiple and grow.

They carried out a number of tests and the products have performed extremely well in the area of repellent surface properties and reduction in the growth of bacteria (vegetative bacterial cells) and mould spore that were placed on Permanon treated surfaces.

These tests and controlled experiments have provided the following conclusions:

Conclusions:

A) Extended exposure of *E coli* and *S aureus** to Permanon surface reduces cell viability with no survivors being recovered following a 6h (hour) contact period.

B) The attachment of bacteria could not be assessed due to the inactivation of bacterial cells through exposure to Permanon coatings.

C) Indirect evidence was found to suggest that Permanon coating is sensitive to acidic sanitizers and was removed following one cleaning cycle.

D) Glass slides coated with Permanon All Round Supershine inhibited the growth of mould for up to 3 weeks. There was NO growth of mould over the test period.

*Staphylococcus aureus

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Basic Questions Answered

a) Will bacteria grow on Permanon?

*The results show that **Ecoli** and **S aureus** bacteria after a 6 hour cycle were not viable and that **no bacteria survived on the Permanon treated surface***

b) Will bacteria (type) die if not able to attach to surfaces?

The bacteria attachment rate could not be assessed as the bacteria had not survived.

Note: Although not a finding it would be an assumption that due to their non-survival rate they could not become established or grow on a Permanon treated surface.

c) With proper cleaning regimes will Permanon reduce and/or eliminate the ability of bacteria and differing pathogens to grow?

The indirect evidence was found to suggest that Permanon coating is sensitive to acidic sanitizers and was removed following one cleaning cycle with a highly acidic cleaner.

Note: This finding supports the Permanon instructions that indicate a non-acidic biodegradable cleaning agent will remove dormant or non-surviving bacteria without reducing the repellent properties of Permanon for multiple cleaning cycles. This represents a potentially substantial reduction in toxic, acidic or harsh cleaners when integrated into the cleaning cycles. This in turn represents a potential lessening of health related issues for both the public and employee groups that must work or interact with acidic materials.

d) Will mould grow on Permanon treated areas?

The evidence was found to suggest that Permanon coated areas in wet or high humidity areas would restrict the growth of mould.

Note: No mould growth was detected over a three week period while nutrients were used to encourage their growth over the test period.

Methodology and Process

Methodology

Bacteria cultivation and enumeration

Escherichia coli P36 was cultivated at 37°C for 16h in Tryptic Soy Broth (TSB) supplemented with 30 µg/ml kanamycin. Staphylococcus aureus was grown in BHI at 37°C for 16 h. At the end of the growth period the cells were harvested by centrifugation and resuspended in saline to a final cell density of 9 log cfu/ml. The suspensions were held at 4°C until required.

E. coli was enumerated on TSA supplemented with 50µg/ml kanamycin with S aureus being cultured on BHI agar plates. Both sets of plates were incubated at 37°C for 24 h.

Growth assay on treated surfaces

Stainless steel coupons ($3cm^2$) were autoclaved prior to use. The coupons were sprayed with the Permanon solution then left for 20 mins at room temperature. The test bacteria were then drop-inoculated onto the surface to give a final cell density of 7 log cfu per coupon. A set of coupons (n=3) were removed at time 0, 3 h and 6 h and swab samples taken from the surface using a moistened cotton bud. The swabs were transferred to 10 ml saline and vortexed to release the bacteria from the cotton bud tip. A dilution series was prepared in saline and plated out onto the appropriate agar as described above.

Attachment Assay

The extent of attachment of bacteria was performed as described for the growth assay accept coupon samples were taken at time 0, 30 min, 40 min and 60 min.

Stability of surface coatings to sanitation cycles

Stainless steel coupons were sprayed with Permanon and inoculated with the test bacteria as described above. The inoculated coupons were left for 30 min at room temperature and the bacteria recovered by swabbing. The coupons were then cleaned with a commercial organic acid based sanitizer (Mr Clean™; 2% v/v) with physical pressure being applied using a sterile cloth. A further inoculum (7 log cfu) was deposited on the coupons and left for 30 mins prior to recovering using a swab. The process was repeated twice more and bacterial counts enumerated.

RESULTS

Results (A)

Table 1: Effect of Permanon coating on the growth of E coli and S aureus on treated stainless steel surfaces

	Log cfu/Coupon	
Exposure Time	***E coli* P36**	***S aureus***
0	6.31±0.15	6.26±0.13
3h	2.94±0.19	<0.3
6h	**<0.30**	**<0.3**

Table 2: Effect of Permanon on the attachment of E coli and S aurues to treated stainless steel surfaces

	Log cfu/Coupon	
Exposure Time	***E coli* P36**	***S aureus***
0	3.37±1.64	4.91±0.31
30 min	3.17±0.46	3.33±0.25
40 min	2.99±0.16	3.02±0.08
60 min	<0.3	1.07±1.34

Table 3: Stability of Permanon coating to repeated cleaning cycles.

	Log cfu/Coupon	
Cleaning Cycle	***E coli* P36**	***S aureus***
None	4.05±0.25	5.04±0.29
1	1.0±0	3.33±0.25
2	3.14±0.39	4.19±0.17
3	2.90±0.28	4.20±0.21

Mould Growth – Testing and Experiment

Methodology

Aspergillus niger spores (3 log cfu) were deposited onto Permanon coated slides and periodically (weekly) sprayed with 0.1% nutrient broth . The slides were incubated at room temperature for 3 weeks and visually assessed for mould growth.

Results of testing (weekly)

NO growth of *Aspergillus niger* (mould) was observed on Permanon coated slides over a 3 week trial period.

Experiment Preparation Data

A) Preparing the Microorganisms:

- E-coli p36 suspension of Optical Density = 0.2:

Following the manufacturer's instructions, 15g of Trypton Soy Broth (TSB) powder was weighed, and then it was dissolved in a 500ml bottle of distilled water. Next, the TSB was mixed with the distilled water until the solution was clear. Then, it was autoclaved at 121°C for 15min to be sterile. After autoclaving, the solution was kept in bath water to cool down to around 45ºC; 1ml of Kanamycin (Kan) (15mg Kan/ml distilled water) was added. Then, the TSB Kan was cultured with 1ml of E-Coli p36. After culturing, it was incubated at 37°C/24h. In the next day, the culture was centrifuged at 4500 rpm for 10min at room temperature. Then, the precipitate was collected and transferred to 20ml of Sterile Saline (8g NaCl+1g Tween 80 /L distilled water). At that time, the E-Coli p36 was prepared in liquid stock, thus OD (optical density at 600nm) was adjusted to 0.2 of the liquid cultures was determined by using a spectrophotometer. After it was prepared E-Coli P36 OD at 0.2, a dilution series down to -7 was prepared and then plated out -3, -4, -5, -6 and -7 onto TSA Kan. Then, the plates were incubated at 36°C/24h and the colonies were enumerated. After that, the E-coli p36 was adjusted to 7 log.

- Bacillus Spores 7 log cfu/ml:

The Bacillus Spores were already prepared in liquid stock in our lab. The cfu/ml of Bacillus Spores was prepared in 7 log; a dilution series down to -7 was prepared and then plated out -3, -4, -5, -6 and -7 onto TSA.

B) Preparing the sterile Saline (8g NaCl + 1g Tween /L distilled water):

1. Preparing a sterile Saline in test tubes; each tube had 9ml of sterile Saline :

First, 8g of NaCl and 1g of Tween were weighed, and then they were dissolved in a 1000ml bottle of distilled water until the solution was clear. Nine ml of Saline was transferred into each test tube. Then, each test tube was covered by a lid. After that, the test tubes were autoclaved at 121°C for 15min to be sterile. After autoclaving, the sterile Saline was kept in room temperature to cool down around to 25°C to be ready for using.

2. Preparing a sterile Saline in test tubes; each tube had 10ml of sterile Saline:

The same preparation method as the 9ml sterile saline was used but, instead of transferring 9ml, 10ml was transferred.

3. Preparing a sterile Saline in sterile plastic bags; each bag had 30ml of sterile Saline:

First, 4g of NaCl and 0.5g of Tween were weighed, and then they were dissolved in a 500ml bottle of distilled water until the solution was clear. Then, it was autoclaved at 121°C for 15min to be sterile. After autoclaving, the sterile Saline was kept at room temperature to cool down to around 25°C. Before treating the sample, 30ml of sterile Saline was poured into a sterile bag.

C) Preparing the culture medium:

1. TSA Kan (Tryptic Soy Agar with Kanamycin) for E-coli:

Following the manufacturer's instructions, 20g of Tryptic Soy Agar (TSA) was weighed and dissolved in a 500ml bottle of distilled water. Then, the solution was mixed; it was heated until it was clearly dissolved. After the solution had been dissolved, it was autoclaved at 121°C for 15min. Next, it was kept in bath water at 50°C to cool off. When it cooled down to 50°C, 1ml of Kanamycin (Kan) (15mg Kan/ml distilled water) was added. Finally, the medium culture was poured into sterile Petri plates.

2. TSA (Tryptic Soy Agar) for Bacillus Spores:

Following the manufacturer's instructions, 20g of Tryptic Soy Agar (TSA) was weighed and dissolved in a 500ml bottle of distilled water. After the solution was mixed, it was heated until it was clearly dissolved. After the solution had been dissolved, it was autoclaved at 121°C for 15min. Next, it was kept in bath water at 50°C to cool off. When it cooled down to 50°C, the medium culture was poured into sterile Petri plates.

D) <u>The samples:</u>

1. Contaminated sterile stainless steel coupons surface ($9cm^2$) :

Each sterile stainless steel coupon surface was inoculated with 100µl inoculums of 7log CFU/ml of E-coli p36 or B. Spores. Then, the contamination was gently spread over the surface by a sterile yellow spreader. After that, the sample was kept for 30min to dry.

2. Contaminated sterile plastic cutting board surface ($9cm^2$):

Six squares ($9cm^2$) areas were determined on the surface of a sterile plastic cutting board to be inoculated with 100µl inoculums of 7log CFU/ml of E-coli p36 or B. Spores.

After inoculation, each contamination was gently spread over the surface by a sterile yellow spreader. After that, the sample was kept for 30min to dry.

E) The treatment:

- The Sanitizer: (Sanides Green)

The sanitizer was applied onto the contaminated dry surfaces by using a spray. The distance between the surface of the samples and the spray was around 20-30cm; each surface had evenly one coat of the sanitizer by moving the arm from left to right as Hamed recommended. After spraying, the sanitizer was left on the surfaces for four different exposure times which were: 5 min, 30 min, 3h, and 24h before they were tested.

- The Yellow Surface Protection: (All Round Supershine)

The Yellow protection was applied onto each sterile surface of the sample, and then it was left on for 30 minutes in order to dry. After that, the protected surface of each sample was contaminated as the same procedure I mentioned above. The contamination was left on the surfaces for four different exposure times which were: 5 min, 30 min, 3h, and 24h before they were tested.

F) Testing the samples and control samples:

1- Stainless steel coupon surface:

Each coupon of stain less steel including the control sample was placed into a bag of 30ml of sterile Saline (8g NaCl + 1g Tween /L distilled water), and then it was stomached (manually massaged) to be well diluted. After that, the stainless steel coupon was diluted from -1 to -3, except the sample of Bacillus spores had been heated at 60°C for 10min before it was diluted, and then plated out 0.1ml of 0,-1,-2, and -3 in the TSA Kan for E-coli

and TSA for B. spores. After, the plates were incubated at 37°C/24h for E-coli and at 30°C/24h for Bacillus spores. After incubation of the plates, the colonies were counted.

2- Cutting board surface:

Each determined square on the cutting board including untreated sample; a wet sterile sponge that had been placed into a bag of 30ml of sterile Saline (8g NaCl + 1g Tween /L distilled water) was used to scrub off the contamination. Then, the sponge was placed back into the bag and stomached to be well diluted. After that, the contaminated sponge was diluted from -1 to -3, except the samples of Bacillus spores had been heated at 60°C for 10min before it was diluted, and then plated out 0.1ml of 0,-1,-2, and -3 in the TSA Kan for E-coli and TSA for B. spores. After, the plates were incubated at 37°C/24h for E-coli or incubated at 30°C/24h for B. spores. After incubation of the plates, the colonies were counted.

3- Stainless steel coupon surface attachment of cells:

Each coupon of stainless steel including the control sample was rinsed with 10 ml of sterile Saline while the coupon had been held by a sterile tweezers; the inoculated side of the surface was face up to the rinse, and the rinse was collected in a sterile bag. After rinsing, a sterile Nylon swab stick was wiped on the surface of the coupon, and then the Nylon swab stick was placed into 10 ml of sterile Saline. After wiping the coupon, it was places on TSA agar surface; overlaid with TSA agar, and it was incubated at 37°C/24h for E-coli. Next, the collected rinse was diluted down to -2, and then plated out 0.1ml of 0,-1, and -2 in the TSA Kan for E-coli. The nylon swab stick was diluted down to -2, and then

plated out 0.1ml of 0,-1, and -2 in the TSA Kan. After pleating, they were incubated at 37°C/24h then the colonies were counted.

G) The Results:

(Table 1) The effect of the sanitizer on contaminated surfaces.

Bacteria	Surface	Treatment time	Log_{10} (cfu/cm^2)	Log Reductions
E-coli	Stainless steel coupons	0	7 ± 0.00	0
		5 min		>7
		30 min		>7
		3 h		>7
		24 h		>7
E-coli	Plastic cutting board	0	7 ± 0.00	>7
		5 min		>7
		30 min		>7
		3 h		>7
		24 h		>7
B. Spores	Stainless steel coupons	0	7 ± 0.00	0
		5 min	5.86 ± 0.29	0.39
		30 min	6.21 ± 0.15	0.04
		3 h	6.05 ± 0.11	0.20
		24 h	6.08 ± 0.19	0.17
B. Spores	Plastic cutting board	0	7 ± 0.00	0
		5 min	6.17 ± 0.12	0.13
		30 min	6.01 ± 0.12	0.29
		3 h	6.15 ± 0.14	0.15
		24 h	6.11 ± 0.11	0.19

(Table 2) The effect of the Yellow protection on contaminated surfaces.

Bacteria	Surface	Treatment time	Log_{10} (cfu/cm^2)	Log Reductions
E-coli	Stainless steel coupons	0	7 ± 0.00	0
		5 min	6.90 ± 0.05	0.04
		30 min	6.88 ± 0.11	0.06
		3 h		>7
		24 h		>7
E-coli	Plastic cutting board	0	7 ± 0.00	>7
		5 min	6.75 ± 0.19	0.21
		30 min	4.67 ± 1.19	2.29
		3 h	3.23 ± 0.20	3.73

		24 h		>7
B. Spores	Stainless steel coupons	0	7 ± 0.00	0
		5 min	6.29 ± 0.05	0.14
		30 min	6.45 ± 0.26	- 0.02
		3 h	6.39 ± 0.11	0.04
		24 h	6.89 ± 0.03	- 0.46
B. Spores	Plastic cutting board	0	7 ± 0.00	0
		5 min	6.17 ± 0.14	0.24
		30 min	6.04 ± 0.15	0.37
		3 h	6.32 ± 0.10	0.09
		24 h	6.65 ± 0.28	- 0.24

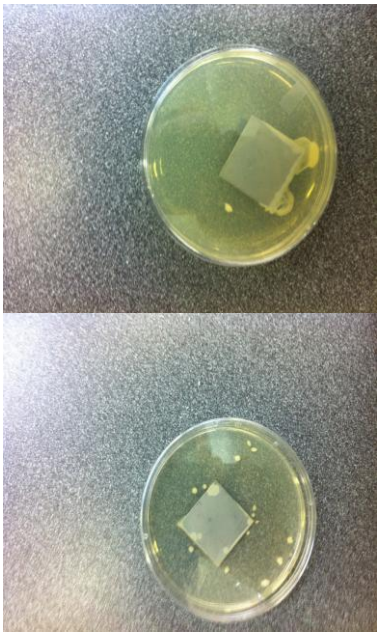
(Table 3) The effect of the sanitizer on attachment of contaminated surfaces.

Bacteria	Surface	Treatment time	Placed on TSA	Rinse collection	10^{-1}	10^{-2}	swab stick	10^{-1}	10^{-2}
E- Coli	Stainless steel	0	Positive	1	0	0	1	0	0
			Positive	3	0	0	2	0	0
		5 min	Positive	0	0	0	0	0	0
			Positive	0	0	0	0	0	0
			Negative	0	0	0	0	0	0
			Negative	0	0	0	0	0	0
			Negative	0	0	0	0	0	0
			Negative	0	0	0	0	0	0
		30 min	Negative	0	0	0	0	0	0
			Negative	0	0	0	0	0	0
			Negative	0	0	0	0	0	0
			Negative	0	0	0	0	0	0
			Negative	0	0	0	0	0	0
			Negative	0	0	0	0	0	0
		3 h	Negative	0	0	0	0	0	0
			Negative	0	0	0	0	0	0
			Negative	0	0	0	0	0	0
			Negative	0	0	0	0	0	0
			Negative	0	0	0	0	0	0
			Negative	0	0	0	0	0	0

- Positive = There was a growth but it was hard to count the colonies (picture 1).
- Negative = There was not any growth.

(Picture 1) Uncountable cells.

D



No growth of *Aspergillus niger* (mould) was observed on Permanon coated slides over a 3 week trial period.

Report Summary

Dr. Warriner and his assistance staff believe further testing of the adherence properties would be of value. They also have received the Toxicity Experiments and Reports from the University Martin Luther in Germany and are reviewing its findings.

It is clear from these experiments and tests at the University of Guelph that Permanon Surface modification properties can positively influence the reduction of mould growth, the reduction of bacteria growth and the related ability for these bacteria to grow and be transmitted.

The ongoing need to modify the surface environments in areas where the worker and public interact to reduce the transmission rates of many bacteria would appear to be supported by the use of Permanon Surface modification nanotech based technologies.

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