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EVALUATION OF THE ANTIMICROBIAL ACTIVITIES OF TITANIUM DIOXIDE-TREATED GLASS SURFACES ACCORDING TO A METHOD BASED UPON JIS Z 2801 : 2000 AND JIS Z 2801 : 2006

This test report is a translation of the test report 903.DEX.10 edited on July 12th 2010

Test report written by: Dr Marlène RICHARD

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BIOTECH-GERMANDE
SAS au capital de 520 000 Euros N° SIRET : 423 865 419 00026 R.C.S Marseille APE : 7120B
Parc Scientifique de Luminy – 163 Avenue de Luminy – case 927 - 13288 MARSEILLE cedex 9
Tel : 33(0)4 91 82 82 40 Fax : 33(0)4 91 82 82 49 Email : biotech.germande@wanadoo.fr

CONTENT

I : DESCRIPTION OF THE STUDY:

Title: Evaluation of the antimicrobial activities of titanium dioxide-treated glass surfaces according to a method based upon JIS Z 2801: 2000 and JIS Z 2801: 2006.

Study n°: 903.DEX.10

Sponsor: DEXPERT
VALORAY Industrie
1, place Paul Verlaine

Contact: Mr DUPRAT

Test period: From 21/06/2010 to 12/07/2010

Study manager: Dr Marlène RICHARD

Tests done by: Isabelle SEVEROVIC

Test laboratory: Laboratoire BIOTECH-GERMANDE
Parc Scientifique de Luminy
163 Avenue de Luminy – Case 927
13288 Marseille Cedex 9

II : AIM OF THE STUDY:

Determine, according to the experimental conditions described in the standards JIS Z 2801: 2000[(1)] and JIS Z 2801: 2006[(2)], the ability of titanium dioxide-treated glass surfaces to reduce within 24 hours at 22°C of at least 10^2 times the number of viable microorganisms spread on test pieces.

III : MATERIAL:

a) Test pieces:

3 titanium dioxide-treated glass surfaces (i.e surface with antimicrobial agent) and 6 untreated glass surfaces are necessary to evaluate the antimicrobial activity for each microorganism tested.

b) Microbial strains :

Pseudomonas aeruginosa CIP 103467
Staphylococcus aureus CIP 4.83
Escherichia coli CIP 54127
Aspergillus niger IP 1431.83 spores
Bacillus cereus CIP 105151 spores

The conditions of preservation of the microbial strains used for the determination of the bactericidal, mycobactericidal, sporicidal and fungicidal activities are those described in the European standard NF EN 12353: 2006[(3)].

c) Maintaining and counting medium:

For *Pseudomonas aeruginosa* CIP 103467, *Staphylococcus aureus* CIP 4.83, *Escherichia coli* CIP 54127 and *Bacillus cereus* CIP 105151 spores:
Trypticase soja agar:
TS agar (BIOMERIEUX, 51044)---40 g
Distilled water: ---l000 ml
Steam sterilized at 121°C for 21 minutes.

For *Aspergillus niger* IP 1431.83:
Malt extract agar:
Soja peptone(SIGMA P-1265) :---3 g
Malt ectract (SIGMA M0383) : ---30 g
Agar (SIGMA A5306) : ---15 g
Distilled water: ---l000 ml
Steam sterilized at 121°C for 21 minutes.

d) Recovery solution:

Tween 80 (SIGMA P17-54) :---50 ml
Sodium thiosulfate (SIGMA S85-03) : ---10 g
Saponin (SIGMA S79-00) : ---5 g
Lecithin (SIGMA P53-94) : ---10 g
Trypticase soja broth: ---500 ml
Steam sterilized at 121°C for 21 minutes.

e) Diluent :

Test suspensions are prepared in phosphate buffer 100 mM, pH 7.0.

i) Stock solution:
Phosphate buffer 10X (1000mM, pH 7.0):
Dibasic sodium phosphate, Na_2HPO_4 $7H_2O$:---144.5g
Monobasic potassium phosphate, KH_2PO_4:---71.2g
Distilled water: ---1000 ml
Steam sterilized at 121°C for 21 minutes.

ii) Working solution:
Phosphate buffer 1X (100mM, pH 7.0):
Phosphate buffer 10X (1000mM, pH 7.0) :---100 ml
Distilled water: ---1000 ml

IV : METHOD :

a) Test suspensions :

For each microorganism, the test suspension is prepared in the diluent (see III.e) and adjusted in order to contain between $2.5x10^5$ and $10x10^5$ cfu/ml, according to the JIS Z 2801: 2000(1) requirements. The number of viable microorganisms in the test suspension (Tc) is verified for each assay using a validated and specific counting method for each microorganism.

b) Test pieces contamination:

For each microorganism, test pieces (i.e 3 titanium dioxide-treated glass surfaces and 6 untreated test pieces), are placed in a sterile Petri dish with the treated surface facing up. 400 µl of the test suspension are deposited onto each test pieces and then cover by two cover glasses (22 x 40 mm) in order to spread the test suspension on a 1760 mm² surface.

It should be noted that the use of cover glasses for the spreading of the test suspension is a limiting factor in the efficacy of the process since they are limiting the amount of oxygen available to activate the photocatalysis process generating the antimicrobial activity of these titanium dioxide-treated surfaces.

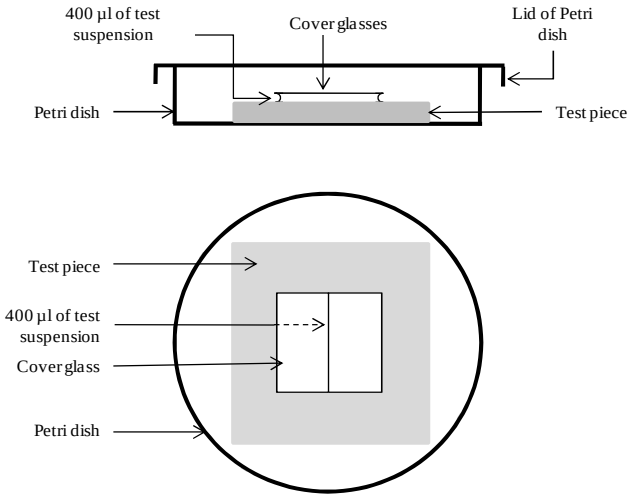

Figure 1 : Instillation of inoculums between the test piece and cover glasses

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c) Incubation of the inoculated test pieces:

After contamination, 3 titanium dioxide-treated glass surfaces and 3 out of 6 untreated surfaces are incubated at room temperature (22°C) for 24±1 hours in open Petri dishes under laminar flow of a safety cabinet and under a light intensity of about 1000 lux. In parallel, the 3 other untreated test pieces are immediately washed out in order to determine the initial contamination level of the test pieces (see IV.d).

d) Determination of the contamination level of the test pieces:

Immediately after contamination or after 24±1 hours of incubation of the test pieces, the residual number of viable microorganisms remaining on the test pieces are determined. Firstly, the cover glasses are carefully removed from the surface and transferred into a test tube containing 10 ml of recovery solution and approximately 1 ml of glass beads from 0.25 to 0.50 mm in diameter. The test tubes containing the cover glasses are submitted to a manual agitation in order to remove the microorganisms from the surface. In a second step, the surface test piece is recovered with 5 ml of recovery solution and then scraped with a cell scraper and rinsed with additional 5 ml of recovery solution. The volume of the recovery solution containing the microorganisms collected in the Petri dish is transferred into the test tube containing the corresponding cover glasses. The number of viable microorganisms present per milliliter of test mixture is determined by successive tenfold dilution and inclusion of 1ml of each dilution in the counting medium specific of the test microorganism.
After incubation at the specific temperature and time of the test microorganism, colonies are counted and the results are expressed as the number of cfu per test piece.

e) Test conditions :

According to JIS Z 2801: 2000[(1)], the tests are satisfactory if the three following test conditions are met:

i) Logarithmic value of the number of viable microorganisms immediately after contamination on untreated test pieces:

$$(L_{max} - L_{min})/(L_{mean}) \leq 0,2$$

Where L_{max}: Maximum logarithm of the number of viable microorganisms on untreated test pieces;

L_{min}: Minimum logarithm of the number of viable microorganisms on untreated test pieces;

L_{mean}: Average of the logarithm of the number of viable microorganisms on three untreated test pieces.

ii) The average of the number of viable microorganisms immediately after contamination of untreated test pieces shall be within the range of $1.0x10^5$ (5,0 $\log_{10}$) and $4.0x10^5$ cfu/test piece (5,6 $\log_{10}$).

iii) The number of viable microorganisms on an untreated test piece after 24 hours shall not be less than $1.0x10^4$ cfu/test piece (4 log).

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f) Expression of antimicrobial activity :

When test conditions are validated, the antimicrobial activity of the antimicrobial test pieces is calculated as follows:

$$R = [\log (B/A) - \log (C/A)] = [\log (B/C)]$$

With R: Value of antimicrobial activity;

A: average of the number of viable microorganisms immediately after contamination on the untreated test pieces;

B: average of the number of viable microorganisms after 24 hours on the untreated test pieces;

C: average of the number of viable microorganisms after 24 hours on the antimicrobial test pieces.

V : RESULTS :

a) Test conditions validation:

Table I : Validation of the number of viable microorganisms on untreated glass surfaces immediately after contamination: $(L_{max} - L_{min})/(L_{mean}) \leq 0,2$; L_{max}: maximum logarithm of the number of viable microorganisms on untreated test pieces; L_{min}: minimum logarithm of the number of viable microorganisms on untreated test pieces and L_{mean}: average of the logarithm of the number of viable microorganisms on three untreated test pieces.

	Assay 1 (L_{max})	Assay 2 (L_{min})	Assay 3	L_{mean}	$(L_{max} - L_{min})/(L_{mean})$
Pseudomonas aeruginosa CIP 103467	5.09	5.00	5.03	5.04	0.016
Staphylococcus aureus CIP 4.83	5.17	4.83	5.07	5.05	0.065
Escherichia coli CIP 54127	5.06	4.94	5.02	5.01	0.020
Aspergillus niger IP 1431.83 spores	4.27	4.05	4.12	4.15	0.051
Bacillus cereus CIP 105151 spores	5.13	5.02	5.04	5.06	0.021

For each test microorganism:

- ✓ $(L_{max} - L_{min})/(L_{meann})$ is lower than 0,2 (table I);
- ✓ The average of the number of viable microorganisms present immediately after contamination on an untreated test pieces is within 5.0 $\log_{10}$ ($1.0x10^5$ cfu/test piece) and 5.6 $\log_{10}$ ($4.0x10^5$ cfu/test piece) for *Pseudomonas aeruginosa* CIP 103467, *Staphylococcus aureus* CIP 4.83, *Escherichia coli* CIP 54127 and *Bacillus cereus* CIP 105151 spores.
  For *Aspergillus niger* IP 1431.83, the average number of viable microorganisms present immediately after contamination on an untreated test pieces is slightly lower than the minimal recommended value ($1.4x10^4$ cfu/test piece instead of $1.0x10^5$ cfu/test piece). This slight deviation from JIS Z 2801: 2000 requirements do not affect the validity of the test conditions since for each test microorganism, the average number of

viable microorganisms found on the untreated test pieces after 24 hours incubation is greater than $1.0x10^4$ cfu/test piece (table II, B).

The test conditions are therefore validated for each microorganism.

b) Main test :

Table II : Evaluation of the antimicrobial activity of titanium dioxide-treated glass surfaces against each test microorganism for a 24±1 hours contact time at 22°C. A: average of the number of viable microorganisms immediately after contamination on the untreated test pieces; B: average of the number of viable microorganisms after 24 hours on the untreated test pieces; C: average of the number of viable microorganisms after 24 hours on the antimicrobial test pieces and R: Value of antimicrobial activity.

	A (Nb. cfu/test piece)	**B** (Nb. cfu/test piece)	**C** (Nb. cfu/test piece)	**R** [Log (B/C)]
Pseudomonas aeruginosa CIP 103467	$1.1x10^5$	$3.7x10^5$	< 14	> **4.4**
Staphylococcus aureus CIP 4.83	$1.2x10^5$	$6.3x10^4$	$1.6x10^4$	0.9
Escherichia coli CIP 54127	$1.0x10^5$	$7.8x10^4$	< 14	> **3.7**
Aspergillus niger IP 1431.83 spores	$1.4x10^4$	$7.8x10^4$	$7.5x10^3$	1.0
Bacillus cereus CIP 105151 spores	$1.2x10^5$	$1.3x10^5$	$1.2x10^5$	0.0

According to the results presented in table II, the antimicrobial activity of the titanium dioxide-treated glass surfaces (cf. table II, C) leads to a reduction of at least 10^2 times the number of viable microorganisms when the test microorganisms are: *Pseudomonas aeruginosa* CIP 103467 and *Escherichia coli* CIP 54127. For those two strains, the logarithmic reductions are respectively >4.4 $\log_{10}$ and > 3.7 $\log_{10}$.

For *Aspergillus niger* IP 1431.83 and *Staphylococcus aureus* CIP 4.83, the reduction of the number of viable microorganisms (respectively 0.9 $\log_{10}$ and 1.0 $\log_{10}$) remains below the standard requirements (2 $\log_{10}$).

For *Bacillus cereus* CIP 105151 spores, the reduction of the number of viable microorganisms is zero.

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VI : CONCLUSIONS :

Under the experimental conditions described in this test report (see IV), the results show that titanium dioxide-treated glass surfaces induce a reduction of at least 10^2 times the number of viable microorganisms deposited on the surface test after a 24 hours contact time at 22°C under light intensity of about 1000 lux, when the test strains are *Pseudomonas aeruginosa* CIP 103467 and *Escherichia coli* CIP 54127.

Even if they are lower than the JIS Z 2801: 2000(1) and JIS Z 2801: 2006(2) requirements, a slight antimicrobial activity is also observed, in the same test conditions, for *Staphylococcus aureus* CIP 4.83 (reduction of 0.9 $\log_{10}$) and *Aspergillus niger* IP 1431.83 spores (reduction of 1.0 $\log_{10}$). However, no antimicrobial activity was observed on *Bacillus cereus* CIP 105151 spores.

VII : REFERENCES :

1. JIS Z 2801: 2000 – Antimicrobial products: Test for antimicrobial activity and efficacy.
2. JIS Z 2801 : 2006 – Antimicrobial products : Test for antimicrobial activity and efficacy, Amendment 1
3. NF EN 12353: 2006 – Preservation of test organisms used for the determination of bactericidal, mycobactericidal, sporicidal and fungicidal activity.

VIII : STATEMENT OF GOOD LABORATORY PRACTICE:

The study was conducted according to NF EN ISO/CEI 17025 (2005) General requirements for the competence of testing and calibration laboratories.
The Quality Assurance Unit (QAU) has reviewed this report and determined it accurately describes the procedures used and that the results and conclusions herein accurately reflect the raw data from the study. Applicable Standard Operating Procedures and Good Laboratory Practice were followed in this study.
The original records of this report, the notebooks, protocol, and final study report are stored in the archives of Biotech-Germande "903.DEX.10".

Marlène RICHARD
Lab deputy manager
Signature  Date 13/07/2011

Christine AH-DIP
Quality Assurance Unit manager
Signature  Date 13/07/2011

Lionel PINEAU
Managing director
Signature  Date 13/07/2011

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