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MEASUREMENT OF ANTIBACTERIAL ACTIVITY ON NON-POROUS SURFACES ACCORDING TO ISO 22196:2011

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Assay: Measurement of antibacterial activity on non-porous surfaces according to ISO 22196:2011

Test beginning: 02/12/2020

Test conclusion: 18/12/2020

Sample description: - TEST SURFACES: n°30 samples of laminated wood (dimensions: 50 x 50 mm of 10 mm max of thickness), disinfected with alcohol 70% and divided into 6 groups of 10 specimens each.
Group 1: surfaces were treated with antibacterial solution (denominated HALOS 5%, batch from July 2020), by electrostatic application for 2 secs. The samples were dried at room temperature for at least 2 hours.
The remaining 20 samples were electrosprayed for 2 secs with HALOS 5%, then divided into 2 groups of 10 specimens each prior to the assay. Group 2: 10 surfaces were washed 5 times with 1 ml ammonia 1%. Group 3: 10 surfaces were washed 5 times with 1 ml sodium hypochlorite 0.9%.

- CONTROL SURFACES: n°10 samples of laminated wood (dimensions: 50 x 50 mm of 10 mm max of thickness) disinfected with alcohol 70%, but non treated with HALOS, were taken as negative control

1. GENERAL

1.1 SUMMARY OF THE ISO 22196

The ISO 22196 specifies a method of evaluating the antibacterial activity of antibacterial-treated plastics, and other non-porous surfaces.

The ISO 22196 was first edited in 2007 and describes a method to quantitatively test the ability of plastics to inhibit the growth of microorganisms (Bacteriostatic effect) or kill them (Bactericidal effect), over a 24 hour period of contact. The second edition of the method, released in 2011, extends its applicability to other Non-Porous Surfaces, no longer limiting it to only plastic surfaces.

The ISO 22196 is a relatively sensitive assay, able to detect low-level antimicrobial effects exerted over long periods of time.

The ISO 22196 was modeled after JIS Z 2801. The two methods are essentially the same.

Building materials are excluded, except where they are used in the same manner as treated articles.

Antibacterial-treated textile products are excluded, even if the surfaces are coated or laminated.

Photocatalytic materials and products are excluded.

The assay is not intended to be used to evaluate the effects and propagation of bacteria on non-porous surfaces without antibacterial treatments.

Secondary effects of antibacterial treatments, such as the prevention of biodeterioration and odor, are not covered by the standard, which is not intended to be used or referenced as a method to document or claim biodegradability of, for instance, plastics materials.

1.2 OVERVIEW OF THE ISO 22196 PROCEDURE

- The test microorganism is prepared, usually by growth in a liquid culture medium. Test microorganism refers to the mandatory list of microbes that must be used in the

test to determine the antimicrobial activity of the product. The mandatory microorganisms are assumed to represent all microbes in its group. Per the method, two representative microorganisms are specified, *S. aureus* and *E. coli*.

- The suspension of the test microorganism is standardized by dilution in a nutritive broth. this affords microorganisms the potential to grow during the test.
- Control and test surfaces are inoculated with microorganisms. The test requires at least 3 specimens from each treated test material and 6 specimens of untreated material. Half of the untreated test specimens are used to measure the viability of bacterial cells immediately after inoculation. The other half are used to measure the viability of bacterial cells after 24-hour period incubation.
- The microbial inoculum is covered with a thin, sterile film. The films are used to spread the inoculum all over, but not beyond, the tested surface area, to prevent the surface from evaporation and to ensure close contact with the antimicrobial surface.
- Parallel controls are run to provide adequate comparisons at both the start of the test as well as after the contact time, in this case 24 hours.
- Microbial concentrations are determined at "time zero" by elution followed by dilution and plating.
- A control is run to verify that the neutralization/elution method effectively neutralizes the antimicrobial agent in the antimicrobial surface being tested.
- Inoculated, covered control and antimicrobial test surfaces are incubated in a humid environment for 24 hours.
- After incubation, microbial concentrations are determined. The reduction of microorganisms compared to initial concentrations and to the control surface is calculated. Based on these reduction calculations, this assay allows us to interpret whether the test substance is bacteriostatic, having the ability to inhibit the growth of microorganisms, or if the test substance is bactericidal, having the ability to kill them.

1.3 STRENGTHS OF THE ISO 22196 TEST

- The method is quantitative and results are reproducible, provided the inoculum does not spill off of the target area after being covered with the thin film.
- The method tests for both bacteriostatic (growth-inhibiting) and bactericidal (bacteria-killing) properties.

- Microbial concentrations are standardized, and bacteria are provided with nutrients during the incubation period, which provides them with ample opportunity to grow if surfaces are not sufficiently antimicrobial. This is in contrast to certain other antimicrobial tests, where microbes are "incubated" in non-nutritive suspensions, which itself may be stressful over long periods.

- The test method includes options to inoculation, such as the volume of the inoculum, to mediate for surfaces that do not lend themselves to the normal testing parameters. This adds flexibility to the method and allows it to be used to evaluate very different surfaces with the same objective, to be antimicrobial.

- The method includes a "pass/fail" criterion for the calculated levels of antimicrobial activity observed in test samples, making determinations of antimicrobial activity less discretionary.

1.4 WEAKNESSES OF THE ISO 22196 TEST

- The ISO 22196 method is not necessarily representative of actual surface contamination events. Moreover, the surface is kept wet (usually for a period of 24 hours). Most of the time, microbial contaminants dry quickly onto surfaces. This limits the time that an aqueous medium is available to facilitate interaction between the antimicrobial surface and microorganisms.

- Although the second edition of this method extends its applicability to other non-porous surfaces, it still excludes a number of surfaces that can be evaluated under this International Standard; these include building materials, textile products that have been treated to be non-porous, and photocatalytic materials.

In conclusion. the ISO 22196 test is an excellent method to quantify the antimicrobial activity level of an antimicrobial surface. Among the various tests for antimicrobial activity of surfaces, this has emerged as one of the industry standards

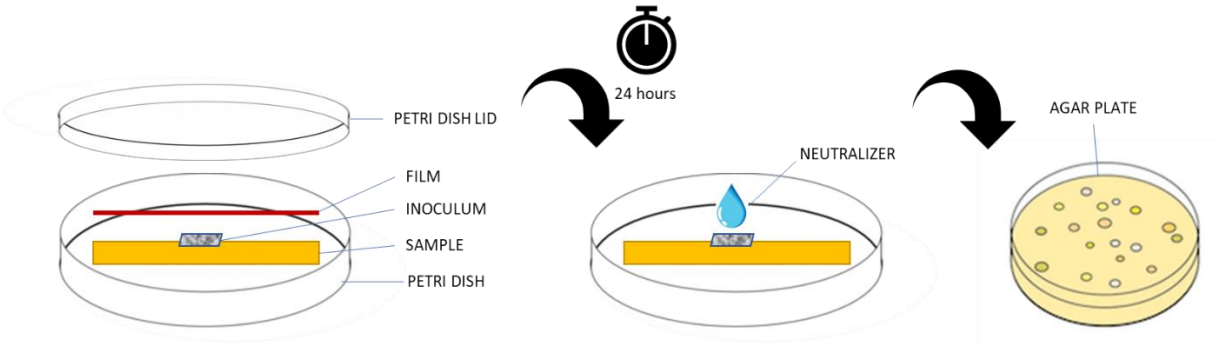

DIAGRAM 1. Schematic representation of the ISO 22196 procedure

2. EXPERIMENTAL

A) Type of sample:

Test Material: n°30 samples of laminated wood (dimensions: 50 x 50 mm of 10 mm max of thickness), first disinfected with alcohol 70% and divided into 3 groups of 10 specimens each.

Group 1: surfaces were treated with antibacterial solution (denominated HALOS 5%), by electrostatic application for 2 secs. The samples were dried at room temperature for at least 2 hours.
Laboratory code: REC3 – 5% July

The remaining 20 samples were electrosprayed for 2 secs with HALOS 5%, dried at room temperature for at least 2 hours and then divided into 2 groups of 10 specimens each prior to the assay.

Group 2: surfaces were washed 5 times with 1 ml ammonia 1%.
Laboratory code: REC3 – 5% NH3

Group 3: surfaces were washed 5 times with 1 ml sodium hypochlorite 0.9%.
Laboratory code: REC3 – 5% Cl

B) Test strains:

- Staphylococcus aureus ATCC 6538, batch number 929105, expiry date 29/07/2021
- Escherichia coli ATCC 8739, batch number 940161, expiry date 18/08/2021

C) Testing conditions:

- Preparation of the test specimens: specimens of laminated wood with antibacterial treatment were supplied cut into squares of 100 x 100 mm of 10 mm max of thickness. As requested, the pieces were not washed with ethanol. Untreated surfaces to be used as control were also supplied.

- Contact time: 24 h ± 1 h

- Diluent: Nutrient Broth 1/500 for strains preparation
  Phosphate-buffered physiological saline: 0.85% of sodium chloride

- Neutralizer: SCDLP broth
  17,0 g of casein peptone, 3,0 g of soybean peptone, 5,0 g of sodium chloride, 2,5 g of disodium hydrogen phosphate, 2,5 g of glucose and 1,0 g of lecithin in 1 000 ml of distilled or deionized water.

- Incubation medium: Tryptic Soy Agar (TSA)

- Incubation of the inoculated test specimens: 35°C ± 1°C, 90% relative humidity for 24 h ± 1 h, damp condition

- Incubation of the plates for the viable bacteria count: 35°C ± 1°C, 90% relative humidity for 48 h

D) Procedure for evaluating the antibacterial activity on non-porous surfaces according to ISO 22196:

The test was carried out in 5 replicates for the test material and for each test strain.
A bacterial suspension was prepared at a concentration (ufc/ml) > 1,2 x 10^5 for E. coli and and for S. aureus.
0.4 ml of bacterial cell suspension (200 µl *Escherichia Coli* and 200 µl *Staphylococcus Aureus*, bacterial concentration about 10^5 ufc/ml) was pipetted on the surface of test specimen placed into a separate sterile Petri dish for each bacterial strain, with the test surface uppermost.

The test inoculum was covered with a piece of inert film (polycarbonate film), as described in ISO 22196:2011 and then the lid replaced on the Petri dish.
Petri dishes containing the inoculated test specimens were incubated at a temperature of 35 ± 1°C and at a relative humidity of 90% for 24 ± 1 h. Next, 10 ml of neutralizer is added to the petri dish. A 10-fold serial dilution is performed on the neutralizer. The count of the viable bacteria recovered was performed, placing 1 ml of the recovered SCDLP from the test specimen and of each dilution into separate sterile Petri dishes. 15 ml of plate count agar were poured into each Petri dish. All petri dishes were incubated at a temperature of 35 ± 1°C and at a relative humidity of 90% for 48 h.

Immediately after inoculation three untreated test specimens were processed in the same way.
The value obtained from this bacterial count was used to determine the recovery rate of the bacteria from the test specimens under investigation.
After incubation, for each test specimen, the number of viable bacteria recovered (cfu/surface) was determined and the antibacterial activity was calculated using Equation:

$$R = (U_t - U_0) - (A_t - U_0) = U_t - A_t$$

Where:

R is the antibacterial activity;
U_0 is the average of the common logarithm of the number of viable bacteria, in cells/cm^2, recovered from the untreated test specimens immediately after inoculation;
U_t is the average number of viable bacteria, in cells/cm^2, recovered from the untreated test specimens after 24 h;
A_t is the average number of viable bacteria, in cells/cm^2, recovered from the treated test specimens after 24 h.

7) Results and conclusions:

Table 1: Summarizing table of the recovery immediately after inoculation and after 24 h from untreated specimens (control samples)

U_0: Average of the common logarithm of the number of viable bacteria, in cells/cm^2, recovered from the untreated test specimens immediately after inoculation	U_t = average of the common logarithm of the number of viable bacteria, in cells/cm^2, recovered from the untreated test specimens after 24 h
170,000	280,000
log U_0 = 5.24	log U_t = 5.44

Table 2: Summarizing table of the recovery after 24 h from test samples and calculation of their antimicrobial activity

Sample	A_t (cells/cm^2)	log A_t	R Antibacterial activity $U_t - A_t$	Reduction of microbial load (%)
REC3 – 5% NH3	570	2.76	2.68	99.79
REC3 – 5% Cl	320	2.50	2.94	99.88

On the basis of data collected and shown in tables 1 and 2, the antibacterial surfaces coated with HALOS 5% and repeatedly washed superficially with commonly used detergents (ammonia 1% and sodium hypochlorite 0.9%) showed a very good antibacterial efficacy against strains of Staphylococcus Aureus ATCC 6538 and Escherichia Coli ATCC 8739, with a reduction of microbial load higher than 99%. These results may indicate that the antibacterial coating (HALOS 5%) is not removed by the application of the cleaning agents, or/and that there is an additive antibacterial efficacy of HALOS 5% and the cleaning agents. All the surfaces tested by ISO 22196: 2011 were imaged by reflected light microscopy (4 x magnification) to check for the integrity of the antibacterial coating. Figure 1 shows optical microscopy pictures of the uncoated surfaces, where a certain roughness of the material can be observed. When the samples are coated with HALOS 5% (figure 2), the surfaces appear smoother compared to the untreated samples, indicating the effective deposition of the antibacterial coating HALOS 5%. After repeated superficial washes with ammonia 1% (figure 3) and sodium hypochlorite 0.9% (figure 4), the smooth aspect of the surfaces is maintained, indicating that the antibacterial coating resists water-based solution of ammonia and sodium hypochlorite.

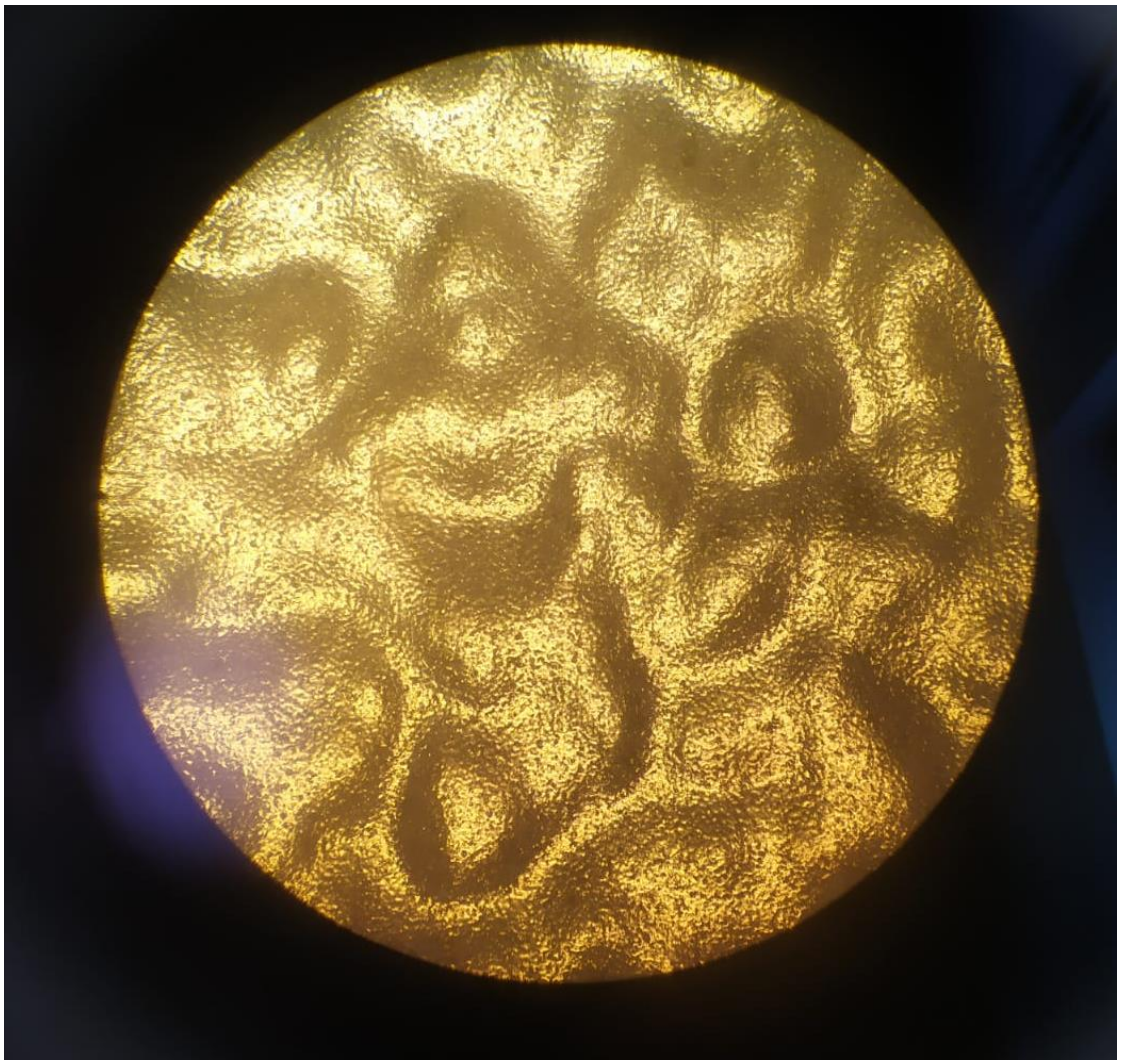

Figure 1. Reflected light microscopy picture (4x magnification) of untreated laminated wood.

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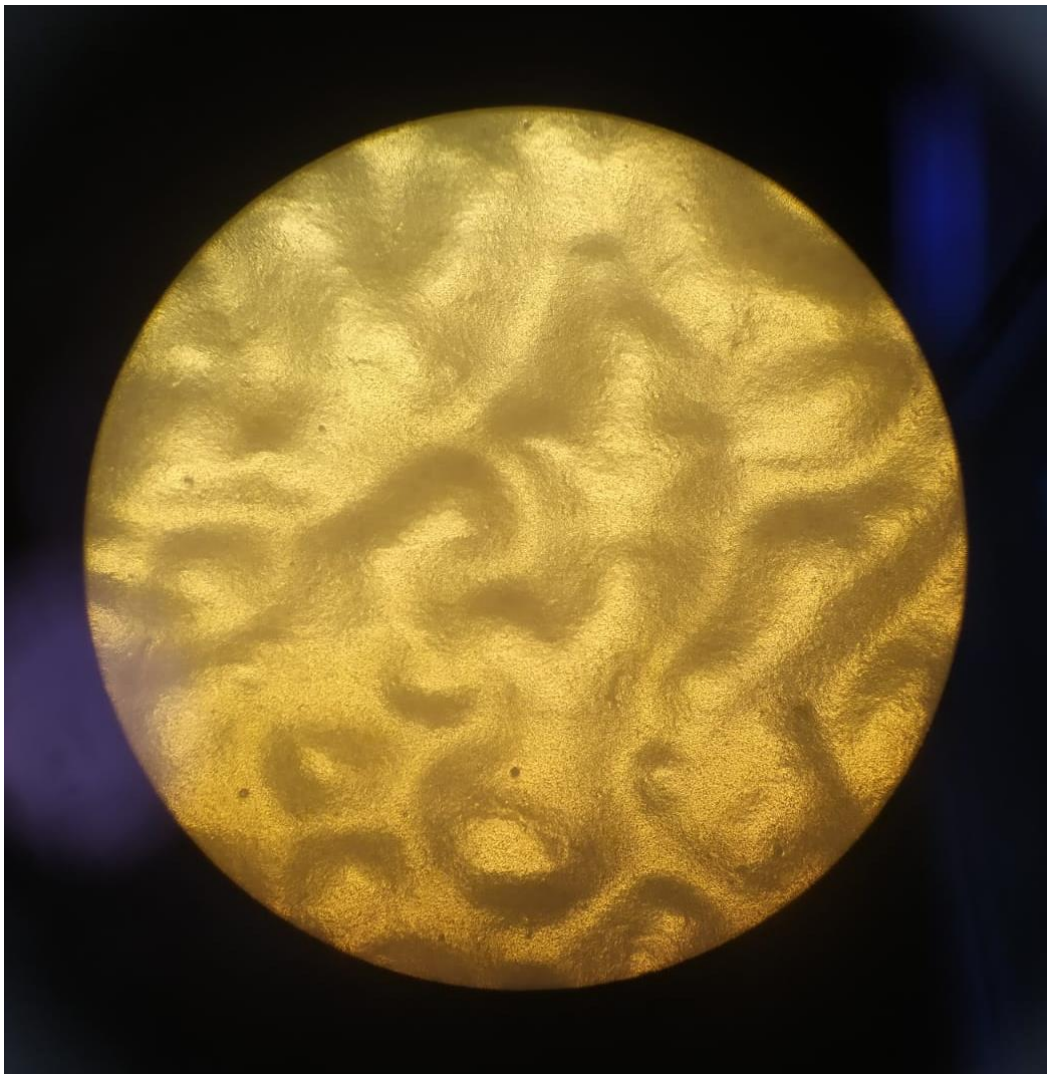

Figure 2. Reflected light microscopy picture (4x magnification) of laminated wood treated with HALOS 5% by electrospray for 2 secs and dried at room temperature for at least 2 hours.

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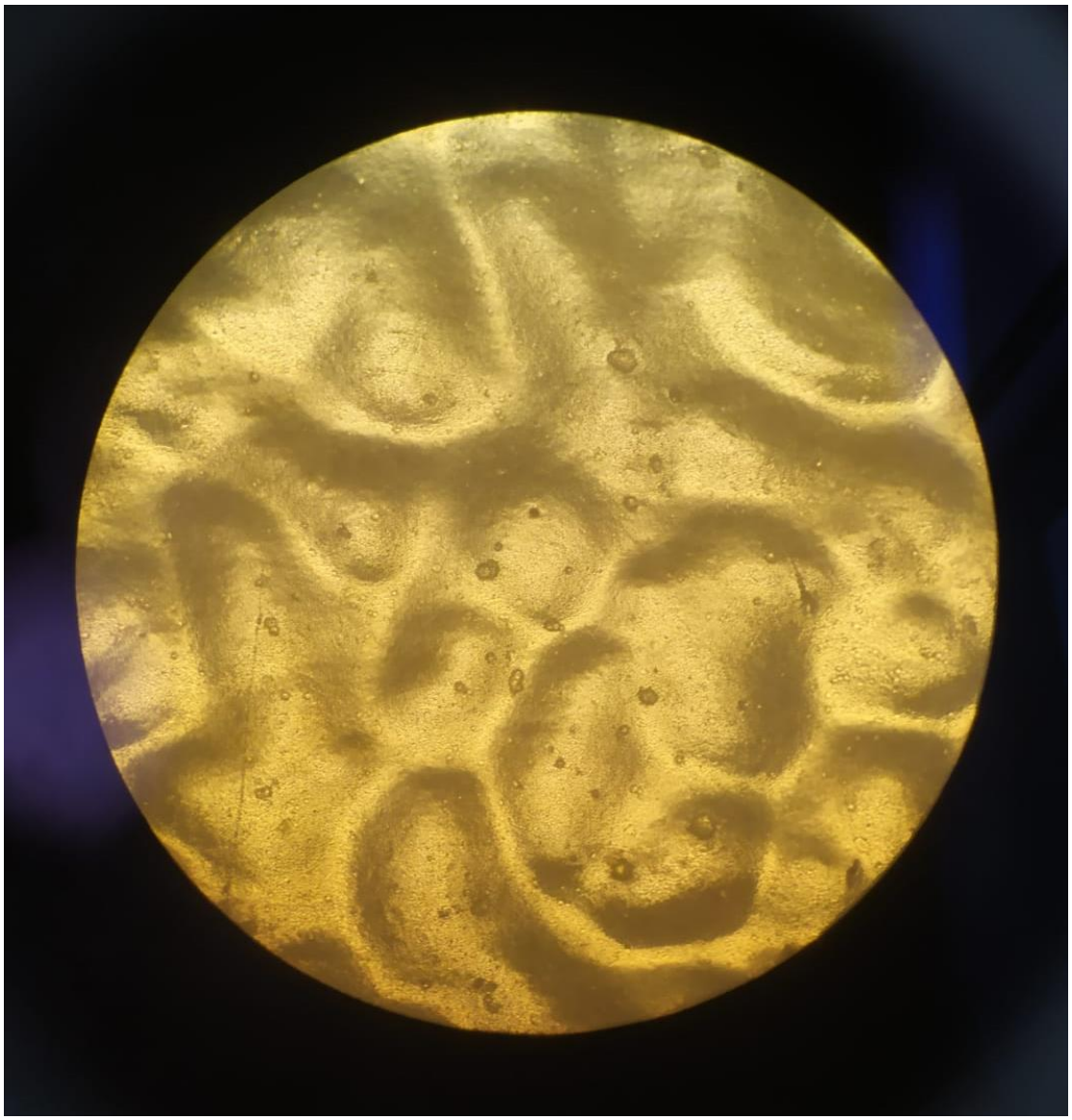

Figure 3. Reflected light microscopy picture (4x magnification) of laminated wood treated with HALOS 5% by electrospray for 2 secs, dried at room temperature for at least 2 hours and washed 5 times with 1 ml ammonia 1%.

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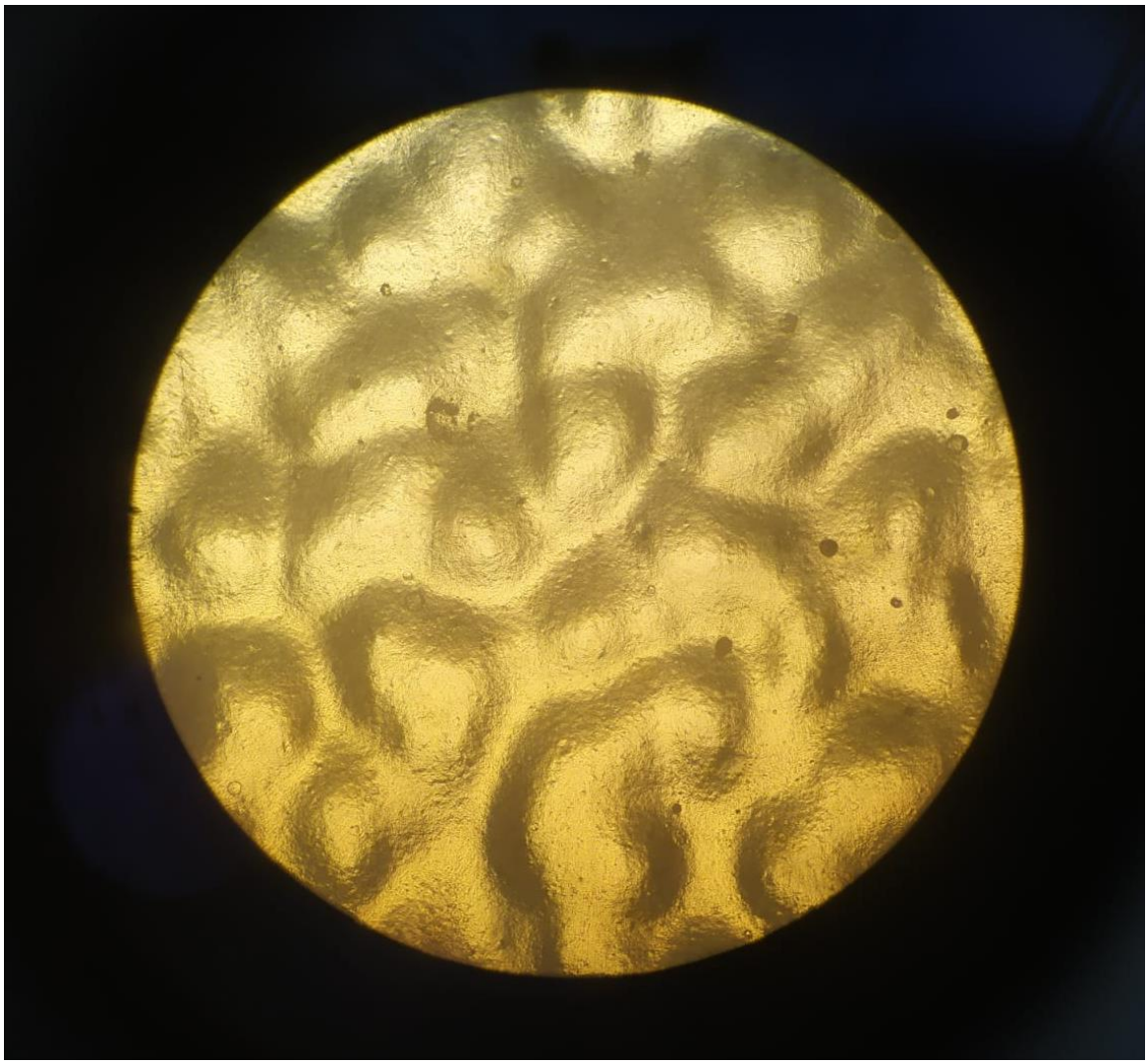

Figure 4. Reflected light microscopy picture (4x magnification) of laminated wood treated with HALOS 5% by electrospray for 2 secs, dried at room temperature for at least 2 hours and washed 5 times with 1 ml sodium hypochlorite 0.9 %.

Camerino, 7th January 2021

Project Principal Investigator

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