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METHOD FOR TESTING THE ANTIMICROBIAL CHARACTER OF THE MATERIALS AND THEIR FITTING TO THE SCOPE

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Abstract: *With the advent of antimicrobial materials it has become a necessity to develop methods for determining their antimicrobial nature. Due to the novelty of the concept, new methods have been developed and those already implemented are in a continuous process of improvement. This paper gives an overview of the methods and can be seen as a guide, so as the applied testing methodology is optimally applied, depending on the nature of the antimicrobial material sought and thus adequate for its intended purpose.*

Key words: *antimicrobial materials, methods.*

1. Introduction

Being in the era of antibiotic resistance, as Dr. Fanner appreciated [6], the opportunity of utilizing antimicrobial materials is increasing in many fields.

Due to this increasing demand, it has become a necessity to develop methods for determining the antimicrobial character of materials, thus new specialized laboratories are becoming available in the face of the new challenges.

National and international standards associations have understood the need for standardized methods to determine the antimicrobial nature of the materials.

In the aforementioned framework, in 2000, the Japanese Standards Association published in JIS (Japanese Industrial Standard) the JIS Z 2801: 2000 standard - Antimicrobial products - Test for antimicrobial activity and efficacy, aimed to regulate the types of antimicrobial tests and the conditions under which they are performed, but this standard only refers to plastics and textiles [17].

Currently, besides the already standardized methods, new methods for determining the antimicrobial potential of the materials are continuously being developed and published.

To highlight the efficacy of anti-microbial materials, the test methods must be well adapted to their purpose and provide reproducible results.

2. Classification of the Determination Methods for the Antimicrobial Character of Materials

Depending on the result of the tests, methods for determining the antimicrobial character of the materials are classified into quantitative and qualitative methods.

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In terms of other evaluation criteria, both quantitative and qualitative methods can be sub-classified as follows: depending on the specific method of analysis, the tests can be physico-chemical and micro-biological; in accordance to the biological entities described; there are fungal, bacterial and viral tests; as referring to the existance of documented references, there are standardized methods and unstandardized methods of analysis.

3. Quantitative Methods for Determination of the Antimicrobial Character of Materials

The quantitative methods indicate the contamination level of the studied materials by quantifying the analysis results and due to this fact these methods are the most commonly used.

3.1. ASTM E2149 Test for Irregularly Shaped Antimicrobial Surfaces

This is a microbiological quantitative and standardized method and representing a bacterial test. It is destined for the analysis of irregularly shaped surfaces of the materials and it is frequently used in the antimicrobial materials industry, although it presents some disadvantages. The principle of this method consists of immersing the material in a suspension of known initial composition, which is to be determined at specific time intervals ranging from 1h to 24 h [12].

The contact time of the material with the liquid suspension is very important in the case of this method, and the standard does not specify the contact time interval after which a material can be considered as antimicrobial. Generally, according to other reference documents, a material can be considered as antimicrobial only if the initial concentration of the bacteria suspension has been significantly reduced, by comparing to a reference sample. In the case of this method, great care should be taken for the antimicrobial agents from the material not to migrate in the bacterial suspension, in which case the results of the analysis being declared as invalid [7].

3.2. ASTM E2180 Test for Hydrophobic Antimicrobial Surfaces

This microbiological, quantitative and standardized method is also a bacterial test. The ASTM E2180 method has been developed for the antimicrobial agents incorporated into polymers or hydrophobic materials efficiency testing.

The principle of this method consists in the formation of a pseudo-film at the surface of the material. The pseudo-film is actually an agar medium inoculated with bacterial suspensions of known concentration. For comparison, a reference material is treated in the same conditions. The samples are incubated for 24 hours, after which the results are interpreted [14].

The method specially applies to hydrophobic materials and it is not recommended for the determination of the efficiency of the surfaces treated against the biofilm cells.

3.3. EPA Copper Sanitization Test

This is a microbiological, quantitative and non-standardized method, but it is frequently used by ATL (Antimicrobial Test Laboratories) from Texas, USA, for the sanitation degree of copper-based materials and alloys determination [8].

The principle of this method consists of inoculating the surfaces with standardized test solutions and after this, the materials are left to dry for 20-40 minutes. The test is performed in triplicate. Microorganism concentration reduction by comparing with the initial concentration of the standard

indicates the antimicrobial potential of the tested surface.

The strength of this method is that it takes into account the drying of the microorganisms. Moreover, the method is targeted towards the typical micro-organisms that can be seen on these surfaces. The method cannot be applied to waxed, painted or varnished surfaces. For research and development purposes, this method can be adapted to test alternative technologies (quats, silver, Peroxides etc.) [9].

3.4. ISO 22196 Test for Antimicrobial Activity of Plastics

This is a microbiological, quantitative method, standardized according to JIS Z 2801. It is modeled after ISO 22196 method and represents a bacterial test. The principle consists in coating the surfaces with a nutrient broth inoculated with a standardized microbial suspension. The nutrient broth consists of 3.0 g of beef extract, 10 g of peptone and 5.0 g of sodium chloride dissolved in 1000 mL of pure distilled water.

The pH value of the fluid is adjusted with sodium hydroxide in the 7 to 7.2 units' interval. The broth is poured onto the tested material placed in the center of a Petri dish. Then, on the culture media 0.4 mL of the bacterial test inoculum is applied. The tests are usually performed in triplicate. The Petri dishes are subjected to incubation for 24 h at 35±1 °C. After incubation the antimicrobial efficacy is established in relation to the initial concentration of bacterial inoculum [15].

This method is most often used for plastics due to the reproducibility of results.

3.5. AATCC TM100

AATCC TM100 is a quantitative, standardized microbiological method, usually applied to antimicrobial textile materials. AATCC TM100 requires only one sample for testing, while the JIS L 1902 and ISO 20743 standards recommend the testing in triplicate [16], [18]. The method was applied by the US Army for socks and military outfit testing [1]. A disadvantage of this method is the usage of a high-level nutrient medium for bacteria, which does not happen in real bacteria-growing media [5].

3.6. Contact Plate Method

This microbiological method was developed on the basis of quality standards in order to determine specific micro-organisms.

The contact plate method applies to both bacterial and fungal tests. In general, contact plates are used to determine the sanitation level of solid and flat surfaces. But in research, this method can be adapted also to the testing of liquids by contact plate immersion. Contact plates are fitted on each side with a slide containing a culture medium. After sampling, the slides are placed in sterile covered plastic vials, provided by the supplier (Figure 1).

Fig. 1. *Bacteria determination test example*

The advantage of the method is that the contact plates are ready to use and allows the simultaneous determination of two types of germs.

Bacteria, fungi and yeasts from the tested surfaces are sampled on the surface of the testing slides. The tubes in which the slides are placed are incubated for 24-48 h.

Results are expressed in CFU/cm^2 (Figure 2).

Fig. 2. *Fungi determination test example*

This analysis method offers fast, reliable results and does not require qualified personal.

3.7. Turbidity or Optical Density

This quantitative physico-chemical method represents also a bacterial test. Adapting this method in the field of microbiology is possible due to the correlation between turbidity and concentration of bacterial suspensions. For example, turbidity of a bacterial suspension of *E. coli* of approximately 3.0 CFU/mL is equivalent to 1 McFarland turbidity standard [2], [4]. By using this method the material is tested indirectly, analyzing either the washing water or a bacterial suspension of known concentration. The tested solid anti-microbial materials are placed in sterile water or bacterial suspension of known concentration. The method requires a long contact time between the material and the fluid medium in order for the results to be valid.

For this analysis either turbidimeters or spectrophotometers could be used in order to determine the optical density of the suspensions, at wavelengths between 580 and 660 nm.

3.8. The Filtering Membrane Method

The filtering membrane is a micro-biological, quantitative method and represents a bacterial test. It is a standardized method for determining water quality, but can be used also for determining the antimicrobial activity of the materials by analyzing the washing waters. The principle of this method consists in filtering known volumes of water samples through a membrane with 0.45 μm diameter pores. Bacteria from the water samples remain on the surface of the membrane filter and then the loaded membranes are placed with sterile forceps on different selective culture media. They are subjected to incubation at optimum temperatures for development of different bacteria strains. The results are expressed in CFU (colony forming units) (Figure 3).

This method presents a major disadvantage: by washing the material of interest it is possible that not all the bacteria are present in the washing water, because the natural phenomenon of bacterial adherence.

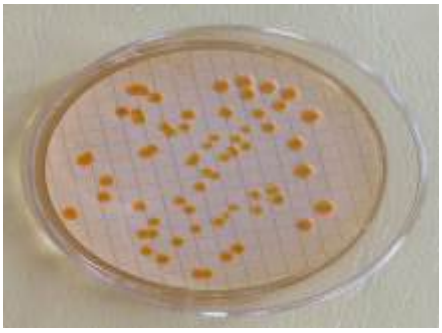

Fig. 3. *Coliform bacteria determination from washing water test*

4. Qualitative Methods for Determination of the Antimicrobial Character of the Materials

The qualitative methods do not offer quantifiable results. In general, the results are expressed as “negative” or “positive”. The qualitative methods could offer valuable information regarding the

antimicrobial properties of materials. The results obtained by these methods are based on chromatics and inhibition areas.

4.1. ASTM E1428 "Pink Stain Test"

It is a standardized, qualitative microbiological method, using the microorganism *Streptoverticullium reticulum*. The principle of the method consists in the appearance of pink spots on the material introduced in plates seeded with a specific growth medium [13].

The method can be used for any flat-surfaced synthetic polymeric material. It is not recommended for dark-colored materials, as staining is difficult to identify. Another disadvantage of the method is that it is not specific to a certain type of microorganism. Obtaining the final results after 14 days of incubation is also a minus [7].

4.2. AATCC TM147

This microbiological method, also called qualitative Kirby-Bauer Qualitative Test for Growth Inhibition since it has been adapted by Kirby-Bauer and frequently used in medicine.

The principle of the AATCC TMI147 method is the obtaining of a inhibition area around the sample exhibiting antimicrobial character [10].

The method can be applied to all solids (wood, polymers, and textiles).

AATCC TM147 is used to test both bacterial and fungal activity. An agar medium contained in a Petri plate is seeded with a known microbial inoculum culture. The material to be analyzed is placed in the middle of the Petri dish. After 18-24 h of incubation, antimicrobial materials develop an inhibition area around them (Figure 4). If the material does not present antimicrobial activity, no inhibition area occurs, as can be seen from Figure 5 [3].

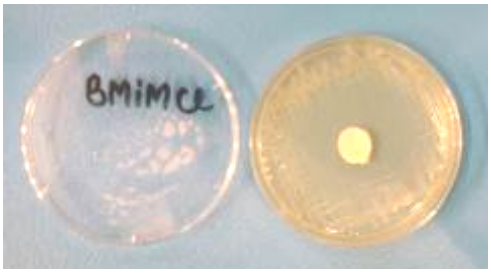

Fig. 4. *Inhibition area promoted by a polymer sample treated with an antimicrobial agent*

Fig. 5. *Absence of the inhibition zone for the untreated polymer samples*

Even if the method is qualitative, a correct evaluation could be realized by measuring the inhibition area. The measurements could be compared only if the samples present the same diameter.

As a function of the inhibition area, the antimicrobial activity level of the tested sample could be easily evaluated, as it can be observed from Figures 6 and 7.

The method is relatively rapid and can be used for a wide range of materials. Even if it is only a qualitative method, still it could offer indications regarding the level of antimicrobial efficiency.

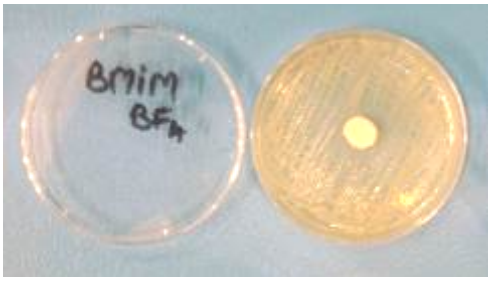

Fig. 6. *Low level of microbial activity*

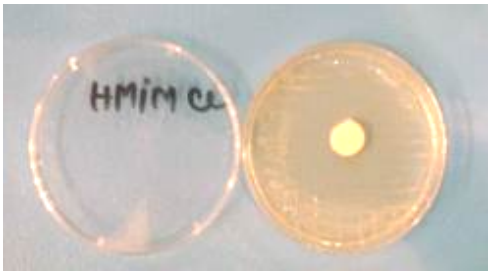

Fig. 7. *High level of microbial activity*

4.3. AATCC TM30 (Part III and IV)

Method AATCC TM30 (Part III) [14] is a typical qualitative fungal test. The sample is placed in a Petri dish containing dextrose-agar medium innoculated with spores of *Aspergillus niger*. Samples thus prepared were incubated for seven days, the time needed for the microorganism Aspergillus niger to attain maturation.

In most cases it is necessary to use a microscope for correct interpretation of the results. Although rare, in some cases an inhibition area around the analyzed material could be recorded.

AATCC TM30 part IV [11] method is less aggressive than the previous and makes a better discrimination between treated and untreated samples. It is used for determining the anti-mould efficiency of the material against several types of moulds by using a suspension of spores which is sprayed on the tested sample. Samples thus prepared are placed in a closed glass recipient and incubated [5].

4.4. SwabCheck Hygiene Test

SwabCheck is a qualitative and non-standardized bacterial test.

The surface subjected to microbial analysis is whipped with a cellulose pad. Thus, any bacteria resident on the tested surface is transferred via the cellulose pad into a specific culture medium containing an indicator dye.

The tube containing the culture medium is incubated at 37 °C for 24 hours. A single bacterium is sufficient to determine a change in the color of the indicator, indicating that SwabCheck is 1000 times more sensible than the conventional ATP method [9]. This precision is of uttermost importance in the food industry.

Colour modification from red to yellow indicates the presence of a microbiological load. The colour change is based on the reaction of the acid from the culture medium with the indicator dye. A rapid change in colour indicates a high level of bacterial contamination of the surface [7].

Fig. 8. *SwabCheck method sampling example*

The advantage of the SwabCheck method is represented by the possibility of sample collection from hard to reach places (Figures 8, 9), where usually micro-organisms reside.

SwabCheck is useful in determining the sanitation levels of surfaces from the food and beverages processing units, as well as from laboratories and so forth.

The advantage of SwabCheck consists in the fact that it is sterile and exists in a ready to use form. Also, it is easy to handle, offers quick results and has a long shelf life.

SwabCheck is used only as an indication for the sanitation degree for various types

of surfaces, but an evaluation of the contamination can be established by the rate of colour modification of the medium. This method is easy to use and does not require qualified personal.

It is not recommended for materials with grains or fine grooves where the cellulose swab cannot reach.

Fig. 9. *SwabCheck tests examples*

5. Conclusions

The determination of the antimicrobial character of the materials is a time-consuming complex process, requiring attention, care and thorough documentation. The establishment of the working method must be performed in concordance with the material's properties and use. So, it is necessary to know the purpose for which the material was created and what are the user expectations regarding the material. The chosen method must simulate as accurately as possible the real-world situations. For reproducible results with a high degree of confidence, a validation of the used method is absolutely necessary.

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References

1. Johnson, A.: *AATCC TM Clarification*. US Army Natick Soldier RD&E Center, Natick, MA, USA.
2. Noumedem, J.A.K., Tamokou, J.D., Teke, G.N., Momo, R.C., Kuete, V.: *Phytochemical Analysis, Antimicrobial and Radical-Scavenging Properties of Acalypha Manniana Leaves*. In: SpringerPlus **2** (2013) No. 503. doi: 10.1186/2193-1801-2-503
3. Patachia, S., Damian, N.: *Cryogels Based on Poly(Vinyl Alcohol)/Ionic Liquids: From Obtaining to Antimicrobial Activity*. In: Soft Materials **12** (2014) No. 4, p. 371-379. DOI:10.1080/1539445X.2014.933113.
4. Rojas, J.J., Ochoa, V.J., Ocampo, S.A., Munoz, J.F.: *Screening for Antimicrobial Activity of Ten Medicinal Plants Used in Colombian Folkloric Medicine: A Possible Alternative in the Treatment of Non-Nosocomial Infections*. In: BMC Complementary and Alternative Medicine **6** (2006) No. 2, doi:I0.II86/1472-6882-6-2.
5. Swofford, H.W.: *An Overview of Antimicrobial Testing for Textile Applications,* In: Microban International, USA, AATCC Review (2010).
6. Tanner, B.: *Antimicrobial Fabrics-Issues and Opportunities in the Era of Antibiotic Resistance.* In: AATCC Review **9 (**2009), p. 30-33.
7. *** Hygiene Swabcheck, Ready to Use - Thomas Scientific (2014). Source: www.thomassci.com/Supplies/.../Hygiene-Swabcheck-Ready-To-U. Accessed: 15-09-2014.
8. http://www.epa.gov/oppad001/pdf_files/test_method_copper_alloy_surfaces.pdf. Accessed: 20-09-2014.

9. http://www.antimicrobialtestlaboratories.com. Accessed: 17-09-2014.
10. AATCC Tehnical Manual **85** (2010), p. 251-252. Accessed: 10.09.2014.
11. AATCC Tehnical Manual **85** (2010), p. 76-79. Accessed: 15.09.2014.
12. *** ASTM E2149: *Standard Test Method for Determining the Antimicrobial Activity of Immobilized Antimicrobial Agents under Dynamic Contact Conditions.* Accessed: 10.09.2014.
13. *** ASTM E1428 "Pink Stain Test": *Standard Test Method for Evaluating the Performance of Antimicrobials in or on Polymeric Solids against Staining by Streptoverticillium reticulum.* Accessed: 12.09.2014.
14. *** ASTM E2180: *Standard Test Method for Determining the Activity of Incoporated Antimicrobial Agents in Polymeric or Hydrophobic Materials.* Accessed: 10.09.2014.
15. *** ISO 22196: *Measurement of Antibacterial Activity on Plastic Surface.* Accessed: 10.09.2014.
16. *** ISO 20743: *Textiles - Determination of Antibacterial Activity of Antibacterial Finished Products,* 1st Edition, 2007. Accessed: 10.09.2014.
17. *** Japanese Industrial Standard, JIS Z 2801:2000*: Antimicrobial Products - Test for Antimicrobial Activity and Efficacy*. Accessed: 05.09.2014.
18. *** Japanese Industrial Standard, JIS L 1902:2002 (JAFET/JSA): *Testing for Antibacterial Activity and Efficacy on Textile Products*. Accessed: 11.08.2014.