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Measurement method of anti-biofilm activity on plastic and other non-porous surfaces

Méthode de mesure de l'activité anti-biofilm sur le plastique et autres surfaces non poreuses

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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This document was prepared by Technical Committee ISO/TC 61, *Plastics*, Subcommittee SC 6, *Ageing, chemical and environmental resistance*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Biofilms are known to be compositions of substances such as polysaccharides, lipids, and nucleic acids produced by microorganisms and the cells of microorganisms, and are widely found in our living environment as visible harms such as slimes, where water is present.

ISO 22196 on antibacterial test and ISO 21702 on antiviral test are the test methods for evaluating "invisible hygiene" on the surface of non-porous products. Similarly, ISO 20743 on antibacterial test and ISO 18184 on antiviral test are the test methods for evaluating the same of porous products.

This document describes how to evaluate products that are treated to suppress biofilm formations on the non-porous surfaces.

Measurement method of anti-biofilm activity on plastic and other non-porous surfaces

1 Scope

This document specifies a test method to evaluate the anti-biofilm activity of anti-biofilm treated plastics and other non-porous surfaces of products, including intermediate products. It is applicable to products such as plastics, coating materials, ceramics, stainless steels and rubber. Textile and photocatalytic materials are out of its scope.

This method is intended to be a screening test for material development, and it is not expected to reflect effects observed in the actual environment in which materials will be deployed. A "Crystal violet staining – Absorbance measurement assay" is used to quantify the amount of biofilm formation in this document.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
biofilm
microbial cells and their metabolites such as polysaccharides, proteins, lipids and nucleic acids, firmly attached to the material surface of the product in water

Note 1 to entry: In this document, crystal violet is used to stain the biofilm.

3.2
anti-biofilm
controlling the formation of *biofilm* (3.1) attached to the material surface of the product

3.3
anti-biofilm activity
activity in suppressing formation of *biofilm* (3.1) on the material surface

4 Materials

4.1 Bacterial strain to be used for the tests

The test strain is *Staphylococcus epidermidis* ATCC35984.

Other species of bacteria may be used after appropriate validation, as the importance of the species chosen can differ depending on the target application. If other species are used, the name of the species, its strain number and the specific reason for their use shall be included in the test report.

4.2 Reagents

Water shall be distilled or deionized and shall have a conductivity of <1 µS/cm.

All reagents shall be of an analytical grade and/or of a grade appropriate for microbiological purposes.

4.3 Culture media and solution

4.3.1 General

The culture medium specified in 4.3.2, 4.3.3, 4.3.4 and 4.3.5 shall be used. The medium may be obtained from commercial suppliers, where it shall be prepared in accordance with the manufacturer's instructions.

The quantity of the culture medium can be changed according to the intended test volume, but the composition of the culture medium should not be changed.

4.3.2 Nutrient agar (NA)

Prepare NA by dissolving 5,0 g of meat extract, 10,0 g of peptone, 5,0 g of sodium chloride and 15,0 g of agar powder in 1 000 ml of distilled or deionized water. Heat, with stirring, on a hotplate or in a boiling-water bath until the agar has dissolved. Adjust the pH to a value between 7,0 and 7,2 (at 25 °C) with sodium hydroxide or hydrochloric acid. Sterilize by autoclaving (see 6.2). If it is not used immediately after the preparation, store it at 5 °C to 10 °C. NA that has been kept for one month or longer after the preparation shall not be used.

4.3.3 Suspension medium-1/5 Tryptic soy broth (1/5TSB)

Prepare 1/5 TSB by dissolving 17,0 g of a pancreatic digest of casein, 3,0 g of papaic digest of soybean, 5,0 g of sodium chloride, 2,5 g of dipotassium phosphate and 2,5 g of dextrose in 1 000 ml of distilled or deionized water. Dilute the trypticase soy broth with distilled or deionized water 1:4 volumetrically and adjust the pH to a value between 7,1 and 7,5 (at 25 °C) with sodium hydroxide or hydrochloric acid. Sterilize by autoclaving (see 6.2). If it is not used immediately after the preparation, store it at 5 °C to 10 °C. 1/5 TSB that has been kept for one week or longer after the preparation shall not be used.

4.3.4 Plate count agar

Prepare a plate count agar by dissolving 2,5 g of a yeast extract, 5,0 g of tryptone, 1,0 g of glucose and 15,0 g of agar powder in 1 000 ml of distilled or deionized water. Heat, with stirring, on a hotplate or in a boiling-water bath until the agar has dissolved. Adjust the pH to a value between 7,0 and 7,2 (at 25 °C) with sodium hydroxide or hydrochloric acid. Sterilize by autoclaving (see 6.2). If it is not used immediately after the preparation, store it at 5 °C to 10 °C. Plate count agar that has been kept for one month or longer after the preparation shall not be used.

4.3.5 Slant culture medium

Slants are prepared using 6 ml to 10 ml of molten NA poured into a suitable-capped test tube. Sterilize by autoclaving (see 6.2). After the sterilization, place the test tube at an angle of about 15° to the horizontal direction and allow the contents to become solid. If it is not used immediately after the preparation, store it at 5 °C to 10 °C. Slant culture medium kept for one month or longer after the preparation shall not be used.

4.3.6 0,1 % Crystal violet aqueous solution

Prepare a crystal violet aqueous solution by dissolving 1,0 g of crystal violet in 1 000 ml of distilled or deionized water.

If any residues are observed in the solution, they must be removed by filtering the solution through a qualitative filter paper, grade No 2 (5.14), until a clear filtrate is obtained.

4.3.7 1,0 % Sodium dodecyl sulfate aqueous solution

Prepare a sodium dodecyl sulfate aqueous solution by dissolving 10,0 g of sodium dodecyl sulfate in 1 000 ml of distilled or deionized water.

5 Apparatus

Unless otherwise specified, use the following apparatus and materials.

5.1 Dry-heat sterilizer, capable of maintaining the temperature at a value between 160 °C and 180 °C within ±2 °C of the set point at equilibrium conditions.

5.2 Autoclave, capable of maintaining a temperature of (121 ± 2) °C and a pressure of (103 ± 5) kPa.

5.3 Hotplate with stirrer, or **hot-water bath**.

5.4 pH-meter, capable of measuring to ±0,2 units.

5.5 Balance, capable of weighing to ±0,01 g.

5.6 Pipetter, capable of setting a plastic pipette.

5.7 Plastic pipette, sterile, and capable of measuring with capacities of 25 ml ± 0,25 ml.

5.8 Freezer, capable of operating at a temperature of – (80 ± 2) °C or – (20 ± 2) °C.

5.9 Incubator, capable of maintaining the temperature within ±1 °C of the set point at equilibrium conditions.

5.10 Clean bench, for microbial test.

5.11 Spectrophotometer, capable of measuring at 590 nm.

5.12 Reciprocal liner shaker, for microbial test. Swing width: (30 ± 5) mm.

5.13 Water-soluble non-woven fabric, a polyvinyl alcohol non-woven fabric.

5.14 Qualitative filter paper, Grade No.2.

5.15 Test tubes.

5.16 Sterile container, with an outer diameter of 63 mm to 65 mm, a depth of 31 mm to 35 mm, an inner volume of 50 ml to 60 ml.

5.17 Stoppered Erlenmeyer flasks or **media bottles**, as required for preparation of media.

6 Sterilization of apparatus and storage of stock cultures

6.1 Dry-heat sterilization

Place the objects to be sterilized in a dry-heat sterilizer, referring to the temperature and time given in Table 1. The used temperature or time may differ according to the objects.

Table 1 — Condition of dry-heat sterilization

Temperature	Minimum sterilization time
180 °C	30 minutes
170 °C	60 minutes
160 °C	120 minutes

6.2 High-pressure steam sterilization

Place the objects to be sterilized in an autoclave and maintain at (121 ± 2) °C for at least 15 min.

6.3 Preparation of glassware

Wash all glassware well with alkali or neutral detergent, then rinse well with distilled or deionized water. Sterilize using the dry-heat sterilizer or the autoclave prior to use.

6.4 Maintenance of stock cultures

A stock culture shall be stored at 5 °C to 10 °C on an appropriate medium and transferred monthly. After five transfers or if more than one month has passed between the transfers, the stock culture shall be discarded and replaced with a fresh culture. The initial passage removed from the original master strain that is obtained from culture collection is counted as the first transfer.

7 Procedure

7.1 Biofilm formation on the surface of the test specimens

7.1.1 Pre-culture of bacteria

Using a sterile inoculating loop, transfer bacteria from the stock culture to the slant culture medium and incubate at (35 ± 1) °C for 24 h to 48 h. Pour 20 ml of 1/5 TSB into a 100 ml Erlenmeyer flask. Apply an inoculating loop to transfer the bacteria and inoculate it in the broth. Incubate under the following conditions:

— Temperature: 35 °C ± 2 °C;

— Rate of shaking: 110 min^{-1} and 3 cm width by reciprocal liner shaker;

— Incubation time: 18 h to 24 h.

NOTE The incubated slant culture can be stored at 5 °C to 10 °C for a week, if not used immediately.

7.1.2 Preparation of test specimens

The test shall be performed on at least three specimens from each treated test material and untreated test material.

The size of the test specimen is expected to be a flat, 30 mm ± 2 mm square.

The test specimens shall be sterile prior to the test. The sterilization method is selected so as not to affect the effect of the test specimen. For example, a high-pressure steam sterilization is one option.

7.1.3 Preparation of the inoculated incubating medium

Ensure that the test bacteria in pre-culture (see 7.1.1) are evenly dispersed, and estimate the number of bacteria directly using a microscope and a counting chamber, or another appropriate method such as spectrophotometer. Dilute this suspension with 1/5 TSB, to obtain a bacterial cell density that is between $1{,}0\times10^3$ cells/ml and $1{,}0\times10^4$ cells/ml. This suspension is used as the inoculated incubating medium.

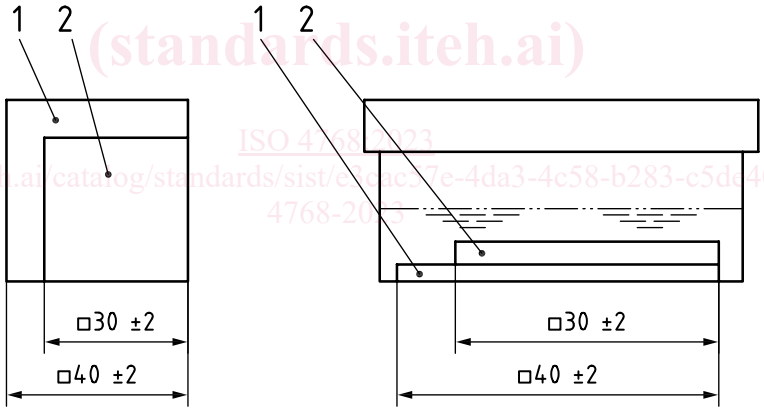
7.1.4 Inoculation of test specimens

The surface to be tested is the surface of the product. The edge faces of the product shall not be tested. Each test specimen prepared, in accordance with 7.1.2, is attached to a glass plate of 40 mm ± 2 mm (square) and placed into a separate sterile vessel with the treated surface facing upwards. See Figure 1.

It is necessary to fix them to the glass plate with an adhesive that does not affect the growth of microorganisms.

Pipette 20 ml of the inoculated incubating medium prepared in accordance with 7.1.3 into each of the vessels containing the test specimens. The upper surface of the test specimen shall be covered with at least a 5 mm depth of the inoculated incubating medium. If necessary, the volume of the inoculated incubating medium shall be increased to achieve this.

Dimensions in millimetres



Key

1 glass plate (40 mm ± 2 mm, square)

2 test specimen (30 mm ± 2 mm, square)

Figure 1 — Inoculation of test specimen

7.1.5 Incubation of the inoculated test specimens

Unless otherwise specified, incubate the vessels containing the test specimens (see 7.1.4) at a temperature of (35 ± 1) °C for (48 ± 1) h.

The concentration of the diluted TSB may be changed from 1/5 TSB depending on the effect of the agent contained in the treated test material.

NOTE For example, if the effect of the agent is so strong that no growth of the test bacterium is observed in the growth medium above the test specimens (due to elution of the active substance etc), the concentration of the TSB can be increased until growth is achieved (e.g. 1/2 TSB or undiluted).