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Quantitative determination of antibacterial activity of ceramic tile surfaces — Test methods —

Part 2: Ceramic tile surfaces with incorporated photocatalytic antibacterial agents

Détermination quantitative de l'activité antibactérienne des surfaces des carreaux céramiques — Méthodes d'essai —

Partie 2: Carreaux céramiques incorporant des agents antibactériens photocatalytiques en surface

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Contents

Page

Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 189, *Ceramic tile*.

A list of all parts in the ISO 17721 series can be found on the ISO website.

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.

Quantitative determination of antibacterial activity of ceramic tile surfaces — Test methods —

Part 2:
Ceramic tile surfaces with incorporated photocatalytic antibacterial agents

1 Scope

This document specifies test methods for evaluating the antibacterial activity of glazed and unglazed ceramic tile surfaces with incorporated photocatalytic antibacterial agents.

Secondary effects on ceramic tile surfaces due to photocatalytic antibacterial treatments, such as changes in chemical resistance, stain resistance or small colour differences, are not covered by this document. For chemical resistance refer to ISO 10545-13, for stain resistance refer to ISO 10545-14 and for colour differences refer to ISO 10545-16.

Other types of performance of photocatalytic ceramics, i.e. decomposition of water contaminants, self-cleaning, antifogging and air purification, are not covered by this document. It is also not intended to be used to evaluate ceramic surfaces that have been treated with topical disinfectants or agents that can offer residual activity for limited periods.

Any results obtained with this document will always refer to this document and the conditions used. Results obtained with this document indicate antibacterial activity under the specified experimental conditions used herein, and do not reflect activity under other circumstances where a variety of factors, such as temperature, humidity, different bacterial species, nutrient conditions, etc., are considered.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 17721-1, *Quantitative determination of antibacterial activity of ceramic tile surfaces – Test methods – Part1: Ceramic tile surfaces with incorporated antibacterial agents*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 17721-1 and the following apply.

ISO and IEC maintain terminological 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
photocatalyst
substance that carries out many functions based on oxidization and reduction reactions under ultraviolet (UV) irradiation, including decomposition and removal of air and water contaminants, deodorization, and antibacterial, self-cleaning and antifogging actions

4 Materials

WARNING — Handling and manipulation of microorganisms which are potentially hazardous requires a high degree of technical competence and may be subject to current national legislation and regulations. Only personnel trained in microbiological techniques should carry out such tests. Appropriate practices for disinfection, sterilization and laboratory hygiene must be strictly observed.

4.1 Bacterial strains

Bacteria used for the tests are:

a) *Escherichia coli*;

b) *Staphylococcus aureus*.

The bacterial strains to be used in the test are listed in Table 1 and are stored by entities that are registered under the World Federation for Culture Collections or of the Japan Society for Culture Collections. Other bacterial species may be tested with this test method, details of the species used, culture conditions and testing process shall be described in detail in the test report.

Transfer of cultures should be performed aseptically in safety cabinets. Inoculate each strain onto a nutrient agar medium slant using a sterile inoculation loop; incubate for 18 h - 24 h at 37 °C ± 1 °C, and then store at 5 °C – 10 °C. Subculture the strains by repeating the process within 30 d. The maximum number of subcultures from the original strain from the culture collection is 10. Discard slant cultures appropriately after 30 d.

NOTE 1 In the case of bacteria stored at -80 °C and lyophilized cultures, the maximum number of subcultures from the original strain is 10.

NOTE 2 If necessary, additional tests with other bacterial strains can be performed.

Table 1 — Bacterial strains and culture collections

Bacteria	Culture collections
E. coli	ATCC 8739, DSM 1576, NBRC 3972, CIP 53.126, NCIB 8545
S. aureus	ATCC 6538P, DSM 346, NBRC 12732, CIP 53.156, NCIB 8625

4.2 Culture media and solutions

Any water used shall be distilled or deionized and have a conductivity of <1 µS/cm. All reagents shall be of analytical grade and/or of a grade appropriate for microbiological purposes.

4.2.1 Non-ionic surfactant

The non-ionic surfactant shall be polyoxyethylene sorbitan monooleate (polysorbate 80).

4.2.2 Nutrient broth

For 1 000 ml of purified water, take 3,0 g of meat extract, 10,0 g of peptone and 5,0 g of sodium chloride, put them in a flask and dissolve them thoroughly. When the contents are thoroughly dissolved, use a solution of sodium hydroxide or hydrochloric acid to bring the pH to (7,0 ± 0,1) at 25 °C. Sterilize in an autoclave at 121 °C ± 2 °C for at least 15 min. After preparation, if nutrient broth is not used immediately, store it at 5 °C to 10 °C. Storage for long periods should be avoided. Other suitable media such as tryptic soy agar (TSA) and tryptic soy broth (TSB) can also be used for the growth and quantification steps.

4.2.3 1/500 nutrient broth

For 1 000 ml of purified water, take 3,0 g of meat extract, 10,0 g of peptone and 5,0 g of sodium chloride, put them in a flask and dissolve them thoroughly. Dilute this medium 500 times using distilled or deionized water, and set the pH to (7,0 ± 0,2) using hydrochloric acid solution or sodium hydroxide solution. Sterilize in an autoclave at 121 °C ± 2 °C for at least 15 min. After preparation, if 1/500 nutrient broth is not used immediately, store it at 5 °C to 10 °C.

Other culture media may be selected based on the requirements for the bacteria selected for the testing. Details of the selected media shall be included in the test report under culture conditions.

Prepared culture media may be stored at 5 °C - 10 °C for up to one month.

4.2.4 Nutrient agar

For 1 000 ml of purified water, take 3,0 g of meat extract, 5,0 g of peptone and 15,0 g of agar powder, put them in a flask and mix. Heat the flask in boiling water to dissolve the contents thoroughly. Use a 0,1 mol/l solution of sodium hydroxide to bring the pH to (6,8 ± 0,2) at 25 °C. Sterilize in an autoclave at 121 °C ± 2 °C for at least 15 min. After preparation, if nutrient agar is not used immediately, store it at 5 °C to 10 °C. Keep the medium temperature between 45 °C and 48 °C when mixing with a bacterial suspension.

Other culture media may be selected based on the requirements for the bacteria selected for the testing. Details of the selected media shall be included in the test report under culture conditions. Prepared culture media may be stored at 5 °C - 10 °C for up to one month.

4.2.5 Soybean-casein digest broth with lecithin and polysorbate 80 (SCDLP)

For 1 000 ml of purified water, take 17,0 g of casein peptone, 3,0 g of soybean peptone, 5,0 g of sodium chloride, 2,5 g of phosphoric acid monopotassium dehydrogenate, 2,5 g of glucose and 1,0 g of lecithin, put them in a flask and dissolve them. Add 7,0 g of non-ionic surfactant and dissolve it. Use a solution of sodium hydroxide or hydrochloric acid to bring the pH to (7,0 ± 0,2) at 25 °C. Sterilize in an autoclave at 121 °C ± 2 °C for at least 15 min. After preparation, if SCDLP is not used immediately, store it at 5 °C to 10 °C.

Prepared SCDLP solution may be stored at 5 °C - 10 °C for up to one month.

4.2.6 Phosphate buffer saline (PBS)

For 1 000 ml of purified water, take 34,0 g of potassium dihydrogen phosphate, put it in a flask and dissolve it thoroughly. Use a solution of sodium hydroxide to bring the pH to (7,0 ± 0,2) at 25 °C. Sterilize in an autoclave at 121 °C ± 2 °C for at least 15 min. After preparation, if PBS is not used immediately, store it at 5 °C to 10 °C.

Prepared PBS solution may be stored at 5 °C - 10 °C for up to one month.

5 Test specimen

5.1 Size

Reduce samples to test pieces of a size approximately (50 ± 2) mm × (50 ± 2) mm square or as close to these dimensions as practical. Six treated specimens are required to conduct one full test. Test specimen shall be sterilized based on the appropriate sterilization process selected for the test sample. All surfaces of the test specimen shall be sterilized.

NOTE 1 When it is difficult or impossible to cut (50 ± 2) mm long (up to 10 mm thickness) squares, it is acceptable to use a different specimen size as long as the specimen surface can be covered with a 400 mm^2 to 1 600 mm^2 film.

NOTE 2 When the specimen surface is stained with organic contaminant, it is acceptable to first eliminate the contaminant by exposure to a 1,0 mW/cm^2 light source within the limit of 24 h.

5.2 Control

To explain the results and to validate the test, proper controls shall be included in the experimental set up. These controls should be devoid of any antibacterial properties and establish a baseline for technical performance by minimizing misleading results caused by the following.

— Day-to-day variations in laboratory conditions and test organism viability.

— Sample-to-sample variations within the formulation of commercial glazes where different sources are used for the glaze components and normal fluctuations occur in the mined materials. These variations frequently result in inconsistent effects on microbial proliferation, making it difficult to assess the efficacy of designed changes to the formula.

— The inherent water absorbing nature of ceramic surfaces that can cause organism death by desiccation and consequent misrepresentation of the antibacterial activity of the glaze itself.

Non-treated test specimens, i.e. test specimens which do not have any antibacterial treatment, should be used as controls. Nine non-treated control samples are required to conduct one full test.

When non-treated specimens cannot be provided, use glass panes instead. Care should be taken to avoid microbial contamination and cross-contamination among specimens.

5.3 Precondition

Control and test specimens should be appropriately sterilized before the precondition step. Prepare separate sample conditioning beds for the test samples and controls. Line the lower half of a sterile 150 mm × 150 mm Petri dish with a grade #1 Whatman filter paper size 150 mm or equivalent

Wet the filter paper thoroughly with sterile deionized water. Place the test pieces together on the wet filter paper. Label the outside of each dish with sufficient sample information to maintain unique sample identity. Further condition the samples by placing these beds in an incubator maintained at 37 °C ± 2 °C and ≥75 % RH for 18 h-24 h. Following this period, keep the filter paper wet through the time of incubation.

5.4 Number of test specimen

At a minimum, 9 non-treated and 6 treated test specimens will be included in the testing. Additional specimen may be included and details of the number of specimens used shall be included in the test report.

6 Procedure

6.1 Preparation of test inoculum

Transfer the stored bacteria to the nutrient agar slant using a sterile inoculating loop and incubate at 37 °C ± 1 °C for 16 h to 24 h. Transfer the bacteria to a new nutrient agar slant and incubate at 37 °C ± 1 °C for 16 h to 20 h. Uniformly disperse a small quantity of test bacteria in 1/500 NB with a platinum loop, and measure the bacteria count using the optical microscope observation method or any other adequate method.

Suitably dilute this bacteria suspension with 1/500 NB to obtain a count of 6,7×10^5 cfu/ml – 2,6x10^6 cfu/ml and use the result as the bacterial suspension for the test.

If the test bacteria suspension is not to be used immediately, store it at 4 °C and use it within 4 h.

NOTE See Figure 1 for a flowchart of the photocatalytic test procedure.

6.2 Adhesive film

The adhesive film is inert and non-water absorbent with good sealing properties, with a transparency rate ≥85 % in the 340 nm to 380 nm range. The sheets are cut with dimensions of 40 mm ± 2 mm. The film should not affect bacterial growth and can be made of polyethylene, polypropylene or polyester [poly(ethylene terephthalate)]. Film that is 0,05 mm to 0,10 mm thick is recommended.

NOTE Films cut from Stomacher bags are also suitable.

6.3 Inoculation of test specimens

Precondition 9 non-treated control specimens and 6 treated test specimens, when non-treated specimens cannot be provided, use glass panes instead. Collect exactly 0,15 ml of test bacterial suspension with a sterilized pipette and drip it onto each test piece. Put a film on top of the dripped suspension and lightly push to get the suspension to spread to the whole film surface, while taking care that no suspension leaks out of the film edge.

The regulated suspension quantity can create leakage of suspension from the film edge or might not be enough to spread the suspension uniformly. In such a case, it is acceptable to reduce down to half the quantity of suspension or increase to twice the quantity of suspension. However, when the volume of test inoculum is changed, the concentration of the bacterial cells in the inoculum shall be adjusted to provide the same number of bacterial cells as when the normal volume of test inoculum is applied.

6.4 Recovery of bacteria from non-treated control specimens at time t = 0 h

Immediately after inoculation, process half of the untreated control test specimens by placing each test piece into 10 ml of either SCDLP broth (see 4.2.5) or a suitable, validated neutralizer. Shake vigorously for 60 s ± 5 s to ensure total and uniform removal of the test organisms from the film and test piece.

Other methods for recovery, such as the use of a stomacher bag, can be utilized. In addition, if a lower limit of detection is required, then the amount of neutralizer added can be reduced, however the entire sample shall be covered with neutralizer broth before proceeding.

6.5 UV irradiation conditions

Ultraviolet (UV) irradiation is attained by using a black light blue (BLB) lamp that has a wavelength of 351 nm with blue glass for absorbing visible light. The irradiation intensity shall be measurable at the test sample position. The UV radiometer shall be calibrated for the light source to be used or corrected to ascertain sensitivity within the wavelength range to be absorbed by the photocatalytic test piece.

Choice of UV intensity depends upon the circumstances of where the photocatalytic ceramic tile has been applied. Table 2 gives a range of UV intensities with reference to real life examples.

Table 2 — UV irradiation intensity reference for the test procedure

UV intensity	Example
0,25 mW/cm^2	Beside the window in during daytime
0,10 mW/cm^2	In the room (inside, about 1,5 m from the window) during daytime, by the window during early morning or before sunset
0,01 mW/cm^2	In the room (inside, about 3 m from the window) during daytime
0,001 mW/cm^2	In the room without a window (only indoor light), in the room at night (only indoor light)

Arrange the BLB lamp in a dark room/chamber and measure the irradiation intensity using the UV radiometer. Place the Petri dishes containing 3 non-treated and 3 photocatalytic treated test specimens on the photoelectric sensor of the UV radiometer. Desired UV intensity can be achieved by adjusting the

distance between test specimens and BLB lamp or by diffusing UV light from the BLB lamp by using appropriate UV filtering material (per ISO 14605) such as a punched metal sheet.

NOTE The maximum UV intensity is 0,25 mW/cm^2 to avoid damage by UV irradiation only. The minimum UV intensity of the photoelectric sensor at present is 0,001 mW/cm^2.

During the irradiation step, the Petri dish lid should be removed as UV light does not penetrate the material. To preserve the humidity, high transparency glass may be used.

Expose to UV light the Petri dishes containing the specimens (4 non-treated and 4 photocatalytic treated test specimens) with bacterial suspension for the desired contact time period. See below for different contact times. One or more of the contact time points may be selected for the testing. Each time point shall be considered as one test. Controls should also be included for different time points. Selected time point(s) and all results shall be included in the test report.

Section A: contact time of 30 min

Section B: contact time of 1 h

Section C: contact time of 4 h

Section D: contact time of 8 h

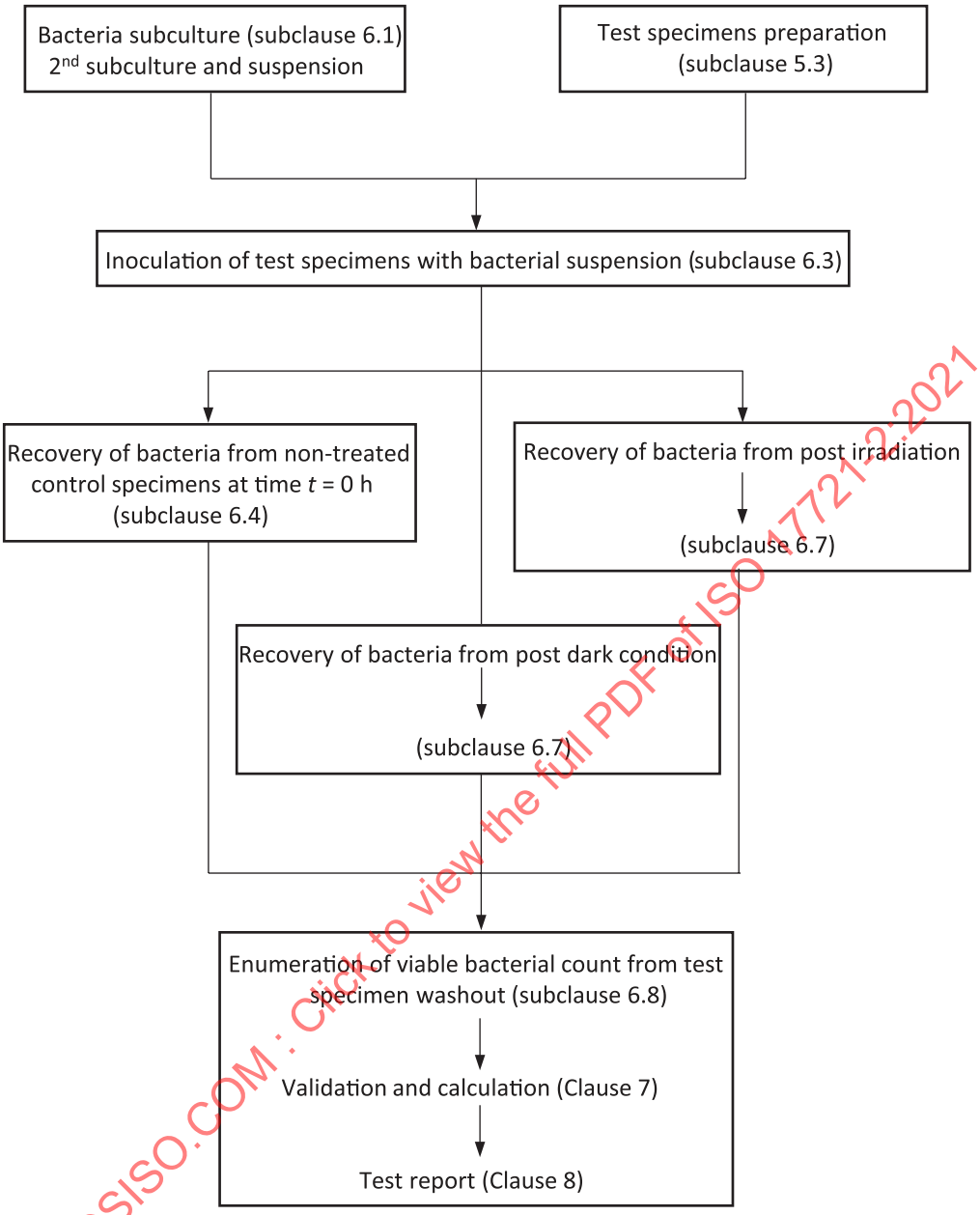

Figure 1 — Flowchart for photocatalytic test procedure

6.6 Dark conditions

Keep the Petri dishes containing the remaining inoculated specimens (3 non-treated and 3 photocatalytic treated test specimens) with bacterial suspension in a dark place, for the same time as in 6.4.

6.7 Recovery of bacteria from post irradiation and dark condition test specimens

Perform the washout for the irradiated and dark condition non-treated and treated test specimens as described in 6.4.

6.8 Enumeration of viable bacterial count from test specimen washout

Serial 10-fold dilutions of the washout solution are carried out in PBS by taking 1 ml of washout and adding it to 9 ml of PBS or other suitable buffer and vortexed for even distribution. 1 ml from this

solution is added to 9 ml of PBS in another tube and vortexed. This process is repeated to obtain a series of 10-fold dilutions from the washout.

Enumeration of bacterial counts is performed by the pour plate method. 1 ml from each dilution series and washout is added to two Petri dishes. 15 ml - 20 ml of molten nutrient agar maintained at 45 °C - 48 °C is added to the Petri dishes and the agar allowed to solidify at room temperature. Invert the Petri dishes and incubate at 37 °C ± 1 °C for 24 h – 48 h.

Other enumeration techniques, such as those applying luminescence can be used determining the viable bacterial count. Method of enumeration in such cases shall be recorded in the test report. Along with the enumeration method, justification and viability of the enumeration method selected will be mentioned in the report.

Petri dishes containing colonies in the range of 30 – 300 are counted and recorded. Concentration of bacteria in the washout solution is obtained by Formula (1) and expressed to two significant digits.

$$P = Z \times R \tag{1}$$

where

P is the bacteria concentration in the washout (cfu/ml);

Z is the average number of colonies recorded in the duplicate Petri dishes;

R is the dilution factor.

When the number of viable bacteria is less than 30 in the Petri dishes with 1 ml of washout solution, the cell number is used to calculate the average number. When the number of viable bacteria is less than 1 in the Petri dishes with 1 ml of washout solution, the average number is taken as 1.

7 Validation and calculations

7.1 General

The test results are calculated as follows. The calculated values are usually rounded to the second decimal place in accordance with ISO 80000-1.

7.2 Criteria for a valid test

Applying Formula (2), calculate the number of viable bacteria using the concentration of bacteria obtained from 6.8.

$$N = P \times V \tag{2}$$

where

N is the number of cells of bacteria;

P is the bacteria concentration in the washout (cfu/ml);

V is the volume of SCDLP used as washout.

A test is considered valid if it fulfils all of the following criteria. If one or more of these criteria are not fulfilled, the test is considered as not valid and shall be performed again.

1) The average number of viable bacteria of non-treated specimens after inoculation shall be within the10^5 to 4×10^5 cells range for all 4 specimens.

