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Chemical disinfectants and antiseptics — Quantitative non-porous surface test for the evaluation of bactericidal and/or fungicidal activity of chemical disinfectants used in food, industrial, domestic and institutional areas — Test method and requirements without mechanical action (phase 2, step 2)

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National foreword

This British Standard is the UK implementation of EN 13697:2015. It supersedes BS EN 13697:2001 which is withdrawn.

The UK participation in its preparation was entrusted to Technical Committee CH/216, Chemical disinfectants and antiseptics.

A list of organizations represented on this committee can be obtained on request to its secretary.

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Chemical disinfectants and antiseptics - Quantitative non-porous surface test for the evaluation of bactericidal and/or fungicidal activity of chemical disinfectants used in food, industrial, domestic and institutional areas - Test method and requirements without mechanical action (phase 2, step 2)

Antiseptiques et désinfectants chimiques - Essai quantitatif de surface non-poreuse pour l'évaluation de l'activité bactéricide et/ou fongicide des désinfectants chimiques utilisés dans le domaine de l'agro-alimentaire, dans l'industrie, dans les domaines domestiques et en collectivité - Méthode d'essai sans action mécanique et prescriptions (phase 2/étape 2)

Chemische Desinfektionsmittel und Antiseptika - Quantitativer Oberflächen-Versuch nicht poröser Oberflächen zur Bestimmung der bakteriziden und/oder fungiziden Wirkung chemischer Desinfektionsmittel in den Bereichen Lebensmittel, Industrie, Haushalt und öffentliche Einrichtungen - Prüfverfahren und Anforderungen ohne mechanische Behandlung (Phase 2, Stufe 2)

This European Standard was approved by CEN on 20 January 2015.

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CEN-CENELEC Management Centre: Avenue Marnix 17, B-1000 Brussels

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Foreword

This document (EN 13697:2015) has been prepared by Technical Committee CEN/TC 216 “Chemical disinfectants and antiseptics”, the secretariat of which is held by AFNOR.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by October 2015 and conflicting national standards shall be withdrawn at the latest by October 2015.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. CEN [and/or CENELEC] shall not be held responsible for identifying any or all such patent rights.

This document supersedes EN 13697:2001.

The changes between this edition and EN 13697:2001 are the following:

— interfering substance has been changed from 0,03 % bovine albumin to 0,85 % skimmed milk (see Clause 4, Table 1) for *Pseudomonas aeruginosa* under clean conditions only;

— *A. brasiliensis* (ex A. niger) spore preparation has been updated in order to harmonize this step with the QST fungicidal test method amendment issued in 2012 (see 5.4.1.3 b));

— Calculations of the weighed means and of the results have been modified in order to be harmonized with new CEN TC 216 standards (see 5.4.1.5, 5.5.2, 5.5.3 and 5.6);

— Other paragraphs have been harmonized to new CEN TC 216 standards (e.g. preparation of hard water, 5.2.2.7).

Results obtained from the previous standard for *Aspergillus niger* need to be repeated to take into account the new spore morphology requirement and the change in interfering substance.

According to the CEN-CENELEC Internal Regulations, the national standards organizations of the following countries are bound to implement this European Standard: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, Former Yugoslav Republic of Macedonia, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and the United Kingdom.

Introduction

This European Standard describes a surface test method for establishing whether a product proposed as a disinfectant in the fields described in Clause 1 has or does not have bactericidal and/or fungicidal or yeasticidal activity on non-porous surfaces.

This European Standard has been revised in order to modify the interfering substance under “clean conditions” adopted for *P. aeruginosa*; in order to modify the calculation of N, NC, NT, Nc, Na and consequently the final results and to harmonize the standard with the other recent CEN TC 216 standards.

The laboratory test closely simulates practical conditions of application. Chosen conditions (contact time, temperature, organisms on surfaces ...) reflect parameters which are found in practical situations including conditions which may influence the action of disinfectants. Each use concentration found from this test corresponds to defined experimental conditions.

The conditions are intended to cover general purposes and to allow reference between laboratories and product types.

However, for some applications the recommendations of use of a product can differ and therefore additional test conditions need to be used.

1 Scope

This European Standard specifies a test method (phase 2/step 2) and the minimum requirements for bactericidal and/or fungicidal or yeasticidal activity of chemical disinfectants that form a homogeneous physically stable preparation in hard water or – in the case of ready-to-use products – with water in food, industrial, domestic and institutional areas, excluding areas and situations where disinfection is medically indicated and excluding products used on living tissues.

The scope of this European Standard applies at least to the following:

a) Processing, distribution and retailing of:

   1) Food of animal origin:

      i) milk and milk products;

      ii) meat and meat products;

      iii) fish, seafood and products;

      iv) eggs and egg products;

      v) animal feeds;

      vi) etc.

   2) Food of vegetable origin:

      i) beverages;

      ii) fruits, vegetables and derivatives (including sugar distillery);

      iii) flour, milling and backing;

      iv) animal feeds;

      v) etc.

b) Institutional and domestic areas:

   1) catering establishments;

   2) public areas;

   3) public transports;

   4) schools;

   5) nurseries;

   6) shops;

   7) sports rooms;

   8) waste container (bins);

9) hotels;

10) dwellings;

11) clinically non sensitive areas of hospitals;

12) offices;

13) etc.

c) Other industrial areas:

1) packaging material;

2) biotechnology (yeast, proteins, enzymes...);

3) pharmaceutical;

4) cosmetics and toiletries;

5) textiles;

6) space industry, computer industry;

7) etc.

Using this European Standard, it is possible to determine the bactericidal or fungicidal or yeasticidal activity of the undiluted product. As three concentrations are tested, in the active to non active range, dilution of the product is required and, therefore, the product forms a homogeneous stable preparation in hard water.

EN 14885 specifies in detail the relationship of the various tests to one another and to use recommendations.

NOTE 1 The method described is intended to determine the activity of commercial formulations or active substances on bacteria and/or fungi in the conditions in which they are used.

NOTE 2 This method cannot be used to evaluate the activity of products against mycobacteria.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN 12353, *Chemical disinfectants and antiseptics - Preservation of test organisms used for the determination of bactericidal (including Legionella), mycobactericidal, sporicidal, fungicidal and virucidal (including bacteriophages) activity*

EN 14885, *Chemical disinfectants and antiseptics - Application of European Standards for chemical disinfectants and antiseptics*

ISO 4793, *Laboratory sintered (fritted) filters - Porosity grading, classification and designation*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in EN 14885 apply.

4 Requirements

The product shall demonstrate at least a 4 decimal log (lg) reduction for bacteria and at least a 3 decimal log (lg) reduction for fungi, when tested in accordance with Table 1 and 5.5.1.

Table 1 — Obligatory and additional conditions

Test Conditions	Bactericidal activity on non-porous surfaces without mechanical action	Yeasticidal activity on non-porous surfaces without mechanical action	Fungicidal activity on non-porous surfaces without mechanical action
Test organism (see 5.2.1) **obligatory**	*Enterococcus hirae* *Escherichia coli* *Pseudomonas aeruginosa* *Staphylococcus aureus*	*Candida albicans*	*Candida albicans* *Aspergillus brasiliensis (ex A. niger)*
example	*Salmonella typhimurium* *Lactobacillus brevis* *Enterobacter cloacae*	*Saccharomyces cerevisiae* (for breweries) *Saccharomyces cerevisiae var. diastaticus* (for breweries)	any relevant test organism
Test temperature **obligatory**	Between 18 °C ± 1 °C and 25 °C ± 1 °C	Between 18 °C ± 1 °C and 25 °C ± 1 °C	Between 18 °C ± 1 °C and 25 °C ± 1 °C
additional	4 °C ± 1 °C; 10 °C ± 1 °C; 40 °C ± 1 °C	4 °C ± 1 °C; 10 °C ± 1 °C; 40 °C ± 1 °C	4 °C ± 1 °C; 10 °C ± 1 °C; 40 °C ± 1 °C
Contact time **obligatory**	5 min ± 10 s	15 min ± 10 s	15 min ± 10 s
additional	1 min ± 5 s; 15 min ± 10 s; 30 min ± 10 s; 60 min ± 10 s	1 min ± 5 s; 5 min ± 10 s; 30 min ± 10 s; 60 min ± 10 s	1 min ± 5 s; 5 min ± 10 s; 30 min ± 10 s; 60 min ± 10 s
Interfering substance **obligatory** clean conditions	0,3 g/l Bovine Albumin for *Staphylococcus aureus*, *Enterococcus hirae* and *Escherichia coli* ; 8,5 g/l skim milk for *Pseudomonas aeruginosa*	0,3 g/l Bovine Albumin for *C. albicans*	0,3 g/l Bovine Albumin for *C. albicans* and *A. brasiliensis*
dirty conditions	3,0 g/l Bovine Albumin for *Staphylococcus aureus*, *Enterococcus hirae, Pseudomonas aeruginosa* and *Escherichia coli*	3,0 g/l Bovine Albumin for *C. albicans*	3,0 g/l Bovine Albumin for *C. albicans* and *A. brasiliensis*
additional	any relevant substance	any relevant substance	any relevant substance
Log reduction from a water control (decimal log)	≥ 4Log	≥ 3Log	≥ 3Log

The obligatory contact times for surface disinfectants stated in Table 1 were chosen to enable comparison of standard conditions. The referenced test conditions are by no means intended as requirements for the use of a product, nor as requirements for the evaluation and acceptance of products by regulatory authorities.

The recommended contact time for the use of the product is within the responsibility of the manufacturer.

Where appropriate (specific purposes), additional specific bactericidal/yeasticidal/fungicidal activity shall be determined under other conditions of time, temperature, additional strains and interfering substances in order to take into account intended specific use conditions.

NOTE For the additional conditions, the concentration defined as a result can be lower than the one obtained under the obligatory test conditions.

5 Test methods

5.1 Principle

A test suspension of bacteria or fungi in a solution of interfering substances is inoculated onto a test stainless steel surface and dried. A prepared sample of the product under test is applied in a manner which covers the dried film. The surface is maintained at a specified temperature for a defined period of time. The surface is transferred to a previously validated neutralization medium so that the action of the disinfectant is immediately neutralized. The number of surviving organisms which can be recovered from the surface is determined quantitatively.

The number of bacteria or fungi on a surface treated with hard water in place of the disinfectant is also determined and the reduction in viable counts attributed to the product is calculated by difference.

5.2 Materials and reagents

5.2.1 Test organisms

The bactericidal activity shall be evaluated using the following four strains:

— *Pseudomonas aeruginosa* ATCC 15 442[1)];

— *Staphylococcus aureus* ATCC 6 538;

— *Enterococcus hirae* ATCC 10 541;

— *Escherichia coli* ATCC 10 536.

The fungicidal or yeasticidal activity shall be evaluated using the following two strains:

— *Candida albicans* ATCC 10 231;

— *Aspergillus brasiliensis (ex A. niger)* ATCC 16 404.

If required for specific applications, additional strains may be chosen from, for example:

— *Salmonella typhimurium* ATCC 13 311;

— *Lactobacillus brevis* DSM 6 235;

— *Enterobacter cloacae* DSM 6 234;

— *Saccharomyces cerevisiae* (for breweries) or ATCC 9 763 or DSM 1 333;

— *Saccharomyces cerevisiae* var. *diastaticus* (for breweries) DSM 70 487.

NOTE See Annex A for corresponding strain numbers in some other culture collections.

If additional strains are used, they shall be incubated under optimum growth conditions (temperature, time, atmosphere) and noted in the test report.

If the additional strains selected do not correspond to the specified strains, their suitability for supplying inocula of sufficient concentration shall be verified. If the additional strains tested are not classified at a

1) ATCC 15 442, ATCC 6 538, ATCC 10 541, ATCC 10 536, ATCC 10 231, ATCC 16 404 and ATCC 13311 are the collection numbers of strains supplied by the American Type Culture Collections. This information is given for the convenience of users of this standard and does not constitute an endorsement by CEN of the product named. Equivalent products can be used if they can be shown to lead to the same results.

reference centre, their identification characteristics shall be stated. In addition, they shall be held by the testing laboratory or national culture under a reference for 5 years.

5.2.2 Culture media and reagents

5.2.2.1 General

The reagents shall be of analytical grade and/or appropriate for microbiological purposes.

5.2.2.2 Water

The water shall be free from substances that are toxic or inhibiting to bacteria and fungi. It shall be freshly glass distilled and not demineralized water.

Sterilize in the autoclave (see 5.3.2.1).

NOTE 1 If the water is sterilized during sterilization of the reagents, this is not necessary.

NOTE 2 If distilled water of adequate quality is not available, water for injectable preparation (see European Pharmacopoeia) can be used.

5.2.2.3 Tryptone Soya Agar (TSA)

For maintenance of bacterial strains and performance of viable counts.

Tryptone, pancreatic digest of casein	15,0 g
Soya peptone, papaic digest of Soybean meal	5,0 g
NaCl	5,0 g
Agar	15,0 g
Water (see 5.2.2.2)	1 000,0 ml

Sterilize in the autoclave (see 5.3.2.1). After sterilization, the pH of the medium shall be equivalent to 7,2 ± 0,2 when measured at 20 ° C.

5.2.2.4 Malt extract agar (MEA)

For maintenance of fungal strains, sporulation and performance of viable counts.

Malt extract (food grade, e.g. Cristomalt powder from Difal)	30,0 g
Agar	15,0 g
Water (see 5.2.2.2)	1 000,0 ml

The malt extract should be food grade (e.g. Cristomalt powder from Difal) or equivalent that is not highly purified and not only based on maltose (e.g. Malt extract from OXOID) [2]. However, if there are problems producing at least 75 % spiny spores see 5.4.1.4.2.

2) This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of the product named. Equivalent products may be used if they can be shown to lead to the same results.

Sterilize in the autoclave [5.3.2.1a)]. After sterilization, the pH of the medium shall be equivalent to 5,6 ± 0,2 when measured at (20 ± 1) °C.

In case of encountering problems with neutralization (5.5.2.3 and 5.5.2.4), it may be necessary to add neutralizer to the MEA. Annex B gives guidance on the neutralizers that may be used.

5.2.2.5 Diluent

Tryptone sodium chloride solution:

Tryptone, pancreatic digest of casein	1,0 g
NaCl	8,5 g
Water (see 5.2.2.2)	1 000,0 ml

Sterilize in the autoclave (see 5.3.2.1). After sterilization the pH shall be equivalent to 7,0 ± 0,2 when measured at 20 °C.

5.2.2.6 Neutralizer

The neutralizer shall be validated for the product under test in accordance with 5.5.2.3 and 5.5.2.4. The neutralizer shall be sterile.

NOTE Information on neutralizers that have been found to be suitable for some categories of products is given in Annex B.

5.2.2.7 Hard water for dilution of the products

Hard water for dilution of products shall be prepared as follows:

— solution A: Dissolve 19,84 g anhydrous $MgCl_2$ and 46,24 g anhydrous $CaCl_2$ in water (see 5.2.2.2) and dilute to 1 000 ml. Sterilize by membrane filtration (5.3.2.19) or in the autoclave (5.3.2.1 a). Autoclaving – if used - may cause a loss of liquid. In this case, make up to 1 000 ml with water (5.2.2.2) under aseptic conditions. Store the solution in the refrigerator (5.3.2.15) for no longer than one month.

— solution B: Dissolve 35,02 g $NaHC0_3$ in water (see 5.2.2.2) and dilute to 1 000 ml. 1 000 ml. Sterilize by membrane filtration (5.3.2.19). Store the solution in the refrigerator (5.3.2.15) for no longer than one week.

Add at least 600 ml water (see 5.2.2.2) to 6,0 ml of solution A in a 1 000 ml volumetric flask, then add 8,0 ml solution B. Mix and dilute to 1 000 ml with water (see 5.2.2.2).

Sterilize by passing through a filter with a maximum effective pore size of 0,45 μm.

The pH of the hard water shall be 7,0 ± 0,2, when measured at (20 ± 1) °C (5.3.2.6). If necessary, adjust the pH by using a solution of approximately 40 g/l (about 1 mol/l) of sodium hydroxide (NaOH) or approximately 36,5 g/l (about 1 mol/l) of hydrochloric acid (HCl).

The hard water shall be freshly prepared under aseptic conditions and used within 12 h.

NOTE When preparing the test product solutions (5.4.2), the addition of the product to the hard water produces a different final water hardness expressed as calcium carbonate ($CaCO_3$) in each test tube. In any case, the final hardness is lower than 375 mg/l of calcium carbonate.

5.2.2.8 Interfering substances

5.2.2.8.1 General

The interfering substance shall be chosen according to the conditions of use laid down for the product.

The interfering substance shall be sterile and prepared at 2 times its final concentration in the test.

For the additional interfering substances, the ionic composition (e.g. pH, calcium and/or magnesium hardness) and chemical composition (e.g. mineral substances, protein, carbohydrates, lipids, detergents) shall be fully defined.

NOTE The term “interfering substance” is used even if it contains more than one substance.

The method of preparation and sterilization together with the composition shall be noted in the test report (see 5.7).

5.2.2.8.2 Bovine albumin and skimmed solutions

Bovine albumin solutions for the test conditions shall be prepared as follows:

— Preparation for clean conditions:

  — dissolve 0,06 g of bovine albumin (Cohn fraction V for Dubos medium) in 100 ml of water (see 5.2.2.2);

  — sterilize by 0,45 µm membrane filtration (see 5.3.2.19), keep in the refrigerator and use within one month.

The final concentration of bovine albumin in the test procedure (see 5.5.2) is 0,3 g/l.

Skim milk solution for the test conditions shall be prepared as follows:

— Skimmed milk (clean conditions for *Pseudomonas aeruginosa* only), guaranteed free of antibiotics or additives, shall be reconstituted at a rate of 100 g powder per litre of water (see 5.2.2.2). The working solution shall be prepared as follows:

  — prepare a solution of 1,70 % (*V/V*) in water (see 5.2.2.2) by adding 17 parts of the 10 % stock solution (100 g skimmed milk in 1 l water) to 83 parts of water;

  — sterilize for 30 min at 105 °C ± 3 °C (or 5 min at 121 °C ± 3 °C).

The final concentration of milk in the test procedure (see 5.5.2) is 8,5 g/l of reconstituted milk.

— Preparation for dirty conditions;

  — dissolve 0,60 g of bovine albumin (Cohn fraction V for Dubos medium) in 100 ml of water (see 5.2.2.2);

  — sterilize by 0,45 µm membrane filtration (see 5.3.2.19), keep in the refrigerator and use within one month.

The final concentration of bovine albumin in the test procedure (see 5.5.2) is 3,0 g/l.

In addition, other interfering substances for chemical disinfectants with detergent properties (therefore simulating additional dirty conditions for specific uses) can be chosen from:

5.2.2.8.3 Milk (dairies)

Skimmed milk, guaranteed free of antibiotics or additives, shall be reconstituted at a rate of 100 g powder per litre of water (see 5.2.2.2). The working solution shall be prepared as follows:

— prepare a solution of 2,0 % (*V/V*) in water (see 5.2.2.2) by adding 2 parts of reconstituted milk to 98 parts of water;

— sterilize for 30 min at 105 °C ± 3 °C (or 5 min at 121 °C ± 3 °C).

The final concentration of milk in the test procedure (see 5.5.2) shall be 1,0 % (*V/V*) of reconstituted milk.

5.2.2.8.4 Yeast extract (breweries)

Dehydrated yeast extract for bacteriology, shall be prepared as follows:

— prepare a 20 g/l solution in water (see 5.2.2.2), adjust to pH 7,0 ± 0,2 with sodium hydroxide;

— sterilize in the autoclave (see 5.3.1).

The final concentration of yeast extract in the test procedure (see 5.5.2) shall be 10 g/l.

5.2.2.8.5 Sucrose (beverage, soft drink industries)

Prepare a 20 g/l solution in water (see 5.2.2.2), sterilize by membrane filtration.

The final concentration of sucrose in the test procedure (see 5.5.2) is 10 g/l.

5.2.2.8.6 pH 5,0 and pH 9,0 buffer solutions (clean-in-place)

The buffer solution shall be described in the test report and pH values shall be recorded. The final pH in the test shall be equal to 5,0 ± 0,2 or 9,0 ± 0,2.

5.2.2.9 Sodium lauryl sulphate (cosmetics or cosmetic industries)

Prepare a 10 g/l solution of sodium lauryl sulphate in water (see 5.2.2.2). Sterilize in the autoclave (see 5.3.1).

The final concentration of sodium lauryl sulphate in the test procedure (see 5.5.2) is 5 g/l.

5.2.3 Test surface

Stainless steel discs (2 cm diameter discs) 304 with grade 2b finish on both sides. The surfaces should be flat.

The surfaces should be used only once.

Prior to use the surfaces should be placed in a beaker (minimum size: 50 ml) containing not less than 20 ml of 5 % (*V/V*) Decon® [3] for 60 min. Immediately rinse the discs with running freshly distilled water for 10 s.

The surface shall not be allowed to dry to any extent. The discs shall only be handled with forceps. Rinse the discs with water (see 5.2.2.2) for a further 10 s to ensure complete removal of the surfactant. To supply a satisfactory flow of water, a sterilized fluid dispensing pressure vessel with suitable hose and connectors or other suitable method can be used and regulated to supply approximately 2 000 ml per min. To sterilize, place

3) Decon® is an example of a suitable product available commercially. This information is given for the convenience of users of this standard and does not constitute an endorsement by CEN of this product.

the clean disc in a bath containing 70 % (*V/V*) iso-propanol for 15 min. Remove the disc and dry by evaporation under laminar air flow.

5.3 Apparatus and glassware

5.3.1 General

Sterilize all glassware and parts of apparatus that will come into contact with the culture media and reagents or the sample, except those which are supplied sterile, by one of the following methods:

a) in the autoclave (see 5.3.2.1) by maintaining it at 121_{0}^{+3} °C for a minimum holding time of 15 min;

b) in the dry heat sterilizer (see 5.3.2.1) by maintaining it at 180 °C for a minimum holding time of 30 min, at 170 °C for a minimum holding time of 1 h or at 160 °C for a minimum holding time of 2 h.

5.3.2 Usual microbiological laboratory equipment [4] and in particular, the following:

5.3.2.1 Apparatus for sterilization:

a) For moist heat sterilization, an autoclave capable of being maintained at 121_{0}^{+3} °C for 15 min;

b) for dry heat treatment, a hot air oven capable of being maintained at 180 °C for a minimum holding time of 30 min, at 170 °C for a minimum holding time of 1 h or at 160 °C for a minimum holding time of 2 h.

5.3.2.2 Temperature controlled cabinet.

5.3.2.3 Water baths capable of being controlled at 20 °C ± 1 °C, at 45 °C ± 1 °C and at additional test temperatures θ ± 1 °C (see 5.5.1).

5.3.2.4 Incubator (for bactericidal activity), capable of being controlled at either 36 °C ± 1 °C or 37 °C ± 1 °C. An incubator at 37 °C ± 1 °C may be used if an incubator at 36 °C ± 1 °C is not available.

5.3.2.5 Incubator (for fungicidal or yeasticidal activity), capable of being controlled at 30 °C ± 1 °C.

5.3.2.6 pH meter, having an accuracy of calibration of 0,1 pH units at 20 °C ± 1 °C.

5.3.2.7 Stopwatch.

5.3.2.8 Vortex mixer (mechanical shaker or electromechanical agitator, i.e. Vortex® mixer [5]).

5.3.2.9 Containers: Test tubes, culture bottles or flasks of suitable capacity.

5.3.2.10 Graduated pipettes of nominal capacities 10 ml, 1 ml, 0,1 ml and 0,05 ml, or calibrated automatic pipettes.

5.3.2.11 Petri dishes of size 90 mm to 100 mm.

5.3.2.12 Glass beads (Diameter: ≤ 5 mm).

5.3.2.13 Volumetric flasks.

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4) Disposable equipment is an acceptable alternative to reusable glassware.

5) Vortex® in an example of a suitable product available commercially. This information is given for the convenience of users of this standard and does not constitute an endorsement by CEN of this product.

5.3.2.14 Mechanical shaker.

5.3.2.15 Refrigerator capable of being controlled at 2 °C to 8 °C.

5.3.2.16 Forceps.

5.3.2.17 Microbiological filtered laminar air flow cabinet.

5.3.2.18 Fritted filter: Porosity of 40 µm to 100 µm (see ISO 4793).

5.3.2.19 Membrane filtration apparatus, constructed of a material compatible with the substances to be filtered.

The apparatus shall have a filter holder of at least 50 ml volume. It shall be suitable for use with filters of diameter 47 mm to 50 mm and 0,45 µm pore size for sterilization of hard water (5.2.2.7).

5.4 Preparation of test organism suspensions and product test solutions

5.4.1 Test organism suspensions

5.4.1.1 Stock cultures of test organisms

Stocks cultures shall be kept in accordance with the requirements of EN 12353.

5.4.1.2 Working culture of test organisms

a) Bacteria:

In order to prepare the working culture of the test organism, subculture from the stock culture (see 5.4.1.1) by streaking on TSA slopes (see 5.2.2.3) and incubate (see 5.3.2.4). After 18 h to 24 h, prepare a second subculture from the first subculture in the same way and incubate for 18 h to 24 h. From this second subculture, a third subculture may be produced in the same way.

NOTE 1 The second and/or third subculture are the working culture(s).

If it is not possible to prepare the second subculture on a particular day, a 48 h subculture may be used for subsequent subculturing, provided that the subculture has been kept in the incubator during the 48 h period. In these circumstances, prepare a further 24 h subculture after proceeding. Do not take a fourth subculture.

For additional strains (see 5.2.1), any departure from this method of culturing the bacteria or preparing the suspensions shall be noted, giving the reasons in the test report.

b) fungi:

In order to prepare the working culture of *Candida albicans,* subculture from the stock culture (see 5.4.1.1) by streaking onto MEA slopes (see 5.2.2.4) and incubate (see 5.3.2.5). After 42 h to 48 h, prepare a second subculture from the first subculture in the same way and incubate for 42 h to 48 h. From this second subculture, a third subculture may be produced in the same way.

NOTE 2 The second and/or third subculture are the working culture(s).

For *Aspergillus brasiliensis* (previously *A. niger*) (5.2.1), use only the first subculture grown on MEA (5.2.2.4) in Petri dishes or flasks with ventilated caps (5.3.2.17) and incubate at 30 °C ± 1 °C for 7 d to 9 d. No further subculturing is needed. Do not stack the Petri dishes during the incubation to improve the temperature homogenization.

At the end of incubation, all the cultures shall show a dark brown or black surface. Cultures with appearance of rare and small white or grey areas might be kept.

5.4.1.3 Test suspensions

a) Bacterial test suspension:

Take 10 ml of diluent (see 5.2.2.5) and place in a 100 ml flask with 5 g of glass beads (see 5.3.2.12). Take the working culture (see 5.4.1.2) and transfer loopfuls of the cells into the diluent. The cells should be suspended in the diluent by immersing the loop in the diluent and rubbing it against the side of the flask to dislodge the cells. Shake the flask for 3 min using a mechanical shaker (see 5.3.2.14). Aspirate the suspension from the glass beads and transfer to another flask. Adjust the number of cells in the suspension to $1,5 \times 10^8$ cfu/ml to $5,0 \times 10^8$ cfu/ml using the diluent, estimating the numbers of units by means of spectrophotometer or any other suitable means. Maintain this suspension in the water bath at 20 °C ± 1 °C and use within 2 h.

b) fungal test suspension:

1) *Candida albicans*:

Take 10 ml of diluent (see 5.2.2.5) and place in a 100 ml flask with 5 g of glass beads. Take the working culture (see 5.4.1.2) and transfer loopfuls of the cells into the diluent. The cells should be suspended in the diluent by immersing the loop in the diluent and rubbing it against the side of the flask to dislodge the cells. Shake the flask for 3 min using a mechanical shaker (5.3.2.14). Aspirate the suspension from the glass beads and transfer to another flask. Adjust the number of cells in the suspension to $1,5 \times 10^7$ cfu/ml to $5,0 \times 10^7$ cfu/ml using the diluent, estimating the numbers of units by means of a spectrophotometer or other suitable technique. Maintain this suspension in the water bath at 20 °C ± 1 °C and use within 2 h.

2) *Aspergillus brasiliensis (ex A. niger)*:

Take the working culture (see 5.4.1.2) and suspend the cells in 10 ml of sterile 0,05 % w/v polysorbate 80 [6)] solution in water (see 5.2.2.2). Using a sterile glass rod or spatula detach the conidiospores from the culture surface. Transfer the suspension into a flask and gently shake by hand for one minute together with glass beads (see 5.3.2.12). The suspension is filtered through a fritted filter (see 5.3.2.18).

Carry out a microscopic examination under x 400 magnification immediately after the preparation to show:

— the presence of a high concentration (at least 75 % of spiny spores) of characteristic mature spores, i.e. spiny spores (versus smooth spores);

— the absence of spore germination (check at least 10 fields of view);

— the absence of mycelia fragments (check at least 10 fields of view).

If germinated spores are present, discard the suspension.

If mycelia are present, proceed to a 2nd fritted filtration.

If mycelia are still present, discard the suspension.

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6) Analytical quality, non hydrolysed in accordance with European Pharmacopoeia, Volume 1. TWEEN 80® is an example of a suitable product available commercially. This information is given for the convenience of users of this standard and does not constitute an endorsement by CEN of this product.

If 75 % spiny spores are not achieved it may be due to the *Aspergillus brasiliensis (ex A. niger)* culture or the media used to produce these spores. In this situation, it will be necessary to obtain the culture from another culture collection and/or use a MEA from a different supplier.

Adjust the number of spores in the suspension to 1,5 × 10^7 cfu/ml to 5,0 × 10^7 cfu/ml using the diluent (5.2.2.5), estimating the number of cfu by any suitable means. Use the suspension within 4 h in a water bath controlled at 20 °C ± 1 °C (5.3.2.3). In any case, adjust the temperature according to 5.5.1 only immediately before the start of the test (5.5.2).

The use of a cell counting device for adjusting the number of cells is highly recommended. When using a suitable counting chamber, follow the instructions explicitly.

For counting, prepare 10^{-5} and 10^{-6} dilutions of the test suspension using diluent (5.2.2.5). Mix (5.3.2.8).

Take a sample of 1,0 ml of each dilution in duplicate and inoculate using the pour plate or the spread plate technique.

a) When using the pour plate technique, transfer about half of each 1,0 ml sample into separate Petri dishes (i.e. in duplicate = four plates) and add 15 ml to 20 ml of melted MEA (5.2.2.4),cooled to (45 ± 1) °C.

b) When using the spread plate technique, spread about one quarter of each 1,0 ml sample on an appropriate number (at least four) of surface dried plates containing MEA (5.2.2.4) (i.e. in duplicate – at least eight plates).

This test suspension shall not be stored more than 2 d at 2 °C to 8 °C.

The test suspension shall be mixed (see 5.3.2.8) immediately before use to re-suspend the spores.

5.4.1.4 Counting of bacterial and fungal test suspensions

Dilute the adjusted bacterial suspensions (see 5.4.1.3) by 10^{-6} (serial dilutions) and 10^{-7} and the fungal suspension (see 5.4.1.3) by 10^{-5} and 10^{-6} using diluent (see 5.2.2.5). Mix the suspension (see 5.3.2.8).

Take a sample of 1,0 ml of each dilution in duplicate and inoculate pour plates. Pipette each 1,0 ml sample into separate Petri dishes (see 5.3.2.11) and add 15 ml to 20 ml melted TSA (see 5.2.2.2) for the bacteria and 15 ml to 20 ml melted MEA (see 5.2.2.3) for the fungi, cooled to 45 °C ± 1 °C.

a) Counting of bacterial test suspensions

   1) For the bacterial strains, incubate the plates at 36 °C ± 1 °C or at 37 ° ± 1 °C for 24 h. Discard any plates which are not countable for any reason. Incubate the plates for a further 24 h. Do not recount plates which no longer show countable colonies. Recount the remaining plates.

b) Counting of fungal test suspensions

   1) For the fungal strains, incubate the plates at 30 °C ± 1 °C for 24 h (*Candida albicans*), for 42 h to 48 h (*Aspergillus brasiliensis (ex A. niger)*). Discard any plates which are not countable for any reason. Count the plates and determine the number of colony forming units. Incubate the plates for a further 24 h. Do not recount plates which no longer show well-separated colonies. Recount the remaining plates. For *Aspergillus brasiliensis (ex A. niger)*, continue incubation for a further 20 h to 24 h and if necessary a further 20 h to 24 h, provided the number of countable colonies (discrete colonies) is increasing.

Determine the highest number of colonies for each 1 ml sample.

For incubation and counting, see 5.4.1.5.

5.4.1.5 Counting the weighed mean of the test suspensions

a) Discard any plates that are not countable for any reason. Count the plates and determine the total number of cfu. Do not recount plates that no longer show well-separated colonies. Recount the remaining plates. If the number has increased, use only the higher number for further evaluation.

b) Only the plates showing a number of colonies included in a 15-300 for bacteria and yeast and 15-150 for mould range were used to perform the result calculation. A deviation of 10 % is accepted, so the limits are 14 and 330 for bacteria and yeast and 14-165 for mould.

$$N = \text{Log} \frac{0{,}025 \times c}{(n_1 + 0{,}1 n_2) \times d} \qquad (1)$$

where

c is the sum of the *V*c values taken into account;

*n*1 is the number of *V*c values taken into account in the lower dilution, i.e. 10^{-6};

*n*2 is the number of *V*c values taken into account in the higher dilution, i.e. 10^{-7};

d is the dilution factor corresponding to the lower dilution (10^{-6}).

5.4.2 Product test solutions

Details of samples of the product as received shall be recorded.

Product test solutions shall be prepared in hard water (see 5.2.2.7) at minimum three different concentrations to include one concentration in the active range and one concentration in the non active range.

For solid products, dissolve the product as received by weighing at least 1 g ± 10 mg of the product in a volumetric flask and dilute with hard water (see 5.2.2.7) Subsequent dilutions shall be prepared in volumetric flasks (see 5.3.2.13) on a volume/volume basis in hard water (see 5.2.2.7).

For liquid products, dilutions of the product shall be prepared in hard water (see 5.2.2.7) on a volume/volume basis using volumetric flasks (see 5.3.2.13).

The product as received may be used as one of the product test solutions. For products supplied in a ready to use state, water (see 5.2.2.2) shall be used to prepare dilutions.

When the product is diluted in hard water, it shall give a physically homogeneous stable preparation.

The product test solution and dilutions of it shall be prepared freshly and used within 2 h.

The concentration of the product stated in the test report shall be the test concentration. Record the test concentration in terms of volume per volume or weight per volume.

5.5 Procedure

5.5.1 Choice of experimental conditions

The selection of contact temperature, contact time and interfering substances shall be carried out according to the practical use considered for the product (see Clause 4 Table 1) as follows:

a) test temperature; θ (°C):

   1) the test temperature shall be at room temperature between the range (18 ± 1) °C and (25 ± 1) °C;

   2) additional temperatures may be chosen from 4 °C ± 1 °C or 10 °C ± 1 °C or 40 °C ± 1 °C. A temperature controlled chamber should be used for temperatures other than ambient.

b) contact time; t (min):

   1) the contact time to be tested is 5 min ± 10 s for bacteria and 15 min ± 10 s for fungi;

   2) additional times may be chosen from 1 min ± 10 s, 5 min ± 10 s, 15 min ± 10 s, 30 min ± 10 s and 60 min ± 10 s.

c) strains:

   1) strains shall be as given in 5.2.1.

d) in case of interfering substances:

   1) the obligatory interfering substance to be tested is 0.3 g/l bovine albumin (5.2.2.8.2) or 0,85 % skim milk in case of *Pseudomonas aeruginosa* for clean conditions (5.2.2.8.2) or 3.0 g/l bovine albumin (5.2.2.8.2) for dirty conditions according to Clause 4, Table 1 and practical applications;

   2) in the case of additional requirements other interfering substances (see 5.2.2.8.3 to 5.2.2.8.6) may be chosen according to the application field of the product.

The product shall not cause the formation of any observable precipitate in the experimental conditions used.

5.5.2 Test procedure

5.5.2.1 Test "*Nd*" – determination of microbicidal concentrations

The procedure for determining microbicidal concentrations is as follows:

a) To prepare the microbial test suspension pipette 1,0 ml of the interfering substance (5.2.2.8) into a tube. Add 1,0 ml of the test suspension (5.4.1.3). Start the stopwatch immediately, mix and place the tube in a water bath or temperature controlled cabinet (5.3.2.2) at the at the chosen test temperature θ °C ± 1 °C for 2 min ± 10 s.

   Immediately before addition, the test suspension should be well mixed to fully re-suspend the organisms.

b) Place the test surfaces (5.2.3) in a sterile Petri dish and ensure that the dish is in a horizontal position. Prepare the test surfaces by inoculating 0,05 ml of the microbial test suspension (5.5.2.1 a) on to each test surface. Dry the surfaces at 37 °C until they are visibly dry. It is understood that drying of the test surfaces will occur at different rates due to the ambient conditions of the laboratory and the design of the incubator (e.g. with or without a fan). For this reason no time duration is given and the minimum required time for the surfaces to become visibly dry should be established for each laboratory. The drying time should not exceed 60 min and if it does, alternative drying conditions shall be used. Allow the test surfaces to equilibrate with the chosen test temperature θ °C ± 1°C.

c) Pipette 0,1 ml of each product test solution (5.4.2) to be tested on to separate dried surfaces ensuring that the dried inoculum is totally covered by the test product. Place the surfaces in a temperature controlled cabinet (5.3.2.2) at the chosen test temperature θ °C ± 1°C and contact time *t*.

d) At the end of *t*, transfer each of the surfaces (*Nd*) to a separate container containing 10 ml of neutralizer (5.2.2.6) together with sufficient glass beads (for example 5 g) to support the surface. The surfaces

should be placed with the inoculated surface downwards in contact with the beads. Shake the containers for minimum 1 min. The shaking should be sufficiently vigorous to ensure that the test surface moves constantly over the beads. After the neutralization time of 5 min ± 10 s prepare a series of 10-fold dilutions from 10^{-1} to 10^{-2} of the neutralized mixture in the diluent (5.2.2.5). Take a 1,0 ml sample of the neutralized mixture and each of the dilutions in duplicate and inoculate using pour plate or spread plate technique.

1) When using the pour plate technique, pipette each 1,0 ml sample into separate Petri dishes and add 15 ml to 20 ml of melted TSA (5.2.2.3) for the bacteria and MEA (5.2.2.4) for the fungi, cooled to 45 °C ± 1 °C.

2) When using the spread plate technique, spread each 1,0 ml sample – divided into portions of approximately equal size – on an appropriate number (at least two) of surface dried plates containing TSA (5.2.2.3).

For incubation and counting, see 5.5.3.

e) Recover the test surface (Nts), let the neutralizer drain off and rinse with 10 ml of water (5.2.2.2). Transfer to a Petri dish containing 10 ml of solidified TSA (5.2.2.3) for bacteria and Petri dish containing 10 ml of solidified MEA (5.2.2.4) for fungi and place on top of the agar, test side uppermost. Add 10 ml of TSA (5.2.2.3) and/or MEA (5.2.2.4) melted and cooled to 45 °C.

f) Perform the procedure a) to e) using the other product test solutions at the same time.

g) Perform the procedure a) to f) applying the other obligatory and if appropriate other additional experimental conditions (5.5.1).

5.5.2.2 Water control "*Nc*"

The procedure for determining the water control is as follows:

a) Place one test surface (5.2.3) in a sterile Petri dish and ensure that the dish is in a horizontal position. Inoculate 0,05 ml of the microbial test suspension [5.5.2.1 a)] on to the test surface. Dry the surface at 37 °C until it is visibly dry [5.5.2.1 b)].

b) For the water control (*Nc*), pipette 0,1 ml of hard water (5.2.2.7) or water (5.2.2.2) in the case of ready-to use products on to the test surface ensuring that the dried inoculum is totally covered by the water. Place the surface in a temperature controlled cabinet (5.3.2.2) at the chosen test temperature θ ± 1.°C.

c) At the end of *t*, transfer the surface "*Nc*" into a container containing 10 ml of neutralizer (5.2.2.6) together with sufficient glass beads (for example 5 g) to support the surface. The surfaces should be placed with the inoculated surface downwards in contact with the beads. Shake the containers for minimum 1 min. The shaking should be sufficiently vigorous to ensure that the test surface moves constantly over the beads. After a neutralization time of 5 min ± 10 s prepare a series of 10-fold dilutions for the bacterial strains prepare 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilutions and for the fungal strains 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} of the neutralised mixture in the diluent (5.2.2.5). Take a 1,0 ml sample of these dilutions in duplicate and inoculate using the pour plate or the spread plate technique.

d) Recover the test surface ("Nts"), let the neutralizer drain off and rinse with 10 ml of water (5.2.2.2). Transfer to a Petri dish containing 10 ml of solidified TSA (5.2.2.3) for bacteria and Petri dish containing 10 ml of solidified MEA (5.2.2.4) for fungi and place on top of the agar, test side uppermost. Add 10 ml of TSA (5.2.2.3) and/or MEA (5.2.2.4) melted and cooled to 45 °C. For incubation and counting, see 5.5.3..

5.5.2.3 Neutralizer control “NC” - verification of the absence of toxicity of the neutralizer

To verify the absence of toxicity of the neutralizer, the procedure is as follows:

a) Prepare one inoculated test surface [5.5.2.1 a) and b)].

b) Pipette 10 ml of neutralizer (5.2.2.6) into a container with sufficient glass beads (for example 5 g) to support the surface. Then add 0,1 ml of hard water (5.2.2.7) or water (5.2.2.2) in the case of ready-to-use products. Mix and leave in contact for 5 min ± 10 s at 20 °C ± 1 °C.

c) At the end of the neutralization time transfer the inoculated and dried test surface into the container and place the inoculated surface own wards in contact with the beads (for example 5 g). Shake the containers for minimum 1 min. The shaking should be sufficiently vigorous to ensure that the test surface moves constantly over the beads.

d) After a neutralization time of 5 min ± 10 s prepare a series of 10-fold dilutions of the neutralized mixture “*NC*” in the diluent (5.2.2.5) to produce 10^{-3} to 10^{-5} dilutions. Take a sample of 1.0 ml of each of the dilutions in duplicate and inoculate using the pour plate or the spread plate technique [5.5.2.2 d)]. For incubation and counting, see 5.5.3.

5.5.2.4 Method validation “NT” - dilution-neutralization validation

To validate the dilution neutralization method, the procedure is as follows:

a) Prepare one test surface [5.5.2.1 a) and b)].

b) Pipette 10 ml of neutralizer (5.2.2.7) into a container with sufficient glass beads to support the surface. Then add 0,1 ml of the highest product concentration used in the test (5.5.2.2). Mix and leave in contact for 5 min ± 10 s at 20 °C ± 1 °C.

c) At the end of the neutralization time transfer the inoculated test surface into the container and place the inoculated surface downwards in contact with the beads (for example 5 g). Shake the containers for minimum 1 min. The shaking should be sufficiently vigorous to ensure that the test surface moves constantly over the beads.

d) After 5 min ± 10 s prepare a series of 10-fold dilutions of the neutralized mixture “*NT*” in the diluent (5.2.2.4) to produce 10^{-3} to 10^{-5} dilutions. Take samples of 1,0 ml of each of the dilutions in duplicate and inoculate using pour plate or spread plate technique.

5.5.3 Counting of the test mixtures

For the bacterial strains, incubate the plates at 36 °C ± 1 °C or at 37 °C ± 1 °C for 24 h.

For the fungal strains, incubate the plates at 30 °C ± 1 °C for 24 h (*Candida albicans*), for 42 h to 48 h (Aspergillus *brasiliensis (ex A. niger)*). Discard any plates which are not countable for any reason. Count the plates and determine the number of colony forming units. Incubate the plates for a further 24 h. Do not recount plates which no longer show well-separated colonies. Recount the remaining plates. For *Aspergillus brasiliensis (ex A. niger)*, continue incubation for a further 20 h to 24 h and if necessary a further 20 h to 24 h, provided the number of countable colonies (discrete colonies) is increasing.

Record the number of colony forming units (Nts) remaining on the test surface.

Discard any plates that are not countable for any reason. Count the plates and determine the total number of cfu. Do not recount plates that no longer show well-separated colonies. Recount the remaining plates. If the number has increased, use only the higher number for further evaluation.

Only the plates showing a number of colonies included in a 15-300 for bacteria and yeast and 15-150 for mould range were used to perform the result calculation. A deviation of 10 % is accepted, so the limits are 14 and 330 for bacteria and yeast and 14-165 for mould.

In the assay, where the number of cfu on every plate counted is < 14, the number of cfu/ml should be recorded as < $1,4x10^2$ (<2,15 Log). Where the number of cfu on every plate counted is > 330 or 165, the number of cfu/ml should be recorded as > $3,3x10^5$ (>5,52 Log) or > $1,6 \times 10^5$, (>5,22 Log).

Calculate *N*c and *Nd* the log number of cfu recovered from the test surface using the following equation:

$$Nd\ (or\ Nc) = \log (c \times 10/n \times d) \quad (2)$$

where

c is the sum of *Vc* values taken into account;

n is the number of *Vc* values taken into account;

d is the dilution taken into account.

If one or both of the duplicate *Vc* values are either below the lower or above the upper limit, express the results as “less than” or “more than”. In particular, in the assay, where the number of cfu on every plate counted is < 14, the number of cfu/ml should be recorded as < $1,4x10^2$ (<2,15 Log). Where the number of cfu on every plate counted is > 330 or 165, the number of cfu/ml should be recorded as > $3,3x10^5$ (>5,52 Log) or > $1,6 \times 10^5$, (>5,22 Log).

If both of the duplicate *Vc* values are zero, it is assumed that the count (in ufc) is less than 5/ml in the neutralization medium and an *Na* value of < 0,10 should be used.

Calculate *NC* and *NT* the log number of cfu recovered from the test surface using the following equation:

$$NC, NT = \log (c \times 10/n \times d) \quad (3)$$

where

c is the sum of *Vc* values taken into account;

n is the number of *Vc* values taken into account;

d is the dilution taken into account.

5.6 Calculation and expression of results

5.6.1 Elaboration of data: counting of weighed average values

Rounding of the results shall be performed by rounding the exponential values to two significant ciphers.

When rounding ciphers: if the last cipher is higher than or equal to ‘5’ the previous cipher is increased of a unit; if the last cipher is lower than ‘5’ the previous cipher remains unchanged; proceed this way until reaching two significant ciphers.

Log values are rounded to two decimal significant ciphers as shown in Annex B and Annex C, Table C.1.

If observed counts from two consecutive dilutions fall within the 14-330 or 14-165 range, then a weighted average value is calculated as follows:

$$(m+m'+n+n')/2.2xVxd \quad (4)$$

where

m, m' are the two replicas at the lower dilution expressed as cfu;

n, n' are the two replicas at the higher dilution expressed as cfu;

V is the volume of the inoculated into the plate expressed in ml;

d is the lower dilution factor.

In case not all the replicas at each dilution fall within the 14-330 or 14-165 range, the weighted average will take this into account as follows:

Case 1: only m and n fall within the 14-330 or 14-165 range, the formula will change as follows:

$$(m+n)/1.1xVxd \quad (5)$$

Case 2: only m, m' and n fall within the 14-330 or 14-165 range, the formula will change as follows:

$$(m+m'+n)/2.1xVxd \quad (6)$$

Case 3: only m, n and n' fall within the 14-330 or 14-165 range, the formula will change as follows:

$$(m+n+n')/1.2xVxd \quad (7)$$

Case 4: only m falls within the 14-330 or 14-165 range, the formula will change as follows:

$$m/Vxd \quad (8)$$

5.6.2 Verification of methodology

For each test, check that:

a) the mean counts form duplicate plate used for calculation of *N*, *Nc*, *Nd*, *NC*, *NT* are between 14 and 330 for bacteria and yeast strains and 14 and 165 for mould strains;

b) $6,57 \leq N \leq 7,10$ Logs for bacteria;

c) $5,57 \leq N \leq 6,10$ Logs for fungi;

d) Nc ≥ 6,27 Logs for bacteria;

e) Nc ≥ 5,27 Logs for fungi;

f) NC > 0,5 Nc;

g) NT > 0,5 Nc is not greater than ± 0,3;

h) Nts is less than 100 cfu/ml for active concentrations. If not, the recovery of microorganisms has not been sufficient. For non active concentrations, Nts may be not countable;

i) control of weighted mean counts: quotient is not lower than 5 and not higher than 15. It applies only to N calculation.

5.6.3 Expression of results

5.6.3.1 Reduction

The reduction (*R)* is expressed in logarithm.

For each test organism record the number of cfu/ml in the test procedure for microbicidal activity of the product (5.5.2.2) and the control procedure (5.5.2.3)." For each product concentration and each experimental condition, calculate and record the decimal log (lg) reduction separately using the equation:

$$R = Nc - Nd \quad (9)$$

5.6.4 Conclusion

5.6.4.1 Activity on non-porous surfaces for general purposes

Bactericidal activity on surfaces for general purposes is characterized by the concentration of the tested product for which criteria 5.6.1 and 5.6.2 are met and for which a 4 log or more reduction in viability is demonstrated under the required test conditions: 5 min ± 10 s, 18 °C and 25 °C and clean or dirty conditions (see Clause 4), when the test organisms are *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus hirae* and *Escherichia coli*.

Fungicidal activity on surfaces for general purposes is characterized by the concentration of the tested product for which criteria 5.6.1 and 5.6.2 are met and for which a 3 log or more reduction in viability is demonstrated under the required test conditions: 15 min ± 10 s, 20 °C ± 1 °C and clean or dirty conditions (see Clause 4), when the test organisms are *Candida albicans* and *Aspergillus brasiliensis (ex A. niger)*.

Yeasticidal activity on surfaces for general purposes is characterized by the concentration of the tested product for which criteria 5.6.1 and 5.6.2 are met and for which a 3 log or more reduction in viability is demonstrated under the required test conditions: 15 min ± 10 s, 20 °C ± 1 °C and clean or dirty conditions (see Clause 4), when the test organisms are *Candida albicans*.

Bactericidal and fungicidal or yeasticidal activity on surfaces for general purposes is characterized by the concentration of the tested product for which criteria 5.6.1 and 5.6.2 are met and:

— for which a 4 log or more reduction in viability is demonstrated under the required test conditions: 5 min ± 10 s, 20 °C ± 1 °C and clean or dirty conditions (see Clause 4), when the test organisms are *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus hirae* and *Escherichia coli*;

— for which a 3 log or more reduction in viability is demonstrated under the required test conditions: 15 min ± 10 s, 20 °C ± 1 °C and clean or dirty conditions (see Clause 4), when the test organisms are *Candida albicans* and *Aspergillus brasiliensis (ex A. niger)*;

— for which a 3 log or more reduction in viability is demonstrated under the required test conditions: 15 min ± 10 s, and 20 °C ± 1 °C and clean or dirty conditions (see Clause 4), when the test organisms are *Candida albicans*.

5.6.4.2 Activity on non-porous surfaces for specific purposes

Bactericidal and/or fungicidal or yeasticidal activity on surfaces for specific purposes is characterized by the concentration of the tested product for which criteria 5.6.1 and 5.6.2 are met and for which:

— a 4 log or more reduction in viability is demonstrated, when the test organisms are *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus hirae* and *Escherichia coli*, and if required additional test organisms;

and/or for which:

— a 3 log or more reduction in viability is demonstrated, when the test organisms are *Candida albicans* and *Aspergillus brasiliensis (ex A. niger)*, and if required additional test organisms;

— a 3 log or more reduction in viability is demonstrated, when the test organisms are *Candida albicans*, and if required additional test organisms

under additional conditions: t in minutes, θ in degrees °C, clean or dirty conditions and additional interfering substances (see 5.5.1).

5.7 Test report

The test report shall refer to this European Standard.

The test report shall state, at least, the following information:

a) identification of the laboratory;

b) identification of the sample:

   1) name of the product;

   2) batch number;

   3) manufacturer;

   4) date of delivery:

   5) storage conditions;

   6) active substance(s) and its/their concentration(s) (optional);

c) experimental conditions:

   1) period of analysis;

   2) product diluent used during the test;

   3) product test concentrations;

   4) appearance product dilutions;

   5) contact time(s);

   6) test temperature(s);

   7) interfering substance;

   8) reaction between the inoculum in the presence of interfering substances and product;

   9) temperature of incubation;

10) neutralizer of the bacterial or fungal strains used;

11) identification of the bacterial and/or fungal strains used;

12) identification of the test surface;

d) operating procedure:

1) full details for the test for validation of the neutralization medium shall be given;

e) test results:

1) validation tests;

2) evaluation of bactericidal and/or fungicidal and/or yeasticidal activity;

f) conclusion;

g) locality, date and identified signature(s).

NOTE An example of a typical test report is given in Table C.1.

Annex A
(informative)

Corresponding referenced strains

Pseudomonas aeruginosa :	ATCC	15 442
	CIP	103 467
	DSM	939
	NCIB	10 421
Staphylococcus aureus:	ATCC	6 538
	CIP	4.83
	DSM	799
	NCTC	10 788
	NCIB	9 518
Escherichia coli:	ATCC	10 536
	CIP	54 127
	DSM	682
	NCTC	10 418
	NCIMB	8 879
Enterococcus hirae:	ATCC	10 541
	CIP	5 855
	DSM	3320
	NCIMB	8 192
Salmonella typhimurium:	ATCC	13 311
	CIP	5 858
	NCTC	74
Lactobacillus brevis:	DSM	6 235
	CIP	103 474
Enterobacter cloacae:	DSM	6 234
	CIP	104 674
Saccharomyces cerevisiae:	ATCC	9 763

	IP	143 283
	DSM	1 333
	CBS	5 900
Candida albicans:	ATCC	10 231
	IP	4 872
	DSM	1386
	CBS	6 431
	NCTC	3 179
Aspergillus brasiliensis (ex A. niger):	ATCC	16 404
	DSM	1 988
	CBS	733.88
	IP	1 431.83
	NCTC	2 275
	CMI	149 007

Annex B
(informative)

Neutralizers

Any of the following neutralizers can be used:

— lecithin 3 g/l; polysorbate 80 [7)] 30 g/l; sodium thiosulphate 5 g/l; L-histidine 1 g/l; saponine 30 g/l in diluent (see 5.2.2.4) or in phosphate buffer 0,25 mol/l at 1 % (*V/V*);

— phosphate buffer 0,25 mol/l:

 — $KH_2\ PO_4$ 34 g;

 — water (see 5.2.2.2) 500 ml;

 — adjusted to pH 7,2 ± 0,2 with 1 mol/l NaOH;

 — water (see 5.2.2.2) up to 1 000 ml;

 — sterilized in an autoclave (see 5.3.1);

— fresh egg yolk diluted to 5 % (*V/V*) or 0,5 % (*V/V*);

— 30 g/l polysorbate 80[7)]; 4 g/l sodium lauryl sulphate ; lecithin 3g/l;

— 5 % (*V/V*) fresh egg yolk; 40 g/l polysorbate 80[7)];

— 7 % (*V/V*) ethylene oxide condensate of fatty alcohol ; 20 g/l lecithin; 4 % (*V/V*) polysorbate 80[7)];

— 4 % (*V/V*) ethylene oxide condensate of fatty alcohol; 4 g/l lecithin;

— 30 g/l polysorbate 80[7)]; lecithin 3 g/l; L-histidine 1 g/l;

— glycine as a function of concentration of product;

— 30 g/l polysorbate 80[7)]; lecithin 3 g/l;

— phospholipid emulsion (commercial) at 50 mg/ml (diluted 1 to 10);

— sodium thioglycollate at 0,5 g/l or 5 g/l;

— L cysteine at 0,8 g/l or 1,5 g/l;

— thiomalic acid at 0,075 % (*V/V*) (adjusted to pH 7 with NaOH);

— sodium thiosulphate at 5 g/l;

---

7) Analytical quality, non-hydrolysed in accordance with European Pharmacopoeia volume 1. TWEEN 80® is an example of a suitable product available commercially. This information is given for the convenience of users of this standard and does not constitute an endorsement by CEN of this product.

— catalase or peroxidase: One unit for of these enzymes catalyses the decomposition of 1 µmol of hydrogen peroxide per minutes at 25 °C and at pH 7;

— polysorbate 80[7)] 30 g/l ; saponin 30 g/l ; L-histidine 1 g/l ; L-cysteine 1g/l.

The above list is not exhaustive and other neutralizers may be tried.

Annex C
(informative)

Expression of results with the dilution-neutralization method

Table C.1 — Test results

Test organisms	Bacterial or fungal test suspension : N (See 5.4.1.3)	Validation test : NT (See Annex A)	Validation test : NC (See Annex A)	Water control : Nc (See 5.5.3)	Test procedure at concentrations % (*V/V*) (See 5.5.2.1) 0.5	0.75	1.00
Pseudomonas aeruginosa ATCC 15442	10^{-6} : 229 ;216 10^{-7} : 20 ;17 N : 6,74	10^{-3} : > 330 ; > 330 10^{-4} : 123 ;118 10^{-5} : 9 ;13 NT : 7,08	10^{-3} : > 330 ; > 330 10^{-4} : 137 ;142 10^{-5} : 11 ;4 NC : 7,14	10^{-3} : > 330 ; > 330 10^{-4} : 153 ;123 10^{-5} : 14 ;9 Nc : 7,14 Nts : > 100	10^{-0} : > 330 ; > 330 10^{-1} : 160 ;138 10^{-2} : 13 ;17 Nd : 4,18 Nts : > 100 R : 2,96	10^{-0} : 0 ;0 10^{-1} : 0 ;0 10^{-2} : 0 ;0 Nd : < 0,10 Nts : 12 R: > 6,04	10^{-0} : 0 ;0 10^{-1} : 0 ;0 10^{-2} : 0 ;0 Nd : < 0,10 Nts : 0 R: > 7,04
Escherichia coli ATCC 10536	10^{-6} : 230 ;210 10^{-7} : 23 ;19 N : 6,74	10^{-3} : > 330 ; > 330 10^{-4} : 132 ;113 10^{-5} : 9 ;2 NT : 7,09	10^{-3} : > 330 ; > 330 10^{-4} : 143 ;122 10^{-5} : 22 ;11 NC : 7,14	10^{-3} : > 330 ; > 330 10^{-4} : 155 ;121 10^{-5} : 18 ;23 Nc : 7,15 Nts : > 100	10^{-0} : > 330 ; > 330 10^{-1} : 166 ;144 10^{-2} : 22 ;18 Nd : 4,20 Nts : > 100 R: 2,95	10^{-0} : 210 ;198 10^{-1} : 27 ;19 10^{-2} : 5 ;2 Nd : 3,65 Nts : 0 R: 3,50	10^{-0} : 0 ;0 10^{-1} : 0 ;0 10^{-2} : 0 ;0 Nd : < 0,10 Nts : 0 R: > 7,05

Staphylococcus aureus ATCC 6538	10^{-6} : 227 ;202 10^{-7} : 18 ;23 N : 6,72	10^{-3} : > 330 ; > 330 10^{-4} : 166 ;134 10^{-5} : 15 ;11 NT : 7,18	10^{-3} : > 330 ; > 330 10^{-4} : 158 ;144 10^{-5} : 13 ;8 NC : 7,18	10^{-3} : > 330 ; > 330 10^{-4} : 162 ;146 10^{-5} : 12 ;16 Nc : 7,19 Nts : > 100	10^{-0} : > 330 ; > 330 10^{-1} : 265 ;240 10^{-2} : 33 ;28 Nd : 4,41 Nts : > 100 R: 2,78	10^{-0} : > 330 ; > 330 10^{-1} : 63 ;52 10^{-2} : 4 ;7 Nd : 3,76 Nts : 23 R: 3,43	10^{-0} : 0 ;0 10^{-1} : 0 ;0 10^{-2} : 0 ;0 Nd : < 0,10 Nts : 0 R: > 7,09
Enterococcus hirae ATCC 10541	10^{-6} : 235 ;255 10^{-7} : 29 ;31 N : 7,09	10^{-3} : > 330 ; > 330 10^{-4} : 198 ;178 10^{-5} : 27 ;18 NT : 7,28	10^{-3} : > 330 ; > 330 10^{-4} : 201 ;187 10^{-5} : 17 ;24 NC : 7,29	10^{-3} : > 330 ; > 330 10^{-4} : 194 ;179 10^{-5} : 23 ;19 Nc : 7,28 Nts : > 100	10^{-0} : > 330 ; > 330 10^{-1} : > 300 ; > 300 10^{-2} : 127 ;132 Nd : 5,11 Nts : > 100 R: …	10^{-0} : > 330 ; > 330 10^{-1} : 128 ;145 10^{-2} : 9 ;13 Nd : 4,14 Nts : 8 R: …	10^{-0} : 0 ;0 10^{-1} : 0 ;0 10^{-2} : 0 ;0 Nd : < 0,10 Nts : 0 R: > …

Annex D
(informative)

Bactericidal activity in surfaces in general use conditions (for clean conditions)

a) Identification of the test laboratory .... Besson test house;

b) Identification of the sample:

Name of the product .... Z

Batch number 94–71–51

Manufacturer .... Centipede Formulations Inc

Date of delivery 1994–02–11

Storage conditions .... room temperature and darkness

Product diluent recommended by the manufacturer for use... Potable water

Active substance(s) and its (their) concentration(s) (optional).. Not indicated

c) Test method and its validation:

Method .... Dilution neutralization;

Neutralizer .... 30 g/l, lecithin, sterilized in the autoclave ;

d) Experimental conditions:

Period of analysis .... 1994–02–20 to 1994–03–12

Product diluent used during the test...................... sterile hard water 300 mg/kg $CaCO_3$

Product test concentrations .... 2 g/l, 4 g/l and 8 g/l

Appearance product dilutions............................... colourless, clear solution

Interfering substances... 0.3 g/l of bovine albumin (0.85 % skimmed milk fo Pseudomonas aeruginosa)

Test temperature .... θ = between 18 °C and 25 °C

Contact time .... t = 5 min ± 10 s

Temperature of incubation .... 37 °C ± 1 °C

Identification of the bacterial strains used............. *Pseudomonas aeruginosa* ATCC 15 442
Escherichia coli ATCC 10 536
Staphylococcus aureus ATCC 6 538
Enterococcus hirae ATCC 10 541

e) Test results:

See Table C.1.

f) Conclusion:

In accordance with this European Standard the batch 94–71–51 of the product Z, when diluted at 1 % (*V*/*V*), in hard water, possesses bactericidal activity on surfaces in 5 min at 20 °C under clean conditions 0,3 g/l bovine albumin for referenced strains *Escherichia coli*, *Staphylococcus aureus* a *Enterococcus hirae* and 0,85 % skimmed milk for reference strain *Pseudomonas aeruginosa*.

g) Locality, date and identified signature.

NOTE The test product, batch N° and manufacturer are given as imaginary examples only.

Annex E
(informative)

Precision of the test result

The formula used for the calculation of the precision of the test results can be found in EN 1040:2005, Annex E. The number of repetitions which give a precision of the reduction factor need to be established by appropriate collaborative studies. A guidance to the interpretation of the test results concerning their precision and the number of test repetitions is given in the EN 14885.

Bibliography

[1] EN 1040:2005, *Chemical disinfectants and antiseptics - Quantitative suspension test for the evaluation of basic bactericidal activity of chemical disinfectants and antiseptics - Test method and requirements (phase 1)*

[2] EN 10088-1, *Stainless steels - Part 1: List of stainless steels*

[3] EN 10088-2, *Stainless steels - Part 2: Technical delivery conditions for sheet/plate and strip of corrosion resisting steels for general purposes*

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