SLOVENSKI STANDARD
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Kemična razkužila in antiseptiki - Kvantitativni suspenzijski preskus za vrednotenje osnovnega fungicidnega delovanja ali osnovnega delovanja kemičnih razkužil in antiseptikov na kvasovke - Preskusna metoda in zahteve (faza 1)

Chemical disinfectants and antiseptics - Quantitative suspension test for the evaluation of basic fungicidal or basic yeasticidal activity of chemical disinfectants and antiseptics - Test method and requirements (phase 1)

Chemische Desinfektionsmittel und Antiseptika - Quantitativer Suspensionsversuch zur Bestimmung der fungiziden oder levuroziden Wirkung (Basistest) chemischer Desinfektionsmittel und Antiseptika - Prüfverfahren und Anforderungen (Phase 1)

Antiseptiques et désinfectants chimiques - Essai quantitatif de suspension pour l'évaluation de l'activité fongicide ou levuricide de base des antiseptiques et des désinfectants chimiques - Méthode d'essai et prescriptions (phase 1)

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Chemical disinfectants and antiseptics - Quantitative suspension test for the evaluation of basic fungicidal or basic yeasticidal activity of chemical disinfectants and antiseptics - Test method and requirements (phase 1)

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This European Standard was approved by CEN on 28 July 2005.

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Foreword

This European Standard (EN 1275:2005) 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of an identical text or by endorsement, at the latest by June 2006, and conflicting national standards shall be withdrawn at the latest by June 2006.

This European Standard supersedes EN 1275:1997.

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

Introduction

This European Standard specifies a suspension test for establishing whether a chemical disinfectant or antiseptic does or does not have a *basic* fungicidal or a *basic* yeasticidal activity in the fields described in the scope. The acceptability of a product for a defined purpose cannot be determined from this test method. Therefore products are subjected to further testing by relevant tests specified in European Standards to evaluate their activity under conditions appropriate to their intended use. These European Standards have been or will be developed by CEN TC 216.

1 Scope

This European Standard specifies a test method and the minimum requirements for basic fungicidal or basic yeasticidal activity of chemical disinfectant and antiseptic products that form a homogeneous, physically stable preparation when diluted with water. Products can only be tested at a concentration of 80 % or less as some dilution is always produced by adding the test organisms and water.

This European Standard applies to active substances (antifungal biocides) and to formulations under development that are planned to be used in food, industrial, domestic and institutional, medical and veterinary areas. It applies also to the evaluation of fungicidal or yeasticidal activity of chemical antiseptics and disinfectants when appropriate standards are not available.

NOTE 1 This European Standard does not evaluate the activity of a product for an intended use.

NOTE 2 This method corresponds to a phase 1 test (Annex F).

2 Normative references

The following referenced documents are indispensable for the application of this European Standard. 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 microbial strains used for the determination of bactericidal and fungicidal activity*

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

3 Terms and definitions

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

3.1
product
chemical agent or formulation used as chemical disinfectant or antiseptic

3.2
fungicide
product that kills fungi (moulds and yeasts) and their spores under defined conditions

NOTE The adjective derived from "fungicide" is "fungicidal".

3.3
fungicidal activity
capability of a product to produce a reduction in the number of viable vegetative yeast cells and mould spores of relevant test organisms under defined conditions

3.4
fungistatic activity
capability of a product to inhibit the growth of fungi (moulds and/or yeasts) under defined conditions

3.5
yeasticide
product that kills yeasts under defined conditions

NOTE The adjective derived from "yeasticide" is "yeasticidal".

3.6
yeasticidal activity
capability of a product to produce a reduction in the number of viable yeast cells of relevant test organisms under defined conditions

4 Requirements

The product, shall demonstrate at least a 4 decimal log (lg) reduction when tested in accordance with **Clause 5.**

The fungicidal activity shall be evaluated using at least the following obligatory experimental test conditions: two test organisms (*Candida albicans* – vegetative cells and *Aspergillus niger* – spores), 20 °C, 15 min.

The yeasticidal activity shall be evaluated using at least the following obligatory experimental test conditions: one test organism (*Candida albicans* – vegetative cells), 20 °C, 15 min.

Where indicated, fungicidal or yeasticidal activity could be determined applying additional contact times, temperatures and test organisms in accordance with **5.2.1** and **5.5.1.1**.

NOTE 1 For these additional conditions, the concentration defined as a result can be lower than the one obtained under the obligatory test conditions.

NOTE 2 At the concentration defined as a result, it is not necessary to demonstrate a 4 lg reduction with the obligatory test conditions.

5 Test method

5.1 Principle

5.1.1 A sample of the product as delivered (highest test concentration = 80 %) and/or diluted with water is added to a test suspension of fungi (yeast cells or mould spores). The mixture is maintained at (20 ± 1) °C for 15 min ± 10 s (obligatory test conditions). At the end of this contact time, an aliquot is taken, and the fungicidal and/or the fungistatic activity in this portion is immediately neutralized or suppressed by a validated method. The method of choice is dilution-neutralization. If a suitable neutralizer cannot be found, membrane filtration is used. The numbers of surviving fungi in each sample are determined and the reduction is calculated.

5.1.2 The test is performed using the vegetative cells of *Candida albicans* and the spores of *Aspergillus niger* (fungicidal activity) or only the vegetative cells of *Candida albicans* (yeasticidal activity) as test organisms (obligatory test conditions).

5.1.3 Additional and optional contact times and temperatures are specified. Additional test organisms can be used.

5.2 Materials and reagents

5.2.1 Test organisms

The fungicidal activity shall be evaluated using the following strains as test organisms:[1)]

— *Candida albicans* ATCC 10231;

— *Aspergillus niger* ATCC 16404.

The yeasticidal activity shall be evaluated using only *Candida albicans*.

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

The required incubation temperature for these test organisms is (30 ± 1) °C (**5.3.2.3**).

If additional test organisms are used, they shall be incubated under optimum growth conditions (temperature, time, atmosphere, media) noted in the test report. If the additional test organisms selected do not correspond to the specified strains, their suitability for supplying the required inocula shall be verified. If these additional test organisms are not classified at a reference centre, their identification characteristics shall be stated. In addition, they shall be held by the testing laboratory or national culture collection under a reference for five years.

5.2.2 Culture media and reagents

5.2.2.1 General

All weights of chemical substances given in this European Standard refer to the anhydrous salts. Hydrated forms may be used as an alternative, but the weights required shall be adjusted to allow for consequent molecular weight differences.

The reagents shall be of analytical grade and/or appropriate for microbiological purposes. They shall be free from substances that are toxic or inhibitory to the test organisms.

NOTE 1 To improve reproducibility, it is recommended that commercially available dehydrated material is used for the preparation of culture media. The manufacturer's instructions relating to the preparation of these products should be rigorously followed.

NOTE 2 For each culture medium and reagent, a limitation for use should be fixed.

5.2.2.2 Water

The water shall be freshly glass-distilled water and not demineralized water.

Sterilize in the autoclave [**5.3.2.1** a)].

NOTE 1 Sterilization is not necessary if the water is used e.g. for preparation of culture media and subsequently sterilized.

NOTE 2 If distilled water of adequate quality is not available, water for injections (see bibliographic reference [1]) can be used.

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1) The ATCC numbers are the collection numbers of strains supplied by the American Type Culture Collection (ATCC). 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.

5.2.2.3 Malt Extract Agar (MEA)

Malt extract agar, consisting of:

Malt extract	30,0 g
Soya peptone, papaic digest of soybean meal	3,0 g
Agar	15,0 g
Water (**5.2.2.2**)	to 1 000,0 ml

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

NOTE In case of encountering problems with neutralization (**5.5.1.2** and **5.5.1.3**), it may be necessary to add neutralizer to the MEA. Annex B gives guidance on the neutralizers that may be used.

5.2.2.4 Diluent

Tryptone sodium chloride solution, consisting of:

Tryptone, pancreatic digest of casein	1,0 g
Sodium chloride (NaCl)	8,5 g
Water (**5.2.2.2**)	to 1 000,0 ml

Sterilize in the autoclave [**5.3.2.1** a)]. After sterilization, the pH of the diluent shall be equivalent to 7,0 ± 0,2 when measured at (20 ± 1) °C.

5.2.2.5 Neutralizer

The neutralizer shall be validated for the product being tested in accordance with **5.5.1.2**, **5.5.1.3** and **5.5.2**. It shall be sterile.

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

5.2.2.6 Rinsing liquid (for membrane filtration)

The rinsing liquid shall be validated for the product being tested in accordance with **5.5.1.2**, **5.5.1.3** and **5.5.3.** It shall be sterile, compatible with the filter membrane and capable of filtration through the filter membrane under the test conditions described in **5.5.3**.

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

5.3 Apparatus and glassware

5.3.1 General

Sterilize all glassware and parts of the 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) by moist heat, in the autoclave [**5.3.2.1** a)];

b) by dry heat, in the hot air oven [**5.3.2.1** b)].

5.3.2 Usual microbiological laboratory equipment [2] 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^{+3}_{0}) °C for a minimum holding time of 15 min;

b) for dry heat sterilization, a hot air oven capable of being maintained at (180^{+5}_{0})°C for a minimum holding time of 30 min, at (170^{+5}_{0}) °C for a minimum holding time of 1 h or at (160^{+5}_{0})°C for a minimum holding time of 2 h.

5.3.2.2 Water baths, capable of being controlled at (20 ± 1) °C, at (45 ± 1) °C (to maintain melted MEA in case of pour plate technique) and at additional test temperatures ± 1 °C (**5.5.1**).

5.3.2.3 Incubator, capable of being controlled at (30 ± 1) °C.

5.3.2.4 pH-meter, having an inaccuracy of calibration of no more than ± 0,1 pH units at (20 ± 1) °C.

NOTE A puncture electrode or a flat membrane electrode should be used for measuring the pH of the agar media (**5.2.2.3**).

5.3.2.5 Stopwatch

5.3.2.6 Shakers

a) Electromechanical agitator, e.g. Vortex® mixer[3]

b) Mechanical shaker

5.3.2.7 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 the membrane filtration method (**5.5.3**).

The vacuum source used shall give an even filtration flow rate. In order to obtain a uniform distribution of the micro-organisms over the membrane and to prevent overlong filtration, the device shall be set so as to obtain the filtration of 100 ml of rinsing liquid in 20 s to 40 s.

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2) Disposable sterile equipment is an acceptable alternative to reusable glassware.

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

5.3.2.8 **Refrigerator,** capable of being controlled at 2 °C to 8 °C.

5.3.2.9 **Graduated pipettes**, of nominal capacities 10 ml, 1 ml and 0,1 ml, or calibrated automatic pipettes.

5.3.2.10 **Petri dishes (plates)**, of size 90 mm to 100 mm.

5.3.2.11 **Glass beads**, 3 mm to 4 mm in diameter.

5.3.2.12 **Volumetric flasks**.

5.3.2.13 **Fritted filter,** with porosity of 40 µm to 100 µm according to ISO 4793.

5.3.2.14 **Centrifuge** (2 000 g_N).

5.3.2.15 **Roux bottles or similar flasks**.

5.4 Preparation of test organism suspensions and product test solutions

5.4.1 Test organism suspensions (test and validation suspension)

5.4.1.1 General

For each test organism, two different suspensions have to be prepared: the “test suspension” to perform the test and the “validation suspension” to perform the controls and method validation.

5.4.1.2 Preservation and stock cultures of test organisms

The test organisms and their stock cultures shall be prepared and kept in accordance with EN 12353.

5.4.1.3 Working culture of test organisms

5.4.1.3.1 Candida albicans (yeast)

In order to prepare the working culture of *Candida albicans* (**5.2.1**), prepare a subculture from the stock culture (**5.4.1.2**) by streaking onto MEA (**5.2.2.3**) slopes or plates (**5.3.2.10**) and incubate (**5.3.2.3**). 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. The second and (if produced) third subcultures are the working cultures.

If it is not possible to prepare the second subculture on a particular day, a 72 h subculture may be used for subsequent subculturing, provided that the subculture has been kept in the incubator (**5.3.2.3**) during the 72 h period.

Never produce and use a fourth subculture.

5.4.1.3.2 *Aspergillus niger* (mould)

For *Aspergillus niger* (**5.2.1**), use only the first subculture grown on MEA (**5.2.2.3**) in Roux bottles (**5.3.2.15**) and incubate for 9 d to 11 d. No further subculturing is needed.

5.4.1.3.3 Other test organisms (yeasts or moulds)

For additional test organisms, any departure from this method of culturing the yeast or the mould or of preparing the suspensions shall be noted, giving the reasons in the test report.

5.4.1.4 Test suspension ("N")

5.4.1.4.1 *Candida albicans*

The procedure for preparing the *Candida albicans* test suspension is as follows:

a) take 10 ml of diluent (**5.2.2.4**) and place in a 100 ml flask with 5 g of glass beads (**5.3.2.11**). Take the working culture (**5.4.1.3.1**) and transfer loopfuls of the cells into the diluent (**5.2.2.4**). The cells should be suspended in the diluent by rubbing the loop against the wet wall of the flask to dislodge the cells before immersing in the diluent. Shake the flask for 3 min using a mechanical shaker [**5.3.2.6**b)]. Aspirate the suspension from the glass beads and transfer to another tube;

b) adjust the number of cells in the suspension to 1,5 x 10^7 cfu/ml [4)] to 5,0 x 10^7 cfu/ml using diluent (**5.2.2.4**), estimating the number of cfu by any suitable means. Maintain this test suspension in the water bath at the test temperature θ [**5.5.1.1** a)] and use within 2 h;

NOTE The use of a spectrophotometer for adjusting the number of cells is highly recommended (approximately 620 nm wavelength — cuvette 10 mm path length). Each laboratory should therefore produce calibration data for each test organism knowing that suitable values of optical density are generally found between 0,200 and 0,350. A colorimeter is a suitable alternative.

c) for counting, prepare 10^{-5} and 10^{-6} dilutions of the test suspension using diluent (**5.2.2.4**). Mix [**5.3.2.6**a)].

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

1) When using the pour plate technique, transfer each 1,0 ml sample into separate Petri dishes and add 15 ml to 20 ml melted MEA (**5.2.2.3**), cooled to (45 ± 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 MEA (**5.2.2.3**).

For incubation and counting, see **5.4.1.6**.

5.4.1.4.2 *Aspergillus niger*

The procedure for preparing the *Aspergillus niger* test suspension is as follows:

a) take the working culture (**5.4.1.3.2**) and suspend the spores in 10 ml of sterile 0,05 % (w/v) polysorbate 80 solution in water (**5.2.2.2**). Using a 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 5 g of glass beads (**5.3.2.11**). Filter the suspension through a fritted filter (**5.3.2.13**);

b) carry out a microscopic examination under x 400 magnification immediately after the preparation and just before the test, to show the absence of mycelia fragments and spore germination (check at least ten fields of view for absence of both). If germinated spores are present, discard the suspension.

If mycelia are present, set up a washing process (centrifugation) as follows. Transfer the filtered suspension to centrifuge tubes. The filtered suspension is centrifuged (**5.3.2.14**) at 2 000 g_N for 20 min. The conidiospores are washed at least twice by resuspension in diluent (**5.2.2.4**) and subsequent centrifugation. If mycelia are still present, repeat the washing process;

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4) cfu/ml = colony-forming unit(s) per millilitre.