

SLOVENSKI STANDARD

SIST EN 13624:2022

01-januar-2022

Nadomešča:
SIST EN 13624:2013

Kemična razkužila in antiseptiki - Kvantitativni suspenzijski preskus za vrednotenje fungicidnega delovanja ali delovanja na kvasovke v humani medicini - Preskusna metoda in zahteve (faza 2, stopnja 1)

Chemical disinfectants and antiseptics - Quantitative suspension test for the evaluation of fungicidal or yeasticidal activity in the medical area - Test method and requirements (phase 2, step 1)

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Chemische Desinfektionsmittel und Antiseptika - Quantitativer Suspensionsversuch zur Bestimmung der fungiziden oder levuroziden Wirkung im humanmedizinischen Bereich - Prüfverfahren und Anforderungen (Phase 2, Stufe 1)

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Désinfectants chimiques et antiseptiques - Essai quantitatif de suspension pour l'évaluation de l'activité fongicide ou levuricide en médecine - Méthode d'essai et prescriptions (phase 2, étape 1)

Ta slovenski standard je istoveten z: EN 13624:2021

ICS:

11.080.20 Dezinfektanti in antiseptiki Disinfectants and antiseptics

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EUROPEAN STANDARD

EN 13624

NORME EUROPÉENNE

EUROPÄISCHE NORM

November 2021

ICS 11.080.20

Supersedes EN 13624:2013

English Version

Chemical disinfectants and antiseptics - Quantitative suspension test for the evaluation of fungicidal or yeasticidal activity in the medical area - Test method and requirements (phase 2, step 1)

Désinfectants chimiques et antiseptiques - Essai quantitatif de suspension pour l'évaluation de l'activité fongicide ou levuricide en médecine - Méthode d'essai et exigences (phase 2, étape 1)

Chemische Desinfektionsmittel und Antiseptika - Quantitativer Suspensionsversuch zur Bestimmung der fungiziden oder levuroziden Wirkung im humanmedizinischen Bereich - Prüfverfahren und Anforderungen (Phase 2, Stufe 1)

This European Standard was approved by CEN on 3 October 2021.

CEN members are bound to comply with the CEN/CENELEC Internal Regulations which stipulate the conditions for giving this European Standard the status of a national standard without any alteration. Up-to-date lists and bibliographical references concerning such national standards may be obtained on application to the CEN-CENELEC Management Centre or to any CEN member.

This European Standard exists in three official versions (English, French, German). A version in any other language made by translation under the responsibility of a CEN member into its own language and notified to the CEN-CENELEC Management Centre has the same status as the official versions.

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EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

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European foreword

This document (EN 13624:2021) 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 May 2022, and conflicting national standards shall be withdrawn at the latest by May 2022.

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

This document supersedes EN 13624:2013.

The document was revised to adapt it to the latest state of science, to correct errors and ambiguities, to harmonize the structure and wording with other tests of CEN/TC 216 existing or in preparation and to improve the readability of the standard and thereby make it more understandable. The following is a list of significant technical changes since the last edition:

- De-harmonization of the standard (Annex ZA and references to MDR/MDD deleted);
- Textile disinfection was added (see Table 1, 5.5.1.3);
- The amounts for the dirty conditions for the modified method for ready-to-use products were reduced (5.2.2.8.4 b));
- Clarification that a neutralization time of 10 s shall be used for all products with contact times of 10 min or shorter (5.5.2.2. c) and 5.5.2.5 b));
- Explanation for alternative controls for chemo-thermal disinfection (5.5.2.3);
- The reference to CIP numbers for the fungi strains were deleted and the UMIP numbers were added (Annex A);
- Harmonization of the text with EN 13727;
- Correction of editorial mistakes.

The changes of this revision have no impact on the test results obtained with reference to the version EN 13624:2013. Those results are still valid.

Any feedback and questions on this document should be directed to the users’ national standards body. A complete listing of these bodies can be found on the CEN website.

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

Introduction

This document specifies a suspension test for establishing whether a chemical disinfectant or an antiseptic has a fungicidal or yeasticidal activity in the area and fields described in the scope.

This laboratory test takes into account practical conditions of application of the product including contact time, temperature, test organisms and interfering substances, i.e. conditions which can influence its action in practical situations. Each utilization concentration of the chemical disinfectant or antiseptic found by this test corresponds to the chosen experimental conditions.

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1 Scope

This document specifies a test method and the minimum requirements for fungicidal or yeasticidal activity of chemical disinfectant and antiseptic products that form a homogeneous, physically stable preparation when diluted with hard water, or – in the case of ready-to-use products – with water. Products can only be tested at a concentration of 80 % or less (97 % with a modified method for special cases) as some dilution is always produced by adding the test organisms and interfering substance.

This document applies to products that are used in the medical area in the fields of hygienic handrub, hygienic handwash, surgical handrub, surgical handwash, instrument disinfection by immersion, and surface disinfection by wiping, spraying, flooding or other means.

This document applies to areas and situations where disinfection or antiseptics is medically indicated. Such indications occur in patient care, for example:

- in hospitals, in community medical facilities and in dental institutions;
- in clinics of schools, of kindergartens and of nursing homes;

and can occur in the workplace and in the home. It can also include services such as laundries and kitchens supplying products directly for the patients.

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

NOTE 2 This method corresponds to a phase 2 step 1 test.

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

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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.

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 4793:1980, *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.

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

- IEC Electropedia: available at <https://www.electropedia.org/>
- ISO Online browsing platform: available at <https://www.iso.org/obp>

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4 Requirements

The product shall demonstrate at least a 4 decimal logarithms (lg) reduction (for hygienic handwash at least a 2 lg reduction), when tested in accordance with Table 1 and Clause 5.

Table 1 — Minimum and additional test conditions

Test conditions	Hygienic handrub and handwash	Surgical handrub and handwash	Instrument disinfection	Surface disinfection	Textile disinfection ^d
Minimum spectrum of test organisms	<i>Candida albicans</i> (vegetative cells)	<i>Candida albicans</i> (vegetative cells)	a) fungicidal activity: <i>Aspergillus brasiliensis</i> (conidiospores) <i>Candida albicans</i> (veg. cells) b) yeasticidal activity: <i>Candida albicans</i> (veg. cells)	a) fungicidal activity: <i>Aspergillus brasiliensis</i> (conidiospores) <i>Candida albicans</i> (veg. cells) b) yeasticidal activity: <i>Candida albicans</i> (veg. cells)	a) fungicidal activity: <i>Aspergillus brasiliensis</i> (conidiospores) <i>Candida albicans</i> (veg. cells) b) yeasticidal activity: <i>Candida albicans</i> (veg. cells)
additional	Any relevant test organism				
Test temperature	at 20 °C	at 20 °C	generally between 20°C and 50°C for <i>Candida spec.</i> and between 20°C and 60°C for <i>Aspergillus spec.</i>	between 4 °C and 30 °C	generally between 20°C and 50°C for <i>Candida spec.</i> and between 20°C and 60°C for <i>Aspergillus spec.</i> ^e
Contact time	according to the manufacturer's recommendation,				
	but between 30 s and 60 s	1 min and 5 min	but no longer than 60 min	5 min or 60 min ^a	
Interfering substance	0,3 g/l bovine albumin solution (hygienic handrub) ^b	0,3 g/l bovine albumin solution (surgical handrub) ^b	0,3 g/l bovine albumin solution	0,3 g/l bovine albumin solution	
clean conditions			and/or	and/or	
dirty conditions	3,0 g/l bovine albumin solution plus 3,0 ml/l erythrocytes (hygienic handwash) ^c	3,0 g/l bovine albumin solution plus 3,0 ml/l erythrocytes (surgical handwash) ^c	3,0 g/l bovine albumin solution plus 3,0 ml/l erythrocytes	3,0 g/l bovine albumin solution plus 3,0 ml/l erythrocytes	3,0 g/l bovine albumin solution plus 3,0 ml/l erythrocytes
additional	—	—	any relevant substance	any relevant substance	—
NOTE For the additional conditions, the concentration defined as a result can be lower than the one obtained under the minimum test conditions.					

Test conditions	Hygienic handrub and handwash	Surgical handrub and handwash	Instrument disinfection	Surface disinfection	Textile disinfection ^d
<p>a The contact times for surface disinfectants stated in this table are chosen on the basis of the practical conditions of the product. The recommended contact time for the use of the product is within the responsibility of the manufacturer. Products intended to disinfect surfaces that are likely to come into contact with the patient and/or the medical staff and surfaces, which are frequently touched by different people, leading to the transmission of microorganisms to the patient, shall be tested with a contact time of maximum 5 min. The same applies where the contact time of the product shall be limited for practical reasons. Products for other surfaces than stated above may be tested with a contact time of maximum 60 min.</p> <p>b Hygienic and surgical handrub shall be tested as a minimum under clean conditions.</p> <p>c Hygienic and surgical handwash shall be tested as a minimum under dirty conditions.</p> <p>d For further information see EN 16616:2015, Clause 4.</p> <p>e For further information regarding validity at elevated temperatures see EN 14885.</p>					

5 Test method

5.1 Principle

5.1.1 A sample of the product as delivered and/or diluted with hard water (or water for ready to use products) is added to a test suspension of fungi (yeast cells or mould spores) in a solution of an interfering substance. The mixture is maintained at the temperature and the contact time specified in Clause 4 and 5.5.1.1. At the end of this contact time, an aliquot is taken; the fungicidal and/or the fungistatic action 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.

NOTE Handwash products are always prediluted with hard water (5.2.2.7). The resulting solution is regarded as a ready-to-use product (5.4.2).

5.1.2 The test is performed using the vegetative cells of *Candida albicans* and the conidiospores of *Aspergillus brasiliensis* (fungicidal activity) or only the vegetative cells of *Candida albicans* (yeast activity) as test-organisms (Clause 4, Table 1).

5.1.3 Additional contact times and temperatures are specified (Clause 4, Table 1). Additional interfering substances and test organisms may be used.

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5.2 Materials and reagents

5.2.1 Test organisms

The fungicidal activity shall be evaluated using the following strains as test organisms selected according to Clause 4 (Table 1)¹:

- *Candida albicans* ATCC 10231;
- *Aspergillus brasiliensis* (former “*A. niger*”) ATCC 16404.

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

Refer to Annex A for strain reference 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

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All weights of chemical substances given in this document 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.

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.

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

All specified pH values are measured at (20 ± 1) °C.

5.2.2.2 Water

The water shall be freshly glass-distilled water and not demineralized water. If distilled water of adequate quality is not available, water for injections (see bibliographic reference [2]) can be used.

Sterilize in the autoclave [5.3.2.1 a)]. Sterilization is not necessary if the water is used, e.g. for preparation of culture media and subsequently sterilized.

See 5.2.2.7 for the procedure to prepare hard water.

¹ The ATCC numbers are the collection numbers of strains supplied by these culture collections. This information is given for the convenience of users of this document 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 [food grade (e.g. Christomalt powder from Difal) or an equivalent extract that is not highly purified and not only based on maltose (e.g. Malt extract from OXOID)] ²	30,0 g
Agar	15,0 g
Water (5.2.2.2)	to 1 000,0 ml

Sterilize in the autoclave (5.3.1). After sterilization, the pH (5.3.2.4) of the medium shall be equivalent to $5,6 \pm 0,2$.

In case of encountering problems with neutralization (5.5.1.2 and 5.5.1.3), it may be necessary to add neutralizer to MEA. Annex B gives guidance on the neutralizers that may be used. It is recommended not to use neutralizer that causes opalescence in the agar.

If there are problems with producing at least 75 % spiny conidiospores, see 5.4.1.4.2.

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 (5.3.2.4) of the diluent shall be equivalent to $7,0 \pm 0,2$.

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.

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.

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

² This Malt extract from OXOID is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by CEN of this product.

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For the preparation of 1 l of hard water, the procedure is as follows:

- prepare solution A: dissolve 19,84 g magnesium chloride (MgCl_2) and 46,24 g calcium chloride (CaCl_2) in water (5.2.2.2) and dilute to 1 000 ml. Sterilize by membrane filtration (5.3.2.7) 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 (5.3.2.8) for no longer than one month;
- prepare solution B: dissolve 35,02 g sodium bicarbonate (NaHCO_3) in water (5.2.2.2) and dilute to 1 000 ml. Sterilize by membrane filtration (5.3.2.7). Store the solution in the refrigerator (5.3.2.8) for no longer than one week;
- place 600 ml to 700 ml of water (5.2.2.2) in a 1 000 ml volumetric flask (5.3.2.12) and add 6,0 ml (5.3.2.9) of solution A, then 8,0 ml of solution B. Mix and dilute to 1 000 ml with water (5.2.2.2). The pH (5.3.2.4) of the hard water shall be $7,0 \pm 0,2$. 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 product test solutions (5.4.2), the addition of the product to the hard water produces different final water hardness in each test tube. In any case, the final hardness expressed as calcium carbonate (CaCO_3) is in the test tube lower than 375 mg/l.

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5.2.2.8 Interfering substance**5.2.2.8.1 General**

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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 10 times its final concentration in the test (50 times in the case of the modified method, see 5.2.2.8.4).

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

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

5.2.2.8.2 Clean conditions (bovine albumin solution – low concentration)

Dissolve 0,30 g of bovine albumin fraction V (suitable for microbiological purposes) in 100 ml of diluent (5.2.2.4).

Sterilize by membrane filtration (5.3.2.7), keep in a refrigerator (5.3.2.8) and use within one month.

The final concentration of the bovine albumin in the test procedure (5.5) shall be 0,3 g/l.

5.2.2.8.3 Dirty conditions (mixture of bovine albumin solutions – high concentration with sheep erythrocytes)

Dissolve 3,00 g of bovine albumin fraction V (suitable for microbiological purposes) in 97 ml of diluent (5.2.2.4).

Sterilize by membrane filtration (5.3.2.7).

Prepare at least 8,0 ml fresh defibrinated sheep blood (5.2.2.9). Centrifuge the erythrocytes at 800 g_N for 10 min (5.3.2.13). After discarding the supernatant, resuspend erythrocytes in diluent (5.2.2.4). Repeat this procedure at least 3 times, until the supernatant is colourless.

Resuspend 3 ml of the packed sheep erythrocytes in the 97 ml of sterilized bovine albumin solution (see above). To avoid later contamination, this mixture should be split in portions probably needed per day and kept in separate containers for a maximum of 7 d in a refrigerator (5.3.2.8).

The final concentration of bovine albumin and sheep erythrocytes in the test procedure (5.5) shall be 3 g/l and 3 ml/l respectively.

5.2.2.8.4 Clean and dirty conditions for the modified method for ready-to-use products (5.5.4)

Follow the procedures for preparation according to 5.2.2.8.2 and 5.2.2.8.3, but prepare the interfering substance in fivefold higher concentrations, for the dirty conditions maximum 50 ml to avoid problems with the filtration.

- a) Clean conditions (5.2.2.8.2) – dissolve 1,50 g bovine albumin (instead of 0,3 g) in 100 ml of diluent;
- b) Dirty conditions (5.2.2.8.3) – dissolve 7,5 g bovine albumin (instead of 1,5 g) in 42,5 ml of diluent (instead of 48,5 ml). Prepare at least 20 ml (instead of 4,0 ml) sheep blood. Resuspend 7,5 ml (instead of 1,5 ml) of the packed sheep erythrocytes in 42,5 ml of sterilized bovine albumin solution to obtain 50 ml.

5.2.2.9 Defibrinated sheep blood

The defibrinated sheep blood should be sterile (aseptic blood-letting and preparation), pooled from more than one sheep and can be acquired from a commercial supplier.

5.3 Apparatus and glassware

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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 equipment³, and, in particular, the following:

5.3.2.1 Apparatus for moist and dry heat sterilization:

- a) for moist heat sterilization, an autoclave capable of being maintained at (121^{+3}) °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}) °C for a minimum holding time of 30 min, at (170^{+5}) °C for a minimum holding time of 1 h or at (160^{+5}) °C for a minimum holding time of 2 h.

³ Disposable sterile equipment is an acceptable alternative to reusable glassware.

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5.3.2.2 Water baths, capable of being controlled at $(20 \pm 1) ^\circ\text{C}$, at $(45 \pm 1) ^\circ\text{C}$ (to maintain melted MEA in case of pour plate technique) and at additional test temperatures $\pm 1 ^\circ\text{C}$ (5.5.1).

5.3.2.3 Incubator, capable of being controlled at $(30 \pm 1) ^\circ\text{C}$.

5.3.2.4 pH-meter, having an inaccuracy of calibration of no more than $\pm 0,1$ pH units at $(20 \pm 1) ^\circ\text{C}$. 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[®] mixer⁴;

b) Mechanical shaker.

5.3.2.7 Membrane filtration apparatus, constructed of a material compatible with the substances to be filtered, with a filter holder of at least 50 ml volume, and suitable for use of filters of diameter 47 mm to 50 mm and with a maximum pore size of $0,45 \mu\text{m}$ for sterilization of hard water (5.2.2.7), bovine albumin (5.2.2.8.2, 5.2.2.8.3 and 5.2.2.8.4), and if the membrane filtration method is used (5.5.3).

The vacuum source used shall give an even filtration flow rate. In order to obtain a uniform distribution of the microorganisms 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.

5.3.2.8 Refrigerator, capable of being controlled at $2 ^\circ\text{C}$ to $8 ^\circ\text{C}$.

5.3.2.9 Graduated pipettes, of nominal capacities 10 ml and 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 (diameter 3 mm to 4 mm).

5.3.2.12 Volumetric flasks.

5.3.2.13 Centrifuge ($800 g_N$).

5.3.2.14 Flasks with ventilated caps, Roux bottles or similar flasks.

5.3.2.15 Fritted filters: Porosity of $40 \mu\text{m}$ to $100 \mu\text{m}$ according to ISO 4793:1980, Clause 2.

⁴ Vortex[®] is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by CEN of this product.

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), subculture from the stock culture (5.4.1.2) by streaking onto MEA (5.2.2.3) slopes or plates 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 subculture 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 sub-culturing, 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 brasiliensis* (mould)

For *Aspergillus brasiliensis* (5.2.1) use only the first subculture grown on MEA (5.2.2.3) in Petri dishes (5.3.2.10) or flasks with ventilated caps (5.3.2.14) and incubate for 7 d to 9 d. No further subculturing is needed. Stacking the Petri dishes during the incubation could result in inhomogeneous temperature.

At the end of incubation, all the cultures have to show a dark brown or black surface with only a few small white or grey spots. Examples of appropriate and inappropriate cultures are given in Figures 1 and 2.

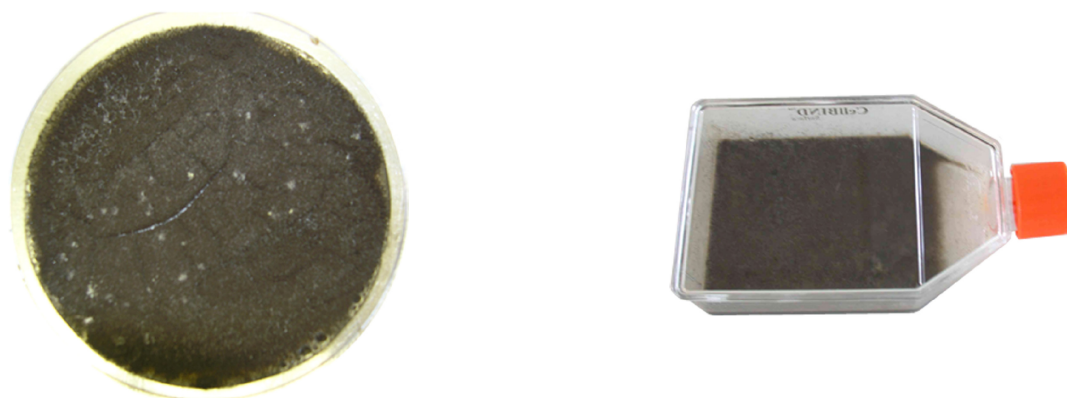


Figure 1 — Photos N°1: Examples of appropriate cultures of *A.brasiliensis* ATCC 16404 after 7 d of incubation at 30 °C