BS EN 13624:2013

BSI Standards Publication

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)

bsi.

...making excellence a habit.™

AVA Document Sharing Hub

Full document details: BS EN 13624:2013

National foreword

This British Standard is the UK implementation of EN 13624:2013. It supersedes BS EN 13624:2003 which is withdrawn.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.

This publication does not purport to include all the necessary provisions of a contract. Users are responsible for its correct application.

© The British Standards Institution 2013.
Published by BSI Standards Limited 2013

ISBN 978 0 580 69791 3

ICS 11.080.20

Compliance with a British Standard cannot confer immunity from legal obligations.

This British Standard was published under the authority of the Standards Policy and Strategy Committee on 31 October 2013.

Amendments issued since publication

Date	Text affected

Documents
Document Sharing Hub

Full document details: BS EN 13624:2013

EUROPEAN STANDARD

NORME EUROPÉENNE

EUROPÄISCHE NORM

EN 13624

September 2013

ICS 11.080.20

Supersedes EN 13624:2003

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 prescriptions (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 August 2013.

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.

CEN members are the national standards bodies of 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 United Kingdom.

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

Management Centre: Avenue Marnix 17, B-1000 Brussels

© 2013 CEN All rights of exploitation in any form and by any means reserved worldwide for CEN national Members.

Ref. No. EN 13624:2013: E

Documents
Document Sharing Hub

Full document details: BS EN 13624:2013

Contents

Page

Foreword 3
Introduction 4
1 Scope 5
2 Normative references 5
3 Terms and definitions 5
4 Requirements 5
5 Test method 7
5.1 Principle 7
5.2 Materials and reagents 7
5.3 Apparatus and glassware 10
5.4 Preparation of test organism suspensions and product test solutions 12
5.5 Procedure for assessing the fungicidal and yeasticidal activity of the product 17
5.6 Experimental data and calculation 24
5.7 Verification of methodology 30
5.8 Expression of results and precision 30
5.9 Interpretation of results – conclusion 31
5.10 Test report 32
Annex A (informative) **Referenced strains in national collections** 35
Annex B (informative) **Neutralizers and rinsing liquids** 36
Annex C (informative) **Graphical representation of test procedures** 38
C.1 Dilution-neutralization method 38
C.2 Membrane filtration method 40
C.3 Dilution-neutralization method (modified method for ready-to-use products) 42
C.4 Membrane filtration method (modified method for ready-to-use products) 44
Annex D (informative) **Example of a typical test report** 46
Annex E (informative) **Precision of the test result** 50
Annex ZA (informative) **Relationship between this European Standard and the Essential Requirements of EU Directive 93/42/EEC** 53
Bibliography 54

Documents
Document Sharing Hub
Full document details: BS EN 13624:2013

Foreword

This document (EN 13624:2013) 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 March 2014, and conflicting national standards shall be withdrawn at the latest by March 2014.

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 13624:2003.

The document was revised to adapt it to the latest state of science, to correct errors and ambiguities, to harmonise 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:

— The Scope was expanded for the following fields of application within the medical area, i.e. products for surgical and/or hygienic handrub and/or handwash and disinfectants for other surfaces than instrument surfaces.

— “Obligatory test conditions” were replaced by “minimum test conditions” (test temperatures and contact times can be chosen within limits) that have to be performed to pass the test.

— An additional modified method is described to test ready-to-use products in a higher concentration than 80 %, i.e. 97 %.

— The quality of the cultured conidiospores of *Aspergillus brasiliensis* is described in greater detail (media, limits and the control methods) resulting from work done in WG 3 of CEN/TC 216.

— The neutralization time was shortened to 10 s for products with contact times of 10 min or less.

— The Annex ZA was reformulated to more accurately describe the relationship with the Medical Device Directive.

Data obtained using the former version of EN 13624 may still be used, if the quality of the conidiospores of *Aspergillus brasiliensis* had been controlled and had met the requirements in this standard (5.4.1.4.2).

This document has been prepared under a mandate given to CEN by the European Commission and the European Free Trade Association, and supports essential requirements of EU Directive(s).

For relationship with EU Directive(s), see informative Annex ZA, which is an integral part of this document.

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, 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.

Documents
Document Sharing Hub
Full document details: BS EN 13624:2013

Introduction

This European Standard 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 may influence its action in practical situations. Each utilisation concentration of the chemical disinfectant or antiseptic found by this test corresponds to the chosen experimental conditions.

Documents
Document Sharing Hub
Full document details: BS EN 13624:2013

1 Scope

This European Standard 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 European Standard 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 European Standard applies to areas and situations where disinfection or antisepsis 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 may occur in the workplace and in the home. It may 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”.

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 4793:1980, *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.

4 Requirements

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

AVA Documents
Document Sharing Hub

Full document details: BS EN 13624:2013

Table 1 — Minimum and additional test conditions

Test conditions	Hygienic handrub and handwash	Surgical handrub and handwash	Instrument disinfection	Surface disinfection
Minimum spectrum of test organisms	*Candida albicans* (vegetative cells)	*Candida albicans* (vegetative cells)	a) **fungicidal activity**: *Aspergillus brasiliensis* (conidiospores) *Candida albicans* (veg. cells) b) **yeasticidal activity**: *Candida albicans* (veg. cells)	a) **fungicidal activity**: *Aspergillus brasiliensis* (conidiospores) *Candida albicans* (veg. cells) b) **yeasticidal activity**: *Candida albicans* (veg. cells)
additional	Any relevant test organism			
Test temperature	according to the manufacturer's recommendation, but at/ between			
	20 °C	20 °C	20 °C and 70 °C	4 °C and 30 °C
Contact time	according to the manufacturer's recommendation,			
	but between		but no longer than	
	30 s and 60 s	1 min and 5 min	60 min	5 min or 60 min[a]
Interfering substance clean conditions	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
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 and handwash)[c]	and/or 3,0 g/l bovine albumin solution plus 3,0 ml/l erythrocytes	and/or 3,0 g/l bovine albumin solution plus 3,0 ml/l erythrocytes
b) 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.

[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.

[b] Hygienic and surgical handrub shall be tested as a minimum under clean conditions.

[c] Hygienic and surgical handwash shall be tested as a minimum under dirty conditions.

Documents
Document Sharing Hub
Full document details: BS EN 13624:2013

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* (yeasticidal 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.

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*.

NOTE See 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.

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 European Standard and does not constitute an endorsement by CEN of the product named.

Documents
Document Sharing Hub
Full document details: BS EN 13624:2013

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.

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

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

See 5.2.2.7 for the procedure to prepare hard water.

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

Sterilise in the autoclave (5.3.1). After sterilisation, the pH (5.3.2.4) of the medium shall be equivalent to 5,6 ± 0,2.

In case of an 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:

---

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 European Standard and does not constitute an endorsement by CEN of this product.

Documents
Document Sharing Hub
Full document details: BS EN 13624:2013

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

Sterilise in the autoclave [5.3.2.1 a)]. After sterilisation, the pH (5.3.2.4) of the diluent shall be equivalent to 7,0 ± 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.

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.

5.2.2.7 Hard water for dilution of products

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. Sterilise 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. Sterilise 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 ± 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.

5.2.2.8 Interfering substance

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

Documents
Document Sharing Hub
Full document details: BS EN 13624:2013

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).

Sterilise 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).

Sterilise 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 sterilised 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.

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 15,0 g bovine albumin (instead of 3,0 g) in 85 ml of diluent (instead of 97 ml).

   Prepare at least 40 ml (instead of 8,0 ml) sheep blood. Resuspend 15 ml (instead of 3,0 ml) of the packed sheep erythrocytes in 85 ml of sterilised bovine albumin solution (see above).

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

5.3.1 General

Sterilise 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:

Documents
Document Sharing Hub
Full document details: BS EN 13624:2013

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[3)]

and, in particular, the following:

5.3.2.1 Apparatus for moist and dry heat sterilisation:

a) for moist heat sterilisation, an autoclave capable of being maintained at (121^{+3}_{0}) °C for a minimum holding time of 15 min;

b) for dry heat sterilisation, 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. 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[4)];

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 0,45 µm pore size for sterilisation 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 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.

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 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.

---

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

4) 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.

Documents
Document Sharing Hub
Full document details: BS EN 13624:2013

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 µm to 100 µm according to ISO 4793:1980, Clause 2.

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* (previously *A. niger*) (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.

Documents
Document Sharing Hub
Full document details: BS EN 13624:2013

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

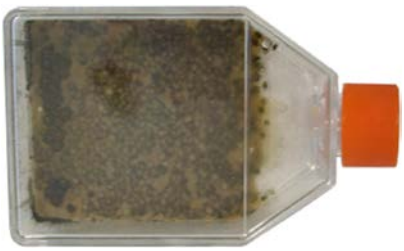

Figure 2 — Photo N°2: Example of inappropriate (not usable) culture of *A.brasiliensis* ATCC 16404 after 7 d of incubation at 30 °C

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*

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 a tube.

b) Adjust the number of cells in the suspension to 1,5 x 10^7 cfu/ml[5)] to 5,0 x 10^7 cfu/ml using diluent (5.2.2.4) (1,5 x 10^8 cfu/ml to 5,0 x 10^8 cfu/ml in the case of the modified method, 5.5.4), estimating the number of cfu by any suitable means. Maintain this test suspension in the water bath at 20 °C and use within 2 h. Adjust the temperature according to 5.5.1.1 a) and 5.5.1.4 only immediately before the start of the test.

   The use of a spectrophotometer for adjusting the number of cells is highly recommended (about 620 nm wavelength – cuvette 10 mm path length). Each laboratory should therefore produce calibration data knowing that suitable values of optical density are generally found between 0,200 and 0,350. To achieve reproducible results of this measurement it may be necessary to dilute the test suspension, e.g. 1+9.

---

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

Documents
Document Sharing Hub
Full document details: BS EN 13624:2013

NOTE A colorimeter is a suitable alternative.

c) For counting, prepare 10^{-5} and 10^{-6} dilutions (10^{-6} and 10^{-7} dilutions in the case of the modified method – 5.5.4) of the test suspension using diluent (5.2.2.4). Mix [(5.3.2.6a)].

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).

The technique used for counting of the test suspension has to be used for all other countings, 5.4.1.5 d), 5.5.2.2 c) and d), 5.5.2.3 b), 5.5.2.4 b) and 5.5.2.5 b).

For incubation and counting, see 5.4.1.6.

5.4.1.4.2 *Aspergillus brasiliensis*

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

a) Take the working culture (5.4.1.3.2) and suspend the conidiospores 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 (5.3.2.15);

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

1) the presence of a high concentration of characteristic mature conidiospores, i.e. spiny conidiospores (versus smooth spores). See Figure 3 and Figure 4.

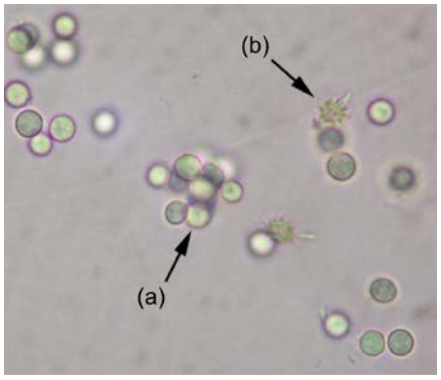

Figure 3 — Photo N°3 Observation of conidiospores under light microscope: Presence of smooth (a) and spiny (b) spores [inappropriate (not usable) suspension]

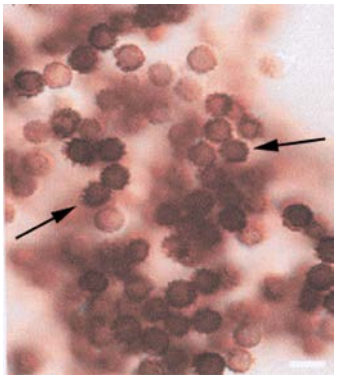

Figure 4 — Photo N°4 Observation of conidiospores under light microscope: High concentration of characteristic mature spores with spiny aspect (appropriate suspension)

If there are less than 75 % spiny conidiospores it may be due to the *Aspergillus* strain used or to the media. Therefore use another strain, preferably from another supplier, and/or try out another media;

Documents
Document Sharing Hub
Full document details: BS EN 13624:2013

2) the absence of conidiospore germination (check at least ten fields of view);

3) if germinated conidiospores are present, discard the suspension;

4) the absence of mycelia fragments (check at least ten fields of view).

If mycelia are present, proceed to a second fritted filtration. If mycelia are still present, discard the suspension.

d) Adjust the number of conidiospores in the suspension to $1{,}5 \times 10^7$ cfu/ml to $5{,}0 \times 10^7$ cfu/ml ($1{,}5 \times 10^8$ cfu/ml to $5{,}0 \times 10^8$ cfu/ml in the case of the modified method – 5.5.4) using the diluent (5.2.2.4), estimating the number of cfu by any suitable means. Use the suspension within 4 h in a water bath controlled at (20 ± 1) °C (5.3.2.2). In any case, adjust the temperature according to 5.5.1.4 only immediately before the start of the test (5.5.2, 5.5.3 or 5.5.4).

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.

Each laboratory should therefore produce calibration data to establish the relationship between the counts obtained using the counting device and the counts (5.4.1.6) obtained by the pour plate or the spread plate technique [d)]. Experienced laboratories found a better fit to the required number of conidiospores when the conidiospore suspension count in the device was 10 % to 50 % higher than the number aimed at.

e) For counting, prepare 10^{-5} and 10^{-6} dilutions (10^{-6} and 10^{-7} dilutions in the case of the modified method 5.5.4) 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 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 (5.2.2.3), cooled to (45 ± 1) °C;

2) 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 (5.2.2.3) (i.e. in duplicate – at least eight plates).

The technique used for counting of the test suspension has to be used for all other countings, 5.4.1.5 d), 5.5.2.2 c) and d), 5.5.2.3 b), 5.5.2.4 b) and 5.5.2.5 b).

For incubation and counting, see 5.4.1.6.

5.4.1.5 Validation suspension (N_V and N_{VB})

a) To prepare the validation suspension, dilute the test suspension N (see 5.4.1.4.1 and 5.4.1.4.2) with the diluent (5.2.2.4) to obtain $3{,}0 \times 10^2$ cfu/ml to $1{,}6 \times 10^3$ cfu/ml (about one fourth (1 + 3) of the 10^{-4} dilution).

NOTE In the case of the modified method (5.5.4) the procedure is the same, but one fourth (1+3) of the 10^{-4} dilution resulting in $3{,}0 \times 10^3$ cfu/ml to $1{,}6 \times 10^4$ cfu/ml.

b) For the neutralizer control N_{VB} (5.5.2.4) dilute the test suspension (5.4.1.4) with the diluent to obtain $3{,}0 \times 10^4$ cfu/ml to $1{,}6 \times 10^5$ cfu/ml [about one fourth (1+3) of the 10^{-2} dilution].

c) Maintain and use these validation suspensions (N_V and N_{VB}) the same way as the test suspension [5.4.1.4.1 b) and 5.4.1.4.2 c)].

d) For counting prepare with diluent (5.2.2.4) a 10^{-1} dilution, in the case of the modified method (5.5.4) a

Documents
Document Sharing Hub
Full document details: BS EN 13624:2013

e) 10^{-2} dilution and in the case of the neutralizer control N_{VB} [see b)] a 10^{-3} dilution. Mix [5.3.2.6 a)]. Take a sample of 1,0 ml in duplicate and inoculate using the pour plate or the spread plate technique [with *Candida albicans*, see 5.4.1.4.1 c); with *Aspergillus brasiliensis*, see 5.4.1.4.2 d)].

For incubation and counting, see 5.4.1.6.

5.4.1.6 Incubation and counting of the test and the validation suspensions

a) Incubate (5.3.2.3) the plates for 42 h to 48 h. Discard any plates which are not countable (for any reason). Count the plates and determine the number of cfu. Only for *Aspergillus brasiliensis*: incubate the plates for a further 20 h to 24 h and – if the number of colonies has increased – for a third additional period of 20 h to 24 h. Do not recount plates which no longer show well separated colonies. Recount the remaining plates. If the number has increased, use only the higher number for further evaluation.

b) Note for each plate the exact number of colonies but record > 165 (for moulds) or > 330 (for yeasts) for any counts higher than 165 and 330 respectively and determine the V_C-values according to 5.6.2.2.

c) Calculate the numbers of cfu/ml in the test suspension N and in the validation suspensions N_V and N_{VB} (neutralizer control 5.5.2.4) using the method given in 5.6.2.3 and 5.6.2.5. Verify according to 5.7.

5.4.2 Product test solutions

The concentration of a product test solution shall be 1,25 times the desired test concentration because it is diluted to 80 % during the test and the method validation (5.5.2 or 5.5.3).

Product test solutions shall be prepared in hard water (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 (5.8.2). The product as received may be used as one of the product test solutions; in this case the highest tested concentration is 80 %. Ready to use products may be tested at 97 % [modified method (5.5.4.)] In this case, the “real test concentration” is 97 %.

Dilutions of ready-to-use products shall be prepared in water (5.2.2.2) instead of hard water. Handwash products are always prediluted with hard water (5.2.2.7) to achieve a 62,5 % solution. The solution simulates the addition of tap water in practice (1:1). Such a product is nevertheless regarded as a “ready-to-use product”. The modified method (5.5.4) cannot be used since 62,5 % represents the highest accepted concentration (50 %), multiplied by 1,25.

For solid products, dissolve the product as received by weighing at least 1,0 g ± 10 mg of the product in a volumetric flask and filling up with hard water (5.2.2.7). Subsequent dilutions (= lower concentrations) shall be prepared in volumetric flasks (5.3.2.12) on a volume/volume basis in hard water (5.2.2.7).

For liquid products, dilutions of the product shall be prepared with hard water in volumetric flasks (5.3.2.12) on a volume/volume basis.

The product test solutions shall be prepared freshly and used in the test within 2 h. They shall give a physically homogenous preparation, stable during the whole procedure. If during the procedure a visible inhomogeneity appears due to the formation of a precipitate or flocculate (for example, through the addition of the interfering substance), it shall be recorded in the test report.

NOTE Counting micro-organisms embedded in a precipitate or flocculate is difficult and unreliable.

The concentration of the product stated in the test report shall be the desired test concentration. Record the test concentration in terms of mass per volume or volume per volume and details of the product sample as received.

Documents
Document Sharing Hub
Full document details: BS EN 13624:2013