BS EN 1657:2024

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

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National foreword

This British Standard is the UK implementation of EN 1657:2024. It supersedes BS EN 1657:2016, which is withdrawn.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 committee manager.

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Published by BSI Standards Limited 2024

ISBN 978 0 539 23188 5

ICS 11.080.20; 71.100.35

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This British Standard was published under the authority of the Standards Policy and Strategy Committee on 31 December 2024.

Amendments/corrigenda issued since publication

Date	Text affected

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EUROPEAN STANDARD

NORME EUROPÉENNE

EUROPÄISCHE NORM

EN 1657

December 2024

ICS 71.100.35

Supersedes EN 1657:2016

English Version

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

Antiseptiques et désinfectants chimiques - Essai quantitatif de suspension pour l'évaluation de l'activité fongicide ou levuricide des antiseptiques et des désinfectants chimiques utilisés dans le domaine vétérinaire - Méthode d'essai et exigences (phase 2, étape 1)

Chemische Desinfektionsmittel und Antiseptika - Quantitativer Suspensionsversuch zur Bestimmung der fungiziden oder levuroziden Wirkung chemischer Desinfektionsmittel und Antiseptika für den Veterinärbereich - Prüfverfahren und Anforderungen (Phase 2, Stufe 1)

This European Standard was approved by CEN on 18 November 2024.

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Ref. No. EN 1657:2024 E

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European foreword

This document (EN 1657:2024) 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 2025, and conflicting national standards shall be withdrawn at the latest by June 2025.

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 1657:2016.

This document was revised to correct obvious 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 obligatory conditions have been removed and the preparation of the milk interfering substance has been amended.

The data obtained using the 2016 version of EN 1657 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.

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Introduction

This document specifies a suspension test method for establishing whether a chemical disinfectant or antiseptic has a fungicidal or yeasticidal activity in the areas 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.

The conditions are intended to cover general purposes and to allow reference between laboratories and product types. Each utilization concentration of the chemical disinfectant or antiseptic found by this test corresponds to defined experimental conditions. However, for some applications the recommendations of use of a product may differ and therefore additional test conditions need to be used.

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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, as some dilution is always produced by adding the test organisms and interfering substance.

This document applies to products that are used in the veterinary area – i.e. in the breeding, husbandry, production, veterinary care facilities, transport and disposal of all animals except when in the food chain following death and entry into processing industry. This document also applies to products used for teat disinfection.

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 under the conditions in which they are used.

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

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, definitions and abbreviated terms

3.1 Terms and definitions

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

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

— ISO Online Browsing Platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

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3.2 Symbols and abbreviated terms

For the purposes of this document, the following symbols and abbreviated terms apply.

c	is the sum of V_C values taken into account
cfu	Colony forming units
n	is the number of V_C values taken into account
N	Test Suspension
Nv	Validation suspension
Na	Test
A	Control A (Experimental conditions control)
B	Control B (Neutralization control)
C	Control C (Dilution neutralization validation)
N_0	Test at time 0
Nv_0	Validation suspension at time 0
R	Reduction
Vc	is the number of cfu counted per sample of 1,0ml

4 Requirements

The product shall demonstrate at least a 4 decimal log (lg) reduction when diluted with hard water (5.2.2.7) or – in the case of ready-to-use products – with water (5.2.2.2) and tested in accordance with Table 1 and Clause 5 under simulated low-level soiling (3,0 g/l bovine albumin) or high level soiling (10,0 g/l yeast extract and 10,0 g/l bovine albumin) or 10,0 g/l milk for teat disinfectants or 3,0 g/l bovine albumin for pre-milking teat disinfectants in additional test conditions.

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Table 1 —Test conditions

Test conditions	Fungicidal activity	Yeasticidal activity	Yeasticidal activity for teat disinfectants
Test organisms	*Aspergillus brasiliensis* *Candida albicans*	*Candida albicans*	*Candida albicans*
additional	any relevant test organism	any relevant test organism	any relevant test organism
Test temperature	At intervals of 5°C		
Minimum	5°C ± 1°C	5°C ± 1°C	20°C ± 1°C
Maximum	40°C ± 1°C	40°C ± 1°C	30°C ± 1°C
Contact time Minimum Maximum	At intervals of 30 s from 30 s to 5 min and at intervals of 5 min from 5 min to 120 min		
	1 min ± 5 s	1 min ± 5 s	1 min ± 5 s for post-milking teat disinfectants 30 s ± 5 s for pre-milking teat disinfectants
	120 min ± 10 s	120 min ± 10 s	5 min ± 10 s for post-milking teat disinfectants 3 min ± 10 s for pre-milking teat disinfectants
Interfering substance			
low level soiling high level soiling	3,0 g/l bovine albumin 10 g/l yeast extract plus 10 g/l bovine albumin	3,0 g/l bovine albumin 10 g/l yeast extract plus 10 g/l bovine albumin	Post milking: 10,0 g/l of reconstituted milk Pre-milking: 3,0 g/l bovine albumin
additional	any relevant substance	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 standard test conditions. The recommended test conditions for the use of the product are within the responsibility of the manufacturer.			

Any additional specific fungicidal or yeasticidal activity shall be determined in accordance with 5.2.1 and 5.5.1.1 in order to take into account intended specific use conditions.

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 temperature θ for the test contact time t in accordance with Table 1. At the end of this contact time, an aliquot is taken, and the fungicidal/yeasticidal and/or the fungistatic/yeaststatic activity in this portion is immediately neutralized or suppressed by a validated

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method. The method of choice is dilution-neutralization. If a suitable neutralizer cannot be found, membrane filtration is used. The number of surviving fungi in each sample are determined and the reduction is calculated.

5.2 Materials and reagents

5.2.1 Test organisms

The fungicidal activity shall be evaluated using the following strains as test organisms:

— *Candida albicans*;

— *Aspergillus brasiliensis*.

(formerly *Aspergillus niger*)

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

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

The required incubation temperature for these test organisms is 30 °C± 1 °C (see 5.3.2.3). The same temperature shall be used for all incubations performed during a test and its control and validation.

If additional test organisms are used, they shall be kept and used under optimum growth conditions (temperature, time, atmosphere, media) and 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 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.

If additional strains do not grow on the media (5.2.2.3) or cannot be used with diluent (5.2.2.4) alternative media shall be used and shall be reported as well as alternative incubation conditions.

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

Ready to use media may be used if it complies with the required specification.

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

All specified pH values are measured at 20 °C± 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 [1]) can be used.

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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.

NOTE 1 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 [a]	30,0 g
Agar	15,0 g
Water (5.2.2.2)	to 1 000,0 ml

[a] The malt extract should be of food grade (Cristomalt poudre from Difal is recommended) or equivalent that is not highly purified and not only based on maltose (Malt extract from OXOID is recommended)[1].

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 °C ± 1 °C.

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 °C ± 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.

5.2.2.7 Hard water for dilution of products

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

[1] 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.

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— 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 of the hard water shall be 7,0 ± 0,2, when measured at 20 °C ± 1 °C (5.3.2.4). 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 a different final water hardness in each test tube. In any case the final hardness is lower than 375 mg/l of calcium carbonate ($CaCO_3$) in the test tube.

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 10 times its final concentration in the test.

For any additional interfering substance 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 Low-level and pre-milking soiling (bovine albumin solution)

Dissolve 3,0 g of bovine albumin fraction V (suitable for microbiological purposes) in 90 ml of water (5.2.2.2) in a 100ml volumetric flask (5.3.2.12). Make up to the mark with water.

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

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

5.2.2.8.3 High-level soiling (mixture of bovine albumin solution with yeast extract)

Dissolve 50,0 g yeast extract powder in 150 ml of water (5.2.2.2) in a 250 ml volumetric flask (5.3.2.12) and allow foam to collapse. Make up to the mark with water (5.2.2.2). Transfer to a clean dry bottle and sterilize in an autoclave [5.3.2.1 a)]. Allow to cool to 20 °C ± 1 °C.

Pipette 25 ml of this solution into a 50 ml volumetric flask (5.3.2.12) and add 10 ml of water (5.2.2.2). Dissolve 5,0 g of bovine albumin fraction V (suitable for microbiological purposes) in the solution with

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shaking and allow foam to collapse. Make up to the mark with water (5.2.2.2), sterilize by membrane filtration (5.3.2.7), keep in the refrigerator (5.3.2.8) and use within one month.

The final concentration in the test procedure (5.5) is 10,0 g/l yeast extract and 10,0 g/l bovine albumin.

5.2.2.8.4 Milk for teat disinfectants

Skimmed milk, guaranteed free of antibiotics and additives and reconstituted at a rate of 100 g powder per litre of water (5.2.2.2), shall be prepared as follows:

— prepare a solution of 10,0 % (v/v) in water (5.2.2.2) by adding 10 parts of reconstituted milk to 90 parts of water. Heat for 30 min at (105 ± 3) °C [or 5 min at (121 ± 3) °C].

The final concentration of reconstituted milk in the test procedure (5.5) is 1,0 % (v/v) of reconstituted milk.

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 the specified temperatures and at 45 °C ± 1 °C (to maintain melted MEA in case of pour plate technique).

5.3.2.3 Incubator, capable of being controlled at the specified temperatures ± 1 °C.

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

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

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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 sterilization of hard water (5.2.2.7) and bovine albumin (5.2.2.8), 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 °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 Flasks with ventilated caps.

5.3.2.15 Microscope capable of x 400 magnification

5.3.2.16 Containers; test tubes, culture bottles or flasks of suitable capacity.

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 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 working stock cultures of test organisms

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

[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.

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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 brasiliensis (mould)

For *Aspergillus brasiliensis* (5.2.1), use only the first subculture grown on MEA (5.2.2.3) in Petri dishes or flasks with ventilated caps (5.3.2.14) and incubate for 7 days to 9 days at 30 °C ± 1°C. 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 have to show a dark brown or black surface. Cultures with rare and small white or grey areas may be used (see Figure 1).

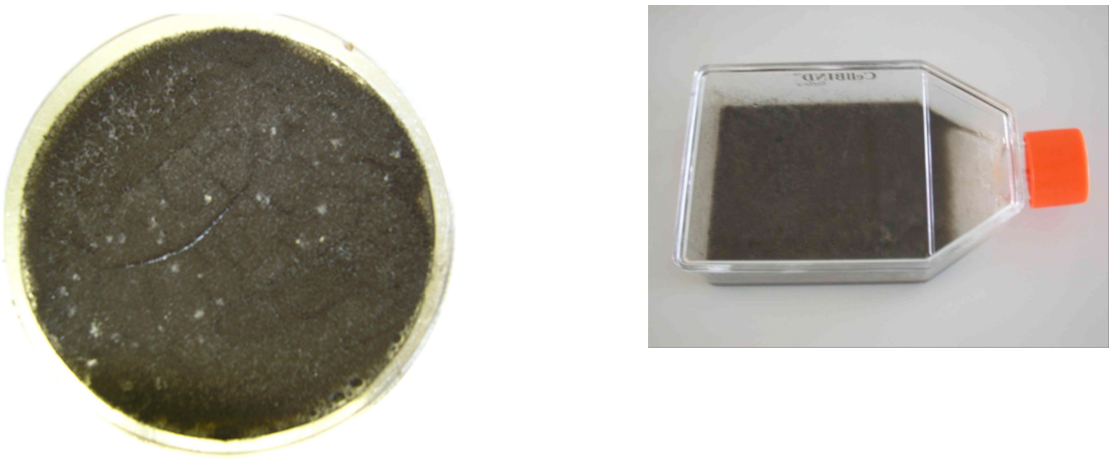

Figure 1 — Photo No 1: *A. brasiliensis* ATCC 16404 after 7 d of incubation at 30°C

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