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BSI Standards Publication

Aerospace series — Paints and varnishes — Determination of resistance to microbial growth

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National foreword

This British Standard is the UK implementation of EN 4159:2011.

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

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Luft- und Raumfahrt - Beschichtungsstoffe - Bestimmung der Widerstandsfähigkeit gegen Schimmelwachstum

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EUROPEAN COMMITTEE FOR STANDARDIZATION
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Foreword

This document (EN 4159:2011) has been prepared by the Aerospace and Defence Industries Association of Europe - Standardization (ASD-STAN).

After enquiries and votes carried out in accordance with the rules of this Association, this Standard has received the approval of the National Associations and the Official Services of the member countries of ASD, prior to its presentation to CEN.

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of an identical text or by endorsement, at the latest by June 2012, and conflicting national standards shall be withdrawn at the latest by June 2012.

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Introduction

Certain fungi are known to be capable of proliferating in fuel systems which can cause corrosion and blockage. Conidiospores are the dispersal form of these fungi. Germination of conidia is the first stage in proliferation of the fungus. If the conidiospore cannot germinate, there can be no proliferation and no blockage of fuel lines, ducts etc.

This method should be performed only by persons qualified in the microbiology of fungi.

The standard can be used to assess the effectiveness of new candidate coating systems in inhibiting microbial (fungal) growth.

1 Scope

This European Standard specifies a method to assess the ability of biocide-containing coatings to prevent the germination of conidiospores of certain fungi known to be capable of proliferating in fuel systems for aerospace applications.

2 Terms and definitions

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

2.1
Conidiospores
single-celled structures produced by the mycelial [mould] form of the fungus

NOTE Conidiospores are spherical or nearly spherical resting cells, i.e. cells which may be dispersed readily but which do not proliferate. However, conidiospores may germinate if they encounter suitable conditions of moisture and nutrients. On germination, a conidiospore produces a long tube-like outgrowth which then forms dense branching structures [mycelia] which may block fuel ducts etc. A suitable coating will prevent germination of conidiospores. A coating which prevents germination of conidiospores is considered to have fungistatic activity. This fungistatic activity may be assessed quantitatively by assessing the success rate of germination of conidiospores under standard conditions (see below) to determine whether the test coating delays or prevents germination of conidiospores when compared with a coating which is known to possess no fungistatic activity. Laboratories which undertake work to this method should first obtain the test fungi (see 5) and perform control experiments to satisfy themselves that they can follow the process of germination of conidiospores. These initial experiments may be performed by placing the agarose gel (see below) on the surface of sterile plastic petri dishes rather than on the surface of coated test panels, as is done in the present method.

3 Principle

3.1 Conidiospores are placed on a gel within a few millimetres of the panel/coating under test. Under the test conditions a high proportion of these conidia germinate [begin growth] rapidly unless some material in the coating diffuses through the gel and prevents germination.

3.2 The success rate of germination, after any given interval of exposure to the coating, is expressed as the number of cells that have germinated divided by the number of cells examined (germinated + nongerminated). The success rate of germination is determined from time to time, beginning when the conidiospores are first exposed to the coating under test. Examination is made using a microscope [100×]. This allows ready distinction between ungerminated conidiospores [approximately spherical] and the long filamentous outgrowth that is the result of germination.

3.3 The results obtained with conidiospores exposed to test coatings are to be compared with results of conidiospores exposed to coatings that contain no inhibitor.

4 Apparatus

4.1 Incubator, capable of maintaining (25 ± 1) °C.

4.2 Autoclave suitable for sterilization of microbiological growth media, i.e. capable of heating the media to 121 °C for 15 min.

4.3 Water bath, set to (45 ± 1) °C.

4.4 Microscope, magnification 100×, and glass microscope slides.

4.5 Plastic disposable petri dishes -- 90 mm to 100 mm diameter.

4.6 Sterile microbiological loops. Commercially available disposable plastic loops (stated to carry 10 µl) are suitable.

4.7 Haemocytometer (blood cell counting chamber) Neubauer ruling.

4.8 Funnel, loosely plugged with nonabsorbent cotton wool.

4.9 Balance, toploading, 0,1 g resolution.

4.10 Micropipetting device (with disposable sterile tips) to deliver 0,010 ml.

4.11 Test fungi, to be obtained from national culture collections:

4.11.1 Amorphotheca resinae (also known as Cladosporium resinae)

4.11.2 Aspergillus niger

4.11.3 National culture collections contain several different strains of each of these fungi. The laboratory should choose a suitable strain (e.g. one isolated from aeronautical fuel tank) by reference to the information supplied by the culture collection.

4.12 Microbiological growth media – Rose Bengal Chloramphenicol Agar. The Oxoid product Rose Bengal Chloramphenicol Agar is suitable.

4.13 Agarose – The product [catalogue No. A0567] of the Sigma Chemical Company is suitable.

4.14 D-glucose – The Sigma product [catalogue No. G7528] is suitable.

5 Specimen

5.1 Test panels are panels coated on one face only with a paint suspected of having fungistatic activity. Control panels are similar panels coated on one face only with a similar paint which lacks any fungistatic additive. These coatings must be applied according to the manufacturer's instructions. The panel edges may be left uncoated. Panels that are 10 mm × 20 mm (length × width) are suitable. Panels should be less than 10 mm thick. Panels are stored at room temperature.

5.2 Three test panels and three control panels are required.

6 Procedure

6.1 Test methods

6.1.1 Preparation of agarose + glucose gel

To 100 ml distilled water in a 250 ml conical flask, add 2 g agarose and 1 g D-glucose. Heat to boiling and swirl thoroughly to obtain a clear solution. Cool the solution in a 45 °C water bath. Pour (20 ± 1) g of the cooled solution into a petri dish and allow the solution to set at room temperature to form a clear colourless gel.

6.1.2 Application of gel to coating

Ensure that the test specimens are at room temperature. Cut the gel formed in 6.1.1 so as to fit, approximately, the specimens under test. Place the cut gel on the coated surface of the specimen and trim any excess. Place the gel/specimen in a petri dish and store it at 25 °C for 22 h to 24 h.

6.1.3 Preparation of spore suspension

Ten days prior to test, make up Rose Bengal Chloramphenicol Agar according to the manufacturer's directions. Pour (15 ± 1) ml of the sterilized and equilibrated [45 °C] medium into each of an appropriate number of petri dishes (90 mm diameter) and allow the agar to form a gel. Seed the prepared medium with the fungus under test. Incubate this culture (see 5.1) for 10 days.

Harvest conidiospores from the culture by passing a sterile microbiological loop gently over the surface of the fungus and then swirling the loop in a few millilitres of sterile water. Shake the suspension thoroughly to disaggregate conidiospores.

Determine the number of conidiospores [per ml of suspension] using a blood cell counting chamber [haemocytometer] according to the manufacturer's directions. A suitable suspension will be made up almost entirely [> 95 %] of conidiospores, the remainder being hyphal fragments readily distinguishible from conidiospores. Remove hyphal fragments by passing the suspension through a funnel loosely plugged with sterile cotton wool. Adjust the suspension to 10^6 conidiospores per ml by either adding water or centrifuging the suspension and resuspending the conidiospores in a suitable volume of sterile water. Use this suspension within two hours of the time it was formed.

6.1.4 Application of suspension to gel and storage at 25 °C

After the specimens have been stored for the required time (see 6.1.2), apply 0,010 ml of suspension (see 6.1.3) to the gel surface using a micropipettor. Then use a sterile microbiological loop to spread the suspension uniformly on the gel surface. The surface should appear to be dry within a few minutes.

Use a razor blade to cut a piece [approximately 5 mm × 5 mm] of gel for microscopic examination [for time = zero]. Mount this on a microscope slide. Place the remainder of the gel + coating + panel in its petri dish and store at 25 °C. The required relative humidity during storage at 25 °C is attained by placing the specimens under test in petri dishes held in loose fitting plastic bags. Each such bag should also contain a petri dish which contains sterile filter paper saturated with sterile distilled water.

6.1.5 Microscopic examination

Examine the slide prepared in 6.1.4 at a magnification of 100×. Count all cells [whether they are conidia or hyphal fragments] in several microscopic fields so that > 100 cells are examined and counted. At this point all conidia should be [approximately] spherical, with no evidence of germination [i.e. no evidence of hyphal outgrowth from the conidiospore]. Discard the sample after examination.

6.1.6 Time course of germination

Follow the time course of germination in the preparations placed in the incubator [6.1.4] by taking a sample from time to time for microscopic examination as described in 6.1.4 and 6.1.5. Take samples at the beginning ($T = 0$ when the conidiospores are spread on the agarose surface) and at 24 h and 48 h thereafter. Extensive germination (success rate > 50 %) should be evident at 24 hours in the conidiospores exposed to the control panel. If this is not observed, then the experiment must be repeated using a conidiospore preparation which gives the expected rapid and abundant germination. If germination is seen to be prevented by the coatings under test during this first 48 hours, then further samples may be taken to ask whether germination may be observed during this extended period. These subsequent samplings may be done at intervals of 4 d to 7 d. See Table 1 as an example. The test intervals and the overall duration of exposure of the fungi to the test panels shall be agreed with the organization which requested the test work.

Discard each sample after examination. Some typical results follow [Table 1]. The number of germinated conidiospores [of Amorphotheca resinae] seen among the stated total number of conidiospores examined is shown for a coating known to be ineffective [Coating A], a coating known to be effective [Coating B], a new coating under development [Coating C]. The trials ended at day 19.

Table 1 — Germinated conidia/total conidia examined

Day	Coating A	Coating B	Coating C
0	0/208	0/208	0/208
1	67/103	0/208	0/211
2	Not tested	0/210	0/217
6	–	0/215	0/210
13	–	23/217 [a]	0/250
19	–	12/216	0/225

[a] Some germination was seen at days 13 and 19 among conidiospores exposed to Coating B. Further growth of the germinated conidia did not occur. Germination was not observed in conidiospores exposed to Coating C.

Coating A did not prevent or delay germination, as seen by the day 1 result. Extensive fungal growth followed, such that microscopic examination was not practicable at day 2 or later. The results for Coating A are similar to results obtained when the coating is omitted, i.e. when the agarose slab is placed on a plastic surface [of a petri dish] rather than on a coated metal panel.

Similar results [not shown] were obtained for the fungus Aspergillus niger. However, germination was not observed over the full 19 days of the experiment. The germination (day 13) seen for A. resinae on Coating B was not observed for A. niger.

It is reasonable to conclude from the results shown that:

1) the conidiospore suspension contained many conidiospores that were capable of rapid germination and that;

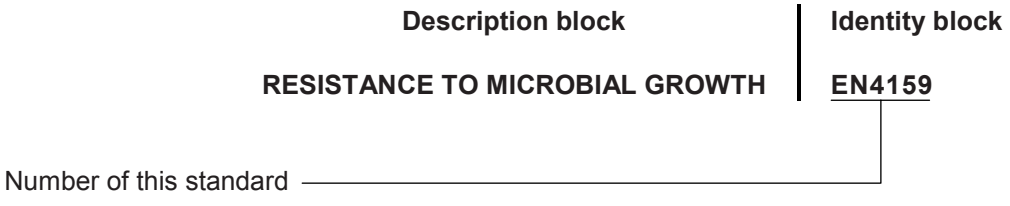
2) the coating [B] previously known to be effective as a fungistatic agent prevented germination for 19 d and;

3) that the new coating also prevented germination for the 19 day duration of the trials.

These trials were carried out in parallel, using portions of the same original conidiospore suspension, the same original agarose preparation, and the same conditions of incubation and examination. The three sets of results are strictly comparable.

7 Designation

EXAMPLE



8 Test report

The test report should include the following information:

a) Identification of the coatings under test: supplier's code number, batch number, date of manufacture, etc.;

b) Reference to this European Standard (EN 4159);

c) The number of specimens tested;

d) A list of the fungi used, with reference to their origin in national culture collections;

e) The results obtained using the methods and materials specified. The success rate of germination in the test samples shall be specified and the success rate of germination in the control samples shall be specified, with a statement as to the duration of exposure of conidiospores to the coating;

f) Any special observations that may influence interpretation of the results stated;

g) Date of tests and identity of persons who performed the tests;

h) Signature of inspector.

Bibliography

[1] EN 2334, *Aerospace series — Chromic-sulphuric acid pickle of aluminium and aluminium alloys*

[2] EN 2802, *Aerospace series — Aluminium alloy AL-P7475-T761 — Sheet and strip — 0,6 mm ≤ a ≤ 6 mm*

[3] EN 3333, *Aerospace series — Aluminium alloy AL-P7475-T762 — Sheet and strip — 0,6 mm ≤ a ≤ 6 mm*

[4] EN ISO 1513, *Paints and varnishes - Examination and preparation of test samples (ISO 1513)*

[5] EN ISO 3696, *Water for analytical laboratory use — Specification and test methods (ISO 3696)*

[6] EN ISO 15528, *Paints, varnishes and raw materials for paints and varnishes — Sampling (ISO 15528)*

[7] www.oxoid.com, *Oxoid microbiological media*

[8] www.sigma-aldrich.com, *Agarose*

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