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Pulp, paper and board — Microbiological examination —

Part 1: Enumeration of bacteria and bacterial spores based on disintegration

Pâtes, papiers et cartons — Analyse microbienne —

Partie 1: Dénombrement des bactéries et des spores bactériennes basé sur la désintégration

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT) see the following URL: Foreword - Supplementary information

The committee responsible for this document is ISO/TC 6, *Paper, board and pulps*, Subcommittee SC 2, *Test methods and quality specifications for paper and board*.

This third edition cancels and replaces the second edition (ISO 8784-1:2005), which has been technically revised.

The second edition was applicable to yeast and mould, as well as bacteria. The following main changes have been made with respect to the previous edition:

— This third edition is only applicable to bacteria and bacterial spores, and no longer applicable to yeast and mould;

— incubation temperature changed from (37 °C ± 1 °C) to (32 °C ± 2 °C) (9.4);

— 2 parallel determinations are to be made (Clause 8 and Clause 9);

— the result can be reported "as received" in addition to reporting on a dry-mass basis (8.2, 11.1, and Clause 12).

ISO 8784 consists of the following parts, under the general title *Pulp, paper and board — Microbiological examination*:

— *Part 1: Enumeration of bacteria and bacterial spores based on disintegration*

Introduction

This part of ISO 8784, which deals with the microbiological examination of dry market pulp, paper, and paperboard, is broadly based on ISO 4833[1] although the conditions are not identical. However, it provides specific amplification where necessary. It is intended for the estimation of colony-forming units, CFU, aerobic bacteria, and bacterial spores.

Because of the exacting techniques required in aseptic procedures, reproducible good quality results can only be ensured by skilled microbiological technicians.

Pulp, paper and board — Microbiological examination —

Part 1: Enumeration of bacteria and bacterial spores based on disintegration

1 Scope

This part of ISO 8784 specifies a method for determining the total number of colony-forming units of bacteria and bacterial spores in dry market pulp, paper, and paperboard after disintegration. The enumeration relates to specific media.

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.

ISO 186:2002, *Paper and board — Sampling to determine average quality*

ISO 7213:1981, *Pulps — Sampling for testing*

ISO 638:2008, *Paper, board and pulps — Determination of dry matter content — Oven-drying method*

3 Terms and definitions

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

3.1
bacteria
microscopic, single-celled organisms that possess a prokaryotic type of cell structure, which reproduce by fission and are able to grow under the test conditions specified in this part of ISO 8784

3.2
bacterial spores
highly resistant, dormant structures

EXAMPLE Endospores from certain genera of bacteria.

3.3
total bacterial count
number of colony-forming units (CFU) of bacteria and bacterial spores formed after incubation in a standard culture medium, under the test conditions specified in this part of ISO 8784

3.4
spore count
number of colony-forming units (CFU) of bacterial spores formed after incubation in a standard culture medium, under the test conditions specified in this part of ISO 8784

4 Principle

This poured plate method involves enumeration of colonies in a standard culture medium. A fibre suspension, prepared from paper, paperboard, or pulp samples, is plated in agar. Two parallel determinations are made. For enumeration of bacterial spores, the fibre suspension is heated for 10 min at 80 °C prior to plating. The plates are incubated at 32 °C for 48 h. The total numbers of bacteria or bacterial spores are enumerated by counting the colonies formed in the agar.

The mean value of 2 parallel determinations is calculated and the results are expressed as the number of CFU per gram of sample.

5 Culture media and diluents

5.1 General

All substrates and diluents shall be appropriately sterilized. When preparing the culture medium, make sure that the ingredients are completely dissolved by mixing while heating prior to dispensing into suitable containers for sterilization. See ISO 11133[2] for quality assurance and guidelines on preparation and production of culture media.

5.2 Water

When water is mentioned in a formula, use distilled water or purified water, see ISO 11133[2].

5.3 Culture media for total bacteria count and spore count

Culture medium shall be prepared as follows, or from commercially available dehydrated culture media according to the manufacturer's instructions. Ready-to-use medium may be used when its composition is comparable to that given in this part of ISO 8784. To test the performance of the medium, see ISO 11133[2].

Plate count agar (PCA) composition per litre:

Tryptone	5,0 g
Yeast Extract	2,5 g
Dextrose	1,0 g
Agar	15,0 g
Water	1 000 ml
Final pH	7,0 ± 0,2

If PCA is not available, Tryptone glucose extract (TGE) agar may be used (see A.3). The use of TGE as an alternative culture medium is acceptable if it gives comparable results as the standard culture medium. The culture medium used shall be stated in the test report (see Clause 12).

5.4 Diluents

Ringer's solution (see A.1) is preferred, although other isotonic solutions may be used. Ringer's tablets are commercially available.

To facilitate the release of cells from the fibres, it is recommended to add 20 µl of Tween 80 (see A.2) per litre to the Ringer's solution prior to sterilization by autoclaving.

The diluent used and if Tween 80 has been added, shall be stated in the test report (see Clause 12).

6 Apparatus and equipment

6.1 General

All laboratory equipment and parts of the equipment in direct contact with the sample and the diluent or the culture medium shall be sterilized.

NOTE For advice on standard microbiological equipment, see ISO 7218[4].

6.2 List of equipment

6.2.1 Use ordinary microbiological laboratory equipment, and the following.

6.2.2 Suitable wrapping material, e.g. aluminium foil (non-coated and inert), ready-to-use envelopes of different sizes or self-closing plastic bags, all of which are commercially available.

6.2.3 Disintegrator, high speed electrical blender with metal (preferably stainless steel) or glass cup that can be sterilized.

NOTE Other homogenizing system with equivalent efficiency may be used.

6.2.4 Incubator, capable of maintaining a constant temperature of 32 °C ± 2 °C.

6.2.5 Petri dishes, having a diameter of 90 mm (standard) or 140 mm to 150 mm (alternative).

6.2.6 Pipettes, of wide-mouth type suitable volume.

The width of the mouth must be large enough so that a 1 % fibre suspension can easily be drawn into the pipette tip.

NOTE A suitable volume is 10 ml or 50 ml.

6.2.7 Water bath, capable of maintaining a temperature of 80 °C ± 2 °C.

6.2.8 Colony-counting equipment or magnifying device, with a magnification between 1,5 × and 2,5 × shall be used.

NOTE The use of an additional lens might be necessary to increase the magnification, up to 10 ×, to facilitate the counting of pin-point bacterial colony-forming units and also to ensure that no other particles except bacterial colonies are counted (see Clause 10).

6.2.9 Balance, with an accuracy of 0,01 g.

6.2.10 Sterilizing unit, an autoclave capable of sterilization at 121 °C.

7 Sampling

Make sure that the sampling procedure is performed using aseptic techniques.

If the sample is to represent a lot of paper or paperboard, the sampling shall be in accordance with ISO 186:2002. From each unit of paper or paperboard to be sampled, cut several top layers and discard them to eliminate surface contamination. Use a sterile knife to cut through several layers of the paper or board sample, producing a stack of sheets. Discard the top sheet.

If the sample is to represent a lot of pulp, the sampling shall be in accordance with ISO 7213:1981. From each unit of dry market pulp to be sampled, discard several top sheets from each bale to eliminate surface contamination.

In other cases, sample a sufficient number of units so that the test material is representative of the paper, the paperboard, or the dry market pulp to be tested. In all sampling and examination procedures, make sure that the test material taken is representative of the sample received.

Ideally, a sample should contain at least four sheets, each of them having a minimum size of 200 mm × 250 mm of dry market pulp, paper, or paperboard (at least 2 sheets for testing and 2 protective sheets).

NOTE For paperboard or thicker material, it might be sufficient to use only 1 sheet for each parallel determination. For thinner paper, more than 2 sheets can be used for each parallel determination.

After sampling, wrap the unexposed test material in suitable wrapping material (6.2.2).

8 Preparation of the test material

8.1 General

Preferably, conduct the procedure under aseptic conditions. A laminar flow hood is recommended for plating. Unwrap the test material under aseptic conditions. Remove the protective sheets on the top and bottom of the sample stack without touching the test sheets in the centre of the sample stack.

The procedure in 8.3 and 8.4 shall be repeated for the 2 parallel determinations.

8.2 Determination of dry-matter content

If the result is to be reported on a dry-mass basis, determine the dry-matter content of the test material, *X*, in accordance with ISO 638:2008.

If the result is to be reported on an "as received"-mass basis (not on a dry-mass basis), omit the determination of dry-matter content and report accordingly [see 11.1 and Clause 12 j)].

8.3 Weighing

Two parallel determinations are made (8.1).

Place a closed Petri dish (6.2.5) on the pan of the balance and determine its tare mass.

With sterile tweezers, hold the sheet or sheets along one edge in one hand, trim and discard the remaining edges with sterile scissors. Cut the sample material into small pieces. Weigh a sufficient amount of the test material (mass approximately 2 g to 3 g), *m*, into the Petri dish, to be able to prepare a fibre suspension having a concentration of 1 %.

NOTE 1 For practical reasons, it can be useful to cut a sufficient amount of small pieces to be able to repeat the test.

NOTE 2 In order to get a short disintegration time it might be useful to cut pieces that are smaller than 5 mm.

Transfer the test material aseptically to the disintegrator jar (6.2.3).

8.4 Disintegration

2 parallel determinations are made (8.1).

Use cooled diluent solution (5.4) to prevent overheating (increase of the suspension temperature above 45 °C) during disintegration. Ensure the sterility of the disintegrator jar (6.2.3) for each test material.

Disintegrate the test material (8.3) in diluent solution (5.4), of a volume, *V*, needed to obtain a 1 % fibre suspension (for 2,0 g use 200 ml, and for 3,0 g use 300 ml). Disintegrate until the suspension is free from fibre clumps.

Other homogenizing systems with equivalent efficiency may be used, and shall be stated in the test report. If it is difficult to obtain a fibre suspension free from fibre clumps by using a disintegrator, some other suitable equipment may be used in addition. If so, the equipment used shall be stated in the test report.

9 Determination of the total bacterial count and spore count

9.1 General

The procedure in 9.2, 9.3, and 9.4 shall be repeated for the 2 parallel determinations.

The procedure shall be carried out in aseptic conditions. The work area shall be cleaned with a suitable disinfectant. If available, a laminar flow hood is recommended for plating.

The procedures for the determination of the total bacterial count and spore count are similar, except that for the determination of bacterial spores the disintegrated test material should be heat-treated as described in 9.3.

After disintegration of the sample, add the fibre suspension to Petri dishes. When using the wide-mouth pipette tip, ensure that no fibre clumps remain in the pipette tip.

NOTE Bacteria and spores might be attached to fibres, and if an inhomogeneous fibre suspension is added to the Petri dishes, the colony counts can be incorrect.

9.2 Plating for total bacterial count

This procedure shall be repeated for the 2 parallel determinations (9.1).

9.2.1 Immediately after disintegration, with a sterile wide mouth pipette (6.2.6), distribute 10 ml, *v*, of the 1 % fibre suspension among 5 sterile standard Petri dishes (6.2.5), i.e. should be as close to 2 ml as possible per 90 mm plate. This will represent 0,1 g of the test material. Within less than 5 min, pour into each plate 15 ml to 20 ml of the selected culture medium (5.3) cooled to approximately 45 °C. Immediately after the addition, rotate the plate with agitation to obtain a uniform distribution of fibre throughout the culture medium. Avoid a swirling motion since the colonies will not be separated this way. It is important that all clumps are broken up, in order that the plates may be examined easily and more accurately. The detection limit is 10 CFU/g.

If the alternative Petri dishes (140 mm to 150 mm) are used, with a wide mouth pipette (6.2.6), distribute 50 ml, *v*, of the 1 % fibre suspension among 5 sterile Petri dishes (6.2.5), i.e. approximately 10 ml per plate. This will represent 0,5 g of test material. In this case, the detection limit is 2 CFU/g.

9.2.2 If a higher dilution is required, add 10 ml of the 1 % fibre suspension to 90 ml of the diluent solution (5.4). Shake the suspension vigorously and plate 10 ml of this suspension as described in 9.2.1. Repeat this procedure (tenfold dilution for each step) until an appropriate dilution is reached.

NOTE If higher counts are expected it might be helpful to distribute 10 ml of the 1 % fibre suspension among five alternative Petri dishes (140 mm to 150 mm) to facilitate counting the number of colony-forming units (CFU) of bacteria.

9.2.3 After plating, allow the agar to solidify at room temperature.

Check the sterility of the culture medium and the diluents by pouring control plates.