INTERNATIONAL STANDARD

ISO 8784-1

Second edition
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Pulp, paper and board — Microbiological examination —

Part 1: Total count of bacteria, yeast and mould based on disintegration

Pâte, papier et carton — Analyse microbienne

Partie 1: Dénombrement total des bactéries, levures et moisissures basé sur la désintégration

Reference number
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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

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

ISO 8784-1 was prepared by Technical Committee ISO/TC 6, *Paper, board an pulps*, Subcommittee SC 2, *Test methods and quality specifications for paper and board*.

This second edition cancels and replaces the first edition (ISO 8784-1:1987), which has been technically revised. The first edition was only applicable to paper and board and only the bacterial colonies were determined. The second edition is also applicable to pulp (dry market pulp), and yeast and mould as well as bacteria are determined. The incubation conditions have been changed in the second edition (37 °C, 48 h for bacteria, 30 °C, 5 days for yeast and mould) compared to the conditions stated in the first edition (30 °C, 72 h).

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

— *Part 1: Total count of bacteria, yeast and mould based on disintegration*

— *Part 2: Surface method for enumeration of microbes on paperboard*

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, without any attempt to isolate species of particular public-health significance.

Because of the exacting techniques required in aseptic procedures, reproducible results can only be secured by skilled microbiological technicians. In addition, health risks may arise from the employment of inadequately trained staff.

Pulp, paper and board — Microbiological examination —

Part 1: Total count of bacteria, yeast and mould based on disintegration

1 Scope

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

This part of ISO 8784 is applicable to most kinds of paper and paperboard, especially those grades intended to come into contact with foodstuffs.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

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

ISO 287, *Paper and board — Determination of moisture content — Oven-drying method*

ISO 638, *Pulps — Determination of dry matter content*

ISO 7213, *Pulps — Sampling for testing*

3 Terms and definitions

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

3.1
microbiological count
number of colony-forming units (CFU) formed in a standard culture medium, after incubation under the conditions specified

4 Principle

Preparation of culture plates from specified dilutions of a suspension of the sample on a specified culture medium. For the determination of bacteria, aerobic incubation of the culture plates for 48 h at 37 °C. For the determination of yeast and mould, aerobic incubation of the culture plates for 5 days at 30 °C. At the end of the incubation time, counting the CFU on the plates.

From the number of colonies counted, the number of CFU per gram of the sample is calculated.

5 Substrates and diluents

All substrates and diluents shall be appropriately sterilized. When preparing the culture medium, make sure that the ingredients are completely dissolved prior to dispensing into suitable containers and sterilization.

5.1 Nutrient substrate

5.1.1 Tryptone Glucose Extract Agar (TGEA)

Use TGEA for bacterial counts. Composition per litre:

Beef extract	3,0 g
Tryptone	5,0 g
Dextrose (*d*-glucose)	1,0 g
Agar	15,0 g
pH of the ready-made medium	7,0

If TGEA is not available, Plate Count Agar (PCA) may be substituted. The use of PCA shall be stated in the test report. The composition of the PCA medium is the same as TGEA, except that beef extract (3 g) is substituted with 2,5 g yeast extract.

5.1.2 Potato Dextrose Agar (PDA)

Use PDA for yeast and mould counts. Composition per litre:

Potatoes, infusion from	200 g
Dextrose	20 g
Agar	15 g
pH of the ready-made medium	5,6

Acidification of PDA: Acidify the medium with drops of 10 % sterile tartaric acid to reach a pH of 3,5 ± 0,1. After adding the tartaric acid to the medium, mix without foaming, and prepare poured plates as usual. Do not heat the medium after the acid has been added, since heating in the acid state will hydrolyse the agar and destroy its solidifying properties.

It is frequently desirable in making yeast and mould counts to inhibit bacterial growth by acidifying the PDA medium, and it is recommended that the reaction of the medium be reduced to pH (3,5 ± 0,1) subsequent to sterilization. The growth of bacteria can alternatively be inhibited by addition of a bactericide. The use of a bactericide shall be stated in the test report.

NOTE The Tryptone Glucose Extract Agar (TGEA) and the Potato Dextrose Agar (PDA) are commercially available in dehydrated form. When using the dehydrated medium, follow the instructions printed on the container.

5.2 Standard diluent: 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.

6 Equipment

All equipment in direct contact with the sample or the diluents shall be sterilized. Use ordinary microbiological laboratory equipment, and the following.

6.1 Colony-counting equipment, fitted with a lens having a magnifying power of at least 1,5 times. The use of an additional lens in conjunction with the lens on the colony counter may be necessary to increase the magnification to 8 to 10 times, to facilitate the counting of pin-point bacterial colony-forming units.

6.2 Disintegrators, with metal or glass jars of about 500 ml capacity, fitted with a high-speed impeller near the bottom and fitted with a cap or lid; or other suitable disintegrator which ensures disintegration of the sample. Place an aluminium foil hood over the cap of each disintegrator jar prior to sterilization.

6.3 Incubator, capable of maintaining temperatures of (30 ± 1) °C and (37 ± 1) °C.

6.4 Petri dishes, having a diameter of 150 mm.

6.5 Pipettes, of suitable wide-mouth type.

6.6 Aluminium foil, envelopes, or self-closing plastic bags, for sampling. Samples can be wrapped as such in aluminium foil, in ready-to-use sterile envelopes of different sizes or self-closing plastic bags, of which all are commercially available.

NOTE Petri dishes having a diameter of 90 mm may be used. However, the size 150 mm is recommended to make it easier to separate the colonies from the material.

7 Sampling

Make sure that the sampling procedure is performed aseptically.

If the sample is to represent a lot of paper or paperboard, the sampling shall be in accordance with ISO 186[1]. If the sample is to represent a lot of pulp, the sampling shall be in accordance with ISO 7213. From each unit of dry market pulp to be sampled, discard several top sheets from each bale to eliminate surface contamination. From each unit of paper or paperboard to be sampled, cut away several top layers and discard them to eliminate surface contamination. Use a sterile knife and cut a bunch of sheets (see the third paragraph of this clause). Discard the top sheet.

In other cases, sample a sufficient number of units so that the test material is representative of the paper or 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 five sheets, each of them having a minimum size of 200 mm × 250 mm of dry market pulp, paper or paperboard (at least 3 sheets for testing and 2 protective sheets).

After sampling, wrap the unexposed test material in suitable wrapping material (6.6).

8 Preparation of the test material

Preferably, the procedure is conducted in a sterile hood. Weighing takes place outside the hood. Unwrap the test material under aseptic conditions and remove the protective sheets without touching the test sheets.

8.1 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 287 or ISO 638, as relevant.

If the result is to be reported on an “as received”-mass basis, the determination of dry-matter content shall be omitted (also, see Note 1 to 11.1).

8.2 Weighing

Place a closed Petri dish (6.4) on the pan of the balance and determine its tare mass.

With a pair of sterile tweezers, hold the edge of the test sheets in one hand, trim and discard the edges with sterile scissors. Weigh a sufficient amount of the test material (mass approximately 2 g to 3 g) into the Petri dish, to be able to prepare a fibre suspension having a concentration of 1 % (see 9.2).

In the weighed material (8.2), make a series of cuts between 5 mm and 10 mm apart. Hold the material using a pair of tweezers and cut squares directly into the disintegrator jar (6.2), by making a series of cuts perpendicular to those made previously.

9 Procedure

9.1 Conditions

Run the whole procedure under aseptic conditions.

9.2 Disintegration

Cool the sterile Ringer's solution (5.2) to prevent the temperature from exceeding 45 °C in the disintegrator jar (if many tests are run on the same disintegrator within a short time).

Use a separate sterile disintegrator jar (6.2) for each test material and disintegrate the weighed material (8.2) in a volume of the cooled sterile Ringer's solution, adjusted to the weighed material to obtain a 1 % fibre suspension (for 2,0 g use 200 ml, for 3,0 g use 300 ml). Disintegrate until the suspension is free from fibre clumps.

When adding the weighed material (8.2) or the standard diluent (5.2) to the disintegrator jar, do not touch the metal cap with the hands, but lift it by grasping the aluminium hood placed over the cap before sterilization. Lift both the hood and the metal cap only enough to permit access to the jar. Allow the aluminium hood to remain on the cap all the time to prevent possible contamination through any opening in the cap or around the top of the jar.

If several test materials are studied, sterilise the disintegrator between each test material.

NOTE If it is difficult (by using a disintegrator) to obtain a fibre suspension free from fibre clumps, a stomacher may be used. The use of a stomacher must, however, be reported.

9.3 Plating

The room in which the material is weighed and the plating is carried out shall be free of air currents and dust. About 30 min before plating, sponge off the surface of the worktable with a suitable disinfectant.

NOTE 1 Ideally, this procedure is carried out in a sterile hood.

Immediately after the disintegration, plate the fibre suspension from 9.2. With a 50 ml sterile, wide-mouth pipette (6.5), distribute 50 ml of the 1 % fibre suspension among 5 sterile Petri dishes (6.4), i.e. approximately 10 ml in each of the 5 Petri dishes. Within less than 5 min, add to each inoculated plate 30 ml of the selected culture media (5.1) cooled to approximately 45 °C. Immediately after the addition, move the plates individually to disperse clumps of fibres and to obtain a uniform distribution of fibre throughout the culture medium. This is accomplished by moving the plates carefully from side to side and back and forth. Avoid a swirling motion since the colonies will not be separated this way. It is important that all clumps be broken up, in order that the plates may be examined easily and more accurately.

NOTE 2 If Petri dishes of 90 mm in diameter are used, use a 10 ml pipette (6.5) and distribute a total volume of 10 ml of the fibre suspension among the 5 Petri dishes (approximately 2 ml in each Petri dish) and add between 15 ml and 20 ml of the selected culture media.

NOTE 3 If it is suspected that the test material has a high microbiological load, prepare and plate additional, more diluted suspensions. Prepare serial dilutions according to Figure B.1.

To check the sterility of the culture medium, diluents and air contamination, prepare one control plate from each container of culture medium used. Also check the sterility of the disintegrator for each test material.

Allow the plates to solidify.

9.4 Incubation

After solidification, invert and store the Petri dishes in an incubator (6.3) at (37 ± 1) °C for (48 ± 3) h for bacteria, and (30 ± 1) °C for 5 days for yeast and mould.

10 Enumeration of the colonies

Before examining the sample plates, record the absence of colonies on the control plates. If any of the control plates are contaminated, the whole procedure has to be repeated.

After incubation, examine the sample plates for the presence and number of colony-forming units (CFU) using the colony-counting equipment (6.1). Examine and make a record of the number of colonies found on each sample plate.

For statistical reasons, the ideal count should be between 15 colonies and 300 colonies per plate. However, this limit is not always appropriate for the low counts expected.

NOTE 1 Agglomerations of coating can be mistaken for bacterial colonies. Use suitable techniques to confirm suspect colonies.

NOTE 2 Not all colonies which grow on Tryptone Glucose Extract Agar (TGEA) will be bacteria, and not all colonies which grow on Potato Dextrose Agar (PDA) will be yeast and mould.

11 Calculation and report

11.1 Calculation

Add the sum of colony counts of the 5 Petri dishes together, n.

Calculate the total colony number, N, per gram dry mass of the sample, according to the equation:

$$N = \frac{n \cdot V \cdot f}{v \cdot m \cdot x} \cdot 100 \qquad (1)$$

where

N is the total colony number, in CFU per gram dry mass of the sample;

n is the added sum of colony counts of the 5 Petri dishes together, in CFU;

V is the volume of the fibre suspension, in millilitres;

f is the dilution factor (if no dilution is performed, the factor is 1);

v is the volume of the plated fibre suspension (here 50 ml), in millilitres;

m is the mass of the weighed test material, in grams;

x is the dry-matter content of the test material as determined in 8.1, as a percentage.

EXAMPLE 3 g of test material was disintegrated in 300 ml of Ringer's solution and the dry-matter content of the sample was 92 % (determined according to ISO 287). The suspension was plated without any further dilution, i.e. f = 1.

Colony counts of 5 Petri dishes, each inoculated with 10 ml of the fibre suspension (50 ml in total), gave the following result:

79 86 82 89 67 Added sum = 403

$$N = \frac{403 \cdot 300 \cdot 1}{50 \cdot 3 \cdot 92} \cdot 100 = 876{,}1$$ which is equivalent to $8{,}76 \times 10^2$ CFU per gram dry mass of the sample.

NOTE 1 If the total colony number is to be reported per gram sample "as received", i.e. not on a dry-mass basis, omit x from Equation (1). This deviation from the standard procedure must be reported.

NOTE 2 For estimation of small numbers, see ISO 4833[2].

11.2 No colonies

If the original suspension contains no colonies, report the result as follows:

"Less than 10 CFU per gram of dry mass of sample".

11.3 Report

Report the results as the number of CFU per gram of sample with two significant figures, using the specified substrate.

12 Precision

Microbiologists agree that there is an intrinsic error of about 10 % in normal plate counts. This method employs plate counts and, therefore, this error is understood among microbiologists. A sample plated out at the same time by several competent microbiologists showed about 5 % deviation from each other.

13 Test report

The test report shall include the following information:

a) reference to this part of ISO 8784;

b) identification of the sample, unit and/or lot tested;

c) date and place of testing;

d) results expressed as number of CFU per gram on TGEA, PCA or PDA;

e) any deviation from the procedure specified in this part of ISO 8784.