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# Pulp, paper and board — Microbiological examination —

## Part 3: Enumeration of yeast and mould based on disintegration



*Pâtes, papiers et cartons — Analyse microbiologique —*

*Partie 3: Dénombrement des levures et des moisissures basé sur la désintégration*



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ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Email: copyright@iso.org
Website: www.iso.org

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



A list of all parts in the ISO 8784 series can be found on the ISO website.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

# Pulp, paper and board — Microbiological examination —

# Part 3:
# Enumeration of yeast and mould based on disintegration

## 1 Scope

This document specifies a method for determining the total number of colony-forming units of yeast and mould in dry market pulp, paper and paperboard after disintegration. The enumeration relates to specific media.

## 2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements 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 638-1, *Paper, board, pulps and cellulosic nanomaterials - Determination of dry matter content by oven-drying method - Part 1: Materials in solid form*

ISO 7213, *Pulps — Sampling for testing*



## 3 Terms and definitions

For the purposes of this document, the following terms and definitions 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/

**3.1**
**yeast**
mesophilic aerobic microorganism which, at 25 °C using mycological agar medium under the conditions described in this document, develops matte or shiny round colonies on the surface of the medium, usually having a regular outline and a more or less convex surface

Note 1 to entry: Yeasts within, rather than on, a medium develop round, lenticular, colonies.

**3.2**
**mould**
mesophilic aerobic filamentous microorganism which, on the surface of mycological agar medium under the conditions described in this document, usually develops flat or fluffy spreading propagules/germs or colonies often with coloured fruiting or sporing structures

Note 1 to entry: Moulds within, rather than on, a medium can develop round, lenticular, colonies.

**3.3**
**yeast and mould count**
number of colony-forming units (CFU) of *yeast* (3.1) and *mould* (3.2) formed after incubation in a standard culture medium, under the test conditions specified in this document

# 4 Principle

This pour 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.

The plates are incubated at 25 °C ± 1 °C for 5 d. The total numbers of yeast and mould are enumerated by counting the colonies formed in the agar. 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 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.

## 5.3 Culture media for total yeast and mould 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 document. To test the performance of the medium, see ISO 11133.

Sabouraud dextrose chloramphenicol agar medium (SDCA) composition per litre:



— dextrose 40,0 g

— peptic digest of animal tissue 5,0 g

— pancreatic digest of casein 5,0 g

— chloramphenicol 0,050 g

— agar 15,0 g

— water 1 000 ml

Dissolve the components (including the chloramphenicol) or the dehydrated complete medium in the water by heating. Dispense the medium into suitable containers. Sterilize in an autoclave at 121 °C for 15 min. After sterilization the pH shall be equivalent to 5,6 ± 0,2 when measured at room temperature.

## 5.4 Diluents

Ringer's solution ¼ strength

Composition per litre:

— Sodium chloride (NaCl) 2,250 g;

— Potassium chloride (KCl) 0,105 g;

— Calcium chloride ($CaCl_2$) 6 $H_2O$ 0,120 g;

— Sodium hydrogen carbonate ($NaHCO_3$) 0,050 g;

— Water 1 000 ml.

NOTE 1 Ringer's solution is preferred, although other isotonic solutions can be used. See ISO 6887-1.

NOTE 2 Ringer's tablets are commercially available.

### 5.5 Non-ionic surfactant

Poly (oxyethylene)-sorbitan monooleate (Tween 80).

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

## 6 Apparatus and equipment

### 6.1 General

Disposable apparatus is an acceptable alternative to re-usable glassware and plastic if it has suitable specifications.

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

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



Usual microbiological laboratory equipment, and in particular the following.

### 6.2 Apparatus for dry sterilisation (oven) and wet sterilization (autoclave).

See ISO 7218.



**6.3 Biological safety cabinet**, class II.

**6.4 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.

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

NOTE Other homogenizing system with equivalent efficiency can be used.

**6.6 Incubator**, capable of maintaining a constant temperature of 25 °C ± 1 °C.

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

**6.8 Pipette**, of wide-mouth type and 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.

**6.9 Colony-counting equipment or magnifying device**, with a magnification between 1,5 × and 2,5 ×.

**6.10 Balance**, with an accuracy of ±0,01 g.

**6.11 Cutting devices,** such as scissors, scalpels, and/or knives.

## 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. 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. 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 determination. For thinner paper, more than 2 sheets can be used for determination.



After sampling, wrap the unexposed test material in suitable wrapping material (6.4).

## 8 Preparation of the test material



### 8.1 General

Preferably, conduct the procedure under aseptic conditions. A laminar flow hood is recommended for cutting/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.

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 638-1.

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).

### 8.2 Weighing

Place a closed Petri dish (6.7) 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 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 (6.5). Ensure the sterility of the disintegration jar (6.5) for each test material.

### 8.3 Disintegration

Use cooled diluent solution adjusted to room temperature (5.4). Avoid overheating (increase of the suspension temperature above 45 °C) during disintegration due to its effects on number of microorganisms. Ensure the sterility of the disintegration jar (6.5) for each test material.

Disintegrate the test material (8.2) in diluent solution (5.4) to obtain a 1 % fibre suspension (for 2,0 g of test material, the volume of diluent should be 200 ml, and for 3,0 g it should be 300 ml). Disintegrate the suspension until it would be free from fibre clumps.

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

## 9 Determination of yeast and mould count

### 9.1 General

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.

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

NOTE Yeast and mould 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 yeast and mould count

**9.2.1** Immediately after disintegration, with a sterile wide mouth pipette (6.8), distribute 10 ml, *v*, of the 1 % fibre suspension evenly among 5 sterile standard Petri dishes (6.7), i.e. approximately 2 ml per each plate (90 mm). This will represent 0,1 g of the test material.



**9.2.2** 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.

Allow the agar to solidify at room temperature.

Check the sterility of the culture medium, the diluents and condition of test by pouring control plates in the same condition as test material.

**9.2.3** 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 9.2.2.

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 yeast and mould.

### 9.3 Incubation

After the agar has solidified, invert the Petri dishes and incubate them (6.6) at 25 °C ± 1 °C for 3 to 5 days.

## 10 Enumeration of the colonies

Before examining the incubated sample Petri dishes, check the control plates for the presence of colonies of yeast and mould. If any of the control plates are contaminated, the whole procedure shall be repeated.

Count the number of colony-forming units (CFU) of yeast and mould using the magnifying device, if appropriate (6.9). Record the number of colonies found on each incubated Petri dish.

If necessary, carry out an examination with a binocular magnifier or with a microscope in order to distinguish between cells of yeasts or moulds and bacteria from colonies.

For statistical reasons, it is preferable to select Petri dishes with between 15 to 150 colonies for enumeration of yeast and mould count. For undiluted samples, 15 or fewer colonies are acceptable.

If more than one third of a plate is covered with swarming yeast and mould, the plate shall be discarded. If more than two plates are discarded, the test procedure shall be repeated from 8.2.

If, after repeating the test, more than two plates are discarded, the overall result shall be reported as "uncountable due to swarming yeast and mould".

Read the plates between 3 d and 5 d of incubation. Select the dishes (9.3) containing less than 150 colonies and count these colonies. Observe the plates after 3 days and in order to ensure of proper growth count the colonies after 5 d. If fast-growing moulds are a problem, count colonies after 3 d and again after 5 d of incubation.



NOTE 1 Enumeration methods for yeasts and especially moulds are imprecise because they consist of a mixture of mycelium and asexual and sexual spores. Numbers of colony-forming units depend on the degree of fragmentation of mycelium and the proportion of spores able to grow on the plating medium.

NOTE 2 Non-linearity of counts from dilution plating often occurs, i.e. 10-fold dilutions of samples often do not result in 10-fold reductions in numbers of colonies recovered on plating media. This has been attributed to fragmentation of mycelia and breaking of spore clumps during dilution in addition to competitive inhibition when large numbers of colonies are present on plates.



**CAUTION — The spores of moulds disperse in the air with great facility, handle the Petri dishes with care to avoid development of satellite colonies which would give an overestimation of population in the sample.**

Count the colonies of yeasts and the colonies of moulds separately, if necessary.

## 11 Calculation and report

### 11.1 Calculation

For the total yeast and mould count, sum up the number of yeast and mould colonies, $n$, from the 5 Petri dishes plated for yeast and mould count (9.2).

Calculate the total yeast and mould count, as $N_{rec}$, per gram (as received) of the sample, according to Formula (1):

$$N_{rec} = \frac{n \times V \times f}{v \times m} \quad (1)$$

where

$N_{rec}$ is the total yeast and mould count, in CFU per gram (as received) of the sample;

$n$ is the sum of colonies on the 5 respective Petri dishes, in CFU;