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Microbiology of the food chain — Horizontal method for the enumeration of microorganisms —

Part 1: Colony count at 30 °C by the pour plate technique

Microbiologie de la chaîne alimentaire — Méthode horizontale pour le dénombrement des micro-organismes —

Partie 1: Comptage des colonies à 30 °C par la technique d'ensemencement en profondeur

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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, www.iso.org/directives.

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The committee responsible for this document is ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

This first edition, together with ISO 4833-2, cancels and replaces ISO 4833:2003.

ISO 4833 consists of the following parts, under the general title *Microbiology of the food chain — Horizontal method for the enumeration of microorganisms*:

— *Part 1: Colony count at 30 °C by the pour plate technique*

— *Part 2: Colony count at 30 °C by the surface plating technique*

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Microbiology of the food chain — Horizontal method for the enumeration of microorganisms —

Part 1:
Colony count at 30 °C by the pour plate technique

1 Scope

This part of ISO 4833 specifies a horizontal method for enumeration of microorganisms that are able to grow and form colonies in a solid medium after aerobic incubation at 30 °C. The method is applicable to:

a) products intended for human consumption and for animal feed;

b) environmental samples in the area of food and feed production and handling.

This part of ISO 4833 is applicable to:

1) products that require a reliable count when a low limit of detection is specified (below 10^2/g or 10^2/ml for liquid samples or below 10^3/g for solid samples);

2) products expected to contain spreading colonies that obscure colonies of other organisms, e.g. milk and milk products likely to contain spreading *Bacillus* spp.

The applicability of this part of ISO 4833 to the examination of certain fermented food and animal feeds is limited and other media or incubation conditions can be more appropriate. However, this method can be applied to such products even though it is possible that the predominant microorganisms in those products are not detected effectively.

For some matrices, the method specified in this part of ISO 4833 can give different results to those obtained using the method specified in ISO 4833-2.

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 6887 (all parts), *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination*

ISO 7218, *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations*

ISO 11133, *Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media*

3 Terms and definitions

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

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3.1
microorganism
entity of microscopic size, encompassing bacteria, fungi, protozoa and viruses

[SOURCE: ISO/TS 11139:2006,[3] 2.26]

Note 1 to entry: For the purposes of this part of ISO 4833, microorganisms are bacteria, yeasts and moulds that are able to produce colonies under the conditions specified in this part of ISO 4833.

4 Principle

A specified quantity of the liquid test sample, or a specified quantity of an initial suspension in the case of other products, is dispensed into an empty Petri dish and mixed with a specified molten agar culture medium to form a poured plate.

Other plates are prepared under the same conditions using decimal dilutions of the test sample or of the initial suspension.

The plates are incubated under aerobic conditions at 30 °C for 72 h.

The number of microorganisms per gram or per millilitre of the test sample is calculated from the number of colonies obtained in the plates containing fewer than 300 colonies.

5 Culture media and diluents

5.1 General

Follow ISO 11133 for preparation, production and performance testing of culture media.

5.2 Diluents

Use the diluent(s) specified in ISO 6887 for the product concerned or the specific International Standard dealing with the product under examination.

5.3 **Agar medium**: plate count agar (PCA)

5.3.1 Composition

Enzymatic digestion of casein	5,0 g
Yeast extract	2,5 g
Glucose, anhydrous ($C_6H_{12}O_6$)	1,0 g
Agar[a]	9 g to 18 g
Water	1 000 ml

[a] Depending on the gel strength of the agar.

When dairy products are examined, add skimmed milk powder at 1,0 g/l of the culture medium. The skimmed milk powder shall be free from inhibitory substances.

5.3.2 Preparation

Dissolve the components or the dehydrated complete medium in the water, by heating if necessary. Mix thoroughly and leave to stand for several minutes.

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Adjust the pH (6.4), if necessary, so that after sterilization it is 7,0 ± 0,2 at 25 °C.

Dispense the medium into tubes, flasks or bottles (6.8) of suitable capacity. Sterilize in an autoclave (6.1) at 121 °C for 15 min.

If the medium is to be used immediately, cool it to 44 °C to 47 °C in a water bath (6.3) before use. If not, store it in the dark at a temperature of (5 ± 3) °C for no longer than 3 months, under conditions which do not allow any change in its composition and properties.

Before beginning the microbiological examination, completely melt the medium, then cool it to 44 °C to 47 °C in a water bath (6.3) before use. See ISO 11133.

Use the molten agar as soon as possible; it should not be retained for more than 4 h.

5.3.3 Performance testing of the culture medium

5.3.3.1 General

Plate count agar is a non-selective medium, used in this part of ISO 4833 as a pour plate. Productivity shall be tested according to ISO 11133.

5.3.3.2 Productivity

Incubation	(30 ± 1) °C for (72 ± 3) h
Control strains	*Escherichia coli* WDCM 00013 or WDCM 00012[a] [World Data Centre for Micro-organisms (WDCM)]
	Bacillus subtilis subsp. *spizizenii* WDCM 00003[a]
	Staphylococcus aureus WDCM 00032 or WDCM 00034
Reference medium	Tryptone soya agar
Control method	Quantitative
Criterion	Productivity ratio (PR) ≥0,7

[a] The strains to be used as a minimum by the user laboratory. See Reference [7] for information on culture collection strain numbers and contact details.

5.4 Overlay medium (if necessary; see 9.2.7)

5.4.1 Composition

Agar[a]	12 g to 18 g
Water	1 000 ml

[a] Depending on the gel strength of the agar.

5.4.2 Preparation

Add the agar to the water and heat to boiling, stirring frequently until the agar is completely dissolved, or steam for about 30 min.

Adjust the pH (6.4), if necessary, so that after sterilization it is 7,0 ± 0,2 at 25 °C.

Dispense the medium into tubes, flasks or bottles (6.8) of appropriate capacity.

Sterilize in an autoclave at 121 °C for 15 min.

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If the medium is to be used immediately, cool it to 44 °C to 47 °C in a water bath (6.3) before use. If not, store it in the dark at a temperature of (5 ± 3) °C for no longer than 3 months, under conditions which do not allow any change in its composition and properties.

Before beginning the microbiological examination, completely melt the medium then cool it to 44 °C to 47 °C in a water bath (6.3) before use. See ISO 11133.

6 Apparatus

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

Usual microbiological laboratory equipment (see ISO 7218) and in particular the following.

6.1 **Oven** for dry sterilization or **autoclave** for wet sterilization, used in accordance with ISO 7218.

6.2 **Incubator**, capable of being maintained at (30 ± 1) °C.

6.3 **Water bath**, capable of being maintained at 44 °C to 47 °C.

6.4 **pH-meter**, accurate to within ±0,1 pH unit at 25 °C.

6.5 **Petri dishes**, made of glass or plastic, of diameter 90 mm to 100 mm.

6.6 **Total delivery graduated pipettes**, of nominal capacity 1 ml, graduated in 0,1 ml divisions, ISO 835[1] class A, or automatic pipettes, ISO 8655-2,[2] with use of sterile tips.

6.7 **Colony-counting equipment** (optional), consisting of an illuminated base and, optionally, a mechanical or electronic digital counter.

6.8 **Tubes, flasks** or **bottles**, of appropriate capacity and not greater than 500 ml.

7 Sampling

Sampling is not part of the method specified in this part of ISO 4833. See the specific International Standard dealing with the product concerned. If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject.

It is important the laboratory receive a truly representative sample which has not been damaged or changed during transport or storage.

8 Preparation of test sample

Prepare the test sample in accordance with the specific International Standard appropriate to the product concerned.

9 Procedure

9.1 Test portion, initial suspension and dilutions

Follow the specifications of ISO 6887 or the specific International Standard appropriate to the product concerned.

9.2 Inoculation and incubation

9.2.1 Take two sterile Petri dishes (6.5). Transfer to each dish, by means of a sterile pipette (6.6), 1 ml of the test sample if liquid, or 1 ml of the initial suspension (10^{-1} dilution) in the case of other products. If plates from more than one dilution are prepared, this may be reduced to one dish (ISO 7218).

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9.2.2 Take one other sterile Petri dish (6.5). Use another sterile pipette (6.6) to dispense 1 ml of the 10^{-1} dilution (liquid product) or 1 ml of the 10^{-2} dilution (other products).

9.2.3 If necessary, repeat the procedure with the further dilutions, using a new sterile pipette for each decimal dilution.

9.2.4 If appropriate and possible, select only the critical dilutions steps (at least two consecutive decimal dilutions) for the inoculation of the Petri dishes that will give colony counts of between 10 and 300 colonies per plate.

9.2.5 Pour about 12 ml to 15 ml of the plate count agar (5.3) at 44 °C to 47 °C into each Petri dish. The time elapsed between the end of the preparation of the initial suspension (or of the 10^{-1} dilution if the product is liquid) and the moment when the medium (5.3) is poured into the dishes shall not exceed 45 min.

9.2.6 Carefully mix the inoculum with the medium by rotating the Petri dishes and allow the mixture to solidify by leaving the Petri dishes standing on a cool horizontal surface.

9.2.7 After complete solidification, and only in the case where it is suspected that the product under examination contains microorganisms whose colonies overgrow the surface of the medium, pour about 4 ml of the overlay medium (5.4) or plate count agar (5.3) at 44 °C to 47 °C on to the surface of the inoculated medium. Allow to solidify as specified in 9.2.6.

9.2.8 Invert the prepared plates and place them in the incubator (6.2) at (30 ± 1) °C, in accordance with ISO 7218. Incubate for (72 ± 3) h.

9.3 Counting of colonies

9.3.1 After the specified incubation period (9.2.8), retain the plates with, if possible, fewer than 300 colonies. Count the colonies on the plates, using the colony-counting equipment (6.7) if necessary. Examine the dishes under subdued light. It is important that pinpoint colonies be included in the count; however, it is essential that the operator avoid mistaking particles of undissolved or precipitated matter in dishes for pinpoint colonies. Examine doubtful objects carefully, using higher magnification where required, in order to distinguish colonies from foreign matter.

9.3.2 Spreading colonies shall be considered as single colonies. If less than one-quarter of the dish is overgrown by spreading, count the colonies on the unaffected part of the dish and calculate the corresponding number of the entire dish. If more than one-quarter is overgrown by spreading colonies, discard the count.

10 Expression of results

10.1 Method of calculation

Follow the procedure specified in ISO 7218.

10.2 Precision

10.2.1 General

Precision data have been evaluated for dishes containing more than 15 and fewer than 300 colonies. The precision data depend on the flora association and the sample matrix. The data presented are derived from collaborative studies (see References [4]–[6]) and are valid for raw and pasteurized milk. These data may be used as estimates when colony counts in other products are determined.

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10.2.2 Repeatability

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will not be greater than the repeatability limit, $r = 0{,}25$, in $\log_{10}N$, where N is the number of microorganisms per millilitre (corresponding to 1,8 on the normal scale in microorganisms per millilitre).

NOTE This repeatability limit derives from collaborative studies of raw and pasteurized milk (see References [4]–[6]) and can be used for such products.

10.2.3 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will not be greater than the reproducibility limit, $R = 0{,}45$, in $\log_{10}N$, where N is the number of microorganisms per millilitre (corresponding to 2,8 on the normal scale in microorganisms per millilitre).

NOTE This reproducibility limit derives from collaborative studies of raw and pasteurized milk (see References [4]–[6]) and can be used for such products.

10.3 Interpretation of test results

10.3.1 General

In the examples (10.3.2 and 10.3.3), the average precision data, a probability level of 95 % and the analysis of one sample are considered. It should be noted that, under practical conditions, the average of several samples is often used. The figures are expressed in numbers of microorganisms per millilitre.

10.3.2 Repeatability conditions

First result: $10^5 = 100\,000$

The difference between the first and the second result should not be greater than $0{,}25\log_{10}N$.

Second result: Lower limit: $10^{4,75} = 56\,000$

Upper limit: $10^{5,25} = 178\,000$

The difference between the first and the second result is acceptable if the second result is not lower than 56 000 or not higher than 178 000.

10.3.3 Reproducibility conditions

Results obtained in the first laboratory (average of duplicate determination): $10^5 = 100\,000$

The difference between the first and the second result obtained in the second laboratory should not be greater than 0,45 $\log_{10}N$ units:

Second results: Lower limit: $10^{4,55} = 36\,000$

Upper limit: $10^{5,45} = 280\,000$

The difference between the results obtained by the first and the second laboratory is acceptable, if the second laboratory obtains a result which is not lower than 36 000 and not higher that 280 000.

Annex A shows the calculation and use of the critical difference (CD) to interpret results.

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11 Test report

The test report shall contain at least the following information:

a) all information necessary for the complete identification of the sample;

b) the sampling method used, if known;

c) the test method used, with reference to this part of ISO 4833 (ISO 4833-1:2013);

d) all operating details not specified in this part of ISO 4833, or regarded as optional, together with details of any incidents which may have influenced the test result(s);

e) the test result(s) obtained;

f) If the repeatability has been checked, the final quoted result obtained.

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