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

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

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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).

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. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 34, *Food products,* Subcommittee SC 9, *Microbiology.*

This second edition cancels and replaces the first edition (ISO 17410:2001), which has been technically revised. It also replaces ISO 6730:2005 | IDF 101:2005[2] and ISO 8552:2004 | IDF 132:2004[4]. The main changes compared with the previous edition are as follows:

— the surface-plating technique is used, as opposed to the pour-plate technique used in ISO 6730:2005 | IDF 101:2005 and ISO 8552:2004 | IDF 132:2004, as psychrotrophic microorganisms are sensitive to heat;

— one horizontal method is used for the enumeration of psychrotrophic microorganisms in a) products intended for human consumption, b) products intended for animal feeding, c) environmental samples in the area of food and feed production, handling, and d) samples from the primary production stage;

— the rapid method has been included as an annex for the estimation of psychrotrophic plate count in raw and pasteurized milk (originating from ISO 8552:2004 | IDF 132:2004);

— performance testing of the culture medium, plate count agar (PCA), has been introduced;

— the expression of results has been changed to be in accordance with ISO 7218.

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.

Introduction

Psychrotrophic microorganisms are able to grow at low temperatures. These microorganisms may cause decay of refrigerated foods (except gas-packaged foods) due to odour and taste deviations. Some psychrotrophic microorganisms present in raw milk are also capable of producing heat stable enzymes. When heated (pasteurization or sterilization) these enzymes are insufficiently inactivated, causing quality defects in the heated product (fat or protein degradation).

For the revision of this document, no performance characteristics were included due to the lack of data and the fact that an interlaboratory study was not organized for the described method, since psychrotrophic microorganisms are a group of microorganisms that are mainly used for process monitoring and considered to be non-pathogenic.

The main technical changes listed in the Foreword, introduced in this document compared to ISO 17410:2001, are considered as minor (see ISO 17468[6]).

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

1 Scope

This document specifies a horizontal method for the enumeration of psychrotrophic microorganisms that are able to grow and form colonies on a solid agar culture medium after aerobic incubation at 6,5 °C.

This document is applicable to

— products intended for human consumption,

— products intended for animal feeding,

— environmental samples in the area of food and feed production, handling, and

— samples from the primary production stage.

NOTE Annex B specifies a rapid method for the estimated enumeration of psychrotrophic microorganisms in raw and pasteurized milk.

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 835, *Laboratory glassware — Graduated pipettes*

ISO 6887 (all parts), *Microbiology of the food chain — 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 8655-2, *Piston-operated volumetric apparatus — Part 2: Piston pipettes*

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.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1
psychrotrophic microorganism
entity of microscopic size, encompassing bacteria, yeasts and moulds

Note 1 to entry: Psychrotrophic microorganisms are bacteria, yeasts and moulds that are able to produce colonies under the conditions specified in this document.

4 Principle

A specified quantity of a test sample (liquid products), or a specified quantity of an initial suspension in the case of other products (non-liquid products), is surface-plated on a solid agar culture medium contained in Petri dishes.

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 6,5 °C for 10 days.

Annex B specifies a method for the rapid estimation of psychrotrophic plate count in raw and pasteurized milk by incubating plates at 21 °C for 25 h. However, it should be noted that not all microorganisms able to produce colonies at 6,5 °C will produce colonies if incubated at 21 °C.

The number of psychrotrophic microorganisms per gram of sample or the number of psychrotrophic microorganisms per millilitre of sample is calculated from the number of colonies obtained on the plates containing fewer than 150 colonies.

5 Culture media and reagents

Follow current laboratory practices in accordance with ISO 7218. The composition of culture media and reagents and their preparation are specified in Annex A. For performance testing of culture media, follow the procedures in accordance with ISO 11133 and Annex A.

6 Equipment and consumables

Disposable equipment is an acceptable alternative to reusable glassware if it has suitable specifications.

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

6.1 Colony-counting device (optional), consisting, for example, of an illuminated base with a dark background, fitted with a magnifying screen to aid colony detection, and (optionally) a mechanical or electronic digital counter.

6.2 Sterile spreader or **spatula**, made of glass, plastic or steel, for spreading inoculated material on the surface of the culture medium.

NOTE A spreader made of glass can be made from a glass rod of about 3,5 mm in diameter, shaped like a hockey stick about 20 cm long, bent at right angles about 3 cm from one end and flattened at the ends by heating.

6.3 Incubator, capable of operating at a temperature of 6,5 °C ± 1 °C.

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

6.5 Petri dishes, made of glass or plastic, of approximately 90 mm or 140 mm diameter.

6.6 pH meter, accuracy to within ±0,1 pH unit at 25 °C.

6.7 Refrigerator, capable of operating at 5 °C ± 3 °C.

6.8 Total delivery graduated pipettes, sterile, of nominal capacities 0,1 ml and 1 ml, ISO 835 class A, or automated pipettes, ISO 8655-2 with use of sterile tips.

6.9 Tubes, bottles or **flasks**, of appropriate capacity, for preparation, sterilization and, if necessary, storage of culture media.

6.10 Water bath, or **similar apparatus**, capable of operating at a temperature of 47 °C to 50 °C and of boiling water.

7 Sampling

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

Recommended sampling techniques are given in the following documents:

— ISO/TS 17728 for food and animal feed[8];

— ISO 707 for milk and milk products[1];

— ISO 6887-3 for fish and fishery products[3];

— ISO 13307 for the primary production stage[5];

— ISO 17604 for carcasses[7];

— ISO 18593 for surfaces[9].

It is important that the laboratory receives a sample that is representative. The sample should not have been damaged or changed during transport or storage.

8 Preparation of test sample

Prepare the test sample from the laboratory sample in accordance with the specific International Standard dealing with the product concerned: follow the procedures specified in ISO 6887 (all parts). If there is no specific International Standard available, it is recommended that the parties concerned come to an agreement on this subject.

9 Procedure

9.1 Test portion, initial suspension and dilutions

Use the method described in ISO 6887 (all parts) or the specific International Standard appropriate to the product concerned.

9.2 Inoculation and incubation

9.2.1 As surface inoculation is used for this method, the plates shall be dried before use, in accordance with ISO 11133, until the droplets have disappeared from the surface of the agar culture medium. Do not over-dry the plates.

Transfer, using a sterile pipette (6.8), 0,1 ml of the test sample if the product is liquid or of the initial suspension in the case of other products (non-liquid products) to the centre of one Petri dish (6.5) containing the plate count agar medium (see A.3).

If only one dilution is performed, then two plates shall be used (see ISO 7218).

To detect low microbial counts, the limit of detection may be increased by a factor of 10 by analysing 1,0 ml of the test sample (liquid product) or 1,0 ml of the initial suspension (non-liquid product). This is achieved by spreading 1,0 ml of the inoculum either over the surface of one large Petri dish (140 mm diameter) or over the surface of three small Petri dishes (90 mm diameter). If two plates per dilution are needed, prepare them using two large ones or six small ones.

9.2.2 Take one other Petri dish (6.5) containing the plate count agar medium (see A.3). Use another sterile pipette (6.8) to dispense 0,1 ml of the next decimal dilution (the colonies to be counted will then be present in a dilution step of 10^{-1} in the case of liquid products and 10^{-2} in the case of non-liquid products).

9.2.3 If necessary, repeat the procedure (see 9.2.2) with further dilutions.

9.2.4 Using a spreader or spatula (6.2), carefully spread the inoculum evenly over the agar surface as quickly as possible after dispensing without touching the sidewalls of the Petri dish (6.5). Allow the inoculum to be absorbed by the agar with the lids on for approximately 15 min at room temperature and check if the agar surface is dry before incubation.

A control plate without inoculum should be included for checking microbial contamination of the plate count agar plates used.

It is possible to use the same spreader (6.2) for all dilutions from the same test sample beginning with the highest dilution and progressing in order to the dilution having the greatest amount of test material.

9.2.5 Invert the plates and place them in the incubator (6.3) at 6,5 °C, in accordance with ISO 7218. Incubate for 10 days.

9.3 Counting of colonies

9.3.1 Following the period of incubation (see 9.2.5), count the colonies from the first (lowest) dilution with no more than 150 colonies. Count the colonies on the plates using the colony-counting device (6.1), if necessary. Examine the plates under subdued light. It is important that pinpoint colonies are included in the count; however, it is essential that the operator avoids mistaking particles of undissolved or precipitated matter in the plates for pinpoint colonies. Examine doubtful objects carefully, using higher magnification where required, in order to distinguish colonies from foreign matter.

NOTE A single clone of microorganisms multiplying in the presence of nutrients to form a visible growth on the solid agar culture medium is called a bacterial colony. The sizes of colonies, measured in millimetres are: pinpoint (≤ 1 mm), small (2 mm to 3 mm), medium (4 mm to 5 mm) and large (> 5 mm)[10].

9.3.2 Consider spreading colonies or a chain of overlapping or at least connected colonies as single colonies. If less than one-quarter of the plate is covered by spreading growth, count the colonies on the unaffected part and calculate the theoretical number for the entire plate by extrapolation. Discard the count if more than one-quarter is overgrown.

NOTE In cases of spreading colonies, an overlay can be used (see ISO 7218).

10 Expression of results

For calculation of the results, follow the procedure(s) in accordance with ISO 7218. Calculate and report the results as the number of psychrotrophic microorganisms in cfu per gram, millilitre, per square centimetre or per sampling device.

In cases where low counts (less than 10 colonies, no colonies or other special cases) of the target organism have been detected, follow ISO 7218 for the expression of results.

11 Test report

The test report shall specify the following:

— the test method used, with reference to this document, i.e. ISO 17410;

— the sampling method used, if known;

— the size of the test portion and/or the nature of the objects examined;

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

— any deviations from this document;

— all information necessary for the complete identification of the sample;

— the test result(s) obtained;

— the date of the test.

If the rapid method for the estimated enumeration of psychrotrophic microorganisms is used for raw or pasteurized milk samples, in accordance with Annex B, specify this in the test report.

12 Quality assurance

The laboratory should have a clearly defined quality control system to ensure that the equipment, reagents and techniques are suitable for the method. The use of positive controls, negative controls and blanks are part of the method. Performance testing of culture media is specified in Annex A and described in ISO 11133.

Annex A
(normative)

Culture media and reagents

A.1 General

The general specifications of ISO 11133 are applicable to the preparation and performance testing of the culture media described in this annex. If culture media or reagents are prepared from dehydrated complete media/reagents or if ready-to-use media/reagents are used, follow the manufacturer's instructions regarding preparation, storage conditions, expiry date and use.

The shelf life of the media indicated in this annex has been determined in some studies. The user shall verify these under their own storage conditions (in accordance with ISO 11133).

Performance testing of culture media is described in A.3.4.

A.2 Diluents

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

A.3 Agar culture medium: plate count agar (PCA)

A.3.1 Composition

Table A.1 provides an overview of the composition.

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

Table A.1 — Composition

Enzymatic digest 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.	

A.3.2 Preparation

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

Adjust the pH (6.6), if necessary, so that after sterilization it is 7,0 ± 0,2 at 25 °C.

Dispense the agar culture medium into tubes, bottles or flasks (6.9) of suitable capacity.

Sterilize for 15 min in the autoclave (6.4) set at 121 °C.

If the agar culture medium is to be used immediately, cool it in a water bath (6.10) maintained at 47 °C to 50 °C before use. If not, allow the agar culture medium to solidify in the tube, bottle or flask (6.9) and store it in the dark at a temperature of 5 °C (6.7) for no longer than three months, under conditions that do not allow any changes in its composition and properties. Before use, melt the agar culture medium completely in a boiling water bath, then cool it in the water bath (6.10) maintained at 47 °C to 50 °C.

A.3.3 Preparation of agar plates

Pour 18 ml to 20 ml of the agar culture medium into sterile Petri dishes with a diameter of 90 mm or pour 45 ml to 50 ml of the agar culture medium into sterile Petri dishes with a diameter of 140 mm (6.5), to obtain agar plates of at least 3 mm thickness, and allow to solidify.

The plates may be stored at 5 °C (6.7) for up to four weeks.

To facilitate uniform spreading, the surface of the plates containing the solidified agar culture medium should be dried in accordance with ISO 11133 so that the inoculum is absorbed within 15 min.

A.3.4 Performance testing

Plate count agar (PCA) is a non-selective agar culture medium, used as a pre-poured plate for surface inoculation. Productivity shall be tested according to ISO 11133.

Table A.2 — Performance testing for the quality assurance of PCA

Medium	Function	Incubation	Control strains	WDCM numbers[a]	Reference medium	Method of control	Criteria[c]	Characteristic reaction
PCA	Productivity	10 days/ 6,5 °C ± 1 °C	*Pseudomonas fluorescens* *Yersinia enterocolitica* subsp. *palearctica*	00115[b] 00216	Tryptone soya agar	Quantitative	$P_R \geq 0,7$	—

[a] Refer to the reference strain catalogue on http://www.wfcc.info for information on culture collection strain numbers and contact details; World Data Centre for Microorganisms (WDCM).

[b] Strain to be used as a minimum.

[c] P_R is productivity ratio.

Annex B
(informative)

Rapid method for the estimated enumeration of psychrotrophic microorganisms in raw or pasteurized milk

B.1 General

This annex specifies the procedure which can be used for the estimation of psychrotrophic microorganisms in raw or pasteurized milk (i.e. the rapid method).

B.2 Equipment and consumables

For the rapid method, the equipment and consumables are used as described in Clause 6, with the addition of the following.

B.2.1 **Incubator**, capable of operating at a temperature of 21 °C ± 1 °C.

B.2.2 **Water bath**, or **similar apparatus**, capable of operating at a temperature of 44 °C to 47 °C and of boiling water.

B.3 Procedure

B.3.1 General

For the rapid method, a similar procedure is followed as described in Clause 9. The differences are the use of the pour-plate technique, the incubation of the plates and the maximum number of colonies to count (300 cfu). Plates are incubated for 25 h ± 1 h in an incubator set at 21 °C (B.2.1).

B.3.2 Inoculation and incubation

B.3.2.1 Take two sterile Petri dishes (6.5). Transfer to each dish, by means of a sterile pipette (6.8), 1 ml of the liquid test sample. If plates of more than one dilution are prepared, this may be reduced to one dish per dilution (see ISO 7218).

B.3.2.2 Take one other sterile Petri dish (6.5). Use another sterile pipette (6.8) to dispense 1 ml of the next decimal dilution.

B.3.2.3 If necessary, repeat the procedure (see B.3.2.2) with further dilutions, using a new sterile pipette (6.8) for each decimal dilution.

B.3.2.4 If appropriate and possible, select only the critical dilution steps (at least two consecutive decimal dilutions) for the inoculation of the Petri dishes (6.5) that will give colony counts of between 10 and 300 colonies.

B.3.2.5 Pour about 12 ml to 15 ml of the plate count agar medium (see A.3) at 44 °C to 47 °C (B.2.2) into each Petri dish[11]. The time elapsed between the end of the preparation of the initial suspension and the moment when the medium (see A.3) is poured into the Petri dishes (6.5) shall not exceed 45 min.