INTERNATIONAL STANDARD

ISO 4832

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Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of coliforms — Colony-count technique

Microbiologie des aliments — Méthode horizontale pour le dénombrement des coliformes — Méthode par comptage des colonies

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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 4832 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

This third edition of ISO 4832 cancels and replaces ISO 4832:1991 and ISO 5541-1:1986. The main changes are follows:

— the alternative procedure of incubation at 35 °C has been deleted (see 4.2);

— a confirmation test in brilliant green lactose bile broth has been introduced (see 5.4 and 9.4).

Considering the nature of the changes to the previous edition of this International Standard, it is considered that the validation of alternative methods based on ISO 4832:1991 is not affected by this revision.

Introduction

Because of the large variety of food and feed products, this horizontal method may not be appropriate in every detail for certain products. In this case, different methods which are specific to these products may be used if absolutely necessary for justified technical reasons. Nevertheless, every attempt should be made to apply this horizontal method as far as possible.

When this International Standard is next reviewed, account will be taken of all information then available regarding the extent to which this horizontal method has been followed and the reasons for deviations from this method in the case of particular products.

The harmonization of test methods cannot be immediate, and for certain groups of products International Standards and/or national standards may already exist that do not comply with this horizontal method. It is hoped that when such standards are reviewed, they will be changed to comply with this International Standard so that eventually the only remaining departures from this horizontal method will be those necessary for well-established technical reasons.

The technique described in this International Standard is more precise than that described in ISO 4831[1], but does not allow a microbiological examination to be carried out on such a large test portion. It is therefore the preferred method when large numbers of coliforms are present. Moreover, since the definition of "coliforms" adopted in the two documents is different, the microorganisms enumerated are not necessarily the same. For any particular product, the method to be chosen will be specified in the International Standard dealing with that product.

For the purposes of a practicable test method, the definition of "coliforms" given in Clause 3 and used as the basis for the procedure is not necessarily identical to corresponding definitions given in other published texts. The method described in this International Standard will, on average, detect only about 90 % of strains of the microorganisms referred to in other publications as "(presumptive) coliforms" (e.g. certain strains of *Citrobacter*, *Enterobacter*, *Klebsiella*) (see Reference [2]).

Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of coliforms — Colony-count technique

1 Scope

This International Standard gives general guidelines for the enumeration of coliforms. It is applicable to

— products intended for human consumption and for the feeding of animals, and

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

by means of the technique of counting colonies after incubation on a solid medium at 30 °C or at 37 °C.

NOTE The temperature is subject to agreement between the parties concerned. In the case of milk and milk products, the temperature of incubation is 30 °C.

This technique is recommended when the number of colonies sought is expected to be more than 100 per millilitre or per gram of the test sample.

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

ISO 7218:—[1)], *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations*

ISO 8261, *Milk and milk products — General guidance for the preparation of test samples, initial suspensions and decimal dilutions for microbiological examination*

ISO/TS 11133-1, *Microbiology of food and animal feeding stuffs — Guidelines on preparation and production of culture media — Part 1: General guidelines on quality assurance for the preparation of culture media in the laboratory*

ISO/TS 11133-2:2003, *Microbiology of food and animal feeding stuffs — Guidelines on preparation and production of culture media — Part 2: Practical guidelines on performance testing of culture media*

1) To be published.

3 Terms and definitions

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

3.1
coliforms
bacteria which, at the specified temperature (i.e. 30 °C or 37 °C, as agreed) form characteristic colonies in crystal violet neutral red bile lactose agar, and which in the confirmation test cause fermentation of lactose with the production of gas under the test conditions specified in this International Standard

4 Principle

4.1 Two poured plates are prepared using a solid selective culture medium, with a specified quantity of the test sample if the initial product is liquid, or with a specified quantity of an initial suspension in the case of other products.

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

4.2 The plates are incubated at 30 °C or 37 °C (as agreed) for 24 h.

4.3 The characteristic colonies are counted and, if required, a number of colonies are confirmed by fermentation of lactose.

4.4 The number of coliforms per millilitre or per gram of sample is calculated from the number of characteristic colonies obtained in the plates chosen (see ISO 7218).

5 Culture media and diluents

5.1 General

See ISO 7218, ISO/TS 11133-1 and ISO/TS 11133-2 for the preparation, production and performance testing of culture media.

5.2 Diluents

See ISO 6887 (relevant part), ISO 8261 or the specific International Standard dealing with the product under examination.

5.3 Solid selective medium: Crystal violet neutral red bile lactose (VRBL) agar

5.3.1 Composition

Enzymatic digest of animal tissues	7 g
Yeast extract	3 g
Lactose ($C_{12}H_{22}O_{11}\cdot H_2O$)	10 g
Sodium chloride	5 g
Bile salts	1,5 g
Neutral red	0,03 g
Crystal violet	0,002 g
Agar[a]	12 g to 18 g
Water	1 000 ml
[a] Depending of the gel strength of the agar.	

5.3.2 Preparation

Proceed as follows in order to conserve the selectivity power and specificity of the medium.

Thoroughly mix the components or the dehydrated complete medium in the water and leave to stand for several minutes. Adjust the pH so that, after boiling, it is 7,4 ± 0,2 at 25 °C. Heat until boiling, stirring from time to time.

Allow to boil for 2 min. Immediately cool the medium in the water bath (6.5) at 44 °C to 47 °C.

To avoid overheating, do not heat the medium for too long nor reheat it. Consequently, do not sterilize it in the autoclave, and check the sterility of the medium at the time of use (see 9.2.2).

Use the medium within 4 h of its preparation.

5.3.3 Performance testing for the quality assurance of the culture medium

For the definitions of selectivity and productivity, refer to ISO/TS 11133-1. Performance testing relating to crystal violet neutral red bile lactose (VRBL) agar is given in ISO/TS 11133-2:2003, Table B.1.

5.4 Confirmation medium: Brilliant green lactose bile broth

5.4.1 Composition

Enzymatic digest of casein	10 g
Lactose ($C_{12}H_{22}O_{11}·H_2O$)	10 g
Dehydrated ox bile	20 g
Brilliant green	0,013 3 g
Water	1 000 ml

5.4.2 Preparation

Dissolve the components of the dehydrated complete medium in the water by heating gently if necessary in a water bath (6.5). If necessary, adjust the pH so that, after sterilization, it is 7,2 ± 0,2 at 25 °C.

Dispense the medium, in quantities of 10 ml, in test tubes (6.7) containing Durham tubes (6.8). Sterilize in an autoclave (6.1) at 121 °C for 15 min. The Durham tubes shall not contain air bubbles after sterilization.

6 Apparatus and glassware

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

6.1 Apparatus for dry sterilization (oven) or **wet sterilization** (autoclave).

See ISO 7218.

6.2 Incubator, capable of operating at 30 °C ± 1 °C or 37 °C ± 1 °C.

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

6.4 Total-delivery pipettes, having nominal capacities of 1 ml.

6.5 Water bath, or similar apparatus, capable of operating at 44 °C to 47 °C or at 100 °C.

6.6 Colony-counting equipment, consisting of an illuminated base and a mechanical or electronic digital counter.

6.7 Test tubes, of dimensions approximately 16 mm × 160 mm.

6.8 Durham tubes, of dimensions appropriate for use with the test tubes (6.7).

6.9 Bottles or **flasks**, for boiling and storage of culture media.

6.10 pH-meter, accurate to ±0,1 pH unit at 25 °C.

6.11 Loop, of platinum-iridium or nickel-chromium, approximately 3 mm in diameter, or disposable loops.

7 Sampling

Sampling should have been carried out in accordance with the specific International Standard appropriate to the product concerned. If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject.

8 Preparation of test sample

Prepare the test sample in accordance with ISO 6887 (relevant part), ISO 8261 or the specific International Standard appropriate to the product concerned. If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject.

9 Procedure

9.1 Test portion, initial suspension and dilutions

Prepare the test portion, initial suspension (primary dilution) and further dilutions in accordance with ISO 6887 (relevant part), ISO 8261 or the specific International Standard appropriate to the product concerned.

9.2 Inoculation and incubation

9.2.1 Prepare two dishes for the liquid product and/or from each dilution chosen. Transfer, with a sterile pipette (6.4), 1 ml of liquid product or the appropriate dilutions to the centre of each dish. Use another sterile pipette to inoculate each dilution into the dishes.

9.2.2 Pour about 15 ml of the VRBL medium (5.3), at 44 °C to 47 °C, into each Petri dish. The time elapsing 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 is poured into the dishes shall not exceed 15 min.

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

Also prepare a control plate with 15 ml of the medium for checking its sterility.

9.2.3 After complete solidification, pour about 4 ml of the VRBL medium (5.3), at 44 °C to 47 °C, onto the surface of the inoculated medium. Allow to solidify as described above.

9.2.4 Invert the prepared dishes and incubate them in the incubator (6.2) set at 30 °C or 37 °C (as agreed) for 24 h ± 2 h.

9.3 Enumeration

After the specified period of incubation (see 9.2.4), select the Petri dishes with, if possible, 10 or more colonies and fewer than 150 colonies. Count, using the colony-counting equipment (6.6), the purplish red colonies with a diameter of at least 0,5 mm (sometimes surrounded by a reddish zone of precipitated bile). These are considered as typical colonies of coliforms and do not require further confirmation.

For details of the colony-count technique, see ISO 7218.

Also count and confirm atypical colonies (e.g. of smaller size), and all colonies derived from milk products that contain sugars other than lactose, immediately after the incubation period according to 9.4. Conversion of sugars other than lactose may result in colonies with an appearance that looks similar to the typical coliforms.

NOTE The appearance of a reddish zone of precipitated bile around the colonies depends on the type of coliform and the quality of the medium.

9.4 Confirmation

Inoculate five colonies of each atypical type, if available, into tubes of brilliant green lactose bile broth (5.4). Incubate the tubes in the incubator (6.2) set at 30 °C or 37 °C (as agreed) for 24 h ± 2 h. Consider as coliforms colonies that show gas formation in the Durham tube. Take the results into account in the calculation (Clause 10).

10 Expression of results

See ISO 7218.

11 Precision

Given a Poisson distribution of microorganisms in the substrate, the confidence limits of this method vary according to the count of colonies examined from ± 16 % to ± 52 % (see Reference [3]). In practice, even greater variation may be found. In various collaborative studies, the standard deviation of the repeatability (s_r) appeared to be 0,20 log units, the standard deviation of the reproducibility (s_R) appeared to be 0,35 log units (see References [4] and [5]).

More information about the confidence limits is given in ISO 7218.

12 Test report

The test report shall specify:

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

— the sampling method used, if known;

— the test method used, with reference to this International Standard;

— all operating details not specified in this International Standard, or regarded as optional, together with details of any incident which may have influenced the result(s);

— the test result(s) obtained;

— if the repeatability has been checked, the final quoted results obtained.