INTERNATIONAL STANDARD

ISO 4832

Second edition
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Microbiology — General guidance for the enumeration of coliforms — Colony count technique

Microbiologie — Directives générales pour le dénombrement des coliformes — Méthode par comptage des colonies

Reference number
ISO 4832:1991(E)

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.

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.

International Standard ISO 4832 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*.

This second edition cancels and replaces the first edition (ISO 4832:1978), of which it constitutes a technical revision.

Annex A forms an integral part of this International Standard.

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Introduction

0.1 This International Standard is intended to provide general guidance for the examination of products not dealt with by existing International Standards and for reference for bodies preparing microbiological methods of test for application to foods or to animal feeding stuffs. Because of the large variety of products within this field of application, these guidelines may not be appropriate for some products in every detail, and for some other products it may be necessary to use different methods. Nevertheless, it is hoped that in all cases every attempt will be made to apply the guidelines provided as far as possible and that deviations from them will only be made if absolutely necessary for technical reasons.

When this International Standard is next reviewed, account will be taken of all information then available regarding the extent to which the guidelines have been followed and the reasons for deviation from them 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 these guidelines. In cases where International Standards already exist for the product to be tested, they should be followed, but 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 these guidelines will be those necessary for well-established technical reasons.

0.2 The technique described in this International Standard is more precise than that described in ISO 4831:1990, *Microbiology — General guidance for the enumeration of coliforms — Most probable number technique*, 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 micro-organisms 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.

0.3 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 with 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 micro-organisms referred to in other publications as "(presumptive) coliforms" (e.g. certain strains of *Citrobacter*, *Enterobacter*, *Klebsiella)*. (See Edwards, P.R. and Ewing, H.W. *Identification of Enterobacteriaceae,* 3rd edition, Burgess Publishing Company, Minneapolis, Minnesota, USA, 1972.)

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Microbiology — General guidance for the enumeration of coliforms — Colony count technique

1 Scope

This International Standard gives general guidelines for the enumeration of coliforms present in products intended for human consumption or feeding of animals, by means of the technique of counting colonies on a solid medium, after incubation at 30 °C, 35 °C or 37 °C, this temperature forming the subject of agreement between the parties concerned.

NOTE 1 The incubation temperature of 30 °C is used when the aim of the enumeration is technological; the temperature of 35 °C or 37 °C is used when the aim of the enumeration is more in the field of public health.

2 Normative references

The following standards contain provisions which, through reference in this text, constitute provisions of this International Standard. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 6887:1983, *Microbiology — General guidance for the preparation of dilutions for microbiological examination.*

ISO 7218:1985, *Microbiology — General guidance for microbiological examinations.*

3 Definition

For the purposes of this International Standard, the following definition applies.

coliforms: Bacteria which, at the specified temperature (i.e. 30 °C, 35 °C or 37 °C, as agreed) form characteristic colonies in crystal violet neutral red bile lactose agar under the test conditions specified in this International Standard.

4 Principle

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

Preparation of other pairs of poured plates, under the same conditions, using decimal dilutions of the test sample or of the initial suspension.

4.2 Incubation of the plates at 30 °C, 35 °C or 37 °C (as agreed) for 24 h.

4.3 Calculation of the number of coliforms per millilitre or per gram of sample from the number of characteristic colonies obtained in the plates chosen (see 10.1).

5 Culture medium and dilution fluid

5.1 General

For current laboratory practice, see ISO 7218.

5.2 Dilution fluid

See ISO 6887 and the specific International Standard dealing with the product under examination.

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

Composition

peptone	7 g
yeast extract	3 g
lactose	10 g
sodium chloride	5 g
bile salts	1,5 g
neutral red	0,03 g
crystal violet	0,002 g
agar	12 g to 18 g[1)]
water	1 000 ml

Preparation

Proceed as follows in order to conserve the selective 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 at 25 °C. Bring to the boil, stirring from time to time.

Allow to boil for 2 min. Immediately cool the medium in the water-bath (6.5) set at 45 °C.

Avoid overheating the medium, heating it for too long or reheating it. Consequently, do not sterilize in the autoclave, and check the sterility of the medium at the time of use (see 9.2.2).

Use the medium within 3 h of its preparation.

6 Apparatus and glassware

NOTE 2 Disposable apparatus is an acceptable alternative to reusable glassware if it has suitable specifications.

Usual microbiological laboratory equipment 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, 35 °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 a nominal capacity of 1 ml.

6.5 Water-bath, or similar apparatus, capable of operating at 45 °C ± 0,5 °C.

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

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

7 Sampling

Sampling shall 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 the test sample

Prepare the test sample in accordance with 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.

9 Procedure

9.1 Test portion, initial suspension and dilutions

See ISO 6887 and the specific International Standard appropriate to the product concerned.

9.2 Inoculation and incubation

9.2.1 Take two sterile Petri dishes (6.3). Using a sterile pipette (6.4), transfer to each dish 1 ml of the test sample, if the product is liquid, or 1 ml of the initial suspension in the case of other products.

Take two other sterile Petri dishes. Using a fresh sterile pipette, transfer to each dish 1 ml of the first decimal dilution (10^{-1}) of the test sample, if the product is liquid, or 1 ml of the first decimal dilution (10^{-2}) of the initial suspension in the case of other products.

Repeat the procedure described with the further dilutions, using a fresh sterile pipette for each decimal dilution.

1) According to the gel strength of the agar.

9.2.2 Pour about 15 ml of the VRBL medium (5.3), at 45 °C ± 0,5 °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 (5.3) 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 45 °C ± 0,5 °C, on to 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 set at 30 °C, 35 °C or 37 °C (as agreed) for 24 h ± 2 h.

9.3 Counting of the colonies

After the specified period of incubation (see 9.2.4), count, using the colony counting equipment (6.6), the characteristic coliform colonies in each dish containing not more than 150 colonies[2] whether characteristic or not.

NOTE 3 After incubation for 24 h, characteristic colonies are purplish red colonies having a diameter of 0,5 mm or greater and sometimes surrounded by a reddish zone of precipitated bile.

10 Expression of results

10.1 Method of calculation

10.1.1 General case — Dishes containing between 15 and 150 characteristic colonies

Retain dishes containing not more than 150 characteristic colonies at two consecutive dilutions. It is necessary that one of these dishes contains at least 15 characteristic colonies.

Calculate the number N of coliforms per millilitre or per gram of product, depending on the case, using the following equation:

$$N = \frac{\sum C}{(n_1 + 0{,}1n_2)d}$$

where

$\sum C$ is the sum of the characteristic colonies counted on all the dishes retained;

n_1 is the number of dishes retained in the first dilution;

n_2 is the number of dishes retained in the second dilution;

d is the dilution factor corresponding to the first dilution.

Round the result calculated to two significant figures.

Take as the result the number of coliforms per millilitre or per gram of product, expressed as a number between 1,0 and 9,9 multiplied by 10^x, where x is the appropriate power of 10.

EXAMPLE

A coliform count at 30 °C gave the following results:

— at the first dilution retained (10^{-2}): 83 and 97 characteristic colonies

— at the second dilution retained (10^{-3}): 13 and 8 characteristic colonies

$$N = \frac{\sum C}{(n_1 + 0{,}1n_2)d}$$

$$= \frac{83 + 97 + 13 + 8}{(2 + 0{,}1 \times 2) \times 10^{-2}}$$

$$= \frac{201}{0{,}022} = 9136$$

Rounding the result as specified above gives 9 100 or 9,1 × 10^3 coliforms per millilitre or per gram of product.

10.1.2 Case where each dish contains less than 15 characteristic colonies

If each of the dishes retained contains less than 15 characteristic colonies, calculate the estimated number N_E of coliforms using the equation given in 10.1.1.

EXAMPLE

A coliform count at 30 °C gave the following results:

— at the 10^{-4} dilution: 140 and 145 colonies, of which 5 and 3 colonies respectively were characteristic

2) Above this number there is a risk that coliform colonies will have an atypical appearance.

— at the 10^{-5} dilution: 11 and 8 colonies, of which 0 and 1 colonies respectively were characteristic

$$N_E = \frac{5 + 3 + 0 + 1}{(2 + 0{,}1 \times 2) \times 10^{-4}}$$

$$= \frac{9}{2{,}2 \times 10^{-4}} = 40000$$

Rounding the result as specified in 10.1.1 gives $4{,}0 \times 10^4$ coliforms per millilitre or per gram of product.

10.1.3 Estimation of small numbers

If the two dishes, corresponding to the test sample (liquid products) or the initial suspension (other products), contain less than 15 characteristic colonies, report the result as follows:

— less than 15 coliforms per millilitre (liquid products);

— less than $15 \times 1/d$ coliforms per gram (other products), where d is the dilution factor of the initial suspension.

10.1.4 No characteristic colonies

If the two dishes, corresponding to the test sample (liquid products) or the initial suspension (other products), contain no characteristic colonies, report the result as follows:

— less than 1 coliform per millilitre (liquid products);

— less than $1 \times 1/d$ coliform per gram (other products), where d is the dilution factor of the initial suspension.

10.2 Precision

10.2.1 Dishes containing between 15 and 150 characteristic colonies (see 10.1.1)

For statistical reasons alone, in 95 % of cases the confidence limits of this method vary from ± 16 % to ± 52 % (Cowell and Morisetti, *J. Sci. Fd. Agric.*, 1969, Vol. 20, p. 573). In practice, even greater variation may be found especially among results obtained by different microbiologists.

10.2.2 Each dish contains less than 15 characteristic colonies (see 10.1.2)

Refer to table A.1. To obtain the confidence limits, multiply the lower and upper limits given by $1/d$, where d is the dilution factor.

10.2.3 Estimation of small numbers (see 10.1.3)

The confidence limits for the estimation of small numbers of coliforms are given in table A.1.

11 Test report

The test report shall specify the method used, the aim (technological or public health) of the test and the temperature chosen, and the results obtained. It shall also mention all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the results.

The test report shall include all information necessary for the complete identification of the sample.

Annex A
(normative)

Confidence limits for the estimation of small numbers of colonies

The confidence limits at the 95 % level for the estimation of small numbers, when the number of characteristic colonies on dishes retained is less than 15, are given in table A.1.

Table A.1

Number of coliforms	Confidence limits at the 95 % level	
	lower	upper
1	< 1	2
2	< 1	4
3	< 1	5
4	1	6
5	2	9
6	2	10
7	2	12
8	3	13
9	4	14
10	4	16
11	5	18
12	6	19
13	7	20
14	7	21
15	8	23