INTERNATIONAL STANDARD

ISO 4831

Second edition
1991-03-01

Microbiology — General guidance for the enumeration of coliforms — Most probable number technique

Microbiologie — Directives générales pour le dénombrement des coliformes — Technique du nombre le plus probable

Reference number
ISO 4831:1991(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 4831 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*.

This second edition cancels and replaces the first edition (ISO 4831:1978), of which it constitutes a technical revision.

Annexes A and B form an integral part of this International Standard.

International Organization for Standardization
Case Postale 56 • CH-1211 Genève 20 • Switzerland

Printed in Switzerland

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 less precise than that described in ISO 4832:1990, *Microbiology — General guidance for the enumeration of coliforms — Colony count technique*, but allows a microbiological examination to be carried out on a larger test portion, thus permitting a lower number of coliforms per gram or per millilitre of product to be detected. 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. A proportion of strains of the micro-organisms described in other published texts as "coliforms" (including *Escherichia coli*) fail to produce enough gas to be detectable by use of a Durham tube (i.e. "anaerogenic strains"). Therefore, the method described in this International Standard will not detect all 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, W.H. *Identification of Enterobacteriaceae*, 3rd edition, Burgess Publishing Company, Minneapolis, Minnesota, USA, 1972.)

Microbiology — General guidance for the enumeration of coliforms — Most probable number 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 calculation of the most probable number (MPN) after incubation at 30 °C, 35 °C or 37 °C in a liquid medium, 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.

A limitation on the applicability of this International Standard is imposed by the method's susceptibility to a large degree of variability. The method should be applied and the results interpreted in the light of the information given in 10.4.

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 6579:1990, *Microbiology — General guidance on methods for the detection of Salmonella.*

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) cause fermentation of lactose with the production of gas under the test conditions specified in this International Standard.

4 Principle

4.1 Inoculation of three tubes of double-strength liquid selective enrichment medium [see 5.3 a)] 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.

4.2 Inoculation of three tubes of single-strength liquid selective enrichment medium [see 5.3 b)] 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.

Then, under the same conditions, inoculation of further tubes of medium 5.3 b) with decimal dilutions of the test sample or of the initial suspension.

4.3 Incubation at 30 °C, 35 °C or 37 °C (as agreed) of the tubes containing double-strength medium [5.3 a)] for 24 h and of the tubes containing single-strength medium [5.3 b)] for 24 h or 48 h, and examination of these tubes.

4.4 Inoculation of a series of tubes of the confirmation medium (5.4), with the cultures from the tubes of medium 5.3 a) and with those cultures from the first series of tubes of medium 5.3 b) in which opacity or gas formation has been noted.

4.5 Incubation at 30 °C, 35 °C or 37 °C (as agreed) for 24 h or 48 h, and examination of the new series of tubes (4.4).

4.6 Calculation of the most probable number of coliforms per millilitre or per gram of sample (i.e. the MPN), from the number of tubes in the new series (4.5) showing gas formation, using a table for determination of most probable numbers.

5 Culture media 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 Lauryl sulfate tryptose broth (selective enrichment medium)

Composition

	a) double-strength medium	b) single-strength medium
tryptose	40 g	20 g
lactose	10 g	5 g
dipotassium hydrogen phosphate (K_2HPO_4)	5,5 g	2,75 g
potassium dihydrogen phosphate (KH_2PO_4)	5,5 g	2,75 g
sodium chloride	10 g	5 g
sodium lauryl sulfate	0,2 g	0,1 g
water	1 000 ml	1 000 ml

Preparation

Dissolve the components or the dehydrated complete medium in the water, by heating if necessary.

Adjust the pH, if necessary, so that after sterilization it is 6,8 at 25 °C.

Dispense the media in quantities of 10 ml into tubes of dimensions 16 mm × 160 mm (6.4) containing Durham tubes (6.5) in the case of single-strength medium, and into test tubes of dimensions 20 mm × 200 mm (6.4) [not containing Durham tubes (6.5)] in the case of the double-strength medium.

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

The Durham tubes shall not contain air bubbles after sterilization.

5.4 Lactose bile brilliant green broth (confirmation medium)

Composition

peptone	10 g
lactose	10 g
dehydrated ox bile	20 g
brilliant green[1]	0,013 3 g
water	1 000 ml

Preparation

Dissolve the components or the dehydrated complete medium in the water, by heating if necessary.

Adjust the pH, if necessary, so that after sterilization it is 7,2 at 25 °C.

Dispense the medium, in quantities of 10 ml, in 16 mm × 160 mm test tubes (6.4) containing Durham tubes (6.5).

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

The Durham tubes shall not contain air bubbles after sterilization.

NOTE 2 Because the dehydrated complete medium may not always produce the expected result, its performance should be checked before use.

6 Apparatus and glassware

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

1) Corresponding to the specifications given in ISO 6579:1981, annex C.

6.4 Test tubes, of dimensions approximately 16 mm × 160 mm and 20 mm × 200 mm, or **bottles** of suitable capacity.

6.5 Durham tubes, of a size suitable for use in the test tubes of dimensions 16 mm × 160 mm (6.4).

6.6 Total delivery pipettes, having nominal capacities of 1 ml and 10 ml.

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 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 (see the diagram in annex A)

Where several samples taken from the same batch are to be examined, carry out the following operations for each sample.

9.1 Test portion, initial suspension and dilutions

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

Prepare a sufficient number of dilutions to ensure that all the tubes corresponding to the final dilution will yield a negative result.

9.2 Inoculation[2] and incubation

9.2.1 Take three tubes of double-strength selective enrichment medium [5.3a)]. Using a sterile pipette (6.6), transfer to each of these tubes 10 ml of the test sample, if liquid, or 10 ml of the initial suspension, in the case of other products.

9.2.2 Then take three tubes of single-strength selective enrichment medium [5.3b)]. Using a fresh sterile pipette (6.6), transfer to each of these tubes 1 ml of the test sample, if liquid, or 1 ml of the initial suspension, in the case of other products.

9.2.3 For each of the further dilutions (from 10^{-1} or 10^{-2}, according to the test sample), continue as described in 9.2.2. Use a fresh sterile pipette for each dilution. Carefully mix the inoculum and the medium.

9.2.4 Leave the tubes of double-strength medium (9.2.1) in the incubator (6.2) set at 30 °C, 35 °C or 37 °C (as agreed) for 24 h ± 2 h.

9.2.5 Leave the tubes of single-strength medium (9.2.2 and 9.2.3) in the incubator (6.2) at 30 °C, 35 °C or 37 °C (as agreed) for 24 h ± 2 h or, if neither gas formation nor opacity preventing the observation of gas formation is observed at this stage, for 48 h ± 2 h.

9.3 Confirmation

9.3.1 From each of the incubated tubes from 9.2.4, inoculate with a loop (6.3) a tube of confirmation medium (5.4). Incubate in the incubator set at 30 °C, 35 °C or 37 °C (as agreed) for 24 h ± 2 h or, if gas formation is not observed at this stage, for 48 h ± 2 h.

9.3.2 Carry out the same procedure for the incubated tubes from 9.2.5 showing gas formation or opacity, when either of these features is first observed (i.e. after 24 h ± 2 h or after 48 h ± 2 h).

9.4 Interpretation

For each dilution, count the total number of tubes in which gas formation is observed in 9.3 (positive tubes) after 24 h ± 2 h and (if used) 48 h ± 2 h.

---

2) It is envisaged that there is a combination of three tubes for each dilution series. For some products and/or each time that results of greater accuracy are required, it is necessary to inoculate series consisting of five tubes (see table B.2).

10 Expression of results

10.1 Selection of dilutions[3)]

For each sample examined, select three consecutive dilutions in accordance with one of the three following cases, whichever is appropriate.

10.1.1 Case 1 — At least one dilution yields three positive tubes

Select the highest dilution (i.e. that having the lowest sample concentration) yielding three positive tubes, together with the next two higher dilutions (i.e. those having sample concentrations of 1/10 and 1/100 of that of the first dilution selected) (see table 1, example 1).

See also 10.1.3.

If sufficient further dilutions were made beyond the highest dilution yielding three positive tubes, select instead the three highest dilutions in the series (i.e. those having the lowest sample concentration) (see table 1, example 2).

10.1.2 Case 2 — No dilution yields three positive tubes

Case 1 cannot be applied. Select the three highest dilutions in the series (i.e. those having the lowest sample concentration) amongst which at least one positive result was obtained (see table 1, example 3).

See also 10.1.3.

10.1.3 Special cases

In all cases where more than one of the three dilutions selected in accordance with 10.1.1 and 10.1.2 does not yield positive tubes, select from these dilutions the lowest one not yielding positive tubes (i.e. that having the highest sample concentration) and the two next lower dilutions in the series (i.e. those having sample concentrations of ten times and one hundred times that of the first dilution selected) (see table 1, examples 4 and 5), except when positive tubes are only found at the level of the first dilution prepared from the sample. In this last case, it is necessary to select the first three dilutions for calculation of the MPN even though this series includes two dilutions yielding no positive tube.

See the examples given in table 1.

10.2 Determination of MPN index

10.2.1 According to the number of samples examined per batch, check, using table B.1 or table B.2, whether the sequences of numbers of positive tubes corresponding to the dilutions selected in accordance with 10.1 are statistically acceptable. Acceptability depends both on the number of samples examined and on the decision whether or not to accept category 2 results.

Thus, for example, if only category 1 results are accepted, the sequence 221 is acceptable only when 10 samples (of the batch concerned) have been examined. However, if the less likely category 2 results are also accepted, the sequence 221 is also acceptable when only 2, 3 or 5 samples have been examined. However, when the sequence 221 is the result of a single examination, it is never acceptable.

Table 1 — Examples of the selection of positive results for the calculation of the MPN

Example	Number of positive tubes obtained from three incubated tubes for the following amounts of sample inoculated per tube[1)]					MPN[2)]	
	Liquid product / Other products	10 ml / 1 g	1 ml / 10^{-1} g	10^{-1} ml / 10^{-2} g	10^{-2} ml / 10^{-3} g	10^{-3} ml / 10^{-4} g	Liquid product ml^{-1}	Other products g^{-1}
1		3	3	2	1	0	$1,5 \times 10^{1}$	$1,5 \times 10^{2}$
2		3	3	3	0		$2,4 \times 10^{1}$	$2,4 \times 10^{2}$
3		2	2	1	1	0	7,4	$7,4 \times 10^{1}$
4		3	3	0	0	0	2,4	$2,4 \times 10^{1}$
5		2	2	0	1	0	$2,1 \times 10^{-1}$	2,1

1) _____, combination selected.

2) Calculated using the MPN index for three tubes (table B.1).

3) In this subclause, the initial suspension and, if necessary, the test sample are considered as dilutions.

10.2.2 For each sequence found to be acceptable in accordance with 10.2.1, obtain the MPN index from table B.1 or table B.2.

10.3 Calculation of most probable number (MPN)

Obtain the number of coliforms per millilitre or per gram by multiplying the MPN index (see 10.2) by the reciprocal of the lowest dilution selected (i.e. that having the highest sample concentration).

When the lowest dilution selected corresponds to the tubes prepared with double-strength medium (inoculation with 10 ml), first divide the MPN index by 10.

Express the result as a number between 1,0 and 9,9 multiplied by 10^x, where x is the appropriate power of 10.

10.4 Precision

It is recognized that wide variations in results may occur with the MPN technique. Results obtained using this method should therefore be used with caution.

Confidence limits are given in table B.1 and table B.2.

EXAMPLE

For a solid sample, in 95 % of the cases, the confidence limits vary from 13 to 200 coliforms per gram for an MPN of $7,4 \times 10^1$ coliforms per gram and from 4 to 99 coliforms per gram for an MPN of $2,4 \times 10^1$ coliforms per gram.

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.