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Mazdoor Kisan Shakti Sangathan
“The Right to Information, The Right to Live”

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Jawaharlal Nehru
“Step Out From the Old to the New”

Indian Standard

MICROBIOLOGY OF FOOD AND ANIMAL FEEDING STUFFS — HORIZONTAL METHOD FOR THE DETECTION AND ENUMERATION OF COLIFORMS

PART 2  MOST PROBABLE NUMBER TECHNIQUE

( Second Revision )

ICS 07.100.30

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BUREAU OF INDIAN STANDARDS
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NEW DELHI 110002
NATIONAL FOREWORD

This Indian Standard (Part 2) (Second Revision) which is identical with ISO 4831 : 2006 ‘Microbiology of food and animal feeding stuffs — Horizontal method for the detection and enumeration of coliforms — Most probable number technique’ issued by the International Organization for Standardization (ISO) was adopted by the Bureau of Indian Standards on the recommendation of the Food Hygiene, Safety Management and Other Systems Sectional Committee and approval of the Food and Agriculture Division Council.

This standard was originally published in 1969. The first revision of this standard was undertaken in 2002 to align with the earlier versions of ISO Standards on the subject, namely, ISO 4831 : 1991 ‘Microbiology — General guidance for the enumeration of coliforms — Most probable number technique’ and ISO 4832 : 1991 ‘Microbiology — General guidance for the enumeration of coliforms — Colony count technique’. Accordingly, first revision of Part 1 of IS 5401 covered general guidance for enumeration of coliforms by colony-count technique, which was identical with ISO 4832 : 1991 and first revision of Part 2 of IS 5401 covered general guidance for enumeration of coliforms by most probable number technique, which was identical with ISO 4831 : 1991. The second revision of this standard has been undertaken to align it with the latest version of the International Standard, namely, ISO 4831 : 2006.

The text of ISO Standard has been approved as suitable for publication as an Indian Standard without deviations. Certain conventions are, however, not identical to those used in Indian Standards. Attention is particularly drawn to the following:

a) Wherever the words ‘International Standard’ appear referring to this standard, they should be read as ‘Indian Standard’.

b) Comma (,) has been used as a decimal marker while in Indian Standards, the current practice is to use a point (.) as the decimal marker.

In this adopted standard, reference appear to the following International Standard for which Indian Standard also exists. The corresponding Indian Standard which is to be substituted in its place is listed below along with its degree of equivalence for the edition indicated:

<table>
<thead>
<tr>
<th>International Standard</th>
<th>Corresponding Indian Standard</th>
<th>Degree of Equivalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISO 6887-1 : 1999 Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions</td>
<td>IS 10232 : 2003 General rules for the preparation of initial suspension and decimal dilutions for microbiological examination of foods (first revision)</td>
<td>Identical</td>
</tr>
</tbody>
</table>

The technical committee has reviewed the provisions of the following International Standards referred in this adopted standard and has decided that they are acceptable for use in conjunction with this standard:

<table>
<thead>
<tr>
<th>International Standard</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISO 6887-2 : 2003</td>
<td>Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 2: Specific rules for the preparation of meat and meat products</td>
</tr>
</tbody>
</table>

(Continued on third cover)
1 Scope

This International Standard gives general guidelines for the detection and 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.

Enumeration is carried out by calculation of the most probable number (MPN) after incubation in a liquid medium at 30 °C or 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 enumeration method is applicable when the number sought is expected to be in the range 1 to 100 per millilitre or per gram of test sample.

A limitation on the applicability of this International Standard is imposed by the susceptibility of the method to a large degree of variability. The information given in Clause 11 provides guidance on the applicability of the method and on the interpretation of the results.

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, 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

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

3.2 detection of coliforms
determination of the presence or absence of these bacteria, in a particular quantity of product, when tests are carried out in accordance with the method specified in this International Standard

3.3 enumeration of coliforms
most probable number of coliforms found per millilitre or per gram of the test sample, when the test is carried out in accordance with the method specified in this International Standard

4 Principle

4.1 Detection of coliforms

4.1.1 A tube of selective enrichment broth is inoculated with the test portion and incubated at 30 °C or 37 °C (as agreed) for 24 h or 48 h.

4.1.2 A tube of confirmation medium is inoculated from the tube obtained in 4.1.1 when opacity and/or gas formation has been noted, and incubated at 30 °C or 37 °C (as agreed) for 24 h or 48 h.

4.1.3 The presence of coliforms is confirmed in the case that opacity and gas formation have been noted after examination of the tube obtained in 4.1.2.

4.2 Enumeration by the MPN technique

4.2.1 Three tubes of double-strength liquid selective enrichment medium are inoculated 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.

4.2.2 Three tubes of single-strength liquid selective enrichment medium are inoculated 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, further tubes of single-strength medium are inoculated with decimal dilutions of the test sample or of the initial suspension.

4.2.3 The tubes containing double-strength selective enrichment medium are incubated at 30 °C or 37 °C (as agreed) for 24 h, and the tubes containing single-strength medium are incubated for 24 h or 48 h, after which period these tubes are examined for gas formation or opacity preventing the detection of gas formation.

4.2.4 A series of tubes of the confirmation medium are inoculated with the cultures from the tubes of double-strength selective enrichment medium, and with the cultures from the tubes of single-strength selective enrichment medium in which gas formation or opacity preventing the detection of gas formation has been noted.

4.2.5 The tubes from 4.2.4 are incubated at 30 °C or 37 °C (as agreed) for 24 h or 48 h and the tubes are examined for gas formation.

4.2.6 The most probable number of coliforms per millilitre or per gram of sample (i.e. the MPN) is calculated from the number of tubes in the new series (4.2.5) showing gas formation. A table for determination of most probable numbers is used.
5 Culture media and diluents

5.1 General


5.2 Diluents

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

5.3 Selective enrichment medium: Lauryl sulfate tryptose broth

5.3.1 Composition

<table>
<thead>
<tr>
<th>Component</th>
<th>a) Double-strength medium</th>
<th>b) Single-strength medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymatic digest of milk and animal proteins</td>
<td>40 g</td>
<td>20 g</td>
</tr>
<tr>
<td>Lactose ($C_{12}H_{22}O_{11} \cdot H_2O$)</td>
<td>10 g</td>
<td>5 g</td>
</tr>
<tr>
<td>Dipotassium hydrogen phosphate ($K_2HPO_4$)</td>
<td>5,5 g</td>
<td>2,75 g</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate ($KH_2PO_4$)</td>
<td>5,5 g</td>
<td>2,75 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>10 g</td>
<td>5 g</td>
</tr>
<tr>
<td>Sodium lauryl sulfate</td>
<td>0,2 g</td>
<td>0,1 g</td>
</tr>
<tr>
<td>Water</td>
<td>1 000 ml</td>
<td>1 000 ml</td>
</tr>
</tbody>
</table>

5.3.2 Preparation

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

Adjust the pH, if necessary, so that after sterilization it is $6,8 \pm 0,2$ at 25 °C.

Dispense the media in quantities of 10 ml into tubes of dimensions of approximately $16 \text{ mm} \times 160 \text{ mm}$ (6.4) containing Durham tubes (6.5) in the case of single-strength medium, and into test tubes of dimensions of approximately $20 \text{ mm} \times 200 \text{ mm}$ (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.

5.3.3 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 lauryl sulfate tryptose broth is given in ISO/TS 11133-2:2003, Table B.1.
5.4 Confirmation medium: Brilliant green lactose bile broth

5.4.1 Composition

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymatic digest of casein</td>
<td>10 g</td>
</tr>
<tr>
<td>Lactose ( \text{C}<em>{12}\text{H}</em>{22}\text{O}<em>{11}\cdot\text{H}</em>{2}\text{O} )</td>
<td>10 g</td>
</tr>
<tr>
<td>Dehydrated ox bile</td>
<td>20 g</td>
</tr>
<tr>
<td>Brilliant green</td>
<td>0.0133 g</td>
</tr>
<tr>
<td>Water</td>
<td>1 000 ml</td>
</tr>
</tbody>
</table>

5.4.2 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 \(7.2 \pm 0.2\) at 25 °C.

Dispense the medium in quantities of 10 ml in test tubes of approximately 16 mm × 160 mm (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.

5.4.3 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 lactose bile brilliant green broth is given in ISO/TS 11133-2:2003, Table B.1.

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

6.4 Test tubes, of dimensions approximately 16 mm × 160 mm and 20 mm × 200 mm.

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 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 the 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 (see Annex A)

9.1 Detection method (see Figure A.1)

9.1.1 Test portion and initial suspension

See ISO 6887 (relevant part), ISO 8261 or the specific International Standard appropriate to the product concerned.

9.1.2 Inoculation and incubation

9.1.2.1 Depending on the limit of detection that is required, \(x\) ml of the test sample if liquid, or \(x\) ml of the initial suspension in the case of other products, is transferred to a tube containing 10 ml of double-strength selective enrichment medium [5.3.1a)] when \(1 \text{ ml} < x < 10 \text{ ml}\), or to a tube containing 10 ml of single-strength selective enrichment medium [5.3.1b)] when \(x \leq 1 \text{ ml}\).

9.1.2.2 Leave the tube of double-strength medium (9.1.2.1) in the incubator (6.2) set at 30 °C or 37 °C (as agreed) for 24 h ± 2 h.

9.1.2.3 Leave the tube of single-strength medium (9.1.2.1) in the incubator (6.2) at 30 °C or 37 °C (as agreed) for 24 h ± 2 h or, if neither gas formation nor opacity preventing the detection of gas formation is observed at this stage, continue incubation for another 24 h ± 2 h.

9.1.3 Confirmation (see Figure A.3)

9.1.3.1 From the incubated tube from 9.1.2.2, inoculate with a loop (6.3) a tube of confirmation medium (5.4). Incubate in the incubator (6.2) set at 30 °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.

9.1.3.2 Carry out the same procedure as described in 9.1.3.1 for the incubated tubes from 9.1.2.3 showing gas formation, or opacity preventing the detection of gas formation, when either of these features is first observed (i.e. after 24 h ± 2 h or after 48 h ± 2 h).

9.1.4 Interpretation (see Figure A.1)

A tube from 9.1.3.1 or 9.1.3.2 in which gas formation is observed after 24 h ± 2 h or 48 h ± 2 h is considered as a positive tube.
9.2 Enumeration method (MPN) (see Figure A.2)

9.2.1 Test portion, initial suspension and dilutions

See ISO 6887 (relevant part), ISO 8261 or 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 yield a negative result.

9.2.2 Inoculation and incubation

9.2.2.1 It is usual that there is a combination of three tubes for each dilution series. However, for some products and/or each time that results of greater accuracy are required, it may be necessary to inoculate series consisting of more then three tubes (e.g. five tubes). For these cases, for the calculation of the MPN see the relevant tables included in ISO 7218.

9.2.2.2 Take three tubes of double-strength selective enrichment medium [5.3.1a)]. 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.

9.2.2.3 Then take three tubes of single-strength selective enrichment medium [5.3.1b)]. 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.

9.2.2.4 For each of the further dilutions, continue as described in 9.2.2.3. Use a fresh sterile pipette for each dilution. Carefully mix the inoculum and the medium.

9.2.2.5 Leave the tubes of double-strength medium (9.2.2.2) in the incubator (6.2) set at 30 °C or 37 °C (as agreed) for 24 h ± 2 h.

9.2.2.6 Leave the tubes of single-strength medium (9.2.2.3 and 9.2.2.4) in the incubator (6.2) set at 30 °C or 37 °C (as agreed) for 24 h ± 2 h or, if neither gas formation nor opacity preventing the detection of gas formation is observed at this stage, continue incubation for another 24 h ± 2 h.

9.2.3 Confirmation (see Figure A.3)

9.2.3.1 From each of the incubated tubes from 9.2.2.5, inoculate with a loop (6.3) a tube of confirmation medium (5.4). Incubate in the incubator (6.2) set at 30 °C or 37 °C (as agreed) for 24 h ± 2 h or, if gas formation is not observed at this stage, continue incubation for another 24 h ± 2 h.

9.2.3.2 Carry out the same procedure as described in 9.2.3.1 for the incubated tubes from 9.2.2.6 showing gas formation, or opacity preventing the detection of gas formation, when either of these features is first observed (i.e. after 24 h ± 2 h or after 48 h ± 2 h).

9.2.4 Interpretation (see Figure A.2)

For each dilution, count the total number of tubes in which gas formation is observed in 9.2.3 (positive tubes) after 24 h ± 2 h and (if used) 48 h ± 2 h.

10 Calculation and expression of results

In accordance with the results of the interpretation (see 9.1.4), indicate the presence or absence of coliforms in a test portion of \(x\) g or \(x\) ml of product (see ISO 7218).

Calculate the most probable number from the number of positive tubes at each dilution. See ISO 7218.
11 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 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;
— the aim of the test and the incubation temperature used;
— 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.
Flowcharts for procedure

Figure A.1 — Detection method
Figure A.2 — Enumeration method
Figure A.3 — Details of the confirmation stage
Bibliography

[1] ISO 4832, *Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of coliforms — Colony-count technique*

In reporting the results of a test or analysis made in accordance with this standard, if the final value, observed or calculated, is to be rounded off, it shall be done in accordance with IS 2 : 1960 ‘Rules for rounding off numerical values (revised)’.

NATIONAL EXPLANATORY NOTE

At 5.3.2 and 5.4.2, there is mention about ‘sterilize’ in an autoclave set at 121°C for 15 min’ which can be achieved by maintaining pressure of 103 kN/m² (15 psi) for 15 min. Therefore, ‘sterilize in an autoclave set at 121°C for 15 min’ may also be termed as ‘sterilize in an autoclave set at 121°C for 15 min maintained at a corresponding pressure of 103 kN/m² (15 psi) in 5.3.2, 5.4.2 and elsewhere in this standard.'
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Review of Indian Standards

Amendments are issued to standards as the need arises on the basis of comments. Standards are also reviewed periodically; a standard alongwith amendments is reaffirmed when such review indicates that no changes are needed; if the review indicates that changes are needed, it is taken up for revision. Users of Indian Standards should ascertain that they are in possession of the latest amendments or edition by referring to the latest issue of ‘BIS Catalogue’ and ‘Standards: Monthly Additions’.

This Indian Standard has been developed from Doc No.: FAD 15 (1819).

Amendments Issued Since Publication

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