IS 5402 (2012): Microbiology of Food and Animal Feeding Stuffs – Horizontal Method for the Enumeration of Micro-Organisms – Colony-Count Technique at 30o C [FAD 15: Food Hygiene, Safety Management and Other Systems]

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भारतीय मानक

खाद्य एवं पशु आहार सामग्री की सूक्ष्म जैविकी — सूक्ष्मजीवों की गणना के लिए समस्तर पद्धति — 30°C पर कोलोनी-गणना तकनीक

(दूसरा पुनरीक्षण)

Indian Standard

MICROBIOLOGY OF FOOD AND ANIMAL FEEDING STUFFS — HORIZONTAL METHOD FOR THE ENUMERATION OF MICRO-ORGANISMS — COLONY-COUNT TECHNIQUE AT 30°C

(Second Revision)

ICS 07.100.30

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NEW DELHI 110002

April 2012 **Price Group 5**

Food Hygiene, Safety Management and Other Systems Sectional Committee, FAD 15

NATIONAL FOREWORD

This Indian Standard (Second Revision) which is identical with ISO 4833 : 2003 'Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of microorganisms — Colony-count technique at 30 °C' 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 it with the earlier versions of ISO Standard on the subject, namely, ISO 4833 : 1991 'Microbiology — General guidance for the enumeration of micro-organisms — Colony-count technique at 30°C'. The second revision of this standard has been undertaken to align it with the latest version of the International Standard, namely, ISO 4833 : 2003.

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:

International Standard	*Corresponding Indian Standard*	*Degree of Equivalence*
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	IS 10232 : 2003 General rules for the preparation of initial suspension and decimal dilutions for microbiological examination of foods (*first revision*)	Identical

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:

International Standard	*Title*
ISO 6887-2 : 2003	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
ISO 6887-3 : 2003	Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 3: Specific rules for the preparation of fish and fishery products

(*Continued on third cover*)

Indian Standard

MICROBIOLOGY OF FOOD AND ANIMAL FEEDING STUFFS — HORIZONTAL METHOD FOR THE ENUMERATION OF MICRO-ORGANISMS — COLONY-COUNT TECHNIQUE AT 30°C

(Second Revision)

1 Scope

This International Standard specifies a horizontal method for the enumeration of microorganisms, by counting the colonies growing in a solid medium after aerobic incubation at 30 °C. Subject to the limitations discussed in the introduction, this International Standard is applicable to products intended for human consumption or the feeding of animals.

The applicability of this International Standard to the examination of certain fermented food and animal feeding stuffs is limited. For the examination of fermented food and animal feeding stuffs, other media and/or incubation conditions might be more appropriate.

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 7218:1996, *Microbiology of food and animal feeding stuffs — General rules 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 Term and definition

For the purposes of this document, the following term and definition applies.

3.1
microorganism
bacteria, yeast and mould-forming countable colony, produced under the conditions specified in this International Standard

4 Principle

4.1 Two poured plates are prepared using a specified culture medium and 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.

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 aerobically incubated at 30 °C for 72 h.

4.3 The number of microorganisms per millilitre or per gram of sample is calculated from the number of colonies obtained on selected plates (see Clause 10).

5 Culture media and diluents

For current laboratory practice, see ISO 7218 and ISO/TS 11133-1.

5.1 Diluents

See the relevant part of ISO 6887.

5.2 Plate count agar (PCA)

5.2.1 Composition

Enzymatic digestion of casein	5,0 g
Yeast extract	2,5 g
Glucose, anhydrous ($C_6H_{12}O_6$)	1,0 g
Agar[1)]	9 g to 18 g
Water	1 000 ml

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

5.2.2 Preparation

5.2.2.1 Preparation from commercial dehydrated complete medium

Follow the manufacturer's instructions and add, if necessary, the skimmed milk powder (see 5.2.1).

Adjust the pH, if necessary, so that after sterilization it is 7,0 $\pm$ 0,2 at 25 °C.

5.2.2.2 Preparation from dehydrated basic components

Dissolve and disperse in the water, in the following order: the yeast extract, the enzymatic digestion of casein, the glucose and, if necessary, the skimmed milk powder. Heating the water will assist this procedure.

Add the agar and heat to boiling, stirring frequently until the agar is completely dissolved.

1) Depending on the gel strength of the agar.

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

5.2.2.3 Distribution, sterilization and storage

Dispense the medium into test tubes (6.8), in quantities of 12 ml to 15 ml per tube, or into flasks or bottles (6.8) of capacity not greater than 500 ml.

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

If the medium is to be used immediately, cool it to 44 °C to 47 °C in a water bath (6.5) before use. If not, store it in the dark at a temperature of 3 °C ± 2 °C for no longer than 3 months, under conditions which do not allow any change in its composition and properties.

Before beginning the microbiological examination, in order to avoid any delay when pouring the medium, completely melt the medium, then cool it to 44 °C to 47 °C in a water bath (6.5) before use.

In order to check the temperature of the agar, it is recommended to place a thermometer into a portion of 15 g/l agar control solution in a separate container identical to that used for the medium. The temperature control solution should be exposed to the same heating and cooling operations as the medium itself.

5.2.3 Performance testing for the quality assurance of the culture medium

To test the performance of the medium, see ISO/TS 11133-1.

5.3 Overlay medium (if necessary; see 9.2.7)

5.3.1 Composition

Agar[1)]	12 g to 18 g
Water	1 000 ml

5.3.2 Preparation

Add the agar to the water and heat to boiling, stirring frequently until the agar is completely dissolved, or steam for about 30 min.

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

5.3.3 Distribution, sterilization and storage

Dispense the medium into test tubes (6.8) in quantities of 4 ml per tube, or into flasks or bottles (6.8) of appropriate capacity.

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

If the medium is to be used immediately, cool it to 44 °C to 47 °C in a water bath (6.5) before use. If not, store it in the dark at a temperature of 3 °C ± 2 °C for no longer than 3 months, under conditions which do not allow any change in its composition and properties.

Before beginning the microbiological examination, in order to avoid any delay when pouring the medium, completely melt the medium, then cool it to 44 °C to 47 °C in a water bath (6.5) before use.

6 Apparatus and glassware

Disposable glassware is an acceptable alternative to re-usable 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.

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

6.4 Pipettes, of nominal capacity 1 ml.

6.5 Water bath, capable of operating at 44 °C to 47 °C.

6.6 Colony-counting equipment, for example, consisting of an illuminated base with a dark background, fitted with magnifying lens of suitable magnification of about 1,5 × may be used and a mechanical or electronic digital counter.

6.7 pH-meter, having an accuracy of calibration of ± 0,1 pH unit at 25 °C.

6.8 Test tubes, flasks or **bottles,** of appropriate capacity and not greater than 500 ml.

7 Sampling

It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage.

Sampling is not part of the method specified in this International Standard. See 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.

8 Preparation of test sample

Prepare the test sample in accordance with the relevant part of ISO 6887, or ISO 8261, and the specified 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 the relevant part of ISO 6887 and the specific International Standard dealing with the product concerned.

9.2 Inoculation and incubation

9.2.1 Take two sterile Petri dishes (6.3). Transfer to each dish, by means of a sterile pipette (6.4) 1 ml of the test sample, if liquid, or 1 ml of the initial suspension in the case of other products (10^{-1} dilution).

9.2.2 Take two other sterile Petri dishes (6.3). Transfer to each dish, by means of another sterile pipette (6.4), 1 ml of the 10^{-1} dilution (liquid product) or 1 ml of the 10^{-2} dilution (other products).

9.2.3 If necessary, repeat the procedure with the further dilutions, using a new sterile pipette for each decimal dilution.

9.2.4 If appropriate and possible, select only the critical dilutions steps (at least two consecutive decimal dilutions) for the inoculation of the Petri dishes that will give colony counts of between 15 and 300 colonies per plate.

9.2.5 Pour about 12 ml to 15 ml of the plate count agar (5.2) 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 (5.2) is poured into the dishes shall not exceed 45 min.

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

9.2.7 After complete solidification, and only in the case where it is suspected that the product under examination contains microorganisms whose colonies will overgrow the surface of the medium, pour about 4 ml of the overlay medium (5.3) at 44 °C to 47 °C onto the surface of the inoculated medium. Allow to solidify as described above.

9.2.8 Invert the prepared dishes and place them in the incubator (6.2) at 30 °C ± 1 °C for 72 h ± 3 h. Do not stack the dishes more than six high. Stacks of dishes should be separated from one another and from the walls and top of the incubator.

9.3 Counting of colonies

9.3.1 After the specified incubation period (9.2.8), count the colonies on the plates (10.1), using the colony-counting equipment (6.6) if necessary. Examine the dishes under subdued light. It is important that pinpoint colonies should be included in the count, but it is essential that the operator avoid mistaking particles of undissolved or precipitated matter in dishes for pinpoint colonies. Examine doubtful objects carefully, using higher magnification where required, in order to distinguish colonies from foreign matter.

9.3.2 Spreading colonies shall be considered as single colonies. If less than one-quarter of the dish is overgrown by spreading, count the colonies on the unaffected part of the dish and calculate the corresponding number of the entire dish. If more than one-quarter is overgrown by spreading colonies, discard the count.

10 Expression of results

10.1 Method of calculation

See Amendment 1 to ISO 7218:1996.

10.2 Precision

10.2.1 General

The precision data were evaluated for dishes containing more than 15 and fewer than 300 colonies. The precision data depend on the flora association and the sample matrix. The data presented are derived from collaborative studies (see references [1], [2] and [3]) and are valid for raw and pasteurized milk. They may be used as estimates when colony counts in other products are determined.

10.2.2 Repeatability

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, should not be greater than the repeatability limit, $r = 0{,}25$, in $\log_{10}$ microorganisms per millilitre (corresponding to 1,8 on the normal scale in microorganisms per millilitre).

NOTE This repeatability limit was derived from collaborative studies for raw and pasteurized milk (see references [1], [2] and [3]) and may be used for such products.

10.2.3 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, should not be greater than the reproducibility limit, $R = 0{,}45$, in $\log_{10}$ microorganisms per millilitre (corresponding to 2,8 on the normal scale in microorganisms per millilitre).

NOTE This reproducibility limit was derived from collaborative studies for raw and pasteurized milk (see references [1], [2] and [3]) and may be used for such products.

10.3 Interpretation of test results

In the following examples, the average precision data, a probability level of 95 % and the analysis of one sample are considered. It should be noted that, under practical conditions, the average of several samples is often used. The figures are indicated in microorganisms per millilitre.

a) **Repeatability conditions**

First result: $10^5 = 100\ 000$

The difference between the first and the second result should not be greater than 0,25 $\log_{10}$ units.

Second result: $\log 10^{4,75} = 56\ 000$ or

$\log 10^{5,25} = 178\ 000$

The difference between the first and the second result is acceptable if the second result is not lower than 56 000 or not higher than 178 000.

b) **Reproducibility conditions**

Results obtained in the first laboratory (average of duplicate determination): $10^5 = 100\ 000$

The difference between the first and the second result obtained in the second laboratory should not be greater than 0,45 $\log_{10}$ units:

Second results: $\log 10^{4,55} = 36\ 000$, or

$\log 10^{5,45} = 280\ 000$

The difference between the results obtained by the first and the second laboratory is acceptable, if the second laboratory obtains a result which is not lower than 36 000 and not higher that 280 000.

Annex A shows the calculation and use of the critical difference (CD) to interpret results.

10.4 Confidence limits

See ISO 7218.

11 Test report

The test report shall specify:

a) all information necessary for the complete identification of the sample;

b) the sampling method used, if known;

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

d) 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;

e) the test results obtained.

Annex A
(informative)

Use of the Critical Difference (CD) for the interpretation of results

In the following examples, the average precision data, a probability level of 95 % and the analysis of one sample are considered. It should be noted that, under practical conditions, the average of several samples is often used. The figures are indicated in microorganisms per millilitre.

a) **Reproducibility conditions**

Results obtained in the first laboratory (average of duplicate determination): $10^5 = 100\ 000$

The difference between this result and a result obtained by a second laboratory (average of n determinations; $n = 2$ in this example) is acceptable if it does not exceed the critical difference (CD), in $\log_{10}$ units:

$$\mathrm{CD} = \sqrt{R^2 - r^2\left(1 - \frac{1}{n}\right)} = \sqrt{R - \frac{r^2}{2}} = \sqrt{0{,}45 - \frac{0{,}25^2}{2}} = 0{,}41$$

where

r is the repeatability limit;

R is the reproducibility limit.

The difference between the results obtained by the first and the second laboratory is acceptable if the second laboratory obtains a result which is not lower than $10^{4{,}59} = 39\ 000$ or not higher that $10^{5{,}41} = 257\ 000$.

b) **Comparison with a limit** (one-sided test)

Limit: $10^5 = 100\ 000$

The difference between the limit and the laboratory result (average of n determinations; $n = 2$ in this example) has to be compared to the critical difference limit (CDL):

$$\mathrm{CDL} = 0{,}84\sqrt{2} \times \sqrt{R^2 - r^2\left(1 - \frac{1}{n}\right)} = 0{,}84\sqrt{2} \times \sqrt{R^2 - \frac{r^2}{2}} = 0{,}24$$

Test results up to $10^{5{,}24} = 174\ 000$ do not indicate non-compliance with the limit.

Bibliography

[1] PITON, C., GRAPPIN, R. A model for statistical evaluation of precision parameters of microbiological methods: Application to dry rehydratable film methods and IDG reference methods for enumeration of total aerobic mesophilic flora and coliforms in raw milk. *J. AOAC*, **74**, 1991, pp. 92-103

[2] SCOTTER, S., ALDRIDGE, M., BACK, J., WOOd, R. Validation of European Community methods for microbiological and chemical analysis of raw and heat-treated milk. *J. Assoc. Publ. Analyst*., **29**, 1993, pp. 1-32

[3] DAHMS, S., WEISS, H. Estimation of precision values for microbiological reference methods: Standardized pour plate technique. *Milchwiss.*, **53**, 1988, pp. 555-559

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International Standard	*Title*
ISO 6887-4 : 2003	Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 4: Specific rules for the preparation of products other than milk and milk products, meat and meat products, and fish and fishery products
ISO 7218 : 1996	Microbiology of food and animal feeding stuffs — General rules for microbiological examinations
ISO 8261 : 2001	Milk and milk products — General guidance for the preparation of test samples, initial suspensions and decimal dilutions for microbiological examination
ISO/TS 11133-1 : 2000	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

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 clause **5.2.2.3** and **5.3.3**, there is mention about 'sterilze' in an autoclave set at 121°C for 15 min' which can be achieved by maintaining pressure of 103 kN/m^2 (I5 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/m^2 (15 psi)' in clause **5.2.2.3**, **5.3.3** 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'.

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Amendments Issued Since Publication

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