TC 34

INTERNATIONAL STANDARD ISO 4832

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Microbiology — General guidance for enumeration of coliforms — Colony count technique at 30 °C

Microbiologie — Directives générales pour le dénombrement des coliformes — Méthode par comptage des colonies obtenues à 30 °C

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FOREWORD

ISO (the International Organization for Standardization) is a worldwide federation of national standards institutes (ISO member bodies). The work of developing International Standards is carried out through ISO technical committees. Every member body interested in a subject for which a technical committee has been set up 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.

Draft International Standards adopted by the technical committees are circulated to the member bodies for approval before their acceptance as International Standards by the ISO Council.

International Standard ISO 4832 was developped by Technical Committee ISO/TC 34, *Agricultural food products*, and was circulated to the member bodies in July 1976.

It has been approved by the member bodies of the following countries :

Australia	Hungary	Romania
Austria	India	South Africa, Rep. of
Bulgaria	Iran	Spain
Canada	Ireland	Turkey
Chile	Israel	United Kingdom
Czechoslovakia	Mexico	U.S.A.
France	Netherlands	Yugoslavia
Germany	Poland	
Ghana	Portugal	

The member bodies of the following countries expressed disapproval of the document on technical grounds :

New Zealand
Thailand

Printed in Switzerland

Microbiology — General guidance for enumeration of coliforms — Colony count technique at 30 °C

0 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 the consideration of bodies preparing reference 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 the 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, but does not allow a microbiological examination to be carried out on such a large test portion. It will therefore be 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 by this International Standard will, on average, detect only about 90 % of strains of the micro-organisms referred to in other publications as "(presumptive) coliforms" (*Citrobacter, Enterobacter, Klebsiella*)[1].

1 SCOPE AND FIELD OF APPLICATION

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.

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 10.2.

2 REFERENCES

ISO 2293, *Meat and meat products — Aerobic count at 30 °C (Reference method).*

ISO 3565, *Meat and meat products — Detection of* salmonellae *(Reference method).*

ISO 4831, *Microbiology — General guidance for enumeration of coliforms — Most probable number technique at* 30 °C.

ISO . . ., *Microbiology — General guidance for preparation of dilutions.*[2]

3 DEFINITION

For the purpose of this International Standard, the following definition applies :

coliforms : Bacteria which, at 30 °C, form characteristic colonies in crystal violet neutral red bile lactose agar under the operational conditions described.

1) See Edwards. P. R., and Ewing, W.H. (1972) : *"Identification of* Enterobacteriaceae", 3rd edition, Burgess Publishing Company, Minneapolis, Minnesota, U.S.A.

2) In preparation.

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 for 24 h.

4.3 Calculation of the number of micro-organisms per millilitre or per gram of sample from the number of characteristic colonies obtained in plates at dilution levels chosen so as to give a significant result.

5 SAMPLING

Carry out sampling in conformity with the International Standard dealing with the product concerned.

6 APPARATUS AND GLASSWARE

Usual microbiological laboratory equipment, and in particular :

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

Apparatus which will enter into contact with the culture medium, the dilution fluid or the sample, except for apparatus that is supplied sterile (particularly plastics apparatus), shall be sterilized either

- by being kept at 170 to 175 °C for not less than 1 h in an oven, or
- by being kept at 121 ± 1 °C for not less than 20 min in an autoclave.

6.2 Incubator, capable of being controlled at 30 ± 1 °C.

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

6.4 Total delivery pipettes (blow-out pipettes), having a nominal capacity of 1 ml.

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

6.6 Colony counting equipment consisting of an illuminated base with a dark background fitted with a magnifying lens to be used at a magnification of 1,5 diameters, and a mechanical or electronic digital counter.

6.7 pH meter.

7 CULTURE MEDIUM AND DILUTION FLUID

7.1 Basic materials

In order to improve the ~~reproducibility~~ precision of the results, it is recommended that, for the preparation of the culture medium, dehydrated basic components or a complete dehydrated medium should be used. The manufacturer's instructions shall be rigorously followed.

The chemicals used shall be of analytical quality.

The water used shall be distilled or deionized, and shall be free from substances that might inhibit growth of coliforms under the test conditions.

7.2 Dilution fluid

Use a peptone-based dilution fluid containing sodium chloride, buffered or not; for example, peptone-saline dilution fluid (see 5.3 of ISO 2293) or buffered peptone water (see 6.2.1 of ISO 3565).

If the dilution fluid is not used immediately, it shall be kept in the dark at a temperature between 0 and + 5 °C and in conditions that prevent any change in its composition. It shall not be kept for longer than 1 month.

7.3 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 in powder or flake form	9 to 18 g[1)]
water	1 000 ml

Preparation

Proceed as follows in order to conserve the selective power and specificity of the medium. Dissolve the components or the dehydrated complete medium in the water and leave to stand for several minutes. Then mix vigorously and adjust the pH (checking with the pH meter) so that, after boiling, it is 7,4 ± 0,1 at 25 °C. Bring to the boil, stirring from time to time.

Allow to boil for 2 min. Immediately cool the medium to 45 ± 0,5 °C, in the water bath (6.5).

Avoid overheating the medium or 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 (9.2.1.3).

Use the medium within 3 h of its preparation.

1) According to the directions of the manufacturer.

8 PREPARATION OF THE TEST SAMPLE

Refer to the particular International Standard dealing with the product under examination. If an International Standard is not available, it is recommended that agreement be reached on this subject by the parties concerned.

9 PROCEDURE

9.1 Test portion, initial suspension and dilutions

Refer to ISO . . . (see clause 2) and to the International Standard dealing with the product under examination.

Prepare the initial suspension and the dilutions using a dilution fluid meeting the requirements given in 7.2.

9.2 Counting technique

9.2.1 *Inoculation*

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

9.2.1.2 Take two other sterile Petri dishes. Using a new sterile pipette, transfer to each dish 1 ml of the 1/10 dilution (liquid product) or 1 ml of the 1/100 dilution (other products).

Repeat the procedure described in the preceding paragraph with the other dilutions.

9.2.1.3 Pour about 12 ml of the VRBL medium (7.3), at 45 ± 0,5 °C, into each Petri dish. The time elapsing between the end of the preparation of the initial suspension (or of the 1/10 dilution if the product is liquid) and the moment when the medium (7.3) 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.

Prepare a control plate, with 12 ml of the medium, for checking its sterility.

9.2.1.4 After complete solidification, pour about 4 ml of the VRBL medium (7.3), at 45 ± 0,5 °C, on to the surface of the inoculated medium. Allow to solidify as described above.

9.2.2 *Incubation*

Invert the prepared dishes and place them in the incubator (6.2) at 30 ± 1 °C. Leave them for 24 ± 2 h.

9.2.3 *Interpretation*

After the specified period of incubation (see 9.2.2), count, using the colony counting equipment (6.6), the characteristic coliform colonies in each dish containing not more than 150 colonies[1] (whether characteristic or not).

NOTE — Characteristic colonies are dark red colonies having a diameter of 0,5 mm or greater, after incubation for 24 h at 30 °C.

10 EXPRESSION OF RESULTS

10.1 Method of calculation

Proceed as described in 10.1.1, 10.1.2. 10.1.3 or 10.1.4, according to the number of colonies counted in accordance with 9.2.3.

10.1.1 If one or both dishes corresponding to a certain dilution contain between 15 and 150 characteristic colonies, calculate the arithmetic mean of the number of colonies counted in the two dishes.

Retain only two significant figures, proceeding as follows :

— if the number is less than 100, round it to the nearest multiple of 5;

— if the number is greater than 100 and ends in a 5, round it to the nearest multiple of 20;

— if the number is greater than 100 and does not end in a 5, round it to the nearest multiple of 10.

Multiply this value by the reciprocal of the corresponding dilution to obtain the number of coliforms per millilitre or per gram of product, according to the circumstances. Express this result as a number between 1,0 and 9,9 multiplied by 10^n, n being the appropriate power of 10.

10.1.2 If there are dishes containing between 15 and 150 characteristic colonies at two consecutive dilutions, calculate the number of coliforms for each dilution as specified in 10.1.1, and take as the result the arithmetic mean of the two values obtained, except when the ratio of the higher value to the lower value is greater than 2; in this case, take the lower value as the result.

10.1.3 If there are fewer than 15 characteristic colonies in dishes corresponding to the test sample (liquid product) or to the initial suspension (other products), report the result as :

— fewer than 15 coliforms per millilitre (liquid product), or

— fewer than 15 × s coliforms per gram (other products), the dilution of the initial suspension being 1/s.

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

10.1.4 If there are no characteristic colonies in dishes corresponding to the test sample (liquid product) or to the initial suspension (other products), report the result as :

— fewer than 1 coliform per millilitre (liquid product), or

— fewer than 1 × s coliforms per gram (other products), the dilution of the initial suspension being 1/s.

10.2 Precision of the method

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

11 TEST REPORT

The test report shall show the method used and the results obtained. It shall also mention all operating conditions not specified in this International Standard or regarded as optional, as well as any circumstance that may have influenced the result.

The report shall include all details required for complete identification of the sample.