INTERNATIONAL STANDARD

ISO 7932

Second edition
1993-10-15

Microbiology — General guidance for the enumeration of *Bacillus cereus* — Colony-count technique at 30 °C

Microbiologie — Directives générales pour le dénombrement de Bacillus cereus *— Méthode par comptage des colonies à* 30 °C

Reference number
ISO 7932:1993(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 7932 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Sub-Committee SC 9, *Microbiology*.

This second edition cancels and replaces the first edition (ISO 7932:1987), which has been technically revised.

Annex A forms an integral part of this International Standard. Annex B is for information only.

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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 microbiological examination of food products not dealt with by existing International Standards and to be taken into account by organizations 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 in every detail for certain products 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 For the purposes of a practicable test method, the definition of *Bacillus cereus* given in clause 3 and used as a basis for the procedure does not exclusively describe strains of *B. cereus*. In particular, the confirmatory tests are inadequate to distinguish between *B. cereus* and other closely related but less commonly encountered bacillus species such as *B. anthracis, B. thuringiensis, B. mycoides,* etc.

0.3 It appears that the spores of many, if not most, strains of *B. cereus* germinate readily on the surface of culture media employed for enumeration. In most cases there does not seem to be a need for heat shock treatment to provoke germination. Sometimes a heat shock procedure is desirable, for example for spore counts or to inhibit growth of vegetative bacterial cells. In such cases, treatment of 15 min at 70 °C is recommended.

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Microbiology — General guidance for the enumeration of *Bacillus cereus* — Colony-count technique at 30 °C

1 Scope

This International Standard gives general guidance for the enumeration of viable *Bacillus cereus* in products intended for human consumption or animal feeding stuffs by means of the colony-count technique at 30 °C.

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.

3.1 *Bacillus cereus*: A microorganism that forms colonies on the surface of a selective culture medium and which gives positive confirmation reactions under the conditions specified in this International Standard.

4 Principle

4.1 Surface plating, on a solid selective culture medium contained in Petri dishes, of a specified quantity of the test sample if the initial product is liquid, or of a specified quantity of an initial suspension in the case of other products.

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

4.2 Aerobic incubation of the plates at 30 °C for 18 h to 48 h.

4.3 Calculation of the number of *B. cereus* per gram or per millilitre of sample from the number of confirmed colonies obtained on plates at dilution levels chosen so as to give a significant result, and confirmation according to the tests specified.

5 Dilution fluid, culture media and reagents

5.1 General

For current laboratory practice, see ISO 7218.

NOTE 1 Commercially prepared ready-to-use reagents may be used.

5.2 Dilution fluid

See ISO 6887 and any specific standard dealing with the product to be examined.

5.3 Agar medium (see reference [1] in annex B)

5.3.1 Base medium

5.3.1.1 Composition

Beef extract	1,0 g
Peptone	10,0 g
D-Mannitol	10,0 g
Sodium chloride (NaCl)	10,0 g
Phenol red	0,025 g
Agar	12 to 18 g [1]
Water	900 ml

1) Depending on the gel strength of the agar.

5.3.1.2 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 at 25 °C.

Dispense the medium in quantities of 90 ml into flasks (6.7) of appropriate capacity.

Sterilize for 15 min in an autoclave (6.1) set at 121 °C.

5.3.2 Polymyxin B solution

5.3.2.1 Composition

Polymyxin B sulfate	10^6 I.U.
Water	100 ml

5.3.2.2 Preparation

Dissolve the polymyxin B sulfate in the water. Sterilize by filtration.

5.3.3 Egg yolk emulsion

Use fresh hens' eggs with their shells intact. Wash the eggs, using a brush, in liquid detergent. Rinse under running water, dip in 95 % (*V/V*) ethanol for 30 s and dry. Using aseptic procedures, break each egg and separate the yolk from the white by repeatedly transferring the yolk from one half of the egg shell to the other. Put the yolks into a sterile measuring cylinder and add four parts by volume of sterile water. Transfer aseptically into a sterile flask (6.7) and mix vigorously.

Heat the mixture for 2 h in a water bath (6.4) set at 45 °C. Then leave for 18 h to 24 h at 0 °C to 5 °C to allow a precipitate to form.

Collect the supernatant emulsion aseptically.

The emulsion may be stored at 0 °C to 5 °C for not longer than 72 h.

5.3.4 Complete medium (MYP agar)

5.3.4.1 Composition

Base medium (5.3.1)	90 ml
Polymyxin B solution (5.3.2)	1,0 ml
Egg yolk emulsion (5.3.3)	10,0 ml

5.3.4.2 Preparation

Melt the base medium and cool it in a water bath (6.4) set at 50 °C.

Add the other liquids, mixing well after each addition.

Cool the complete medium in a water bath (6.4) at 45 °C.

5.3.5 Preparation of agar plates for enumeration

Pour 15 ml to 20 ml portions of the complete medium (5.3.4) into sterile Petri dishes (6.8) and allow to solidify.

The plates may be stored prior to drying at between 0 °C and 5 °C for up to 4 days.

Immediately before use, dry the plates, preferably with the lids off and the agar surface downwards, in a drying cabinet or incubator (6.2) set between 37 °C and 55 °C until the agar surface is dry.

5.3.6 Preparation of agar plates for isolation

Pour portions of about 15 ml of the base medium (5.3.1), previously melted, cooled and kept in a water bath (6.4) at 45 °C, into sterile Petri dishes (6.8) and allow to solidify.

Immediately before use, dry the plates, preferably with the lids off and the agar surface downwards, in a drying cabinet or incubator (6.2) set between 37 °C and 55 °C until the agar surface is dry.

5.4 Glucose agar

5.4.1 Composition

Tryptone	10,0 g
Yeast extract	1,5 g
Glucose	10,0 g
Sodium chloride (NaCl)	5,0 g
Bromocresol purple	0,015 g
Agar	12 to 18 g [1]
Water	1 000 ml

1) Depending on the gel strength of the agar.

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 7,0 at 25 °C.

Dispense the culture medium in quantities of 15 ml into test tubes (6.7).

Sterilize for 15 min in an autoclave (6.1) set at 121 °C.

Immediately before use, melt the medium in a boiling water bath or flowing steam for 10 min, then cool rapidly to about 30 °C, keeping the tubes in a vertical position.

5.5 Voges-Proskauer (VP) medium

5.5.1 Composition

Peptone	7,0 g
Glucose	5,0 g
Dipotassium hydrogen phosphate (K_2HPO_4)	5,0 g
Sodium chloride (NaCl)	5,0 g
Water	1 000 ml

5.5.2 Preparation

Dissolve the components or the dehydrated complete medium in the water.

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

Dispense the medium in quantities of 5 ml into test tubes (6.7).

Sterilize for 15 min in an autoclave (6.1) set at 121 °C.

5.6 Voges-Proskauer (VP) test reagents

5.6.1 α-Naphthol solution

5.6.1.1 Composition

α-Naphthol	5,0 g
Ethanol, 95 % (*V/V*)	100 ml

5.6.1.2 Preparation

Dissolve the α-naphthol in the ethanol.

Store at between 0 °C and 5 °C in a hermetically sealed brown culture flask.

5.6.2 Potassium hydroxide solution

5.6.2.1 Composition

Potassium hydroxide (KOH)	40,0 g
Water	100 ml

5.6.2.2 Preparation

Dissolve the potassium hydroxide slowly in the water.

5.6.3 **Creatine**, crystalline.

5.7 Nitrate medium

5.7.1 Composition

Peptone	5,0 g
Beef extract	3,0 g
Potassium nitrate (KNO_3)	1,0 g
Water	1 000 ml

5.7.2 Preparation

Dissolve the components or the dehydrated complete medium in the water.

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

Dispense the medium in quantities of 5 ml into test tubes (6.7).

Sterilize for 15 min in an autoclave (6.1) set at 121 °C.

5.8 Nitrite reagent

5.8.1 5-Amino-2-naphthalenesulfonic acid (5-2 ANSA) solution

5.8.1.1 Composition

5-2 ANSA	0,1 g
Acetic acid (2,6 mol/l)	100 ml

5.8.1.2 Preparation

Dissolve the 5-2 ANSA in the acetic acid and filter through paper.[1)]

Store in a well-stoppered brown culture flask (preferably with a bulb-type dropper) between 0 °C and 5 °C.

NOTE 2 If 5-2 ANSA is not available, it is possible to use the following alternative reagent:

Solution A: sulfanilic acid, 0,8 g
acetic acid (5 mol/l), 100 ml; and

Solution B: α-naphthol, 0,5 g
acetic acid (5 mol/l), 100 ml.

5.8.2 Sulfanilic acid solution

5.8.2.1 Composition

Sulfanilic acid	0,4 g
Acetic acid (2,6 mol/l)	100 ml

5.8.2.2 Preparation

Dissolve the sulfanilic acid in the acetic acid and filter through paper.[1)]

Store in a well-stoppered brown culture flask (preferably with a bulb-type dropper) between 0 °C and 5 °C.

5.8.3 Preparation of the complete reagent

Just prior to use, mix equal volumes of the two acid solutions (5.8.1 and 5.8.2).

Discard immediately any unused complete reagent.

5.9 Zinc dust

6 Apparatus and glassware

NOTE 3 Disposable apparatus is an acceptable alternative to reusable glassware if it has similar 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 Drying cabinet or **incubator**, ventilated by convection, for drying the agar plates, capable of operating between 37 °C ± 1 °C and 55 °C ± 1 °C.

6.3 Incubator, capable of operating at 30 °C ± 1 °C.

6.4 Water baths, capable of being maintained at 45 °C ± 0,5 °C and 50 °C ± 1 °C.

6.5 Loops, made of platinum/iridium or nickel/chromium wire, approximately 3 mm in diameter, and **stab-inoculation wires** of the same material.

6.6 pH-meter, accurate to within ± 0,1 pH units at 25 °C.

6.7 Test tubes, in particular of diameter 18 mm and length 180 mm, and **culture flasks**[2)] for the sterilization and conservation of the culture media.

6.8 Petri dishes, made of glass or plastic, of diameter 90 mm to 100 mm or, if necessary, 140 mm.

6.9 Graduated pipettes, calibrated for bacteriological use only, of nominal capacities 10 ml and 1 ml, graduated respectively in divisions of 0,5 ml and 0,1 ml, and with an outflow opening of nominal diameter 2 mm to 3 mm.

6.10 Spreaders (hockey-stick type), made of glass or plastic rod of diameter approximately 3,5 mm and length 20 cm, bent at right angles about 3 cm from one end; the cut ends shall be made smooth by heating.

1) Whatman No. 41 filter paper is a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

2) Culture flasks with non-toxic metal screw-caps may be used.

6.11 Rubber bulbs, or other type of safety device, suitable for use with the graduated pipettes.

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. If there is no specific International Standard dealing with sampling of the product concerned, 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 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

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 Transfer, by means of a sterile pipette (6.9), 0,1 ml of the test sample if the product is liquid, or of the initial suspension in the case of other products, to each of two agar plates (5.3.5). Repeat the procedure using further decimal dilutions if necessary.

NOTE 4 When, for certain products, it is desirable to estimate low numbers of *B. cereus*, the limits of detection can be raised by a factor of 10 by examining 1,0 ml of the test sample if the initial product is liquid, or 1,0 ml of the initial suspension for the other products. Distribute the 1 ml of inoculum either on the surface of a large Petri dish (140 mm) or over the surface of three small dishes (90 mm) using a sterile spreader (6.10). In both cases, prepare duplicates by using two large plates or six small plates.

9.2.2 Carefully spread the inoculum as quickly as possible over the surface of the agar plate without touching the sides of the dish with the speader (6.10). Use a fresh sterile spreader for each plate. Leave the plates with the lids on for about 15 min at ambient temperature for the inoculum to be absorbed into the agar.

9.2.3 Invert the prepared plates (9.2.2) and incubate them for 18 h to 24 h in an incubator (6.3) set at 30 °C. If colonies are not clearly visible, incubate the plates for an additional 24 h before counting.

9.3 Counting of the colonies

After the period of incubation (9.2.3), select plates, preferably at two successive dilutions, containing less than 150 colonies.

Count the presumptive *B. cereus* colonies on each plate. The presumptive colonies are large, pink (indicating that mannitol fermentation has not occurred, see note 5) and generally surrounded by a zone of precipitation (indicating the production of lecithinase, see note 6).

If there are less than 15 characteristic colonies on plates inoculated with the liquid product or the lowest dilution for other products, it is possible to make an estimated count as described in 10.2.2.

NOTES

5 If the plates contain numerous mannitol-fermenting microorganisms leading to the production of acid, then the characteristic pink colour of *B. cereus* colonies may be reduced or disappear entirely.

6 Some strains of *B. cereus* produce only little or no lecithinase. Colonies of these strains will not be surrounded by a precipitation zone. These colonies should also be subjected to confirmation tests.

7 If a 1,0 ml inoculum was spread over three plates (see note 4 in 9.2.1), treat these plates as one in all subsequent counting and confirmation procedures.

9.4 Confirmation

9.4.1 Selection and purification of colonies for confirmation

Select five presumptive colonies from each plate selected as in 9.3. If there are less than five colonies on the plate, take all presumptive colonies present. Confirm these colonies as specified in 9.4.2 to 9.4.4.

If the plates are overcrowded and it is not possible to select well-isolated colonies, streak five presumptive colonies on plates with complete medium (5.3.4). Incubate in an incubator (6.3) set at 30 °C for 18 h to 24 h.

Select from each plate at least one well-isolated colony with a pink colour. Confirm this colony as specified in 9.4.2 to 9.4.4.

9.4.2 Glucose agar medium

Inoculate selected colonies (9.4.1), using a stab-inoculation wire (6.5), centrally into tubes containing