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МЕЖДУНАРОДНАЯ ОРГАНИЗАЦИЯ ПО СТАНДАРТИЗАЦИИ

Microbiology — General guidance for 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

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Foreword

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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. They are approved in accordance with ISO procedures requiring at least 75 % approval by the member bodies voting.

International Standard ISO 7932 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*.

Users should note that all International Standards undergo revision from time to time and that any reference made herein to any other International Standard implies its latest edition, unless otherwise stated.

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

0 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 consideration by 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 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. cereus* var. *mycoides*, etc.

0.3 It appears that the spores of many, if not most, strains of *Bacillus 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 micro-organisms. In such cases a treatment of 15 min at 70 °C is recommended.

1 Scope and field of application

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

2 Reference

ISO 6887, *Microbiology — General guidance for the preparation of dilutions for microbiological examination.*

3 Definition

For the purposes of this International Standard, the following definition applies.

Bacillus cereus: A micro-organism 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 to 48 h.

4.3 Calculation of the number of *Bacillus 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 Diluent, media and reagents

5.1 Basic materials.

In order to improve the reproducibility of the results, it is recommended that, for the preparation of the culture medium, dehydrated basic components, or complete dehydrated media

and, for the egg yolk emulsion, a commercially available preparation, be used. The manufacturer's instructions shall be rigorously followed.

The chemical products used shall be of recognized analytical quality.

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

Measurements of pH shall be made using a pH meter (6.6), set at a temperature of 25 °C.

If the diluent and culture media are not used immediately, they shall, unless otherwise specified, be stored in the dark at between 0 and 5 °C for a maximum of one month, in conditions which do not produce any change in their composition.

5.2 Diluent.

See ISO 6887 and any International Standard dealing with the product under examination.

5.3 Agar medium. [1)]

5.3.1 Base medium.

Table 1

Component	Quantity
Beef extract	1,0 g
Peptone	10,0 g
D-Mannitol ($C_6H_{14}O_6$)	10,0 g
Sodium chloride (NaCl)	10,0 g
Phenol red ($C_{19}H_{14}O_3S$)	0,025 g
Agar	12 to 18 g *
Water	1 000 ml

* According to the manufacturer's instructions.

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

Adjust the pH so that after sterilization it is 7,2.

Transfer the medium in quantities of 90 ml to flasks (6.7) of appropriate capacity. Sterilize the medium for 15 min at 121 ± 1 °C.

5.3.2 Polymyxin B, emulsion.

Table 2

Component	Quantity
Polymyxin B sulfate	0,100 g
Water	100 ml

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

5.3.3 Egg yolk, emulsion.

Use fresh hen's 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 transfering 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.

Put the mixture in a water-bath (6.4), maintained at 45 ± 0,5 °C, for 2 h and leave for 18 to 24 h at 0 to 5 °C to allow a precipitate to form.

Collect the supernatant emulsion aseptically.

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

5.3.4 Complete medium (MYP agar).

Table 3

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

Melt the base medium and cool it in a water-bath (6.4) controlled at 50 ± 1 °C.

Add the other liquids, mixing well after each addition.

5.3.5 Preparation of agar plates for enumeration.

Add to sterile Petri dishes (6.8) 15 to 20 ml of the complete medium (5.3.4), cooled and kept in a water-bath (6.4) at 45 ± 0,5 °C, and allow to solidify.

The plates may be stored prior to drying at between 0 and 5 °C for up to 24 h.

Dry the plates, preferably with the lids off and the agar surface downwards, in a drying cabinet, oven or incubator (6.2) at 50 ± 1 °C for 30 min.

5.3.6 Preparation of agar plates for isolation.

Transfer 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 ± 0,5 °C, to 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, oven or incubator (6.2) at 50 ± 1 °C for 30 min.

If prepared in advance, the undried plates shall not be kept longer than 4 h at room temperature or 1 day at between 0 and 5 °C.

1) Mossel, D. A. A., Koopman, M. J. and Jongerius, E. *Appl. Microbiol.*, 1967 (Vol. 15), pp. 650-653.

5.4 Glucose-agar.

Table 4

Component	Quantity
Tryptone	10,0 g
Yeast extract	1,5 g
Glucose ($C_6H_{12}O_6$)	10,0 g
Sodium chloride (NaCl)	5,0 g
Bromocresol purple ($C_{21}H_{15}Br_2NaO_2S$)	0,015 g
Agar	12 to 18 g *
Water	1 000 ml

* According to the manufacturer's instructions.

Dissolve the components or the complete dehydrated medium in the water by boiling. Adjust the pH so that after sterilization it is 7,0. Transfer the culture medium in quantities of 15 ml to test tubes (6.7). Sterilize the medium for 15 min at 121 °C.

Just prior to 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.

Table 5

Component	Quantity
Peptone	7,0 g
Glucose ($C_6H_{12}O_6$)	5,0 g
Dipotassium hydrogenorthophosphate (K_2HPO_4)	5,0 g
Sodium chloride (NaCl)	5,0 g
Water	1 000 ml

Dissolve the components or the complete dehydrated medium in the water. Adjust the pH so that after sterilization it is 7,0. Transfer the medium to test tubes (6.7) in 5 ml quantities and sterilize at 121 °C for 15 min.

5.6 Voges-Proskauer (VP) test reagents.

5.6.1 α-Naphthol, solution.

Table 6

Component	Quantity
α-Naphthol ($C_{10}H_8O$)	5,0 g
Ethanol 96 % (*V/V*)	100 ml

Dissolve the α-naphthol in the ethanol.

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

5.6.2 Potassium hydroxide, solution.

Table 7

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

Dissolve the potassium hydroxide in the water.

5.6.3 Creatine ($C_4H_{10}N_3O$), crystals.

5.7 Nitrate medium.

Table 8

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

Dissolve the components or the complete dehydrated medium in the water. Adjust the pH so that after sterilization it is 7,0. Transfer the medium to test tubes (6.7) in 5 ml quantities. Sterilize at 121 °C for 15 min.

5.8 Nitrite reagent.

5.8.1 5-Amino-2-naphthalene sulfonic acid (5-2 ANSA), solution.

Table 9

Component	Quantity
5-2 ANSA ($C_{10}H_9NO_3S$)	0,1 g
Acetic acid ($C_2H_4O_2$), 15 % (*V/V*)	100 ml

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 and 5 °C.

5.8.2 Sulfanilic acid, solution.

Table 10

Component	Quantity
Sulfanilic acid ($C_6H_7NO_3S$)	0,4 g
Acetic acid ($C_2H_4O_2$), 15 % (*V/V*)	100 ml

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

1) A suitable filter paper is Whatman No. 41 or equivalent. This information is given for the convenience of the user of this International Standard and does not constitute an endorsement of this product by ISO.

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

5.8.3 Preparation of the complete reagent.

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

Discard unused complete reagent immediately.

NOTE — If 5-2 ANSA (5.8.1) is not available, it is possible to use the reagents shown in table 11.

Table 11

Component	Quantity
Solution A	
Sulfanilic acid ($C_6H_7NO_3S$) Acetic acid ($C_2H_4O_2$), 5 mol/l	0,8 g 100 ml
Solution B	
α-Naphthol ($C_{10}H_8O$) Acetic acid ($C_2H_4O_2$), 5 mol/l	0,5 g 100 ml

5.9 Zinc dust.

6 Apparatus and glassware

NOTE — Disposable apparatus is an acceptable alternative to reusable glassware if it has suitable specifications.

Usual microbiological laboratory equipment, and in particular:

6.1 Apparatus for dry sterilization (oven) or wet sterilization (autoclave) (autoclave either operating separately or being a part of a general apparatus for the preparation and distribution of media).

Sterilize apparatus that will come into contact with the culture media, the diluent or the sample, particularly plastic apparatus, except for apparatus that is supplied sterile, by one of the following methods:

a) in the oven (6.1) by maintaining it at 170 to 175 °C for not less than 1 h;

b) in the autoclave (6.1) at 121 ± 1 °C for not less than 20 min.

6.2 Drying cabinet, oven or incubator, ventilated by convection, capable of being maintained at 50 ± 1 °C.

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

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

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

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

6.7 Test tubes, in particular of diameter 18 mm and length 180 mm, and **culture flasks**[1] for the sterilization and conservation of the culture media.

6.8 Petri dishes, of diameter 90 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 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.

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

7 Sampling

Carry out sampling in accordance with the specific International Standard appropriate to the product concerned.

If no specific International Standard exists, it is recommended that agreement be reached on this subject by the parties concerned.

8 Preparation of the test sample

See the specific International Standard appropriate to the product concerned.

If no specific International Standard exists, it is recommended that agreement be reached on this subject by the parties concerned.

9 Procedure

9.1 Test portion, initial suspension and dilutions

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

9.2 Inoculation and incubation

9.2.1 Transfer, by means of a fresh 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.

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

NOTE — When, for certain products, it is desirable to estimate low numbers of *Bacillus 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 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 Incubate the plates (9.2.2) bottom uppermost for 18 to 24 h in an incubator (6.3) at 30 ± 1 °C. If colonies are not clearly visible, incubate the plates for an additional 24 h before counting.

9.3 Interpretation

After the period of incubation (9.2.3), select plates, preferably at two successive dilutions, containing between 15 and 150 colonies.

Count the presumptive *Bacillus cereus* colonies on each plate. The presumptive colonies are large, pink (indicating that mannitol fermentation has not occurred, see note 1) and generally surrounded by a zone of precipitation (indicating the production of lecithinase, see note 2).

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 10.1.6.

NOTES

1 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.

2 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.

3 If a 1,0 ml inoculum was spread over three plates (see the note to 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, 9.4.3 and 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 MYP complete medium (5.3.4). Incubate in an incubator (6.3) at 30 ± 1 °C for 18 to 24 h. Select from each plate at least one well-separated colony with a pink colour. Confirm this colony as specified in 9.4.2, 9.4.3 and 9.4.4.

9.4.2 Glucose-agar medium

Stab inoculate selected colonies (9.4.1) using a stab-inoculation wire (6.5) centrally into tubes containing freshly heated glucose-agar (5.4). Incubate in an incubator (6.3) at 30 ± 1 °C for 24 h.

A yellow colour through the whole tube, usually accompanied by the formation of gas, indicates a positive reaction.

9.4.3 Medium for Voges-Proskauer reaction

Inoculate selected colonies (9.4.1) into tubes containing VP-medium (5.5). Incubate in an incubator (6.3) at 30 ± 1 °C for 24 h. Transfer from each tube 1 ml of the culture to a clean tube to test for acetylmethylcarbinol. Add 0,2 ml of potassium hydroxide solution (5.6.2), 0,6 ml of α-naphthol solution (5.6.1) and a few crystals of creatine (5.6.3). Shake vigorously and leave to stand for 1 h. Formation of an eosin pink colour indicates a positive reaction.

If the reaction is negative, reincubate the tubes containing VP-medium for an additional 24 h and check again for acetylmethylcarbinol as described above.

9.4.4 Nitrate medium

Inoculate selected colonies (9.4.1) into tubes containing nitrate medium (5.7). Incubate in an incubator (6.3) at 30 ± 1 °C for 24 h.

Test for the reduction of nitrate to nitrite by adding 0,2 to 0,5 ml of nitrite reagent (5.8; see note 2) to each tube with a pipette (6.9) equipped with a rubber bulb (6.11).

Formation of red colour indicates the reduction of nitrate to nitrite. If no red colour is formed within 15 min add a small amount of zinc dust (5.9) and leave for 10 min. If after the addition of zinc dust a red colour is formed, the confirmatory test was negative.

NOTES

1 In the interests of safety, it is desirable to carry out this test under a fume hood.

2 When using solutions of sulfanilic acid and α-naphthol as indicated in the note to 5.8, test for the presence of nitrite by adding 2 drops of sulfanilic acid (solution A) and 2 drops of α-naphthol (solution B) to 3 ml of the culture.

9.4.5 Interpretation of the biochemical tests

Table 12

Test	Result to confirm *Bacillus cereus*
MYP agar medium (9.4.1)	Formation of pink colonies, surrounded by precipitate (see notes 1 and 2 to 9.3)
Glucose-agar medium (9.4.2)	The glucose is fermented, producing a yellow colour and usually gas
VP medium (9.4.3)	The production of acetylmethylcarbinol (acetoin) is positive and gives an eosin pink colour with reagents
Nitrate medium (9.4.4)	The reduction of nitrate to nitrite is positive and gives a red colour with reagents

10 Expression of results

10.1 Calculation

10.1.1 If at least 80 % of the selected colonies are confirmed (9.4.5), take as the number of *Bacillus cereus* the number given by the count made in 9.3.

10.1.2 In all other cases, calculate the number of *Bacillus cereus* from the percentage of *Bacillus cereus* obtained in 9.3 which are confirmed (9.4.5).

10.1.3 For plates containing between 15 and 150 characteristic colonies at two consecutive dilutions, calculate the number of *Bacillus cereus* for each dilution as specified in 10.1.1 and 10.1.2 and take as the result the arithmetic mean of the two values obtained. If the ratio of the higher value to the lower value is greater than 2, take the lower value as the result.

10.1.4 Calculate the average number of *Bacillus cereus* from the counts obtained on the duplicate plates (10.1.1 and 10.1.2) or from the two consecutive dilutions in the case of 10.1.3.

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 5, round it to the nearest multiple of 20;

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

Multiply the value obtained by the reciprocal of the inoculum volume (see note 3 to 9.3) and then by the reciprocal of the corresponding dilution of the test sample to obtain the number of *Bacillus cereus* per millilitre or per gram of product, according to the case.

Express the result as a number between 1,0 and 9,9 multiplied by 10^x, where x is the appropriate power of 10. An example of the calculation is given in 10.2.

10.1.5 For estimating low numbers, take the average of the counts of confirmed characteristic colonies (9.4.5) and round to the next highest whole number.

10.1.6 If the average number of confirmed colonies, as calculated in 10.1.4, is less than 15 from the plates inoculated with the test sample (liquid products) or the initial suspension (other products), report the result as follows:

a) for liquid products:

— less than $15 \times n_e$ *Bacillus cereus* per millilitre, where n_e is given by the equation

$$n_e = \frac{1}{V}$$

in which V is the volume of inoculum;

b) for other products:

— less than $15 \times N_e$ *Bacillus cereus* per gram, where N_e is given by the equation

$$N_e = \frac{1}{V} \times \frac{1}{d}$$

in which d is the dilution of the test sample;

c) for estimating low numbers in liquid products:

$m \times n_e$ *Bacillus cereus* per millilitre

where m is the average number of confirmed colonies;

d) for estimating low numbers in other products:

$m \times N_e$ *Bacillus cereus* per gram

Confidence intervals for the estimation of low numbers [c) and d)] are given in the annex.

10.1.7 If there are no confirmed colonies on plates corresponding to the test sample (liquid product) or the initial suspension (other products), report the result as

a) for liquid products:

— less than $1 \times n_e$ *Bacillus cereus* per millilitre

b) for other products:

— less than $1 \times N_e$ *Bacillus cereus* per gram

10.2 Example of calculation of *Bacillus cereus* count

For this example, the 0,1 ml of inoculum of the 10^{-2} dilution of the sample gave 65 and 85 characteristic colonies on each dish (9.3).

All five colonies selected from the plate containing 65 colonies were confirmed (9.4.5) and, therefore, all 65 colonies were considered to be *Bacillus cereus*.

Three of the five colonies selected from the plate containing 85 colonies were confirmed (9.4.5) and therefore 60 % of the 85, i.e. 51 colonies, were considered as *Bacillus cereus*.

The average count (10.1.4) is

$$\frac{65 + 51}{2} = 58 \text{ } \textit{Bacillus cereus}$$

The average count, m, of 58 is rounded to the nearest multiple of 5, i.e. 60.

The number of *Bacillus cereus* per gram or per millilitre is

$$m \times \frac{1}{V} \times \frac{1}{d}$$

$$= 60 \times \frac{1}{0,1} \times \frac{1}{10^{-2}}$$

$$= 60 \times 10 \times 10^{2}$$

$$= 60 \times 10^{3}$$

$$= 6,0 \times 10^{4}$$ *Bacillus cereus* per gram or per millilitre

10.3 Precision of the count (10.1.4)

For statistical reasons, the confidence intervals of this method vary, in 95 % of cases, from ± 16 % to ± 52 % [1]. In practice, even greater variation may be found, especially between results obtained by different microbiologists.

11 Test report

The test report shall show the method used, the incubation period and the results obtained, indicating clearly the method of expression used. It shall also mention any operating details not specified in this International Standard, or regarded as optional, together with details of incidents likely to have influenced the results.

The test report shall include all the information necessary for the complete identification of the sample.

1) Cowell and Morisetti. *J. Sci. Food Agric.*, 1969 (Vol. 20), p. 573.