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Microbiology of food and animal feeding stuffs — Horizontal method for the determination of low numbers of presumptive *Bacillus cereus* — Most probable number technique and detection method

Microbiologie des aliments — Méthode horizontale pour le dénombrement de Bacillus cereus *présumés en petit nombre — Technique du nombre le plus probable et méthode de recherche*

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Foreword

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International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. 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.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 21871 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

Introduction

Because of the large variety of food and feed products, this horizontal method may not be appropriate in every detail for certain products. In this case, different methods, which are specific to these products, may be used if absolutely necessary for justified technical reasons. Nevertheless, every attempt should be made to apply this horizontal method as far as possible.

When this International Standard is next reviewed, account will be taken of all information then available regarding the extent to which this horizontal method has been followed and the reasons for deviations from this method 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 this horizontal method. 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 this horizontal method will be those necessary for well-established technical reasons.

Microbiology of food and animal feeding stuffs — Horizontal method for the determination of low numbers of presumptive *Bacillus cereus* — Most probable number technique and detection method

1 Scope

This International Standard specifies a horizontal method for the detection or the enumeration of low numbers of viable presumptive *Bacillus cereus* by means of the most probable number technique. This International Standard is applicable to

— products intended for human consumption and the feeding of animals, and

— environmental samples in the area of food production and food handling.

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 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*

ISO/TS 11133-2, *Microbiology of food and animal feeding stuffs — Guidelines on preparation and production of culture media — Part 2: Practical guidelines on performance testing of culture media*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
presumptive *Bacillus cereus*
microorganism that forms typical or atypical colonies on the surface of selective culture media and which gives positive confirmation reactions under the conditions specified in this International Standard

NOTE For the purpose of a practical test method, this definition of presumptive *Bacillus cereus*, used as a basis for the procedure, does not exclusively describe strains of *Bacillus cereus*. In particular, the confirmatory test is inadequate to distinguish between *Bacillus cereus* and other closely related but less commonly encountered *Bacillus* species such as *Bacillus weihenstephanensis*, *Bacillus anthracis*, *Bacillus thuringiensis*, *Bacillus mycoides* and *Bacillus pseudomycoides*.

4 Principle

4.1 Enumeration method

4.1.1 Inoculation of three tubes of double-strength liquid selective enrichment medium [5.3.1.1 a)] with a specified quantity of the primary dilution (initial suspension).

4.1.2 Inoculation of three tubes of single-strength liquid selective enrichment medium [5.3.1.1 b)] with a specified quantity of the primary dilution (initial suspension). Then, under the same conditions, inoculation of single-strength liquid selective enrichment medium [5.3.1.1 b)] with specified quantities of decimal dilutions of the primary dilution (initial suspension).

4.1.3 Incubation of the tubes of double and single-strength liquid selective enrichment medium (5.3) for 48 h at 30 °C.

4.1.4 Inoculation of the solid selective medium (5.4 or 5.5) from the liquid selective enrichment medium (5.3).

4.1.5 Incubation of the solid selective medium (5.4 or 5.5) for 18 h to 48 h at 37 °C (5.4) or 30 °C (5.5) and examination of the plates to check for the presence of colonies which, from their characteristics, are considered to be presumptive *Bacillus cereus*.

4.1.6 Confirmation of suspected colonies by means of haemolysis (9.1.5.3) or by microscopic examination (9.1.5.4).

4.1.7 Calculation of the most probable number of presumptive *Bacillus cereus* per gram or per millilitre of sample from selected dilutions by reference to most probable number tables.

4.2 Detection method

4.2.1 Inoculation of a liquid selective enrichment medium (5.3) with a specified quantity of the initial suspension of the test sample.

4.2.2 Incubation of the tube for 48 h at 30 °C.

4.2.3 Inoculation of a solid selective medium (5.4 or 5.5) from the liquid selective enrichment medium (5.3).

4.2.4 Incubation of the solid selective medium (5.4 or 5.5) for 18 h to 48 h at 37 °C (5.4) or 30 °C (5.5) and examination of the plates to check for the presence of colonies which, from their characteristics, are considered to be presumptive *Bacillus cereus*.

4.2.5 Confirmation of suspected colonies by means of haemolysis (9.1.5.3) or by microscopic examination (9.1.5.4).

4.2.6 The results are given as the “presence” or “absence” of presumptive *Bacillus cereus* in grams or millilitres of product.

5 Diluent, culture media and reagents

5.1 General

For current laboratory practice, see ISO 7218, ISO/TS 11133-1 and ISO/TS 11133-2.

5.2 Diluent

See ISO 6887 (all parts), ISO 8261 and any specific standard dealing with the product to be examined.

5.3 Liquid selective enrichment medium: Tryptone soya polymyxin broth (TSPB) (see Reference [1])

5.3.1 Base medium

5.3.1.1 Composition

	a) Double-strength medium	b) Single-strength medium
Enzymatic digest of casein	34,0 g	17,0 g
Enzymatic digest of soya	6,0 g	3,0 g
Sodium chloride (NaCl)	10,0 g	5,0 g
Glucose	5,0 g	2,5 g
Dipotassium hydrogen phosphate (K_2HPO_4)	5,0 g	2,5 g
Water	1 000 ml	1 000 ml

5.3.1.2 Preparation

Dissolve the ingredients or the complete base medium in the water by heating and shaking. Adjust the pH, if necessary, so that after sterilization it is 7,3 ± 0,2 at 25 °C.

Dispense the media in quantities of 10 ml [double-strength medium (5.3.1.1 a)] and 9 ml [single-strength medium (5.3.1.1 b)] into tubes of appropriate capacity [e.g. 16 mm × 160 mm (6.7)].

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

5.3.2 Polymyxin B sulfate solution

5.3.2.1 Composition

Polymyxin B sulfate	500 000 units (equivalent to about 0,05 g)
Water	50 ml

5.3.2.2 Preparation

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

5.3.3 Complete medium

Immediately before use, add 200 µl (double-strength medium) or 100 µl (single-strength medium) of the polymyxin B sulfate solution (5.3.2) to each of the tubes containing base medium (5.3.1).

5.3.4 Performance testing for the quality assurance of the culture medium

For the definition of selectivity and productivity refer to ISO/TS 11133-2. Table 1 introduces the performance testing relating to tryptone soya polymyxin broth (TSPB):

Table 1 — Performance testing of Tryptone soya polymyxin broth (TSPB)

Function	Incubation	Strains of control	Method of control	Criteria	Characteristic reactions
Productivity	48 h at 30 °C	*B. cereus* ATCC 11778 or same strain registered in other collections	Semi-quantitative	⩾ 10 cfu on PEMBA or MYP	Characteristic colonies on PEMBA or MYP (see 5.4.5 or 5.5.6)
Selectivity	48 h at 30 °C	*E. coli* ATCC 25922 or 8739 or same strain registered in other collections	Semi-quantitative	Total inhibition	—

5.4 Solid selective medium: Polymyxin pyruvate egg yolk mannitol bromothymol blue agar (PEMBA) (see Reference [2])

5.4.1 Base medium

5.4.1.1 Composition

Enzymatic digest of casein	1,0 g
D-Mannitol	10,0 g
Sodium pyruvate	10,0 g
Magnesium sulfate, $MgSO_4 \cdot 7\ H_2O$	0,1 g
Sodium chloride	2,0 g
Disodium hydrogen phosphate (Na_2HPO_4)	2,5 g
Potassium dihydrogen phosphate (KH_2PO_4)	0,25 g
Bromothymol blue	0,12 g
Agar	9 g to 18 g [a]
Water	940 ml

[a] Depending on the gel strength of the agar.

5.4.1.2 Preparation

Dissolve the components or the dehydrated complete base medium in the water by heating and shaking.

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

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

5.4.2 Polymyxin B sulfate solution

Prepare as described in 5.3.2.

5.4.3 Egg yolk emulsion

Use fresh clean hen's eggs with their shells intact. Wash the eggs, using a brush, in liquid detergent. Rinse under running water, dip in 70 % volume fraction of 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 47 °C. Then leave for 18 h to 24 h at 3 °C ± 2 °C to allow a precipitate to form.

Collect the supernatant emulsion aseptically.

The emulsion may be stored at 3 °C ± 2 °C for not longer than 72 h.

Both solid selective media described in this International Standard were originally prepared from the 20 % egg yolk emulsion as described in Reference [3]. Ready-to-use egg yolk emulsions are commercially available, in some cases with a different concentration. These may be used. However, the manufacturer's instructions are to be followed, especially in relation to shelf life. In addition, steps are to be taken to ensure that the emulsion concerned is suitable for use in the culture media described in 5.4 and 5.5.

5.4.4 Complete medium (PEMBA agar)

5.4.4.1 Composition

Base medium (5.4.1)	940 ml
Polymyxin B sulfate solution (5.4.2)	10 ml
Egg yolk emulsion (5.4.3)	50 ml

5.4.4.2 Preparation

Melt the base medium and cool it in a water bath (6.4) set at 47 °C.

Heat the other constituents to the same temperature and then add them individually while stirring continuously.

5.4.4.3 Preparation of the agar plates

Transfer about 12,5 ml aliquots of the complete medium to Petri dishes (6.9) and leave them to solidify.

NOTE For technical reasons [2], 12,5 ml are used instead of the usual 15 ml.

The plates may be stored, prior to drying, at 3 °C ± 2 °C for up to 4 d.

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 25 °C and 50 °C until the surface of the agar is dry.

5.4.5 Performance testing for the quality assurance of the culture medium

For the definition of selectivity and productivity refer to ISO/TS 11133-2. Table 2 introduces the performance testing relating to polymyxin pyruvate egg yolk mannitol bromothymol blue agar (PEMBA).

Table 2 — Performance testing of polymyxin pyruvate egg yolk mannitol bromothymol blue agar (PEMBA)

Function	Incubation	Strains of control	Method of control	Criteria	Characteristic reactions
Productivity	18 h to 48 h at 37 °C	*B. cereus* ATCC 11778 or same strain registered in other collections	Qualitative	Good growth	Turquoise blue colonies with precipitation halo
Selectivity	18 h at 48 h at 37 °C	*E. coli* ATCC 25922 or 8739 or same strain registered in other collections	Qualitative	Total inhibition	—