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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*

Reference number
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Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
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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*.

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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.

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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

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*.

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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.

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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):

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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

Component	Amount
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.

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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	—

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5.5 Solid selective medium: Mannitol egg yolk polymyxin agar (MYP) (see Reference [4])

5.5.1 Base medium

5.5.1.1 Composition

Component	Quantity
Meat extract	1,0 g
Enzymatic digest of casein	10,0 g
D-Mannitol	10,0 g
Sodium chloride (NaCl)	10,0 g
Phenol red	0,025 g
Agar	9 g to 18 g [a]
Water	900 ml

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

5.5.1.2 Preparation

Dissolve the components or the dehydrated complete 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.

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

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

5.5.2 Polymyxin B sulfate solution

Prepare as described in 5.3.2.

5.5.3 Egg yolk emulsion

Prepare as described in 5.4.3.

5.5.4 Complete medium (MYP agar)

5.5.4.1 Composition

Component	Quantity
Base medium (5.5.1)	90,0 ml
Polymyxin B sulfate solution (5.5.2)	1,0 ml
Egg yolk emulsion (5.5.3)	10,0 ml

5.5.4.2 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.

5.5.5 Preparation of agar plates

Pour 15 ml to 20 ml portions of the complete medium (5.5.4) into sterile Petri dishes (6.9) and allow to solidify.

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

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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 60 °C until the agar surface is dry.

5.5.6 Performance testing for the quality assurance of the culture medium

For the definition of selectivity and productivity refer to ISO/TS 11133-2. Table 3 introduces the performance testing relating to mannitol egg yolk polymyxin agar (MYP)

Table 3 — Performance testing relating to mannitol egg yolk polymyxin agar (MYP)

Function	Incubation	Control strains	Method of control	Criteria	Characteristic reactions
Productivity	24 h to 48 h at 30 °C	*B. cereus* ATCC 11778 or same strain registered in other collections	Qualitative	Good growth	Pink colonies with precipitation halo
Selectivity	48 h at 30 °C	*E. coli* ATCC 25922 or 8739 or same strain registered in other collections	Qualitative	Total inhibition	—

5.6 Staining solutions for microscopic identification

5.6.1 Malachite green oxalate solution

5.6.1.1 Composition

Malachite green oxalate	5,0 g
Water	100 ml

5.6.1.2 Preparation

Dissolve the malachite green oxalate in the water.

5.6.2 Sudan black B solution

5.6.2.1 Composition

Sudan black B	0,3 g
Ethanol, 70 % (volume fraction)	100 ml

5.6.2.2 Preparation

Dissolve the Sudan black B in the ethanol.

5.6.3 Xylene

5.6.4 Safranin solution

5.6.4.1 Composition

Safranin	0,5 g

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Water	100 ml

5.6.4.2 Preparation

Dissolve the safranin in water.

5.7 Sheep blood agar

5.7.1 Base medium

5.7.1.1 Composition

Enzymatic digest of casein	15 g
Enzymatic digest of soya	5 g
Sodium chloride (NaCl)	5 g
Agar	9 g to 18 g [a]
Water	1 000 ml

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

5.7.1.2 Preparation

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

Adjust the pH, if necessary, so that after sterilization it is pH 7,3 ± 0,2 at 25 °C.

Dispense into flasks (6.7) and sterilize in an autoclave (6.1) at 121 °C for 15 min.

5.7.2 Sheep blood without fibrin

5.7.3 Complete medium

5.7.3.1 Composition

Base medium (5.7.1)	100 ml
Sheep blood without fibrin (5.7.2)	5 ml to 7 ml

5.7.3.2 Preparation

After cooling to 47 °C, add to the base medium (5.7.1) the sheep blood without fibrin (5.7.2). Mix.

Pour approximately 15-ml portions of the complete medium (5.7.3) into sterile Petri dishes (6.9) and allow to solidify.

6 Apparatus and glassware

Usual microbiological laboratory equipment (see ISO 7218) and, in particular, the following.

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

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6.2 Drying cabinet or **incubator**, ventilated by convection, for drying the agar plates, capable of being maintained between 25 °C and 50 °C.

6.3 Incubator, capable of being maintained at 30 °C ± 1 °C or 37 °C ± 1 °C.

6.4 Water bath, capable of being maintained at 47 °C ± 2 °C and approximately 80 °C.

6.5 Loops, made of platinum/iridium or nickel/chromium wire or plastic, approximately 3 mm in diameter.

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

6.7 Test tubes of sufficient dimensions with a capacity suitably in excess of 20 ml (e.g. 16 mm × 160 mm), and **culture flasks** for the sterilization and conservation of the culture media.

6.8 Vortex mixer.

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

6.10 Graduated pipettes, of nominal capacities 10 ml and 1 ml, graduated respectively in 0,5 ml and 0,1 ml.

6.11 Microscope, with oil-immersion objective.

6.12 Glass microscope slides, measuring approximately 76 mm × 26 mm.

6.13 Fine-pored filter paper, e. g. Whatman No. 41®[1)].

6.14 Flasks, of appropriate size for the detection method if larger volumes of the test portion have to be examined (see 9.2.2).

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 applicable part of ISO 6887 or ISO 8261 or 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 Enumeration method

9.1.1 Test portion, initial suspension and dilutions

See the applicable part of ISO 6887 depending on the product concerned, or ISO 8261.

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1) Whatman No. 41 is an example of 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.

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NOTE For enumeration of presumptive *Bacillus cereus* spores only, the primary dilution can be heated at 80 °C for 10 min in a water bath (6.4).

9.1.2 Inoculation and incubation

Inoculate three tubes each containing the double-strength medium [5.3.1.1 a)] with 10 ml each of the primary dilution (initial suspension) and mix the portions with the medium using a test tube mixing apparatus (6.8). These test portions correspond to 1 g of sample per tube.

Inoculate three tubes containing the single-strength medium [5.3.1.1 b)] with 1 ml each of the primary dilution (initial suspension) (equal to 0,1 g of sample per tube) or of the further dilutions (equal 0,01 g, 0,001 g, …, of sample per tube) and mix the portions with the medium using a test tube mixing apparatus (6.8).

Incubate the inoculated tubes in an incubator (6.3) at 30 °C for 48 h ± 4 h.

9.1.3 Subculture

After thorough mixing using a test tube mixing apparatus (6.8) streak an inoculation loop (6.5) of culture from each of the tubes onto the surface of polymyxin pyruvate egg yolk mannitol bromothymol blue agar (PEMBA) (5.4) or mannitol egg yolk polymyxin agar (MYP) (5.5).

Incubate the inoculated plates with the lid downwards at 37 °C (PEMBA) or 30 °C (MYP) for 18 h to 24 h. If the colonies cannot be clearly assessed, continue incubating the plates for up to additional 24 h. If PEMBA is used, the further incubation may also be carried out at room temperature.

9.1.4 Selection of plates

9.1.4.1 General

After incubation is complete, examine the plates for the presence of typical or atypical colonies.

9.1.4.2 Typical colonies

On **PEMBA**, typical colonies of presumptive *Bacillus cereus* are about 2 mm to 5 mm in size, have an irregular edge which is between ragged and root-like with ground glass surface, are turquoise to peacock blue, possibly with a greyish white colony centre against a blue background, and have a precipitation halo (egg yolk reaction) up to 5 mm wide.

On **MYP**, typical colonies are 2 mm to 5 mm in size and are ragged. They have a pink coloration against a crimson background and are surrounded by a precipitation halo (egg yolk reaction) up to 5 mm wide.

9.1.4.3 Atypical colonies

If the plates have a high content of background flora which ferments mannitol, the characteristic coloration of the colonies and background may be reduced or no longer visible. In addition, some presumptive *Bacillus cereus* strains have only a slight egg yolk reaction or none at all. In such cases and in any other doubtful cases, these colonies should also be submitted to the confirmation.

9.1.5 Confirmation

9.1.5.1 General

Typical colonies (9.1.4.2) and atypical colonies (9.1.4.3) on PEMBA or MYP shall be confirmed by means of the haemolysis test on sheep blood agar. Alternatively, typical colonies (9.1.4.2) and atypical colonies (9.1.4.3) on PEMBA (but not on MYP) may be confirmed by means of a microscopic examination.

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9.1.5.2 Selection and purification of colonies for confirmation

Select three colonies from each plate selected as in 9.1.4. If there are less than three colonies on the plate, take all colonies present. Confirm these colonies as specified in 9.1.5.3 or 9.1.5.4.

If the plates are overcrowded and it is not possible to select well-isolated colonies, take colony material from three points and streak it out on plates containing solid selective medium (5.4 or 5.5). Incubate in an incubator (6.3) set at 37 °C (PEMBA) or 30 °C (MYP) for 18 h to 24 h. Select from each plate at least one well-isolated colony. Confirm these colonies as specified in 9.1.5.3 or 9.1.5.4.

9.1.5.3 Confirmation by haemolysis test on sheep blood agar (MYP or PEMBA)

Streak the selected colonies (9.1.4.2 or 9.1.4.3) from MYP or PEMBA onto the surface of sheep blood agar (5.7) in a manner which allows well-separated colonies to develop.

Incubate at 30 °C for 24 h and read haemolysis reaction.

Each colony surrounded by a cleared zone is considered to be haemolysis-positive.

9.1.5.4 Microscopic confirmation (PEMBA)

9.1.5.4.1 Staining

Transfer some material from the centre of the colony in the case of cultures 24 h old or from the periphery in the case of older cultures, using an inoculation loop (6.5), to a degreased microscope slide (6.12) and triturate it in a small drop of water. Dry it in air and fix it by heating. Then stain the spores over boiling water with malachite green solution (5.6.1). After 2 min, rinse off excess dye with water, dry the microscope slide and cover it with a layer of Sudan black B solution (5.6.2) to stain intracellular fat. Allow the action to proceed for 15 min, then wash with xylene (5.6.3), dry with filter paper (6.13) and re-stain with safranin solution (5.6.4) to stain the sporangia. After 20 s, pour off excess dye, rinse with water and dry in air.

9.1.5.4.2 Microscopic examination

Examine the slide under a microscope (6.11) using immersion oil. As a rule, the brick-shaped cells of presumptive *Bacillus cereus* are arranged in chains and are 4 µm to 5 µm long, 1 µm to 1,5 µm wide and contain fairly large amounts of intracellular fat which is stained black. The green stained spores may be central or subterminal, but they never distend the red stained sporangia.

9.2 Detection method

9.2.1 Test portion and initial suspension

See the applicable part of ISO 6887 depending on the product concerned, or ISO 8261.

9.2.2 Inoculation and incubation

Add 1 ml of the initial suspension to 9 ml of single-strength TSPB (5.3) (i.e. 0,1 g or 0,1 ml of the sample) or 10 ml of the initial suspension to 10 ml of double-strength TSPB (5.3) (i.e. 1 g or 1 ml of the sample). For larger volumes of test portions, prepare the initial suspension by adding x ml or x g to $9x$ ml of the diluent (see the applicable part of ISO 6887 or ISO 8261) then add the entire initial suspension to $90x$ ml of single-strength TSPB (5.3) (e. g. add 5 ml or 5 g of the sample to 45 ml of the diluent, and add this entire initial suspension to 450 ml of single-strength TSPB).

Incubate the inoculated tube (6.7) or flask (6.14) in an incubator (6.3) at 30 °C for 48 h ± 4 h.

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9.2.3 Subculture

After thorough mixing, if possible using a Vortex mixer (6.8), streak an inoculation loop (6.5) of culture from the tube or flask onto the surface of polymyxin pyruvate egg yolk mannitol bromothymol blue agar (PEMBA) (5.4) or mannitol egg yolk polymyxin agar (MYP) (5.5). Then proceed as described under 9.1.3, second paragraph.

9.2.4 Selection of plates

Proceed as described under 9.1.4.

9.2.5 Confirmation

Proceed as described under 9.1.5.

10 Calculation and expression of results

10.1 Enumeration method for the determination of the most probable number (MPN)

For each dilution of liquid selective enrichment medium inoculated (9.1.2), record the number of tubes in which the presence of presumptive *Bacillus cereus* has been confirmed (9.1.5). Designate these as positive tubes.

See ISO 7218 for the determination of the most probable number (MPN) and for the expression of results.

10.2 Detection method

In accordance with the interpretation of the results, report the presence or absence of presumptive *Bacillus cereus* in the test portion, specifying the mass in grams, or the volume in millilitres, of the test sample.

11 Test report

The test report shall specify the method used (detection or enumeration, used media) and the results obtained, indicating clearly the method of expression used. It shall also mention 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.

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

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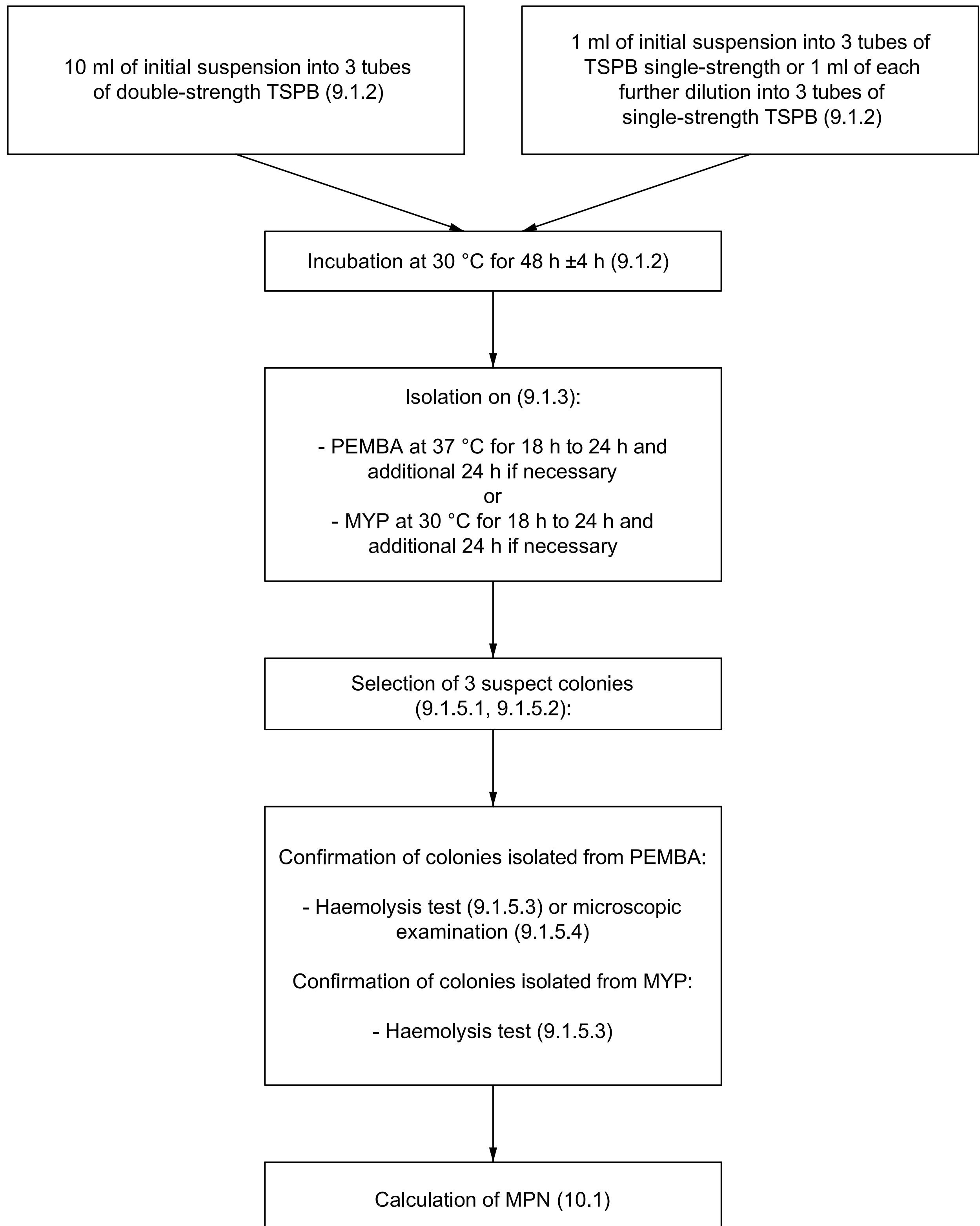
Annex A
(normative)

Diagram of enumeration procedure



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