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Microbiology of the food chain — Enumeration of *Brochothrix* spp. — Colony-count technique

Microbiologie de la chaine alimentaire — Dénombrement de Brochothrix *spp. — Technique par comptage des colonies obtenues*

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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.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation on the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see the following URL: www.iso.org/iso/foreword.html.

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

This second edition cancels and replaces the first edition (ISO 13722:1996), which has been technically revised.

The main changes compared to the previous edition are as follows:

— the method specified in this document has been aligned with the NMKL (Nordic Committee on Food Analysis) method described in Reference [8];

— the title has been changed from 'Meat and meat products' into 'Microbiology of the food chain' and from 'Enumeration of *Brochothrix thermosphacta*' into 'Enumeration of *Brochothrix* spp.', and the scope has been changed accordingly;

— actidione (cycloheximide) has been omitted from the enumeration medium because of its toxicity;

— the catalase test has been added as confirmation test;

— validation data from an NMKL study[8] have been included (Clause 11).

Microbiology of the food chain — Enumeration of *Brochothrix* spp. — Colony-count technique

1 Scope

This document specifies a method for the enumeration of viable *Brochothrix* spp. by means of a colony-count technique.

This document is especially applicable to meat and meat products, but is also suitable for the examination of the following:

— other products intended for human consumption,

— products intended for animal feeding,

— environmental samples in the area of food and feed production, handling, and

— samples from the primary production stage.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements 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 the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination*

ISO 7218, *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations*

ISO 11133, *Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media*

3 Terms and definitions

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

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— IEC Electropedia: available at http://www.electropedia.org/

— ISO Online browsing platform: available at http://www.iso.org/obp

3.1
***Brochothrix* spp.**
Gram-positive bacteria which form characteristic oxidase-negative and catalase-positive colonies on a solid selective medium [streptomycin sulfate/thallium acetate agar (STAA)] under the test conditions specified in this document

Note 1 to entry: To date only two species of *Brochothrix* have been described, *Br. thermosphacta* and *Br. campestris*. *Br. thermosphacta* has often been reported in meat and meat products, but *Br. campestris* has so far only been identified in samples from soil and grass.[9,10] The method described in this document cannot differentiate between these two species.

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 the 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 Incubation of the plates between 22 °C and 25 °C for 48 h.

4.3 Subjection of the colonies to confirmation tests.

4.4 From the number of colonies confirmed, calculation of the number of *Brochothrix* spp. per millilitre or per gram of sample from colonies obtained on plates at dilution levels chosen so as to give the most reliable result.

5 Culture media and reagents

Follow current laboratory practices as specified in ISO 7218.

Carry out the performance testing of culture media as specified in ISO 11133.

The composition of culture media and reagents and their preparation are specified in Annex A.

6 Equipment and consumables

Disposable equipment is an acceptable alternative to reusable glassware if it has suitable specifications. Usual microbiological laboratory equipment, as specified in ISO 7218, and, in particular, the following.

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

6.2 **Incubator** or **drying cabinet**, capable of operating between 25 °C and 50 °C.

6.3 **Incubator**, capable of operating between 22 °C and 25 °C.

6.4 **Petri dishes**, made of glass or plastic, of diameter 90 mm to 100 mm.

6.5 **pH-meter**, accurate to within ±0,1 pH unit at 25 °C.

6.6 **Spreaders** (hockey-stick type), length of approximately 20 cm, bent at right angles about 3 cm from one end.

6.7 **Total-delivery graduated pipettes**, of 1 ml nominal capacity, graduated in divisions of 0,1 ml or automatic pipettes.

6.8 **Water bath**, capable of being maintained at 47 °C ± 2 °C.

6.9 **Wires**, made of platinum, or **rods**, made of glass or plastic, approximately 3 mm in diameter.

7 Sampling

Sampling is not part of the method specified in this document. See the specific International Standard dealing with the product concerned. If there is no specific International Standard dealing with the

sampling of the product concerned, it is recommended that the parties concerned come to an agreement on this subject.

Recommended sampling techniques are given in:

— ISO/TS 17728[3] for food and animal feed;

— ISO 13307[1] for primary production stage;

— ISO 17604[2] for carcasses;

— ISO 18593[4] for environmental samples.

It is important that the laboratory receives a sample that is representative and the sample should not be damaged or changed during transport or storage.

8 Preparation of test sample

Prepare the test sample from the laboratory sample in accordance with the specific International Standard dealing with the product concerned; refer to the ISO 6887 series or the specific International Standard appropriate to the product concerned. If there is no specific International Standard, it is recommended that the parties concerned reach agreement on this subject.

9 Procedure

9.1 Test portion, initial suspension and dilutions

They shall be prepared in accordance with the relevant part of ISO 6887.

9.2 Inoculation and incubation

9.2.1 Transfer, by means of a sterile pipette (6.7) 0,1 ml of the test sample if the product is liquid, or of the initial suspension in the case of other products, to the STAA plate (A.2.5). Repeat the procedure using further decimal dilutions, if necessary. If only the initial suspension is used, also prepare duplicate plates using an additional agar plate.

9.2.2 Carefully spread the liquid as quickly as possible over the surface of the agar plate without touching the sides of the dish with the spreader (6.6). Use a fresh sterile spreader for each plate. Leave the plates with the lids on for about 15 min at ambient temperature for the liquid to be absorbed into the agar.

9.2.3 Invert the prepared plates (9.2.2) and incubate them for 48 h ± 4 h in the incubator (6.3) between 22 °C and 25 °C.

9.3 Counting of the colonies

After the specified period of incubation (9.2.3), count the characteristic colonies on each dish containing 10 to 150 colonies. Characteristic colonies are shiny, round or circular colonies of diameter 1 mm or larger and have an off-white colour.

9.4 Confirmation

9.4.1 General

For general information about confirmation tests refer to ISO 7218.

Select 5 colonies from each Petri dish with 10 to 150 characteristic colonies (9.3). Perform the oxidase test (9.4.2) and the catalase test (9.4.3) for each colony to confirm the presence of *Brochothrix* spp.

9.4.2 Oxidase test

Pseudomonads are able to grow on STAA medium. They shall be differentiated from *Brochothrix* spp. by performing an oxidase test as follows.

Moisten a piece of filter paper with the oxidase reagent (A.3). Take a sample of the bacterial culture obtained from the STAA medium using a platinum wire or glass or plastic rod (6.9) (a nickel/chrome wire gives false positives) and deposit it on the moistened filter paper. Oxidase-positive colonies appear within 15 s as purple colonies. *Brochothrix* spp. are oxidase-negative.

9.4.3 Catalase test

Certain lactic acid bacteria may produce characteristic colonies on STAA medium. Lactic acid bacteria are differentiated from *Brochothrix* spp. by performing the catalase test as follows.

For each colony selected, deposit a loop of culture into a drop of hydrogen peroxide solution (A.4) on a clean microscope slide. The test is positive if bubbles appear within 30 s. *Brochothrix* spp. colonies give a positive catalase reaction.

10 Expression of results

Carry out the expression of results as specified in ISO 7218.

Calculate and report the counts of *Brochothrix* spp. in cfu per gram or millilitre.

11 Performance characteristics of the method

11.1 Interlaboratory study

The NMKL method has been validated in a collaborative study[8]. The data from the study reported in this document are courtesy of NMKL.

Fifteen laboratories analysed 10 samples of lyophilized *Br. thermosphacta* cultures mixed with an unspecified bacterial flora from meat or fish. The participating laboratories analysed the samples using the method described in the NMKL standard, without the use of the catalase test for confirmation of typical colonies.

11.2 Repeatability and reproducibility limit

The results of the study demonstrated a good repeatability of the method, having a standard deviation of 0,13 cfu/g. The reproducibility of the method had a standard deviation of 0,22 cfu/g.

NOTE The interlaboratory study carried out by NMKL did not include any strains of *Br. campestris.*

12 Test report

The test report shall specify:

— the test method used, with reference to this document, i.e. ISO 13722;

— the sampling method used, if known;

— all operating conditions not specified in this document, or regarded as optional, together with details of any incidents which may have influenced the test result(s);

— any deviation in the media or the incubation conditions used;

— all information necessary for the complete identification of the sample;

— the test result(s) obtained.

Annex A
(normative)

Culture media and reagents

A.1 General

The general specifications of ISO 11133 are applicable to the preparation and performance testing of the culture media described in this annex. If culture media or reagents are prepared from dehydrated complete media/reagents or if ready-to-use media/reagents are used, follow the manufacturer's instructions regarding preparation, storage conditions, expiry date and use.

The shelf lives of the media indicated in this annex have been shown in some studies. The user should verify these under their own storage conditions (see ISO 11133).

A.2 Solid selective medium: streptomycin sulfate/thallium acetate agar (STAA)

A.2.1 Base medium

A.2.1.1 Composition

The original recipe[6,7] for STAA included cycloheximide (actidione) at 50 µg/ml. This compound is toxic and should therefore be omitted, although it is still available commercially as part of the dehydrated or ready-poured medium. When high numbers of yeasts and/or filamentous fungi are expected, the less hazardous antifungal compound, amphotericin B (10 µg/ml) is preferred.

Enzymatic digest of animal tissues	20,0 g
Yeast extract	2,0 g
Glycerol	15,0 g
Dipotassium hydrogen phosphate (K_2HPO_4)	1,0 g
Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	1,0 g
Agar[a]	9 g to 18 g
Water	900 ml
[a] Depending on the gel strength of the agar.	

A.2.1.2 Preparation

Dissolve the components or the dehydrated complete medium in the water by boiling. Adjust the pH so that after sterilization it is 7,0 ± 0,2 at 25 °C.

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

A.2.2 Streptomycin sulfate solution

A.2.2.1 Composition

Streptomycin sulfate	1,0 g
Water	100 ml

A.2.2.2 Preparation

Dissolve the streptomycin sulfate in the water. Sterilize by filtration.

A.2.3 Thallium acetate solution

WARNING — Thallium acetate has a high toxicity. Take appropriate procedures to prevent contamination of the operator and the environment when using this chemical and its solution.

A.2.3.1 Composition

Thallium acetate	250 mg
Water	100 ml

A.2.3.2 Preparation

Dissolve the thallium acetate in the water. Sterilize by filtration.

A.2.4 Complete medium

A.2.4.1 Composition

Base medium (A.2.1)	900 ml
Streptomycin sulfate solution (A.2.2)	50 ml
Thallium acetate solution (A.2.3)	20 ml

A.2.4.2 Preparation

Melt the base medium and cool it in a water bath (6.8) set at 47 °C. Using sterile conditions, warm an aliquot of the other liquids (A.2.2 and A.2.3) to 47 °C in the water bath. Add the stipulated volumes (A.2.4.1) of these solutions to the cooled medium, mixing well between each addition.

A.2.5 Preparation of agar plates for enumeration

Pour 15 ml to 20 ml portions of the complete medium (A.2.4) into sterile Petri dishes (6.4) and allow to solidify. The plates may be stored prior to drying at between 5 °C ± 3 °C for up to 1 week. Immediately before use, dry the agar plates, preferably with the lids removed and with the agar surfaces facing downwards, in the incubator (6.2) set at a temperature between 25 °C and 50 °C, until the droplets have disappeared from the surface of the medium. Do not dry them any further. The agar plates can also be dried in a drying cabinet for 30 min with half-open lids, or overnight with the lids in place. Ready-prepared agar plates are available commercially. Store and use them according to the manufacturer's instructions.

A.2.6 Performance testing of streptomycin sulfate/thallium acetate agar (STAA)

For the definition of productivity and selectivity and for the method of control refer to ISO 11133.

For the performance testing of STAA, see Table A.1.