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Dried milk — Enumeration of the specially thermoresistant spores of thermophilic bacteria

Lait sec — Dénombrement des spores spécialement thermorésistantes des bactéries thermophiles

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ISO/TS 27265 | IDF/RM 228 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF). It is being published jointly by ISO and IDF.

Foreword

IDF (the International Dairy Federation) is a non-profit organization representing the dairy sector worldwide. IDF membership comprises National Committees in every member country as well as regional dairy associations having signed a formal agreement on cooperation with IDF. All members of IDF have the right to be represented on the IDF Standing Committees carrying out the technical work. IDF collaborates with ISO in the development of standard methods of analysis and sampling for milk and milk products.

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All work was carried out by the Joint ISO-IDF Action Team on *Harmonization* of the Standing Committee on *Microbiological methods of analysis* under the aegis of its project leaders, Ms S. Miller (NZ) and Mr B. Hill (NZ).

Dried milk — Enumeration of the specially thermoresistant spores of thermophilic bacteria

1 Scope

This Technical Specification specifies a method for the enumeration of colony-forming units (CFU) of specially thermoresistant spores of thermophilic bacteria in dried milk products by using a colony-count technique at 55 °C after heating the sample at 106 °C.

The applicability of this Technical Specification is limited to dried whole milk, skim milk, and buttermilk products that are destined to be recombined and used in the manufacture of sterilized (e.g. UHT or retort-treated) milk products.

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

ISO 6887-5, *Microbiology of food and animal feeding stuffs — Preparation test samples, initial suspension and decimal dilutions for microbiological examination — Part 5: Specific rules for the preparation of milk and milk products* [1)]

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*

3 Terms and definitions

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

3.1
specially thermoresistant spores of thermophilic bacteria
bacterial spores surviving a heat treatment of 106 °C for 30 min forming colonies aerobically at 55 °C in a non-selective medium under the conditions specified in this Technical Specification

1) Supersedes ISO 8261|IDF 122.

4 Principle

A specified quantity of an initial suspension of the test sample is heat treated at 106 °C for 30 min.

Poured Petri dishes are prepared using a specified non-selective culture medium and a specified quantity of the heat-treated initial suspension.

Other dishes may be prepared, under the same conditions, using decimal dilutions of the heat-treated initial suspension.

The dishes are incubated aerobically at 55 °C for 48 h.

The number of CFU of specially thermoresistant spores of thermophilic bacteria per gram of product is calculated from the number of colonies on dishes chosen at dilution levels so as to give a significant result.

The specially thermoresistant spores of thermophilic bacteria enumerated by the method have the potential to survive commercial sterilization processes (e.g. UHT or retort processing).

5 BCP plate count skim milk agar with 0,2 % mass fraction starch

For general guidance, see ISO 7218.

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and distilled or demineralized water or water of equivalent purity.

5.1 Bromocresol purple

5.1.1 Components

Bromocresol purple	4 g
Ethanol 95 % volume fraction	100 ml

5.1.2 Preparation

Dissolve the bromocresol purple in the ethanol. Store the solution in a securely stoppered bottle to prevent evaporation.

5.2 Complete medium

5.2.1 Components

Tryptone	5,0 g
Yeast extract	2,5 g
Glucose monohydrate	1,0 g
Skimmed milk powder [a]	1,0 g
Agar	8 g to 15 g [b]
Starch, soluble [c]	2,0 g
Bromocresol purple [c] (5.1)	1 ml
Water	1 000 ml

[a] The skimmed milk powder shall be free from inhibitory substances.

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

[c] Additional components added to plate count skim milk agar.

5.2.2 Preparation

Dissolve each component in the water by heating to boiling while stirring frequently until all are completely dissolved.

Adjust the pH, if necessary, so that after sterilization it is 7,0 ± 0,2 at 25 °C. Sterilize in an autoclave at 121 °C ± 1 °C for 15 min.

The medium may also be prepared from a suitable commercial, dehydrated plate count skim milk agar to which the additional components are added.

6 Apparatus

Usual microbiological laboratory apparatus, the apparatus required for the preparation of test samples and dilutions as specified in ISO 7218 and ISO 6887-5 and, in particular the following.

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

6.1 Incubator, capable of being maintained at 55 °C ± 1 °C.

6.2 Water baths, capable of being maintained at temperatures of between 15 °C and 25 °C and at 45 °C ± 1 °C.

6.3 pH-meter, temperature-compensated, capable of being read to ± 0,1 pH units at 25 °C.

6.4 Sample heating equipment, pressure vessel or small autoclave capable of raising the sample temperature from 100 °C to 105,5 °C in < 6 min, and of maintaining the temperature of a pilot tube at 106 °C ± 0,5 °C throughout the treatment period. The pressure vessel shall be capable of being rapidly cooled to 100 °C after completion of the heat treatment. Generally that requires this vessel has a steam vent.

Unless already installed, the pressure vessel requires modification (e.g. by attaching a compression fitting with rubber seals to the lid) so that a temperature sensor can pass through the lid into the vessel.

A detailed description of an example for constructing a suitable pressure vessel is given in Annex A.

6.5 Digital thermometer, fitted with a narrow gauge temperature sensor which is accurate to 106 °C ± 0,1 °C and able to pass through the compression fitting into the pressure vessel lid.

6.6 Heat source, gas or electric equipment.

6.7 Test-tubes, screw-capped and of sufficient capacity to leave adequate head-space for mixing 10 ml of test sample. The screw cap of the pilot tube (see 8.2.2) should be so designed that the temperature sensor (6.5) used can be fitted hermetically through it.

6.8 Test-tube rack, capable of fitting in the pressure vessel shown in Annex A.

6.9 Timers (× 2).

6.10 Graduated pipettes, plugged with cotton wool, calibrated and capable of delivering 1 ml ± 0,02 ml and 10 ml ± 0,2 ml respectively.

6.11 Petri dishes, of diameter 85 mm to 100 mm.

6.12 Colony-counting equipment, consisting of an illuminated base with a dark background, fitted with a magnifying lens suitable for using magnification of at least 1,5 times, and a mechanical or electronic digital counter.

7 Sampling

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 707 | IDF 50 [1].

A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage.

8 Procedure

8.1 Preparation of the test sample and primary dilution

Prepare the primary dilution (10^{-1}) in accordance with ISO 6887-5.

8.2 Sample heat treatment

8.2.1 Using a sterile pipette (6.10), transfer 10 ml of primary dilution (10^{-1}) of each test sample into a test-tube (6.7). Close the tube but do not tighten the lid firmly.

8.2.2 Prepare a pilot tube containing 10 ml of primary dilution (10^{-1}) of one of the test samples by the same procedure as in 8.2.1.

8.2.3 Place the pressure vessel (6.4) containing the appropriate volume of water on the heat source (6.6) and heat the water to boiling.

8.2.4 Assemble the sample tubes and pilot tube in the test-tube rack (6.8). Place the temperature sensor (6.5) through the screw cap of the pilot tube and fully immerse it in the sample. Close the tube.

8.2.5 When the water is boiling, place the rack with the tubes in the pressure vessel and start one of the timers (6.9). Ensure that the pilot tube temperature sensor is still immersed in the sample and the chamber temperature sensor is within the chamber. Close the lid of the pressure vessel with the steam vent open.

8.2.6 When the sample temperature in the pilot tube reaches 100 °C, close the steam vent.

8.2.7 When the pilot tube temperature reaches 105,5 °C, stop the timer and record the time. The time should not exceed 6 min.

8.2.8 When the pilot tube temperature reaches 105,5 °C, start the second timer (6.9).

8.2.9 Allow the temperature to rise so that the sample in the pilot tube reaches 106 °C ± 0,5 °C. Stabilize at this temperature for the duration of the test by reducing the heat input from the heat source so as to ensure that the correct temperature is maintained and unnecessary water evaporation is prevented.

8.2.10 After 30 min have elapsed since starting the second timer in 8.2.8, turn off the heat source. Remove the pressure vessel from the heat source and release the pressure immediately by cautiously opening the steam vent so as to achieve a rapid fall in temperature. Open the pressure vessel as soon as the pilot tube temperature falls below 100 °C. Immediately transfer the sample rack to a water bath (6.2) maintained at a temperature of between 15 °C and 25 °C for cooling down the sample.

8.3 Inoculation and incubation

8.3.1 After the samples have been cooled, prepare six sterile Petri dishes (6.11). Using a sterile pipette (6.10), transfer 1 ml of heated test sample (10^{-1} dilution) to three of the six dishes by which approximately one-third of sample is used for each dish.

NOTE This approach reduces agar opacity and thereby ensures that all colonies are readily visible.

Repeat this procedure for the other three remaining dishes.

8.3.2 Prepare a 10^{-2} dilution of the heated test sample. Take two further sterile Petri dishes (6.11). Using another sterile pipette, transfer 1 ml of 10^{-2} dilution to each of the two dishes.

8.3.3 If necessary, repeat this operation using further decimal dilutions according to ISO 6887-5.

8.3.4 Pour about 15 ml of medium (Clause 5), previously melted and maintained in the water bath (6.2) at 45 °C, into each of the prepared Petri dishes.

8.3.5 Carefully mix the inoculum with the medium by rotating the Petri dishes. Allow the mixture to solidify by leaving the Petri dishes to stand on a cool horizontal surface.

8.3.6 The time between the preparation of the first dilution and mixing the inoculum with the medium shall not exceed 15 min.

8.3.7 Prepare a sufficient number of control plates in order to check sterility.

8.3.8 After the dishes thus prepared have been inverted, place them in the incubator (6.1) at 55 °C for 48 h ± 2 h. To minimize evaporation, place the dishes in a sealed plastic bag.

8.4 Counting of colonies

8.4.1 After the specified period of incubation (8.3.8), count the colonies on each Petri dish under subdued light using the colony-counting equipment (6.12). Ensure pinpoint colonies are included.

The bromocresol purple added to the medium (Clause 5), improves colony visibility. However, colonies may be purple or yellow depending on whether acid has been produced. Therefore, count all colonies on the plate.

8.4.2 The operator shall avoid mistaking particles of undissolved or precipitated matter in dishes for pinpoint colonies.

Examine doubtful objects carefully by using higher magnification, if required, so as to distinguish colonies from foreign matter.

8.4.3 Consider spreading colonies as single colonies. If less than one-quarter of the dish is overgrown by spreading colonies, count the colonies on the non-overgrown part of the dish. Calculate the corresponding number for the entire dish.

If more than one-quarter of the dish is overgrown by spreading colonies, discard the count.

8.4.4 When 1 ml of sample (10^{-1} dilution) has been distributed over three dishes, record the total number of colonies counted on the individual dishes as the count for that particular dilution.

8.4.5 Retain dishes and record the results where the CFU counts for a single dish (or the cumulative counts for three dishes) are less than 300. Also ensure that all counts in the range of interest (including those being outside the statistically valid range) are recorded.

8.5 Calculation and expression of test results

See ISO 7218 for the calculation and expression of test results.

9 Repeatability

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, shall in not more than 5 % of cases be greater than 0,4 lg n [2)], where n is the CFU count.

NOTE For repeatability definitions, see ISO 5725-1 [2].

If the repeatability requirements are not met, investigate the possible sources of error.

10 Test report

The test report shall contain at least the following information:

a) all information required for the complete identification of the sample;

b) the sampling method used, if known;

c) the test method used, with reference to this Technical Specification;

d) all operating details not specified in this Technical Specification, or regarded as optional, together with details of any incidents that may have influenced the test results;

e) the test results obtained, clearly mentioning the method of expression used.

2) lg n = $\log_{10} n$

Annex A
(informative)

Modification of pressure cooker for performing the thermophilic spore test

A.1 Pressure vessel, of capacity 6 l, capable of being maintained at 106 °C ± 0,5 °C, e.g. a modified "Chef's Design" [3] pressure cooker (see Figure A.1).

Key

1 proportional relief valve [Burkert type 2832 [3]]
2 digital thermometer — Pilot tube temperature
3 controller [Burkert type 8625-2 [3]] showing chamber temperature
4 "Chef's Design" [3] pressure cooker

Figure A.1 — Experimental set-up for monitoring the pilot tube temperature

A.2 Pressure control. Modify the pressure cooker as follows so as to maintain the temperature in the vessel at 106 °C ± 0,5 °C (see Figure A.1).

Fit a direct acting proportional relief valve to the lid of the pressure cooker. This valve allows steam release when the temperature in the chamber approaches the maximum temperature allowed for the test.

Govern the opening and closing of the relief valve by a control unit monitoring the temperature in the pressure cooker via a Pt-100 temperature sensor (see Figure A.2).

3) Example of a suitable product available commercially. This information is given for the convenience of the user of this Technical Specification and does not constitute an endorsement by either ISO or IDF of this product. Other products may be used provided that they can be shown to give similar results.

Weld two compression fittings (with rubber seals) to the lid of the pressure cooker so that the Pt-100 temperature sensor and the pilot tube temperature sensor (6.4) can pass through them into the pressure cooker chamber (see Figure A.2).

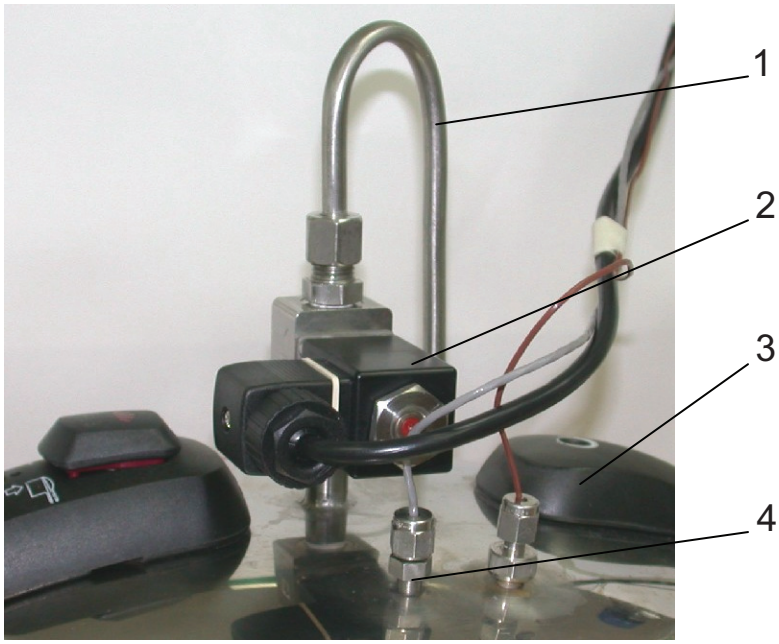
The following equipment is suitable for achieving the appropriate pressure and temperature control:

a) proportional direct-acting relief valve [Burkert type 2832 [4)]];

b) controller [Burkert type 8625-2 [4)]].

Programme the controller so that the temperature of the sample in the pilot tube is maintained at 106 °C ± 0,5 °C.

When using the Burkert 8625-2 [4)] controller, adjustment of the temperature setting, the amplification factor, and pulse time are key parameters to ensure that 106 °C ± 0,5 °C is maintained throughout the 30 min of the test heat treatment.



Key

1 Steam release tube
2 Proportional relief valve [Burkert type 2832 [4)]]
3 Compression fitting (with rubber seal) — Pilot tube temperature sensor
4 Compression fitting (with rubber seal) — Pt-100 chamber temperature sensor

Figure A.2 — Proportional relief valve and compression fittings that allow the Pt-100 and temperature sensor to enter the chamber

4) Example of a suitable product available commercially. This information is given for the convenience of the user of this Technical Specification and does not constitute an endorsement by either ISO or IDF of this product. Other products may be used provided that they can be shown to give similar results.

A.3 Test-tube rack. Figure A.3 shows a rack constructed to maximize the number of tubes that can be fitted in the pressure cooker.

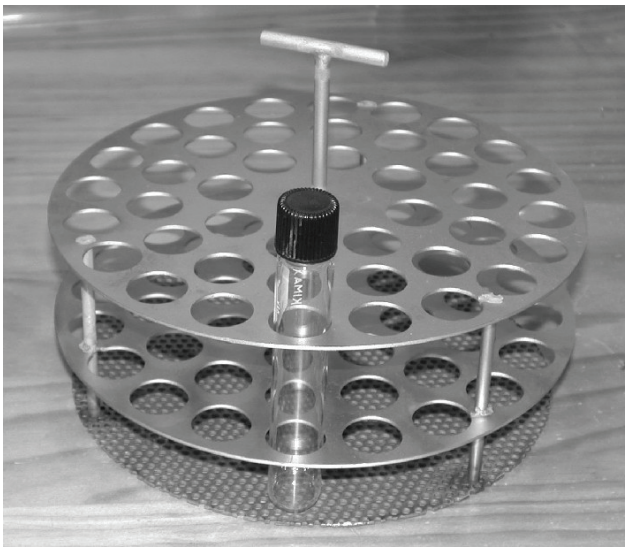


Figure A.3 — Test-tube rack to fit pressure cooker

Bibliography

[1] ISO 707 | IDF 50, *Milk and milk products — Guidance on sampling*

[2] ISO 5725-1, *Accuracy (trueness and precision) of measurement methods and results — Part 1: General principles and definitions*

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