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Milk — Enumeration of colony-forming units of psychrotrophic microorganisms — Colony-count technique at 6,5 °C

Lait — Dénombrement des unités formant colonie de micro-organismes psychrotrophes — Technique par comptage des colonies à 6,5 °C

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Foreword

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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 6730⏐IDF 101 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.

This edition of ISO 6730⏐IDF 101 cancels and replaces ISO 6730:1992, of which it constitutes a minor revision.

Foreword

IDF (the International Dairy Federation) is a worldwide federation of the dairy sector with a National Committee in every member country. Every National Committee has 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.

Draft International Standards adopted by the Action Teams and Standing Committees are circulated to the National Committees for voting. Publication as an International Standard requires approval by at least 50 % of the IDF National Committees casting a vote.

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ISO 6730 | IDF 101 was prepared by the International Dairy Federation (IDF) and Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*. It is being published jointly by IDF and ISO.

All work was carried out by the Joint ISO/IDF/AOAC Group of Experts on *Non-pathogenic contaminants with classical techniques* (E 22), under the aegis of its chairman, Mr H. Asperger (AT).

This edition cancels and replaces IDF 101A:1991.

Milk — Enumeration of colony-forming units of psychrotrophic microorganisms — Colony-count technique at 6,5 °C

1 Scope

This International Standard specifies a method for the enumeration of colony-forming units (CFU) of psychrotrophic microorganisms in raw and heat-treated milk by means of the colony-count technique at 6,5 °C.

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 6887-1:1999, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions*

ISO 7218:1996, *Microbiology of food and animal feeding stuffs — General rules for microbiological examinations*

ISO 8261:2001, *Milk and milk products — General guidance for the preparation of test samples, initial suspensions and decimal dilutions for microbiological examination*

3 Terms and definitions

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

3.1
psychrotrophic microorganisms
bacteria, yeasts and moulds forming countable colonies under the conditions specified in this International Standard

4 Principle

4.1 Poured plates are prepared using a specified culture medium and a specified quantity of the test sample. Other plates are prepared under the same conditions, using decimal dilutions of the test sample.

4.2 The plates are aerobically incubated at 6,5 °C for 10 days.

4.3 The number of colony-forming units (CFU) of microorganisms per millilitre of sample is calculated from the number of colonies obtained on plates chosen at dilution levels so as to give a significant result.

5 Diluents and culture medium

5.1 General

For general guidance, see ISO 7218.

5.2 Basic materials

See ISO 8261.

5.3 Diluents for general use

See ISO 8261.

5.4 Distribution, sterilization and storage

See ISO 8261.

5.5 Culture medium

5.5.1 Components

Tryptone	5,0 g
Yeast extract	2,5 g
Glucose monohydrate ($C_6H_{12}O_6 \cdot H_2O$)	1,0 g
Skimmed milk powder [a]	1,0 g
Agar	10 g to 15 g [b]
Water	1 000 ml

[a] The skimmed milk powder shall be free from inhibitory substances. This should be proved by comparative tests using skimmed milk powder known to be free from such substances.

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

5.5.2 Preparation

5.5.2.1 Preparation from commercial dehydrated complete medium

Follow the manufacturer's instructions but, in all cases, add the skimmed milk powder, even if the manufacturer considers such an addition unnecessary.

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

5.5.2.2 Preparation from dehydrated basic components

Dissolve and disperse in the water, in the following order: the yeast extract, tryptone, glucose and, finally, the skimmed milk powder.

NOTE Heating the water will assist in this procedure.

Add the agar and heat to boiling, stirring frequently until the agar is completely dissolved, or steam for about 30 min. If the solution is not clear, filter it through filter paper.

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

5.5.2.3 Distribution, sterilization and storage

Dispense the medium into test tubes (6.9) in quantities of 12 ml to 15 ml per tube, or into flasks or bottles (6.9) in quantities of 100 ml to 150 ml.

Sterilize in an autoclave (6.1) at 121 °C ± 1 °C for 15 min. If the medium is to be used immediately, cool it to 45 °C in the water bath (6.5). If not, before beginning the microbiological examination, in order to avoid any delay when pouring the medium, completely melt the medium in a hot water bath (6.6) then cool it to 45 °C in the water bath (6.5). (See also 8.5.4.)

Store the medium in the dark at a temperature between 0 °C and +5 °C for no longer than 3 months after preparation.

In order to check the temperature of the agar, it is recommended to place a thermometer into a portion of 15 g/l agar solution in a separate container identical to that used for the medium. This temperature control solution should be exposed to the same heating and cooling operations as the medium itself.

6 Apparatus and glassware

CAUTION — Sterilize all apparatus that will come into contact with the test sample, the diluent, the dilutions or the culture medium in accordance with ISO 8261.

Disposable apparatus is an acceptable alternative to re-usable glassware if it has suitable specifications.

Usual microbiological laboratory equipment, the apparatus required for the preparation of test samples and dilutions as specified in ISO 8261 and, in particular, the following.

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

See ISO 7218.

6.2 Incubator, capable of operating at 6,5 °C ± 0,5 °C.

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

6.4 Graduated pipettes, plugged with cotton wool, calibrated to deliver 1 ml ± 0,02 ml or 10 ml ± 0,2 ml or 11 ml ± 0,2 ml.

6.5 Water bath, capable of operating at 45 °C ± 1 °C.

6.6 Water bath, capable of operating at more than 100 °C.

6.7 Colony-counting equipment, consisting of an illuminated base with a dark background, fitted with a magnifying lens to be used at a magnification of ×1,5 and a mechanical or electronic digital counter.

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

6.9 Test tubes, of approximately 20 ml capacity (or flasks or bottles of suitable capacity), and **flasks** or **bottles** of 150 ml to 250 ml capacity, for sterilization and storage of the culture medium.

Bottles or flasks with non-toxic metal screw caps may be used.

7 Sampling

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

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

8 Procedure

8.1 General

In order to improve the precision of the method, the preparation of dilutions should be carefully standardized. Factors that affect precision are

— type of blending equipments,

— blending time,

— diluent,

— time allowed for large particles to settle, and

— mixing time allowed in the preparation of decimal dilutions.

CAUTION — Usual aseptic precautions shall be taken. The operations described in 8.2 and 8.3 shall not be carried out in sunlight.

8.2 Preparation of the test sample and primary dilution

See ISO 8261:2001, 8.2.

8.3 Further decimal dilutions

See ISO 6887-1 and ISO 8261:2001, 8.3.

Other dilution series may be used (e.g. a primary dilution of 10 ml of test sample in 90 ml of diluent, or 11 ml of test sample in 99 ml of diluent). The accuracy and precision of the method are greater when the larger quantities of sample and diluent are used.

8.4 Duration of the procedure

See ISO 6887-1.

8.5 Inoculation and incubation

8.5.1 Take two sterile Petri dishes (6.3). Transfer to each dish, by means of a sterile pipette (6.4), 1 ml of the test sample.

8.5.2 Take two further sterile Petri dishes. Transfer to each dish, by means of another sterile pipette, 1 ml of the 10^{-1} dilution of the test sample.

8.5.3 If necessary, repeat this operation using further decimal dilutions.

8.5.4 Check that the temperature of the culture medium (5.5) does not exceed 46 °C.

If the culture medium is at a temperature greater than 46 °C, it may damage or kill the psychrotrophic microflora of the sample. If any damage is expected, spread plating plus a low incubation temperature should be used.

Pour 12 ml to 15 ml of the culture medium (5.5) into each Petri dish.

If 15 ml is insufficient to obtain a homogeneous distribution of the organisms, a volume of 20 ml should be used.

8.5.5 Carefully mix the inoculum with the medium by rotating the Petri dishes, and allow the mixture to solidify by leaving the Petri dishes to stand on a cool horizontal surface.

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

8.5.7 Prepare a sufficient number of control plates to check the sterility.

8.5.8 Invert the prepared dishes and place them in the incubator (6.2) set at 6,5 °C for 10 days.

To prevent spreading, some precautions should be taken, such as

— the addition of an overlayer of culture medium after solidifying, or

— the addition of a drop of glycerol on filter paper in the lid of the dish.

8.5.9 Do not stack the dishes more than six high. Separate the stacks of dishes from one another and from the walls and top of the incubator.

8.6 Interpretation

8.6.1 Count the colonies on each plate (see 9.1), using the colony-counting equipment (6.7).

Examine the plates in subdued light. It is important that pinpoint colonies be included in the count but it is essential that the operator avoid mistaking particles of undissolved or precipitated matter in dishes for pinpoint colonies. Examine doubtful objects carefully, using higher magnification where required, to distinguish colonies from foreign matter.

8.6.2 Spreading colonies shall be considered as single colonies. If less than one-quarter of the surface is overgrown by spreading colonies, count the colonies on the unaffected part of the plate and calculate the corresponding number for the entire plate. If more than one-quarter of the surface is overgrown by spreading colonies, discard the count.

9 Expression of results

9.1 Retain dishes containing at least 10 colonies and not more than 300 colonies.

Calculate the number of CFU of microorganisms, N, per millilitre of milk, using the following formula:

$$N = \frac{\Sigma C}{\left(n_1 + 0{,}1\, n_2\right) d}$$

where

ΣC is the sum of colonies counted on all the dishes retained;

n_1 is the number of dishes retained in the first dilution resulting in 10 to 300 colonies;

n_2 is the number of dishes retained in the second dilution resulting in 10 to 300 colonies;

d is the dilution factor corresponding to the first dilution.

9.2 If there are more than two countable dilutions resulting in 10 to 300 colonies, the formula should be modified to take into account the further dilutions. For three dilutions, the formula becomes

$$N = \frac{\Sigma C}{\left(n_1 + 0{,}1\, n_2 + 0{,}01\, n_3\right) d}$$

where n_3 is the number of dishes retained in the third dilution resulting in 10 to 300 colonies.

9.3 Round the result obtained to two significant figures. When the number to be rounded is 5, with no further significant figures, round the number immediately to the left of the 5 to give an even figure. For example, 28 500 is rounded to 28 000, and 11 500 is rounded to 12 000.

Take as the result the number of CFU of psychrotrophic microorganisms per millilitre of milk, expressed as a number between 1,0 and 9,9 multiplied by 10^x, where x is the appropriate power of 10.

EXAMPLE A count of the CFU of microorganisms gave the following results (two Petri dishes per dilution were incubated):

— at the first dilution retained (10^{-2}), 168 and 215 colonies,

— at the second dilution retained (10^{-3}), 14 and 25 colonies,

$$N = \frac{\Sigma C}{\left(n_1 + 0{,}1\, n_2\right) d} = \frac{168 + 215 + 14 + 25}{[2 + (0{,}1 \times 2)] \times 10^{-2}} = \frac{422}{0{,}022} = 19\,182$$

Rounding the result as specified above gives 19 000 or $1{,}9 \times 10^4$ CFU psychrotrophic microorganisms per millilitre of milk.

9.4 If the two dishes corresponding to the test sample contain fewer than 10 colonies, report the result as "less than $10 \times 1/d$ CFU of psychrotrophic microorganisms per millilitre of milk", where d is the dilution factor of the lowest dilution.

9.5 If there are only dishes containing more than 300 colonies, calculate an estimated count from dishes having a count nearest to 300 colonies and multiply this number by the reciprocal of the value corresponding to the highest dilution. Report the result as the "estimated number of colony-forming units of psychrotrophic microorganisms per millilitre of milk".

10 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, will in not more than 5 % of cases be greater than 30 % of the lower result.

If the repeatability requirements are not met in 5 % or more of the cases, an investigation into possible sources of error should be considered.

NOTE Repeatability definitions are given in ISO 5725-1.

11 Test report

The test report shall specify:

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

b) the sampling method used, if known;

c) the test method used, with reference to this International Standard;

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

e) the test result(s) obtained, or, if the repeatability has been checked, the final quoted result obtained.

Bibliography

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

[2] ISO 5725-1:1994, *Accuracy (trueness and precision) of measurement methods and results — Part 1: General principles and definitions*

[3] ISO 5725-2:1994, A*ccuracy (trueness and precision) of measurement methods and results — Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method*

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