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EAS 68-3 (2007) (English): Milk and milk products — Methods of microbiological examination Part 3: Enumeration of colony-forming units of yeasts and/or moulds Colony-count technique at 25 °C

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EAST AFRICAN STANDARD

Milk and milk products — Methods of microbiological examination Part 3: Enumeration of colony-forming units of yeasts and/or moulds — Colony-count technique at 25 °C

EAST AFRICAN COMMUNITY

First Edition 2006

Foreword

Development of the East African Standards has been necessitated by the need for harmonizing requirements governing quality of products and services in East Africa. It is envisaged that through harmonized standardization, trade barriers which are encountered when goods and services are exchanged within the Community will be removed.

In order to achieve this objective, the Partner States in the Community through their National Bureaux of Standards, have established an East African Standards Committee.

The Committee is composed of representatives of the National Standards Bodies in Partner States, together with the representatives from the private sectors and consumer organizations. Draft East African Standards are circulated to stakeholders through the National Standards Bodies in the Partner States. The comments received are discussed and incorporated before finalization of standards, in accordance with the procedures of the Community.

East African Standards are subject to review, to keep pace with technological advances. Users of the East African Standards are therefore expected to ensure that they always have the latest versions of the standards they are implementing.

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Milk and milk products — Enumeration of colony-forming units of yeasts and/or moulds — Colony-count technique at 25 °C

Lait et produits laitiers — Dénombrement des unités formant colonie de levures et/ou moisissures — Comptage des colonies à 25 °C

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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 6611 | IDF 94 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF), in collaboration with AOAC International. It is being published jointly by ISO and IDF and separately by AOAC International.

This edition of ISO 6611 | IDF 94 cancels and replaces ISO 6611:1992, 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 and AOAC International 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 National Committees casting a vote.

ISO 6611 | IDF 94 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF), in collaboration with AOAC International. It is being published jointly by ISO and IDF and separately by AOAC International.

All work was carried out by the Joint ISO/IDF/AOAC Group of Experts, *Enumeration of yeasts and moulds in dairy products* (E34), under the aegis of its chairman, Mr J.J. Devoyod (FR).

This edition of ISO 6611 | IDF 94 cancels and replaces IDF 94B:1990, of which it constitutes a minor revision.

Milk and milk products — Enumeration of colony-forming units of yeasts and/or moulds — Colony-count technique at 25 °C

1 Scope

This International Standard specifies a method for the detection and enumeration of colony-forming units (CFU) of viable yeasts and/or moulds in milk and milk products by means of the colony-count technique at 25 °C.

The method is applicable to

— milk, liquid milk products,

— dried milk, dried sweet whey, dried buttermilk, lactose,

— cheese,

— acid casein, lactic casein, rennet casein,

— caseinate, acid whey powder,

— butter,

— frozen milk products (including edible ices),

— custard, desserts, fermented milk and cream.

NOTE This method is not suitable for a large number of thermolabile yeasts (in fresh cheese). In such cases the agar-surface-plating method is preferred.

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, *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, *Microbiology of food and animal feeding stuffs — General rules for microbiological examinations*

ISO 8261 | IDF 122:2001, *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
yeasts and moulds
microorganisms which at 25 °C form colonies in a selective medium under the conditions specified in this International Standard

4 Principle

4.1 Poured plates are prepared using a specified selective culture medium and a specified quantity of the test sample if the initial product is liquid, or of an initial suspension in the case of other products.

Other plates are prepared, under the same conditions, using decimal dilutions of the test sample or of the initial suspension.

4.2 The plates are aerobically incubated at 25 °C for 5 days.

4.3 The number of colony-forming units (CFU) of yeasts and/or moulds per gram or per millilitre of product 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

For general guidance, see ISO 7218.

5.1 Basic materials

See ISO 8261|IDF 122.

5.1.1 Diluents

For diluents for general use and diluents for special purposes, see ISO 8261|IDF 122.

5.1.2 Distribution, sterilization and storage of diluents

See ISO 8261|IDF 122.

5.2 Yeast extract/dextrose/oxytetracycline/agar medium

5.2.1 Basic medium

5.2.1.1 Components

Yeast extract powder	5,0 g
Dextrose ($C_6H_{12}O_6$)	20,0 g
Agar	10 g to 15 g[a]
Water	900 ml

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

5.2.1.2 Preparation

Dissolve the components or dehydrated complete medium in the water, by heating if necessary.

Adjust the pH, if necessary, so that after sterilization it is 6,6 at 25 °C.

Sterilize in an autoclave (6.1) at 121 °C ± 1 °C for 15 min.

5.2.2 Oxytetracycline hydrochloride solution

5.2.2.1 Components

Oxytetracycline hydrochloride ($C_{22}H_{30}O_{11}$·HCl)	50 mg
Water	50 ml

5.2.2.2 Preparation

Dissolve the oxytetracycline hydrochloride in the water. The solution shall be freshly prepared before use. Sterilize the solution by means of filtration.

5.2.3 Complete medium

5.2.3.1 Components

Oxytetracycline hydrochloride solution	10 ml
Basic medium	90 ml

5.2.3.2 Preparation

Cool the sterilized basic medium (5.2.1) to 45 °C. Just before use, bring the oxytetracycline hydrochloride solution (5.2.2) to 45 °C and add 10 ml of this solution aseptically to 90 ml of the basic medium.

5.3 Yeast extract/dextrose/chloramphenicol/agar medium

5.3.1 Components

Yeast extract powder	5,0 g
Dextrose ($C_6H_{12}O_6$)	20,0 g
Chloramphenicol ($C_{11}H_{12}Cl_2N_2O_5$)	0,1 g[a]
Agar	12 g to 15 g[b]
Water	1 000 ml

[a] In order to obtain a final concentration of 100 µg/ml of medium.

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

5.3.2 Preparation

Dissolve the components in the water by heating, if necessary.

Adjust the pH, if necessary, so that after sterilization it is 6,6 at 25 °C.

Dispense the agar medium into suitable containers (6.8).

Sterilize in an autoclave (6.1) at 121 °C ± 1 °C for 15 min.

6 Apparatus and glassware

CAUTION — Sterilize all apparatus that will come into contact with the test sample, the diluents, the dilutions or the culture medium in accordance with ISO 8261|IDF 122:2001, 6.1.

Disposable apparatus is an acceptable alternative to reusable 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|IDF 122 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 25 °C ± 1 °C.

6.3 Petri dishes, 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 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.

6.7 pH-meter, temperature-compensated, accurate to ± 0,1 pH units at 25 °C.

6.8 Culture bottles or **flasks**.

Bottles or flasks with non-toxic metal screwcaps 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.

In cheeses that are matured with a yeast or mould coat, it may be desirable to exclude the coat from the sample for analysis. In these instances, the coat may be removed using a sterile scalpel or knife before sampling is commenced.

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 as follows:

— type of blending equipment;

— blending time;

— diluent;

— time allowed for large particles to settle;

— 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 | IDF 122.

8.3 Further decimal dilutions

See ISO 8261 | IDF 122.

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, if liquid, or 1 ml of the initial suspension in the case of other products.

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 (liquid product) or 1 ml of the 10^{-2} dilution (other products).

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

8.5.4 Pour about 15 ml of the medium containing oxytetracycline hydrochloride (5.2) or the medium containing chloramphenicol (5.3), previously melted and maintained at 45 °C in the water bath (6.5), into each Petri dish.

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 After inverting the prepared dishes (8.5.5), place them (while keeping in an upright position) in the incubator (6.2) set at 25 °C for 5 days.

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

— the addition of an overlayer of culture medium after resolidifying, 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 dish. Avoid including the occasional bacterial colony that may have grown. If required, distinguish between colonies of yeasts and colonies of moulds on the basis of morphological characteristics. See 8.7.

8.6.2 Retain only dishes containing at least 10 and not more than 150 colonies. If parts of the dishes are overgrown by moulds, or if it is difficult to count well-isolated colonies, count colonies on dishes at the next higher dilution, even though their number may be less than 10. In the latter case, proceed as in 9.2.

8.7 Confirmation

The identity of any pinpoint or doubtful colonies shall be investigated by microscopic examination.

If necessary, confirm at least $\sqrt{n}$ of the colonies microscopically, where n is the number of colonies counted.

9 Expression of results

9.1 Retain dishes containing at least 10 and not more than 150 colonies.

Calculate the number of CFU of yeasts and/or moulds, N, per gram or per millilitre of product, using the following formula:

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

where

$\sum C$ is the sum of colonies counted on the dishes retained;

n_1 is the number of dishes retained in the first dilution resulting in between 10 and 150 colonies;

n_2 is the number of dishes retained in the second dilution resulting in between 10 and 150 colonies;

d is the dilution factor corresponding of the first dilution.

If there are more than two countable dilutions resulting in between 10 and 150 colonies, the formula should be modified to take into account the further dilutions. For three dilutions, the formula becomes

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

where n_3 is the number of dishes retained in the third dilution resulting in between 10 and 150 colonies.

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 yeasts and/or moulds per millilitre or per gram of product, 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 yeasts and/or moulds gave the following results (two Petri dishes per dilution were incubated):

— at the first dilution retained (10^{-2}), 83 and 97 colonies;

— at the second dilution retained (10^{-3}), 33 and 28 colonies:

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

$$= \frac{83 + 97 + 33 + 28}{[2 + (0{,}1 \times 2)]10^{-2}} = \frac{241}{0{,}022} = 10\,954$$

Rounding the results as specified in 9.1 gives 11 000 or $1{,}1 \times 10^4$ CFU of yeasts and/or moulds per gram or per millilitre of product.

9.2 If the two dishes corresponding to the test sample (liquid products) or the initial suspension (other products) contain less than 10 colonies, report the result as follows:

— less than 10 CFU of yeasts and/or moulds per millilitre (liquid products);

— less than $10 \times 1/d$ CFU of yeast and/or moulds per gram (other products), where d is the dilution factor of the initial suspension.

9.3 If there are only dishes containing more than 150 colonies, calculate an estimated count from dishes having a count nearest to 150 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 yeasts and/or moulds per gram or per millilitre of product".

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, an investigation into possible sources of error should be carried out.

NOTE For repeatability definitions, see 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 results;

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

Bibliography

[1] ISO 707, *Milk and milk products — Guidance on sampling*[1)]

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

[3] ISO 5725-2, 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*

1) Equivalent to IDF 50.

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