INTERNATIONAL STANDARD

ISO 6611

First edition
1992-02-01

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

Reference number
ISO 6611:1992(E)

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.

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.

International Standard ISO 6611 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Sub-Committee SC 5, *Milk and milk products*, in collaboration with the International Dairy Federation (IDF) and the Association of Official Analytical Chemists (AOAC), and will also be published by these organizations.

Annex A of this International Standard is for information only.

International Organization for Standardization
Case Postale 56 • CH-1211 Genève 20 • Switzerland
Printed in Switzerland

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 1 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 standards contain provisions which, through reference in this text, constitute provisions of this International Standard. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 7218:1985, *Microbiology — General guidance for microbiological examinations.*

ISO 8261:1989, *Milk and milk products — Preparation of test samples and dilutions for microbiological examination.*

3 Definition

For the purposes of this International Standard, the following definition applies.

3.1 yeasts and moulds: Micro-organisms which at 25 °C form colonies in a selective medium under the conditions specified in this International Standard.

4 Principle

4.1 Preparation of poured plates 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.

Preparation of other plates, under the same conditions, using decimal dilutions of the test sample or of the initial suspension.

4.2 Aerobic incubation of the plates at 25 °C for 5 days.

4.3 Calculation of the number of colony-forming units (CFU) of yeasts and/or moulds per gram or per millilitre of product 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 Diluents for special purposes

See ISO 8261.

5.5 Distribution, sterilization and storage of diluents

See ISO 8261.

5.6 Yeast extract/dextrose/oxytetracycline/agar medium

5.6.1 Basic medium

5.6.1.1 Components

Yeast extract powder	5,0 g
Dextrose ($C_6H_{12}O_6$)	20,0 g
Agar	10 g to 15 g [1]
Water	900 ml

1) According to the gel strength of the agar.

5.6.1.2 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.

5.6.2 Oxytetracycline solution

5.6.2.1 Components

Oxytetracycline hydrochloride ($C_{22}H_{30}N_2O_{11}$)·HCl	50 mg
Water	50 mg

5.6.2.2 Preparation

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

5.6.3 Complete medium

5.6.3.1 Components

Oxytetracycline hydrochloride	10 ml
Basic medium	90 ml

5.6.3.2 Preparation

Cool the sterilized basic medium (5.6.1) to 45 °C. Just before use, bring the oxytetracycline solution (5.6.2) to 45 °C and add 10 ml of this solution aseptically to 90 ml of the basic medium.

5.7 Yeast extract/dextrose/chloramphenicol/agar medium

5.7.1 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 [1]
Agar	12 g to 15 g [2]
Water	1 000 ml

1) In order to obtain a final concentration of 100 µg/ml of medium.

2) According to the gel strength of the agar.

5.7.2 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 the 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 8261:1989, 6.1.

NOTE 2 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 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 Temperature-compensated pH-meter, accurate to ± 0,1 pH units at 25 °C.

6.8 Culture bottles or **flasks**

NOTE 3 Bottles or flasks with non-toxic metal screw-caps may be used.

7 Sampling

Sampling should have been carried out in accordance with ISO 707.

NOTE 4 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

NOTE 5 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 8.1 and 8.2 shall not be carried out in sunlight.

8.1 Preparation of the test sample and primary dilution

See ISO 8261.

8.2 Further decimal dilutions

See ISO 8261.

8.3 Duration of the procedure

See ISO 8261:1989, 8.3.

8.4 Inoculation and incubation

8.4.1 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.

8.4.2 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).

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

8.4.4 Pour about 15 ml of the medium containing oxytetracycline (5.6) or the medium containing chloramphenicol (5.7), previously melted and maintained at 45 °C in the water-bath (6.5), into each Petri dish.

8.4.5 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.

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

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

8.4.8 Invert the prepared dishes (8.4.5) and place them in the incubator (6.2) set at 25 °C for 4 days.

NOTE 6 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.

8.4.9 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.

8.5 Interpretation

8.5.1 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 8.6.

8.5.2 Retain only dishes containing between 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.

8.6 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 10 to 150 colonies;

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

d is the dilution factor corresponding to the first dilution.

NOTE 7 If there are more than two countable dilutions resulting in 10 to 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 10 to 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 result 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 yeasts 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".

10 Repeatability

The absolute difference between two 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 not be greater than 30 % of the lower result.

NOTES

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

9 For repeatability definitions, see ISO 5725.

11 Test report

The test report shall specify the method used 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.

Annex A
(informative)

Bibliography

[1] ISO 707:1985, *Milk and milk products — Methods of sampling.*

[2] ISO 5725:1986, *Precision of test methods — Determination of repeatability and reproducibility for a standard test method by inter-laboratory tests.*

This page intentionally left blank