INTERNATIONAL STANDARD

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Cereals, pulses and derived products — Enumeration of bacteria, yeasts and moulds

Céréales, légumineuses et produits dérivés — Dénombrement des bactéries, levures et moisissures

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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.

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 7698 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*.

Annex A of this International Standard is for information only.

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Introduction

The enumeration of bacteria, yeasts and moulds in cereals, pulses and their derived products enables determination of their micro-organisms content and/or, depending on the objective, determination of the nature of the microflora present. The objective may be, for example, to determine the quality in terms of hygiene of a lot, to study the dynamic progression of a microflora in order to evaluate the efficiency of a particular type of storage, or to evaluate the impact of a physical or chemical treatment on the microflora.

In this type of analysis, which is usually carried out on ground products, fragments of thallus are taken into account, but it is the fungal spores in particular that are enumerated, and more so where the species present are highly sporulating. This method constitutes an effective means of following the progression of micro-organisms (in cereals, pulses and derived products) of a particular evolutionary series provided that the identification of those species which allow the evolution to be followed has been carried out. Under these conditions, which relate more to experimentation than to the testing of unknown lots, the values given by the enumeration correlate quite well with those given by more technological criteria (e.g. fat acidity and germinative capacity).

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Cereals, pulses and derived products — Enumeration of bacteria, yeasts and moulds

1 Scope

This International Standard specifies a method for the enumeration of bacteria, yeasts and moulds in cereals, pulses and their immediate derived products (flour, semolina, bran, etc.).

It takes into account the standards giving general guidance, notably ISO 7954[1], prepared by subcommittee 9, *Microbiology*, of ISO/TC 34, *Agricultural food products*.

Reference should be made to ISO 7218[2] for the good laboratory practice for microbiological examinations.

NOTE 1 Owing to the nature of yeasts and moulds, the enumeration is subject to certain imprecisions.

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 950:1979, *Cereals — Sampling (as grain)*.

ISO 2170:1980, *Cereals and pulses — Sampling of milled products*.

ISO 6887:1983, *Microbiology — General guidance for the preparation of dilutions for microbiological examination*.

3 Definitions

For the purposes of this International Standard, the following definitions apply.

3.1 bacteria: Mesophilic micro-organisms, either aerobic or facultatively anaerobic, which at 30 °C develop within and on the surface of an agar medium under the conditions described in this International Standard.

3.2 yeasts: Mesophilic aerobic micro-organisms which, at 25 °C using mycological agar medium under the conditions described in this International Standard, either

a) on the surface of the medium, develop matt or shiny round colonies usually having a regular outline and a more or less convex surface, or

b) within the medium, develop round, lenticular, colonies.

3.3 moulds: Mesophilic aerobic filamentous micro-organisms which, on the surface of mycological agar medium under the conditions described in this International Standard, usually develop flat or fluffy spreading colonies often with coloured fruiting or sporing structures.

4 Principle

4.1 Preparation of duplicated poured plates of each of the two specified culture media (5.3.1 and 5.3.2) containing a specified quantity of an initial suspension.

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

4.2 Aerobic incubation of plates containing the agar medium for enumerating bacteria (5.3.1) at 30 °C for 3 days.

Aerobic incubation of plates containing the agar medium for enumerating yeasts and moulds (5.3.2) at 25 °C for 3 days, 4 days or 5 days.

4.3 Calculation of the number of bacteria from the number of colonies present on selected plates of the agar medium (5.3.1).

Calculation of the number of yeasts and/or moulds from the number of colonies present on selected plates of the agar medium (5.3.2).

5 Diluent, culture media and other products

5.1 Basic materials

In order to improve the reproducibility of the results, it is recommended that, for the preparation of the diluent and culture media, dehydrated basic components or complete dehydrated media be used. Similarly, commercially prepared reagents may be used. The manufacturer's instructions shall be rigorously followed.

The chemical products used shall be of recognized analytical grade.

The water used shall be distilled or deionized, and shall be free from substances that might inhibit the growth of micro-organisms (bacteria, yeasts and moulds) under the test conditions.

If the diluent and media are not used immediately, they shall, unless otherwise stated, be stored in the dark at a temperature between 0 °C and + 5 °C, for no longer than one month, in conditions that prevent any change in their composition.

5.2 Diluent

Peptone-sodium chloride solution, see ISO 6887.

NOTE 2 To improve the homogeneity of spore suspensions, a surface-active agent such as sorbitol oleic ester (Tween 80) may be added at a concentration of 0,033 g per litre of diluent.

5.3 Culture media

5.3.1 Agar medium for enumerating bacteria

Composition

tryptone[1]	5,0 g
dehydrated yeast extract	2,5 g
anhydrous dextrose	1,0 g
agar	9 g to 18 g[2]
water	1 000 ml

NOTE 3 If required, an inhibitor product such as actidione (cycloheximide) or natamycin (pimaricin) may be added to the culture medium, in quantities of 0,1 g/l, to prevent the development of yeasts.

Preparation

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

Dispense the medium into the test tubes (6.9) in quantities of 15 ml per tube or into flasks or bottles (6.9), of a suitable capacity, to approximately half the volume of the flask or bottle.

Sterilize the medium in the autoclave (6.1) at 121 °C ± 1 °C for 20 min.

If the medium is to be used immediately, cool it before use in the water-bath (6.7) set at 45 °C ± 0,5 °C.

If not, before beginning the microbiological examination, in order to avoid delay when pouring the agar, completely melt the medium in a boiling water-bath, then cool it before use in the water-bath (6.7) set at 45 °C ± 0,5 °C.

5.3.2 Agar medium for enumerating yeasts and moulds (yeast extract-dextrose-chloramphenicol-agar medium)

Composition

yeast extract	5 g
dextrose ($C_6H_{12}O_6$)	20 g
chloramphenicol ($C_{11}H_{12}Cl_2N_2O_5$)	0,1 g
agar	9 g to 18 g[2]
water	1 000 ml

1) This term is only used at present by certain producers of media. Any other casein digest giving comparable results may be used.

2) According to the manufacturer's instructions.

Preparation

Dissolve the components in the water by boiling. If necessary, adjust the pH so that after sterilization it is 6,6 at 25 °C.

Dispense the medium into containers (6.9) of appropriate capacity.

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

If the medium is to be used immediately, cool it before use in the water-bath (6.7) set at 45 °C ± 0,5 °C.

If not, before beginning the microbiological examination, in order to avoid delay when pouring the agar, completely melt the medium in a boiling water-bath, then cool it before use in the water-bath (6.7) set at 45 °C ± 0,5 °C.

NOTE 4 Chloramphenicol may be replaced by oxytetracycline ($C_{22}H_{30}N_2O_{11}$). In this case, prepare the basic medium as described above, omitting the chloramphenicol. Dispense it in quantities of 100 ml and sterilize. Prepare also a 0,1 % (*m*/*m*) solution of oxytetracycline hydrochloride in water and sterilize by filtration. Just prior to use, add 10 ml of this solution aseptically to 100 ml of the basic medium which has been melted and maintained at 45 °C ± 0,5 °C.

6 Apparatus and glassware

NOTE 5 Disposable apparatus is an acceptable alternative to reusable glassware, if it has suitable specifications.

Usual microbiological laboratory equipment and, in particular, the following.

6.1 Apparatus for dry sterilization (oven) or **wet sterilization (autoclave)** (autoclave either operating separately or being part of a general apparatus for the preparation and distribution of media).

Sterilize apparatus that will come into contact with the diluent, the culture media or the sample, particularly plastic apparatus, except for apparatus that is supplied sterile, by one of the following methods:

a) in the oven (6.1) by maintaining it at 170 °C to 175 °C for not less than 1 h;

b) in the autoclave (6.1) by maintaining it at 121 °C ± 1 °C for not less than 20 min.

6.2 Blending equipment.

In accordance with ISO 6887, one of the following shall be used:

a) a **rotary blender**, preferably with top drive, operating at a rotational frequency between 8 000 min^{-1} and 45 000 min^{-1}, with glass or metal bowls, preferably fitted with lids, resistant to the conditions of sterilization;

b) a **peristaltic-type blender** (Stomacher), with sterile plastic bags.

NOTE 6 The bowls or plastic bags should have sufficient capacity to allow the sample to be properly mixed with the appropriate amount of diluent. In general, the volume of the container should be equal to about twice the volume of the sample plus diluent.

6.3 Mixer, vortex-type, for mixing the contents of the test tubes, flasks or bottles (dilutions of the initial suspension).

6.4 Incubators, capable of being controlled at 30 °C ± 1 °C and at 25 °C ± 1 °C.

6.5 Petri dishes, of 90 mm to 100 mm diameter.

6.6 Graduated pipettes, calibrated for bacteriological use only, of nominal capacities 10 ml and 1 ml, graduated in divisions of 0,5 ml and 0,1 ml respectively, and with an outflow opening of 2 mm to 3 mm.

6.7 Water-bath, or similar equipment, capable of being controlled at 45 °C ± 0,5 °C.

6.8 pH meter, accurate to ± 0,1 pH unit at 25 °C.

6.9 Test tubes, in particular of dimensions 20 mm × 200 mm, or **flasks** or **bottles**, of 0,5 l and 1 l capacity.

7 Sampling

Sampling shall have been carried out in accordance with ISO 950 or ISO 2170, as appropriate.

The amount of laboratory sample shall be greater than the mass of the test portion required for the analyses, as shown in table 1 (9.1), to enable repetition of the analyses if necessary.

If it is not possible to perform the analysis as soon as the samples are received at the laboratory, they may be stored at approximately 10 °C for a maximum period of 48 h. This fact shall be mentioned in the test report. Under no circumstances shall the samples be frozen.

8 Preparation of the test sample

Thoroughly mix the laboratory sample before taking representative test samples.

NOTE 7 Owing to the nature of the products covered by this International Standard, the micro-organisms are frequently unevenly distributed throughout the test sample

and it is desirable to carry out separate determinations on at least three and preferably five test portions from the test sample where an accurate assessment of the microbial population is required.

9 Procedure

9.1 Test portion

Weigh, to the nearest 0,1 g, the mass of the test portion specified in table 1 into

a) for products in category 1, the bowl of a rotary blender [6.2 a)], or

b) for products in category 2, a plastic bag of a Stomacher [6.2 b)].

Table 1

Category	Product	Mass of test portion g	Volume of diluent ml
1	Grain or seed	40	360
2	Milled products (flour, semolina, bran, ...)	20	180

9.2 Preparation of the initial suspension

Add to the test portion (9.1) the corresponding volume of diluent specified in table 1.

Leave the test portion and diluent in contact for 30 min. Then, either

a) operate the rotary blender [6.2 a)] for a sufficient time to give a total number of revolutions of 15 000 to 20 000 (even with the slowest blender, this time shall not exceed 2,5 min), or

b) operate the Stomacher [6.2 b)] for 2 min.

9.3 Preparation of the dilutions

Prepare the dilutions in accordance with ISO 6887.

Before each aliquot portion is taken for the inoculation, mix the contents of the test tube using a vortex-type mixer (6.3).

9.4 Inoculation

9.4.1 Take four sterile Petri dishes (6.5). Using a sterile pipette, transfer to each dish 1 ml of the initial suspension (10^{-1} dilution) (9.2).

9.4.2 Take four other sterile Petri dishes. Using a new sterile pipette, transfer to each dish 1 ml of the 10^{-2} dilution (9.3).

Repeat this procedure using further dilutions as required (see 9.6.1 and 9.6.2).

9.4.3 For each group of four dishes, pour into two dishes approximately 15 ml of the agar medium 5.3.1 and into the other two dishes approximately 15 ml of the agar medium 5.3.2.

Carefully and thoroughly mix the inoculum and the medium, and leave to solidify, with the dishes standing on a cool horizontal surface.

Also prepare two control dishes, one containing approximately 15 ml of the agar medium 5.3.1 and the other containing approximately 15 ml of the agar medium 5.3.2, to check their sterility.

9.5 Incubation

9.5.1 Bacteria

Invert the plates containing the agar medium 5.3.1 and place them in the incubator (6.4) controlled at 30 °C ± 1 °C for 3 days.

9.5.2 Yeasts and moulds

Place the plates, which may be upright or inverted, containing the agar medium 5.3.2 in the incubator (6.4) controlled at 25 °C ± 1 °C for 5 days.

9.6 Interpretation

9.6.1 Enumeration of colonies of bacteria

Examine the plates after the specified incubation period (9.5.1).

Carry out the enumeration of the colonies for each plate of the agar medium 5.3.1 containing not more than 300 colonies. It is necessary that one of these plates contains at least 15 colonies.

9.6.2 Enumeration of colonies of yeasts and/or moulds

Count the colonies on each plate after 3 days, 4 days and 5 days of incubation. After 5 days, retain those plates containing fewer than 150 colonies. If parts of the plates are overgrown with moulds, or if it is difficult to count well-isolated colonies, retain the counts obtained after 4 days or even 3 days of incubation. In this event, the incubation period of 3 days or 4 days shall be stated in the test report.

The distinction between yeast colonies and mould thalli is made through macroscopic examination. However, in ambiguous cases, perform a micro-

scopic examination of the "colonies": yeast colonies are generally composed of ovoid or round cells, whereas thalli show mycelian filaments.

If required, carry out a microscopic examination in order to distinguish, according to their morphology, the colonies of yeasts and moulds from colonies of bacteria.

10 Expression of results

10.1 Calculation

10.1.1 Calculate the number of micro-organisms, i.e. bacteria, yeasts and/or moulds, per gram of product using the formula

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

where

$\sum C$ is the sum of colonies on all plates counted and retained at two successive dilutions;

d is the dilution from which the first counts were obtained (e.g. 10^{-2});

n_1 is the number of plates counted and retained in the first dilution;

n_2 is the number of plates counted and retained in the second dilution.

10.1.2 Round the result obtained in 10.1.1 to two significant figures. When the number to be rounded is 5, with no further significant figures, round the number to give an even number immediately to the left: for example 28 500 is rounded to 28 000; 11 500 is rounded to 12 000.

10.1.3 Express the result as a number between 1,0 and 9,9 multiplied by 10^x, where x is the appropriate power of 10.

If there are no colonies on the plates from the initial suspension (9.4.1), the number of micro-organisms, i.e. bacteria, yeasts and/or moulds, per gram of product shall be reported as less than 10.

10.2 Example of calculation

A yeasts and moulds count gave the following results (two Petri dishes per dilution were incubated):

10^{-2} dilution: 105 and 97 colonies

10^{-3} dilution: 18 and 23 colonies

$$\frac{\sum C}{(n_1 + 0{,}1n_2)d} = \frac{105 + 97 + 18 + 23}{[2 + (0{,}1 \times 2)]10^{-2}} = \frac{243}{0{,}022}$$

$$= 11\,045$$

Rounding the result as specified 10.1.2 gives 11 000.

The estimated number of yeasts and moulds per gram of product is therefore $1{,}1 \times 10^4$.

10.3 Precision

For statistical reasons, in 95 % of cases the confidence levels for this method vary from ± 16 % to ± 52 % [3]. In practice, even larger variations may be observed, in particular between the results obtained by different microbiologists.

11 Test report

The test report shall show the method used, the incubation period and the results obtained, indicating clearly the method of expression used. It shall also mention any operating details not specified in this International Standard, or regarded as optional, together with details of any incidents likely to have influenced the results.

The test report shall include all the information necessary for the complete identification of the sample.