INTERNATIONAL STANDARD

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Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of yeasts and moulds —

Part 1:
Colony count technique in products with water activity greater than 0,95

Microbiologie des aliments — Méthode horizontale pour le dénombrement des levures et moisissures —

Partie 1: Technique par comptage des colonies dans les produits à activité d'eau supérieure à 0,95

Reference number
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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 21527-1 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

ISO 21527 consists of the following parts, under the general title *Microbiology of food and animal feedings stuffs — Horizontal method for the enumeration of yeasts and moulds*:

— *Part 1: Colony count technique in products with water activity greater than 0,95*

— *Part 2: Colony count technique in products with water activity less than or equal to 0,95*

This part of ISO 21527, together with ISO 21527-2, cancel and replace ISO 7698:1990, ISO 7954:1987 and ISO 13681:1995.

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Introduction

Because of the large variety of food and feed products, the applications of the horizontal method specified in ISO 21527 (all parts) may not be appropriate for certain products. In this case, different methods, which are specific to these products, may be used if absolutely necessary for justified technical reasons. Nevertheless, every attempt shall be made to apply the horizontal method as specified in ISO 21527 (all parts) as far as possible.

When ISO 21527 (all parts) is next reviewed, account will be taken of all information then available regarding the extent to which the horizontal method has been followed and the reasons for deviations from this method in the case of particular products.

The harmonization of test methods cannot be immediate, and for certain groups of products International Standards and/or national standards may already exist that do not comply with the horizontal method as specified in ISO 21527 (all parts). It is hoped that when such standards are reviewed they will be changed to comply with ISO 21527 (all parts) so that eventually the only remaining departures from this horizontal method will be those necessary for well-established technical reasons.

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Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of yeasts and moulds —

Part 1: Colony count technique in products with water activity greater than 0,95

WARNING — It is essential that enumeration of moulds is carried out with the greatest care to protect the operator and to prevent contamination of the atmosphere with mould spores.

1 Scope

This part of ISO 21527 specifies a horizontal method for the enumeration of viable yeasts and moulds in products intended for human consumption or feeding of animals that have a water activity greater than 0,95 [eggs, meat, dairy products (except milk powder), fruits, vegetables, fresh pastes, etc.], by means of the colony count technique at 25 °C ± 1 °C (References [1], [2]).

This part of ISO 21527 does not allow the enumeration of mould spores. Neither the identification of fungal flora nor the examination of foods for mycotoxins lie within the scope of this part of ISO 21527. The method specified in this part of ISO 21527 is not suitable for enumeration of heat-resistant fungi, such as *Byssochlamys fulva* or *Byssochlamys nivea*, in canned or bottled fruit and vegetables.

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 (all parts), *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination*

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

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

ISO/TS 11133 (all parts), *Microbiology of food and animal feeding stuffs — Guidelines on preparation and production of culture media*

3 Terms and definitions

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

NOTE There are some intermediate forms and the distinction between a **yeast** (3.1) and a **mould** (3.2) can be arbitrary.

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3.1
yeast
mesophilic aerobic microorganism which, at 25 °C using mycological agar medium under the conditions described in this part of ISO 21527, develops matt or shiny round **colonies** (3.4) on the surface of the medium, usually having a regular outline and a more or less convex surface

NOTE Yeasts within, rather than on, a medium develop round, lenticular, colonies.

3.2
mould
mesophilic aerobic filamentous microorganism which, on the surface of mycological agar medium under the conditions described in this part of ISO 21527, usually develops flat or fluffy spreading **propagules/germs** (3.3) or **colonies** (3.4) often with coloured fruiting or sporing structures.

NOTE Moulds within, rather than on, a medium can develop round, lenticular, colonies.

3.3
propagule
germ
viable entity capable of growth in a nutrient medium

EXAMPLE Vegetative cell, group of cells, spore, spore cluster, or a piece of fungal mycelium.

[ISO 6107-6:2004, 65]

3.4
colony
localized visible accumulation of microbial mass developed on or in a solid nutrient medium from a viable particle

[ISO 6107-6:2004, 15]

4 Principle

4.1 Surface-inoculated plates are prepared using a specified selective culture medium. Depending on the expected number of colonies, a specified quantity of the sample (if the product is liquid), or of an initial suspension (in the case of other products), or decimal dilutions of the sample/suspension are used.

Additional plates can be prepared under the same conditions, using decimal dilutions of the test sample or of the initial suspension.

4.2 The plates are then aerobically incubated at 25 °C ± 1 °C for 5 d. If necessary, the agar plates are left to stand in diffuse daylight for 1 d to 2 d.

4.3 Colonies/propagules are then counted and, if required (to distinguish yeast colonies from bacterial colonies), the identity of any doubtful colonies is confirmed by examination with a binocular magnifier or microscope.

4.4 The number of yeasts and moulds per gram or per millilitre of sample is calculated from the number of colonies/propagules/germs obtained on plates chosen at dilution levels producing countable colonies. Moulds and yeasts are counted separately, if necessary.

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5 Diluent and culture medium

For current laboratory practice, see ISO 6887 (all parts) and ISO 8261.

5.1 Diluent

5.1.1 General

See ISO 6887 (all parts), ISO 8261 and the specific International Standard dealing with the product concerned.

NOTE It is possible to add surface-active agents such as sodium poly(oxyethylene)sorbatitanmonooleate [1)] [0,05 % (mass concentration)] to diluents to reduce clumping of mould spores and conidia (Reference [2]).

Except for specific preparation of the test sample, the use of 0,1 % (mass concentration) peptone water broth as diluent is recommended.

5.1.2 Composition of 0,1 % (mass concentration) peptone water broth

Enzymatic digest of animal or vegetal tissues	1,0 g
Water	1 000 ml

5.1.3 Preparation of 0,1 % (mass concentration) peptone water broth

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

If necessary, adjust the pH so that, after sterilization, it is 7,0 ± 0,2 at 25 °C.

5.2 Culture medium

5.2.1 Dichloran-rose bengal chloramphenicol agar (DRBC) (References [3], [4])

5.2.1.1 Composition

Enzymatic digest of animal and plant tissues	5,0 g
D-Glucose ($C_6H_{12}O_6$)	10,0 g
Potassium dihydrogenphosphate (KH_2PO_4)	1,0 g
Magnesium sulfate ($MgSO_4 \cdot H_2O$)	0,5 g
Dichloran (2,6-dichloro-4-nitroaniline)	0,002 g
Rose bengal	0,025 g
Agar	12 g to 15 g [a]
Chloramphenicol	0,1 g
Water, distilled or deionized	1 000 ml

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

1) Tween 80 is an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

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5.2.1.2 Preparation

5.2.1.2.1 General

Suspend all the ingredients except chloramphenicol in the water and bring to the boil to dissolve completely. If necessary, adjust the pH (6.4) so that after sterilization it is 5,6 ± 0,2 at 25 °C.

Add 10 ml of a 1 % (mass concentration) solution of chloramphenicol in ethanol and mix. Dispense the medium in quantities into suitable containers (6.5) of suitable capacity. Sterilize by autoclaving at 121 °C for 15 min.

Immediately, cool the medium in a water bath (6.3) maintained at a temperature of 44 °C to 47 °C. Cool to below 50 °C and dispense 15 ml amounts into sterile Petri dishes (6.6).

Allow the medium to solidify, and dry, if necessary, the surface of the plates as described in ISO 7218 and ISO/TS 11133 (all parts).

Use immediately, or store in the dark, according to ISO/TS 11133 (all parts) until required.

CAUTION — Avoid exposure of the medium to light, since cytotoxic breakdown products can result in underestimation of mycoflora in samples.

5.2.1.2.2 Optional addition of chlortetracycline hydrochloride

Where bacterial overgrowth may be a problem (e.g. raw meats), chloramphenicol (50 mg/l) and chlortetracycline (50 mg/l) are recommended. In this case, prepare the basic medium as described above, with only chloramphenicol 50 mg, dispense it in quantities of 100 ml and sterilize. Prepare also a 0,1 % (mass concentration) solution of chlortetracycline hydrochloride in water (relatively unstable in solution, it must be freshly prepared) and sterilize by filtration. Just prior to use, add 5 ml of this solution aseptically to 100 ml of the basic medium, and pour plates. Gentamicin is not recommended, as it has been reported to cause inhibition of some yeast species.

5.2.1.2.3 Optional addition of trace elements

In order for moulds to exhibit their full morphology, particularly any pigments they normally produce, they need trace elements that may not be present in DRBC. To identify moulds on this medium, add the following trace element solution at 1 ml per litre of the medium, prior to autoclaving: $ZnSO_4 \cdot 7H_2O$ 1g; $CuSO_4 \cdot 5H_2O$ 0,5 g; water, distilled or deionized 100 ml (Reference [1]).

5.2.1.2.4 Optional addition of Tergitol[2)]

In order to avoid overgrowth of Mucoraceae on agar plates, the addition of Tergitol[2)] (1 ml/l) to the culture medium is recommended.

5.2.1.3 Performance testing for the quality assurance of the culture medium

5.2.1.3.1 General

DRBC is a solid medium. Productivity and selectivity shall be tested according to ISO/TS 11133 (all parts) according to the following specifications:

2) Example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

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5.2.1.3.2 Productivity

Incubation:	5 d at 25 °C ± 1 °C
Strains:	*Saccharomyces cerevisiae* ATCC 9763 *Candida albicans* ATCC 10231 *Aspergillus niger* ATCC 16404 *Mucor racemosus* ATCC 42647 or strains recorded as equivalent in other fungal collections
Reference media:	media batch SDA (Sabouraud dextrose agar) already validated
Control method:	quantitative
Criteria:	productivity ratio, $P_R \geqslant 0,5$
Characteristic reaction:	characteristic colony/propagules/germs according to each species

5.2.1.3.3 Selectivity

Incubation:	5 d at 25 °C ± 1 °C
Strains:	*Escherichia coli* ATCC 25922 or *Bacillus subtilis* ATCC 6633 or strains recorded as equivalent in other bacterial collections
Control method:	qualitative
Criteria:	total inhibition

6 Apparatus and glassware

Disposable apparatus is an acceptable alternative to reusable glassware, provided that it has suitable specifications.

Usual microbiological laboratory equipment (see ISO 7218) and, in particular, the following.

6.1 Incubator, capable of operating at 25 °C ± 1 °C.

6.2 Total delivery pipettes, sterile, of nominal capacity 1 ml, and graduated in divisions of 0,1 ml.

6.3 Water bath, or similar apparatus, capable of operating at 44 °C to 47 °C.

6.4 pH meter, accurate to 0,1 pH units at 25 °C.

6.5 Bottles, flasks and tubes, for boiling and storage of culture media, and for making of dilutions.

6.6 Petri dishes, sterile, of glass or plastic, with a diameter 90 mm to 100 mm.

6.7 Microscope, for distinguishing yeast from bacterial cells (bright field, magnification of 250 to 1 000 times).

6.8 Spreaders, made of glass or plastic (of diameter less than 2 mm and length 80 mm). Diameter should not exceed 2 mm in order to minimize the amount of samples adhering to them at the end of the spreading procedure.

6.9 Binocular magnifier, for discriminating and differentiation colonies/cells of yeasts and moulds (magnification 6,5 to 50 times).

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7 Sampling

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

Sampling is not part of the method specified in this part of ISO 21527. Sampling should be carried out in accordance with the specific International Standard, appropriate to the product concerned. If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject.

8 Preparation of the test sample

Prepare the test sample in accordance with ISO 6887 (all parts), ISO 7218, ISO 8261 and the specific International Standard dealing with the product concerned. If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject.

9 Procedure

9.1 Test portion, initial suspension and dilutions

Prepare the test portion, initial suspension (primary dilution) and further dilutions in accordance with ISO 6887 (all parts), ISO 7218, ISO 8261 and the specific International Standard appropriate to the product concerned.

Except for specific preparation of the test sample, it is recommended to use 0,1 % peptone water broth (5.1.3) as diluent. Use a peristaltic homogenizer in preference to a blender or shaker.

Due to the rapid sedimentation of spores in the pipette, maintain the pipette (6.2) in a horizontal (not vertical) position when filled with the appropriate volume of initial suspension and dilutions.

Shake the initial suspension and dilutions in order to avoid sedimentation of microorganism-containing particles.

9.2 Inoculation and incubation

9.2.1 On to one DRBC agar plate (5.2.1), using a sterile pipette (6.2), transfer 0,1 ml of the test sample if liquid, or 0,1 ml of the initial suspension in the case of other products (Clause 8).

On to a second DRBC agar plate, using a fresh sterile pipette, transfer 0,1 ml of the first decimal dilution (10^{-1}) dilution (liquid product), or 0,1 ml of the 10^{-2} dilution (other products).

To facilitate enumeration of low populations of yeasts and moulds, volumes of up to 0,3 ml of a 10^{-1} dilution of sample, or of the test sample if liquid, can be spread on to three plates.

Repeat these operations with subsequent dilutions, using a new sterile pipette for each decimal dilution.

NOTE If the presence of fast-growing moulds is suspected, use ISO 21527-2 [6].

9.2.2 Spread the liquid over the surface of the agar plate with a sterile spreader (6.8) until the liquid is completely absorbed into the medium.

Inoculation of plates by the pour-plate method may also be used, but in this case the equivalence of the results shall be validated compared to inoculation on the surface, and the discrimination and differentiation of moulds and yeast are not admissible. The method of spreading out on the surface can give higher enumerations. The spread-plate technique facilitates maximum exposure of cells to atmospheric oxygen and avoids any risk of thermal inactivation of fungal propagules. The results can depend on the type of fungi.

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9.2.3 Incubate the prepared plates (9.2.2) aerobically, lids uppermost, in an upright position in the incubator (6.1) at 25 °C ± 1 °C for 5 d. If necessary, leave the agar plates to stand in diffuse daylight for 1 d to 2 d.

It is recommended to incubate the dishes (6.6) in an open plastic bag in order not to contaminate the incubator in the event of dissemination of the moulds out of the dishes.

9.3 Counting and selection of colonies for confirmation

Read the plates between 2 d and 5 d of incubation. Select the dishes (9.2.3) containing less than 150 colonies/propagules/germs and count these colonies/propagules/germs. If fast-growing moulds are a problem, count colonies/propagules/germs after 2 d and again after 5 d of incubation.

NOTE 1 Enumeration methods for yeasts and especially moulds are imprecise because they consist of a mixture of mycelium and asexual and sexual spores. Numbers of colony-forming units depend on the degree of fragmentation of mycelium and the proportion of spores able to grow on the plating medium.

NOTE 2 Non-linearity of counts from dilution plating often occurs, i.e. 10-fold dilutions of samples often do not result in 10-fold reductions in numbers of colonies recovered on plating media. This has been attributed to fragmentation of mycelia and breaking of spore clumps during dilution in addition to competitive inhibition when large numbers of colonies are present on plates.

CAUTION — The spores of moulds disperse in the air with great facility, handle the Petri dishes with care to avoid development of satellite colonies which would give an overestimation of population in the sample.

If necessary, carry out an examination with a binocular magnifier (6.9) or with a microscope (6.7) in order to distinguish between cells of yeasts or moulds and bacteria from colonies.

Count the colonies of yeasts and the colonies/propagules of moulds separately, if necessary.

For identification of yeast and moulds, select areas of fungal growth and remove for high microscopic examination or inoculate on suitable isolation or identification media.

10 Expression of results and confidence limits

See ISO 7218.

Record the colonies of yeasts and the colonies/propagules of moulds separately, if necessary.

11 Test report

The test report shall include at least the following information:

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 part of ISO 21527;

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.

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[5] ISO 6107-6:2004, *Water quality — Vocabulary — Part 6*

[6] ISO 21527-2, *Microbiology of food and animal feedings stuffs — Horizontal method for the enumeration of yeasts and moulds — Part 2: Colony count technique in products with water activity less than or equal to 0,95*

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