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Microbiology of food and animal feeding 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

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

Partie 2: Technique par comptage des colonies dans les produits à activité d'eau inférieure ou égale à 0,95

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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 21527-2 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 21527-1, cancel and replace ISO 7698:1990, ISO 7954:1987 and ISO 13681:1995.

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.

Microbiology of food and animal feeding 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

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 osmophilic yeasts and xerophilic moulds in products intended for human consumption or feeding of animals that have a water activity less than or equal to 0,95 (dry fruits, cakes, jams, dried meat, salted fish, grains, cereals and cereal products, flours, nuts, spices and condiments, etc. [Annex A]), by means of the colony count technique at 25 °C ± 1 °C (Reference [3]).

This part of ISO 21527 does not apply to dehydrated products with water activity less than or equal to 0,60 (dehydrated cereals, oleaginous products, spices, leguminous plants, seeds, powders for instant drinks, dry products for domestic animals, etc.) and does not allow the enumeration of mould spores (Reference [3]). 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 halophilic xerophilic fungi (i.e. *Polypaecilum pisce, Basipetospora halophila)* such as may be found in dried fish.

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*

ISO 21527-1, *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*

3 Terms and definitions

For the purposes of this document the terms and definitions given in ISO 21527-1 and the following apply.

3.1
osmophilic yeast
xerophilic mould
fungus which is capable of growth at a water activity less than or equal to 0,95

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 to 7 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.

5 Diluent and culture medium

For current laboratory practice, see ISO/TS 11133 (all parts).

5.1 Diluent

5.1.1 General

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

The use of a diluent containing a sufficient amount of solute [e.g. a 20 % to 35 % (mass concentration) solution of glycerol or D-glucose] is recommended to minimize osmotic shock to xerophilic mould and osmophilic yeast cells when serial dilutions are made prior to plating (References [1], [3]).

NOTE It is possible to add surface-active agents such as sodium poly(oxyethylene)sorbitanmonooleate [1)] [0,05 % (mass concentration)] to diluents to reduce clumping of mould spores and conidia (Reference [3]).

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

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.

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 18 % (mass concentration) glycerol agar (DG18) (References [4], [5], [6])

5.2.1.1 Composition

Casein enzymatic digest	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
Glycerol anhydrous	220 g
Agar	12 g to 15 g [a]
Chloramphenicol	0,1 g
Water, distilled or deionized	1000 ml
[a] Depending on the gel strength of the agar.	

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, 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. Since it is relatively unstable, freshly prepare also a 0,1 % (mass concentration) solution of chlortetracycline hydrochloride in water 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 (Reference [3]).

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 DG18. 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 [2]).

5.2.1.3 Performance testing for the quality assurance of the culture medium

5.2.1.3.1 General

DG18 medium is a solid medium. Productivity and selectivity shall be tested according to ISO/TS 11133 (all parts) according to the following specifications:

5.2.1.3.2 Productivity

Incubation:	5 d at 25 °C ± 1 °C
Strains:	*Saccharomyces cerevisiae* ATCC 9763 *Wallemia sebi* ATCC 42694 *Aspergillus restrictus* ATCC 42693 *Eurotium rubrum* ATCC 42690 or strains recorded as equivalent in other fungal collections
Reference media:	media batch SDA (Sabouraud D-glucose 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 *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, in glass or plastic, with a diameter 90 mm to 100 mm.

6.7 Microscope, for distinguishing yeast from bacterial cells (bright field, of magnification 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 sample adhering to the spreader at the end of the procedure.

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

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 % (mass concentration) peptone water broth (5.1.3) as diluent. Use a peristaltic homogeniser 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 DG18 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 DG18 agar plate, using a fresh sterile pipette, transfer 0,1 ml of the first decimal (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 three plates.

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

For particulate or solid foods, e.g. nuts or grains, direct plating is recommended. Samples of these types of products are surface disinfected in a 0,35 % (1 000 µg/g) sodium hypochlorite solution with a contact time of 2 min, followed by rinsing with sterile distilled water, dried on a sterile paper and then placed on solidified media (References [1, 2]).

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.

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 to 7 d. If necessary, leave the agar plates to stand in diffuse daylight for 1 d to 2 d.

If the presence of *Xeromyces bisporus* is suspected, incubate the plates for 10 d.

It is recommended that the plates be incubated 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

After the specified period of incubation, select the dishes (9.2.3) containing < 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 to 7 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 a 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.

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 part of ISO 21527, or regarded as optional, together with details of any incidents which may have influenced the test results;

e) the test results obtained.

Annex A
(informative)

Examples of water activity according to matrices

Table A.1 gives examples of water activity according to the products to be analyzed.

Table A.1 — Water activity a_w according to matrices

Water activity a_w	Food examples/matrices
$\geqslant$ 0,95	Highly perishable foods (fresh and canned fruits, vegetables, meat, fish), milk, cooked sausages, breads, foods containing up to 4 % of sucrose or 7 % of NaCl (salt)
$\geqslant$ 0,91	Hard cheeses such as Cheddar, cured meat, fruit juice concentrates containing 55 % sucrose or 12 % NaCl
$\geqslant$ 0,87	Fermented sausage, sponge cakes, dry cheese, margarine, foods containing 65 % sucrose or 15 % NaCl (salt)
$\geqslant$ 0,80	Most fruit juice concentrates, condensed milk, syrup, flour, high-sugar cakes, pulses containing 15 % to 17 % moisture
$\geqslant$ 0,75	Jam, marmalade, glace fruits, marzipan, marshmallows, cake
$\geqslant$ 0,65	Rolled oats containing 10 % moisture, jelly, molasses, nuts
$\geqslant$ 0,60	Dried fruits containing 15 % to 20 % moisture, caramel, toffee, honey, bars cereals, dog feed, granulated food, grains, cereals and cereal products
$\geqslant$ 0,50	Noodles containing 12 % moisture, spices containing 10 % moisture
$\geqslant$ 0,40	Whole egg powder containing 5 % moisture, nougat
$\geqslant$ 0,30	Cookies, crackers, bread crusts containing 3 % to 5 % moisture, base of dehydrated sauce
$\geqslant$ 0,03	Whole milk powder containing 2 % to 3 % moisture, dehydrated soups, instant coffee

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