
Cosmetics — Microbiology — Enumeration of yeast and mould

*Cosmétiques — Microbiologie — Dénombrement des levures et des
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.

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 16212 was prepared by Technical Committee ISO/TC 217, *Comestics*.

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Cosmetics — Microbiology — Enumeration of yeast and mould

1 Scope

This International Standard gives general guidelines for enumeration of yeast and mould present in cosmetics by counting the colonies on selective agar medium after aerobic incubation.

In order to ensure product quality and safety for consumers, it is advisable to perform an appropriate microbiological risk analysis so as to determine the types of cosmetic products to which this International Standard is applicable. Products considered to present a low microbiological risk include those with low water activity, hydro-alcoholic products, products with extreme pH values, etc.

Because of the large variety of cosmetic products within this field of application, this method might not be suited to some products in every detail (e.g. certain water-immiscible products). Other methods (e.g. automated) can be used for the test presented here provided that their equivalence has been demonstrated or the method has been otherwise validated.

Yeast enumerated can be identified using suitable identification tests, for example tests described in the standards listed in the Bibliography. Mould enumerated can be identified by other appropriate methods, if necessary.

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 21148, *Cosmetics — Microbiology — General instructions for microbiological examination*

EN 12353, *Chemical disinfectants and antiseptics — Preservation of test organisms used for the determination of bactericidal, mycobactericidal, sporicidal and fungicidal activity*

3 Terms and definitions

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

3.1

yeast

single-cell fungus, which multiplies mainly vegetatively by budding, able to grow under the test conditions specified in this International Standard

3.2

mould

mycelium forming microfungus, including spores and conidia, able to grow under the test conditions specified in this International Standard

3.3

product

portion of an identified cosmetic product received in the laboratory for testing

3.4

sample

portion of the product (at least 1 g or 1 ml) which is used in the test to prepare the initial suspension

3.5

initial suspension

suspension (or solution) of a sample in a defined volume of an appropriate liquid (diluent, neutralizer, broth or combination thereof)

3.6

sample dilution(s)

dilution(s) of the initial suspension

4 Principles

4.1 General

This method involves enumeration of colonies on a selective agar medium. The possible inhibition of fungal growth by the sample shall be neutralized to allow the detection of viable microorganism (see Reference [2]). In all cases and whatever the methodology, the neutralization of the antifungicidal properties of the product shall be checked and validated (see References [3], [5], [6]).

4.2 Plate count

Plate count consists of the following steps. <https://standards.iteh.ai/catalog/standards/sist/faf2e3b-b7dd-4f4d-8190-b3ae250f4a02/iso-16212-2008>

- a) Preparation of poured plates or spread plates, using a specified culture medium, and inoculation of the plates using a defined quantity of the initial suspension or dilution of the product.
- b) Aerobic incubation of the plates at $25\text{ °C} \pm 2,5\text{ °C}$ for 3 d to 5 d.
- c) Counting of the number of colony-forming units (CFU) and calculation of the amount of yeast and mould per millilitre or per gram of product.

NOTE An alternative condition for incubation is $22,5\text{ °C} \pm 2,5\text{ °C}$ for 5 d to 7 d using the culture medium without antibiotic.

4.3 Membrane filtration

Membrane filtration consists of the following steps.

- a) Transfer of a suitable amount of the sample, prepared by a valid method, in the filtration apparatus, wetted with a small volume of an appropriate sterile diluent. Immediate filtration and washing according to the validated procedure. Transfer of the membrane filter on to the surface of the specified agar medium as specified in ISO 21148.
- b) Aerobic incubation of the membranes at $25\text{ °C} \pm 2,5\text{ °C}$ for 3 d to 5 d.
- c) Counting of the number of colony-forming units (CFU) and calculation of the amount of yeast and mould per millilitre or per gram of product.

NOTE An alternative condition for incubation is $22,5\text{ °C} \pm 2,5\text{ °C}$ for 5 d to 7 d using the culture medium without antibiotic.

5 Diluents, neutralizers and culture media

5.1 General

General specifications are given in ISO 21148. When water is mentioned in a formula, use distilled water or purified water as specified in ISO 21148.

The following diluents, neutralizers and culture media are suitable for enumeration of yeasts and moulds. Other diluents, neutralizers and culture media may be used if they have been demonstrated to be suitable for use.

5.2 Neutralizing diluents and diluents

5.2.1 General

The diluent is used to disperse the sample. It may contain neutralizers if the sample to be tested has antifungal properties. The efficacy of the neutralization shall be demonstrated before the determination of the count (see Clause 12). Information relative to suitable neutralizers is given in Annex D.

5.2.2 Neutralizing diluent

5.2.2.1 Fluid casein digest-soy lecithin-polysorbate 20 medium (SCDLP 20 broth)

5.2.2.1.1 Composition

— pancreatic digest of casein	20,0 g
— soy lecithin	5,0 g
— polysorbate 20	40 ml
— water	960 ml

5.2.2.1.2 Preparation

Dissolve the polysorbate 20 in 960 ml of water by mixing while heating in a water bath at $49\text{ °C} \pm 2\text{ °C}$. Add pancreatic digest of casein and soy lecithin. Heat for about 30 min to effect solution. Mix and dispense the medium into suitable containers. Sterilize in the autoclave at 121 °C for 15 min. After sterilization, the pH shall be equivalent to $7,3 \pm 0,2$ when measured at room temperature.

5.2.2.2 Other neutralizing diluents

Other neutralizing diluents may be used as appropriate (see Annex A and Annex D).

5.2.3 Diluent

5.2.3.1 Fluid A

5.2.3.1.1 Composition

— peptic digest of animal tissue	1,0 g
— water	1 000 ml

5.2.3.1.2 Preparation

Dissolve 1 g of peptone in water to make 1 l. Heat with frequent agitation. Dispense into suitable containers. Sterilize in the autoclave at 121 °C for 15 min. After sterilization the pH shall be equivalent to $7,1 \pm 0,2$ when measured at room temperature.

5.2.3.2 Other diluents

Other diluents may be used as appropriate (see Annex B).

5.3 Diluent for yeast suspension (tryptone sodium chloride solution)

5.3.1 Composition

— tryptone, pancreatic digest of casein	1,00 g
— sodium chloride	8,50 g
— water	1 000 ml

5.3.2 Preparation

Dissolve the components in the water by mixing while heating. Dispense into suitable containers. Sterilize in the autoclave at 121 °C for 15 min. After sterilization the pH shall be equivalent to $7,0 \pm 0,2$ when measured at room temperature.

5.4 Culture media

5.4.1 General

Culture media may be prepared as follows, or from dehydrated culture media according to the manufacturer's instructions. Ready-to-use media may be used when their composition and/or growth yields are comparable to those of the formulae given herein.

5.4.2 Sabouraud dextrose chloramphenicol agar medium (SDCA)

5.4.2.1 Composition

— dextrose	40,0 g
— peptic digest of animal tissue	5,0 g
— pancreatic digest of casein	5,0 g
— chloramphenicol	0,050 g
— agar	15,0 g
— water	1 000 ml

5.4.2.2 Preparation

Dissolve the components (including the chloramphenicol) or the dehydrated complete medium in the water by mixing while heating. Dispense the medium into suitable containers. Sterilize in an autoclave at 121 °C for 15 min. After sterilization the pH shall be equivalent to $5,6 \pm 0,2$ when measured at room temperature.

NOTE For known and non-contaminated products (with bacteria), the media are used without chloramphenicol.

5.4.3 Other media

Other media may be used as appropriate (see Annex C).

5.4.4 Agar medium for cultivation of reference strain: Sabouraud dextrose agar medium (SDA)

5.4.4.1 Composition

— dextrose	40,0 g
— peptic digest of animal tissue	5,0 g
— pancreatic digest of casein	5,0 g
— agar	15,0 g
— water	1 000 ml

5.4.4.2 Preparation

Dissolve the components or the dehydrated complete medium in the water by mixing while heating. Dispense the medium into suitable containers. Sterilize in an autoclave at 121 °C for 15 min. After sterilization the pH shall be equivalent to $5,6 \pm 0,2$ when measured at room temperature.

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6 Apparatus and glassware

The laboratory equipment, apparatus and glassware are described in ISO 21148.

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7 Strain of microorganisms

For testing the efficacy of neutralizers, one yeast reference strain is used.

Candida albicans ATCC¹⁾ 10231 or equivalent strain (IP²⁾ 48.72 or NCPF³⁾ 3179 or NBRC⁴⁾ 1594 or KCTC⁵⁾ 17205 or TISTR⁶⁾ 5779) or other equivalent national collection strain.

The selected yeast strain being considered more susceptible to antifungicidal activity than moulds may be accepted as representative of fungi (yeast and mould) for the validation of the methodology. However, in case of specific needs, the test for the efficacy of neutralizers may be performed with an additional mould reference strain, using a suitable protocol for the preparation of a calibrated inoculum (e.g. see EN 13624:2003^[11], 5.4.1.4).

The culture should be reconstituted according to the procedures provided by the supplier of reference strain.

The strain may be kept in the laboratory in accordance with EN 12353.

1) ATCC = American Type Culture Collection.

2) IP = Institut Pasteur.

3) NCPF = National Collection of Pathogenic Fungi.

4) NBRC = National Biological Resource Center.

5) KCTC = Korean Collection for Type Culture.

6) TISTR = Thailand Institute of Scientific and Technological Research.

8 Handling of cosmetic products and laboratory samples

If necessary, store products to be tested at room temperature.

Do not incubate, refrigerate or freeze products (3.3) and samples (3.4) before or after analysis.

Sampling of cosmetic products to be analysed should be carried out as described in ISO 21148. Analyse samples as described in ISO 21148 and according to the procedure described in Clause 9.

9 Procedure

9.1 General recommendation

Use sterile material, equipment and aseptic techniques to prepare the sample, initial suspension and dilutions. In the case of the preparation of the initial suspension, the time that elapses between the end of the preparation and the moment the inoculum comes into contact with the culture medium shall not exceed 45 min, unless specifically mentioned in the established protocols or documents.

9.2 Preparation of the initial suspension

9.2.1 General

The initial suspension is prepared from a sample of at least 1 g or 1 ml of the well-mixed product under test.

Note S , the exact mass or volume of the sample.

The initial suspension is usually a 1:10 dilution. Larger volumes of diluent may be required if high levels of contamination are expected and/or if antifungal properties are still present in the 1:10 dilution.

9.2.2 Water-miscible products

Transfer the sample, S , of product to an appropriate volume (e.g. 9 ml) of neutralizing diluent (see 5.2.2) or diluent (see 5.2.3).

Note d , the dilution factor.

9.2.3 Water-immiscible products

Transfer the sample, S , of product to a suitable container containing a suitable quantity of solubilizing agent (e.g. polysorbate 80 solution). Disperse the sample within the solubilizing agent and add an appropriate volume (e.g. 9 ml) of neutralizing diluent (see 5.2.2) or diluent (see 5.2.3).

Note d , the dilution factor.

9.3 Counting methods

9.3.1 Dilutions for counting methods

Usually, the initial suspension is the first counted dilution. If needed, additional serial dilutions (e.g. 1:10 dilution) may be performed from the initial suspension using the same diluent (according to the expected level of contamination of the product).

Generally, counting is performed using at least two Petri dishes, but it is possible to use only one Petri dish in case of routine testing, or if counts are performed on successive dilutions of the same sample or according to previous results.

9.3.2 Plate-count methods

9.3.2.1 Pour-plate method

In Petri dishes of 85 mm to 100 mm in diameter, add 1 ml of the initial suspension and/or sample dilution prepared as validated (see Clause 12) and pour 15 ml to 20 ml of the melted agar medium (see 5.4.2) kept in a water bath at no more than 48 °C. If larger Petri dishes are used, the amount of agar medium is increased accordingly.

Mix the initial suspension and/or sample dilution with the medium, carefully rotating or tilting the plates sufficiently to disperse them. Allow the mixture in the Petri dishes to solidify on a horizontal surface at room temperature.

9.3.2.2 Surface spread method

In Petri dishes of 85 mm to 100 mm in diameter, put 15 ml to 20 ml of the melted agar medium (see 5.4.2) kept in a water bath at no more than 48 °C. If larger Petri dishes are used, the volume of the agar is increased accordingly.

Allow plates to cool and solidify, for example in a microbiological cabinet or in an incubator. Spread over the surface of the medium a measured volume of not less than 0,1 ml of the initial suspension and/or sample dilution prepared as validated (see Clause 12).

9.3.2.3 Membrane filtration method

Use a membrane having a nominal pore size $\leq 0,45 \mu\text{m}$.

Transfer a suitable amount of the initial suspension or of the sample dilution prepared as validated (preferably representing at least 1 g or 1 ml of the product) on to the membrane.

Filter immediately and wash the membrane (follow the procedure developed during the validation, see Clause 12).

Transfer the membrane on to the surface of the agar medium (see 5.4.2).

9.3.2.4 Incubation

Unless otherwise stated, invert the inoculated dishes and place them in the incubator set at $25 \text{ °C} \pm 2,5 \text{ °C}$ for 3 d to 5 d or use the alternative condition (see Notes in 4.2 and 4.3). After incubation, the dishes shall, if possible, be examined immediately. Alternatively, they may be stored, unless otherwise specified, for up to a maximum of 24 h in the refrigerator at $5 \text{ °C} \pm 3 \text{ °C}$.

NOTE 1 In certain cases, where there is a potential for confusing particles from the product with counted colonies, it can be useful to prepare duplicate dishes containing the same sample dilutions and agar medium which are stored in the refrigerator for comparison with incubated dishes.

NOTE 2 An intermediate check can be performed where both yeast and mould are suspected.

10 Counting of colonies (plate counts and membrane filtration methods)

After incubation, count the colonies:

- in Petri dishes containing 15 colonies to 150 colonies; if less than 15 colonies are counted, see 11.2.3;
- on membranes containing 15 colonies to 150 colonies; if less than 15 colonies are counted, see 11.2.3.