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Cosmetics — Microbiology — Enumeration and detection of aerobic mesophilic bacteria

Cosmétiques — Microbiologie — Dénombrement et détection des bactéries aérobies mésophiles

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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.

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ISO 21149 was prepared by Technical Committee ISO/TC 217, *Cosmetics*.

Cosmetics — Microbiology — Enumeration and detection of aerobic mesophilic bacteria

1 Scope

This International Standard gives general guidelines for enumeration and detection of mesophilic aerobic bacteria present in cosmetics,

— by counting the colonies on agar medium after aerobic incubation, or

— by checking the absence of bacterial growth after enrichment.

Because of the large variety of cosmetic products within this field of application, this method may not be appropriate for some products in every detail (e.g. certain water immiscible products). Other methods (e.g. automated) may be substituted for the tests presented here provided that their equivalence has been demonstrated or the method has been otherwise validated.

If needed, microorganisms enumerated or detected may be identified using suitable identification tests described in the standards given in the Bibliography.

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, extreme pH values, etc.

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 21148:2005, *Cosmetics — Microbiology — General instructions for microbiological examination*

3 Terms and definitions

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

3.1
aerobic mesophilic bacteria
mesophilic bacteria growing aerobically under the conditions specified in this International Standard

NOTE In the described conditions, other types of microorganisms (e.g. yeast, mould) can be detected.

3.2
product
portion of an identified cosmetic product received in the laboratory for testing

3.3
sample
portion of the product (at least 1 g or 1 ml) which is used in the test to prepare the initial suspension

3.4
initial suspension
suspension (or solution) of a sample in a defined volume of an appropriate liquid (diluent, neutralizer, broth or combination of them)

3.5
sample dilution
dilution of the initial suspension

4 Principle

4.1 General

This method involves enumeration of colonies on a non-selective agar medium or by the presence or absence of bacterial growth after enrichment. The possible inhibition of microbial growth by the sample shall be neutralized to allow the detection of viable microorganism [1]. In all cases and whatever the methodology, the neutralization of the antimicrobial properties of the product shall be checked and validated [2] [3] [4].

4.2 Plate count

Plate count consists of the following steps.

— 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.

— Aerobic incubation of the plates at 32,5 °C ± 2,5 °C for 72 h ± 6 h.

— Counting the number of colony forming units (CFU) and calculation of the number of aerobic mesophilic bacteria per millilitre or per gram of product.

4.3 Membrane filtration

Membrane filtration consists of the following steps.

— Transfer a suitable amount of the sample prepared as validated in the filtration apparatus wetted with a small volume of an appropriate sterile diluent, filter immediately and wash according to the validated procedure (see 13.3.4). Transfer the membrane filter onto the surface of the specified agar medium as specified in ISO 21148.

— Aerobic incubation of the membranes at 32,5 °C ± 2,5 °C for 72 h ± 6 h.

— Counting the number of colony forming units (CFU) and calculation of the number of aerobic mesophilic bacteria per millilitre or per gram of product.

4.4 Detection of bacteria by enrichment

Detection of bacteria by enrichment consists of the following steps:

— Incubation at 32,5 °C ± 2,5 °C for at least 20 h of a defined quantity of the initial suspension in a non-selective liquid medium containing suitable neutralizers and/or dispersing agents.

— Transfer of a defined quantity of the previous suspension on non-selective solid agar medium.

— Aerobic incubation at 32,5 °C ± 2,5 °C for 48 h to 72 h.

— Detection of growth and expression of results as “presence/absence” of aerobic mesophilic bacteria per sample S of product.

5 Diluents, neutralizers and culture media

5.1 General

General specifications are given in ISO 21148. When water is used 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 and detection of aerobic mesophilic bacteria. 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 specimen to be tested has antimicrobial properties. The efficacy of the neutralization shall be demonstrated before the determination of the count (see Clause 13). Information relative to suitable neutralizers is given in Annex D.

5.2.2 Neutralizing diluents

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	40,0 ml
Water	960,0 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 °C ± 2 °C. Add pancreatic digest of casein and soy lecithin. Heat for about 30 min to obtain 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 ± 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 ± 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 the bacterial suspension (tryptone sodium chloride solution)

5.3.1 Composition

Tryptone, pancreatic digest of casein	1,0 g
Sodium chloride	8,5 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 ± 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 instructions of the manufacturer. Ready-to-use media may be used when their composition and/or growth yields are comparable to those of the formulas given herein.

5.4.2 Culture media for counting

5.4.2.1 Soybean–casein digest agar medium (SCDA) or tryptic soy agar (TSA)

5.4.2.1.1 Composition

Pancreatic digest of casein	15,0 g
Papaic digest of soybean meal	5,0 g

Sodium chloride	5,0 g
Agar	15,0 g
Water	1 000 ml

5.4.2.1.2 Preparation

Dissolve the components or the dehydrated complete medium in the water by mixing while heating. Dispense the medium into suitable containers. Sterilize in the autoclave at 121 °C for 15 min. After sterilization and cooling down, the pH shall be equivalent to 7,3 ± 0,2 when measured at room temperature.

5.4.2.2 Other media for counting

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

5.4.3 Culture media for detection

5.4.3.1 General

When chosen, an enrichment broth and an agar medium shall be used for bacterial detection.

The enrichment broth is used to disperse the sample and to increase the initial microbial population. It may contain neutralizers if the specimen to be tested has antimicrobial properties.

5.4.3.2 Enrichment broth: Eugon LT 100 broth

5.4.3.2.1 General

This medium contains ingredients

— which neutralize inhibitory substances present in the sample: lecithin and polysorbate 80,

— dispersing agent: octoxynol 9.

5.4.3.2.2 Composition

Pancreatic digest of casein	15,0 g
Papaic digest of soybean meal	5,0 g
L-cystine	0,7 g
Sodium chloride	4,0 g
Sodium sulfite	0,2 g
Glucose	5,5 g
Egg lecithin	1,0 g
Polysorbate 80	5,0 g
Octoxynol 9	1,0 g
Water	1 000 ml

5.4.3.2.3 Preparation

Dissolve successively polysorbate 80, octoxynol 9 and egg lecithin into boiling water until their complete dissolution. Dissolve the other components by mixing while heating. Dispense the medium into suitable containers. Sterilize in the autoclave at 121 °C for 15 min. After sterilization, the pH shall be equivalent to 7,0 ± 0,2 when measured at room temperature.

5.4.3.3 Agar media for detection

5.4.3.3.1 Eugon LT 100 agar medium

5.4.3.3.1.1 Composition

Pancreatic digest of casein	15,0 g
Papaic digest of soybean meal	5,0 g
L-cystine	0,7 g
Sodium chloride	4,0 g
Sodium sulfite	0,2 g
Glucose	5,5 g
Egg lecithin	1,0 g
Polysorbate 80	5,0 g
Octoxynol 9	1,0 g
Agar	15,0 g
Water	1 000 ml

5.4.3.3.1.2 Preparation

Dissolve successively polysorbate 80, octoxynol 9 and egg lecithin into boiling water until their complete dissolution. Dissolve the other components by mixing while heating. Mix gently to avoid foam. Dispense the medium into suitable containers. Sterilize in the autoclave at 121 °C for 15 min. After sterilization and cooling down, the pH shall be equivalent to 7,0 ± 0,2 when measured at room temperature.

5.4.3.3.2 Other agar media for detection

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

5.4.4 Agar medium for cultivation of reference strains

Use soybean-casein digest agar medium (SCDA) or tryptic soy agar (TSA) (5.4.2.1).

6 Apparatus and glassware

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

7 Strains of microorganisms

For testing the efficacy of neutralizers, two strains representative of both Gram negative and Gram positive microorganisms [2] [5], respectively, are used:

— *Pseudomonas aeruginosa* ATCC 9027 (equivalent strain: CIP 82.118 or NCIMB 8626 or NBRC 13275 or KCTC 2513 or other equivalent national collection strain);

— *Staphylococcus aureus* ATCC[1)] 6538 (equivalent strain: CIP[2)] 4.83 or NCIMB[3)] 9518 or NBRC[4)] 13276 or KCTC[5)] 1916 or other equivalent national collection strain).

An alternative to the Gram negative strain may be: *Escherichia coli* ATCC 8739 (equivalent strain: CIP 53.126 or NCIMB 8545 or NBRC 3972 or KCTC 2571 or other equivalent national collection strain).

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

The strains may be kept in the laboratory according to the EN 12353.

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 (3.2) and samples (3.3) before or after analysis.

Sampling of cosmetic products to be analysed should be carried out, as described in ISO 21148. Analyse samples as specified in ISO 21148 and in accordance with the following procedure.

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 an initial suspension, the time which 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 (3.3) of at least 1 g or 1 ml of the well-mixed product (3.2) under test.

Note S the exact mass or volume of the sample.

The initial suspension is usually 1:10 dilution. Larger volumes of diluent or enrichment broth may be required if high levels of contamination are expected and/or if anti-microbial properties are still present in 1:10 dilution.

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1) ATCC = American Type Culture Collection.

2) CIP = Institut Pasteur Collection.

3) NCIMB = National Collection of Industrial and Marine Bacteria.

4) NBRC = National Biological Resource Center.

5) KCTC = Korean Collection for Type Culture.

9.2.2 Water-miscible products

Transfer the sample S of product to an appropriate volume (e.g. 9 ml) of neutralizing diluent (5.2.2) or diluent (5.2.3) or enrichment broth (5.4.3.2), depending on the method used (9.3 or 9.4).

Note the dilution factor d.

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). Disperse the sample within the solubilizing agent and add an appropriate volume (e.g. 9 ml) of neutralizing diluent (5.2.2) or diluent (5.2.3) or enrichment broth (5.4.3.2), depending on the method used (9.3 or 9.4).

Note the dilution factor d.

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 85 mm to 100 mm in diameter, add 1 ml of the initial suspension and/or sample dilution prepared as validated (see Clause 13) and pour 15 ml to 20 ml of the melted agar medium (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 85 mm to 100 mm in diameter, put 15 ml to 20 ml of the melted agar medium (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 13).

9.3.2.3 Membrane filtration method

Use membranes having a nominal pore size no greater than 0,45 µm.

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) onto the membrane. Filter immediately and wash the membrane (follow the procedure developed during the validation, see Clause 13).

Transfer the membrane onto the surface of the agar medium (5.4.2).