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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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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).

9.3.2.4 Incubation

Unless otherwise stated, invert the inoculated dishes and place them in the incubator set at 32,5 °C ± 2,5 °C for 72 h ± 6 h. After incubation, the dishes shall, if possible, be examined immediately. Otherwise, they may be stored, unless otherwise specified, for up to a maximum of 24 h in the refrigerator.

NOTE 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.

9.4 Enrichment

9.4.1 General

The initial suspension is prepared (see 9.2) in the enrichment broth (5.4.3.2) chosen following the procedure developed during the validation (see Clause 13).

9.4.2 Incubation of the sample

9.4.2.1 General

Incubate the initial suspension prepared in broth (5.4.3.2) at 32,5 °C ± 2,5 °C for at least 20 h.

9.4.2.2 Subculture

Using a sterile pipette, transfer 0,1 ml to 0,5 ml of the incubated suspension on the surface of a Petri dish (diameter 85 mm to 100 mm) containing approximately 15 ml to 20 ml of suitable detection agar medium (5.4.2.1). If larger Petri dishes are used, the volume of the agar is increased accordingly.

9.4.2.3 Incubation of the subculture

Do not invert the inoculated plate (or wait for the absorption of the incubated suspension by the agar before inverting) and incubate at 32,5 °C ± 2,5 °C for 48 h to 72 h.

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

After incubation, count the colonies:

— in Petri dishes containing 30 colonies to 300 colonies; if less than 30 colonies are counted, see 12.2.3;

— on membranes containing 15 colonies to 150 colonies; if less than 15 colonies are counted, see 12.2.3.

11 Detection of growth (enrichment method)

After incubation of the subculture, check the agar surface and record the presence or absence of growth.

12 Expression of results

12.1 Method of calculation for plate count

Calculate the number N of microorganisms present in the sample S, using:

— m, the arithmetic mean of the counts obtained from the duplicates in Equation (1)

— c, the number of colonies counted on a single plate in Equation (2), or

— $\bar{x}_c$, the weighted mean of the counts obtained from two successive dilutions in Equation (3),

according to the following equations:

$$N = m/(V \times d) \quad (1)$$

$$N = c/(V \times d) \quad (2)$$

$$N = \bar{x}_c/(V \times d) \quad (3)$$

where

m is the arithmetic mean of the counts obtained from the duplicates;

V is the volume of inoculum applied to each dish, in millilitre;

d is the dilution factor corresponding to the dilution made for the preparation of the initial suspension (9.2) or for the first counted dilution;

c is the number of colonies counted on a single plate;

$\bar{x}_c$ is the weighted mean of the colonies counted from two successive dilutions and is calculated as follows:

$$\bar{x}_c = \frac{\sum c}{n_1 + 0{,}1 n_2}$$

where

$\sum c$ is the sum of colonies counted on all the dishes retained from two successive dilutions;

n_1 is the number of dishes counted for the initial suspension (or for the first counted dilution);

n_2 is the number of dishes counted for the 1/10 dilution of the initial suspension (or for the second counted dilution).

Round off the result calculated to two significant figures. For this, if the last figure is below 5, the preceding figure is not modified; if the last figure is 5 or more, the preceding figure is increased by one unit. Proceed stepwise until two significant figures are obtained. Note the number N obtained.

12.2 Interpretation

12.2.1 The inherent variability of plate count should be taken into account. Two results should only be considered different if the difference exceeds 50 % or, when expressed in log, the difference exceeds 0,3.

For a count to be precise, only plates with more than 30 colonies and less than 300 colonies and membranes with more than 15 colonies and less than 150 colonies should be taken into account. Check that the counts are obtained from dilutions validated according to the chosen method (see Clause 13).

12.2.2 Where the number of CFU is more than 30 and less than 300 on plates or more than 15 and less than 150 on membranes, where S is the mass or the volume of the sample (9.2), express the result as follows:

— if S is at least 1 g or 1 ml, and V is at least 1 ml:

  the number of aerobic mesophilic bacteria per millilitre or per gram of the sample = N/S;

— if S is less than 1 g or 1 ml, and/or V is less than 1 ml:

  the number of aerobic mesophilic bacteria in the sample (note the tested quantity of sample taking into account S and V) is = N.

Express the result as a number between 1,0 and 9,9 multiplied by the appropriate power of 10 (see Example 1, Example 2, Example 3 and Example 7).

12.2.3 Where the number of CFU is less than 30 on plates or 15 on membranes, express the result as follows:

— if S is at least 1 g or 1 ml, and V is at least 1 ml:

  the estimated number of aerobic mesophilic bacteria per millilitre or per gram of the sample is = N/S;

— if S is less than 1 g or 1 ml, and/or V is less than 1 ml:

  the estimated number of aerobic mesophilic bacteria in the sample is = N.

Where S is the mass or the volume of the sample (9.2).

Express the result as a number between 1,0 and 9,9 multiplied by the appropriate power of 10 (see Example 4, Example 5 and Example 6).

12.2.4 Where no colony is observed, the result is reported as follows:

— less than $1/d \times V \times S$ of aerobic mesophilic bacteria per gram or millilitre of the product (S is at least 1 g or 1 ml);

— less than $1/d \times V$ of aerobic mesophilic bacteria in the sample S (note the tested quantity of sample taking into account S and V) (S is less than 1 g or 1 ml);

where d is the dilution factor of the initial suspension (9.2) and V is 1 (for counting with the pour-plate method and for membrane filtration) or 0,1 (for the spread plate method) (see Example 8).

12.3 Examples

12.3.1 EXAMPLE 1 Two dishes for one dilution

S = 1 g or 1 ml; V = 1; counts obtained: for the dilution 10^{-1}, 38 and 42.

For Equation (1):

$N = m/(V \times d)$ = 40/(1 × 10^{-1}) = 40/0,1 = 400 or 4 × 10^2 aerobic mesophilic bacteria per millilitre or per gram of the sample.

12.3.2 EXAMPLE 2 One dish for one dilution

S = 1 g or 1 ml; V = 1; count obtained: for the dilution 10^{-1}: 60.

For Equation (2):

$N = c/(V \times d) = 60/(1 \times 10^{-1)} = 60/0{,}1 = 600$ or 6×10^2 aerobic mesophilic bacteria per millilitre or per gram of the sample.

12.3.3 EXAMPLE 3 Two dishes for two dilutions

S = 1 g or 1 ml; V = 1; counts obtained: for the dilution 10^{-2}, 235 and 282; for the dilution 10^{-3}, 31 and 39.

For Equation (3):

$N = \bar{x}_c/(V \times d) = 235 + 282 + 31 + 39/1(2 + 0{,}1 \times 2) \times 10^{-2} = 587/0{,}022 = 26\,682$.

Rounding the result as specified above gives 27 000 or $2{,}7 \times 10^4$ aerobic mesophilic bacteria per millilitre or per gram of the sample.

12.3.4 EXAMPLE 4 Two membrane filters for one dilution

S = 1 g or 1 ml; V = 1; counts obtained: for the dilution 10^{-1}, 18 and 22.

For Equation (1):

$N = m/(V \times d) = 20/(1 \times 10^{-1}) = 20/0{,}1 = 200$ or 2×10^2 aerobic mesophilic bacteria per millilitre or per gram of the sample.

12.3.5 EXAMPLE 5 One membrane filter for one dilution

S = 1 g or 1 ml; V = 1; count obtained: for the dilution 10^{-1}, 65.

For Equation (2):

$N = c/(V \times d) = 65/(1 \times 10^{-1)} = 65/0{,}1 = 650$ or $6{,}5 \times 10^2$ aerobic mesophilic bacteria per millilitre or per gram of the sample.

12.3.6 EXAMPLE 6 Two membrane filters for two dilutions

S = 1 g or 1 ml; V = 1; counts obtained: for the dilution 10^{-1}, 121 and 105; for the dilution 10^{-2}, 15 and 25.

For Equation (3):

$N = \bar{x}_c/(V \times d) = 121 + 105 + 15 + 25/1\ (2 + 0{,}1 \times 2) \times 110–1 = 266/0{,}22 = 1\,209$.

Rounding the result as specified above gives 1 200 or $1{,}2 \times 10^3$ aerobic mesophilic bacteria per millilitre or per gram of the sample.

12.3.7 EXAMPLE 7 Two dishes for one dilution

S = 1 g or 1 ml; V = 1; counts obtained for the dilution 10^{-1}, 28 and 22.

For Equation (1):

$N = m/(V \times d) = 25/(1 \times 10^{-1}) = 25/0{,}1 = 250$.

The estimated number is 250 or $2,5 \times 10^2$ aerobic mesophilic bacteria per millilitre or per gram of the sample.

12.3.8 Example 8

S = 1 g or 1 ml; V = 1; counts obtained for the dilution 10^{-1}, 0 and 0.

For Equation (1):

$N \leqslant 1/(V \times d)$,

$\leqslant 1/(1 \times 10^{-1})$,

$\leqslant 1/0,1$,

$\leqslant 10$.

The estimated number is less than 10 aerobic mesophilic bacteria per millilitre or per gram of the sample.

12.3.9 Example 9

S = 1 g or 1 ml; V = 1; counts obtained for dilution 10^{-1}, 0 and 3.

For Equation (1):

$N \leqslant m\ (V \times d)$,

$\leqslant 1,5/(1 \times 10^{-1})$,

$\leqslant 1,5/0,1$,

$\leqslant 15$.

The estimated number is less than 15 mesophilic bacteria per millilitre or per gram of the sample.

12.4 Detection after enrichment

In case of growth (see Clause 11), express the result as:

"Presence of aerobic mesophilic bacteria in the sample S",

and proceed by counting using one of the proposed methods (see 9.3).

If no growth is detected (see Clause 11), express results as:

"Absence of aerobic mesophilic bacteria in the sample S".

13 Neutralization of the antimicrobial properties of the product

13.1 General

The different tests described below demonstrate that the microorganisms can grow under the conditions of analysis.

The two strains (see Clause 7) used to demonstrate the validity of these properties are generally sensitive to antimicrobial agents.

13.2 Preparation of inoculum

Prior to the test, and for each strain, inoculate the surface of soybean casein digest agar (SCDA) or other suitable (non-selective, non-neutralizing) medium. Incubate at 32,5 °C ± 2,5 °C for 18 h to 24 h. To harvest the bacterial culture, use a sterile loop, streak the surface of the culture and re-suspend into the diluent for bacterial suspensions (5.3) to obtain a calibrated suspension of about 1×10^8 CFU/ml (e.g. using a spectrophotometer, ISO 21148:—, Annex C). Use this suspension and its dilutions within 2 h.

13.3 Validation of counting methods

13.3.1 Principle

For each strain, mix the neutralized sample (initial suspension, or sample dilution according to the antimicrobial activity or the low solubility of the product) with a dilution of microorganism. Plate on a Petri dish or filter on membrane. After incubation, check the nature of the colonies and compare the count with a control (without the sample).

If the count is less than 50 % (0,3 log) of the control, modify the procedure (diluents, neutralization agents, or combination of both, see Annex D). The inherent variability of plate count should be taken into account. Two results should only be considered different if the difference exceeds 50 %, or when expressed in log the difference exceeds 0,3. Failure of the inoculum to grow invalidates the test unless it is possible to consider that contamination of the product with this microorganism is unlikely.

13.3.2 Validation of the pour-plate method

Mix 9 ml of the initial suspension and/or the sample dilution(s) in neutralizing diluent (or other, see 5.2) with 1 ml of a suspension of microorganisms containing 1 000 CFU/ml to 3 000 CFU/ml. Transfer 1 ml in a Petri dish (preferably in duplicate) 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. In parallel, prepare and plate a control using the same diluent and the same suspension of microorganisms, but without the sample.

After incubation for 24 h to 72 h at 32,5 °C ± 2,5 °C, count the colonies on the plates and compare the counts obtained for the test and of the control. The diluent and the counting method are validated at the 1:10 dilution (when 1 ml of the initial suspension is used) if the validation count is at least 50 % (0,3 log) of the control count.

13.3.3 Validation of the surface spread method

Mix 9 ml of the initial suspension in neutralizing diluent (or other, see 5.1) with 1 ml of a suspension of microorganisms containing 10 000 CFU/ml to 30 000 CFU/ml (or less if 0,5 ml or 1 ml are spread). Spread at least 0,1 ml on a solidified agar plate (5.4.2) (preferably in duplicate). In parallel, prepare and plate a control using the same diluent and the same suspension of microorganisms, but without the sample.

After incubation for 24 h to 72 h at 32,5 °C ± 2,5 °C, count the colonies on the plates and compare the counts obtained for the test and of the control. The diluent and the counting method are validated at the 1:10 dilution (when 1 ml of the initial suspension is used) if the validation count is at least 50% (0,3 log) of the control count.

13.3.4 Validation of the membrane filtration method

Mix to the volume of initial suspension or of the sample dilution used in the test (see 9.3.2.3) a suitable amount of a calibrated suspension of microorganisms corresponding to approximately 100 CFU.

Filter immediately the entire volume and wash the membrane using defined volumes of water (5.1), diluent (5.2.3) or neutralizing diluent (5.2.2). Transfer the membrane onto the surface of a suitable agar medium (5.4.2).

In parallel, prepare a control in the same conditions as above, but without the product. Filter and wash the control in the same conditions

After incubation for 24 h to 72 h at 32,5 °C ± 2,5 °C, count the colonies on the membranes and compare the counts obtained for the test and for the control. The membrane filtration method and the diluent are validated if the validation count is at least 50 % (0,3 log) of the control count.

13.4 Validation of the detection method by enrichment

13.4.1 Procedure

Prepare in tubes containing 9 ml of diluent for bacterial suspensions (5.3) a dilution of each calibrated suspension strain in order to obtain a final count between 100 CFU/ml and 500 CFU/ml. To count the final concentration of viable microorganisms in the standardized suspension, transfer 1 ml of the suspension into a Petri dish 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.

Incubate at 32,5 °C ± 2,5 °C for 20 h to 24 h.

Prepare in duplicate the initial suspension of the sample (3.3) in the conditions chosen for the test (at least 1 g or 1 ml of product, defined volume of enrichment broth (5.4.3.2) in tubes or flasks. In one tube (validation test), introduce aseptically 0,1 ml of standardized suspension of microorganisms. Mix, then incubate each tube (validation test and control) at 32,5 °C ± 2,5 °C for 20 h to 24 h.

For each tube or flask and using a sterile pipette, transfer 0,1 ml to 0,5 ml (same conditions as in the test) of incubated mixture onto the surface of Petri dish (diameter 85 mm to 100 mm) containing approximately 15 ml to 20 ml of suitable agar medium.

Incubate the plates at 32,5 °C ± 2,5 °C for 24 h to 72 h.

13.4.2 Interpretation of results

For each strain, check that the standardized suspension of bacteria contains between 100 CFU/ml and 500 CFU/ml.

The neutralization and the detection method are validated if a growth characteristic, defined as,

— for *Staphylococcus aureus*: culture pigmented in yellow;

— for *Pseudomonas aeruginosa*: greenish to yellowish culture of the inoculated organism occurs on the validation plate and no growth occurs on the control plate.

When growth is detected on the control plate (contaminated products), the neutralization and the detection method are validated if the inoculated microorganism is recovered on the validation plate.

13.5 Interpretation of validation results

Failure of growth on the validation plates indicates that an anti-microbial activity is still present and necessitates a modification of the conditions of the method. This may be accomplished by an increase in the volume of nutrient broth, the quantity of product remaining the same, or by incorporation of a sufficient quantity of inactivating agent in the nutrient broth, or by an appropriate combination of modifications so as to permit the growth of the bacteria.

If, in spite of the incorporation of suitable inactivating agents and a substantial increase in the volume of broth, it is still not possible to recover viable cultures as described above, indicate that the article is not likely to be contaminated with the given species of microorganism.

14 Test report

The test report shall specify the following:

a) all information necessary for the complete identification of the product;

b) the method used;

c) the results obtained;

d) all operating details for the preparation of the initial suspension;

e) the description of the method with the neutralizers and media used;

f) the validation of the method, even if the test has been performed separately;

g) any point not specified in this document, or regarded as optional, together with details of any incidents which may have influenced the results.

Annex A
(informative)

Other neutralizing diluents

A.1 General

Any neutralizing diluent may be used to prepare the initial suspension if it has been checked and validated. The following neutralizing diluents are examples of suitable formula. General information on neutralization is given in Annex D.

A.2 Eugon LT100 liquid broth

See 5.4.3.2.

A.3 Lecithin polysorbate (LP) diluent

A.3.1 Composition

Polypeptone	1,0 g
Egg lecithin	0,7 g
Polysorbate 80	20,0 g
Water	980 ml

A.3.2 Preparation

Mix and dissolve the ingredients by mixing while heating. Cool down until 25 °C before dispensing the solution into suitable containers. Sterilize in the autoclave at 121 °C for 15 min. After sterilization, the pH shall be equivalent to 7,2 ± 0,2 when measured at room temperature.

A.4 Modified Letheen broth [5]

A.4.1 Composition

Peptic digest of meat	20,0 g
Pancreatic digest of casein	5,0 g
Beef extract	5,0 g
Yeast extract	2,0 g
Lecithin	0,7 g
Polysorbate 80	5,0 g
Sodium chloride	5,0 g
Sodium bisulfite	0,1 g
Water	1 000 ml

A.4.2 Preparation

Dissolve polysorbate 80 and lecithin successively 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, the pH shall be equivalent to 7,2 ± 0,2 when measured at room temperature.

Annex B
(informative)

Other diluents

B.1 General

Any diluent may be used to prepare the initial suspension if it has been checked and validated. The following diluent is an example of suitable formula.

B.2 Buffered peptone solution pH 7

B.2.1 Composition

Meat peptone	1,0 g
Sodium chloride	4,3 g
Monopotassium phosphate	3,6 g
Disodium phosphate dihydrate	7,2 g
Water	1 000 ml

B.2.2 Preparation

Dissolve the ingredients in boiling water. Mix. Cool down until 25 °C before dispensing the solution 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.

Annex C
(informative)

Other culture media

C.1 General

Any culture medium may be used if it has been checked and validated. The following media are examples of suitable formula.

C.2 Agar media for counting

C.2.1 Eugon LT 100 agar medium

See 5.4.3.3.1.

C.2.2 LT 100 Agar

C.2.2.1 Composition

Pancreatic digest of casein	15,0 g
Papaic digest of soybean meal	5,0 g
Sodium chloride	5,0 g
Egg lecithin	1,0 g
Polysorbate 80	5,0 g
Octoxynol 9	1,0 g
Agar	15,0 g
Water	1 000 ml

C.2.2.2 Preparation

Dissolve polysorbate 80, octoxynol 9 and egg lecithin successively 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.

C.2.3 Agar added soybean casein digest medium (agar added SCD broth)

C.2.3.1 Composition

Casein peptone	17,0 g
Soybean peptone	3,0 g
Sodium chloride	5,0 g
Dipotassium hydrogen phosphate	2,5 g
Glucose	2,5 g
Agar	15,0 g
Water	1 000 ml

C.2.3.2 Preparation

Dissolve all of these components or dehydrated complete medium successively into boiling water until their complete dissolution. 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 7,2 ± 0,2 when measured at room temperature.

C.3 Enrichment broths

C.3.1 Modified Letheen broth [5]

See A.4.

C.3.2 Soybean-casein-digest-lecithin-polysorbate 80 medium (SCDLP 80 Broth)

C.3.2.1 Composition

Casein peptone	17,0 g
Soybean peptone	3,0 g
Sodium chloride	5,0 g
Dipotassium hydrogen phosphate	2,5 g
Glucose	2,5 g
Lecithin	1,0 g
Polysorbate 80	7,0 g
Water	1 000 ml

C.3.2.2 Preparation

Dissolve all of these components or dehydrated complete medium successively into boiling water until their complete dissolution. 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 7,2 ± 0,2 when measured at room temperature.

C.3.3 D/E neutralizing broth (Dey/Engley neutralizing broth) [5]

C.3.3.1 Composition

Glucose	10,0 g
Soybean lecithin	7,0 g
$Na_2S_2O_3 \cdot 5H_2O$	6,0 g
Polysorbate 80	5,0 g
Pancreatic digest of casein	5,0 g
$NaHSO_3$	2,5 g
Yeast extract	2,5 g
Sodium thioglycollate	1,0 g
Bromcresol purple	0,02 g
Water	1 000 ml

C.3.3.2 Preparation

Dissolve all of these components or dehydrated complete medium successively into boiling water until their complete dissolution. 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 7,6 ± 0,2 when measured at room temperature.

C.4 Soybean-casein-digest-lecithin-polysorbate 80 agar medium (SCDLPA) for detection

C.4.1 Composition

Casein peptone	15,0 g
Soybean peptone	5,0 g
Sodium chloride	5,0 g
Egg lecithin	1,0 g
Polysorbate 80	7,0 g
Agar	15,0 g
Water	1 000 ml

C.4.2 Preparation

Mix all components and dissolve by 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 7,0 ± 0,2 when measured at room temperature.

Annex D
(informative)

Neutralizers of antimicrobial activity of preservatives and rinsing liquids

Preservative	Chemical compounds able to neutralize preservative's antimicrobial activity	Examples of suitable neutralizers and of *rinsing liquids* [6] [7] *(for membrane filtration methods)*
Phenolic compounds: parabens, phenoxyethanol, phenylethanol, etc. Anilides	Lecithin, Polysorbate 80, Ethylene oxide condensate of fatty alcohol Non-ionic surfactants	Polysorbate 80, 30 g/l + lecithin, 3 g/l. Ethylene oxide condensate of fatty alcohol, 7 g/l + lecithin, 20 g/l + polysorbate 80, 4 g/l. D/E neutralizing broth[a]. *Rinsing liquid: distilled water; tryptone, 1 g/l + NaCl 9 g/l; polysorbate 80, 5 g/l.*
Quaternary ammonium compounds Cationic surfactants	Lecithin, saponin, polysorbate 80, sodium dodecyl sulphate Ethylene oxide condensate of fatty alcohol	Polysorbate 80, 30 g/l + sodium dodecyl sulphate, 4 g/l + lecithin, 3 g/l. Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3 g/l. D/E neutralizing broth[a]. *Rinsing liquid : distilled water; tryptone, 1 g/l + NaCl, 9 g/l; polysorbate 80, 5 g/l.*
Aldehydes Formaldehyde-release agents	Glycine, histidine	Lecithin, 3 g/l + polysorbate 80, 30 g/l + L-histidine, 1 g/l. Polysorbate 80, 30 g/l + saponin, 30 g/l + L-histidine, 1 g/l + L-cysteine, 1 g/l. D/E neutralizing broth[a]. *Rinsing liquid: polysorbate 80, 3 g/l + L-histidine 0,5 g/l.*
Oxidizing compounds	Sodium thiosulphate	Sodium thiosulphate, 5 g/l. *Rinsing liquid: sodium thiosulphate, 3 g/l.*
Isothiazolinones, imidazoles	Lecithin, saponin Amines, sulfates, mercaptans, sodium bisulfite, sodium thioglycollate	Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3 g/l. *Rinsing liquid: tryptone, 1 g/l + NaCl, 9 g/l; polysorbate 80, 5 g/l.*
Biguanides	Lecithin, saponin, polysorbate 80	Polysorbate 80, 30 g/l + saponin, 30 g/l + lecithin, 3 g/l. *Rinsing liquid: tryptone, 1 g/l + NaCl, 9 g/l; polysorbate 80, 5 g/l.*
Metallic salts (Cu, Zn, Hg) Organo-mercuric compounds	Sodium bisulphate, L-cysteine Sulfhydryl compounds, thioglycollic acid	Sodium thioglycollate, 0,5 g/l or 5 g/l. L-cysteine, 0,8 g/l or 1,5 g/l. D/E neutralizing broth[a]. *Rinsing liquid: sodium thioglycollate, 0,5 g/l.*

[a] D/E neutralizing broth (Dey/Engley neutralizing broth), see Annex C.

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