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Cosmetics — Microbiology — Detection of specified and non-specified microorganisms

Cosmétiques — Microbiologie — Détection des micro-organismes spécifiés et non spécifiés

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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 18415 was prepared by Technical Committee ISO/TC 217, *Cosmetics*.

Introduction

Microbiological examinations of cosmetic products are carried out according to an appropriate microbiological risk analysis in order to ensure their quality and safety for consumers.

Microbiological risk analysis depends on several parameters such as:

— potential alteration of cosmetic products;

— pathogenicity of microorganisms;

— site of application of the cosmetic product (hair, skin, eyes, mucous membranes);

— type of user (adults, children, including under 3 years).

For cosmetics and other topical products, the detection of skin pathogens such as *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans* may be relevant because they can cause skin or eye infection. The detection of other kinds of microorganisms might be of interest since these microorganisms (including indicators of faecal contamination e.g. *Escherichia coli*) suggest hygienic failure during manufacturing process.

Cosmetics — Microbiology — Detection of specified and non-specified microorganisms

1 Scope

This International Standard gives general guidelines for the detection and identification of specified microorganisms in cosmetic products as well as for the detection and identification of other kinds of aerobic mesophilic non-specified microorganisms in cosmetic products.

Microorganisms considered as specified in this International Standard might differ from country to country according to national practices or regulations. Most of them considered as specified microorganisms include one or more of the following species: *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus* and *Candida albicans*.

In order to ensure product quality and safety for consumers, it is advisable to perform an appropriate microbiological risk analysis in order to determine the types of cosmetic product 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.

The method described in this International Standard is based on the detection of microbial growth in a non-selective liquid medium (enrichment broth) suitable to detect microbial contamination, followed by isolation of microorganisms on non-selective agar media. Other methods can be appropriate depending on the level of detection required.

In this International Standard specific indications are given for identification of *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus* and *Candida albicans*. Other microorganisms that grow under the conditions described in this International Standard, may be identified by using suitable tests according to a general scheme (see Annex A). Other standards (e.g., ISO 18416, ISO 21150, ISO 22717, ISO 22718) may be appropriate.

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

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*

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

3.2
sample
portion of the product (at least 1 g or 1 ml) that is used in the test to prepare the initial suspension

3.3
initial suspension
suspension (or solution) of the sample in a defined volume of an appropriate enrichment broth

3.4
sample dilution
dilution of the initial suspension

3.5
aerobic mesophilic microorganisms
mesophilic bacteria or yeast growing aerobically under the conditions specified in this International Standard

NOTE In the described conditions other types of microorganism (e.g. molds) are detectable.

3.6
specified microorganisms
aerobic mesophilic bacteria or yeast undesirable in a cosmetic product and recognised as a skin pathogen species that may be harmful for human health or as an indication of hygienic failure in the manufacturing process

3.6.1
Pseudomonas aeruginosa
Gram-negative rod (bacilli), motile; smooth colonies pigmented (pigmented brown or greenish)

NOTE 1 The main characteristics for identification are growth on a selective cetrimide agar medium, oxidase positive, production of diffusible fluorescent pigments and production of a soluble phenazine pigment (pyocyanin) in suitable media.

NOTE 2 *Pseudomonas aeruginosa* can be isolated from a wide variety of environmental sources, especially in water and has a very high potential to spoil many different substrates. It can produce infections of human skin or eye areas. It is undesirable in cosmetic products for its potential pathogenicity and its capacity to affect the physico-chemical properties of the cosmetic formula.

3.6.2
Escherichia coli
Gram-negative rod (bacilli), motile, smooth colonies

NOTE 1 The main characteristics are catalase positive, oxidase negative, fermentation of lactose, production of indole, growth on selective medium containing bile salts with characteristic colonies.

NOTE 2 *Escherichia coli can* be isolated from the moist environmental sources (air, water, soil) and is a faecal contamination indicator.

3.6.3
Staphylococus aureus
Gram-positive cocci, mainly aggregated in grape-like clusters, smooth colonies generally pigmented in yellow

NOTE 1 The main characteristics for identification are growth on a specific selective medium, catalase positive, coagulase positive.

NOTE 2 *Staphylococcus aureus* is an opportunistic pathogen for humans, which often can be also present on the skin of healthy individuals without causing them any apparent illness. It is a specified microorganism and undesirable in cosmetic products.

3.6.4
Candida albicans
yeast that forms white to beige, creamy and convex colonies on the surface of a non-selective agar medium

NOTE The main characteristics for identification are production of germ tube and/or pseudomycelium and chlamydospore when the test is performed following the method specified in this International Standard

3.7
non-specified microorganism
aerobic mesophilic bacteria or yeast found in cosmetic products, not defined in 3.6

3.9
enrichment broth
non-selective liquid medium containing suitable neutralizers and/or dispersing agents and validated for the product under test

4 Principle

The first step of the procedure is to perform an enrichment by using a non-selective broth medium to increase the number of microorganisms without the risk of inhibition by the selective ingredients that are present in selective/differential growth media.

The following steps (isolation and identification) are performed according to need by using appropriate conditions of incubation and suitable identification test, as described in this International Standard.

The possible inhibition of microbial growth by the sample shall be neutralized to allow the detection of viable microorganisms [2]. In all cases and whatever the methodology, the neutralization of the antimicrobial properties of the product shall be checked and validated [2],[3],[4].

5 Diluents 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 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. The efficacy of the neutralization shall be demonstrated (see Clause 11). Information relative to suitable neutralizers is given in Annex C.

The enrichment broth (5.3.2.1) or any of the ones listed in Annex B is suitable for checking the presence of specified and non-specified microorganisms in accordance with this International Standard provided that they are validated in accordance with Clause 11.

Other diluents and culture media may be used if it has been demonstrated that they are suitable for use.

5.2 Diluent for the microbial suspension (tryptone sodium chloride solution)

5.2.1 General

The diluent is used for the preparation of bacteria and yeast suspensions used for the validation procedure (see Clause 11).

5.2.2 Composition

— tryptone, pancreatic digest of casein 1,0 g

— sodium chloride 8,5 g

— water 1 000 ml

5.2.3 Preparation

Dissolve the components in 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.

5.3 Culture media

5.3.1 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.

5.3.2 Enrichment broth

5.3.2.1 Eugon LT100 broth

5.3.2.1.1 General

This medium contains ingredients which neutralize inhibitory substances present in the sample: lecithin and polysorbate 80, and dispersing agent: octoxynol 9.

5.3.2.1.2 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

5.3.2.1.3 Preparation

Dissolve the components polysorbate 80, octoxynol 9 and egg lecithin, one after another in boiling water to 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.

5.3.2.2 Other enrichment broths

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

5.3.3 Non-selective agar medium

5.3.3.1 General

This medium is used for the isolation and detection of specified and non-specified microorganisms present in the initial suspension after enrichment and for the preparation of inoculum used in the validation procedure.

5.3.3.2 Soybean-casein digest agar medium (SCDA) or tryptic soy agar (TSA)

5.3.3.2.1 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

5.3.3.2.2 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.

5.3.3.3 Other non-selective agar medium

Other non-selective, non-neutralizing agar media may be used (see Annex B).

6 Apparatus and glassware

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

7 Strains of microorganism

For testing the recovery efficiency of the test conditions, three specified microorganisms are used: two strains representative of both Gram-negative and Gram-positive bacteria and a strain of yeast.

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

— *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);

— *Candida albicans* ATCC 10231 (equivalent strain: IP[6)] 48.72 or NCPF[7)] 3179 or NBRC 1594 or KCTC 17205 or other equivalent national collection strain).

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

The strains can be kept in the laboratory in accordance with 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 (3.1) and samples (3.2) 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 given in Clause 9.

9 Procedure

9.1 General recommendations

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 in an appropriate diluent before transfer in the enrichment broth, the time that elapses between the end of the preparation and the moment the initial suspension and/or sample dilutions come 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 in the enrichment broth

9.2.1 General

The enrichment is prepared from a sample of at least 1 g or 1 ml of the well-mixed product under test, which is dispersed in at least 9 ml of enrichment broth.

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

The method shall be checked to ensure that the composition (neutralizer eventually added) and the volume of the broth perform satisfactorily (see 11.3).

1) American Type Culture Collection.
2) Collection de l'Institut Pasteur.
3) National Collection of Industrial and Marine Bacteria.
4) National Biological Resource Center.
5) Korean Collection for Type Culture.
6) Institut Pasteur.
7) National Collection of Pathogenic Fungi.

NOTE In some cases, and when possible, filtration of the cosmetic product through a membrane which is afterwards immersed in the enrichment broth facilitates the neutralization of the antimicrobial properties of the product (see 11.3.3).

9.2.2 Water-miscible products

Transfer the sample, S, of product to a suitable container containing an appropriate volume (at least 9 ml) of broth.

9.2.3 Water-immiscible products

Transfer the sample, S, of product to a suitable container containing a suitable quantity of dispersing agent (e.g. polysorbate 80).

Disperse the sample within the solubilizing agent and add an appropriate volume (at least 9 ml) of broth.

9.2.4 Filterable products

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

Transfer the sample, S, on to the membrane in a filtration apparatus (see ISO 21148). Filter immediately and wash the membrane using defined volumes of water and/or diluent. Transfer the membrane and immerse into a tube or flask of suitable size containing an appropriate volume (at least 9 ml) of enrichment broth. This preparation is equivalent to an initial suspension.

9.3 Incubation of the initial suspension

Incubate the initial suspension prepared in broth (see 9.2) at 32,5 °C ± 2,5 °C for at least 20 h.

9.4 Isolation of specified and non-specified microorganisms

Using a sterile loop, streak an aliquot of the incubated enrichment broth on to the surface of a Petri dish (diameter 85 mm to 100 mm) containing approximately 15 ml to 20 ml of non-selective agar medium. If larger Petri dishes are used, the volume of the agar is increased accordingly.

Invert the Petri dish and incubate at 32,5 °C ± 2,5 °C for 48 h to 72 h. The procedure for identification of colonies is described below for *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Candida albicans* and for others microorganisms described in 9.9.

9.5 Procedure for identification of the specified microorganism: *Pseudomonas aeruginosa*

9.5.1 Gram staining

Proceed to a Gram staining as described in ISO 21148 on a part of suspect[8)] colony well isolated from a surface of soybean-casein digest agar.

The microscopic observation of the Gram stain shall reveal Gram-negative rods.

9.5.2 Oxidase test

Follow the procedure specified in ISO 21148.

Check for oxidase positive test.

8) Suspect for *Pseudomonas aeruginosa* means smooth colonies generally pigmented greenish to yellowish.

9.5.3 Identification test

Use a suitable identification protocol for non-fermenting Gram-negative rod (i.e. standardized and/or miniaturized biochemical system) with a dedicated database (or equivalent like a catalogue) including the typical characteristics of *Pseudomonas aeruginosa*.

When using an identification kit, follow the instructions given by the supplier (inoculation, incubation, reading) and compare the final result with the database. The name of the microorganism to be identified shall be *Pseudomonas aeruginosa* with a level of confidence considered as appropriate by the identification system.

9.6 Procedure for identification of the specified microorganism: *Escherichia coli*

9.6.1 Gram staining

Proceed to a Gram staining as described in ISO 21148 on a part of suspect[9)] colony well isolated from a surface of soybean-casein digest agar.

The microscopic observation of the Gram stain shall reveal Gram-negative rods.

9.6.2 Oxidase test

Check for oxidase negative test as described in ISO 21148.

9.6.3 Identification test

Use a suitable identification protocol for fermenting Gram-negative rod (i.e. standardized and/or miniaturized biochemical system) with a dedicated database (or equivalent like a catalogue) including the typical characteristics of *Escherichia coli*.

When using an identification kit, follow the instructions given by the supplier (inoculation, incubation, reading) and compare the final result with the database. The name of the microorganism to be identified shall be *Escherichia coli* with a level of confidence considered as appropriate by the identification system.

9.7 Procedure for identification of the specified microorganism: *Staphylococus aureus*

9.7.1 Gram staining

Proceed to a Gram staining as described in ISO 21148 on a part of suspect[10)] colony well isolated from the surface of soybean-casein digest agar.

The microscopic observation of the Gram stain shall reveal Gram-negative rods.

9.7.2 Catalase test

Check for a catalase positive test as described in ISO 21148.

9.7.3 Identification test

Use a suitable identification protocol for Gram positive cocci (i.e. standardized and/or miniaturized biochemical system) with a dedicated database (or equivalent like a catalogue) including the typical characteristics of *Staphylococcus aureus*.

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9) Suspect for *Escherichia coli* means smooth colonies.

10) Suspect for *Staphyloccocus aureus* means smooth colonies generally pigmented in yellow.

When using an identification kit, follow the instructions given by the supplier (inoculation, incubation, reading) and compare the final result with the database. The name of the microorganism to be identified shall be *Staphylococcus aureus* with a level of confidence considered as appropriate by the identification system.

9.8 Procedure for the identification of the specified microorganism: *Candida albicans*

9.8.1 Gram staining

Proceed to a Gram staining as described in ISO 21148 on a part of suspect[11)] colony well isolated from non-selective agar media.

The microscopic observation of the Gram stain shall reveal a violet colour, short ovoid or elongate cells, sometimes with budding cells.

9.8.2 Identification test

Use a suitable identification protocol for yeast (i.e. standardized and/or miniaturized biochemical system) with a dedicated database (or equivalent like a catalogue) including the typical characteristics of *Candida albicans*.

When using an identification kit, follow the instructions given by the supplier (inoculation, incubation, reading) and compare the final result with the database. The name of the microorganism to be identified shall be *Candida albicans* with a level of confidence considered as appropriate by the identification system.

9.9 Procedure for the identification of non-specified microorganisms

9.9.1 Gram staining

Proceed to a Gram staining as described in ISO 21148 on a part of colony well isolated from the surface of soybean-casein digest agar.

Note the morphology and the Gram staining reaction revealed on microscopic observation.

9.9.2 Oxidase test

In the case of a Gram-negative bacilli (rod), proceed to perform an oxidase test as described in ISO 21148 on a part of a microbial colony, well isolated from the surface of a streaked soybean-casein digest agar.

Note the result of the test.

9.9.3 Catalase test

In the case of a Gram-positive coccus, proceed as described in ISO 21148 on a part of a microbial colony, well isolated from the surface of a streaked soybean-casein digest agar.

Note the result of the test.

9.9.4 Identification test

Based on the results of the preliminary tests (see 9.9.1, 9.9.2, 9.9.3), choose a suitable identification protocol (i.e. standardized and/or miniaturized biochemical system) with a dedicated database (or equivalent like a catalogue) including the typical characteristics of the suspected species (see scheme in Annex A).

11) Suspect for *Candida albicans* means smooth colonies generally white pigmented.

When using an identification kit, follow the instructions given by the supplier (inoculation, incubation, reading) and compare the final result with the database. Note the name of the identified microorganism and the level of confidence considered as appropriate by the identification system.

10 Expression of the results

10.1 Detection of specified microorganisms

For each species of specified microorganism, and if the identification of the colonies confirms the presence of this species, express the result as:

— Presence of (*name of the species*) in the sample, S.

If the identification of the colonies doesn't confirm the presence of this species, express results as indicated in 10.2.

10.2 Detection of non-specified microorganisms

If growth is observed after enrichment and if the colonies are isolated and recognized as non-specified microorganisms, express the result as:

— Presence of (*name of the species* and/or main morphological characteristics) in the sample, S, and absence of specified microorganisms.

10.3 Absence of microorganisms

If no growth after enrichment and isolation is observed, express the result as:

— Absence of aerobic mesophilic organisms (specified microorganisms included) in the sample, S.

11 Neutralization of the antimicrobial properties of the product

11.1 General

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

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

11.2 Preparation of inoculum

Prior to the test, and for each strain, inoculate the surface of a 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 microbial culture use a sterile loop, streak the surface of the culture and re-suspend in the diluent for microbial suspensions to obtain a calibrated suspension of about 1×10^8 CFU/ml for the bacteria and about 1×10^6 CFU/ml for the yeast (e.g. using a spectrophotometer, ISO 21148:2005, Annex C). Use this suspension and its dilutions within 2 h.

11.3 Validation of detection method by enrichment

11.3.1 Principle

For each test strain, mix the neutralized sample (initial suspension) with a diluted calibrated suspension of microorganisms, then incubate the mixture. After incubation, streak for isolation on a non-selective agar medium. A non-inoculated control is prepared, incubated and plated in parallel.

After incubation, the validation plates and the control plates are checked for the presence of micro-organisms. Results are interpreted.

11.3.2 Procedure

In tubes of 9 ml of diluent prepare a dilution of the calibrated suspension in order to obtain a final count between 100 CFU per mil and 500 CFU per ml. To count the viable microorganisms in the dilution of the calibrated suspension, transfer 1 ml of the suspension into a Petri dish and pour 15 ml to 20 ml of the melted agar medium 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 in the conditions chosen for the test (at least 1 g or 1 ml of product under test, defined volume of enrichment broth) in a tube or flask. When using the membrane filtration method, filter in duplicate, at least 1 ml of product under test and transfer each membrane into a tube or flask containing the enrichment broth in the conditions chosen for the test.

Introduce aseptically, 0,1 ml of the final dilution of the calibrated suspension of microorganisms into one tube or flask (validation test). Mix, then incubate either tubes or flasks (validation test and non-inoculated control) at 32,5 °C ± 2,5 °C for 20 h to 24 h.

Perform an isolation for each tube or flask (validation test and non-inoculated control). Using a sterile loop, streak an aliquot (same conditions as in the test) of the incubated mixture on the surface of a Petri dish (diameter 85 mm to 100 mm) containing approximately 15 ml to 20 ml of non-selective agar medium. Incubate the plates at 32,5 °C ± 2,5 °C for 24 h to 48 h.

11.3.3 Interpretation of validation results

Confirm that the final dilution of the calibrated suspensions of bacteria or yeast contain between 100 CFU per ml and 500 CFU per ml.

When no growth occurs on the control plate, the neutralization and the detection are validated if characteristic[12)] colonies of the inoculated organisms grow on the validation plates.

When growth is detected on the control plates, the neutralization and the detection are validated if characteristic colonies of the inoculated microorganisms are recovered on the validation plates (possibly a mixed culture with colonies found on the control plate may be present).

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12) Characteristic means:

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

— for *Pseudomonas aeruginosa*: greenish to yellowish culture;

— for *Candida albicans*: convex, smooth and creamy coloured colonies;

— for *Escherichia coli*: smooth colonies.

If no characteristic colonies are detected on the validation plates, whatever the presence of growth on the control plates, the neutralization and the detection are not validated

Failure of growth on the validation plates indicates that antimicrobial 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 enrichment broth, the quantity of product remaining the same, or by incorporation of a sufficient quantity of inactivating agent in the enrichment broth, or by an appropriate combination of modifications so as to permit the growth of bacteria and yeast.

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

12 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;

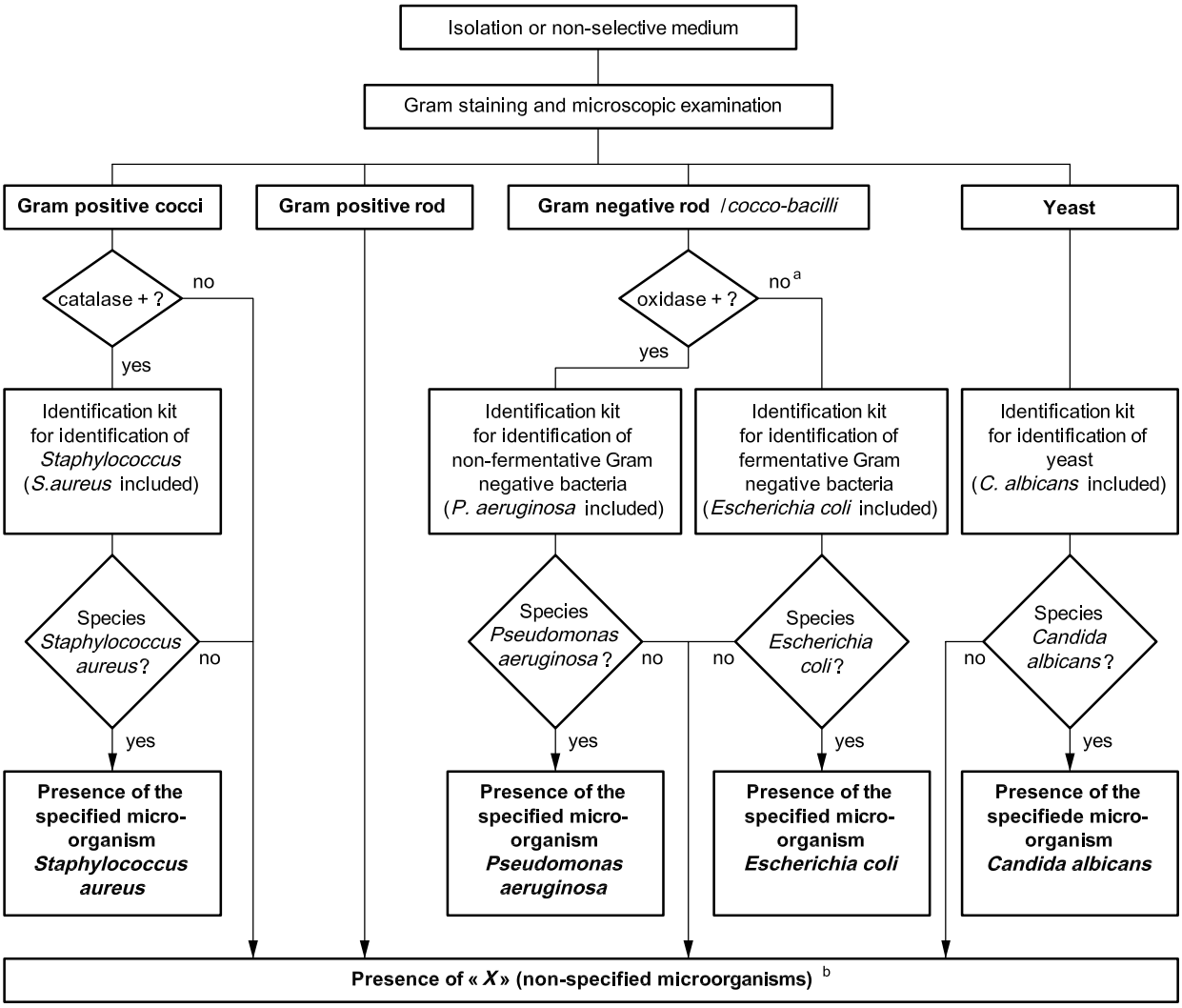
e) the description of the method with the neutralizers and media used;

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 that may have influenced the results.

Annex A
(informative)

General scheme for identification of microorganisms



[a] Some *Pseudomonas* or closer species may be oxidase negative, however identification kits for fermentative bacteria include this type of microorganisms.

[b] The identification of non-specified microorganisms may be useful to locate the source of contamination in a production center or to comply with internal specification.

Annex B
(informative)

Other Media

B.1 Other enrichment broth

B.1.1 Modified letheen broth [4]

B.1.1.1 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

B.1.1.2 Preparation

Dissolve, one after the other in boiling water, polysorbate 80 and lecithin until their complete dissolution. Dissolve the other components by 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.

B.1.2 Fluid casein digest-soy lecithin-polysorbate 20 medium (FCDLP 20)

B.1.2.1 Composition

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

B.1.2.2 Preparation

Dissolve the pancreatic digest of casein and the soy lecithin in 960 ml of water, heating in a water bath at 48 °C to 50 °C for about 30 min to effect dissolution. Add 40 ml of polysorbate 20. Mix and dispense 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.

B.1.3 Soybean-casein-digest-lecithin-polysorbate 80 medium (SCDLP 80 broth)

B.1.3.1 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
- lecithin 1,0 g
- polysorbate 80 7,0 g
- water 1 000 ml

B.1.3.2 Preparation

Dissolve all of these components or dehydrated complete medium one after the other in boiling water until their complete dissolution. 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.

B.1.4 D/E neutralizing broth (Dey/Engley neutralizing broth) [7]

B.1.4.1 Composition

- glucose 10,0 g
- soybean lecithin 7,0 g
- sodium thiosulfate pentahydrate 6,0 g
- polysorbate 80 5,0 g
- pancreatic digest of casein 5,0 g
- sodium bisulfite 2,5 g
- yeast extract 2,5 g
- sodium thioglycollate 1,0 g
- bromcresol purple 0,02 g
- water 1 000 ml

B.1.4.2 Preparation

Dissolve all of these components or dehydrated complete medium one after the other in boiling water until their complete dissolution. Dispense the medium into suitable containers. Sterilize in the autoclave at 121 °C for 15 min. After sterilization, the pH shall be equivalent to 7,6 ± 0,2 when measured at room temperature.

B.2 Other non-selective agar medium — Agar added soybean-casein-digest medium (agar added SCD broth)

B.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
- agar 15,0 g
- water 1 000 ml

B.2.2 Preparation

Dissolve the components or dehydrated complete medium in the water 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 7,3 ± 0,2 when measured at room temperature.

Annex C
(informative)

Neutralizers of antimicrobial activity of preservatives and rinsing liquids

Preservative	Chemical compounds able to neutralize a preservative's antimicrobial activity	Examples of suitable neutralizers and of *rinsing liquids (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.*
Quarternary 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 B.		

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