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Milk and milk products — Method for the enumeration of *Pseudomonas* spp.

Lait et produits laitiers — Méthode de dénombrement des Pseudomonas *spp.*

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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.

In other circumstances, particularly when there is an urgent market requirement for such documents, a technical committee may decide to publish other types of document:

— an ISO Publicly Available Specification (ISO/PAS) represents an agreement between technical experts in an ISO working group and is accepted for publication if it is approved by more than 50 % of the members of the parent committee casting a vote;

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An ISO/PAS or ISO/TS is reviewed after three years in order to decide whether it will be confirmed for a further three years, revised to become an International Standard, or withdrawn. If the ISO/PAS or ISO/TS is confirmed, it is reviewed again after a further three years, at which time it must either be transformed into an International Standard or be withdrawn.

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/TS 11059 | IDF/RM 225 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF). It is being published jointly by ISO and IDF.

Foreword

IDF (the International Dairy Federation) is a non-profit organization representing the dairy sector worldwide. IDF membership comprises National Committees in every member country as well as regional dairy associations having signed a formal agreement on cooperation with IDF. All members of IDF have the right to be represented on the IDF Standing Committees carrying out the technical work. IDF collaborates with ISO in the development of standard methods of analysis and sampling for milk and milk products.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the Action Teams and Standing Committees are circulated to the National Committees for voting. Publication as an International Standard requires approval by at least 50 % of the IDF National Committees casting a vote.

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All work was carried out by the Joint ISO-IDF Action Team on *Microbiological harmonization* of the Standing Committee on *Microbiological methods of analysis* under the aegis of its project leader, Mrs P. Rollier (FR).

Milk and milk products — Method for the enumeration of *Pseudomonas* spp.

1 Scope

This Technical Specification describes a method for the enumeration of *Pseudomonas* spp. in milk and milk products. The method allows the isolation of all pigmented and non-pigmented psychrophilic *Pseudomonas* spp.

This Technical Specification is also applicable to dairy environmental samples.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 6887-5, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 5: Specific rules for the preparation of milk and milk products*

ISO 7218, *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations*

ISO/TS 11133-1, *Microbiology of food and animal feeding stuffs — Guidelines on preparation and production of culture media — Part 1: General guidelines on quality assurance for the preparation of culture media in the laboratory*

ISO/TS 11133-2, *Microbiology of food and animal feeding stuffs — Guidelines on preparation and production of culture media — Part 2: Practical guidelines on performance testing of culture media*

3 Terms and definitions

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

3.1
***Pseudomonas* spp.**
species of bacteria of the genus *Pseudomonas* which form colonies in penicillin and pimaricin agar (PPA) at 25 °C displaying the biochemical characteristics described, when tested as described in this Technical Specification

4 Principle

The surface of a solid selective culture medium is inoculated with a specified quantity of the test sample if the product is liquid, or with a specified quantity of the initial suspension in the case of other products. The inoculation is done, under the same conditions, using decimal dilutions of the test sample or of the initial suspension.

The plates are incubated aerobically at 25 °C for 48 h.

The number of *Pseudomonas* is calculated per millilitre, or per gram, of sample from the number of colonies obtained on plates at dilution levels chosen so as to give a significant result, and after confirmation of the selected colonies by the oxidase test and glucose fermentation test.

5 Diluent, culture media and reagent

5.1 General

For current laboratory practice, see ISO/TS 11133-1 and ISO/TS 11133-2.

5.2 Diluent

See ISO 6887-5.

5.3 Penicillin and pimaricin agar (PPA)

5.3.1 Basic medium

5.3.1.1 Composition

Component	Amount
Enzymatic digest of gelatine	16,0 g
Enzymatic digest of casein	10,0 g
Potassium sulfate (K_2SO_4)	10,0 g
Magnesium chloride ($MgCl_2$)	1,4 g
Agar	12,0 g to 18,0 g [a]
Water	1 000 ml
[a] Depending on the gel strength of the agar.	

5.3.1.2 Preparation

Dissolve the components or the dehydrated complete medium in the water by boiling. Adjust the pH, if necessary, so that after sterilization it is 7,2 ± 0,2 at 25 °C. Dispense the basic medium into flasks or bottles of appropriate capacity. Sterilize the medium in the autoclave (6.1) at 121 °C for 15 min.

5.3.2 Inhibitor solutions

5.3.2.1 Penicillin solution

5.3.2.1.1 Composition

Component	Amount
Penicillin G, potassium salt	10^6 IU
Water	10 ml

5.3.2.1.2 Preparation

Dissolve the penicillin in the water and sterilize the solution by filtration.

The penicillin solution can be stored at 5 °C ± 3 °C for one week or frozen in aliquots at −20 °C for 6 months.

5.3.2.2 Pimaricin solution

5.3.2.2.1 Composition

Component	Amount
Pimaricin (natamycin)	0,1 g
Water	10 ml

5.3.2.2.2 Preparation

Dissolve the pimaricin in the water. Sterilize the solution in the autoclave (6.1) at 110 °C for 20 min.

Pimaricin in solution is not stable, and has to be protected from light. Therefore, use the pimaricin solution on the day of its preparation. Alternatively, the solution can be stored frozen in aliquots at −20 °C for 6 months.

5.3.3 Complete medium

5.3.3.1 Composition

Volume	Final concentration
Basic medium 100 ml	—
Penicillin solution 0,1 ml	100 000 IU/l
Pimaricin solution 0,1 ml	0,01 g/l

5.3.3.2 Preparation

Under aseptic conditions, add the inhibitor solutions to the basic medium, melted and maintained at between 44 °C and 47 °C. Carefully mix the thus prepared complete medium.

5.3.4 Preparation of PPA agar plates

For preparation and drying of the plates, see ISO/TS 11133-1. If prepared in advance, the agar plates can be kept in the dark at 5 °C ± 3 °C for no longer than 1 day.

5.3.5 Performance testing for quality control

5.3.5.1 Productivity

Incubation:	At 25 °C ± 1 °C for 48 h ± 2 h
Strain:	*Pseudomonas fluorescens* ATCC 13525 or *Pseudomonas aeruginosa* ATCC 27853
Reference medium:	Soybean-casein digest agar medium (TSA)
Method of control:	Quantitative
Criteria:	Productivity ratio, $P_R > 0{,}5$ and non-characteristic colonies

5.3.5.2 Selectivity

Incubation:	At 25 °C ± 1 °C for 48 h ± 2 h
Strain:	*Escherichia coli* ATCC 25922 or *Staphylococcus aureus* ATCC 25923
Method of control:	Qualitative
Criteria:	Total inhibition

5.4 Nutrient agar

5.4.1 Composition

Component	Amount
Meat extract	3,0 g
Enzymatic digest of animal tissues	5,0 g
Agar	12,0 g to 18,0 g [a]
Water	1 000 ml

[a] Depending on the gel strength of the agar.

5.4.2 Preparation

Dissolve the dehydrated components or the dehydrated complete medium in the water by boiling. Adjust the pH, if necessary, so that after sterilization it is 7,0 ± 0,2 at 25 °C. Dispense the culture medium into tubes or bottles of appropriate capacity. Sterilize the nutrient agar in the autoclave (6.1) at 121 °C for 15 min.

5.4.3 Preparation of nutrient agar plates

For preparation, drying and storage of the plates, see ISO/TS 11133-1.

5.4.4 Performance testing for quality control

Productivity details are given below.

Incubation:	At 25 °C ± 1 °C for 24 h ± 2 h
Strain:	*Pseudomonas fluorescens* ATCC 13525 or *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922 or *Staphylococcus aureus* ATCC 25923
Reference medium:	Soybean-casein digest agar medium (TSA)
Method of control:	Quantitative
Criteria:	Productivity ratio, $P_R > 0{,}7$ and non-characteristic colonies

5.5 Glucose agar

5.5.1 Composition

Component	Amount
Enzymatic digest of casein	10,0 g
Yeast extract	1,5 g
Sodium chloride	5,0 g
Glucose	10,0 g
Bromocresol purple	0,015 g
Agar	12,0 g to 18,0 g [a]
Water	1 000 ml
[a] Depending on the gel strength of the agar.	

5.5.2 Preparation

Dissolve the components or the dehydrated complete medium in the water, heating if necessary. Adjust the pH, if necessary, so that after sterilization it is 7,0 ± 0,2 at 25 °C.

Dispense the medium in 10 ml amounts into test tubes (6.5). Sterilize the glucose agar in the autoclave (6.1) at 121 °C for 15 min. Leave the tubes in a vertical position.

Just before use, heat in boiling water or flowing steam for 15 min. Then cool rapidly to the incubation temperature.

5.5.3 Performance testing for quality control

5.5.3.1 Positive test

Incubation:	At 25 ± 1 °C for 24 h ± 2 h
Strain:	*Escherichia coli* ATCC 25922
Method of control:	Qualitative
Criteria:	Positive

5.5.3.2 Negative test

Incubation:	At 25 °C ± 1 °C for 24 h ± 2 h
Strain:	*Pseudomonas fluorescens* ATCC 13525 or *Pseudomonas aeruginosa* ATCC 27853
Method of control:	Qualitative
Criteria:	Negative

5.6 Reagent for the detection of oxidase

5.6.1 Composition

Component	Amount
N,N,N′,N′ -Tetramethyl-*p*-phenylenediamine dihydrochloride	1,0 g
Water	100 ml

5.6.2 Preparation

Dissolve the reagent in the water immediately before use.

Commercially available discs or sticks may be used. If so, follow the manufacturer's instructions.

6 Apparatus and glassware

Usual microbiological equipment (see ISO 7218) and, in particular, the following.

6.1 Apparatus for dry sterilization (oven) or **wet sterilization (autoclave)**.

See ISO 7218.

6.2 Incubator, capable of operating at 25 °C ± 1 °C.

6.3 pH-meter.

See ISO 7218.

6.4 Loops, of platinum-iridium or sterile plastic, approximately 3 mm in diameter or **wires** of the same material or **glass rods**.

NOTE A nickel-chromium loop is not suitable for use in the oxidase test (see 8.4.2).

6.5 Test tubes, bottles or **flasks**, of appropriate capacity.

6.6 Total-delivery graduated pipettes, capacity 1 ml, graduated in 0,1 ml divisions, ISO 835[2] class A, or **automatic pipettes**, ISO 8655-2[3], used with sterile disposable tips.

6.7 Petri dishes, made of glass or plastic, of diameter 90 mm to 100 mm.

6.8 Spreaders, made of glass or plastic, e.g. hockey sticks made from a glass rod of diameter approximately 3,5 mm and of length 200 mm, bent at right angles about 30 mm from one end and with the cut ends made smooth by heating.

7 Sampling

A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage (see ISO 7218).

Sampling is not part of the method specified in this Technical Specification. A recommended sampling method is given in ISO 707 | IDF 50[1].

If there is no specific International Standard dealing with sampling of the product concerned, it is recommended that the parties concerned come to an agreement on the subject.

8 Procedure

8.1 Test portion, initial suspension and dilutions

Prepare the initial suspension and dilutions in accordance with ISO 6887-5.

8.2 Inoculation and incubation

8.2.1 Take one PPA plate (5.3.4). Transfer, by means of a pipette (6.6), 0,1 ml of the test sample or 0,1 ml of the initial suspension on to the plate. Take another PPA plate. Transfer, by means of a new sterile pipette, 0,1 ml of the first decimal dilution on to the plate.

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

8.2.2 Spread the liquid over the surface of the agar plate with a sterile spreader (6.8) until the surface is completely dry.

8.2.3 Incubate the prepared plates (8.2.2) with their agar surface facing upwards in the incubator (6.2) at 25 °C ± 1 °C for 48 h ± 2 h.

8.3 Counting and selection of colonies

After the specified incubation period, count the colonies on each plate and retain plates containing less than 150 colonies. Randomly select five colonies from each retained plate for confirmation in 8.4.

8.4 Confirmation

8.4.1 Subculturing

Streak on nutrient agar plates (5.4.3) each of the colonies selected for confirmation.

Incubate these plates in the incubator (6.2) at 25 °C ± 1 °C for 24 h to 48 h. Select a well-isolated colony from each of the incubated plates for biochemical confirmation.

8.4.2 Oxidase reaction

Moisten a piece of filter paper with the oxidase reagent (5.6.2). Using a platinum-iridium wire or a glass or plastic rod (6.4) (a nickel-chromium wire gives false positives), take a sample of the bacterial culture obtained from the nutrient agar medium. Deposit the sample on the moistened filter paper.

In the case of the presence of oxidase, a dark violet to purple colour appears within a period of between 5 s and 30 s. If the colour has not been changed after 30 s, the test is considered negative.

8.4.3 Fermentation of glucose

Stab, by means of a wire (6.4), colonies from the nutrient agar medium into tubes containing glucose agar (5.5.2). Incubate the tubes in the incubator (6.2) at 25 °C ± 1 °C for 24 h ± 3 h without hermetically closing the tubes.

Consider the test to be negative (absence of glucose fermentation) when growth can be observed but no yellow colour develops throughout the content of the tube. Some *Pseudomonas* strains may develop a yellow colour at the agar surface resulting from glucose oxidation.

8.4.4 Interpretation: colonies

Consider colonies showing a positive oxidase reaction and absence of glucose fermentation as *Pseudomonas* colonies.

9 Expression of results

See ISO 7218.

10 Test report

The test report shall contain at least the following information:

a) all information required for the complete identification of the sample;

b) the test method used, with reference to this Technical Specification (Reviewed Method);

c) the results obtained;

d) all operating details not specified in this Technical Specification (Reviewed Method), or regarded as optional, together with details of any incident which may have influenced the result(s).

Bibliography

[1] ISO 707 | IDF 50, *Milk and milk products — Guidance on sampling*

[2] ISO 835, *Laboratory glassware — Graduated pipettes*

[3] ISO 8655-2, *Piston-operated volumetric apparatus — Part 2: Piston pipettes*

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