International Standard ISO 7402

INTERNATIONAL ORGANIZATION FOR STANDARDIZATION•МЕЖДУНАРОДНАЯ ОРГАНИЗАЦИЯ ПО СТАНДАРТИЗАЦИИ•ORGANISATION INTERNATIONALE DE NORMALISATION

Microbiology — General guidance for the enumeration of Enterobacteriaceae without resuscitation — MPN technique and colony count technique

Microbiologie — Directives générales pour le dénombrement sans revivification des Enterobacteriaceae — Technique NPP et méthode par comptage des colonies

First edition — 1985-10-15

UDC 579.67.087.23 : 579.842.1/.2 **Ref. No. ISO 7402-1985 (E)**

Descriptors: microbiological analysis, detection, micro-organisms, enterobacteriaceae, test results, test equipment, bacteria count methods.

Price based on 8 pages

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.

Draft International Standards adopted by the technical committees are circulated to the member bodies for approval before their acceptance as International Standards by the ISO Council. They are approved in accordance with ISO procedures requiring at least 75 % approval by the member bodies voting.

International Standard ISO 7402 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*.

Users should note that all International Standards undergo revision from time to time and that any reference made herein to any other International Standard implies its latest edition, unless otherwise stated.

Printed in Switzerland

Microbiology — General guidance for the enumeration of Enterobacteriaceae without resuscitation — MPN technique and colony count technique

0 Introduction

This International Standard is intended to provide general guidance for the examination of products not dealt with by existing International Standards and for the reference of bodies preparing microbiological methods of test for application to food products or to animal feeding stuffs. In view of the large variety of products within this field of application, these guidelines may not be appropriate for some products in every detail, and for some other products it may be necessary to use different methods. Nevertheless, it is hoped that, in all cases, every attempt will be made to apply these guidelines as far as possible and that deviations from them will only be made if absolutely necessary for technical reasons.

When this International Standard is next reviewed, account will be taken of all information then available regarding the extent to which the guidelines have been followed and the reasons which necessitated deviation from them in the case of particular products.

The harmonization of test methods cannot be immediate, and for certain groups of products, International Standards and/or national standards that do not comply with the guidelines may already exist. In cases where International Standards already exist for the product to be tested, they should be followed, but it is hoped that, when they are reviewed, they will be aligned with this International Standard so that, eventually, the only remaining departures from these guidelines will be those necessary for well established technical reasons.

For any particular product, the method to be used will be specified in the International Standard dealing with that product.

1 Scope and field of application

This International Standard gives general guidance for the enumeration of Enterobacteriaceae present in products intended for human consumption or feeding of animals

— by calculation of the most probable number (MPN) after incubation at 35 °C or 37 °C in liquid medium

— by counting colonies in a solid medium after incubation at 35 °C or 37 °C.

For low numbers, the MPN method is preferable, otherwise the colony count method is preferred.

This International Standard does not include resuscitation procedures and the results should not, therefore, be related to criteria or specifications based on the assumption that resuscitation has been carried out.

A limitation on the applicability of this International Standard is imposed by the susceptibility of the methods to a large degree of variability. The methods should be used and the results interpreted in the light of the information given in 10.3.

2 Reference

ISO 6887, *Microbiology — General guidance for the preparation of dilutions for microbiological examination.*

3 Definitions

For the purpose of this International Standard, the following definitions apply.

3.1 Enterobacteriaceae: Micro-organisms which ferment glucose and show a negative oxidase reaction when the test is carried out according to the method specified.

3.2 count of Enterobacteriaceae: The number of Enterobacteriaceae found per millilitre or per gram of the test sample when the test is carried out according to the method specified.

4 Principle

4.1 Preparation of dilutions

Preparation of decimal dilutions from the test sample.

4.2 Enumeration of Enterobacteriaceae

4.2.1 Most probable number (MPN) technique

NOTE — This technique is recommended when the number sought is expected to be in the range 1 to 100 per millilitre or per gram of the test sample.

Inoculation of three tubes of double-strength medium with a specified quantity of test sample if the product is liquid, or with a specified quantity of the initial suspension in the case of other products.

Inoculation of three tubes of single-strength medium 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. Then, under the same conditions, inoculation of three tubes of single-strength medium with the first decimal dilution of the test sample or of the initial suspension.

Incubation of the tubes at 35 °C or 37 °C [1] for 24 h.

From the number of confirmed positive tubes, calculation of the most probable number of Enterobacteriaceae per millilitre or per gram of the test sample using the MPN table (see annex A).

4.2.2 Colony count technique

NOTE — This technique is recommended when the number sought is expected to be greater than 100 per millilitre or per gram of the test sample.

Inoculation of two plates of violet red bile glucose agar (poured plate technique) with a specified quantity of the test sample if the initial product is liquid, or with a specified quantity of the initial suspension in the case of other products. Covering with an overlayer of the same medium.

Inoculation of other pairs of plates under the same conditions, using decimal dilutions of the test sample or of the initial suspension.

Incubation of the plates at 35 °C or 37 °C [1] for 24 h.

From the number of confirmed typical colonies per plate, calculation of the number of Enterobacteriaceae per millilitre or per gram of the test sample.

5 Diluent, culture media and reagent

5.1 Basic materials

In order to improve the reproducibility of the results, it is recommended that, for the preparation of the diluent and culture media, dehydrated basic components or complete dehydrated media be used. Similarly, commercially prepared reagents may be used. The manufacturer's instructions shall be rigorously followed.

Chemical products shall be of recognized analytical quality.

The water used shall be distilled or deionized water, and shall be free from substances that might inhibit the growth of Enterobacteriaceae under the test conditions.

If the diluent and media are not used immediately, they shall, unless otherwise stated, be kept in the dark at a temperature between 0 and 5 °C, for no longer than 1 month, in conditions that prevent any change in their composition.

5.2 Diluent

See ISO 6887 and any specific standard dealing with the product to be examined.

5.3 Culture media

5.3.1 Buffered brilliant green bile glucose broth (E.E. broth)

Composition	a) Double-strength medium	b) Single-strength medium
Peptone	20,0 g	10,0 g
Glucose	10,0 g	5,0 g
Disodium hydrogenorthophosphate (Na_2HPO_4)	12,90 g	6,45 g
Potassium dihydrogenorthophosphate (KH_2PO_4)	4,0 g	2,0 g
Ox bile, dehydrated	40,0 g	20,0 g
Brilliant green	0,030 g	0,015 g
Water	1 000 ml	1 000 ml

Preparation

Dissolve the components or the complete dehydrated medium in the water by boiling. Do not heat the medium for longer than 30 min. Cool the medium rapidly.

Adjust the pH so that after boiling it is 7,2 at 25 °C.

Transfer 10 ml portions to sterile tubes or bottles (6.5).

1) The temperature should be agreed between the parties concerned and recorded in the test report.

Do not sterilize the medium.

The medium may be stored for up to 1 week at 0 to 5 °C.

5.3.2 Violet red bile glucose agar

Composition

Peptone	7,0	g
Yeast extract	3,0	g
Bile salts	1,5	g
Glucose	10,0	g
Sodium chloride	5,0	g
Neutral red	0,03	g
Crystal violet	0,002	g
Agar	8 to 18	g [1]
Water	1 000	ml

Preparation

Dissolve the components or the complete dehydrated medium in the water by boiling.

Adjust the pH so that after boiling it is 7,4 at 25 °C.

Transfer the culture medium to sterile tubes, flasks or bottles (6.5) of capacity not more than 500 ml.

Do not sterilize the medium.

Prepare this medium just before use (see 9.2.2 and 9.3.1).

Preparation of agar plates (required only for MPN technique, see 9.2.2)

Transfer immediately approximately 15 ml of the culture medium, cooled to approximately 45 °C, to Petri dishes (6.7), and allow to solidify.

Just before use, dry the plates, preferably with the lids off and the agar surface downwards, in the oven (6.3), maintained at 50 °C, for 30 min.

If prepared in advance, the undried plates shall not be kept for longer than 4 h at room temperature or 1 day at 0 to 5 °C.

5.3.3 Glucose agar

Composition

Tryptone	10,0	g
Yeast extract	1,5	g
Glucose	10,0	g
Sodium chloride	5,0	g
Bromocresol purple	0,015	g
Agar	8 to 18	g [1]
Water	1 000	ml

Preparation

Dissolve the components or the complete dehydrated medium in the water by boiling.

Adjust the pH so that after sterilization it is 7,0 at 25 °C.

Transfer the culture medium in quantities of 15 ml to tubes or bottles (6.5).

Sterilize the medium for 15 min at 121 ± 1 °C.

Leave the tubes or bottles in a vertical position.

The medium may be stored for up to 1 week at 0 to 5 °C.

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

5.3.4 Nutrient agar

Composition

Beef extract	3,0	g
Peptone	5,0	g
Agar	8 to 18	g [1]
Water	1 000	ml

Preparation

Dissolve the components or the complete dehydrated medium in the water by boiling.

Adjust the pH so that after sterilization it is 7,0 at 25 °C.

Transfer the culture medium to tubes, bottles or flasks (6.5) of capacity not more than 500 ml.

Sterilize the medium for 15 min at 121 ± 1 °C.

Preparation of agar plates (see 9.4.1)

Transfer portions of about 15 ml of the culture medium, melted and cooled to approximately 45 °C, to Petri dishes (6.7) and allow to solidify.

Just before use, dry the plates, preferably with the lids off and the agar surface downwards, in the oven (6.3), maintained at 50 °C, for 30 min.

If prepared in advance, the undried plates shall not be kept for longer than 4 h at room temperature or 1 day at 0 to 5 °C.

5.4 Oxidase reagent

Composition

N,N,N′,N′-tetramethyl-*p*-phenylene-diamine dihydrochloride	1,0 g
Water	100 ml

1) According to the manufacturer's instructions.

Preparation

Dissolve the reagent in the cold water.

Prepare the reagent just before use.

6 Apparatus and glassware

NOTE — Disposable apparatus is an acceptable alternative to reusable glassware if it has suitable specifications.

Usual microbiological laboratory equipment and in particular:

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

Apparatus that will come into contact with the diluent, culture media, or the sample, except for apparatus that is supplied sterile (in particular plastics apparatus), shall be sterilized by one of the following methods:

a) by being kept at 170 to 175 °C for not less than 1 h in an oven;

b) by being kept at 121 ± 1 °C for not less than 20 min in an autoclave.

6.2 Incubator, capable of being maintained at 35 ± 1 °C or at 37 ± 1 °C.

6.3 Drying cabinet, oven or **incubator,** capable of being maintained at 50 ± 5 °C, for drying the surfaces of agar plates.

6.4 Water-bath, capable of being maintained at 45 °C, for cooling the melted culture media.

6.5 Test-tubes, of dimensions approximately 16 mm × 160 mm and 20 mm × 200 mm, and **flasks** or **bottles,** for the sterilization and storage of the diluent and culture media.

6.6 Petri dishes, made of glass or plastics, of diameter 90 to 100 mm.

6.7 Loop, of diameter approximately 3 mm, and **wire,** of platinum-iridium or nickel-chromium, and/or a **glass rod.**

NOTE — A nickel-chromium loop is not suitable for use in the oxidase test (see 9.4.2.1).

6.8 Total delivery graduated pipettes, of nominal capacities 1 and 10 ml, graduated respectively in 0,1 ml and 0,5 ml divisions, and with an outflow opening of diameter 2 to 3 mm.

6.9 pH-meter, accurate to 0,1 pH unit at 25 °C.

7 Sampling

Carry out sampling in accordance with the specific standard appropriate to the product concerned. If no such specific standard exists, it is recommended that agreement be reached on this subject by the parties concerned.

8 Preparation of the test sample

See the specific standard appropriate to the product concerned. If no such specific standard exists, it is recommended that agreement be reached on this subject by the parties concerned.

9 Procedure

9.1 Test portion, initial suspension and dilutions

See ISO 6887 and any specific standard appropriate to the product concerned.

Prepare a single decimal dilution series from the test sample if the product is liquid, or from the initial suspension in the case of other products.

9.2 MPN technique

9.2.1 Inoculation and incubation

Take three tubes of double-strength medium [5.3.1 a)]. Transfer to each of these tubes, using a pipette (6.8), 10 ml of the test sample if the product is liquid, or 10 ml of the initial suspension in the case of other products.

Take three tubes of single-strength medium [5.3.1 b)]. Transfer to each of these tubes, using another pipette (6.8), 1 ml of the test sample if the product is liquid, or 1 ml of the initial suspension in the case of other products.

Take three more tubes of single-strength medium [5.3.1 b)]. Transfer to each of these tubes, using another pipette (6.8), 1 ml of the first decimal dilution (10^{-1}) of the test sample if the product is liquid, or 1 ml of the first decimal dilution of the initial suspension (10^{-2}) in the case of other products.

Incubate these nine tubes at 35 °C or 37 °C [1] for 24 h.

9.2.2 Isolation

Streak a loopful (6.7) from each of the nine incubated cultures (see 9.2.1) on the violet red bile glucose agar plates (see 5.3.2) and incubate the plates at 35 °C or 37 °C [1] for 24 h.

1) The temperature should be agreed between the parties concerned and recorded in the test report.

9.2.3 Selection of colonies for confirmation

From each of the plates incubated in 9.2.2 on which typical pink to red colonies (with or without precipitation haloes) or colourless, mucoid colonies have developed, select at random five such colonies for biochemical confirmation (see 9.4.2) after subculturing (see 9.4.1).

9.3 Colony count technique

9.3.1 Inoculation and incubation

9.3.1.1 Take two sterile Petri dishes (6.6). Using a sterile pipette (6.8), transfer to each dish 1 ml of the test sample if the product is liquid or 1 ml of the initial suspension in the case of other products.

Take a further two sterile Petri dishes. Using a fresh sterile pipette, transfer to each dish 1 ml of the first decimal dilution (10^{-1}) of the test sample if the product is liquid, or 1 ml of the first decimal dilution of the initial suspension (10^{-2}) in the case of other products.

Repeat this procedure with successive decimal dilutions, using a fresh sterile pipette for each decimal dilution.

9.3.1.2 Pour into each of the dishes approximately 15 ml of the violet red bile glucose agar medium (5.3.2) which has been melted and cooled to approximately 45 °C in the water-bath (6.4). The time elapsing between completion of the preparation of the initial suspension (or of the 10^{-1} dilution if the product is liquid) and the pouring of the medium (5.3.2) into the dishes shall not exceed 15 min.

Carefully mix the inoculum with the medium by horizontal movements of the plates and allow the mixture to solidify by placing the plates on a cool horizontal surface.

9.3.1.3 After solidification of the mixture, add a covering layer of 10 to 15 ml of the violet red bile glucose agar medium (5.3.2), melted and cooled as described in 9.3.1.2, to prevent spreading growth and to obtain semi-anaerobic conditions.

9.3.1.4 Allow the second layer to solidify. Incubate the plates, bottom uppermost, at 35 °C or 37 °C [1] for 24 h.

9.3.2 Counting and selection of colonies

Select, if possible, two plates (9.3.1.4) containing 15 to 150 typical colonies (see 9.2.3) of diameter 0,5 mm or more; count these suspect colonies. Select at random five such colonies from each plate for biochemical confirmation (see 9.4.2) after subculturing (see 9.4.1).

If two successive dilution levels gave plates containing 15 to 150 typical colonies, select the two plates inoculated with the greater amount of test sample.

Consider the determination to be void if half or more than half the surface area of a plate is overgrown. If less than half of the surface area of a plate is overgrown, count the colonies on the clear part and extrapolate so that the number corresponds to the total surface area of the plate.

9.4 Confirmation

9.4.1 Subculturing

Streak on nutrient agar plates (5.3.4) each of the colonies selected for confirmation (see 9.2.3 and 9.3.2).

Incubate these plates at 35 °C or 37 °C [1] for 24 h. Select a well isolated colony from each of the incubated plates for biochemical confirmation (see 9.4.2).

9.4.2 Biochemical confirmation

9.4.2.1 Oxidase reaction

Using the platinum-iridium loop or wire or glass rod (6.7), take a portion of each well isolated colony (9.4.1) and streak on a filter paper moistened with the oxidase reagent (5.4) or on a commercially available disc. A nickel-chromium loop or wire shall not be used.

Consider the test to be negative if the colour has not turned dark purple in 10 s.

9.4.2.2 Fermentation test

Stab, using a wire (6.7), the same colonies selected in 9.4.2.1 into tubes containing glucose agar (5.3.3). Incubate at 35 °C or 37 °C [1] for 24 h.

If a yellow colour develops throughout the contents of the tube, the reaction is regarded as positive. Most strains produce gas.

10 Expression of results

10.1 Calculation of the most probable number (MPN)

10.1.1 Count the number of positive tubes for each dilution.

10.1.2 If one of the selected typical colonies (9.2.3) of a subculture (see 9.4.1) is oxidase-negative and glucose-positive, the tube from which the subculture is derived shall be regarded as being positive.

10.1.3 Using the MPN table (see annex A), determine from the number of positive tubes in the different dilutions, the most probable number (MPN) index.

---

1) The temperature should be agreed between the parties concerned and recorded in the test report.

10.1.4 In the case of liquid products, the number of Enterobacteriaceae per millilitre is calculated by dividing the MPN index by 10. In the case of other products for which initial suspensions are prepared, the number per gram is equal to the MPN index.

10.2 Calculation of colony count

10.2.1 If at least 80 % of the selected typical colonies (see 9.3.2) are oxidase-negative and glucose-positive and thus confirmed as Enterobacteriaceae, the number of micro-organisms present will be the same as that given by the count made in 9.3.2.

10.2.2 In all other cases, the number shall be calculated from the percentage of oxidase-negative and glucose-positive colonies in relation to the total number of selected colonies (see 9.3.2).

10.2.3 Calculate the arithmetic mean of the number of Enterobacteriaceae colonies counted on the duplicate plates in 9.3.2.

10.2.4 Retain only two significant figures, rounding the result as follows:

a) if the number is less than 100, round it to the nearest multiple of 5;

b) if the number is more than 100 and does not end with a 5, round it to the nearest multiple of 10;

c) if the number is more than 100 and ends with a 5, round it to the nearest multiple of 20.

10.2.5 Calculate the number of Enterobacteriaceae per millilitre (in the case of liquid products) or per gram (in the case of other products) by multiplying the number obtained according to 10.2.4 by the dilution factor.

10.2.6 Express the result as a number between 1,0 and 9,9 multiplied by the appropriate power of 10.

In the event of finding less than 15 typical Enterobacteriaceae colonies per plate, express the result as calculated (in 10.2.3 and 10.2.4), noting that the 95 % confidence limits (annex B) widen for smaller numbers of colonies, but that they are still better than those for corresponding MPN counts (see annex A).

10.3 Precision of the method

It is known that wide variations in results may occur with the MPN technique. Results obtained from this method should therefore be used with caution. Confidence limits are given in annex A.

For statistical reasons alone, in 95 % of cases the confidence limits of the colony count technique vary from ±16 % to ±52 % [1]; for colony counts of less than 15 per plate, the confidence limits are given in annex B. In practice, even greater variation may be found, especially among results obtained by different workers.

11 Test report

The test report shall show the method used, the temperature of incubation and the results obtained. It shall also mention any operating details not specified in this International Standard, or regarded as optional, together with details of any incidents likely to have influenced the results.

The test report shall include all the information necessary for the complete identification of the sample.

1) See COWELL and MORISETTI, *J. Sci. Fd. Agric.* **20** : 573.

Annex A

Determination of most probable number

Table 1 — MPN indexes and confidence limits[1)]

Number of positive results			MPN index	Category[2)] when number of tests is 1	2	3	5	10	Confidence limits[3)] ⩾95 %		⩾99 %	
0	0	0	<0,30						0,00	0,94	0,00	1,40
0	0	1	0,30	3	2	2	2	1	0,01	0,95	0,00	1,40
0	1	0	0,30	2	1	1	1	1	0,01	1,00	0,00	1,60
0	1	1	0,61	0	3	3	3	3	0,12	1,70	0,05	2,50
0	2	0	0,62	3	2	2	2	1	0,12	1,70	0,05	2,50
0	3	0	0,94	0	0	0	0	3	0,35	3,50	0,18	4,60
1	0	0	0,36	1	1	1	1	1	0,02	1,70	0,01	2,50
1	0	1	0,72	2	2	1	1	1	0,12	1,70	0,05	2,50
1	0	2	1,1	0	0	0	3	3	0,4	3,5	0,2	4,6
1	1	0	0,74	1	1	1	1	1	0,13	2,00	0,06	2,70
1	1	1	1,1	3	3	2	2	2	0,4	3,5	0,2	4,6
1	2	0	1,1	2	2	1	1	1	0,4	3,5	0,2	4,6
1	2	1	1,5	3	3	3	3	2	0,5	3,8	0,2	5,2
1	3	0	1,6	3	3	3	3	2	0,5	3,8	0,2	5,2
2	0	0	0,92	1	1	1	1	1	0,15	3,50	0,07	4,60
2	0	1	1,4	2	1	1	1	1	0,4	3,5	0,2	4,6
2	0	2	2,0	0	3	3	3	3	0,5	3,8	0,2	5,2
2	1	0	1,5	1	1	1	1	1	0,4	3,8	0,2	5,2
2	1	1	2,0	2	2	1	1	1	0,5	3,8	0,2	5,2
2	1	2	2,7	0	3	3	3	3	0,9	9,4	0,5	14,2
2	2	0	2,1	1	1	1	1	1	0,5	4,0	0,2	5,6
2	2	1	2,8	3	2	2	2	1	0,9	9,4	0,5	14,2
2	2	2	3,5	0	0	0	0	3	0,9	9,4	0,5	14,2
2	3	0	2,9	3	2	2	2	1	0,9	9,4	0,5	14,2
2	3	1	3,6	0	3	3	3	3	0,9	9,4	0,5	14,2
3	0	0	2,3	1	1	1	1	1	0,5	9,4	0,3	14,2
3	0	1	3,8	1	1	1	1	1	0,9	10,4	0,5	15,7
3	0	2	6,4	3	3	2	2	2	1,6	18,1	1,0	25,0
3	1	0	4,3	1	1	1	1	1	0,9	18,1	0,5	25,0
3	1	1	7,5	1	1	1	1	1	1,7	19,9	1,1	27,0
3	1	2	12	3	2	2	2	1	3	36	2	44
3	1	3	16	0	0	0	3	3	3	38	2	52
3	2	0	9,3	1	1	1	1	1	1,8	36,0	1,2	43,0
3	2	1	15	1	1	1	1	1	3	38	2	52
3	2	2	21	2	1	1	1	1	3	40	2	56
3	2	3	29	3	3	3	2	2	9	99	5	152
3	3	0	24	1	1	1	1	1	4	99	3	152
3	3	1	46	1	1	1	1	1	9	198	5	283
3	3	2	110	1	1	1	1	1	20	400	10	570
3	3	3	>110									

1) From De Man, J.C. *Eur. J. Appl. Microbiol. Biotechnol.* **17** 1983 : 301-305.

2) See table 2.

3) The confidence limits given in table 1 are meant only to provide some idea about the influence of statistical variations on results. There will always also be other sources of variation, which may sometimes be even more important.