INTERNATIONAL STANDARD

ISO 9232

IDF 146

First edition
2003-02-01

Yogurt — Identification of characteristic microorganisms (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*)

*Yaourt — Identification des micro-organismes caractéristiques (*Lactobacillus delbrueckii *subsp.* bulgaricus *et* Streptococcus thermophilus*)*

Reference numbers
ISO 9232:2003(E)
IDF 146:2003(E)

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Published in Switzerland

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Foreword

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ISO 9232 | IDF 146 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF), in collaboration with AOAC International. It is being published jointly by ISO and IDF and separately by AOAC International.

Foreword

IDF (the International Dairy Federation) is a worldwide federation of the dairy sector with a National Committee in every member country. Every National Committee has the right to be represented on the IDF Standing Committees carrying out the technical work. IDF collaborates with ISO and AOAC International in the development of standard methods of analysis and sampling for milk and milk products.

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 National Committees casting a vote.

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All work was carried out by the Joint ISO/IDF/AOAC Action Team, *Lactic acid bacteria and starters,* of the Standing Committee on *Microbiological methods of analysis*, under the aegis of its project leader, Prof. B. Bianchi Salvadori (IT).

Yogurt — Identification of characteristic microorganisms (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*)

1 Scope

This International Standard specifies tests for the identification of the characteristic microorganisms in yogurt on the basis of their morphological, cultural and physiological properties.

It is applicable to strains isolated from yogurts in which both characteristic microorganisms are present and viable.

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 6887-1, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions*

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

ISO 7889|IDF 117:2002, *Yogurt — Enumeration of characteristic microorganisms — Colony-count technique at 37 °C*

ISO 8261|IDF 122, *Milk and milk products — General guidance for the preparation of test samples, initial suspensions and decimal dilutions for microbiological examination*

3 Terms and definitions

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

3.1
characteristic microorganisms in yogurt
Lactobacillus delbrueckii subsp. *bulgaricus* and *Streptococcus thermophilus*

4 Principle

4.1 The morphological, cultural and biochemical characteristics of *L. delbrueckii* subsp. *bulgaricus* are determined.

4.2 The morphological, cultural and biochemical characteristics of *S. thermophilus* are determined.

5 Culture media, diluents and reagents

Use only reagents of recognized analytical grade, unless otherwise specified, and glass-distilled or demineralized water or water of equivalent purity. The water used for the preparation of the enzyme solutions should be at least double glass-distilled. See also ISO 6887-1 and ISO 8261|IDF 122. For other materials, see ISO 7889|IDF 117.

5.1 Culture media

Use only freshly prepared culture media which shall not be exposed to direct sunlight. If the prepared culture media are not used immediately, they shall, unless otherwise specified, be cooled and stored at between 2 °C and 4 °C for no longer than 1 week and under conditions which do not produce any change in their composition. As for reagents, see storage conditions in ISO 7218.

5.1.1 Skimmed milk

5.1.1.1 Composition

Low-heat-treated, spray-dried skimmed milk, free from growth inhibitors	100 g
Water up to	1 000 ml

5.1.1.2 Preparation

Dissolve the dried milk in the water. Distribute 10 ml portions of the obtained solution in test tubes of 16 mm × 160 mm (6.5). Sterilize in an autoclave at 110 °C ± 1 °C for 30 min or at 115 °C ± 1 °C for 20 min. After sterilization and before use, check the sterility by incubating the test tubes in the incubator (6.1) set at 37 °C for 3 days.

5.1.2 MRS broth

5.1.2.1 Composition

Peptone 1(tryptic digest of casein)	10,00 g
Meat extract	10,00 g
Yeast extract (dried)	5,00 g
Glucose ($C_6H_{12}O_6$)	20,00 g
Tween 80 (sorbitan mono-oleate)	1,00 ml
Dipotassium hydrogen phosphate (K_2HPO_4)	2,00 g
Sodium acetate trihydrate ($CH_3CO_2Na{\cdot}3H_2O$)	5,00 g
Diammonium citrate [$C_6H_6O_7(NH_4)_2$]	2,00 g
Magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$)	0,20 g
Manganese sulfate tetrahydrate ($MnSO_4{\cdot}4H_2O$)	0,05 g
Water up to	1 000 ml

5.1.2.2 Preparation

Separately dissolve each component in the already boiling water. Cool in a water bath (6.8) to 50 °C. Adjust the pH so that after sterilization it is 6,5 ± 0,2 at 25 °C ± 1 °C, by using a reagent (5.2) and checking with the pH-meter (6.4).

Distribute 20 ml portions of the obtained medium in test tubes of 20 mm × 200 mm (6.5). Sterilize in an autoclave at 121 °C ± 1 °C for 15 min.

NOTE When using commercially available MRS media, the obtained results may differ significantly from one supplier to the other. Therefore, always check commercially MRS medium against the medium prepared as described above.

5.1.3 Basic medium for fermentation tests

5.1.3.1 Composition

Use the composition as described in 5.1.2.1 for the MRS broth, but omitting the meat extract and the glucose component.

5.1.3.2 Preparation

Prepare the basic medium as described in 5.1.2.2 for the MRS broth, using the components described in 5.1.3.1 and adjusting the pH so that after sterilization it is 6,95 ± 0,05 instead of pH 6,5 at 25 °C ± 1 °C.

5.1.4 Culture medium for production of CO_2

5.1.4.1 Composition

Use the composition as described in 5.1.2.1 for the MRS broth, but omitting the meat extract component and replacing the 20 g of glucose with 50 g of glucose.

5.1.4.2 Preparation

Prepare the culture medium as described in 5.1.2.2 for the MRS broth using the components described in 5.1.4.1 and adjusting the pH so that after sterilization it is 6,95 ± 0,05 instead of pH 6,5 at 25 °C ± 1 °C.

Distribute 10 ml portions instead of 20 ml (as described in 5.1.2.2) of the obtained medium in the test tubes of 16 mm × 160 mm (6.5). Sterilize in an autoclave at 121 °C ± 1 °C for 15 min.

5.1.5 Overlay agar

5.1.5.1 Composition

Bacteriological agar	20 g
Water up to	1 000 ml

5.1.5.2 Preparation

Dissolve the agar in the water. Distribute 10 ml portions in tubes of 16 mm × 160 mm (6.5). Sterilize in an autoclave at 121 °C ± 1 °C for 15 min.

5.1.6 Litmus milk

5.1.6.1 Composition

Litmus powder	0,70 g
Skimmed milk (5.1.1) up to	1 000 ml

5.1.6.2 Preparation

Prepare the litmus milk as described in 5.1.1.2 for the skimmed milk, using the components as described in 5.1.6.1.

NOTE Litmus powder or skimmed milk with litmus is commercially available.

5.1.7 M17 broth

5.1.7.1 Basic medium

5.1.7.1.1 Composition

Peptone 1 (tryptic digest of casein)	2,50 g
Peptone 2 (peptic digest of meat)	2,50 g
Peptone 3 (papain digest of soya)	5,00 g
Yeast extract (dried)	2,50 g
Meat extract	5,00 g
β-Glycerophosphate (disodium salt) ($C_3H_7O_6PNa_2$)	19,00 g
Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	0,25 g
Ascorbic acid ($C_6H_8O_6$)	0,50 g
Water up to	950 ml

5.1.7.1.2 Preparation

Separately dissolve the components in the boiling water. Cool on a water bath (6.8) to 50 °C. Adjust the pH so that after sterilization it is 6,8 ± 0,2 at 25 °C ± 1 °C by using a reagents (5.2) and checking with the pH-meter (6.4).

Distribute 19 ml portions of the obtained medium in test tubes of 20 mm × 200 mm (6.5). Sterilize for 15 min in an autoclave at 121 °C ± 1 °C.

5.1.7.2 Lactose solution

5.1.7.2.1 Composition

Lactose ($C_{12}H_{22}O_{11}$)	10 g
Water up to	100 ml

5.1.7.2.2 Preparation

Dissolve the lactose in the water. Sterilize for 15 min in an autoclave at 121 °C ± 1 °C.

5.1.7.3 Complete medium

5.1.7.3.1 Composition

Lactose solution (5.1.7.2)	1 ml
Basic medium (5.1.7.1)	19 ml

5.1.7.3.2 Preparation

Immediately before use, add the lactose solution to the test tubes with the basic medium (5.1.7.1). Mix by swirling.

NOTE When using commercially available M17 media, the obtained results may differ significantly from one supplier to the other. Therefore, always check commercially M17 medium against the medium prepared as described above.

5.1.8 Culture medium for growth in presence of 6,5 % NaCl

5.1.8.1 Composition

Use the composition as described in 5.1.7.1.1 for the M17 broth, but replacing the 19 g of β-glycerophosphate component with 65 g of sodium chloride (NaCl).

5.1.8.2 Preparation

Prepare the culture medium as described in 5.1.7.1.2 for the M17 broth but distributing 10 ml instead of 19 ml (as described in 5.1.7.1.2) of the obtained medium in the test tubes of 16 mm × 160 mm (6.5). Sterilize for 15 min in an autoclave at 121 °C ± 1 °C.

5.2 Reagents for adjustment of pH

5.2.1 Sodium hydroxide solution, c(NaOH) = 0,1 mol/l approximately.

5.2.2 Hydrochloric acid solution, c(HCl) = 0,1 mol/l approximately.

5.3 Reagent for staining, ethanolic solution of methylene blue, 6 g/l.

5.4 Reagent for catalase reaction, hydrogen peroxide (H_2O_2), 1,5 % (volume fraction).

6 Apparatus and glassware

Sterilization of equipment that will come into contact with the test sample or the culture medium shall be carried out in accordance with the requirements of ISO 8261 | IDF 122. The glassware shall be resistant to repeated sterilization.

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

6.1 Incubators, capable of operating at 10 °C ± 1 °C, at 15 °C ± 1 °C, at 37 °C ± 1 °C and at 45 °C ± 1 °C.

6.2 Test tube agitator, for example a vortex mixer.

6.3 Magnifying lens, magnification × 8 to × 10.

6.4 pH-meter, with temperature compensation, accurate to ± 0,1 pH unit at 25 °C ± 1 °C (see also ISO 7218).

6.5 Test tubes, with rubber stoppers or caps, of diameter and length 16 mm × 160 mm and 20 mm × 200 mm, to hold the culture medium.

6.6 Graduated pipettes, for bacteriological use, sterilized and calibrated to the tip, capable of delivering 1 ml ± 0,02 ml and 10 ml ± 0,2 ml (see ISO 6887-1).

Presterilized pipettes made of synthetic materials may be used instead of glass pipettes.

6.7 Glass rod.

6.8 Water baths, capable of operating at 10 °C ± 1 °C, at between 44 °C and 47 °C, at 45 °C ± 1 °C, at 50 °C ± 1 °C, and capable of boiling.

7 Procedure

7.1 Isolation of colonies

Select colonies from the plates used for counting, as obtained according to ISO 7889|IDF 117, by using a magnifying lens (6.3), if needed. Inoculate the stipulated broths to obtain pure cultures after incubation. Incubate in the incubator (6.1) set at 37 °C for 24 h, under the atmospheric conditions described in ISO 7889|IDF 117.

7.2 Phenotypic characteristics required for identification of *L. delbrueckii* subsp. *bulgaricus*

7.2.1 Culture media

7.2.1.1 Use skimmed milk (5.1.1) and MRS broth (5.1.2) for routine cultures and physiological tests, as described below.

7.2.1.2 For fermentation tests, use a commercially manufactured kit, having shown it is appropriate for this purpose with the microorganisms under investigation. Follow the manufacturer's instructions precisely.

NOTE Basic medium for fermentation tests (5.1.3) is supplied with the diagnostic kit and is used to prepare the colony inoculum.

7.2.2 Characteristics to be considered

7.2.2.1 Morphology

Use freshly prepared 24-h pure cultures grown in skimmed milk (5.1.1) in an incubator (6.1) set at 37 °C for 24 h. Stain smears of cultures with methylene blue (5.3) for a few minutes before making a microscope examination. For shape and cell arrangement, refer to Table A.1 in Annex A. Volutin granules should be visible within the cells.

7.2.2.2 Catalase reaction

Mix equal volumes of the MRS broth culture (see 7.1), incubated in the incubator (6.1) set at 37 °C for 18 h to 24 h, with 1,5 % hydrogen peroxide in a test tube (6.5) fitted with a rubber stopper. Prepare a non-inoculated control broth at the same time.

Gently turn the tubes upside down once to favour mixing, and observe for bubbles of oxygen forming in the broth at room temperature over 20 min. *L. delbrueckii* subsp. *bulgaricus* will not produce oxygen. The test is negative if gas is seen in the control tube.

7.2.2.3 Growth in the broth at 15 °C and 45 °C

Use a drop of culture of the test strain, incubated in the MRS broth (see 7.1) in the incubator (6.1) set at 37 °C for 18 h to 24 h, to inoculate two fresh MRS broths (5.1.2.2). One broth should previously have been brought in a water bath (6.8) to 15 °C and the other in another water bath to 45 °C. Incubate one in an incubator (6.1) set at 15 °C and the other in an incubator set at 45 °C for up to 7 days. Observe for turbidity. *L. delbrueckii* subsp. *bulgaricus* does not grow at 15 °C, but does grow at 45 °C giving turbidity.

7.2.2.4 Production of CO_2

Inoculate 10 ml of culture medium (5.1.4) with 0,1 ml of an MRS broth culture of the test strain (see 7.1) incubated in an incubator (6.1) set at 37 °C for 18 h to 24 h. Do not allow the culture to contaminate the inside of the tube above the broth. Cover the surface of the broth with molten overlay agar (5.1.5), precooled to 47 °C ± 1 °C, to a depth of 1 cm. Incubate for 1 week in an incubator (6.1) set at 37 °C. The presence of gas is evident when the agar layer detaches itself from the underlying contents. Under these conditions, *L. delbrueckii* subsp. *bulgaricus* does not produce gas.

7.2.2.5 Fermentation of sugars

For the preparation of the inoculum and reading of the results, follow the directions supplied with the diagnostic kit.

7.2.2.6 Determination of lactic acid enantiomers (optical isomers) in milk cultures

7.2.2.6.1 General

When commercially available diagnostic kits are used, follow the manufacturer's instructions.

7.2.2.6.2 Preparation

Successively subculture twice the pure culture of the test strain in autoclaved skimmed milk (5.1.1) by incubating it in the incubator (6.1) set at 37 °C until the culture has clotted (about 16 h). Examine the culture microscopically for purity using a methylene blue (5.3) stained smear (see ISO 7889|IDF 117:2002, 9.2). Subculture a third time in autoclaved skimmed milk (5.1.1) using a 0,1 ml inoculum. Incubate again in the incubator set at 37 °C for 48 h. After a further microscopic test for purity, homogenize the contents of the test tube with the aid of a glass rod (6.7). Under these conditions, *L. delbrueckii* subsp. *bulgaricus* produces over 95 % D(–) lactic acid. Therefore, determine the contents of lactic acid and lactate enantiomers in the sample cultures according to the method specified in Annex B.

7.2.2.6.3 Calculation

Calculate the D(–) lactic acid content of the sample, using the following equation:

$$w_{\mathrm{D(-)}} = \frac{C_{\mathrm{D(-)}}}{C_{\mathrm{D(-)}} + C_{\mathrm{L(+)}}} \times 100\ \%$$

where

$w_{\mathrm{D(-)}}$ is the mass percentage of the D(–) form in the total lactic acid content of the sample;

$C_{\mathrm{D(-)}}$ is the numerical value of the mass fraction of D(–) lactic acid, in percent;

$C_{\mathrm{L(+)}}$ is the numerical value of the mass fraction of L(+) lactic acid, in percent.

7.2.2.6.4 Expression of results

Express the obtained results to two decimal places.

7.3 Phenotypic characteristics required for identification of *S. thermophilus*

7.3.1 Culture media

For routine cultures and physiological tests, use autoclaved litmus milk (5.1.6.2) and M17 broth (5.1.7.1.2).

7.3.2 Characteristics to be considered

7.3.2.1 Morphology

Use freshly prepared litmus milk cultures (5.1.6.2). Stain smears of cultures with methylene blue (5.3) for a few minutes before making a microscopic examination. For shape and cell arrangement, refer to Table A.2.

7.3.2.2 Catalase reaction

See 7.2.2.2.

Under these conditions, *S. thermophilus* will not produce oxygen. See Table A.2.

7.3.2.3 Growth in litmus milk at 10 °C and 45 °C

Inoculate the test tubes containing litmus milk (5.1.6.2) with a drop of M17 broth culture (see 7.1), pre-incubated overnight in an incubator (6.1) set at 37 °C, of the strains to be tested. Keep the relevant tubes in an incubator set at 10 °C and the others in another incubator set at 45 °C for up to 7 days. At a temperature of 10 °C ± 1 °C *S. thermophilus* does not change litmus milk, but it does at a temperature of 45 °C ± 1 °C according to the pattern ACr (7.3.2.4) (see Table A.2).

7.3.2.4 Action in litmus milk (ACr = acidification, coagulation, reduction)

Litmus milk acidified by *S. thermophilus* turns pink and then coagulates. After coagulation the colour remains pink due to very slow and often incomplete reduction of litmus, with a more intensely coloured upper ring (see Table A.2).

7.3.2.5 Growth in the presence of sodium chloride

Inoculate the test tubes containing culture medium (5.1.8.2) with a drop of M17 broth culture (see 7.1), pre-incubated overnight in an incubator set at 37 °C, of the strains to be tested. Incubate for up to 7 days in an incubator (6.2) set at 37 °C. Under these conditions, no turbidity will develop with *S. thermophilus* (see Table A.2).

7.3.2.6 Utilization of sugars

Table A.1 indicates the most valuable sugars to distinguish *L. delbrueckii* subsp. *bulgaricus* from other species belonging to the taxonomic group of the obligatorily homofermentative lactobacilli. Examine the inoculated commercial test strip for sugar reactions according to the manufacturer's instructions.

8 Expression of results

8.1 Compare the results of the tests described in 7.2 with the main attributes of *L. delbrueckii* subsp. *bulgaricus* given in Table A.1.

8.2 Compare the results of the tests described in 7.3 with the main attributes of *S. thermophilus* given in Table A.2.

9 Test report

The test report shall specify:

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

b) the sampling method used, if known;

c) the test method used, with reference to this International Standard;

d) all operating details not specified in this International Standard, or regarded as optional, together with details of any incident which may have influenced the result(s);

e) the test result(s) obtained.

Annex A
(normative)

Main attributes tables

Table A.1 — Main attributes of *Lactobacillus delbrueckii* subsp. *bulgaricus*, *L. delbrueckii* subsp. *lactis*, *L. helveticus* and *L. acidophilus*

Attributes	*L. delbrueckii* subsp. *bulgaricus*	*L. delbrueckii* subsp. *lactis*	*L. helveticus*	*L. acidophilus*
Morphology	Rods with round ends. Variable according to age, medium, strain. Numerous metachromatic bodies in cells of old culture stained with methylene blue.	As for *L. delbrueckii* subsp. *bulgaricus.*	Polymorphism of old cultures not so marked as with *L. delbrueckii* subsp. *lactis* and *L. delbrueckii* subsp. *bulgaricus*. No metachromatic bodies.	Rods with round ends, singly, in pairs or short chains. No metachromatic bodies.
Catalase reaction	–	–	–	–
CO_2 production from glucose	–	–	–	–
Growth at 15 °C ± 1 °C	–	–	–	–
Growth at 45 °C ± 1 °C	+	+	+	+
Fermented sugars				
— fructose	+	+	+/–	+
— galactose	–	+/–	+	+
— glucose	+	+	+	+
— lactose	+	+	+	+
— maltose	–	+	+/–	+
— mannose	+/–	+	+/–	+
— sucrose	–	+	–	+
— trehalose	–	+	+/–	+/–
— gluconate	–	–	–	–
— cellobiose	–	–	–	+
— esculine	–	–	–	+
Lactic acid enantiomers	D(–)	D(–)	DL	DL

\+ = positive reaction for 90 % or more strains.

– = negative reaction for 90 % or more strains.

+/– = variable reaction, weak, slow or negative.

These sugar fermentation patterns refer to results achievable with a commercially available diagnostic kit for the identification of lactic acid bacteria. See Annex B.

Table A.2 — Main attributes of *S. thermophilus*

Attributes	Appearance	Remarks
Morphology	Spherical or ovoid cells in pairs or long chains, strong polymorphism in old cells	
Catalase reaction	–	
Growth at 10 °C ± 1 °C	–	Lactococci grow at 10 °C.
Growth at 45 °C ± 1 °C	+	Lactococci do not grow at 45 °C. Group D streptococci grow at both 10 °C and 45 °C.
Reaction on litmus milk — rapid acidification — coagulation — very slow and often incomplete	A C	Lactococci and Group D streptococci react RAC.
Reduction of litmus	R	
Growth in presence of NaCl (6,5 %)	–	*S. thermophilus* is very sensitive to sodium chloride. Group D streptococci can grow in the presence of 6,5 % NaCl.
+ = positive reaction for 90 % or more strains. – = reaction always negative.		

Annex B
(normative)

Milk cultures of lactic acid bacteria — Determination of the contents of lactic acid and lactate enantiomers

B.1 General

This annex specifies an enzymatic method for the determination of the enantiomers (optical isomers) of lactic acid and lactates. The method is applicable to skimmed milk cultures of lactobacilli isolated from yogurt.

NOTE This method was elaborated on the basis of the results of a ring test, where instead of single biochemical reagents (B.4.6, B.4.7, B.4.8, B.4.9) a suitable test combination available commercially was used.

B.2 Terms and definitions

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

B.2.1
contents of lactic acid and lactates enantiomers in milk cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus*
mass fractions of substances determined by the procedure specified in this annex

NOTE They are expressed as mass fractions in percent.

B.3 Principle

B.3.1 Sample cultures are prepared in autoclaved skimmed milk. A suitable dilution of samples is then prepared and the fat and proteins are precipitated, followed by filtration.The filtrate is treated with the following enzymes and biochemical substances, added simultaneously but acting in sequence:

a) L-lactate dehydrogenase (L-LDH, EC 1.1.1.27) or D-lactate dehydrogenase (D-LDH, EC 1.1.1.28) in the presence of nicotinamide adenine dinucleotide (NAD) to oxidize lactate to pyruvate and to convert NAD to its reduced form (NADH);

b) glutamate-pyruvate transaminase (GPT, EC 2.6.1.2) in the presence of L-glutamate to transform pyruvate into L-alanine and to convert L-glutamate to α-ketoglutarate.

B.3.2 The content of NADH is spectrometrically determined by reading the absorbance of the test solution at 340 nm.

B.3.3 The contents of lactic acid and lactate enantiomers are stoichiometric with the amount of NADH according to the following reactions:

$$\text{L/D-lactate} + \text{NAD} \rightleftarrows \text{pyruvate} + \text{NADH} + \text{H}^+$$

$$\text{pyruvate} + \text{L-glutamate} + \text{GPT} \rightleftarrows \text{L-alanine} + \alpha\text{-ketoglutarate}$$

B.4 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified, and glass-distilled or demineralized water or water of equivalent purity. The water used for the preparation of the enzyme solutions shall be at least double glass-distilled. See also ISO 6887-1 and ISO 8261 | IDF 122.

B.4.1 Potassium hexacyanoferrate(II) solution

Dissolve 3,6 g of potassium hexacyanoferrate(II) trihydrate ($K_4[Fe(CN)_6]\cdot 3H_2O$) in water in a 100 ml one-mark volumetric flask (B.5.5). Dilute to the mark with water and mix.

B.4.2 Zinc sulfate solution

Dissolve 7,2 g of zinc sulfate heptahydrate ($ZnSO_4\cdot 7H_2O$) in water in a 100 ml one-mark volumetric flask (B.5.5). Dilute to the mark with water and mix.

B.4.3 Sodium hydroxide solution, c(NaOH) = 0,1 mol/l.

Dissolve 0,4 g of sodium hydroxide in water in a 100 ml one-mark volumetric flask (B.5.5). Dilute to the mark with water and mix.

B.4.4 Sodium hydroxide solution, c(NaOH) = 10 mol/l.

Dissolve 40 g of sodium hydroxide (NaOH) in water in a 100 ml one-mark volumetric flask (B.5.5). Dilute to the mark with water and mix.

B.4.5 Buffer solution with pH 10,0: c(glycylglycine) = 0,6 mol/l; c(L-glutamate) = 0,1 mol/l.

Dissolve 4,75 g of glycylglycine and 0,88 g of L-glutamic acid in about 50 ml of water in a conical flask (B.5.4). Adjust to pH 10,0 with about 4,6 ml of 10 mol/l sodium hydroxide solution (B.4.4) and dilute to 60 ml with water. The solution is stable for at least 3 months at +4 °C.

B.4.6 Nicotinamide-adenine dinucleotide solution, c(NAD) = 47 mmol/l approximately.

Dissolve 420 mg of NAD in 12 ml of water in a conical flask (B.5.4). The solution is stable at +4 °C for 3 weeks.

B.4.7 Glutamate-pyruvate transaminase, c(GPT) = 20 mg/ml.

Centrifuge 2 ml of the GPT suspension in the centrifuge (B.5.1) set at a rotational frequency of about 4 000 min^{-1} for 10 min. Suck off 1,0 ml of the clear supernatant and discard. The thus-obtained GPT suspension is stable at +4 °C for at least 1 year.

B.4.8 L-lactate dehydrogenase solution, c(L-LDH) = 10 mg/ml.

Use the L-lactate dehydrogenase solution undiluted. The solution is stable at +4 °C for at least 1 year.

B.4.9 D-lactate dehydrogenase solution, c(D-LDH) = 5 mg/ml.

Use the D-lactate dehydrogenase solution undiluted. The solution is stable at +4 °C for at least 1 year.

B.5 Apparatus

Usual laboratory equipment and, in particular, the following.

B.5.1 Centrifuge, capable of operating at a rotational frequency of 4 000 min^{-1}.

B.5.2 Analytical balance, capable of weighing to the nearest 10 mg.

B.5.3 Test tubes, with rubber stoppers or caps of diameter and length 20 mm × 200 mm.

B.5.4 Conical flasks, of capacities 25 ml, 50 ml or 150 ml.

B.5.5 One-mark volumetric flasks, of capacity 100 ml.

B.5.6 Pipettes, capable of delivering 5 ml, 1 ml, 0,2 ml, 0,05 ml and 0,02 ml.

B.5.7 Graduated pipettes, of capacity 5 ml, with graduation 0,1 ml.

B.5.8 Filter funnels, of diameter about 7 cm.

B.5.9 Pleated filter papers, medium grade, of diameter about 15 cm, free from lactic acid and lactates.

B.5.10 Glass rods.

B.5.11 Plastic paddles, suitable for mixing the contents of spectrometric cells.

B.5.12 Spectrometer, capable of operating at 340 nm.

B.5.13 Disposable UV-grade cells, with optical path length of 10 mm.

B.6 Preparation of test sample

B.6.1 Prepare sample cultures according to 7.2.2.6.

B.6.2 Prepare a “sample blank” by inoculating with 1 % sterile water a test tube of autoclaved skimmed milk prepared according to 5.1.1. Incubate in the incubator (6.1) set at 37 °C for 48 h.

B.6.3 Store prepared sample cultures (B.6.1) refrigerated or deep-frozen until analysis.

B.7 Procedure

CAUTION — Avoid contamination, especially with perspiration since this contains L(+) lactic acid. Care should be taken not to touch the tips of pipettes, plastic rods, filter paper, etc. The use of plastic gloves is recommended.

B.7.1 Test portion

Weigh, to the nearest 10 mg, 2,00 g of the test sample culture (B.6.3) in a volumetric flask (B.5.5)

B.7.2 Blank test

Carry out a blank test on the “sample blank” as prepared in B.6.2 on each fresh lot of skimmed milk powder used. Proceed as specified in B.7.1, B.7.3 and B.7.4 using all the reagents but omitting the test portion.

Use the obtained figure in the calculation (see B.8).

B.7.3 Deproteination

B.7.3.1 Add to the test portion (B.7.1), 50 ml of water and, in the following order, 5,0 ml of the potassium hexacyanoferrate(II) solution (B.4.1), 5,0 ml of the zinc sulfate solution (B.4.2) and 10,0 ml of the sodium hydroxide solution (B.4.3), swirling thoroughly after each addition. Dilute to the 100 ml mark with water and mix. Allow the mixture to stand for 30 min.

B.7.3.2 Filter over filter paper (B.5.9), discarding the first few millilitres. A slightly opalescent filtrate may be used for the test.

B.7.4 Determination

B.7.4.1 General

The amount of L(+) or D(−) lactic acid and lactates present in the cell (B.5.13) should range between 2 µg and 20 µg. The filtrates, therefore, should be sufficiently diluted to yield sample solutions with an L(+) or D(−) enantiomer concentration of between 0,02 g/l and 0,2 g/l.

B.7.4.2 Determination of L(+) lactic acid and lactates

Pipette into each disposable UV-grade cell (B.5.13):

Buffer (B.4.5)	1,00 ml
NAD (B.4.6)	0,20 ml
GPT (B.4.7)	0,02 ml
Water	0,90 ml

Then, at regular time intervals (e.g. 30 s), add 0,10 ml of water (blank) or sample blank (B.6.2), or test sample cultures (B.6.1). Mix thoroughly with plastic paddles (B.5.11).

Read the absorbance of each solution against air (A_1) after 5 min. Start the enzymatic reaction by adding 0,02 ml of L-LDH (B.4.9) to each cell at regular time intervals (e.g. 30 s). Mix thoroughly. On completion of the reaction (after 10 min), read the absorbance of each solution (A_2) again.

Calculate the absorbance differences ($A_2 - A_1$) for blank (ΔA_b), test sample blank (ΔA_{sb}) (B.6.2) and test samples (ΔA_s) (B.6.1).

Subtract the absorbance difference of the blank from the absorbance difference of each sample and sample blank as follows:

$$A_{L(+)} = \Delta A_s - \Delta A_b$$

$$A_{sb} = \Delta A_{sb} - \Delta A_b$$

B.7.4.3 Determination of D(–) lactic acid and lactates

Pipette the same amounts of components following the procedure in B.7.4.2 up to mixing with plastic paddles.

Read the absorbance of each solution against air (A_1) after 5 min. Start the enzymatic reaction by adding 0,05 ml of D-LDH (B.4.9) to each cell at regular time intervals (e.g. 30 s). Mix thoroughly. On completion of the reaction (after 30 min), read the absorbance of each solution (A_2) once again.

Calculate the absorbance differences ($A_2 - A_1$) for blank (ΔA_b), sample blank (ΔA_{sb}) and test samples (ΔA_s).

Subtract the absorbance difference of the blank from the absorbance difference of each test sample and sample blank as follows:

$$A_{D(-)} = \Delta A_s - \Delta A_b$$

$$A_{sb} = \Delta A_{sb} - \Delta A_b$$

B.8 Calculation and expression of results

B.8.1 Calculation

Calculate the concentration, C, of L(+) or D(–) lactic acid in the culture, using the following equation:

$$C = \frac{V_1 \times M_r \times d \times 100}{m \times \varepsilon \times l_p \times V_2 \times 10\,000} \times A$$

where

C is the content of L(+) or D(–) lactic acid per 100 g of culture, in grams;

V_1 is the numerical value of the final volume, in millilitres;

V_2 is the numerical value of the sample volume, in millilitres;

M_r is the numerical value of the relative molecular mass of the substance to be assayed;

A is the numerical value of the absorbance read for each solution (B.7.4.2 and B.7.4.3) against air;

l_p is the numerical value of the optical path length, in centimetres;

ε is the numerical value of the molar absorption coefficient of NADH at 340 nm, that is, 6,3 [l·mmol^{-1} cm^{-1}];

m is the numerical value of the mass, in grams, of the test portion (B.7.1) or sample blank (B.7.2).

d is the numerical value of the dilution factor.

It follows for L(+) lactic acid in the sample:

$$C_{L(+)} = \frac{d \times 2{,}24 \times 90{,}1 \times 100}{m \times \varepsilon \times 1 \times 0{,}1 \times 10\,000} \times A_{L(+)}$$

$$C_{L(+)} = \frac{d \times 20{,}18}{m \times \varepsilon} \times A_{L(+)}$$

where $C_{L(+)}$ is the content of L(+) lactic acid, in grams per 100 g of sample culture (B.6.1).

It follows for L(+) lactic acid in the sample blank:

$$C'_{sb} = \frac{d \times 20{,}18}{m \times \varepsilon} \times A'_{sb}$$

where C'_{sb} is the content of L(+) lactic acid, in grams per 100 g of sample blank (B.6.2).

Subtract the sample blank concentration (C'_{sb}) from each sample culture concentration (C'_s) to correct for any L-lactate present in the substrate:

$$C'_{L(+)} = C'_s - C'_{sb}$$

Similarly, it follows for D(–) lactic acid in the sample:

$$C_{\mathrm{D}(-)} = \frac{d \times 2{,}27 \times 90{,}1 \times 100}{m \times \varepsilon \times 1 \times 0{,}1 \times 10\,000} \times A_{\mathrm{D}(-)}$$

$$C_{\mathrm{D}(-)} = \frac{d \times 20{,}45}{m \times \varepsilon} \times A_{\mathrm{D}(-)}$$

where $C_{\mathrm{D}(-)}$ is the content of D(–) lactic acid, in grams per 100 g of sample culture (B.6.1).

Then, it follows for D(–) lactic acid in the sample blank:

$$C_{\mathrm{sb}} = \frac{d \times 20{,}45}{m \times \varepsilon} \times A_{\mathrm{sb}}$$

where C_{sb} is the content of D(–) lactic acid, in grams per 100 g of sample blank (B.6.2).

Subtract the sample blank concentration (C_{sb}) from each sample culture concentration (C_{s}) to correct for any D-lactate present in the substrate:

$$C'_{\mathrm{D}(-)} = C_{\mathrm{s}} - C_{\mathrm{sb}}$$

B.8.2 Expression of results

Express the results to two decimal places.

B.9 Test report

The test report shall specify:

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

b) the sampling method used, if known;

c) the test method used, with reference to this International Standard;

d) all operating details not specified in this International Standard, or regarded as optional, together with details of any incident which may have influenced the result(s);

e) the test result(s) obtained.

Bibliography

[1] ISO 8069, *Dried milk — Determination of lactic acid and lactates content — Enzymatic method*

[2] 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*

ICS 07.100.30

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