SLOVENSKI STANDARD
SIST EN 13610:2003

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Kemična razkužila – Kvantitativni suspenzijski preskus za ocenjevanje virucidnega delovanja kemičnih razkužil proti bakteriofagom v živilski in drugih industrijah – Preskusna metoda in zahteve (faza 2, stopnja 1)

Chemical disinfectants - Quantitative suspension test for the evaluation of virucidal activity against bacteriophages of chemical disinfectants used in food and industrial areas - Test method and requirements (phase 2, step 1)

Chemische Desinfektionsmittel - Quantitativer Suspensionsversuch zur Bestimmung der viruziden Wirkung gegenüber Bakteriophagen von chemischen Desinfektionsmitteln in den Bereichen Lebensmittel, und Industrie - Prüfverfahren und Anforderung (Phase 2, Stufe 1)

Désinfectants chimiques - Essai quantitatif de suspension pour l'évaluation de l'activité virucide, vis a vis des bactériophages, des désinfectants chimiques utilisés dans les domaines de l'agro-alimentaire et de l'industrie - Méthode d'essai et prescriptions (phase 2, étape 1)

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Chemical disinfectants - Quantitative suspension test for the evaluation of virucidal activity against bacteriophages of chemical disinfectants used in food and industrial areas - Test method and requirements (phase 2, step 1)

Désinfectants chimiques - Essai quantitatif de suspension pour l'évaluation de l'activité virucide contre les bactériophages des désinfectants chimiques utilisés dans le domaine de l'agro-alimentaire et dans l'industrie - Méthode d'essai et exigences (phase 2, étape 1)

Chemische Desinfektionsmittel - Quantitativer Suspensionsversuch zur Bestimmung der viruziden Wirkung gegenüber Bakteriophagen von chemischen Desinfektionsmitteln in den Bereichen Lebensmittel, und Industrie - Prüfverfahren und Anforderung (Phase 2, Stufe 1)

This European Standard was approved by CEN on 23 October 2002.

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This European Standard exists in three official versions (English, French, German). A version in any other language made by translation under the responsibility of a CEN member into its own language and notified to the Management Centre has the same status as the official versions.

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COMITÉ EUROPÉEN DE NORMALISATION
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Foreword

This document (EN 13610:2002) has been prepared by Technical Committee CEN /TC 216, "Antiseptics and chemical disinfectants" the secretariat of which is held by AFNOR.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by June 2003, and conflicting national standards shall be withdrawn at the latest by June 2003.

In this European Standard the Annex A is normative and the Annexes B, C, D and E are informative.

According to the CEN/CENELEC Internal Regulations, the national standards organizations of the following countries are bound to implement this European Standard: Austria, Belgium, Czech Republic, Denmark, Finland, France, Germany, Greece, Iceland, Ireland, Italy, Luxembourg, Malta, Netherlands, Norway, Portugal, Spain, Sweden, Switzerland and the United Kingdom.

Introduction

This European Standard describes a suspension test method intended to establish whether a product proposed as a disinfectant in the fields described in clause 1 has or does not have virucidal activity against bacteriophages.

NOTE Virulent bacteriophages (phages) lytic for (i.e. virulent for) starter cultures of *Lactococcus lactis* subsp. *lactis* used for the production of cheese and other fermented milk products are used as model viruses to test the virucidal activity of a product.

The laboratory test closely simulates practical conditions of application. Chosen conditions (contact time, temperature, viruses [i.e. bacteriophages] in suspension, ...) reflect parameters which are found in practical situations including conditions which may influence the action of disinfectants.

Each utilization concentration found from this test corresponds to defined experimental conditions.

The conditions are intended to cover general purposes and to allow reference between laboratories and product types.

However for some applications the recommendations of use may differ and therefore additional test conditions need to be used.

1 Scope

This European Standard specifies a test method (phase 2, step 1) and requirements for the minimum virucidal activity against bacteriophages of chemical disinfectants that form a homogeneous, physically stable preparation in hard water and that are used in food and industrial areas, excluding areas and situations where disinfection is medically indicated and excluding products used on living tissues.

This European Standard applies at least to the following:

a) processing, distribution and retailing of:

   1) food of animal origin:

      — milk and milk products;

      — meat and meat products;

      — fish, seafood, and their products;

      — eggs and egg products;

      — animal feeds;

      — etc.;

   2) food of vegetable origin:

      — beverages;

      — fruits, vegetables and their derivatives (including sugar, distillery ...);

      — flour, milling and baking;

      — animal feeds;

      — ....;

b) other industrial areas:

      — biotechnology (yeast, proteins, enzymes, ...).

Using this European Standard, it is not possible to determine the virucidal activity against bacteriophages of undiluted product as some dilution is always produced by adding the inoculum and interfering substance.

For chemical disinfectants that can be used without dilution it is not possible to determine whether these products, at a concentration above 80 % have a virucidal activity against bacteriophages.

NOTE The method described is intended to determine the activity of commercial formulations or active substances on viruses (bacteriophages) in the conditions in which they are used.

This European Standard is applicable only to disinfectants, complying with the validation test (see annex A).

2 Normative references

Not applicable.

3 Terms and definitions

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

3.1
product (for chemical disinfection and/or antisepsis)
chemical agent or formulation used as a chemical disinfectant or antiseptic

[EN 1040:1997]

3.2
virucide against bacteriophages
product which inactivates the bacteriophages

NOTE The adjective derived from "virucide" is "virucidal".

3.3
virucidal activity against bacteriophages
capability of a product to reduce the infectivity of intact bacteriophages belonging to reference bacteriophage strains P001 and P008 for at least 4 lg under the conditions defined by this standard

3.4
infectivity of bacteriophages
the ability of a bacteriophage to propagate in a suitable host bacterial cell resulting in the release of bacteriophage progeny

3.5
inactivation of bacteriophage
the reduction of infectivity of a bacteriophage by a product specified as a chemical disinfectant

3.6
reference bacteriophage suspension
bacteriophage suspension of a defined virus strain maintained in reference centers and which should not be passaged more than 10 times

3.7
stock bacteriophage suspension
bacteriophage suspension of a defined strain that has been multiplied on a large scale to obtain a bacteriophage suspension revealing identical characteristics as the reference bacteriophage suspension

NOTE This stock bacteriophage suspension is used to prepare a high-titer test bacteriophage suspension for the bacteriophage inactivation test.

3.8
high-titer bacteriophage suspension
the high-titer bacteriophages suspension obtained from agar plates revealing confluent lysis in the bacterial lawn that is used to prepare the bacteriophage test suspension in the virucidal testing of the disinfectant

3.9
bacteriophage test suspension
the bacteriophage suspension of a defined titer that is used in the virucidal testing of the disinfectant

4 Requirements

The product diluted in hard water when tested in accordance with clause 5 shall demonstrate at least a 4 $\log_{10}$ reduction of infectivity of bacteriophages when tested in the presence of a volume fraction of 1 % acidic whey (prepared from acidified low-fat milk) or optionally in the presence of a volume fraction of 1 % skim milk as the interfering substance according to its practical applications and under the required test conditions (20 °C, 15 min, 2 reference bacteriophage strains).

The virucidal activity against bacteriophages shall be evaluated using the two virulent bacteriophages *Lactococcus lactis* subsp. *lactis* bacteriophage P001 and *Lactococcus lactis* subsp. *lactis* bacteriophage P008.

Both phages shall be propagated on the host strain *Lactococcus lactis* subsp. *lactis* F7/2.

The determined virucidal concentration of the tested product is suggested as being suitable for practical situations of use.

Where appropriate, additional specific virucidal activity against bacteriophages shall be determined under other conditions of time, temperature, additional strains and interfering substances in accordance with (see 5.7.1) in order to take into account intended specific use conditions.

NOTE For these additional conditions, the concentration defined as a result can be lower than the one obtained under the initial test conditions of 20 °C, 15 min, 2 selected bacteriophage reference strains.

5 Test methods

5.1 Principle

5.1.1 A test suspension of bacteriophages in a solution of interfering substances is added to a prepared sample of the product under test diluted in hard water.

The mixture is maintained at 20 °C ± 1 °C for 15 min ± 10 s (required obligatory test conditions).

At the end of the contact time, aliquots are taken and the virucidal activity against bacteriophages in this portion is immediately neutralized or suppressed by a validated method. The method of choice is dilution-neutralization with a validated neutralizer. If a suitable neutralizer is not available for a specific product, removal of the product by molecular sieving (i.e. gel filtration) shall be used.

The number of surviving bacteriophage particles and the number of bacteriophage particles in the test suspension are determined from appropriate dilution series with a factor of 10 prepared in medium in test tubes.

5.1.2 Additional and optional exposure times, temperatures and interfering substances are specified (see 5.7.1).

NOTE 1 The test described is based on an assessment (under specific conditions) which gives a reduction of at least 99,99 % (4 lg) of the infectivity of the different phages after different contact times.

NOTE 2 For principal reasons, the result of an inactivation applied to a viral population is not necessarily equal to 100 %: i.e., one cannot conclude that there is a 100 % inactivation when on conducting the experiment no infectious phage are found within a limit number of sampling.

5.2 Material and reagents

5.2.1 Test organisms

The virucidal activity against bacteriophages shall be evaluated using the two following bacteriophage strains :

— *Lactococcus lactis* subsp. *lactis* bacteriophage P001 DSM 4262 [1];

— *Lactococcus lactis* subsp. *lactis* bacteriophage P008 DSM 10567.

Both phages shall be propagated on the following host strain:

— *Lactococcus lactis* subsp. *lactis* F7/2 DSM 4366.

5.2.2 Culture media and reagents

5.2.2.1 General

The reagents shall be of analytical grade and/or appropriate for microbiological purposes. They shall be free from substances that cause toxic or inactivating effects either to the bacteriophages or to the host bacteria used for counting phage-derived plaques.

NOTE To improve reproducibility, it is recommended that commercially available dehydrated material is used for the preparation of culture media. The manufacturers instructions relating to the preparation of these products should be rigorously followed.

5.2.2.2 Water

The water shall be freshly glass distilled water and not demineralized water.

Sterilize in the autoclave (see 5.3.1).

NOTE 1 If the water is sterilized during the sterilization of the reagents, this is not necessary.

NOTE 2 If distilled water of adequate quality is not available, water for injectable preparation (European Pharmacopoeia) can be used.

5.2.2.3 M17-broth

For maintenance of bacterial host strain, propagation of bacteriophages and for formulation of phage diluent (see 5.2.2.6).

Phytone peptone (from soya meal)	5,00 g
Polypeptone peptone (from casein & animal tissue)	5,00 g
Beef extract powder	5,00 g
Yeast extract	2,50 g
D(+)-lactose	5,00 g
Ascorbic acid	0,50 g
Sodium-ß-glycerophosphate	19,00 g
Magnesium sulfate , 7 H_2O	0,25 g
Water (see 5.2.2.2)	1 000 ml

Sterilize in the autoclave (see 5.3.1). After sterilization the pH of the medium shall be equivalent to 7,0 ± 0,2 when measured at 20 °C. When M17-broth is the diluent for neutralizer formulation (see 5.2.2.12 and Annex B), double

[1] DSM 4262, DSM 10567 and DSM 4366 are the collection numbers of bacteriophage and bacterial strains supplied by the DSMZ (Deutsche Sammlung von Mikroorganismen und ZellKulturen). This information is given for the convenience of users of this standard and does not constitute an endorsement by CEN of the culture collection named. Corresponding strains supplied by other culture collections may be used if they can be shown to lead to the same results.

concentrated M17-broth shall be used for preparation (i.e., all reagents shall be added in double concentration to 1 000 ml water).

5.2.2.4 M17-agar (bottom agar)

Bottom agar for quantitative counting of lysis zones (plaques) obtained from single infective bacteriophage particles in the bacterial lawn of the host bacteria.

Add 15 g of agar to 1 000 ml of M17-broth (see 5.2.2.3). Dissolve the agar by boiling with constant stirring.

Sterilize in the autoclave (see 5.3.1). After sterilization the pH of the medium shall be equivalent to 7,0 ± 0,2 when measured at 20 °C. When the agar is cooled down to 47 °C ± 1 °C, add 10 ml of a sterile 1 mol/l $CaCl_2$-stock solution (see 5.2.2.8). Mix gently and pour 15 ml to 18 ml of agar into Petri dishes (see 5.3.2.10).

5.2.2.5 Overlay agar (top agar, soft agar)

For counting bacteriophages: Dissolve 6,5 g agar in 1 000 ml M17-broth (see 5.2.2.3) and heat until boiling with constant stirring. Dispense the molten agar in test tubes (2,5 to 3 ml each).

Sterilize in the autoclave (see 5.3.1).

NOTE For achieving clear phage-derived lysis zones (plaques) in the lawn of host bacterial cells only well-defined agar should be used which is specified by the supplier for phage enumeration by the overlay technique (see 5.5.2 and 5.5.3).

5.2.2.6 Phage diluent (on basis of ¼ strength Ringer's solution)

For preparing dilution series for titration of phage (counting of phage-derived lysis zones):

— **1/4-strength Ringer's solution:**

  — sodium chloride 2,250 g;
  — potassium chloride 0,105 g;
  — calcium chloride, anhydrous 0,06 g;
  — sodium hydrogen carbonate 0,050 g;
  — water (see 5.2.2.2) to 1 000 ml.

Add 10 ml M17-broth (see 5.2.2.3) to 90 ml of 1/4-strength Ringer's solution.

Sterilize in the autoclave (see 5.3.1). Before use, add 1 ml from an 1 mol/l $CaCl_2$-stock solution (see 5.2.2.8) to 100 ml of the dilution broth.

NOTE Ringer's solution can be prepared from ready-to-use tablets according to the supplier's recommendations.

5.2.2.7 SM-buffer

For resuspension and storage of intact phage particles:

— Tris-HCl 2,4 g;
— NaCl 5,8 g;

— $MgSO_4$, 7 H_2O 2,5 g;

— water (see 5.2.2.2) to 1 000 ml.

Adjust the pH of the buffer to 7,4 ± 0,1. Sterilize in the autoclave (see 5.3.1).

5.2.2.8 $CaCl_2$-stock solutions (1 mol/l and 0,05 mol/l)

Dissolve either 110,99 or 5,55 g anhydrous $CaCl_2$ in water (see 5.2.2.2) and dilute to 1 000 ml to obtain the 1 mol/l or the 0,05 mol/l stock solution, respectively. Sterilize in the autoclave (see 5.3.1).

5.2.2.9 Lactic acid solution (a volume fraction of 10 %)

For acidification of low-fat milk to prepare acidic whey.

Dilute a volume fraction of 90 % stock solution of lactic acid with water (see 5.2.2.2) to obtain a volume fraction of 10 % working solution. For this, 8 parts of water are added to 1 part of stock solution. Sterilize in the autoclave (see 5.3.1).

5.2.2.10 Phosphate-buffered saline

Prepare first a 10 mmol/l sodium phosphate buffer (pH 7,2):

— solution A: 1,42 g anhydrous Na_2HP0_4 are dissolved in water (see 5.2.2.2) and diluted to 1 000 ml with water;

— solution B: 1,20 g anhydrous NaH_2P0_4 are dissolved in water (see 5.2.2.2) and diluted to 1 000 ml with water.

Mix solutions A and B under constant stirring to obtain a final solution with a pH of 7,2.

Dissolve 8,5 g NaCl in 10 mmol/l sodium phosphate buffer (pH 7,2) and dilute to 1 000 ml with this buffer. Sterilize in the autoclave (see 5.3.1).

5.2.2.11 Sephadex® [2] G-25 gel for molecular sieving (i.e. gel filtration)

Resuspend 22 g of Sephadex®[2] G-25 powder in 100 ml phosphate-buffered saline (see 5.2.2.10). Sterilize in the autoclave (see 5.3.1). After cooling down to room temperature, fill 20 ml of the gel suspension into sterile plastic syringes placed in a sterile centrifuge bottle. Remove excess of buffer by centrifugation in a bench-top centrifuge (see 5.3.2.13) at 1 000 x *g* for 10 min under aseptic conditions. Store these ready-to-use units at 4 °C to 8 °C. They shall be used within a 4 h-period.

NOTE Alternatively, commercially available, disposable ready-to-use columns of suitable capacity can be used.

5.2.2.12 Neutralizer

The neutralizer shall be validated for the product under test in accordance with Annex A. The neutralizer shall be sterile.

NOTE Information on neutralizers that have been found to be suitable for some categories of products is given in Annex B.

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[2] Analytical quality of cross-linked dextran beads for molecular sieving (i.e. gel filtration). Sephadex® G-25 is an example of a suitable product available commercially. This information is given for the convenience of users of this standard and does not constitute an endorsement by CEN of this product.

5.2.2.13 Hard water for dilution of products

Hard water shall be prepared as follows:

— solution A: dissolve 19,84 g anhydrous magnesium chloride ($MgCl_2$) or an equivalent of hydrated magnesium chloride and 46,24 g anhydrous calcium chloride ($CaCl_2$) or an equivalent of hydrated calcium chloride in water (see 5.2.2.2) and dilute to 1 000 ml.

  Sterilize in the autoclave (see 5.3.1). Store the solution at 2 °C to 8 °C for no longer than one month;

— solution B: dissolve 35,02 sodium bicarbonate ($NaHCO_3$) in water (see 5.2.2.2) and dilute to 1 000 ml. Sterilize by membrane filtration (see 5.3.2.7). Store the solution at 2 °C to 8 °C for no longer than one week.

Hard Water: For the preparation of 1 litre, place at least 600 ml water (see 5.2.2.2) in a 1 000 ml volumetric flask (see 5.3.2.12) and add 6,0 ml of solution A, then 8,0 ml of solution B. Mix and dilute to 1 000 ml with water (see 5.2.2.2). The pH of the hard water shall be 7,0 ± 0,2.

If necessary adjust the pH by using a solution of approximately 40 g/l (about 1 mol/l) of sodium hydroxide (NaOH) or approximately 36,5 g/l (about 1 mol/l) of hydrochloric acid (HCl).

The hard water shall be freshly prepared under aseptic conditions and used within 12 h.

NOTE When preparing the working culture (see 5.4.2) the addition of the product to this hard water produces a different final water hardness in each test tube.

In any case the final hardness is lower than 300 mg/kg of calcium carbonate ($CaCO_3$) in the test tube.

5.2.2.14 Interfering substances

5.2.2.14.1 General

The ionic composition (pH, calcium and/or magnesium hardness) and chemical composition (mineral substances, protein, glycosides, lipids, detergents ...) shall be fully defined.

The interfering substance shall be chosen according to the conditions of use laid down for the product.

The interfering substance shall be sterile and prepared at 10-times of its final concentration in the test.

The method of preparation and sterilization together with the composition shall be noted in the test report (see 5.9).

5.2.2.14.2 Whey solution

Prepare acidic whey solution from pasteurized low fat milk (1,5 % fat content) for the test conditions as follows:

— add 0,3 ml of a volume fraction of 10 % lactic acid solution (see 5.2.2.9) to 10 ml milk, mix (see 5.3.2.6) and keep the sample for 30 min at room temperature. Mix (see 5.3.2.6) occasionally during this 30 min period. Subsequently sediment the precipitated milk proteins in a bench top centrifuge (see 5.3.2.13) at maximum speed (4 000 x *g* at minimum) for 30 min. Sterilize the supernatant (whey) by membrane filtration (0,45 µm pore size) (see 5.3.2.7) and store at 4 °C to 8 °C;

— to obtain a volume fraction of 10 % working solution which is required as the obligatory interfering substance for the phage suspension test (see 5.7.2), dilute 1 part of acidic whey broth with 9 parts of water (see 5.2.2.2). Store the volume fraction of 10 % whey solution at 4 °C to 8 °C.

The whey solutions shall be stored for up to 1 month at 4 °C to 8 °C. For longer storage periods, they shall be kept frozen at - 18 °C to - 20 °C or lower.