GB

NATIONAL STANDARD OF THE

PEOPLE'S REPUBLIC OF CHINA

GB 4789.14-2014

National Food Safety Standard –

Food Microbiological Examination - Bacillus Cereus

Issued on: December 1, 2014 **Implemented on: May 1, 2015**

Issued by: National Health and Family Planning Commission of the People's Republic of China

Table of Contents

National Food Safety Standard – Food Microbiological Examination - Bacillus Cereus

1 Scope

This Standard specifies the examination method of bacillus cereus in food.

The first method of this Standard applies to the count of bacillus cereus in food which has a high content of bacillus cereus; and the second method applies to the count of bacillus cereus in food which has a low content of bacillus cereus.

2 Equipment and materials

In addition to conventional sterilization and culture equipment for microbiology laboratories, the other equipment and materials are as follows:

a) refrigerators: 2°C ~ 5°C;

b) thermostatic incubators: 30°C ± 1°C, 36°C ± 1°C;

c) homogenizers;

d) electronic balances: sensitivity 0.1 g;

e) sterile conical flasks: 100 mL, 500 mL;

f) sterile pipettes: 1 mL (having 0.01 mL scale), 10 mL (having 0.1 mL scale) or micropipettes and tips;

g) sterile plates: diameter 90 mm;

h) sterile examination tubes: 18 mm ×180 mm;

i) microscopes: 10× ~ 100× (oil immersion lens);

j) L spreader.

3 Culture media and reagents

3.1 Phosphate buffer solution (PBS): see A.1 of Annex A.

3.2 Mannitol yolk polymyxin (MYP) agar: see A.2 of Annex A.

4.2.3 Sample dilution

Absorb 1 mL of the 1:10 sample homogeneous solution of 4.2.2 to add to a dilution tube containing 9 mL of PBS or normal saline; fully mix up to make 1:100 sample homogeneous solution. In accordance with the estimation of sample contamination degree, operate as above and make into ten-fold incremental serial dilution sample homogeneous solution in succession. After each dilution, replace one 1 mL sterile pipette or tip.

4.2.4 Sample inoculation

In accordance with the estimation of sample contamination degree, select 2 ~ 3 sample homogeneous solutions of appropriate dilution (liquid samples can include primary liquid); transfer the inoculum sizes of 0.3 mL, 0.3 mL and 0.4 mL to three MYP agar plates respectively; then use a sterile L spreader to apply them to the whole plates; and pay attention to not touching the edge of the plates. Before use, if there are water drops on the surface of the MYP agar plates, they can be placed in an incubator to dry at 25°C ~ 50°C until the water drops on the surface of the plates disappear.

4.2.5 Isolation and culture

4.2.5.1 Isolation

Under normal conditions, allow plates to stand for 10 min after applying. If sample solution is difficult to absorb, it can be placed in an incubator to culture for 1 h at 30°C ± 1°C; turn over plates after sample homogeneous solution is evenly absorbed; place them upside down in the incubator; culture for 24 h ± 2 h at 30°C ± 1°C. If the colonies are not typical, continue culturing for 24 h ± 2 h before observing. On MYP agar plates, typical colonies are of a faint pink colour (indicating unfermented mannitol) and there are white to pale orchid pink precipitation ring (indicating the production of lecithinase).

4.2.5.2 Pure culture

Select at least 5 typical colonies (select all if less than 5) from each plate (complying with the requirements of 4.4.1.1); inoculate them by streaking in nutrient agar plates for pure culture; culture for 24 h ± 2 h at 30°C ± 1°C to carry out confirmatory test. On nutrient agar plates, typical colonies are offwhite, occasionally yellowish green, non-transparent, ground glass shaped or melting wax shaped in surface roughness, usually flared on the edge and of diameter 4 mm ~ 10 mm.

4.3 Confirmatory appraisal

4.3.1 Dyeing microscopic examination

Pick single colonies of pure culture for Gram's microscopic examination. Bacillus cereus is Gram positive bacillus of size (1 μm ~ 1.3 μm) ×(3 μm ~ 5 μm); the spore is oval which is located at the centre or one end of thallus, not expanding on thallus; both ends of thallus are flat, normally arranged in the shape of short chains or long chains.

Pick single suspicious colonies to streak parallel straight lines 2 cm ~ 3 cm distant on nutrient agar plates which are dried for 1 d ~ 2 d at room temperature; culture for 24 h ~ 48 h at 30°C ± 1°C, not exceeding 72 h. Use the standard strains of bacillus cereus and bacillus mycoides as control to carry out synchronous test. Bacillus mycoides shows the characteristics of root growth. Bacillus cereus shows the characteristics of rough valley growth.

4.3.2.5 Lysozyme tolerance test

Use an inoculating loop to pick one loop of pure strain suspension to inoculate in a lysozyme broth; culture for 24 h at 36°C ± 1°C. Bacillus cereus is capable of growing in the medium (containing 0.001% of lysozyme). In case of any negative reaction, continue to culture for 24 h. Bacillus megaterium does not grow.

4.3.2.6 Protein toxin crystal test

Pick single suspicious colonies of pure culture to inoculate on manganese sulfate nutrient agar plates; culture for 24 h ± 2 h at 31°C ± 1°C; store for 3 d ~ 4 d at room temperature; pick a little of culture to place on the glass slide; and add dropwise distilled water to mix up and form a thin film. After natural drying and low fire fixation, add methyl alcohol to act for 30 s before pour out; dry through a flame; drop 0.5% basic fuchsin fully on the glass slide; place above a flame for heating (steam starts to show but do not let dye solution boil) for 1 min ~ 2 min for 1 min ~ 2 min; remove the flame; replace dye solution to heat for dyeing for 30 s once again; pour out dye solution and use clean tap water to rinse thoroughly and carry out microscopic examination after drying in the air. Observe whether there are free spores (light red) and rhombic protein crystals dyed into dark red. If the production of free spores is not rich, continue to store culture for 2 d ~ 3 d at room temperature before examination. Except bacillus thuringiensis, other bacilli do not generate protein crystals.

4.3.3 Biochemical typing (optional)

Bacillus cereus is classified into different biochemical types in accordance with citrate utilization, nitrate reduction, amylolysis, V-P test reaction and gelatin liquefaction test. See Table 2.

Annex A

Culture media and reagents

A.1 Phosphate buffer solution (PBS)

A.1.1 Composition

Potassium dihydrogen phosphate	34.0 g
Distilled water	500.0 mL

A.1.2 Preparation

Stock solution: weigh 34.0 g of potassium dihydrogen phosphate to dissolve in 500 mL of distilled water; use about 175 mL of 1 mol/L sodium hydroxide solution to adjust pH to 7.2; use distilled water to dilute to 1 000 mL before storing in a refrigerator.

Dilute solution: take 1.25 mL of stock solution; use distilled water to dilute to 1 000 mL; load in appropriate containers; carry out autoclaved sterilization for 15 min at 121°C.

A.2 Mannitol yolk polymyxin (MYP) agar

A.2.1 Composition

Peptone	10.0 g
Beef powder	1.0 g
D-mannitol	10.0 g
Sodium chloride	10.0 g
Agar powder	12.0 ~ 15.0 g
0.2% phenol red solution	13.0 mL
50% egg yolk emulsion	50.0 mL
Polymyxin B	100,000 IU
Distilled water	950.0 mL

A.2.2 Preparation

Add the first five ingredients of A.2.1 to 950 mL of distilled water; heat to dissolve; calibrate pH to 7.3 ± 0.1; add phenol red solution. Load in different bottles, 95 mL per bottle; carry out autoclaved sterilization for 15 min at 121°C. Heat to dissolve agar immediately before use; cool to 50°C; add 5 mL of 50% egg yolk emulsion and 1 mL of polymyxin B of concentration 10,000 IU to each bottle; pour plate after mixing up.

A.2.2.1 50% egg yolk emulsion

Take fresh eggs; use hard brush to clean shell thoroughly; drain off; soak in 70% alcoholic solution for 30 min. Take egg yolks out by sterile operation; add equal amount of sterile normal saline; prepare for use after mixing up.

A.5.3 Result

It is positive if air bubbles are generated within 30 s; it is negative if no air bubble is generated.

A.6 Motility medium

A.6.1 Composition

Casein tryptone (or casein peptone)	10.0 g
Yeast powder	2.5 g
Glucose	5.0 g
Anhydrous disodium hydrogen phosphate	2.5 g
Agar powder	3.0 ~ 5.0 g
Distilled water	1,000.0 mL

A.6.2 Preparation

Dissolve the ingredients mentioned in A.6.1 in distilled water; calibrate pH to 7.2 ± 0.2; heat to dissolve. Load 2 mL ~ 3 mL in each tube. Carry out autoclaved sterilization for 15 min at 121°C as standby.

A.6.3 Test method

Use an inoculating needle to pick culture to inoculate in a motility medium by puncture; culture for 48 h ± 2 h at 30°C ± 1°C. Bacillus cereus shall grow diffusedly along the puncture line, while bacillus mycoides normally grows in “villiform”, forming a honeycomb-shaped diffusion. Motility test can also be checked using the pendant-drop method. Bacillus cereus and bacillus thuringiensis are usually more motile while bacillus anthracis is not motile.

A.7 Nitrate broth

A.7.1 Ingredients

Peptone	5.0 g
Potassium nitrate	0.2 g
Distilled water	1,000.0 mL

A.7.2 Preparation

Dissolve the ingredients mentioned in A.7.1 in distilled water. Calibrate pH to 7.4; load 5 mL in each tube; carry out autoclaved sterilization for 15 min at 121°C.

A.7.3 Nitrate reduction reagent

Solution A: dissolve 0.8 g of sulfanilic acid in 100 mL of 2.5 mol/L acetic acid solution.

Solution B: dissolve 0.5 g of α-Naphthylamine in 100 mL of 2.5 mol/Lacetic acid solution.

A.10 0.5% basic fuchsin

A.10.1 Composition

Basic fuchsin	0.5 g
Ethyl alcohol	20.0 mL
Distilled water	80.0 mL

A.10.2 Preparation

Take 0.5 g of basic fuchsin to dissolve in 20 mL of ethyl alcohol; use distilled water to dilute to 100 mL; use filter paper to filter for storage as standby.

A.11 Motility medium

A.11.1 Composition

Peptone	10.0 g
Beef extract	3.0 g
Agar	4.0 g
Sodium chloride	5.0 g
Distilled water	1,000.0 mL

A.11.2 Preparation

Dissolve the ingredients mentioned in A.11.1 in distilled water. Calibrate pH to 7.2 ±0.2; load in small test tubes; carry out autoclaved sterilization for 15 min at 121°C as standby.

A.12 Sugar fermentation tube

A.12.1 Composition

Beef powder	5.0 g
Peptone	10.0 g
Sodium chloride	3.0 g
Disodium hydrogen phosphate ($Na_2HPO_4 \cdot 12H_2O$)	2.0 g
0.2% bromothymol blue solution	12.0 g
Distilled water	1,000.0 mL

A.12.2 Preparation

A.12.2.1 For sugar fermentation tube, calibrate pH to 7.2 ±0.2 after preparing the ingredients mentioned in A.12.1; add 0.5% of glucose; load in a small test tube with an inverted small tube; carry out autoclaved sterilization for 15 min at 115°C.

A.12.2.2 For other sugar fermentation tubes, load 100 mL in each bottle after preparing the ingredients mentioned in A.12.1; carry out autoclaved sterilization for 15 min at 115°C. Use all sugars to prepare 10% solutions respectively; meanwhile, carry out autoclaved sterilization for 15 min at 115°C. Add 5 mL of sugar solution to 100 mL of medium; load into small test tubes by sterile operation.

defibrinated sheep blood; mix up before pouring plate.

A.15 Lysozyme nutrient broth

A.15.1 Composition

Beef powder	3.0 g
Peptone	5.0 g
Distilled water	990.0 mL
0.1% lysozyme solution	10.0 mL

A.15.2 Preparation

Except lysozyme solution, dissolve the ingredients mentioned in A.15.1 in distilled water. Calibrate pH to 6.8 ± 0.1; load 99 mL in each bottle. Carry out autoclaved sterilization for 15 min at 121°C. Add 1 mL of 0.1% lysozyme solution to each bottle; load into sterile test tubes after mixing up, 2.5 mL each tube. The preparation of 0.1% lysozyme solution: add 0.1 g of lysozyme in 65 mL of sterile 0.1 mol/L hydrochloric acid; boil for 20 min to dissolve with the partition of water; use sterile 0.1 mol/L hydrochloric acid to dilute to 100 mL. Or, weigh 0.1 g of lysozyme to dissolve in 100 mL of sterile distilled water; use nitrocellulose membrane of pore size 0.45 μm for filtration. Test whether it is sterile before use.

A.15.3 Test method

Use an inoculating loop to pick one loop of pure strain suspension to inoculate in a lysozyme broth; culture for 24 h at 36°C ± 1°C. Bacillus cereus is capable of growing in the medium (containing 0.001% lysozyme). In case of a negative reaction, continue to culture for 24 h.

A.16 Simmons citrate medium

A.16.1 Composition

Sodium chloride	5.0 g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.2 g
Ammonium dihydrogen phosphate	1.0 g
Dipotassium hydrogen phosphate	1.0 g
Sodium citrate	1.0 g
Agar powder	12.0 ~ 15.0 g
Distilled water	1,000.0 mL
0.2% bromothymol blue solution	40.0 mL

A.16.2 Preparation

Except bromothymol blue solution and agar, dissolve all ingredients mentioned in A.16.1 10,000.0 mL of distilled water; calibrate pH to 6.8; add agar; heat to dissolve. Then add bromothymol blue solution; load into test tubes after mixing up; carry out autoclaved sterilization for 15 min at 121°C. Make a slant.

This is an excerpt of the PDF (Some pages are marked off intentionally)

------ The End ------