ENUMERATION OF

BACILLUS CEREUS AND

OTHER BACILLUS SPECIES

F 15

Issued by Standards Unit, Evaluations and Standards Laboratory
Specialist and Reference Microbiology Division
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ENUMERATION OF BACILLUS CEREUS AND OTHER BACILLUS SPECIES

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AMENDMENT PROCEDURE

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<th>Issue no. Discarded</th>
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<th>Page</th>
<th>Section(s) involved</th>
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On issue of revised or new pages each controlled document should be updated by the copyholder in the laboratory.
STANDARD OPERATING PROCEDURE FOR THE ENUMERATION OF *BACILLUS CEREUS* AND OTHER *BACILLUS* SPECIES

INTRODUCTION

Scope

The method described is applicable to the enumeration of *B. cereus* and other *Bacillus* species in all food types. The media used will recover most species including all those implicated in food poisoning.

Background

*Bacillus* species in food that is ready-to-eat is generally considered to be unsatisfactory when counts of greater than or equal to $10^4$ colony forming units per gram (cfu/g) are present\(^1\). However, when investigating food poisoning outbreaks, lower counts may also be considered significant. The method below describes the enumeration of *Bacillus cereus* and other *Bacillus* species\(^2-3\).
1.0 PRINCIPLE

Specified volumes of $10^{-1}$ and further dilutions of the test sample as appropriate are surface inoculated onto a selective agar medium with incubation at 30°C for up to 48 hours.

The number of colony forming units (cfu) of total Bacillus species or B. cereus is calculated per gram of sample from the number of typical colonies obtained on the selective agar medium, and subsequently confirmed by biochemical tests.

2.0 DEFINITIONS

For the purposes of this method, the following definitions apply:

**Bacillus species**
Micro-organisms that demonstrate morphology typical of aerobic, Gram positive, spore forming bacilli on selective and non-selective agar media.

**Presumptive Bacillus cereus**
Members of Bacillus species that do not ferment mannitol, usually produce lecithinase, and exhibit β-haemolysis on blood agar.

**Bacillus cereus**
Strains of presumptive Bacillus cereus that display the morphological and biochemical characteristics described in this method.

3.0 SAFETY CONSIDERATIONS

Normal microbiology laboratory precautions apply. Chemical resistant gloves should be worn when making up nitrate reagents A and B.

4.0 EQUIPMENT

Usual laboratory equipment and in addition:

- Top pan balance capable of weighing to 0.1g
- Gravimetric diluter (optional)
- Vortex mixer (optional)
- Spiral plater (optional)
- Incubator at 30°C ± 1°C
- Colony counter (optional)
- Stomacher
- Stomacher bags (sterile)
- Automatic pipettors and sterile pipette tips capable of delivering 10 mL, 1 mL and 0.1 mL volumes (optional)
- Pipettes (sterile total delivery), 10 mL and 1 mL graduated in 0.1 mL volumes (optional)
- Anaerobic cabinet (optional)
- Modified atmosphere jars and gas generation sachets capable of achieving <1% oxygen, 9-13% carbon dioxide (optional)
5.0 CULTURE MEDIA

Equivalent commercial dehydrated media may be used; follow the manufacturer’s instructions.

Peptone saline diluent (Maximum recovery diluent) (optional)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>8.5 g</td>
</tr>
<tr>
<td>Water</td>
<td>1 L</td>
</tr>
</tbody>
</table>

pH 7.0 ± 0.2 at 25°C

Buffered peptone water (optional)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
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<tr>
<td>Peptone</td>
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<tr>
<td>Disodium hydrogen phosphate</td>
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<td>Potassium dihydrogen phosphate</td>
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</tr>
<tr>
<td>Water</td>
<td>1 L</td>
</tr>
</tbody>
</table>

pH 7.2 ± 0.2 at 25°C

Polymixin pyruvate egg yolk mannitol bromothymol blue agar (PEMBA) (optional)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
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<tbody>
<tr>
<td>Peptone</td>
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<tr>
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<tr>
<td>Sodium chloride</td>
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<tr>
<td>Magnesium sulphate</td>
<td>0.1 g</td>
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<tr>
<td>Disodium hydrogen phosphate</td>
<td>2.5 g</td>
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<tr>
<td>Potassium dihydrogen phosphate</td>
<td>0.25 g</td>
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<tr>
<td>Bromothymol blue</td>
<td>0.12 g</td>
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<tr>
<td>Sodium pyruvate</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Egg yolk emulsion</td>
<td>50 mL</td>
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<tr>
<td>Polymixin B</td>
<td>100,000 IU</td>
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<tr>
<td>Agar</td>
<td>14.0 g</td>
</tr>
<tr>
<td>Water</td>
<td>1 L</td>
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pH 7.2 ± 0.2 at 25°C

Mannitol phenol red egg yolk polymixin agar (MEYP) (optional)

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<tbody>
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<tr>
<td>Mannitol</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Phenol red</td>
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</tr>
<tr>
<td>Egg yolk emulsion</td>
<td>100 mL</td>
</tr>
<tr>
<td>Polymixin B</td>
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<td>Agar</td>
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<td>Water</td>
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pH 7.2 ± 0.2 at 25°C
**Blood agar**

Columbia agar with 5% horse blood

**Motility nitrate medium (optional)**

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<tbody>
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<tr>
<td>Meat extract</td>
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<tr>
<td>Galactose</td>
<td>5.0 g</td>
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<tr>
<td>Glycerol</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Potassium nitrate</td>
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<tr>
<td>d-Sodium hydrogen orthophosphate</td>
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<td>Agar</td>
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<td>Water</td>
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pH 7.3 ± 0.2 at 25°C

**Nitrate broth (optional)**

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<tr>
<td>Beef extract</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Potassium nitrate</td>
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</tr>
<tr>
<td>Water</td>
<td>1 L</td>
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pH 7.0 ± 0.2 at 25°C

**Ammonium salt sugars (optional)**

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<tbody>
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<td>Ammonium phosphate</td>
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<tr>
<td>Potassium chloride</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td></td>
</tr>
<tr>
<td>heptahydrate</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Carbohydrate*</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Bromocresol purple</td>
<td>8 mg</td>
</tr>
<tr>
<td>Or Bromothymol blue</td>
<td>40 mg</td>
</tr>
<tr>
<td>Agar</td>
<td>12.0 g</td>
</tr>
<tr>
<td>Water</td>
<td>1 L</td>
</tr>
</tbody>
</table>

pH 6.9 ± 0.2 at 25°C

*Glucose, arabinose, mannitol, xylose
Prepare as slopes.

**Nitrite reagents**

Nitrite reagent A:
5-amino-2-naphthalene-sulphonic acid (0.1% solution in acetic acid 15% (v/v))

Nitrite reagent B:
Sulphanilic acid (0.4% solution in acetic acid 15% (v/v))

**Zinc dust**

**Commercial biochemical test kits (optional)**

**Gram's stain reagents**
6.0 SAMPLE PROCESSING

6.1 Sample preparation and dilutions

Following the procedure described in Standard method F 2, prepare a $10^1$ homogenate in either peptone saline diluent (PSD) or buffered peptone water and further decimal dilutions in PSD as required.

6.2 Inoculation and incubation

Following the procedure described in Standard method F 11, use a spiral plater to inoculate $50 \mu L$ of each dilution onto the surface of a pre-dried polymixin pyruvate egg yolk mannitol agar (PEMBA) plate or mannitol phenol red egg yolk polymixin agar (MEYP) plate.

Alternatively, following the procedure described in Standard Method F 10, inoculate $0.1 mL$ of each dilution onto the centre of a dried selective agar plate. Using a sterile spreader, and starting with the highest dilution, spread the inoculum over the surface of each plate as soon as possible taking care not to touch the sides of the plate.

Leave the plates on the bench for approximately 15 minutes to allow absorption of the inoculum into the agar. Invert the plates and place in an incubator at $30^\circ C$. Examine the plates after incubation for 18 – 24 hours and 42 – 48 hours. Note: *B. cereus* colonies will normally be evident after 24 hours. Subculture at this stage may be preferable to prevent overgrowth occurring during extended incubation. If present, count and record the number of colonies.

6.3 Counting of colonies

**PEMBA**

*B. cereus* appear as large (approximately 5 mm diameter), crenated colonies, a distinctive turquoise to peacock blue colour i.e: mannitol negative, and usually surrounded by a zone of egg yolk precipitate of the same colour (small zones of egg yolk precipitation are of no significance and can be ignored).

Most other members of the Bacillus group, including the Bacillus subtilis-licheniformis group, appear as green or yellow colonies i.e: mannitol positive, and do not produce lecithinase (small zones of precipitation in the egg yolk are of no significance and can be ignored.).

**MEYP**

*B. cereus* produces large pink (mannitol negative) colonies usually surrounded by a zone of egg yolk precipitate (due to precipitation of hydrolysed lecithin).

Most other Bacillus species produce yellow colonies due to the production of acid from mannitol, and are lecithinase negative.

Notes

1. The blue colour on PEMBA or pink colour on MEYP may not be apparent if the food under examination is very acidic or if the plate contains numerous mannitol-fermenting micro-organisms leading to the production of acid.

2. Some strains of *B. cereus* produce little or no lecithinase. Colonies of these strains will not be surrounded by a precipitation zone. Mannitol negative colonies without egg yolk precipitation should also be subjected to confirmation tests.
3. Some members of the Enterobacteriaceae, such as *Proteus*, and many strains of *Staphylococcus aureus* are able to grow on these selective media. However they are easily distinguished by colonial morphology and overall appearance, and by egg yolk clearing instead of precipitation.

After incubation for 42 - 48 hours count the total number of *Bacillus* colonies and the number of suspect *B. cereus* colonies separately. Record counts. If counts exceed $10^4/g$ confirm their identity biochemically.

6.4 Confirmatory tests

6.4.1 Confirmation of presumptive *Bacillus cereus*
Subculture 5 suspect colonies (or all colonies if less than 5) onto PEMBA or MEYP and blood agar and incubate the plates at 30°C for 18-24 hours. Examine for purity. Pure cultures that are blue on PEMBA or pink on MEYP, with or without lecithinase precipitation, and show β-haemolysis on blood agar are confirmed as presumptive *B. cereus*.

6.4.2 Confirmation of *Bacillus cereus*
To confirm the identity of presumptive *B. cereus* colonies subculture to ammonium salt sugars (glucose, mannitol, arabinose and xylose), either motility nitrate medium (by stab inoculation) or nitrate broth, and two blood agar plates. Incubate one blood agar plate anaerobically and all other media aerobically at 30°C for 22 ± 2 hours. Incubate bottled media with caps loose.

Appropriate commercial biochemical test kits may also be used.

*Acid production from carbohydrate media*
This is demonstrated by the development of a yellow colour in the agar medium.

*Nitrate reduction test*
Mix equal volumes of nitrite reagents A and B just before use and test for the presence of nitrite by adding 0.2 – 0.5 mL of this mixture to each tube containing growth in either the motility nitrate medium or the nitrate broth. The formation of a red colour confirms the reduction of nitrate to nitrite. If a red colour does not develop within 15 minutes add a small amount of zinc dust and allow to stand for 10 minutes. If a red colour develops after the addition of zinc dust, no reduction of nitrate to nitrite has taken place.

*Anaerobic growth*
Examine the anaerobic blood agar plate for the presence or absence of growth and record. If growth is very weak compared with the control strain of *B. cereus* consider the result to be negative. Check that growth has occurred on the aerobic agar plate and record.

Presumptive *B. cereus* isolates that produce acid from glucose but not mannitol, xylose or arabinose, reduce nitrates to nitrites and grow anaerobically are confirmed as *B. cereus*.

6.4.3 Confirmation of *Bacillus* species
Subculture 5 suspect colonies (or all colonies if less than 5) onto PEMBA or MEYP and blood agar and incubate the plates at 30°C for 18 - 24 hours. Examine the plates for purity and colonial morphology. Perform a Gram stain when colonial appearance is atypical to confirm cell morphology. *Bacillus* species typically appear as large Gram positive bacilli, with or without visible spores. Record results.

6.4.4 Identification of pathogenic *Bacillus* species
To identify the presence of potentially pathogenic strains of *Bacillus* species, perform the tests described in 8.4.2. Record the reactions. Refer to Table 1 to determine the presence of potentially pathogenic strains.

*Control organisms*
Positive controls:
Bacillus cereus  NCTC 7464
Bacillus subtilis  NCTC 10400

Negative control:
Escherichia coli  NCTC 9001
7.0 CALCULATION OF RESULTS

Counts should be calculated, where possible, using dilutions which give 20 or more colonies on spiral plates and 15 or more colonies on spread plates. Calculate the number of *Bacillus* species or *B. cereus* per gram as follows:

\[
\text{Count per g} = \frac{\text{No. of colonies confirmed}}{\text{No. of colonies tested}} \times \frac{\text{Presumptive count}}{\text{Volume tested} \times \text{dilution}}
\]

8.0 REPORTING OF RESULTS

Report the total count of all *Bacillus* organisms including *B. cereus* as *Bacillus* species. Also report the count of *B. cereus* separately.

If no colonies are present, report as:

- Less than 1.0 x 10^2 cfu/g or mL (0.1 mL surface spread)
- or
- less than 2.0 x 10^2 cfu/g or mL (spiral plate)

If colonies are present, report counts with one figure before and one figure after the decimal point in the form of:

\[a \times 10^b\text{ cfu/g or mL}\]

where \(a\) is never less than 1.0 or greater than 9.9 and \(b\) represents the appropriate power of ten. Round counts up if the last figure is 5 or more and down if the last figure is 4 or less.

E.g: 1920 cfu/g = 1.9 x 10^3 cfu per g
235,000 cfu/g = 2.4 x 10^5 cfu per g

If counts of *Bacillus* species and *B. cereus* are such that identification is not required (<10^4/g), report results as presumptive *Bacillus* species or presumptive *Bacillus cereus*.

If identification of *Bacillus* species has been performed but the *B. subtilis-licheniformis* group not identified, report (not *B. subtilis-licheniformis* group).

If a species belonging to the *B. subtilis-licheniformis* group is identified, report the identity.

9.0 REFERENCE FACILITIES

Confirmation, serotyping and enterotoxin testing of *B. cereus*, identification of *Bacillus* species isolates, and enterotoxin testing of food samples is available at the Food Safety Microbiology Laboratory, Specialist and Reference Microbiology Division, Colindale.

Isolates should be sent to FSML when counts exceed 10^4/g or are associated with food poisoning.
Appendix: Flowchart showing the process for the enumeration of *Bacillus cereus* and other *Bacillus* species

1. Prepare a 10-1 dilution of sample
2. Homogenise by stomaching
3. Prepare further dilutions in peptone saline diluent if required
4. Inoculate 50 µL (spiral plater) or 0.1 mL (surface spread) of each dilution onto PEMBA or MEYP
5. Incubate in aerobic conditions at 30°C and examine after 18-24 and 42-48 hours.
6. Count typical colonies
7. Subculture 5 (or all if <5) typical colonies of each morphological type to PEMBA or MEYP and Blood agar
8. Incubate in aerobic conditions at 30°C for 18-24 hours
9. Examine for absence of mannitol fermentation, presence of haemolysis, and (usually) presence of lecithinase (egg yolk precipitation) (*Bacillus cereus*)
10. Examine for typical colonial morphology (*Bacillus* species)
11. If count exceeds 10^4/g or if implicated in food poisoning perform biochemical testing
12. Calculate the total *Bacillus* species count per gram and the *B.cereus* count per gram
13. Report counts separately
14. If biochemical testing has been performed report the identity
Table 1: Identification of the Bacillus species commonly involved in food poisoning

<table>
<thead>
<tr>
<th></th>
<th><em>B.cereus</em></th>
<th><em>B.pumilus</em></th>
<th><em>B.subtilis</em></th>
<th><em>B.licheniformis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Arabinose</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mannitol</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Xylose</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reduction</td>
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<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Anaerobic growth</td>
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Email: standards@hpa.org.uk
REFERENCES

1. PHLS Advisory Committee for Food and Dairy Products, 2000. Guidelines for the microbiological quality of some ready-to-eat foods sampled at the point of sale. Communicable Disease and Public Health 3 (3) 163-9


7. Health and Safety Executive. 5 steps to risk assessment: a step by step guide to a safer and healthier workplace, IND (G) 163 (L). London: HSE, 1/94


11. ISO 7932: 1993 Amendment 1 Document for public comment. 20.9.99