



BACILLUS CEREUS AGAR BASE (PEMBA) BACILLUS CEREUS SELECTIVE AGAR (PEMBA)

Dehydrated and ready-to-use culture medium and selective supplement

PEMBA: typical *Bacillus cereus* colonies**1 - INTENDED USE**

For the detection and enumeration of *B. cereus* group in foodstuffs and other samples.

2 – COMPOSITION***DEHYDRATED BACILLUS CEREUS AGAR BASE (PEMBA)
TYPICAL FORMULA (AFTER RECONSTITUTION WITH 1 L OF WATER)**

D-mannitol	10.00 g
Peptone	1.00 g
Sodium pyruvate	10.00 g
Sodium chloride	2.00 g
Magnesium sulphate	0.10 g
Potassium dihydrogen phosphate	0.25 g
Disodium hydrogen phosphate	2.50 g
Bromothymol blue	0.12 g
Agar	15.00 g

BACILLUS CEREUS SELECTIVE AGAR (PEMBA) - READY TO USE PLATES

Bacillus Cereus Agar Base (PEMBA)	41 g
Egg Yolk Emulsion 50%	50 mL
Polymyxin B sulphate	100,000 IU
Purified water	950 mL

*The formulas may be adjusted and/or supplemented to meet the required performances criteria.

3 - PRINCIPLE OF THE METHOD AND EXPLANATION OF THE PROCEDURE

Bacillus cereus is a group of ubiquitous facultative anaerobic sporeforming Gram-positive rods commonly found in soil. Group comprises six species: *B. cereus*, *B. thuringiensis*, *B. weihenstephanensis*, *B. mycoides*, *B. pseudo-mycoides*, *B. weihenstephanensis* and *B. anthracis*. *Bacillus cereus* is a foodborne pathogen that can produce two toxins, one heat stable and emetic and the other thermolabile the cause diarrhoea.¹ The infection is caused by ingestion of foods such as meat, rice and vegetables that are contaminated with *B. cereus*, and have been left at room temperature after cooking.

Hoolbrook and Anderson² in 1980 described the use and performance of an improved diagnostic and selective medium, polymyxin pyruvate egg yolk mannitol bromothymol blue agar-Pemba, for the detection of *Bacillus cereus* in foods.

PEMBA is recommended by ISO 21871³ for detection and enumeration of low number of presumptive *B. cereus* in foods.

Bacillus Cereus Agar (PEMBA) contains a peptone that provides carbon, nitrogen, and minerals for microbial growth. This medium relies on the selective inhibitory component polymyxin B and two indicator systems: mannitol and bromothymol blue and egg yolk.

Selectivity is attained with polymyxin B and a critical concentration of nutrients. *B. cereus* will not attack mannitol but dissimilate egg yolk and consequently give rise to typical bacilliform turquoise to peacock blue colonies with halos.⁴ Non-target organisms that ferment mannitol produce acid products and form yellow colonies.

4 - DIRECTIONS FOR MEDIUM PREPARATION

Suspend 20.5 g in 470 mL of cold purified water. Heat to boiling with frequent agitation, sterilize by autoclaving at 121°C for 15 minute and cool to 44-47°C. Reconstitute under aseptic conditions the contents of one vial of Bacillus Cereus Antimicrobial Supplement (Ref. 4240001) with 5 mL of sterile purified water, add to the base medium and mix. Add 25 mL of Egg Yolk Emulsion, (Ref. 4211601), mix well and distribute into sterile Petri dishes.

5 - PHYSICAL CHARACTERISTICS

Dehydrated medium appearance	grey, fine, homogeneous, free-flowing powder
Solution appearance	blue-green, slightly opalescent
Prepared plates appearance	green-blue, opaque
Final pH at 20-25 °C	7.2 ± 0.2

6 - MATERIALS PROVIDED - PACKAGING

Product	Type	REF	Pack
Bacillus Cereus Agar Base (PEMBA)	Dehydrated medium	4011122	500 g (12.2 L)
Bacillus Cereus Selective Agar (PEMBA)	Ready-to-use medium in plates	541112	2 x 10 plates ø 90 mm

7 - MATERIALS REQUIRED BUT NOT PROVIDED

Autoclave, water-bath, sterile loops and pipettes, incubator and laboratory equipment as required, Erlenmeyer flasks, sterile Petri dishes, Egg Yolk Emulsion (REF 4211601), Bacillus Cereus Antimicrobial Supplement (REF 4240001), ancillary culture media and reagents.

8 – SPECIMENS

Products intended for human consumption and the feeding of animals, and environmental samples in the area of food production and food handling. For sample collection, storage, transport and preparation, follow good laboratory practice and refer to applicable International Standards.³





9 - TEST PROCEDURE

ISO 21871,³ recommends the procedure summarised here.

1. Inoculate PEMBA agar plates after selective enrichment in tryptone soya polymyxin broth (TSPB). TSPB tubes can be prepared from Tryptic Soy Broth (REF 402155) with added Bacillus Cereus Antimicrobial Supplement (REF 4240001).
2. For enumeration method inoculate three tubes of double-strength TSPB with 10 mL of the primary dilution and inoculate single-strength TSPB with 1 mL of decimal dilutions of the primary dilution (initial suspension). For detection method inoculate 1 mL of the initial suspension to 9 mL of single-strength TSPB.
3. Incubate the inoculated TSPB tubes at 30 °C for 48 h ± 4 h.
4. Mix well the TSPB tubes and streak an inoculation loop of culture from each of the tubes onto the surface of PEMBA.
5. Incubate the inoculated plates with the lid downwards at 37 °C for 18 h to 24 h.
6. If the colonies cannot be clearly assessed, continue incubating the plates for up to additional 24 h.

10 - READING AND INTERPRETATION

After incubation is complete, examine the plates for the presence of typical or atypical colonies.³

On PEMBA, typical colonies of presumptive *Bacillus cereus* are about 2 mm to 5 mm in size, have an irregular edge which is between ragged and root-like with ground glass surface, are turquoise to peacock blue, possibly with a greyish white colony centre against a blue background, and have a precipitation halo (egg yolk reaction) up to 5 mm wide.

If the plates have a high content of background flora which ferments mannitol, the characteristic coloration of the colonies and background may be reduced or no longer visible. In addition, some presumptive *Bacillus cereus* strains have only a slight egg yolk reaction or none at all. In such cases and in any other doubtful cases, these colonies should also be submitted to the confirmation.

Typical and atypical colonies on PEMBA shall be confirmed by means of the haemolysis test on sheep blood agar and a microscopic examination.

- Streak the selected colonies from PEMBA onto the surface of sheep blood agar in a manner which allows well-separated colonies to develop. Incubate at 30 °C for 24 h and read haemolysis reaction. Each colony surrounded by a cleared zone is considered to be haemolysis-positive.
- Typical and atypical colonies on PEMBA may be confirmed by means of microscopic examination using malachite green solution for staining the spores and Sudan black B solution for staining the intracellular fat globules and re-stain with safranin solution.
- Examine the slide under a microscope using immersion oil. As a rule, the brick-shaped cells of presumptive *Bacillus cereus* are arranged in chains and are 4 µm to 5 µm long, 1 µm to 1,5 µm wide and contain fairly large amounts of intracellular fat which is stained black. The green stained spores may be central or subterminal, but they never distend the red stained sporangia.

Other tests useful for differentiating typical strains of *B. cereus* from other members of the *B. cereus* group:⁵ 1) Microscopic observation (large Gram-positive rods in short-to-long chains; spores are ellipsoidal, central to sub-terminal, and do not swell the sporangium); 2) Glucose fermentation (+); 3) Voges Proskauer Reaction (+); 4) Nitrate reduction (+); 5) Motility test (+); 6) Decomposition of tyrosine (+); 7) Growth in presence of 0.001% lysozyme.

11 - USER QUALITY CONTROL

All manufactured lots of the product are released for sale after the Quality Control has been performed to check the compliance with the specifications. However, the end user can perform its own Quality Control in accordance with the local applicable regulations, in compliance with accreditation requirements and the experience of the Laboratory. Here below are listed some test strains useful for the quality control.

CONTROL STRAINS	INCUBATION T° / T / ATM	EXPECTED RESULTS
<i>B. cereus</i> ATCC 11778	18-24h /30°C/A	growth, turquoise-blue colonies with opaque halo
<i>E. coli</i> ATCC 11775	18-24h /30°C/A	no growth
<i>B. subtilis</i> ATCC 6633	18-24h /30°C/A	growth, yellow colonies without opaque halo

A: aerobic incubation; ATCC is a trademark of American Type Culture Collection

12 – PERFORMANCE CHARACTERISTICS

Prior to release for sale, representative samples of all lots of dehydrated Bacillus Cereus Agar Base, supplemented with Bacillus Cereus Antimicrobial Supplement and ready-to-use plates (Test Batch:TB) are tested for productivity, specificity and selectivity by comparing the results with Tryptic Soy Agar.

The productivity is tested by a quantitative method with the target strain *B. cereus* ATCC 11778: the plates are inoculated with decimal dilutions in saline of a colonies' suspension and incubated at 30 °C for 18-24 hours. The colonies are enumerated on both media and the productivity ratio (Pr) is calculated. If Pr is ≥ 0.5 and if the colonies morphology and colour are typical (turquoise-blue with opaque halo) the results are considered acceptable and conform to the specifications.

Moreover the productivity characteristics are tested by semi-quantitative ecometric technique with the following target strains: *B. cereus* ATCC 14579 and *B. thuringiensis* ATCC 10792. After incubation, the amount of growth and the colony characteristics are evaluated: the target strains exhibit good growth, with turquoise-blue colonies with opaque halo.

The specificity is assessed by semi-quantitative ecometric technique with *B. subtilis* ATCC 6633. After incubation, the amount of growth and the colony characteristics are evaluated: *B. subtilis* exhibits good growth with yellow colonies without opaque halo.

The selectivity is assessed with modified Miles-Misra surface drop method by inoculating the plates with suitable decimal dilutions in saline of a 0.5 McFarland suspension of *E. coli* ATCC 25922. The growth of *E. coli* is totally inhibited.

13-LIMITATIONS OF THE METHODS

- For enumeration of presumptive *Bacillus cereus* spores only, the primary dilution can be heated at 80 °C for 10 min in a water bath.³
- The confirmatory tests may in some instances be inadequate for distinguishing *B. cereus* from culturally similar organisms that could occasionally be encountered in foods. These organisms include 1) the insect pathogen *B. thuringiensis*, which produces protein toxin crystals; 2) *B. mycoides*, which characteristically produces rhizoid colonies on agar media; and 3) *B. anthracis*, which exhibits marked animal pathogenicity and is non-motile. With the exception of *B. thuringiensis*, which is currently being used for insect control on food and forage crops, these organisms are seldom encountered in the routine examination of foods.⁵
- Even if the microbial colonies on the plates are differentiated on the basis of their morphological and chromatic characteristics, it is recommended that biochemical, immunological, molecular, or mass spectrometry testing be performed on isolates, from pure culture, for complete identification.





14 - PRECAUTIONS AND WARNINGS

- The medium base, the supplements and the ready-to-use plates are for microbiological control and for professional use only; they are to be used by adequately trained and qualified laboratory personnel, observing approved biohazard precautions and aseptic techniques.
- The medium base and the supplements shall be used in association according to the described directions. Apply Good Manufacturing Practice in the production process of prepared media.
- Dehydrated media and antibiotics containing supplements must be handled with suitable protection. Before use, consult the Material Safety Data Sheets.
- This culture medium contains raw materials of animal origin. The ante and post mortem controls of the animals and those during the production and distribution cycle of the raw materials, cannot completely guarantee that the product doesn't contain any transmissible pathogen. Therefore, it is recommended that the culture medium be treated as potentially infectious, and handled observing the usual specific precautions: do not ingest, inhale, or allow to come into contact with skin, eyes, mucous membranes. Download the TSE Statement from the website www.biolifeitaliana.it, describing the measures implemented by Biolife Italiana for the risk reduction linked to infectious animal diseases.
- Each plate of this culture medium is for single use only.
- Ready-to-use plates are not to be considered a "sterile product" as they are not subject to terminal sterilization, but a product with controlled bio contamination, within the limits of defined specifications reported on the Quality Control Certificate.
- All laboratory specimens should be considered infectious.
- The laboratory area must be controlled to avoid contaminants such as medium and supplements or microbial agents.
- Sterilize all biohazard waste before disposal. Dispose the unused medium and supplements and the inoculated plates with samples or microbial strains in accordance with current local legislation.
- Do not use the culture medium and the supplement as active ingredients for pharmaceutical preparations or as production materials intended for human and animal consumption.
- The Certificates of Analysis and the Safety Data Sheets are available on the website www.biolifeitaliana.it.
- The information provided in this document has been defined to the best of our knowledge and ability and represents a guideline for the proper use of the product but without obligation or liability. In all cases existing local laws, regulations and standard procedures must be observed for the examination of samples collected from human and animal organic districts, for environmental samples and for products intended for human or animal consumption. Our information does not relieve our customers from their responsibility for checking the suitability of our product for the intended purpose.

15 - STORAGE CONDITIONS AND SHELF LIFE

Ready to use plates

Upon receipt, store plates in their original pack at +2°C /+8°C away from direct light. If properly stored, the plates may be used up to the expiration date. Do not use the plates beyond this date. Plates from opened plastic sachet can be used for 7 days when stored in a clean area at 2-8°C. Do not use the plates if the plastic sachet is damaged or if the dish is broken. Do not use the plates with signs of deterioration (e.g., microbial contamination, dehydration, shrinking or cracking of the medium, atypical colour, excess of moisture).

Dehydrated medium

Upon receipt, store at +10°C /+30°C away from direct light in a dry place. If properly stored, it may be used up to the expiration date. Do not use beyond this date. Avoid opening the bottle in humid places. After use, the container must be tightly closed. Discard the product if the container and/or the cap are damaged, or if the container is not well closed, or in case of evident deterioration of the powder (colour changes, hardening, large lumps).

The user is responsible for the manufacturing and quality control processes of prepared media and the validation of their shelf life, according to the type (plates/bottles) and the applied storage conditions (temperature and packaging). According to ISO 21871 the plates may be stored prior to drying at between 1 °C and 5 °C for up to 4 days.³

16 - REFERENCES

1. Turene C, Alexander DC Bacillus and other Aerobic Endospore-Forming Bacteria. In Carrol KC, Pfaller MA et al. editors. Manual of clinical microbiology, 12th ed. Washington, DC: American Society for Microbiology; 2019.
2. Holbrook R, Anderson JM. An improved selective and diagnostic medium for the isolation of Bacillus cereus in foods. Can J Microbiol 1980; 26: 753-759.
3. ISO 21871:2005 - Microbiology of food and animal feeding stuffs -- Horizontal method for the determination of low numbers of presumptive Bacillus cereus -- Most probable number technique and detection method
4. Baird RM, Corry JEL, Curtis GDW. Pharmacopoeia of Culture Media for Food Microbiology. Proceedings of the 4th International Symposium on Quality Assurance and Quality Control of Microbiological Culture Media, Manchester 4-5 September, 1986. Int J Food Microbiol 1987; 233-234.
5. US Food and Drug Administration. Bacteriological Analytical Manual (BAM) Chapter 14: Bacillus cereus. Content current as of: 06/29/2021

TABLE OF APPLICABLE SYMBOLS

REF or REF Catalogue number	LOT Batch code	Manufacturer	This side up	Store in a dry place	Fragile
Temperature limitation	Content sufficient for <n> tests	Consult Instructions for Use	Use by	Keep away from direct light	For single use only

REVISION HISTORY

Version	Description of changes	Date
Revision 1	Updated layout and content	2022/05
Revision 2	Modifications to chapters 2, 5, 6, 7, 14, 15	2022/12

Note: minor typographical, grammatical, and formatting changes are not included in the revision history.

