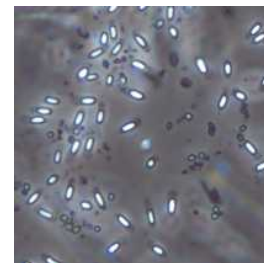


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High-Yield Sporulation and Purification of *Bacillus cereus* and *Bacillus thuringiensis* Spores

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We use this protocol and it's working

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Abstract

This protocol describes a synchronized sporulation method for *Bacillus cereus* and *Bacillus thuringiensis*, optimized for high spore yield and purity. The procedure includes preparation of culture media (BHI, PCA, and Difco Sporulation Medium), stepwise cultivation to induce sporulation, and two complementary spore purification approaches—one simple heat-treatment and washing method, and one enhanced method using a HistoDenz® density gradient. The addition of manganese, iron, and calcium salts enhances sporulation efficiency. The protocol enables the production of spore suspensions with >90% purity as confirmed by phase contrast microscopy. It is suitable for applications in microbiology and biotechnology where controlled, high-quality spore production is required.



Guidelines

- This protocol outlines the synchronized sporulation of *Bacillus cereus* and *Bacillus thuringiensis*, including culturing, sporulation induction, and spore purification via simple and density-gradient methods.
- All reagents, culture media, and consumables must be sterile to ensure culture integrity and prevent contamination.
- Maintain consistent incubation temperatures and agitation speeds, as sporulation efficiency is highly sensitive to environmental conditions.
- Perform microscopy validation (e.g., phase contrast) to assess sporulation efficiency (>90% spores) before proceeding to purification steps.
- Choose the **simple purification method** for rapid downstream applications or use the **enhanced density-gradient method** to achieve highly purified spore preparations.
- Ensure that all centrifugation steps are performed with properly balanced tubes and temperature control when required.

Materials

REAGENTS

- **Meat Extract (ME)**, powdered culture medium ingredient
- **Meat Peptone (MP)**, powdered culture medium ingredient
- **Plate Count Agar (PCA)**, powdered culture medium
- **Brain Heart Infusion (BHI)**, powdered culture medium
- **Potassium Chloride (KCl)** solution, 10% (w/v), sterile-filtered
- **Magnesium Sulfate Heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)** solution, 1.2% (w/v), sterile-filtered
- **Sodium Hydroxide (NaOH)** solution, 1 M
- **Manganese(II) Chloride (MnCl_2)** solution, 0.01 M, sterile-filtered
- **Iron(II) Sulfate (FeSO_4)** solution, 1 mM, sterile-filtered
- **Calcium Nitrate [$\text{Ca}(\text{NO}_3)_2$]** solution, 1 M, sterile-filtered
- **Phosphate-Buffered Saline (PBS)**, pH 7.4
- **PBS with 0.5% Triton X-100**, pH 7.4
- **HistoDenz[®]**
- **Lysozyme**, $\geq 10,000$ U/mg protein
- **Deionized (DI) water**

LABWARE AND EQUIPMENT

- 15 mL glass test tubes
- 200 mL Schott glass media bottles
- 1000 mL Erlenmeyer flasks (with baffles if available)
- 2000 mL flat-bottom glass flasks
- P200 micropipette and sterile tips
- 10 mL disposable plastic pipettes and pipettor
- Stainless steel spatula
- Sterile disposable reagent reservoirs
- Autoclavable glass beads
- Orbital shaker with temperature control
- Phase contrast optical microscope
- Cuvette spectrophotometer (600 nm capability)
- Precision analytical balance (± 0.1 mg)
- Water baths (70°C and 50°C presets)
- Centrifuge (capable of 10,000×g)

MICROORGANISMS

- *Bacillus cereus* stock culture (e.g., ATCC 14579)
- *Bacillus thuringiensis* stock culture (e.g., ATCC 10792)

Safety warnings

- ❗
 - Do not overtighten bottle caps during autoclaving — pressure buildup may cause explosion or deformation of the bottles.
 - *Bacillus cereus* is a biosafety level 2 organism and a known human pathogen. Always handle cultures in accordance with institutional biosafety guidelines.
 - Avoid direct contact with **hot culture media**, glassware, or water baths — use heat-resistant gloves and handle with care.
 - **Histodenz solutions are dense and slippery** — layer carefully to avoid mixing phases during density-gradient setup.
 - Ensure complete removal of supernatants during washing steps to avoid dilution of spore preparations or carryover of toxic reagents.
 - Use sterile technique when adding filtered MnCl_2 , FeSO_4 , and $\text{Ca}(\text{NO}_3)_2$ — contamination at this stage can compromise sporulation efficiency.

Before start

Prepare all stock solutions in advance:

- 10% KCl
- 1.2% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
- 0.01 M MnCl_2 (sterile filtered)
- 1 mM FeSO_4 (sterile filtered)
- 1 M $\text{Ca}(\text{NO}_3)_2$ (sterile filtered)
- Lysozyme working solution (10 $\mu\text{g}/\text{mL}$ in PBS-Triton X-100)
- **Sterilize all glassware and media** by autoclaving at 121°C for 15 minutes unless otherwise indicated.
- **Label all media and bottles** with preparation date, pH (if adjusted), and composition to ensure proper tracking during multi-day cultivation.
- Confirm **availability of a phase contrast microscope** for sporulation assessment.
- Prepare and pre-warm water baths and incubators to their required temperatures prior to beginning timed steps.



Brain Heart Infusion (BHI) liquid bacterial culturing medium preparation (150 mL final volume)

1 Fill a 250mL Schott flask with 150mL DI water and heat in microwave in defrost power setting;



2 Add 5.55 g of BHI powder to DI water and homogenize by agitation;



3 Close the flask and autoclave for 15' at 121°C.



Note: Make sure the screw cap is loose enough to prevent pressure build-up inside the flask during autoclaving.

4 Remove from autoclave and cool to RT. Screw cap tightly and store at 4°C;

PCA solid bacterial culturing medium preparation (500 mL final volume);

5 Dispense glass beads into a 1000 mL flat bottom flask until bottom is covered. Transfer 500 mL of DI water into the flask;

6 Weigh 11.25 g of powdered PCA culture medium and add it to the flask containing DI water;



7 Homogenize the mixture by agitation at RT;



Note: Avoid creating foam;

8 Bring mixture to a boil over a Bunsen burner;



Note: Agitate frequently to avoid overheating the medium on the bottom of the flask. Wear a heat-protecting glove;

9 When mixture becomes clear and homogenous, remove from heat;



Note: Do not place the flask directly on a cold surface to avoid glass shattering. Place it over a cloth or another adequate material.

10 Dispense the culture medium to small glass bottles suitable for autoclaving. Autoclave for 15' at 120°C;





Note: Fill the bottles to half capacity to avoid overflowing during autoclaving.

- 11 If the medium is to be used immediately after, dispense into plastic Petri dishes ($\approx 20\text{mL}$ per dish), otherwise store the medium in the bottles. Store at 4°C ;

Note: When necessary, take the bottle containing the PCA culture medium, melt it in a water bath, and pour it into plastic Petri dishes. The Petri dishes containing the culture medium should be stored inverted at 4°C . If prolonged storage at 4°C is required, keep the dishes inside a sealed plastic bag to prevent desiccation.

Difco Sporulating Medium (DSM) preparation (1000 mL final volume)

- 12 Transfer 500mL of DI water to a 1000 mL Erlenmeyer flask. Add 3.0g Beef Extract powdered culture medium ingredient and 5.0 g of Peptone powdered culture medium ingredient. Homogenize by agitation; 

- 13 Add 10mL 10% KCl and 10mL 1.2% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Homogenize by agitation; 

- 14 Add DI water up to near 1000 mL and correct pH to 7.6 using 1 M NaOH dropwise under stirring. Make up the volume to 1000mL;

- 15 Dispense the culture medium to small glass bottles suitable for autoclaving. Autoclave for 15' at 120°C ;

Note: Fill the bottles to half capacity to avoid overflowing during autoclaving.

- 16 Let medium cool to RT. If the medium is not to be used immediately, screw bottle caps tightly and store bottles at 4°C ;

Note: Incubate a control bottle at 37°C after autoclaving as a sterility check;

Cultivation and sporulation

- 17 Transfer 10 mL of BHI liquid culturing medium to a 15mL glass test tube;

- 18 Transfer 100 μL of *Bacillus cereus* / *thuringiensis* bacterial stock to the tube containing the BHI culture medium;

- 19 Incubate at 37°C for 24h; 



20 Vortex the bacterial culture for 10 seconds and transfer 100 μ L to a petri dish containing PCA culture medium. Inoculate the medium by streak plating;

Note: Use streak inoculation technique to obtain isolated bacterial colonies;

21 Incubate at 37°C for 24h;



22 Using an inoculating loop, remove an isolated bacterial colony from the petri dish containing PCA culture medium and transfer it to a 2000 mL flat bottom flask containing 500 mL of DSM culture medium;

23 Incubate the medium at 30°C on an orbital shaker at 150 rpm for 72h;



24 Centrifuge the culture medium at 4000 x g for 15 minutes and discard the supernatant;

25 Resuspend the pellet and transfer it to a 2000 mL flat bottom flask containing 500 mL of DSM culture medium;

Note: Reintroduction of the pellet into fresh DSM medium increases spore density through subculturing.

26 Add 500 μ L of sterile 0.01M MnCl₂, 500 μ L of 1mM FeSO₄, and 500 μ L of 1M Ca(NO₃)₂ to the DSM culture medium;

Note: Sterilize solutions using a 0.2 μ m syringe filter before adding to culture medium.

27 Incubate the medium at 30°C on an orbital shaker at 150 rpm for 72h;

Note: After 72h visualize the suspension in a phase contrast optical microscope at 400 x and 1000 x magnification. Bacterial spores should amount to >90% of the total microbial mass, identifiable by the presence of refractive ovals;

Spore purification (Simple method)

28 Place the culture in a hot bath at 70°C for 10 minutes;

Note: Thermal shock will eliminate remaining vegetative forms without damaging spore structures.

29 Centrifuge the cultures at 10 000xg for 5 minutes and remove the supernatant; Resuspend the pellet in 15mL of DI water at 4°C;



Note: Repeat this washing step 3 times;

- 30 Resuspend the pellet in 15mL of DI water at 4°C;

Note: For short-term storage, resuspend spores in PBS + 0.5% Triton X-100 at 4°C. For long-term storage, resuspend spores in bacterial freezing medium containing 15% glycerol at -80°C.

Spore purification (Enhanced method)

- 31 Place the culture in a hot bath at 70°C for 10 minutes;

Note: Thermal shock will eliminate remaining vegetative forms without damaging spore structures.

- 32 Perform density-gradient centrifugation

- 32.1 Transfer a 50% HistoDenz[®] (w/v) solution to a 15 mL or 50 mL Falcon tube;

- 32.2 Transfer an equal volume of spore suspension to the falcon tube;

Note: Transfer spore suspension carefully, making sure the two liquid phases are separated.

- 32.3 Centrifuge the falcon tube at 4500 × g for 10 min;

Note: Spores deposit at the bottom or just above the bottom of the 50% HistoDenz layer, or form a pellet at the bottom of the tube, depending on their density and the volume used.

- 32.4 Using a pipette, carefully aspirate the supernatant and stop just before reaching the denser bottom layer or visible pellet;

Note: The supernatant will contain a mixture of immature and damaged spores as well as cellular debris, and should be discarded to enhance the spore suspension's purity.

- 32.5 Resuspend the pellet in 15mL of DI water at 4°C and centrifuge the suspension at 10 000xg for 5 minutes. Remove the supernatant;

Note: Repeat this washing step 3 times;

- 33 Resuspend the spores in PBS-TritonX 0.5% containing lysozyme at 10 µg/mL. Incubate for 1h at 4°C;



34 Store the spore suspension at 4°C in PBS-TritonX 0.5% for 7 days.

Note: After 7 days visualize the suspension in a phase contrast optical microscope. Phase-bright bacterial spores should amount to >95% of the total microbial mass;

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