

Cytotoxicity assay for detection of cereulide produced by emetic *Bacillus cereus*

Elrike Frenzel¹ and Monika Ehling-Schulz^{2*}

¹Department of Molecular Genetics, Rijksuniversiteit Groningen/ Groningen Biomolecular Sciences and Biotechnology Institute, Groningen, The Netherlands; ²Department of Pathobiology, University of Veterinary Medicine Vienna/Institute of Microbiology, Vienna, Austria

*For correspondence: monika.ehling-schulz@vetmeduni.ac.at

[Abstract] The emetic subgroup of *Bacillus cereus* strains produces cereulide, a dodecadepsipeptide (1.2 kDa), which is the causative agent of food poisonings. Cereulide is synthesized by a nonribosomal peptide synthetase (NRPS), the Ces-NRPS (Ehling-Schulz *et al.*, 2006). Cereulide is a cyclic and lipophilic potassium ionophor structurally related to the macrolide antibiotic valinomycin. Both substances act on mitochondria by depolarization and uncoupling of ATP synthesis, and this effect (mitochondrial swelling) is used to quantify cereulide in a HEp-2 cell based viability assay.

In this protocol, which was modified from an assay published by Finlay *et al.* (1999), valinomycin is used as a reference standard for ionophor-induced cytotoxicity, because purified cereulide is not commercially available yet. This assay has been used to quantify cereulide amounts from different *B. cereus* mutants (Lücking *et al.*, 2009; Frenzel *et al.*, 2012) and to estimate cereulide levels extracted from foods (artificially) contaminated with *B. cereus* (Frenzel *et al.*, 2011).

Materials and Reagents

1. *Bacillus cereus* F4810/72 (or other cereulide producing, emetic *B. cereus* strain)
2. Tryptone
3. Yeast extract
4. NaCl
5. HEp-2 cell line (Frenzel *et al.*, 2011)
6. MEM Earle's medium (with 2.2 g/L NaHCO₃, with stable glutamine) (Biochrom, catalog number: FG0325)
7. Penicillin-streptomycin solution (10,000 µg/ml) (Biochrom, catalog number: A2212/3)
8. Sodium pyruvate solution (Biochrom, catalog number: L0473)
9. Fetal bovine serum (FBS) (Biochrom, catalog number: S0113)
10. Phosphate Buffered Saline (PBS) (Biochrom, catalog number: L1825)
11. 99% Ethanol (highest purity grade available)

12. WST-1 cell proliferation reagent (Roche, catalog number: 05015944001)
13. Valinomycin (reference standard) (Sigma-Aldrich, catalog number: 94675)
14. 10x Trypsin/EDTA solution (0.5 %/0.2 %) (Biochrom, catalog number: L2153)
15. LB-Miller (see Recipes)
16. MEM-Earle HEp-2 medium (see Recipes)
17. MEM-Earle HEp-2 medium with ethanol (see Recipes)

Equipment

1. 30 °C incubator with aeration (for liquid bacterial cultures)
2. 37 °C incubator with 5% CO₂ (for HEp-2 cell cultures)
3. Spectrophotometer
4. Table top centrifuge
5. Microplate shaker
6. Microscope and counting chamber for cell culture
7. Microscope and counting chamber for bacteria (e.g. Helber counting chamber; depth: 0.02 mm; small square area: 0.0025 mm²)
8. Multichannel pipet (8 channels, manual or electronic; e.g. Transferpette[®], BrandTech Scientific 10-200 µl, catalog number: 2705410)
9. Microplate reader (multiwell scanning spectrophotometer)
10. 500 ml baffled flask
11. Tissue culture flasks with filter caps, 75 cm² (Biochrom, catalog number: P90076)
12. Safe seal tubes (e.g. Eppendorf, catalog number: 0030120.094) 96-well cell culture plates, polystyrene (Biochrom, catalog number: P92696)

Procedure

1. Cereulide sample preparation (*B. cereus* culture and growth conditions)
 - a. Inoculate 3 ml LB medium with *B. cereus* and incubate at 30 °C, 150 rpm for 16 h (pre-culture).
 - b. After 16 h, determine the cell count (CFU/ml) of the pre-culture with a counting chamber appropriate for bacteria (e.g. Helber counting chamber). Inoculate 100 ml LB in 500 ml baffled flask with 10³ CFU/ml (approx. 1:10,000 dilution; i.e. add approx. 100 µl of 1:100 dilution of bacteria to 100 ml LB). Incubate at 30 °C, 150 rpm for 24 h.
 - c. Cereulide accumulates to stable levels after 24 h of cultivation at 30 °C (Frenzel *et al.*, 2011). Withdraw 1-2 ml samples of *B. cereus* cultures and autoclave in safe seal

tubes (17 min at 121 °C). This step will denature heat-labile enterotoxins and proteases, whereas cereulide remains stable. Store samples at -20 °C.

- d. The remaining part of the *B. cereus* culture is autoclaved (17 min at 121 °C) and disposed.
2. HEp-2 cell based cereulide cytotoxicity assay
 - a. Maintain HEp-2 cells as monolayers in 75 cm² tissue culture flasks at 37 °C in a 5% CO₂ atmosphere. Routinely grow HEp-2 cells in 25 ml MEM-Earle HEp-2 medium and sub-culture cells 1:4 approximately every 2-3 days when they reach 70-80% confluence. To detach adherent cells for sub-culturing, add 5 ml of trypsin/EDTA solution and incubate for 5-10 min at 37 °C. Add 5 ml of MEM-Earle HEp-2 medium and resuspend detached HEp-2 cells. Centrifuge cells (5 min, 150 x g, room temperature) and resuspend pellet in 10 ml MEM-Earle HEp-2 medium. Split cells 1:4 in new tissue culture flask containing 25 ml MEM-Earle HEp-2 medium.
 - b. If cells from point 2a) reach 70-80% confluence, cytotoxicity assay can be performed. Add 90 µl of MEM-Earle HEp-2 medium with ethanol to wells A1 to H1 of a 96-well cell culture plate (see Figure 1). Fill the remaining wells with 50 µl MEM-Earle HEp-2 medium with ethanol.

	1	2	3	4	5	6	7	8	9	10	11	12
A	90 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM
B	90 µl MEM + 10 µl val	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM
C	90 µl MEM + 10 µl val	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM
D	90 µl MEM + 10 µl sample 1	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM
E	90 µl MEM + 10 µl sample 1	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM
F	90 µl MEM + 10 µl sample 2	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM
G	90 µl MEM + 10 µl sample 2	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM
H	90 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM	50 µl MEM

Figure 1. Example for setup of cereulide cytotoxicity 96-well assay plate. MEM = MEM-Earle HEp-2 medium with ethanol; val = valinomycin (5 µg/ml); green= background control wells; orange = 100% cell viability wells without test substance.

- c. Dilute valinomycin stock solution (50 µg/ml) 1:10 with MEM-Earle HEp-2 medium with ethanol. Add 10 µl in wells B1 and C1 (final concentration is 500 ng/ml).
 - d. Two additional samples (prepared as described in point 1 and stored at -20 °C) can be tested on this plate: Add 10 µl of sample 1 to wells D1 and E1; add 10 µl of sample 2 to wells F1 and G1. Rows A and H remain free of samples (background control wells).
 - e. Mix samples and medium thoroughly by repeated pipetting using a multichannel pipet. Remove 50 µl of the wells and perform serial dilutions (1:1) of the samples to column A10-H10. Columns 11 and 12 remain free of sample (100% cell viability control wells).
 - f. Remove growth medium from a HEp-2 culture flask containing a 70-80% confluent monolayer. Wash the cells with 5 ml PBS. Add 5 ml of trypsin/EDTA solution and incubate for 5-10 min at 37 °C.
 - g. Add 5 ml of MEM-Earle HEp-2 medium and resuspend detached HEp-2 cells.
 - h. Centrifuge cells (5 min, 150 x g, room temperature) and resuspend pellet in 10 ml MEM-Earle HEp-2 medium.
 - i. Determine cell count with counting chamber. Adjust the cells count to 3.4×10^5 cells/ml.
 - j. Dispense 150 µl of cells per well ($\sim 5 \times 10^4$ cells/well). Incubate plate for 48 h at 37 °C in a 5% CO₂ atmosphere.
 - k. To save WST-1 reagent, discard 100 µl of culture medium from each well and add 10 µl of WST-1 reagent to the remaining culture to detect mitochondrial activity of HEp-2 cells. Rows A and H remain free of WST-1 for calculation of the background value. Shake plate to distribute proliferation reagent uniformly and incubate plate for 20 min at 37 °C in a 5% CO₂ atmosphere.
 - l. Shake plate thoroughly and measure absorbance in a microplate reader at 450 nm.
3. Calculation of cereulide amounts
- a. Import raw data from absorbance measurement to calculation/statistics program (e.g. Microsoft Excel).
 - b. Perform background subtraction on all wells with the mean of the background control wells (Rows A and H; see Figure 2). Calculate dose-response curve for the internal standard valinomycin [Percent viability inhibition = (mean absorbance test wells / mean absorbance 100% cell viability control wells) x 100]. Determine the 50% inhibitory concentration for valinomycin by linear regression.
 - c. Calculate dose-response curves for samples as given above. Determine the 50% inhibitory concentration for samples (presented by the reciprocal value of the sample dilution that resulted in 50% loss of mitochondrial activity). Calculate cereulide

amounts as valinomycin equivalents (VE) by extrapolating the absorbance value at 50% cell viability inhibition to the dose response curve for valinomycin.

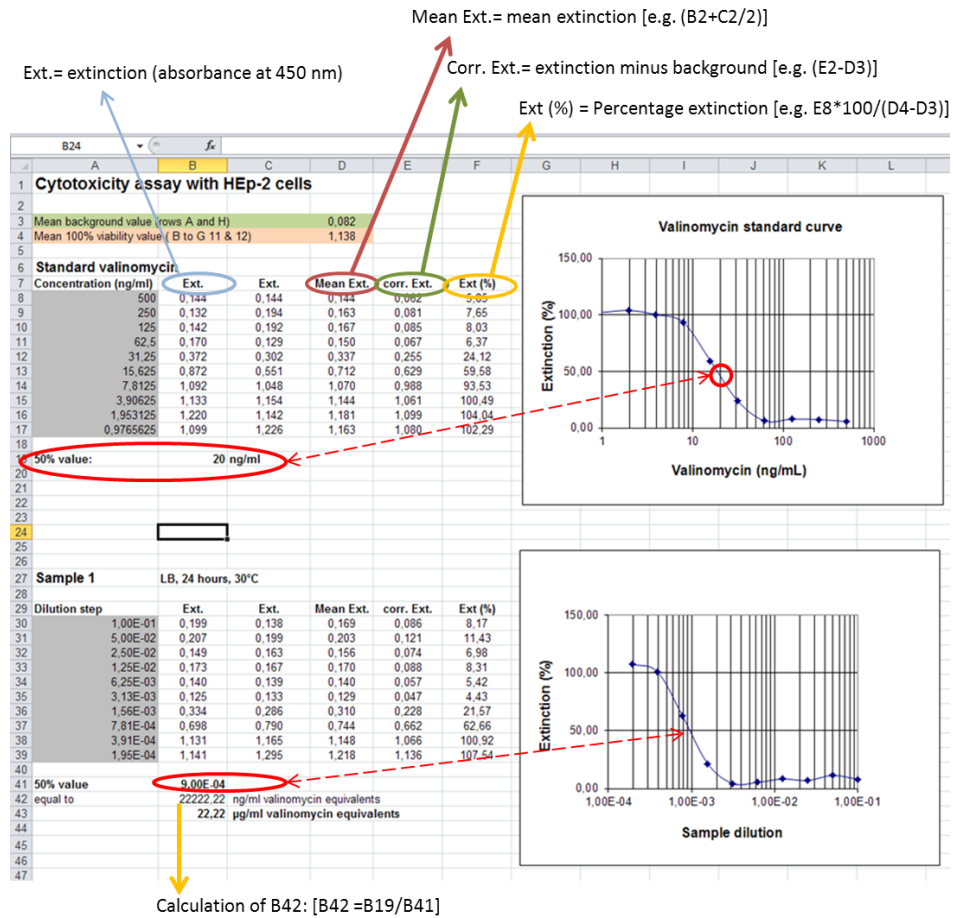


Figure 2. Example for calculation of cereulide concentrations (given in valinomycin equivalents) from dose-response-curves plotted in Microsoft Excel

Recipes

- LB-Miller
 - 10 g tryptone
 - 5 g yeast extract
 - 10 g NaCl
 - Add dH₂O to 1 L
 - Adjust pH to 7.0 ± 0.2
 - Autoclave 121 °C, 15 min
- MEM-Earle HEP-2 medium
 - 488 ml MEM Earle's medium (with 2.2 g/L NaHCO₃, with stable glutamine)

- 2 ml penicillin-streptomycin solution (10,000 µg/ml)
- 5 ml sodium pyruvate solution
- 5 ml FBS
- 3. MEM-Earle HEp-2 medium with ethanol
 - 478 ml MEM Earle's medium (with 2.2 g/L NaHCO₃, with stable glutamine)
 - 2 ml penicillin-streptomycin solution (10,000 µg/ml)
 - 5 ml sodium pyruvate solution
 - 5 ml FBS
 - 10 ml 99% ethanol (solvent for cereulide); highest purity grade available

Acknowledgments

This protocol was adapted from previously published papers: Finlay *et al.* (1999); Lücking *et al.* (2009); and Frenzel *et al.* (2011). This research project was supported by the German Ministry of Economics and Technology (via AiF) and the FEI (Forschungskreis der Ernährungsindustrie e.V., Bonn), projects AiF 15186 N and 16845 N. We thank Prof. Dr. Dr. E. Märklbauer and Dr. R. Dietrich (Ludwig-Maximilians-University Munich, Germany) for kindly providing the HEp-2 cell line.

References

1. Ehling-Schul, M., Vukov, N., Schulz, A., Shaheen, R., Andersson, M., Martlbauer, E., and Scherer, S. (2005). [Identification and partial characterization of the nonribosomal peptide synthetase gene responsible for cereulide production in emetic *Bacillus cereus*](#). *Appl Environ Microbiol* 71, 105-113.
2. Finlay, W. J., Logan, N. A., Sutherland, A. D. (1999). [Semiautomated metabolic staining assay for *Bacillus cereus* emetic toxin](#). *Appl Environ Microbiol* 65, 1811–1812.
3. Frenzel, E., Doll, V., Pauthner, M., Lücking, G., Scherer, S., Ehling-Schulz, M. (2012). [CodY orchestrates the expression of virulence determinants in emetic *Bacillus cereus* by impacting key regulatory circuits](#). *Mol Microbiol* 85 (1), 67-88.
4. Frenzel E., Letzel, T., Scherer, S., Ehling-Schulz, M. (2011). [Inhibition of cereulide toxin synthesis by emetic *Bacillus cereus* via long-chain poly-phosphates](#). *Appl Environ Microbiol* 77, 1475-1482.
5. Lücking, G., Dommel, M. K., Scherer, S., Fouet, A., Ehling-Schulz, M. (2009). [Cereulide synthesis in emetic *Bacillus cereus* is controlled by the transition state regulator AbrB, but not by the virulence regulator PlcR](#). *Microbiology* 155, 922-931.

6. The HEp-2 cell line is a permanent human larynx carcinoma cell line. The cells were kindly provided by Prof. E. Martlbauer and Dr. R. Dietrich (Ludwig Maximilians University Munich, Germany). See Nielsen, C., Casteel, M., Didier, A., Dietrich, R., Martlbauer, E. (2009). [Trichothecene-induced cytotoxicity on human cell lines](#). *Mycotox Res* 25, 77-84.