



Method Validation Report for the Enumeration of *Bacillus Cereus* Using Compact-Dry BC Medium

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Abstract

This study presents a detailed method validation report for the detection and enumeration of *Bacillus cereus* using the Compact Dry *Bacillus cereus* medium, which is a chromogenic and dehydrated culture plate designed for microbiological analysis. The primary objective of this study was to evaluate the analytical performance of the method across multiple food sample categories, including dairy-based products and rice-based food samples. These matrices were intentionally contaminated with known concentrations of *Bacillus cereus* to facilitate controlled validation.

The validation focused on assessing critical performance parameters such as linearity, repeatability, reproducibility, accuracy, and the limit of detection. Artificial contamination was applied to the food samples to achieve defined levels of *Bacillus cereus* cells for precision evaluation. The degree of analytical precision varied depending on the specific type of food matrix tested, reflecting the method's performance in diverse conditions.

The results clearly demonstrated that the Compact Dry *Bacillus cereus* medium consistently supported the selective growth and accurate identification of *Bacillus cereus* colonies, which appeared with a distinct blue green coloration. The method exhibited a high degree of specificity, with negligible interference from non-target microorganisms, and showed excellent reproducibility across different analysts.

Quantitative results obtained using this method closely matched those generated by conventional reference procedures, thereby confirming its reliability. The study concludes that the Compact Dry *Bacillus cereus* medium is a suitable, efficient, and user-friendly alternative for the routine detection and enumeration of *Bacillus cereus* in food safety testing laboratories.

Key words: *Bacillus cereus* enumeration, Method validation, Compact Dry *Bacillus cereus* medium, Food microbiology, Chromogenic culture plates, Food safety testing.



Introduction

Bacillus cereus is a Gram-positive, rod-shaped, spore-forming bacterium that is widely distributed in the environment, including soil, water, and the gastrointestinal tracts of both humans and animals. It is recognized as an opportunistic pathogen responsible for foodborne illnesses in humans, primarily resulting from the ingestion of contaminated food products (Yu et al., 2019). The organism is capable of producing both heat-stable and heat-labile toxins, which are associated with two distinct syndromes: the emetic form caused by cereulide (a heat-stable cyclic peptide), and the diarrheal form associated with enterotoxins such as hemolysin BL (HBL), nonhemolytic enterotoxin (NHE), and cytotoxin K (CytK). (Mohammadi et al., 2022), (Liu et al., 2020)

Due to its widespread occurrence and potential health risks, *Bacillus cereus* represents a significant concern in the food industry. One of its key survival mechanisms is the ability to form endospores, which can endure thermal processing methods such as cooking. These spores may germinate and proliferate if food is stored or handled under suboptimal conditions. Outbreaks are frequently linked to improperly stored or reheated foods, particularly rice, pasta, dairy products, and meat-based items. Epidemiological studies indicate that *Bacillus cereus* contributes to a considerable global burden of foodborne illnesses. In the United States alone, it is estimated to cause approximately 63,400 cases of illness annually. (Mohammadi et al., 2022)

Given these risks, the implementation of rapid, reliable, and efficient detection methods is imperative for effective food safety monitoring. Conventional detection approaches involve culturing on selective media, followed by biochemical and molecular confirmation steps. While these methods are accurate, they are often time-consuming and labour-intensive.

In response to these challenges, the Compact Dry *Bacillus cereus* medium has been developed as a chromogenic, ready-to-use alternative for the direct enumeration of *Bacillus cereus*. This medium allows for the rapid identification of the bacterium based on the development of characteristic blue-green colonies upon incubation.

Method Validation Overview: Validation of the Compact Dry *Bacillus cereus* method is essential to confirm its suitability for diverse food matrices. A study published in the Journal of AOAC International (2023) evaluated this method against the ISO 7932:2004 reference standard across ten different food matrices, including dairy products, meats, and ready-to-eat meals. The findings demonstrated that the Compact Dry method delivered comparable results in terms of repeatability, accuracy, and robustness, with a relative standard deviation of repeatability (RSDr)



of less than or equal to 9.2 percent for 28 out of 30 matrix and inoculum level combinations tested. (Liu et al., 2020)

Current Validation Study: In the present validation study, two food matrices raw cheese and flour, were selected to evaluate the method's performance. These samples were artificially inoculated with known concentrations of *Bacillus cereus* to ensure consistent distribution and achieve desired inoculum levels. Testing was performed by four trained analysts using identical method protocols. The data obtained were used to assess the following analytical parameters:

Linearity: Linearity refers to the ability of the method to produce results that are directly proportional to the concentration of the analyte within a specified range. Any deviation from linear behaviour across the tested range contributes to linearity uncertainty.

Repeatability: Repeatability is defined as the consistency of test results when the same sample is analysed multiple times under the same conditions. In this study, cheese and flour samples spiked with a known concentration of *Bacillus cereus* were tested in ten replicates. The mean, standard deviation, and relative standard deviation were calculated to assess repeatability.

Reproducibility: Reproducibility evaluates the consistency of test results when analyses are performed by different analysts using the same method protocol. Spiked cheese and flour samples were tested by four independent analysts, and the mean, standard deviation, and relative standard deviation were calculated to determine inter-analyst variability.

This rigorous validation confirms the applicability of the Compact Dry *Bacillus cereus* medium for reliable and efficient detection of *Bacillus cereus* in commonly consumed food products. The method's simplicity and rapid turnaround make it a practical solution for routine monitoring in food safety laboratories.

Methodology

Preparation of the Stock Culture: One lyophilized pellet of *Bacillus cereus* strain ATCC 10876, obtained from Microbiologics, was rehydrated using sterile phosphate buffered saline. The buffer was pre-incubated at thirty-five degrees Celsius for thirty minutes prior to inoculation. The resulting suspension was then incubated aerobically at thirty-five degrees Celsius for twenty-four hours to allow for optimal bacterial revival and proliferation. Following incubation, a serial dilution of the culture was performed to verify and quantify the concentration of viable bacterial cells.



Artificial Contamination of Food Samples: Two distinct food matrices, namely cheese and flour, were selected for method validation. These samples were artificially contaminated using the cell suspension prepared in Step One. Each matrix was spiked with graded concentrations of the bacterial suspension, specifically targeting final concentrations ranging from ten to the power of minus five to ten to the power of minus seven colony forming units per millilitre. This approach was employed to simulate various levels of contamination and to assess the performance of the detection method across a realistic microbial load spectrum.

Method Validation Parameters: The analytical method was validated by evaluating the linearity, repeatability, reproducibility, accuracy, limit of detection.

Results

Linearity Results.

The table 01 below presents the viable counts obtained from serial dilutions of the rehydrated *Bacillus cereus* culture. It includes mean values and variability measures to confirm culture consistency.

Table 01: Stock Culture for *Bacillus cereus*

Dilution	Sterile Distilled Water										Mean	Standard Deviation	% Relative Standard
10 ⁻⁵	320	300	330	320	300	310	330	340	300	350	320	17.64	5.51
10 ⁻⁶	34	35	33	34	35	36	33	35	34	36	34.5	1.08	3.13
10 ⁻⁷	3	3	3	3	3	5	3	4	3	4	3.4	0.70	20.57
10 ⁻⁸	0	0	0	0	0	0	0	0	0	0	0	0	0

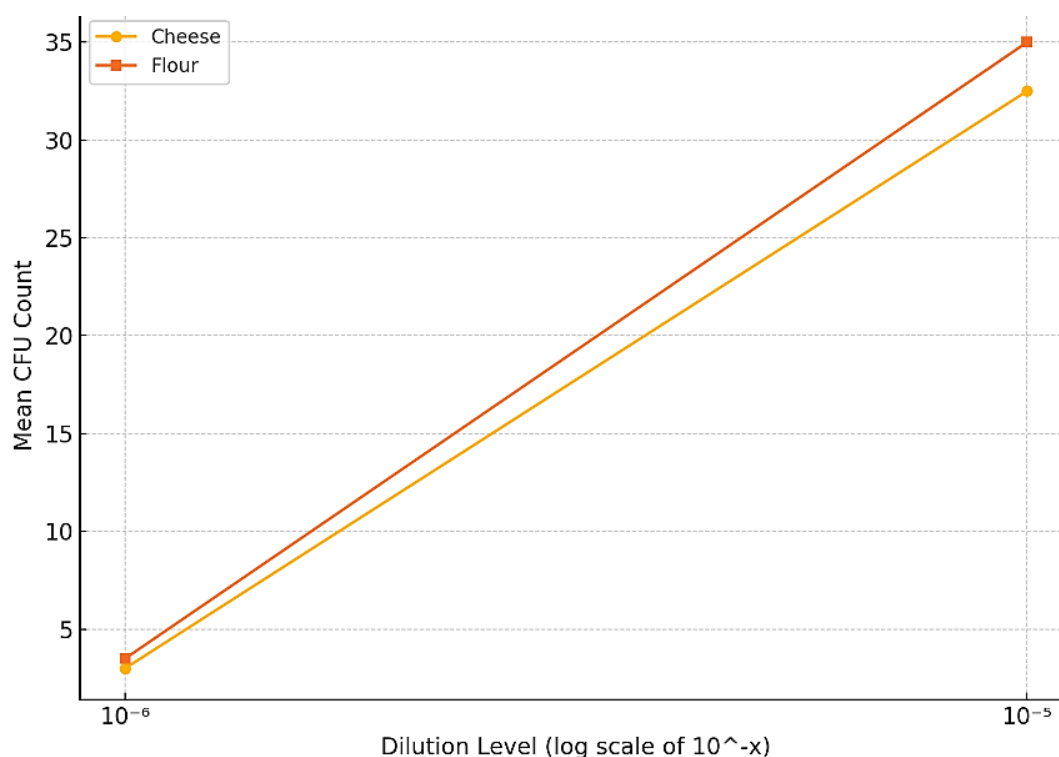
The table 02 below summarizes the microbial counts for cheese and flour across dilution levels. The results demonstrate a consistent linear relationship essential for quantitative analysis.



Samples	Cheese					Flour				
Dilutions	Values		Mean	SD	% RSD	Values		Mean	SD	% RSD
10^{-5}	33	32	32.5	0.71	2.18	34	36	35	1.41	4.05
10^{-6}	3	3	3	0	0	3	4	3.5	0.71	20.20
10^{-7}	0	0	0	0	0	0	0	0	0	0

Table 02: Linearity for Cheese and Flour

The graph 01 below depicts the linearity of response in cheese samples and flour samples across decreasing concentrations. The trend line confirms proportional detection accuracy within the tested range.



Graph 01: Linearity of *Bacillus cereus* in cheese sample and flour sample



Repeatability Results

The tables 03, 04, 05 and 06 below present repeatability data for cheese and flour samples, analysed independently by two analysts. Consistency in results supports method precision under identical conditions.

Table 03: Repeatability for Cheese by Analyst 01

Dilution	Repeatability										Mean	SD	RSD
	1	2	3	4	5	6	7	8	9	10			
10 ⁻⁵	33	36	35	30	32	34	33	31	35	33	33.2	1.88	5.64
10 ⁻⁶	3	3	3	3	2	5	4	3	3	3	3.2	0.78	24.65

Table 04: Repeatability for Cheese by Analyst 02

Dilution	Repeatability										Mean	SD	RSD
	1	2	3	4	5	6	7	8	9	10			
10 ⁻⁵	34	35	33	30	32	36	33	35	32	31	33.1	1.91	5.78
10 ⁻⁶	3	4	3	3	2	5	3	4	3	3	3.3	0.82	24.94

Table 05: Repeatability for Flour by Analyst 01

Dilution	Repeatability										Mean	SD	RSD
	1	2	3	4	5	6	7	8	9	10			
10 ⁻⁵	32	33	30	34	36	32	33	34	32	34	33	1.63	4.95
10 ⁻⁶	3	3	2	4	4	3	3	3	3	5	3.3	0.82	24.95



Table 06: Repeatability for Flour by Analyst 02

Dilution	Repeatability										Mean	SD	RSD
	1	2	3	4	5	6	7	8	9	10			
10 ⁻⁵	35	32	34	36	33	35	32	31	33	35	33.6	1.65	4.90
10 ⁻⁶	3	3	3	5	4	4	3	3	4	3	3.5	0.71	20.20

Reproducibility Results

The table 07 compares reproducibility across analysts for both food matrices. The findings indicate acceptable inter-analyst variability, reinforcing the method's robustness.

Table 07: Reproducibility for Cheese and Flour samples

Dilution	Reproducibility- Cheese									
	Analyst 1		Mean	SD	%RSD	Analyst 2		Mean	SD	%RSD
10 ⁻⁵	35	32	33.5	2.12	6.33	32	34	33	1.41	4.29
10 ⁻⁶	4	3	3.5	0.71	20.20	2	3	2.5	0.71	28.28
Dilution	Reproducibility- Flour									
	Analyst 1		Mean	SD	%RSD	Analyst 2		Mean	SD	%RSD
10 ⁻⁵	35	34	34.5	0.71	2.05	33	30	31.5	2.12	6.73
10 ⁻⁶	4	3	3.5	0.71	20.20	3	3	3	0	0

Limit of Detection

The limit of detection for the enumeration of *Bacillus cereus* using the Compact Dry Bacillus cereus medium was determined to be 10 colony forming units per gram, representing the lowest concentration at which the organism could be reliably detected under the validated test conditions.



Discussion

In the present study, the detection and enumeration of *Bacillus cereus* were validated using the Compact Dry *Bacillus cereus* chromogenic culture plate method across two distinct food matrices which are cheese and flour. The application of both low and high inoculum levels of *Bacillus cereus* enabled a comprehensive assessment of the method's sensitivity and dynamic range under conditions that reflect real-world contamination scenarios. The Compact Dry *Bacillus cereus* plates consistently supported the selective growth of *Bacillus cereus*, with the target organism forming visually distinctive blue-green colonies that allowed for straightforward and accurate enumeration.

The calculated standard deviation and relative standard deviation for both repeatability and reproducibility were within acceptable analytical limits, demonstrating a high degree of precision across different analysts and testing conditions. Collectively, the findings confirm that the Compact Dry *Bacillus cereus* method is both reliable and robust for the detection and quantification of *Bacillus cereus* in complex food matrices. This validates its suitability for use in food industry quality control systems and regulatory compliance monitoring programs.

Conclusion

This study confirms the validity of the Compact Dry *Bacillus cereus* chromogenic plate method for the reliable detection and enumeration of *Bacillus cereus* in food samples. The method demonstrated acceptable precision, accuracy, and sensitivity across cheese and flour matrices. With a clear detection limit of 10 colony forming units per gram, it is suitable for routine microbiological analysis in food safety laboratories and meets the necessary criteria for implementation in quality control and compliance monitoring.

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