FOOD BIOLOGICAL CONTAMINANTS

Efficient Isolation and Identification of *Bacillus cereus* Group

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Bacillus cereus **is a group of ubiquitous facultative anaerobic sporeforming Gram-positive rods commonly found in soil. The spores frequently contaminate a variety of foods, including produce, meat, eggs, and dairy products. Foodborne illnesses associated with toxins produced by *B. cereus* can result in self-limiting diarrhea or vomiting. Plate enumeration methods recommended by recognized food authorities to detect the presence of *B. cereus* in potentially contaminated food products do not inhibit other Gram-positive competitive bacteria. This study evaluated the use of Bacara, a new chromogenic agar, as an efficient method to identify and enumerate *B. cereus* group from food matrixes, even in the presence of background flora. Inclusivity and exclusivity testing was performed using four different selective and differential media for *B. cereus,* including Mannitol Egg Yolk Polymyxin (MYP), Polymyxin Pyruvate Egg-Yolk Mannitol Bromothymol Blue Agar, Bacillus Chromogenic Media, Brilliance, and Bacara. MYP and Bacara were also used in plate enumeration studies to isolate *B. cereus* from artificially contaminated foods.**

Bacillus cereus has been detected and implicated in several contaminated food products and supplements since 1906, when Plazikowski associated the organism associated with food poisoning (1). The current method recommended in the U.S. Food and Drug Administration's *Bacteriological Analytical Manual* (BAM; 2), International Organization for Standardization (ISO; 3, 4), and AOAC *Official Methods of Analysis* (5) for the enumeration and identification of *B. cereus* includes growth on Mannitol Egg Yolk Polymyxin (MYP) media and biochemical characterization of suspect isolates. Problematic issues with MYP, as noted by other authors, include a lack of characteristic colony morphology and production of lecithinase. Characteristic growth may be masked by the presence of background flora, such as *Bacillus* species other than *B. cereus* and *Staphylococcus aureus,* that ferment mannitol and produce lecithinase (6). Overlapping precipitation zones and the propensity of *B. cereus* colonies to coalesce hinders accurate enumeration (7). Lastly, the media must be used within 4 days of preparation (6, 7).

Newer chromogenic media formulations incorporate specific enzyme substrates to allow for the identification of several organisms, including *Escherichia coli, S. aureus,* and *Listeria monocytogenes.* The use of selective media can eliminate the need for subcultures and biochemical testing resulting in more rapid detection and reduced reagent costs (8, 9). Several of the formulations comply with food and water regulatory authorities (8).

Previous *B. cereus* enumeration surveys using isolate panels, as well as food matrixes, have demonstrated the advantage of chromogenic media over traditional plating media, such as MYP or Polymixin Pyruvate Egg-Yolk Mannitol Bromothymol Blue Agar (PEMBA). The chromogenic agar tested in two separate studies clearly illustrated that the chromogenic media were more selective (7, 10). Statistical information from one study comparing 100 *B. cereus* strains on chromogenic media confirmed that only three *B. cereus* strains showed weak or atypical reactions using the chromogenic media, compared to 75 *B. cereus* strains with weak or atypical reactions on PEMBA and 65 with weak or atypical reactions on MYP (10). The goal of this study was to compare the efficiency and accuracy of *B. cereus* enumeration from adulterated food matrixes using MYP and Bacara, a new chromogenic agar from AES Chemunex (Cranberry, NJ).

Materials and Methods

Bacterial Strains and Media

A variety of selective and/or differential media were evaluated using previously characterized bacterial isolates obtained from American Type Culture Collection (Manassas, VA), Bacillus Genetic Stock Center (Columbus, OH), and U.S. Food and Drug Administration (FDA) collections (College Park, MD). The FDA *B. cereus* strains were associated with foodborne outbreaks, but the implicated food sources are unknown.

Isolates were retrieved from frozen glycerol stock cultures, streaked to tryptic soy agar (TSA), and incubated overnight at 30°C. A single colony of each strain was grown overnight in brain heart infusion broth (pH 7.4) with 1 g/L glucose (BHIG), and 10 µL aliquots of the overnight culture were streaked to solid media for isolation. A total of 107 bacterial strains—including 61 *B. cereus* group strains, 14 *Bacillus* sp. not *cereus*, 19 Gram-negative bacterial strains, and 13 Gram-positive bacterial strains—were streaked to selective and differential media (Tables 1 and 2). The media included dehydrated media purchased from Oxoid Ltd (Basingstoke, UK), prepared according to the manufacturer's instructions, MYP, PEMBA, and Brilliance™ agar. Bacillus Chromogenic Media (BCM) was purchased from R&F Laboratories (Downers Grove, IL) and prepared according

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Table 1. Summary of *Bacillus cereus* isolates used in this study; toxin status also shown, if known[a]

B. cereus strain No.	Diarrheal enterotoxin	Emetic toxin PCR result	Comment
A11778	Pos	Neg	Standard media testing strain
A14579	Pos	Neg	Environmental strain
B6A1	Neg	Neg	Wild type isolate; original code T
B6A2	Neg	Neg	Wild type isolate; original code T-HT
B6A3	Pos	Neg	Wild type isolate; original code NRRL-569; streptomycin-resistant
B6A4	Pos	Neg	Streptomycin-resistant mutant of NRRL-569 (B6A3)
B6A8	Pos	Neg	Probiotic strain; orgignal code bactisubtyl
B6A10	Pos	Neg	Wild type isolate; orginal code ATCC13472 and HER 1399
B6A15	Pos	Neg	Wild type isolate; original code ATCC 10987 and NRS 248
B6A17	Pos	Neg	Wild type isolate; original code ATCC 13472
B6A18	Pos	Neg	Wild type isolate; original code ATCC 15816
B6A25	Neg	Neg	Wild type; orginal code GGC-D16B
B6G1	Pos	Neg	gerQA2 mutant; orginal code AM1311
B6G2	Pos	Neg	gerIA5 mutant; original code AM134
B6E1	Pos	Neg	Resistant to teracycline; original code GP7
B6G3	Pos	Neg	ala-1 mutant; original code AM1316
B6A9	Pos	Neg	Wild type isolate
F3995A	Unk	Neg	Food
F3998A	Unk	Neg	Food
F4014A	Unk	Unk	Food
F4034A	Unk	Unk	Food
F4047A	Unk	Unk	Food
F4225A	Pos	Neg	Food
F4226A	Pos	Pos	Food
F4228A	Pos	Neg	Food
F4229A	Pos	Neg	Food
F4230A	Pos	Pos	Food
F13061	Unk	Unk	Food
FM77	Pos	Unk	Food
FTJL14[b]	Pos	Pos	Food
F196668	Pos	Neg	Food
F180WPB	Pos	Neg	Food
F4227A	Pos	Pos	Food
F66	Unk	Unk	Food
F60006	Pos	Pos	Food
F1259	Unk	Unk	Food
F1261	Unk	Unk	Food
F1263	Unk	Unk	Food
F1265	Unk	Unk	Food
F1396	Unk	Unk	Food
F3A	Pos	Neg	Food
F26	Pos	Neg	Food
F96	Pos	Neg	Food
F4A	Pos	Neg	Food
F6A	Pos	Neg	Food
F59	Pos	Neg	Food
FTOL-12	Pos	Neg	Food

[a] American Type Culture Collection (ATCC); B = Bacillus Genetic Stock Center (BGSC); F = U.S. Food and Drug Administration (FDA) collection; Pos = positive; Neg = negative; Unk = unknown enterotoxin status; FDA strains were outbreak strains from unknown food sources.

[b] Bennett et al. (ref. 12).

to the manufacturer's instructions. Bacara was purchased from AES Chemunex as prepared media.

Enterotoxin Detection

The Tecra *Bacillus* Diarrheoal Enterotoxin Visual Immunoassay (3M, St. Paul, MN) is an ELISA screening kit used to detect NheA, a protein associated with the diarrheal symptoms of *B. cereus.* The ELISA kit was used to screen isolates following manufacturer's guidelines for foods. Isolates were also assessed for the presence of the emetic toxin using a PCR assay (primers unpublished). Results as available are listed in Table 1.

Colony Morphology

The growth characteristics recorded included presence or absence of precipitation zone, if applicable, and colony morphology after overnight incubation at 30°C. The expected *B. cereus* colony when grown on MYP is pink, on PEMBA peacock blue, and on Bacara orange-pink. All three formulations include egg yolk. If the bacterium produces lecithinase, the colony is surrounded by a halo or precipitation zone. *B. cereus* colonies grown on Brilliance and BCM are turquoise green.

Table 2. Summary of bacterial strains not *B. cereus* and the growth characteristics noted following overnight incubation at 30°C[a]

Strain No.	Bacterial isolate	MYP	BACARA
F872	*Acinetobacter* sp.	NG	NG
F873	*Acinetobacter* sp.	NG	NG
F825	*Aeromonas hydrophila*	NG	NG
F176	*Bacillus amyloliquifaciens*	Y/HALO	NG
F177	*Bacillus amyloliquifaciens*	Y/HALO	NG
F178	*Bacillus amyloliquifaciens*	NG	NG
F179	*Bacillus amyloliquifaciens*	NG	NG
F180	*Bacillus amyloliquifaciens*	NG	NG
F181	Bacillus amyloliquifaciens	NG	NG
F182	*Bacillus amyloliquifaciens*	NG	NG
F183	*Bacillus amyloliquifaciens*	NG	NG
F_BA663	*Bacillus anthrcis Sterne*	P/NO HALO	O/NO HALO
A61	*Bacillus circulans*	Y/NO HALO	NG
FMYC	*Bacillus mycoides*	Y/HALO	NG
F1267	*Bacillus subtilis*	NG	NG
F1268	*Bacillus subtilis*	Y/HALO	NG
F1269	*Bacillus subtilis*	Y/HALO	NG
F1270	*Bacillus subtilis*	Y/HALO	NG
F1258	*Bacillus thuringiensis*	P/HALO	O/HALO
F1260	*Bacillus thuringiensis*	P/HALO	O/HALO
F1262	*Bacillus thuringiensis*	P/HALO	O/HALO
F1264	*Bacillus thuringiensis*	P/HALO	O/HALO
F1266	*Bacillus thuringiensis*	P/HALO	O/HALO
F1397	*Bacillus thuringiensis*	P/HALO	O/HALO
F1398	*Bacillus thuringiensis*	P/HALO	O/HALO
B6A21	*Bacillus weihenstephanensis*	P/HALO	O/HALO
B6A22	*Bacillus weihenstephanensis*	P/HALO	O/HALO
B6A23	*Bacillus weihenstephanensis*	P/HALO	O/HALO
A64	*Brevibacillus laterosporus*	P/NO HALO	NG
A25408	*Citrobacter freundii*	NG	NG
A43864	*Citrobacter koseri*	NG	NG
A13048	*Enterobacter aerogenes*	NG	NG
A33731	*Enterobacter amnigenus*	NG	NG
A35956	*Enterobacter asburiae*	NG	NG
A35030	*Enterobacter cloaceae*	NG	NG
F611	*Enterobacter sakazakii*	NG	NG
A29212	*Enterococcus faecalis*	Y/NO HALO	O/NO HALO
FNES23	*Enterococcus faecalis*	Y/NO HALO	O/NO HALO
F385	*Escherichia coli*	NG	NG
F386	*Escherichia coli*	NG	NG
F387	*Escherichia coli*	NG	NG

Table 2. (*continued*)

Strain No.	Bacterial Isolate	MYP: 24 h	BACARA: 24 h
F392	*Escherichia coli*	NG	NG
F1403	*Listeria monocytogenes*	Y/HALO	NG
F1404	*Listeria monocytogenes*	Y/HALO	NG
F1405	*Listeria monocytogenes*	Y/HALO	NG
F1406	*Listeria monocytogenes*	Y/HALO	NG
A29948	*Paenibacillus gordonae*	NG	NG
A8509	*Paenibacillus macerans*	Y/NO HALO	NG
A7070	*Paenibacillus polymyxa*	Y/NO HALO	NG
F1	*Salmonella enterica*	NG	NG
F249	*Salmonella enterica*	NG	NG
F336	*Salmonella enterica*	NG	NG
F522	*Salmonella enterica*	NG	NG
F1161	*Shigella sonnei*	NG	NG
A1766	*Staph aureus*	Y/HALO	W/NO HALO
A1767	*Staph aureus*	Y/HALO	W/NO HALO
A12228	*Staph epidermidis*	Y/HALO	NG
A49051	*Staph. intermedius*	P/HALO	P/NO HALO
A14576	*Virgibacillus pantothenicus*	NG	NG

[a] A = American Type Culture Collection (ATCC); B = Bacillus Genetic Stock Center (BGSC); F = U.S. Food and Drug Administration (FDA) collection; NG = no growth; P = pink colony; Y = yellow colony; O = orange colony; W = white colony; HALO = lecithinase positive; NO HALO = lecithinase negative.

Food Adulteration

Food matrixes were artificially contaminated or adulterated with *S. aureus* (ATCC BAA1747), *E. coli* (FDA 392), *Bacillus subtilis* (FDA 1267), and an enterotoxingenic *B. cereus* strain (FDA 4227A) from a foodborne outbreak. The FDA 4227A strain tested positive for the diarrheal enterotoxin and was also presumptively positive for the emetic toxin by PCR screening. Single colonies of each strain were transferred to separate flasks with 50 mL BHIG and incubated overnight on an orbital shaker at 30°C. The *B. cereus* cells were pelleted and resuspended with 10 mL of Butterfield's Phosphate Buffer (BPB), yielding approximately 1×10^9 CFU/mL. Low, medium, and high dilutions (approximately 1×10^{-6}, 1×10^{-4}, and 1×10^{-1} CFU/mL, respectively) of *B. cereus* were prepared with BPB. A mixed culture was prepared using the resuspended pellets of *S. aureus, E. coli,* and *B. subtilis.* The pellets were combined in 20 mL BPB, and 6 mL of the triple species cocktail was combined with 6 mL of one of the *B. cereus* dilutions to spike a variety of food matrixes for enumeration studies, as described in the BAM (2). Briefly, 300 g food samples, including cooked rice, macaroni and cheese dinner, mashed potatoes, milk, dehydrated diet drink, and infant formula, were inoculated and mixed with bacterial cocktails and allowed to equilibrate at 4°C for 48 h. Five replicates of 50 g

Table 3. Average colony counts (CFU/g) of five replicates plated in duplicate and transformed to $\log_{10}$ from low, medium, and high inoculum levels of *B. cereus* from artificially contaminated food samples

	MYP agar			BACARA agar		
Sample	Low	Med	High	Low	Med	High
Rice	ND[a]	ND	ND	2.2	4.1	6.3
Diet drink	ND	ND	6.3	2.1	3.9	7.2
Infant formula	ND	ND	ND	2.0	3.8	6.8
Mac and cheese	ND	ND	ND	2.5	3.4	6.4
Mashed potatoes	ND	ND	5.9	2.8	4.1	6.8
Milk	ND	ND	7.6	2.3	4.5	7.6

[a] ND = No colonies detected.

Table 4. Results of a two-sample *t*-test on the log transformed data

Product	*B. cereus* target level	SD $\log_{10}$ CFU/g: BAM method, MYP	SD $\log_{10}$ CFU/g: New method, BACARA	*P* value
Rice	1×10^{-6}	0.00	0.20	0.0044
Rice	1×10^{-4}	0.00	0.56	0.0004
Rice	1×10^{-1}	0.00	1.19	0.0028
Mac and cheese	1×10^{-6}	0.00	0.30	0.2092
Mac and cheese	1×10^{-4}	0.00	0.06	<0.0001
Mac and cheese	1×10^{-1}	0.92	1.10	0.0841
Potato	1×10^{-6}	0.00	0.38	0.0026
Potato	1×10^{-4}	0.00	0.26	<0.0001
Potato	1×10^{-1}	0.77	1.08	0.0449
Milk	1×10^{-6}	0.00	0.30	0.0004
Milk	1×10^{-4}	0.00	0.32	<0.0001
Milk	1×10^{-1}	0.63	0.11	0.0005
Diet drink	1×10^{-6}	0.00	0.59	0.0433
Diet drink	1×10^{-4}	0.00	0.58	0.0008
Diet drink	1×10^{-1}	1.19	0.82	0.0096
Infant formula	1×10^{-6}	0.00	0.32	0.0264
Infant formula	1×10^{-4}	0.00	0.55	0.0006
Infant formula	1×10^{-1}	0.00	1.68	0.0093

portions of each food sample were homogenized at high speed (18 000–21 000 rpm) for 2 min in a Waring blender with 450 mL BPB. Ten-fold serial dilutions were prepared in BPB; 100 µL was then spread in duplicate to TSA, MYP, and Bacara for overnight incubation at 30°C. Due to a laboratory mishap, one set of plates was evaluated for the dehydrated diet drink.

Plate Enumeration Analysis

All aerobic plate counts inoculated from the six adulterated food matrixes were converted to CFU/g, and the average colony counts of five replicates plated in duplicate were transformed to base-10 logarithms (Table 3). The exception is that one set of plates was recorded for the diet drink. The log-transformed data were further analyzed (Table 4) by a two-sample *t*-test using the R version 2.11.1 software package (11).

Results

Growth characteristics of *B. cereus* strains and *B. cereus* group strains showed typical colony morphology with a zone of precipitation if the media were supplemented with egg yolk. The comparison of BCM and Brilliance with the traditional

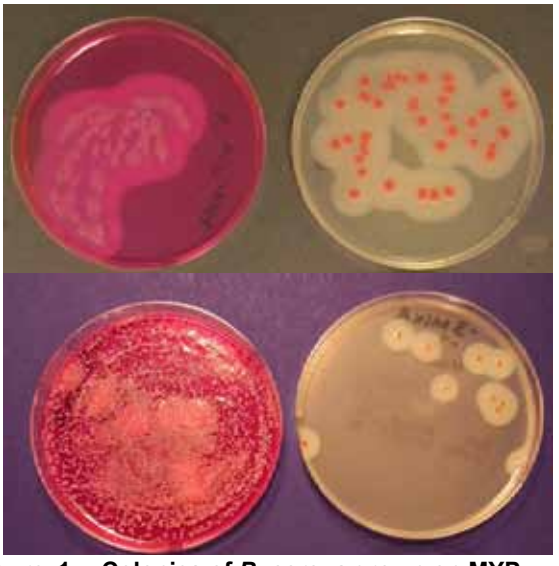

Figure 1. Colonies of *B. cereus* grown on MYP are pink and lecithinase positive (top left), but other bacteria are not inhibited and can obscure detection of *B. cereus* colonies (bottom left). Colonies of *B. cereus* grown on Bacara are pink-orange and are lecithinase positive (top right), but other organisms are inhibited (bottom right).

media recommended by food authorities were in agreement with previous studies (7, 9, 10). However, Bacara was not included in the previous studies.

Colonies of *B. cereus* and *B. cereus* group formed uniform individual pink-orange colonies on Bacara. Furthermore, the same strains with the exception of *B. anthracis* Sterne showed the typical zone of precipitation. The Gram-negative strains, including *Acinetobacter* sp., *E. coli,* and *Enterotobacter* sp., were inhibited on Bacara and MYP (Table 2). The Gram-positive bacterial strains, including *Listeria monocytogenes, Paenibacillus* sp., *Virgibacillus* sp., *Bacillus amyloliquefaciens, B. circulans, B. mycoides, B. subtilis, Brevibacillus laterosporus,* and *Staphylococcus epidermidis,* were also inhibited on Bacara, but some strains did grow on MYP (Table 2). Some of the staphylococci grew on Bacara as either white or pink pinpoint colonies; enterococci isolates grew on Bacara as pink pinpoint colonies. These colonies did not have a zone of precipitation and were easily distinguishable from representative *B. cereus* strains (Table 2).

The bacterial growth on TSA plates from the adulterated food samples inoculated with *B. cereus, B. subtilis, S. aureus,* and *E. coli* was not much different than the growth on MYP plates. The abundant growth of competitive bacteria present in the mixed cultures interfered with the growth, detection, and colony enumeration of *B. cereus.* However, Bacara inhibited the competitive flora, and colonies of *B. cereus* were easily enumerated (Figure 1). The lowest level of *B. cereus* recovered from a mixed culture on Bacara showed a log average count of 2, but the log average count was significantly higher on MYP plates, at 5.9 (Table 3). In fact, colonies of *B. cereus* were generally not detected on MYP plate cultures from the adulterated food due to the presence of competitive bacteria (Table 3).

The results of the statistical analysis showed a significant difference ($P \leq 0.05$) in log CFU/g between MYP and Bacara for

rice, infant formula, mashed potatoes, and milk. Bacara is able to detect *B. cereus* at much lower concentrations than MYP in these food matrixes when they have been artificially contaminated with competitive bacteria and dilutions of the target organism. There was not a significant difference between the log CFU/g for Bacara and MYP for the detection of *B. cereus* in the presence of competitive bacteria from macaroni and cheese (Table 4).

Discussion

Interpretation of MYP plates of the adulterated food samples was complicated by the presence of competitive bacterial flora because detection of discrete mannitol-negative *B. cereus* colonies were masked by the mannitol positive colonies of *S. aureus* and *B. subtilis*. Even enumeration of pure cultures is difficult since the colonies coalesce and the precipitation zones overlap (6). The aerobic plate counts for MYP indicated that the agar was not selective or differential for the growth of *B. cereus* in the presence of competitive bacteria. However, the target organism was grown on the Bacara plates, despite the high numbers of competitive bacteria present in the adulterated food samples. Additionally, the enumeration of *B. cereus* was more efficient and accurate with Bacara than with MYP, because the colonies were discrete and uniform in color and size. The time to detection (≤24 h) without further subculturing or biochemical testing was consistent with other chromogenic media and permitted completion in significantly less time than traditional media (8, 9). As with other chromogenic media, Bacara does not require additional conformational testing and, therefore, would be considered beneficial as a cost- and time-saving measure (8, 9). The statistical analysis shows low *P* values for the food matrixes tested, indicating that the two media, MYP and Bacara, are not equivalent in the detection of *B. cereus* in the presence of competitive flora.

Bacara has been validated by AFNOR (Saint-Davis, France; Certificate No. AES 10/10-0710) for the enumeration of strains of the *B. cereus* group from food matrixes. We concur with the AFNOR laboratories and suggest that Bacara replace MYP as a more rapid method to recover *B. cereus* from contaminated food products.

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