Dynabeads Protein G Antibody Conjugates Combined with Modified Brain Heart Infusion Broth for the Enrichment and Separation of *Bacillus cereus* in Artificially Contaminated Vegetables

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Abstract A modified brain heart infusion (MBHI) broth and a protocol of immunomagnetic separation (IMS) using antibody-coated Dynabeads[®] protein G were developed for the enrichment and separation of *Bacillus cereus* in artificially contaminated vegetable samples. The MBHI consisted of BHI and 0.34 g/L magnesium sulfate, 12.08 g/L sodium pyruvate, 1.82 g/L yeast extract, and polymyxin B. The amount of immunomagnetic beads (IMBs) and immunoreaction time were optimized. The capture efficiency was 58.32% with 0.4 mg IMBs when the immunoreaction time was 20 min. Capture of *B. cereus* by IMBs did not interfere with competing flora. Pre-enrichment IMS was validated with four *B. cereus* strains in artificially contaminated baby sprouts, bean sprouts, lettuce, and spinach at two levels (~0.1 and ~1 CFU/g). We were able to detect and isolate *B. cereus* in 40/40 samples of vegetables contaminated at 0.1 CFU/g with IMS after 6 h of enrichment in MBHI.

Keywords: *Bacillus cereus*, immunomagnetic separation, Dynabeads[®] protein G, enrichment

Introduction

Bacillus cereus, a rod-shaped gram-positive food-borne pathogen, causes diarrheal and emetic types of food poisoning (1,2). It is a typical pathogen commonly found in soil and can easily spread to growing plants. As a result, *B. cereus* contamination becomes a special challenge for the food industry. Furthermore, severe forms of disease caused by emetic *B. cereus* may lead to hospitalization or even death (3-5). Green vegetables implicated in diarrheal syndromes and the possible presence of *B. cereus* on fresh vegetables could be a microbial risk (6,7). Thus, rapid detection methods or technologies to decrease the total assay time for this pathogen are needed.

The traditional plating method using selective media is widely applied to isolate and quantify *B. cereus* in food samples. Usually, poisoned food samples contain a high number of colonies ($>10^5$ *B. cereus* cells/g or mL), but some cases involving lower numbers (10-100/g or mL) have also been reported (8). An enrichment procedure for the samples is thus required to increase the number of target pathogens from the food to detectable levels of at least 10^2 CFU/g of food product (9). However, the enrichment procedure usually needs

more than 18 h, which increases the total assay time (10-12). So a medium that could favor the growth of *B. cereus* in a shorter time is needed to help decrease the total experiment time. Thus, an improved medium using modified brain heart infusion (MBHI) for *B. cereus* was developed in this paper.

The immunomagnetic separation (IMS) method uses immunomagnetic beads (IMBs) as the capture reagents to isolate pathogens from food samples (13-15). IMS could effectively separate target bacteria from competitive microflora in the food samples, thus reducing the sample volume (16,17). This selective separation relies on the specificity of antibody to antigen combined with the magnetic particles. For this study, commercial Dynabeads[®] protein G (Invitrogen, Carlsbad, CA, USA) was conjugated to rabbit polyclonal anti-*B. cereus* antibodies to form IMBs that can capture the bacteria antigen from food samples. Dynabeads[®] protein G are uniform (2.8 μ m) super-paramagnetic beads with recombinant protein G covalently coupled to the surface. Protein G is a cell wall protein with affinity for immunoglobulin (IgG) isolated from a human group G streptococcal strain that could bind all human IgG subclasses and rabbit and mouse IgG (18).

Therefore, the purposes of this work were to (i) develop an enhanced enrichment medium for *B. cereus* based on the best basal broths by comparing the cell count after enrichment in the three broths, (ii) optimize the IMS method by adjusting the coupling rate between antibody to Dynabeads® protein G and immunoreaction times, and (iii) evaluate the improved pre-enrichment IMS method for enrichment and separation of *B. cereus* in artificially contaminated vegetable samples.

Materials and Methods

Preparation of bacterial strains Four strains of *B. cereus* (ATCC 11778, ATCC 12480, ATCC 14579, and ATCC25621), *Escherichia coli* O157:H7 (ATCC 43895), *Staphylococcus aureus* (ATCC 12600), and *Listeria monocytogenes* (ATCC 15313), were used in this study. Frozen stock cultures maintained at 70°C in tryptic soy broth (TSB; Difco, Sparks, MD, USA) containing 20% glycerol were thawed at room temperature. Each strain (0.1 mL) was inoculated into 9 mL of TSB and incubated at 35°C for 18–24 h to reach the stationary phase. The cell suspension was centrifuged at 3,000×g for 10 min at 4°C. The supernatant was removed and the pellet was washed twice with 9 mL 0.1% (w/v) sterile peptone water (PW; Difco) for each wash. The cell suspension was used for inoculation within 48 h. The cell count of each culture was measured by plating 0.1 mL portions of appropriate dilutions onto tryptic soy agar (TSA; Difco) plates and incubated at 35°C for 24 h.

Comparison of basal media for the growth of *B. cereus* BHI (Bacto), buffered peptone water (BPW; Difco), and TSB (Difco) were prepared according to the manufacturer's recommendations. *B. cereus* suspensions (0.1 mL; 10⁵ CFU/mL) were inoculated into 9.9 mL of BHI, BPW, and TSB and incubated at 35°C. Subsequently, suspensions (1 mL) were incubated for 3, 6, 8, 10, 12, and 14 h and serially diluted in 0.1% (w/v) sterile PW, followed by appropriate dilution onto TSA in duplicate. After incubation at 35°C for 24 h, the colonies on agar plates were enumerated.

Preparation of *B. cereus* enrichment medium For preparation of the enrichment medium for *B. cereus*, 7.7 g calf brains, 9.8 g beef heart, 10.0 g proteose peptone, 2.0 g dextrose, 5.0 g sodium chloride, 2.5 g disodium phosphate, 0.34 g magnesium sulfate (Sigma-Aldrich, St. Louis, MO, USA), and 1.82 g yeast extract (YE; Difco) were suspended in 1.0 L distilled water. After the pH was adjusted to 7.4±0.2, the solution was autoclaved at 121°C for 15 min and cooled to 50–55°C. Polymyxin B (Sigma-Aldrich) of 500,000 units was dissolved in 33.3 mL autoclaved distilled water, filter sterilized, and stored in the dark at 4°C until use. Subsequently, 12.5 g sodium pyruvate (Sigma-Aldrich) and 6.67 mL sterile polymyxin B solution were added to 1 L cooled broth (50–55°C). The broth was thoroughly mixed and 9 mL was separated to each sterilized tube for storage at 4°C. Possible

promoters were selected and the concentration of each supplement was evaluated and optimized separately by assaying the growth of *B. cereus*.

Comparison of injured microorganism in MBHI and BHI For acid treatment, an aliquot of 2 mL of a washed cell suspension of *B. cereus* was resuspended in 0.1% PW and 25% (v/v) citric acid solution (pH 1.5) was added to achieve a final pH 4.0. The suspension was kept for 12 h at room temperature. For heat injury treatment, an aliquot of 2 mL of a washed cell suspension of *B. cereus* was resuspended in 0.1% PW and heated at 55°C in a water bath for 10 min. Immediately after the heat treatment, the tubes were cooled in an ice bath for 30 s to stop the damage by heating. For low-temperature injury treatment, an aliquot of 2 mL of a washed cell suspension of *B. cereus* was resuspended in 0.1% PW and kept at 4°C for 5 h. After the treatment, the tubes were treated under running tap water for 1 min. After each of the acid, heat, and low-temperature injury treatments, the cell suspensions were serially diluted in 0.1% PW, and appropriate dilutions (10¹–10² and 10²–10³ CFU/mL) were inoculated into MBHI and BHI for 4 and 6 h incubation at 35°C.

Preparation of Dynabeads® protein G with antibody Immunoaffinity magnetic beads were prepared by coating anti-*B. cereus* antibody ab20556 (4 mg/mL, Abcam, Cambridge, MA, USA) to Dynabeads® protein G (30 mg/mL, Life Technologies AS, Oslo, Norway). The Dynabeads® protein G was resuspended by vortexing for more than 30 s and 1 mg was transferred to a 1.5-mL tube. The tube was placed on a magnet (DynaL MPC™-S; Life Technologies) to separate the beads from the solution and the supernatant was removed. Fifteen micrograms of the anti-*B. cereus* antibody diluted in 200 µL phosphate buffered saline (PBS, pH 7.4, Life Technologies) with 0.02% Tween®-20 was added to the Dynabeads® protein G for conjugation via the fragment crystallizable region. Dynabeads®-Ab complex IMBs were incubated with rotation for 20 min at room temperature and placed on a magnet to remove the supernatant. The IMBs were resuspended in 200 µL PBS with 0.02% Tween®-20 and stored at 4°C.

Capture efficiency calculations The capture efficiency (CE), defined as the percentage fraction of the total bacteria retained on the surface of the beads, was calculated with the equation based on the cells bound to the IMBs (19). The following equation was used for calculating CE: $CE (\%) = (1 - C_u/C_o) \times 100\%$, where C_o is the total number of cells present in the sample (CFU/mL) and C_u is the number of cells unbound to the IMBs (CFU/mL, in supernatant and washed solution).

Procedure and key parameters for immunomagnetic separation *B. cereus* was incubated in TSB at 35°C for 18–24 h and 1 mL was gently pipetted to each 1.5 mL tube to resuspend the IMBs. The mixture was incubated with rotation for 20 min at room temperature and separated by a magnet. An aliquot (0.1 mL) from the supernatant

was spread on Mannitol Egg Yolk-Polymyxin agar (MYP agar; Difco) plates for enumeration after appropriate dilution. The IMBs-bacteria complex was washed three times using 200 μ L PBS for each wash. An aliquot (0.1 mL) from the washing solution was spread on MYP agar plates after appropriate dilution.

The CE of IMBs against *B. cereus* (10^4 CFU/mL) was studied using five amounts of IMBs (0.05, 0.1, 0.2, 0.4, and 0.6 mg) and five immunoreaction times (10, 20, 30, 40, and 50 min).

Specificity test Nonspecific binding of IMBs was tested using three nontarget bacteria of *E. coli* O157:H7, *L. monocytogenes*, and *S. aureus*. An aliquot of 0.25 mL from 10^5 CFU/mL and 10^3 CFU/mL of *B. cereus* was mixed with 0.75 mL from 10^7 CFU/mL of a competitive flora consisting of *E. coli* O157:H7, *L. monocytogenes*, and *S. aureus*. Binding of IMBs against target and nontarget bacteria was tested by the plating method. Inoculum levels of *B. cereus* were plated on MYP agar plates and nontarget bacteria were plated on TSA plates (20).

Food samples preparation and enrichment Baby sprouts, bean sprouts, lettuce, and spinach were purchased from a local retail market in Chuncheon, South Korea, and used for the spiking experiment. Inedible, wilted, and damaged portions were trimmed. The samples were checked for negative *B. cereus* to exclude potential false positives from natural contamination (21). Baby sprouts, bean sprouts, lettuce, and spinach were separately spiked with *B. cereus* ATCC 14579, ATCC 25621, ATCC 11778, and ATCC 12480. For pathogen spiking, *B. cereus* cultured in TSB overnight was diluted tenfold in 0.1% sterile PW. Baby sprouts and bean sprouts were weighed (10 g portions) into stomach bags (Nasco Whirl-pak, Janesville, WI, USA) and inoculated with 0.2 mL of appropriate dilutions of the culture to achieve low levels (~ 0.1 CFU/g) and high levels (~ 1 CFU/g). Lettuce and spinach (10 g portions) were inoculated by depositing droplets on 20 locations with 0.2 mL of appropriate dilutions of the culture to achieve low levels (~ 0.1 CFU/g) and high levels (~ 1 CFU/g). Following inoculation, the inoculated samples were held at 2–5°C overnight prior to analysis to allow time for the organisms to equilibrate with the sample. After equilibration, each inoculated sample was mixed with 90 mL MBHI and stomached for 2 min (Lab-blender 400; Seward, London, UK), followed by enrichment at 35°C. After enrichment for 6 and 24 h, each sample (1 mL) was serially diluted tenfold in 0.1% PW, followed by inoculation with 0.1 mL of the appropriate dilution onto MYP agar. Meanwhile, 1 mL from each sample was mixed with 0.4 mg IMBs prepared according to the IMS procedure. The washed IMBs-bacteria complex was suspended with 0.1 mL PBS and 50 μ L was used for plating on MYP agar. After incubation, up to five typical colonies were picked from each plate and confirmed with real-time PCR targeting the 16S rDNA gene (22). Five enrichment trials were performed for each strain at each contamination level.

Statistical analysis The Design Expert program (version 8.0.5b; Stat-Ease, Inc., Minneapolis, MN, USA) was used for response surface

methodology (RSM) design for developing the MBHI. The effect of supplements magnesium sulfate, sodium citrate, sodium pyruvate, yeast extract, microbial data, and CE were reported as means with standard deviation of the duplicate. One-way ANOVA was used to compare the mean among the groups and the statistical significance threshold was determined as $p < 0.05$ with SPSS (version 21.0; SPSS Inc., Chicago, IL, USA). Fisher's exact probability tests for 2×2 contingency tables were applied to calculate significant differences in total results for food analysis at $p = 0.05$.

Results and Discussion

Selection of basal medium and enrichment supplements The optimal medium for growth of *B. cereus* was selected by comparing BHI, BPW, and TSB at 35°C (Fig. 1). The growth curves showed that *B. cereus* grew better in BHI than in TSB or BPW. When the initial cell number was 3.19 log CFU/mL, it grew up to 6.77, 6.18, and 6.40 log CFU/mL, respectively, in BHI, BPW, and TSB after 6 h incubation. Although there was no significant difference between the colony numbers after incubation in these three media for 6 and 14 h, the colony numbers were slightly higher after incubation in BHI than in TSB or BPW. Therefore, BHI was selected as the basal medium, which is a rich medium using a dehydrated infusion of porcine brain and heart.

The four supplements (magnesium sulfate, sodium citrate, sodium pyruvate, and yeast extract) were separately added to BHI at different concentrations and the growth of *B. cereus* was shown in Fig. 2 (the initial cell number was 1.20 log CFU/mL). The colony numbers of BHI supplemented with magnesium sulfate (0.2–0.4 g/L), sodium pyruvate (7.5–12.5 g/L), and yeast extract (1.5–2.5 g/L) were significantly higher than that of BHI alone ($p < 0.05$). When sodium citrate was added in BHI, the colony numbers were higher with a concentration of 1 g/L, but there were no significant differences with other concentrations compared to BHI alone. The concentration optimization studies for these three supplements were carried out by an RSM with the three accelerators, magnesium sulfate, sodium pyruvate, and yeast extract, at levels of 0.2–0.4, 7.5–12.5, and 1.5–2.5 g/L, respectively (23). Polymyxin B was added as a selective reagent since it was used in the conventional plating of MYP agar and polymyxin pyruvate egg yolk mannitol bromothymol blue (PEMB) agar for inhibiting gram-negative bacteria. According to the analysis, the optimal concentrations of magnesium sulfate, sodium pyruvate, and yeast extract were 0.34, 12.08, and 1.82 g/L, respectively. As reported, magnesium sulfate could promote germination of *B. cereus* and yeast extract was used for promoting the growth of *B. cereus* (24). Sodium pyruvate was added in the medium for facilitating the recovery of injured cells (25). The colony numbers were higher with both uninjured and injured cells using the developed MBHI for 4 and 6 h compared to that using BHI ($p < 0.05$). Exceptions were found for cells injured by acid for 4 and 6 h incubation and for cells injured by heat for 6 h incubation.

Fig. 1. Growth curve of *B. cereus* in BHI, BPW, and TSB for 14 h at 35°C with the inoculum level around 10^3 CFU/mL

For uninjured and injured cells after 4 h enrichment in MBHI, even when the initial cell number was as low as 0.15 log CFU/mL, the

colony number grew to more than 3 log CFU/mL (Table 1). Our developed MBHI supported the log CFU/mL of pure *B. cereus* from 1.12 to 6.23 in 6 h, which saved more time compared to a similar log growth in BPW and simultaneous enrichment broth for 18 h (11,12).

Optimization of the immunomagnetic separation method The amount of IMBs and immunoreaction time, which affect the CE against *B. cereus*, were optimized. As shown in Fig. 3A, CE increased from 5.97% to 55.45% with increases in the amount of IMBs from 0.05 to 0.6 mg ($p < 0.05$). There was no significant difference in CE between the amounts of IMBs from 0.4 to 0.6 mg ($p > 0.05$). Thus, to minimize the use of IMBs, 0.4 mg of IMBs was optimized for subsequent experiments. The CE was calculated based on the unbound cells that were contained in supernatant and wash solutions to avoid underestimation (20,26). As one magnetic particle could have captured more than one cell, or a cluster of magnetic particles could

Fig. 2. Effects of different supplements on the growth of *B. cereus* in BHI for 6 h incubation at 35°C. Data are shown as means of log CFU/mL $\pm$ SD. Bars labeled with different letters at different amounts indicate a significant difference in the cell numbers after incubation ($p < 0.05$).

Table 1. Efficacy of MBHI and BHI in recovering injured cells of *B. cereus* by acidification, heat, and cold

Treatment	Mean log CFU/mL $\pm$ SD				
	Initial cell number	BHI	MBHI	BHI	MBHI
		4 h		6 h	
None (uninjured)	$1.64 \pm 0.15e^{1)}$	$4.43 \pm 0.05d$	$4.71 \pm 0.08c$	$5.92 \pm 0.05b$	$6.25 \pm 0.04a$
	$0.59 \pm 0.16e$	$3.30 \pm 0.14d$	$3.62 \pm 0.05c$	$4.98 \pm 0.03b$	$5.33 \pm 0.15a$
Acidification	$1.65 \pm 0.05c$	$4.15 \pm 0.11b$	$4.40 \pm 0.32b$	$5.12 \pm 0.05a$	$5.41 \pm 0.02a$
	$0.54 \pm 0.09e$	$3.06 \pm 0.03d$	$3.47 \pm 0.02c$	$4.04 \pm 0.06b$	$4.36 \pm 0.12a$
Heat	$1.14 \pm 0.09d$	$3.95 \pm 0.07c$	$4.48 \pm 0.12b$	$5.58 \pm 0.12a$	$5.83 \pm 0.11a$
	$0.15 \pm 0.21e$	$3.00 \pm 0.06d$	$3.41 \pm 0.13c$	$4.29 \pm 0.25b$	$4.79 \pm 0.06a$
Cold	$1.74 \pm 0.27e$	$3.90 \pm 0.04d$	$4.55 \pm 0.04c$	$5.05 \pm 0.14b$	$5.61 \pm 0.02a$
	$0.80 \pm 0.14d$	$3.46 \pm 0.12c$	$3.84 \pm 0.18b$	$4.01 \pm 0.13b$	$4.39 \pm 0.06a$

¹⁾ Within rows, means with different letters are significantly different ($p < 0.05$).

Fig. 3. Effects of the amount of IMBs and immunoreaction time of CE. Data are shown as means of CE $\pm$ SD. Bars labeled with different letters at different amounts and immunoreaction times indicate a significant difference in CE ($p < 0.05$).

be formed due to capture of only one cell by several particles, the calculation method based on the separated cells could be underestimated since the plating method only counts the colonies on the agar plates (27). The CE based on the counts of cells bound to the magnetic beads was less than that based on the unbound cells in the supernatant (19). When the initial amount of bacteria is known, the CE calculated based on unbound cells in the supernatant and washing solution presents useful quantitative results (19,28).

The effect of different immunoreaction times on the CE of IMBs against *B. cereus* at room temperature is shown in Fig. 3B. Immunoreaction times of 10, 20, 30, 40, and 50 min were used with the concentration of 9.8×10^3 CFU/mL. Fig. 3B shows that the CE with 20 min of immunoreaction time was 58.32% and no significant increase in CE ($p > 0.05$) is seen with longer reaction times. The immunoreaction time of 20 min was considered optimal for IMB conjugation with the target bacteria *B. cereus*. Immunoreaction time is an important factor for CE. When Dynabeads[®] protein G was used in the IMS method, 10 min was recommended as the minimum immunoreaction time according to the protocol. Some researchers have used immunoreaction times ranging from 15 to 90 min (17,19,20,26). IMBs generated with Dynabeads[®] protein G showed an increase in CE with an increase in immunoreaction time from 10 to 20 min and no significant increase with longer immunoreaction times from 20 to 50 min.

Specificity test with target and nontarget bacterial strains For the specificity assay of IMBs, 0.25 mL of 7.2×10^3 or 7.2×10^5 CFU/mL of *B. cereus* was mixed with 0.75 mL of constant competitive flora consisting 1.8×10^7 CFU/mL of *E. coli* O157:H7, *L. monocytogenes*, and *S. aureus* to evaluate the specificity of IMBs. There were no significant differences ($p > 0.05$) between the CEs of 40.37 and 38.65% obtained with 7.2×10^3 and 7.2×10^5 CFU/mL of *B. cereus*, respectively, mixed with the competitive flora. When 1.8×10^3 CFU of *B. cereus* was mixed with 6.0×10^6 CFU of *E. coli* O157:H7, *L. monocytogenes*, and *S. aureus*, CE was found to decrease by 17.95%. While using a higher inoculation level of 1.8×10^5 CFU of *B. cereus*, CE decreased by 19.67%. Similar decreases in CE have been reported when the target bacteria were mixed with nontarget bacteria in other studies (29,30). Yang *et al.* (29) reported a similar CE change from 31.3 to 41.9% when the target bacteria were mixed with nontarget bacteria in nanoparticle-based IMS.

Food analysis In this part, the pre-enrichment IMS procedure was evaluated with four different *B. cereus* strains with four vegetable samples: baby sprouts, bean sprouts, lettuce, and spinach. For each strain tested, the samples were inoculated at two levels, a low level (~ 0.1 CFU/g) and a high level (~ 1 CFU/g). Artificially contaminated samples were enriched in MBHI for 6 and 24 h at 35°C, and enrichment samples with and without IMS were plated on MYP agar.

Table 2. Detection of *B. cereus* in artificially contaminated vegetable samples at a low level (0.04-0.10 CFU/g) enriched in MBHI incubated at 35°C for 6 and 24 h

Samples	<i>B. cereus</i> strains (inoculum level CUF/g)	Incubation time	Positive results	Positive results with IMS
Baby sprouts	ATCC 14579 0.04	6 h	7/10	10/10
		24 h	8/10	10/10
Bean sprouts	ATCC 25621 0.10	6 h	5/10	10/10
		24 h	8/10	10/10
Lettuce	ATCC 11778 0.078	6 h	9/10	10/10
		24 h	10/10	10/10
Spinach	ATCC 12480 0.063	6 h	7/10	10/10
		24 h	9/10	10/10
	Total	6 h	28/40c	40/40a ¹⁾
	Total	24 h	35/40b	40/40a

¹⁾Totals with the same letter assigned are not significantly different at $p = 0.05$.

Table 3. Detection of *B. cereus* in artificially contaminated vegetable samples at a high level (0.4-1.0 CFU/g) enriched in MBHI incubated at 35°C for 6 and 24 h

Samples	<i>B. cereus</i> strains (inoculum level CFU/g)	Incubation time	Positive results	Positive results with IMS
Baby sprouts	ATCC 14579 0.4	6 h	7/10	10/10
		24 h	9/10	10/10
Bean sprouts	ATCC 25621 1.0	6 h	6/10	10/10
		24 h	8/10	10/10
Lettuce	ATCC 11778 0.78	6 h	8/10	10/10
		24 h	10/10	10/10
Spinach	ATCC 12480 0.63	6 h	8/10	10/10
		24 h	10/10	10/10
	Total	6 h	29/40b	40/40a ¹⁾
	Total	24 h	37/40a	40/40a

¹⁾Totals with the same letter assigned are not significantly different at $p=0.05$.

With 6 h enrichment for samples inoculated at the low level (~0.1 CFU/g), positive results were obtained for *B. cereus* in 40/40 trials (100%) with IMS and 28/40 (70%) trials without IMS (Table 2). After 24 h enrichment at the low inoculum level, 35/40 (87.5%) trials tested positive without IMS and 40/40 tested positive with IMS. The positive results obtained with IMS were significantly higher than those without IMS ($p<0.05$). Background interference considerably decreased on the plates when IMS was used compared to directly plating on MYP agar, as IMS could effectively separate target bacteria from food samples.

With 6 h enrichment for samples inoculated at the high level (~1 CFU/g), positive results were obtained for *B. cereus* in 40/40 trials (100%) with IMS and 29/40 (72.5%) trials without IMS (Table 3). The positive results obtained with IMS were significantly higher than those without IMS after 6 h enrichment ($p>0.05$). After 24 h enrichment at the low inoculum level, 37/40 (92.5%) trials tested positive without IMS and 40/40 (100%) trials tested positive with IMS. The bean sprouts samples showed poor results and the colonies of *B. cereus* on MYP agar appeared yellow because of high acid formation, which obscured the differentiation (31). However, with IMS, all samples gave positive results. Following enrichment, plating onto the selective media after IMS was more productive than direct plating onto the selective media without IMS (32,33).

In conclusion, an enrichment medium MBHI combined with the IMS procedure was developed for pre-enrichment and efficient separation of *B. cereus* using pure *B. cereus* strains and artificially contaminated vegetable samples. Using the RSM, the optimal concentrations of magnesium sulfate, sodium pyruvate, and yeast extract were determined to be 0.34, 12.08, and 1.82 g/L, respectively. The CE of IMBs generated with the Dynabeads[®] protein G and anti-*B. cereus* antibody was significantly influenced by the amount of IMBs and immunoreaction time. The IMS procedure helped decrease background interference on the agar plates and made the isolation of target bacteria from MYP agar easier. Even at the low inoculum level (~0.1 CFU/g) for the four different *B. cereus* strains in artificially contaminated vegetable samples, all the samples showed positive results with IMS after 6 h enrichment in MBHI.

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