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Combination of Hydrophobic Filtration and Enrichment Methods for Detecting *Bacillus cereus* in Fresh-Cut Cabbage

Sujung Lee[1,2], Yukyung Choi[1,2], Heeyoung Lee[2], Sejeong Kim[1,2], Jeeyeon Lee[1,2], Jimyeong Ha[1,2], Hyemin Oh[1,2], Yewon Lee[1,2], Yujin Kim[1,2], Yohan Yoon[1,2], and Soomin Lee[2*]

[1]*Department of Food and Nutrition, Sookmyung Women's University, Seoul, Korea*
[2]*Risk Analysis Research Center, Sookmyung Women's University, Seoul, Korea*
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ABSTRACT - This study developed a rapid detection method for *Bacillus cereus* in fresh-cut cabbages. Fresh-cut cabbage samples were inoculated at 1-, 2- and 3-Log CFU/g, and pathogens were enriched in tryptic soy broth containing 0.15% polymyxin B at 30°C, 37°C, and 42°C to determine the detection limit and appropriate enrichment temperature for multiplex PCR detection. Enriched bacterial cells in enrichment broth were collected in a hydrophobic filter prior to DNA extraction for multiplex PCR. Filters were resuspended in distilled water, and DNA was extracted from the suspension. DNA samples were further analyzed by multiplex PCR. Detection limit of multiplex PCR was 5-Log CFU/mL. *B. cereus* cell counts were higher ($P < 0.05$) at 42°C than other temperatures. Detection rate of 1-, 2-, and 3-Log CFU/g inoculated samples were 60%, 80%, and 100% after enrichment respectively. However, when enriched samples were filtered with hydrophobic membrane filter, detection rates became 100%, regardless of inoculation level. Results indicate a combination of enrichment with hydrophobic filtration improves rapid detection efficiency of *B. cereus* in fresh-cut cabbage by multiplex PCR.

Key words : *Bacillus cereus*, Fresh-cut cabbage, Multiplex PCR, Enrichment, Hydrophobic filtration

Fresh-cut vegetables that are consumed raw, are being concerned for foodborne pathogens, because they could be contaminated throughout cultivation, irrigation, post harvesting, and packaging[1,2]. As the vegetable consumption has been increased, *Bacillus cereus* has been recognized as one of the most frequently detected foodborne pathogens in fresh-cut vegetables with low concentration about 3-Log CFU/g[1,3]. Thus, the detection method of *B. cereus* should be able to detect low concentration of *B. cereus* and distinguish *B. cereus* from other *B. cereus* group[3-7]. However, conventional methods of *B. cereus* detection require 24~48 h for enumeration in selective culture media and additional 18~48 h for identification[5,8,9].

To complement selective culture methods, polymerase chain reaction (PCR) based detection methods (e.g. real-time PCR and droplet digital PCR) have been developed for rapid detection[10,11]. However, PCR methods were sensitive to inhibitors and used for identification only with isolated colony[12-14]. Ganji et al.[15] reported that *Clostridium perfringens* was identified by PCR when the cell concentration was presented upto 4-Log CFU/g in food samples, and *B. cereus* need to be concentrated upto 3-Log CFU/mL to be detected by multiplex PCR in cold dish samples. Thus, if cell concentrations can increase rapidly, bacteria in samples can be detected directly by PCR, and enrichment method has been accompanied as one of the pretreatment method to apply PCR method[16]. PCR inhibitors that contained in the food and environmental samples can interfere PCR reactions[13]. Wei et al.[17] identified that polysaccharides, polyphenols, and pectin may interfere PCR reactions. To improve the PCR reactions, silica membrane filtration and column chromatography using Chelex and cetrimonium bromide have been used[18,19]. Also, two-step filtration was applied to remove PCR inhibitory substances from beef samples[20]. Nabil et al.[21] conducted hydrophobic membrane filter procedure for concentration of viruses. However, there have been few previous studies on the application of hydrophobic filtration in prior to PCR for the bacteria detection. Therefore, this study developed the efficient detection method for *B. cereus* in fresh-cut cabbages

*Correspondence to: Soomin Lee, Risk Analysis Research Center, Sookmyung Women's University, Seoul 04210, Korea
Tel: 82-2-2077-7585, Fax: 82-2-710-9479
E-mail: slee0719@naver.com

Materials and Methods

Inocula preparation

A single colony of each *B. cereus* strain [*B. cereus* KCTC-

1013, *B. cereus* KCTC1014, *B. cereus* KCTC1092, *B. cereus* KCTC1094, and *B. cereus* KCTC3624] was cultured in 10 mL tryptic soy broth (TSB; Becton, Dickinson and Company, Sparks, MD, USA) at 30°C for 24 h. One tenth milliliter aliquot was then cultured in 10 mL of TSB at 30°C for 24 h. A five-strain mixture (50 mL) of *B. cereus* was centrifuged at 1,912 × g at 4°C for 15 min, and the cell pellet was washed twice with phosphate-buffered saline (PBS, pH 7.4; 0.2 g of KH_2PO_4, 1.5 g of $Na_2HPO_4 \cdot 7H_2O$, 8.0 g of NaCl, and 0.2 g of KCl in 1 L of distilled water). The suspension was then diluted with PBS serially to make 1-, 2-, 3-, 4-, 5-, 6-, and 7-Log CFU/mL of inocula.

Detection limit of *B. cereus* multiplex PCR kit

To determine detection limit with multiplex *B. cereus* PCR kit (Jinsung-UniTech Co., Korea), one-milliliter aliquots of each inoculum level were used to prepare DNA template. The one-milliliter aliquots of the inocula were centrifuged at 14,000 rpm at 4°C for 5 min, and the pellets were resuspended in 30 μL of distilled water. The bacterial suspensions were heated at 100°C for 10 min and centrifuged at 14,000 rpm at 4°C for 3 min. The supernatant was used as DNA template in PCR analysis according to the manufacturer's protocol. Three primers for *cry1* (138 bp), *groEL* (250 bp), and *ces* (405 bp) were included in the multiplex PCR kit (Jinsung-UniTech Co., Korea). *B. cereus* producing emetic toxin was detected by the presence of *groEL* and *ces* gene. *B. cereus,* non-producing emetic toxin, was detected by *groEL* gene. *B. thuringiensis* was indicated by *cry1* and *groEL* gene.

Determination of enrichment temperature

The enrichment broth (TSB with 0.15% polymyxin B) was prepared according to the Bacteriological Analytical Manual[6]. One-mililiter of polymyxin B (Oxoid, England) was added into 150 mL of TSB after filtering with 0.45 μm pore size filter (Hyundai Micro Co., Korea). The inoculum was inoculated into 50 mL TSB with 0.15% polymyxin B (Oxoid, England) to obtain 1-Log CFU/mL, and the media were incubated at 30°C for 0, 3, 6, 9, and 12 h. This process was conducted at other temperatures (37°C and 42°C) in same manners. One-mililiter aliquots of the enriched samples were spread plated on mannitol-egg yolk-polymyxin (MYP ; Becton, Dickinson and Company) agar and incubated at 30°C for 24 h. *B. cereus* colonies on the plates were manually counted.

B. cereus inoculation and detection

The fresh-cut cabbage samples were purchased from a market in Seoul, South Korea. Twenty-gram portions of the cabbage were placed into the filter bag (3M, St. Paul, MN, USA), and 0.1-mL aliquots of the inocula were inoculated at 1-, 2-, and 3-Log CFU/g in each sample. The inoculated samples were rubbed with hands 20 times, and the samples were placed at room temperature for 15 min to allow *B. cereus* attachment. Eighty milliliters of TSB with 0.15% polymyxin B were placed into the cabbage samples. The samples were enriched at 42°C. The temperature was determined by the previous assay. One-milliliter aliquots of the enriched samples were centrifuged at 14,000 rpm at 4°C for 5 min, and the pellet was washed by adding 30 μL distilled water. For non-filtered samples, the suspension was then boiled at 100°C for 10 min. The heated suspension was left at room temperature for 2 min and centrifuged at 14,000 rpm at 4°C for 3 min. For hydrophobic-filtered samples 1-mL aliquots of the enriched samples were filtered through hydrophobic filter membranes with 0.45 μm pore size (Hyundai micro Co., LTD., South Korea). Before filtering, the hydrophobic filter membranes were dipped in 2-propanol (Sigma-Aldrich Co., St. Louis, USA), and they were then assembled with ADVANTEC® filter holder (Toko Roshi Kaisha., LTD., Japan). Two-milliliters of distilled water were filtered for the hydrophobic filter to wash the residues. After the filter membranes were vortexed in 2 mL of distilled water for 1 min, and the filtrates were centrifuged at 14,000 rpm and 4°C for 5 min. Thirty-microliter of distilled water were added into the pellets, and the suspension were boiled at 100°C for 10 min. The heated suspensions were left at room temperature for 2 min and centrifuged at 14,000 rpm and 4°C for 3 min. The supernatants were used as DNA template. To detect *B. cereus* in both non-filtered and hydrophobic filtered samples multiplex PCR was conducted as described above.

Statistical analysis

Data (n = 4) were analyzed with the general linear model procedure of SAS® (SAS ver. 9.2 Institute Inc., USA). The significance of fixed effects was determined by a pairwise t-test at $\alpha = 0.05$.

Results and Discussion

The minimal detection concentration for detection of *B. cereus* by multiplex PCR was about 5-Log CFU/mL (Fig. 1). The result indicates that the *B. cereus* in fresh-cut cabbage samples should be enriched at least up to 5-Log CFU/g to be detected by the multiplex PCR in case *B. cereus* level is lower than that in a fresh-cut cabbage sample. Thus, enrichment temperatures for *B. cereus* enrichment were compared, and *B. cereus* cell counts were generally higher ($p < 0.05$) at 42°C than those at 30°C and 37°C (Table 1). Hence, we decided to use 42°C for enrichment temperature.

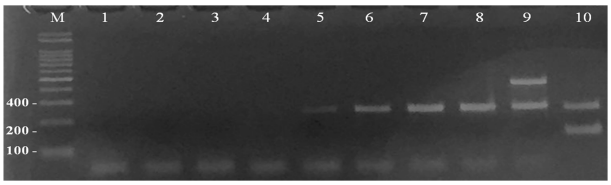

Fig. 1. Detection limit of *Bacillus cereus* multiplex PCR. Lane M: 100-bp ladder; lane 1: < 1 Log CFU/g inoculation; lane 2: 1-Log CFU/g inoculation; lane 3: 2-Log CFU/g inoculation; lane 4: 3-Log CFU/g inoculation; lane 5: 4-Log CFU/g inoculation; lane 6: 5-Log CFU/g inoculation; lane 7: 6-Log CFU/g inoculation; lane 8: 7-Log CFU/g inoculation; lane 9: *B. cereus* positive control; lane 10: *B. thuringiensis* positive control.

Table 1. *Bacillus cereus* cell counts (Log CFU/g; mean ± standard deviation) in enrichment broth samples inoculated at 1-Log CFU/mL (n = 4)

Enrichment time (h)	Incubated temperatures (°C)		
	30	37	42
0	0.9 ± 0.4^{Ac}	0.9 ± 0.4^{Ad}	0.9 ± 0.4^{Ad}
3	2.5 ± 0.4^{Ac}	2.9 ± 0.6^{Acd}	3.2 ± 0.3^{Ac}
6	4.6 ± 0.6^{Bb}	4.8 ± 0.4^{ABbc}	5.4 ± 0.3^{Ab}
9	5.7 ± 1.1^{Aab}	5.9 ± 1.2^{Aab}	6.2 ± 0.9^{Aab}
12	7.3 ± 0.5^{Aa}	7.7 ± 0.1^{Aa}	7.2 ± 0.4^{Aa}

$^{A-B}$Means with the same row with different superscript uppercase letters are significantly different ($p < 0.05$)
$^{a-d}$Means with the same column with different superscript lowercase letters are significantly different ($p < 0.05$)

Table 2. *Bacillus cereus* cell counts (Log CFU/g; mean ± standard deviation) in fresh-cut cabbage samples (n = 5) during enrichment at 42°C

Enrichment time (h)	Inoculation levels (Log CFU/g)		
	1	2	3
0	0.8 ± 0.3	1.7 ± 0.8	2.9 ± 0.2
4	-*	-	5.8 ± 0.2
5	5.4 ± 0.3	6.4 ± 0.3	7.5 ± 0.3
6	6.4 ± 0.6	7.5 ± 0.4	8.2 ± 0.2
7	6.9 ± 0.3	-	-

*: Not tested

When inoculated fresh-cut cabbage samples at 1-Log CFU/g were enriched for 7 h, *B. cereus* was detected with multiplex PCR in three of the five samples (60%), even though *B. cereus* levels were above the detection limit (Table 2 and Fig. 2). *B. cereus* in 2-Log CFU/g inoculated samples were enriched from 1.7 to 7.5 Log CFU/g that was above the detection limit within 6 h, and the pathogen was detected with multiplex PCR in four samples (80%) (Table 2 and Fig. 2). *B. cereus* in 3-Log CFU/g inoculated samples

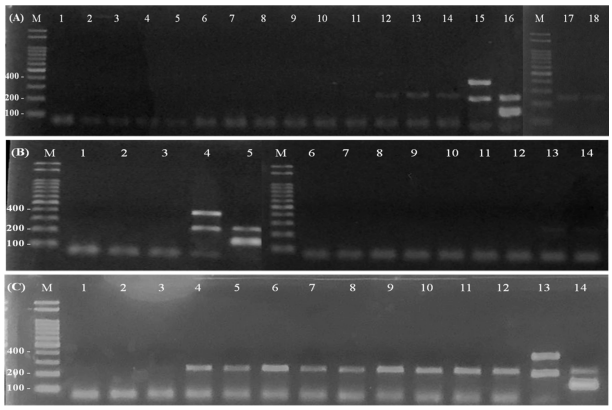

Fig. 2. Detection of *Bacillus cereus* in non-filtered fresh-cut cabbage samples by multiplex PCR. (A) Lane M: 100-bp ladder; lanes 1-2: 3-Log CFU/g inoculated and 4-h enriched samples; lanes 3-4: 1-Log CFU/g inoculated and 5-h enriched samples; lanes 5-6: 2-Log CFU/g inoculated and 5-h enriched samples; lanes 7-8: 3-Log CFU/g inoculated and 5-h enriched samples; lanes 9-10: 1-Log CFU/g inoculated and 6-h enriched samples; lanes 11-12: 2-Log CFU/g inoculated and 6-h enriched samples; lanes 13-14: 3-Log CFU/g inoculated and 6-h enriched samples; lane 15: *B. cereus* positive control; lane 16: *B. thuringiensis* positive control; lanes 17-18: 1-Log CFU/g inoculated and 7-h enriched samples; (B) lane M: 100-bp ladder; lanes 1-3: 1-Log CFU/g inoculated and 4-h enriched samples; lane 4: *B. cereus* positive control; lane 5: *B. thuringiensis* positive control; lanes 6-8: 1-Log CFU/g inoculated and 5-h enriched samples; lanes 9-11: 2-Log CFU/g inoculated and 5-h enriched samples; lanes 12-14: 3-Log CFU/g inoculated and 5-h enriched samples; (C) lane M: 100-bp ladder; lanes 1-3: 1-Log CFU/g inoculated and 6-h enriched samples; lanes 4-6: 2-Log CFU/g inoculated and 6-h enriched samples; lanes 7-9: 3-Log CFU/g inoculated and 6-h enriched samples; lanes 10-12: 1-Log CFU/g inoculated and 7-h enriched samples; lane 13: *B. cereus* positive control; lane 14: *B. thuringiensis* positive control.

were enriched from 2.9 to 8.2 Log CFU/g within 6 h, and the pathogen were detected in all samples (100%) (Table 2 and Fig. 2). These results indicate that enrichment is not sufficient to have accurate detection result with multiplex PCR. Elhariry et al.[22] reported that *B. cereus* spores and vegetative cells could adhere rapidly to cabbage, and that would make the minimal concentration for detection of the multiplex PCR increase. Also, PCR inhibitors such as chlorophyll and pectin could influence detection efficiency of the multiplex PCR[13,23]. In previous report, hydrophobic liquid was used for the NA purification by acting the liquid as an immiscible phase filter (IPF)[24]. Similarly, Wu et al.[23] reported that the hydrophobic filter can effectively eliminate chlorophyll from lettuce samples to detect *E. coli* O157:H7. Thus, to improve the detection efficiency of PCR method, enriched samples were filtered with a hydrophobic filter. For filtered samples with a hydrophobic filter after enrichment, *B. cereus* were detected in all 1-Log CFU/g inoculated

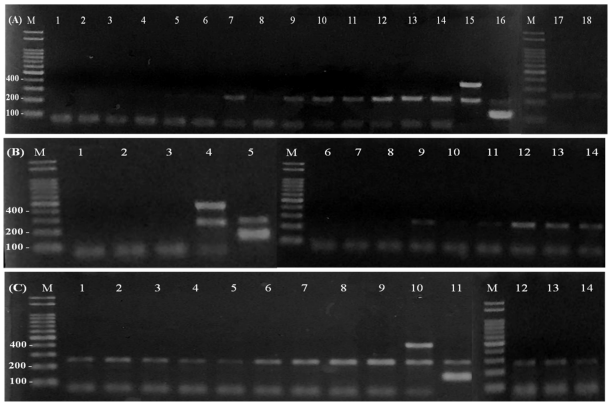

Fig. 3. Detection of *Bacillus cereus* in hydrophobic-filtrated fresh-cut cabbage homogenates by multiplex PCR. (A) Lane M: 100-bp ladder; lanes 1-2: 3-Log CFU/g inoculated and 4-h enriched samples; lanes 3-4: 1-Log CFU/g inoculated and 5-h enriched samples; lanes 5-6: 2-Log CFU/g inoculated and 5-h enriched samples; lanes 7-8: 3-Log CFU/g inoculated and 5-h enriched samples; lanes 9-10: 1-Log CFU/ inoculated and 6-h enriched samples; lanes 11-12: 2-Log CFU/g inoculated and 6-h enriched samples; lanes 13-14: 3-Log CFU/g inoculated and 6-h enriched samples; lane 15: *B. cereus* positive control; lane 16: *B. thuringiensis* positive control; lanes 17-18: 1-Log CFU/g inoculated and 7-h enriched samples; (B) lane M: 100-bp ladder; lanes 1-3: 1-Log CFU/g inoculated and 4-h enriched samples; lane 4: *B. cereus* positive control; lane 5: *B. thuringiensis* positive control; lanes 6-8: 1-Log CFU/g inoculated and 5-h enriched samples; lanes 9-11: 2-Log CFU/g inoculated and 5-h enriched samples; lanes 12-14: 3-Log CFU/g inoculated and 5-h enriched samples; (C) lane M: 100-bp ladder; lanes 1-3: 1-Log CFU/g inoculated and 6-h enriched samples; lanes 4-6: 2-Log CFU/g inoculated and 6-h enriched samples; lanes 7-9: 3-Log CFU/g inoculated and 6-h enriched samples; lane 10: *B. cereus* positive control; lane 11: *B. thuringiensis* positive control; lanes 12-14: 1-Log CFU/g inoculated and 7-h enriched samples;

samples (100%) within 6 h. The bacteria in 2-Log CFU/g inoculated samples were detected in all samples (100%) within 5 h. These results indicate that filtering enriched samples with a hydrophobic filter can improve detection efficiency of *B. cereus* in fresh-cut cabbage with multiplex PCR. Hydrophobic filter removes PCR inhibitors such as chlorophyll from fresh-cut cabbage[23].

In conclusion, a combination of enrichment and hydrophobic filtering fresh-cut cabbage samples improves detection efficiency for *B. cereus* with multiplex PCR.

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국문요약

본 연구에서는 신선편이 양배추 내 *Bacillus cereus*의 최적 증균 온도를 선정하고 증균배양액에 소수성필터를 적용하여 multiplex PCR의 검출률을 확인하였다. *B. cereus* 증균온도는 *B. cereus* 균주 5 개를 혼합하여 1 Log CFU/mL이 되도록 증균배지에 접종하고 30°C, 37°C, 42°C에서 증균한 뒤 3시간 간격으로 MYP agar에 도말한 후 계수하여 선정하였다. 소수성필터 미적용 그룹은 *B. cereus* 균주 5 개를 혼합하여 신선편이 양배추에 접종한 뒤 최적 증균온도에서 증균하였으며, 증균배양액을 가열하여 DNA를 추출한 뒤 multiplex PCR을 진행하였다. 소수성필터 적용 그룹은 증균배양액을 소수성 필터에 적용하고 필터에 있는 균을 멸균증류수로 현탁한 뒤 가열하여 추출된 DNA로 multiplex PCR을 진행하였다. 증균온도 확인 결과, 6시간 증균 시 42°C에서 증균된 샘플(5.4 ± 0.3 Log CFU/mL)과 30°C에서 증균된 샘플(4.6 ± 0.6 Log CFU/mL) 간 유의차가 확인되었다($p < 0.05$). 소수성필터 적용 유무에 따른 multiplex PCR 결과, 1 Log CFU/g 접종된 샘플의 검출률이 소수성 필터 적용 전 60%(3/5)에서 100%(5/5)로 향상되었다. 2 Log CFU/g 접종 샘플은 소수성필터 적용 전 80%(4/5)에서 소수성 필터 적용 후 100%(5/5)로 검출률이 증가하였으나, 3 Log CFU/g 접종 샘플은 소수성 필터 적용 전후 모두 100%(5/5)로 확인되었다. 이상의 결과를 통해 신선편이 양배추 내 *B. cereus* 검출 시 증균배양액에 소수성필터를 적용하고 multiplex PCR을 적용했을 때 신속하고 효율적인 검출이 가능할 것으로 판단된다.

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