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Original Article

Detection of Emetic *Bacillus cereus* from Ready-to-eat Foods in Markets and its Production of Cereulide under Simulated Conditions

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Abstract: *B. cereus*-produced cereulide as an emetic toxin is commonly isolated in starch-based cooked foods. This study examined the prevalence of *B. cereus* from ready-to-eat foods in markets by polymerase chain reaction analysis and determined the relationship between the level of *B. cereus* and the quantity of cereulide in the sample after different storage times and temperatures. The prevalence of general *B. cereus* in 43 starch foods was 32.6%, and the level of *B. cereus* ranged from 0.5 to 1.95 log cfu/g, meeting the Korea Food Code Specifications of 3 log CFU/g of *B. cereus*. No samples revealed emetic *B. cereus.* Fried rice samples were inoculated with a cereulide-producing reference strain, *B. cereus* NCCP 14796, to determine the level of *B. cereus* and the quantity of cereulide in the samples after storage for 0, 4, 6, 8, 20, 24, 30, 48, 72, and 96 h at 7, 25, 35, and 57°C. The average levels of *B. cereus* at 7, 25, 35, and 57°C were 4.38, 7.31, 7.88, and 3.82 log cfu/g, and the levels of cereulide were 150.41, 1680.70, 2652.65, and 77.83 μg/mL, respectively, showing a significant difference according to the incubation time ($P<0.05$) and temperature ($P<0.001$).

Key words: Emetic *Bacillus cereus,* starch foods, cereulide, storage, risk assessment model

I. Introduction

Bacillus cereus is an causative agent of foodborne illness (KMFDS 2016) and is commonly isolated from starch-based convenience foods (Kim *et al.* 2004; Bahk *et al.* 2007), including gimbap, fried rice (Chang *et al.* 2011), and sushi (Kim *et al.* 2008), which are dishes consumed frequently by Koreans as a staple diet. In particular, fried rice has become a globally popular food that is widely consumed in the United States, as well as East-, South and Middle East Asia. On the other hand, *B. cereus* produces an emetic toxin substance, cereulide. The prevalence of emetic *B. cereus* in starch-based foods (e.g., fried rice dishes) has attracted little research attention. Moreover, there is little research on the quantitative analysis of the toxic substance, cereulide.

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B. cereus is a Gram-positive, rod type of facultative bacteria, having mobility with a peritrichous flagellum (Kim & Choi 2009). *B. cereus* is distributed widely in soil and water environments, resulting in a relatively high likelihood of contamination in crops, vegetables, and fruits. The bacteria have also isolated from ready-to-eat foods or convenience foods, such as cooked rice (Kim *et al.* 2008; Chang & Lee 2009) or *gimbap*, which might be treated by improper heating or unhygienic practices from employees in Korea. *B. cereus* has been reported to grow well in starch foods, such as rice dishes, pasta, and noodles, which have a neutral pH (Agata *et al.* 2002; Gadaga *et al.* 2008; Dong 2013).

B. cereus grows at optimal temperatures of 30 to 40°C and can survive temperatures from 7 to 49°C. Moreover, its spores can endure heating at 135°C for 4 h (Agata *et al.* 2002). The optimal pH for the growth of the organism is pH 4.9 to 9.3. In addition, it has a tolerance to *Penicillin*, which it catalyzes to casein and tyrosine to form catalase, showing a positive reaction with egg-yolk and hydrolysis (Agata *et al.* 2002; Jaaskelainen *et al.* 2004).

The outbreaks of foodborne illnesses caused by *B. cereus*

have been attributed to toxic substances comprised of two types depending on the symptoms, diarrheal and emetic (Granum & Lund 1997; Lake *et al.* 2004). The diarrhea-toxin consists of 17 amino acids and is inactivated by heat treatment at 56°C for 5 min (Finlay *et al.* 2002; Haggblom *et al.* 2002). The symptoms of the outbreaks caused by this toxin include vomiting, abdominal pain, nausea, and diarrhea (Kotiranta *et al.* 2000; Lake *et al.* 2004). The substances are associated with meat, poultry, milk, and dairy products (Kang *et al.* 2008).

The emetic toxin, known as cereulide, has a large cyclic structure with four peptide units of amino acids (Haggblom *et al.* 2002; Ankolekar *et al.* 2009). Cereulide is generated immediately before spore formation and is very stable to heat (>126°C for 90 min), acids, and alkalis (Agata *et al.* 2002). In addition, it can be verified by the existence of the cereulide synthesis enzyme (Haggblom *et al.* 2002).

B. cereus-produced cereulide, as an emetic toxin substance, is only isolated from starch-based convenience foods and carries a higher risk to people who consume these foods regularly. On the other hand, there has been little research on the prevalence of *B. cereus* from starch-based foods and the production of cereulide (Kang *et al.* 2008; Lee & Chang 2009; Chang *et al.* 2011; Choi 2015; Lim *et al. 2016*). Therefore, this study performed polymerase chain reaction (PCR) analysis to examine the prevalence of *B. cereus* in cooked foods, including starch convenience foods, particularly emetic *B. cereus*, which produces the cereulide toxin. The specific purposes of the study were as follows: 1) to identify the prevalence of emetic *B. cereus* and generic *B. cereus* in starch-based dishes; and 2) to examine the effects of time, temperature, and pH on the growth of emetic *B. cereus*. In addition, the quantity of cereulide was tested after inoculating fried rice samples with emetic *B. cereus* different simulated conditions: storage for 0, 4, 6, 8, 20, 24, 30, 48, 72, and 96 h at 7, 25, 35, and 57°C.

II. Materials and Methods

1. Materials

1) Prevalence of *B. cereus* in starch foods

Forty-three samples consisting of three types were tested due to being frequently consumed convenience foods for Koran: prepared dishes with homemade recipes, takeout dishes from markets, and delivered dishes from a Chinese restaurant. The first type of samples included cooked rice dishes, such as *bap* (cooked rice), *boribap* (cooked rice with barely), *hyeonmibap* (cooked rice with brown rice), and *chopssalbap* (cooked rice with sticky rice). The second type of samples were commercial products of cooked rice-based dishes (n=2), *gimbap* (made by rolling cooked rice on a piece of laver with meat, egg, and vegetables (e.g., spinach, carrot, cucumber, etc.), lunchbox meals, bread, cake, spaghetti, rice cake, and juk products (type of rice porridge with various vegetables or nuts). The samples were purchased in convenience stores, taken out in an icebox, and brought to the laboratory within 10 min. The last type was delivery foods, such as *deopbap* and fried rice (*bokkeumbap*) dishes from Chinese restaurants, which were located within a 15 to 20 min delivery distance. Immediately after delivery, the samples were treated aseptically and used for the microbiological tests.

2) Storage test on the growth of *B. cereus* in the fried rice dish

For the storage test, a fried rice dish sample was delivered from a Chinese restaurant within a 20 min delivery distance. After delivery, the sample was used immediately for microbial analyses. The sample was divided into 40×50 g portions for the experiments, packaged with a sterilized bag, and stored for 4, 6, 8, 20, 24, 30, 48, 72, and 96 h at 7, 25, 35, and 57°C.

3) Reference strain and experimental conditions for the simulation test

For the inoculation test of *B. cereus*, the delivered samples were sterilized in an autoclave for the aseptic treatments. The reference emetic strain of *B. cereus* NCCP 14796, producing cereulide was obtained from the Korea Centers for Disease Control and Prevention, the department of pathogen defense and research. After the samples were separated into 40 Þ 50 g portions, *B. cereus* NCCP 14796 was inoculated at an initial level of 103~104 cfu/g. The inoculated samples were stored for 4, 6, 8, 20, 24, 30, 48, 72, and 96 h at 7, 25, 35, and 57°C.

2. Microbial analyses

1) Quantification of APC (Aerobic Mesophilic Plate Count)

Samples (25 g) were placed in 225 mL sterile physiological saline water and homogenized vigorously for 2 min using a stomacher (Easymix, Bruz Cedex, France). A sample diluted 10 fold with sterile physiological saline water was prepared, ranging from 10^{-2} to 10^{-7} diluted solutions. A 1 mL sample of the diluted solutions was placed into a petri dish

containing 15 mL of Plate Count Agar (Difco, MI, USA). The plates were incubated for 48 h at 35°C.

2) Quantification of *B. cereus*

Samples (25 g) were placed in 225 mL sterile physiological saline and then homogenized vigorously for 2 min using a stomacher (Easymix, France). A sample diluted 10 fold with sterile physiological saline water was prepared, ranging from 10^{-2} to 10^{-7} diluted samples. Subsequently, 0.2 mL of the diluted sample solution was streaked on five plates of mannitol yolk polymyxin (MYP) agar base containing egg yolk. The plates were incubated for 24±2 h at 30°C, and the number of pink colonies surrounded by a zone of lecithin hydrolysis was counted. A confirmation test was carried out using a PCR assay. The final number of colonies was calculated by the reflection of the positive rate after the confirmation tests.

3) PCR assay

Samples showing a positive reaction on *B. cereus* were tested further to determine if the cereulide synthesis gene was present using the PCR tests. Two types of primers targeting the gyrB and ces genes, designed by the Bioneer company (Daejeon, Korea), were used to detect emetic *B. cereus* separately from the generic *B. cereus*, as shown in <Table 1>. To identify generic *B. cereus*, a single colony from the cultivated sample onto the MYP agar was suspended in 100 μL of sterile distilled water and heated for 20 min at 95°C using a heating block. The suspension was centrifuged at 12,000 rpm for 10 min at 4°C, and 13 μL of the supernatant was used as a DNA template. The PCR mixture (20 μL) contained Top DNA polymerase 1U, dNTP 250 μm, Tris-HCl 10 mM, KCl 30 mM, $MgCl_2$ 1.5 mM (Bioneer, Korea). The PCR reaction conditions were as follows: 30 cycles (94°C, 1 min→58°C, 1.5 min→72°C, 2.5 min)→72°C, 7 min. The amplification results were separated by electrophoresis on a 1% agarose gel in 0.5x TAE buffer. The gels were stained with ethidium bromide and visualized using a UV transilluminator (Entela, USA).

The same experimental procedures reported above except for the PCR reaction conditions were used to detect emetic *B. cereus* containing the cereulide producing gene: 95°C for 15 min→5 cycles (95°C, 60 sec→53°C, 75 sec→72°C, 50 sec)→25 cycles (95°C, 60 sec→58°C, 75 sec→72°C, 50 sec)→72°C, 5 min.

3. Physiochemical analysis

1) pH

Ten gram samples were placed in 90 mL distilled water and homogenized vigorously for 2 min using a stomacher (AES laboratory, France). The pH was measured using a pH meter (Orion, USA).

2) Salinity

Ten gram samples were placed in 90 mL distilled water and homogenized vigorously for 2 min using a stomacher (AES laboratory, France). The salinity was measured using a salt meter (Daeyoon Scale Industrial Co., Korea).

4. Quantitative analysis of cereulide by liquid chromatography/mass spectrometry (LC/MS)

1) Preparation of the cell extracts

LC/MS was performed to quantify the emetic toxin in the samples containing the cereulide synthesis gene according to the procedure reported by Haggblom *et al.* (2002). Biomass from the plates was collected, and cells were lysed using three repeated freeze-thaw cycles and extracted overnight with 98% methanol (10 mL per gram of biomass [wet weight]). The mixture was then centrifuged at 3,800 rpm, and the supernatant was collected. The sample was evaporated to dryness, weighed, and dissolved in 1mL of 98% methanol. Finally, the sample was filtered through a 0.45 μm filter and stored at –20°C until analyzed. LC/MS analysis was performed using a hundredfold dilution.

2) Analytical conditions

Liquid chromatography was performed on an Agilent 1200 series (Agilent Technologies Inc., USA) equipped with a C18 column (Imtakt, Japan) (2.0×30 mm Cadenza). The column was maintained at 40°C, and the sample injection

Table 1. Primers used to detect *B. cereus* and the cereulide-producing gene with PCR

Primer name	Primer sequence (5'-3')	Target gene	Product size	Reference
BC1 BC2r	ATTGGTGACACCGATCAAACA TCATACGTATGGATGTTATTC	gyrB	365	(Yamada *et al.*, 1999)
CesF1 CesR2	GGTGACACATTATCATATAAGGTG GTAAGCGAACCTGTCTGTAACAACA	ces	1,271	(Ehling-Schulz *et al.*, 2004)

Table 2. MRM transitions and conditions of valinomycin and cereulide

Compound	Q1	Q3	DP	EP	CE	CXP
Valinomycin	1128.8	1083.9	121	10	51	30
		343.3			83	18
Cereulide	1170.7	1125.7	131	10	53	30
		357.2			85	18

volume was 2 μL. Mass spectrometry was performed on an AB SCIEX 4000 QTRAP Mass Spectrometry (AB SCIEX, USA) and measured in electrospray ionization (ESI) mode (Ion spray voltage 5500 V, Gas 1 50, Gas 2 45, Temperature 5500, Curtain gas 22, CAD gas medium). The MRM analysis conditions were established because the mass spectrometer operates in MRM (multiple reaction monitoring) <Table 2>. Cereulide carries a high risk and is not commercialized. Therefore, valinomycin structurally similar molecules were used as standards <Figure 1>. The microbial tests, PCR analysis, and chemical analysis were conducted in duplicate. The LC/MS analysis experiments were conducted in triplicate.

5. Statistical analysis

Minitab 16 (Minitab Inc., USA) was used to analyze the two-way variance and compare the APC levels, *B. cereus*, pH, and salinity according to the storage temperature and time. A Pearson correlation was performed to examine the relationship between *B. cereus* and the cereulide concentrations according to the storage temperature and time. A significance test was performed at the 95% confidence interval.

III. Results and Discussion

1. Prevalence of *B. cereus* in starch-based cooked foods

From the experiments with 43 starch-based cooked foods, including ready-to-eat foods, 16 samples showed positive responses on the MYP medium. The PCR tests performed to confirm the generic *B. cereus* and emetic types showed that 32.6% of the samples (14 food items) contained generic *B. cereus*, ranging from 0.5 to 1.95 log cfu/g. Emetic *B. cereus* was not detected in any of the samples <Table 3>.

For the levels of generic *B. cereus*, a cake made with cheese showed the highest levels of *B. cereus* at 1.95 log cfu/g, followed in order by the fried rice dishes with seafood (1.35 log cfu/g) and ham and vegetables (1.30 log cfu/g). Despite having lower levels of *B. cereus*, *deopbap* and *gimbap* also contained *B. cereus* ranging from 0.5 to 1.0 log cfu/g. Steamed rice dishes, including mixed grains, juk, and rice cakes, were not contaminated with *B. cereus*. Lunch boxes, such as chicken mayonnaise, *sanchae-bibimbap*, and curried rice, also did not contain *B. cereus*. Three types of popular spaghetti (carbonara, meatball, and garlic and oil sauce), and bread (e.g., baguette and bagel) also did not contain *B. cereus*. PCR analyses for the cereulide synthetic gene showed that none of the 43 food samples contained *B. cereus* generating the cereulide toxin substance <Figure 2>.

Few studies in Korea have examined *B. cereus* contamination in raw rice. In raw rice samples, *B. cereus* was detected in 25 out of 36 samples (69.4%); two of these samples carried the emetic *B. cereus* (Forghani *et al.* 2016). In a study of the prevalence of emetic toxin-producing *B. cereus* in Korea rice (Kim *et al.* 2014), 37 out of 65 white rice samples (57.8%) were contaminated with *B. cereus* spores, but the emetic toxin was detected in only one sample, showing a 2.7% detection rate. Ankolekar *et al.* (2009) analyzed 178 raw rice samples from retail food stores for the presence of *B. cereus* spores. Spores of the bacillus species were found in 94 (52.8%) of the rice samples, showing a prevalence of hbl enterotoxin (56.6%) and nhe enterotoxin (89.1%). On the other hand, the cereulide synthetase (ces) gene was not identified in any isolates (Ankolekar *et al.* 2009).

In terms of levels of *B. cereus* contamination in cooked rice foods or ready-to-eat foods, *B. cereus* was detected in 16 out of 60 samples in a quantitative test (26.7%) (Kim 2016), and a very low percentage of samples were unsatisfactory with more than 4 log cfu/g of *B. cereus*. In the present study, 14 samples from 43 starch-based cooked foods (32.6%) were contaminated with generic *B. cereus* according to the PCR tests; the contamination level of *B. cereus* of the 14 samples ranged from 0.5 to 1.95 log cfu/g, but none of them was the emetic *B. cereus*. This shows that the limit of *B. cereus* for ready-to-eat food samples of 4 log cfu/g is appropriate (KMFDS 2018).

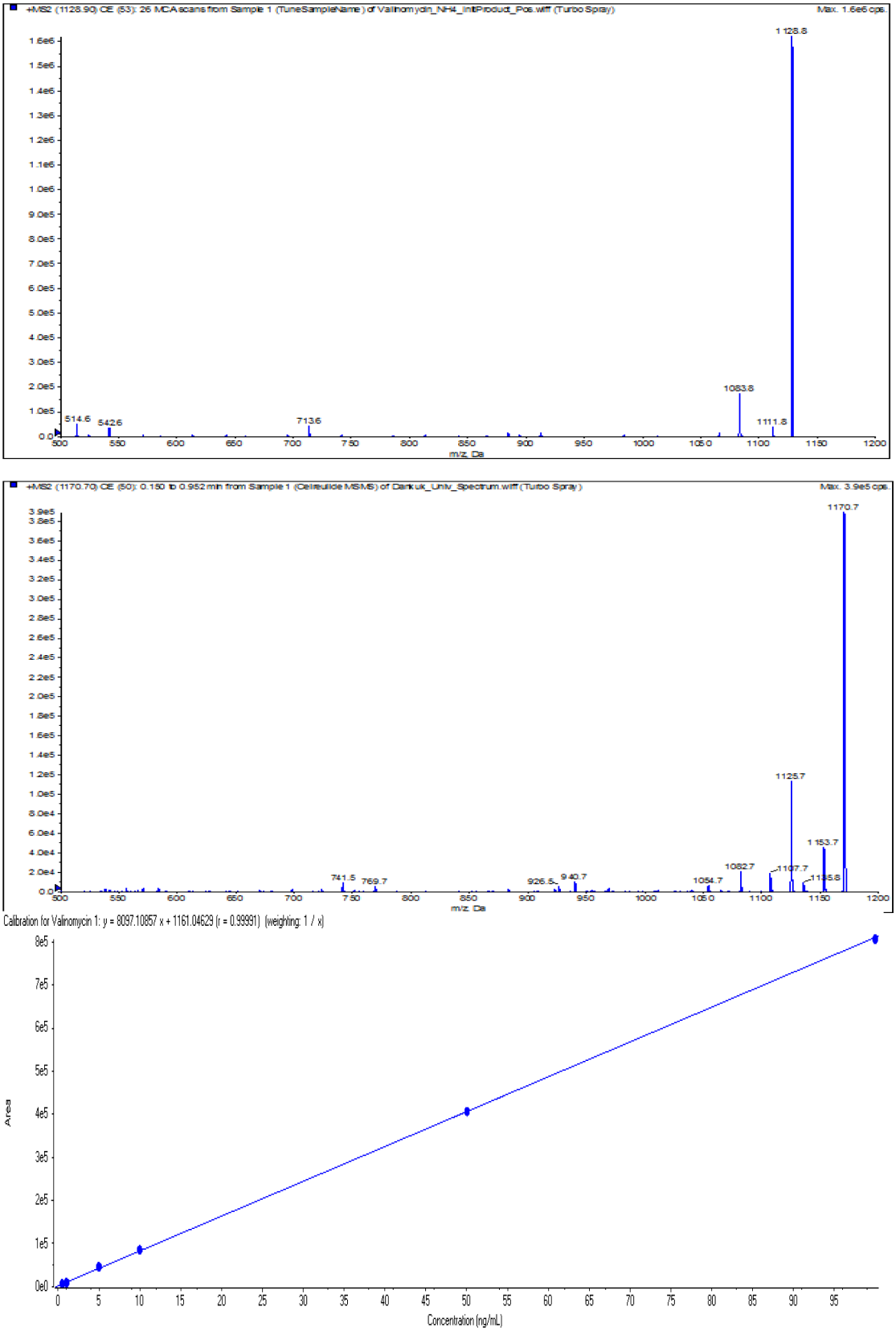

Figure 1. Mass spectrum of valinomycin and cereulide (a) valinomycin (b) cereulide and (c) calibration curve for valinomycin.

<Table 3> Prevalence of *B. cereus* in 43 starch foods (Unit: log cfu/g) (Mean±SD)

	Food	Presumptive test	Confirmed test	Cereulide (ces gene)	Remark
Rice	Boiled rice (n=2)	ND[1)]	ND	ND	Takeout
	Steamed rice	ND	ND	ND	Cooking
	Boiled barley	ND	ND	ND	Cooking
	Brown rice	ND	ND	ND	Cooking
	Boiled glutinous rice	ND	ND	ND	Cooking
Deopbap	Spicy stir-fried pork- *deopbap*	1.00±0.00	1.00±0.00	ND	Delivered
	Squid *deopbap*	ND	ND	ND	Delivered
Gimbap	*Gimbap* (n=2)	1.82±0.74	ND	ND	Takeout
	Kimchi Gimbap (n=2)	1.65±0.92	0.50±0.71	ND	Takeout
	Cheese *Gimbap* (n=2)	1.65±0.92	0.50±0.71	ND	Takeout
	Tuna *Gimbap* (n=2)	2.11±1.14	0.65±0.91	ND	Takeout
	Beef *Gimbap* (n=2)	ND	ND	ND	Takeout
Box lunch	Chicken mayo	ND	ND	ND	Takeout
	Sanchae-bibimbap	ND	ND	ND	Takeout
	Curried rice	ND	ND	ND	Takeout
Fried rice	Kimchi fried rice	2.18±0.00	1.09±0.00	ND	Delivered
	Fried rice with ham and vegetable	1.30±0.00	1.30±0.00	ND	Delivered
	Fried rice (n=2)	1.57±0.81	0.50±0.71	ND	Delivered
	Fried rice with seafood (n=2)	1.35±0.49	1.35±0.49	ND	Delivered
Bread	Baguette	ND	ND	ND	Takeout
	Grain bread	ND	ND	ND	Takeout
	Bagel	ND	ND	ND	Takeout
Cake	Whipped-cream cake	ND	ND	ND	Takeout
	Cheesecake	1.95±0.00	1.95±0.00	ND	Takeout
Spaghetti	Spaghetti with tomato sauce	ND	ND	ND	Takeout
	Spaghetti carbonara	ND	ND	ND	Takeout
	Spaghetti with garlic and oil sauce	ND	ND	ND	Takeout
Rice cake	Jeolpyeon	ND	ND	ND	Takeout
	Injeolmi	ND	ND	ND	Takeout
	Steamed rice cake	ND	ND	ND	Takeout
	Rice cake with beans	ND	ND	ND	Takeout
	Baekseolgi	ND	ND	ND	Takeout
Juk	Vegetable-*juk*	ND	ND	ND	Takeout
	Beef and mushrooms-*juk*	ND	ND	ND	Takeout
	Seafood-*juk*	ND	ND	ND	Takeout

[1)]ND: Not detected

2. Storage tests on the growth of *B. cereus* at different times and temperatures

In the delivered fried rice dishes stored at 7, 25, 35, and 57°C for 96 h, none of the samples stored at 7 and 57°C produced *B. cereus* colonies on the MYP plate. Some samples exposed to 25 and 35°C for more than 20 h showed the presumptive *B. cereus*, but neither generic *B. cereus* nor emetic *B. cereus* was identified by the PCR assay. This suggests that no emetic *B. cereus* existed on the delivered fried rice dishes after 96 h storage at 7, 25, 35, and 57°C. Although the data are not presented in the Table, the samples showed 4.91, 6.22, 6.65, and 4.62 log cfu/g of APC at 7, 25, 35, and 57°C, respectively. Moreover, at those temperatures, the average pH was 7.68, 6.27, 5.94, and 7.24, respectively, and the salinity was 0.04, 0.05, 0.05, and 0.04%, respectively.

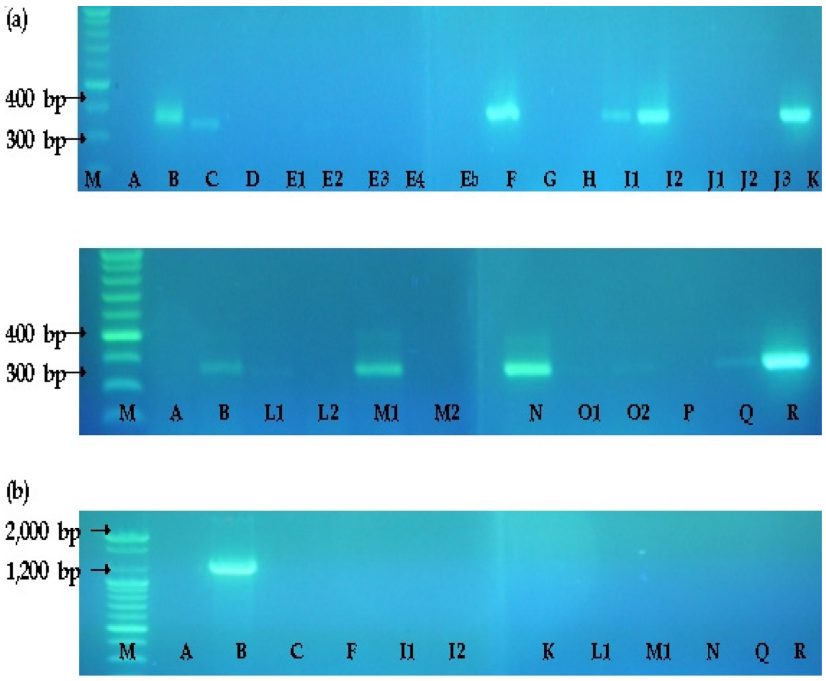

Figure 2. PCR detection of *B. cereus* and cereulide-producing *B. cereus* (a) *B. cereus* and (b) cereulide producing *B. cereus*.
Lane M, size marker; lane A, negative; lane B, *B. cereus* NCCP 14796 used as the cereulide-producing reference strain; lane C, *B. cereus* isolated from spicy stir-fried pork-*deopbap*; lane D, *B. cereus* isolated from *gimbap* (a); lane E, *B. cereus* isolated from *gimbap* (b); lane F, *B. cereus* isolated from *Kimchi gimbap* (a); lane G, *B. cereus* isolated from *Kimchi gimbap* (b); lane H, *B. cereus* isolated from cheese *gimbap* (a); lane I, *B. cereus* isolated from cheese *gimbap* (b); lane J, *B. cereus* isolated from tuna *gimbap* (a); lane K, *B. cereus* isolated from tuna *gimbap* (b); lane L, *B. cereus* isolated from *Kimchi* fried rice; lane M, *B. cereus* isolated from fried rice with ham and vegetable; lane N, *B. cereus* isolated from fried rice (a); lane O, *B. cereus* isolated from fried rice (b); lane P, *B. cereus* isolated from fried rice with seafood (a); lane Q, *B. cereus* isolated from fried rice with seafood (b); lane R, *B. cereus* isolated from cheese cake.

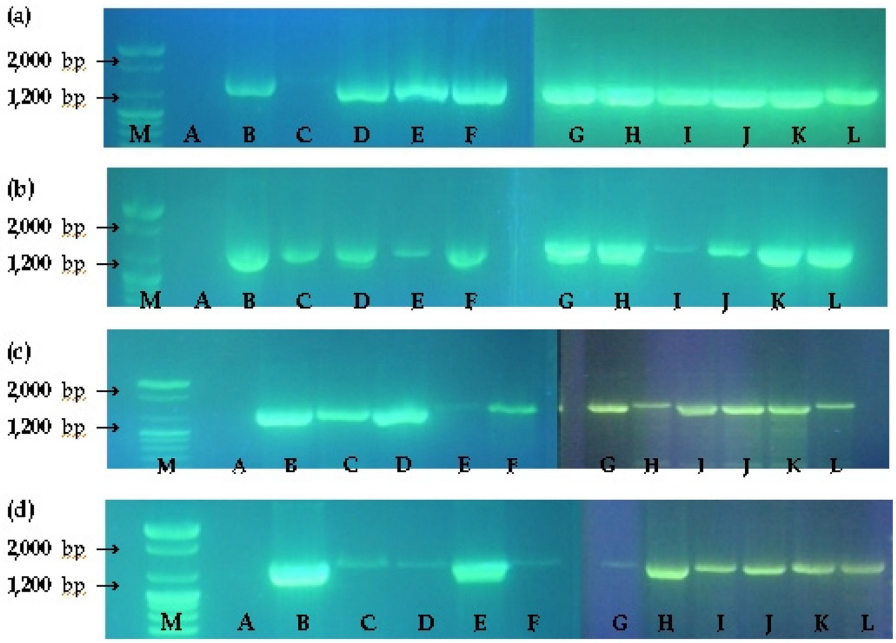

Figure 3. PCR detection of cereulide-producing *B. cereus* (a) 7°C, (b) 25°C, (c) 35°C and (d) 57°C.
Lane M, size marker; lane A, negative; lane B, *B. cereus* NCCP 14796 used as the cereulide-producing reference strain; lane C, *B. cereus* isolated from inoculated fried rice at 0 h; lane D, *B. cereus* isolated from inoculated fried rice at 4 h; lane E, *B. cereus* isolated from inoculated fried rice at 6 h; lane F, *B. cereus* isolated from inoculated fried rice at 8 h; lane G, *B. cereus* isolated from inoculated fried rice at 20 h; lane H, *B. cereus* isolated from inoculated fried rice at 24 h; lane I, *B. cereus* isolated from inoculated fried rice at 30 h; lane J, *B. cereus* isolated from inoculated fried rice at 48 h; lane K, *B. cereus* isolated from inoculated fried rice at 72 h; lane L, *B. cereus* isolated from inoculated fried rice at 96 h.

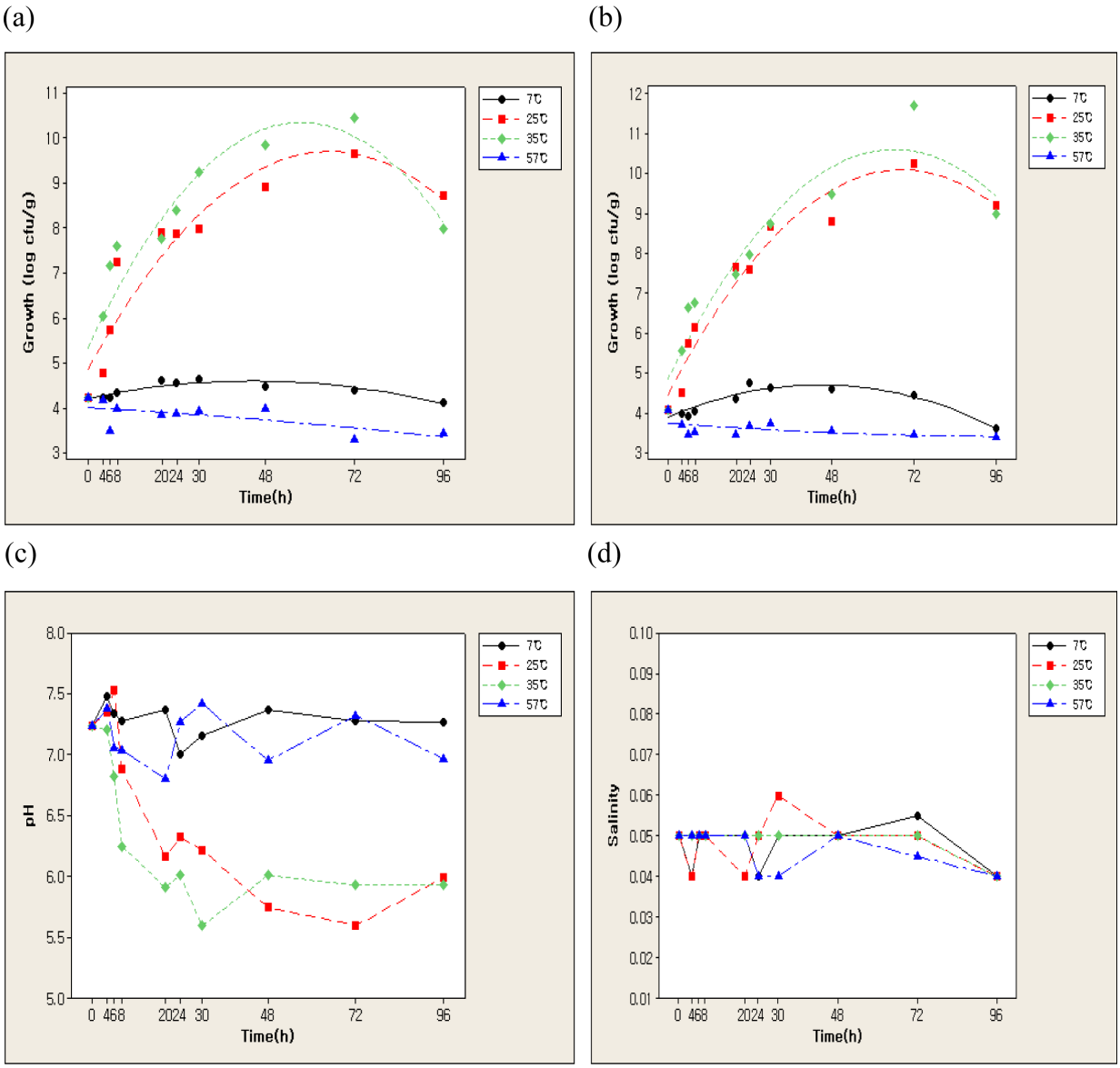

Figure 4. Changes in *B. cereus*, APC, pH, and salinity in the inoculation test of emetic *B. cereus* at different storage temperatures and times (a) Growth of *B. cereus* NCCP 14796 on inoculated fried rice at 7°C, 25°C, 35°C, and 57°C. Scatter diagram made by Minitab 16(Two-way ANOVA, Temp (F=33.80***), Time (F=2.49*) R2=74.15%) (b) Growth of APC on inoculated fried rice at 7°C, 25°C, 35°C, and 57°C. Scatter diagram made by Minitab 16 (Two-way ANOVA, Temp (F=28.27***), Time (F=2.87*) R2=71.67%) (c) Changes in pH on inoculated fried rice at 7°C, 25°C, 35°C, and 57°C. Scatter diagram made by Minitab 16 (Two-way ANOVA, Temp (F=15.72***), Time (F=2.96*) R2=61.29%) (d) Changes in salinity on inoculated fried rice at 7°C, 25°C, 35°C, and 57°C. Scatter diagram made by Minitab 16 (Two-way ANOVA, Temp (F=0.59), Time (F=2.57) R2=24.92%)

3. Growth of *B. cereus* and APC on the fried rice dishes after inoculating emetic *B. cereus* for different times and temperatures

The initial concentration of *B. cereus* NCCP 14,796 inoculated on the fried rice dishes at 0 h was 4.22 log cfu/g. <Figure 3> presents the PCR detection of cereulide from the emetic *B. cereus* of the samples. The average levels of *B. cereus* on the fried rice dishes at 7, 25, 35, and 57°C were 4.38, 7.31, 7.88, and 3.82 log cfu/g, showing rapid growth at 25 and 35°C and a peak curve around 48 h with gradually slower growth to 96 h <Figure 4a>. Two-way ANOVA (Analysis of Variance) showed that the growth of *B. cereus* was affected by the storage temperature ($P<0.001$) and time ($P<0.05$), with a significantly higher R^2 value of 74.15%. Therefore, *B. cereus* grew more rapidly at 25 or 35°C than at 7 or 57°C.

Under the same storage environment, the average level of APC after 96 h at 7, 25, 35, and 57°C was 4.24, 7.27, 7.74,

Table 4. Level of cereulide production depending on the time and temperature conditions (Unit: ng/mL)

	7°C		25°C		35°C		57°C	
Time	DFSM[1)]	Mean±SD	DFSM	Mean±SD	DFSM	Mean±SD	DFSM	Mean±SD
0 h	10^{-2}	1883.25±241.90	10^{-2}	1883.25±241.90	10^{-2}	1883.25±241.90	10^{-2}	1883.25±241.90
4 h	10^{-2}	1504.10±79.48	10^{-3}	1680.70±81.18	10^{-3}	2652.65±86.48	10^{-2}	778.30±400.65
6 h	10^{-2}	1609.10±299.53	10^{-3}	2409.40±159.81	10^{-4}	2740.95±357.10	10^{-2}	1132.60±31.11
8 h[2)]	10^{-2}	1504.15±172.04	10^{-4}	3641.30±403.48	10^{-4}	4845.40±2604.13	10^{-2}	1116.70±111.30

1)Dilution factor on solid media
2)F values were not calculated because of biomass taken from different dilution factors of solid media

and 3.60 log cfu/g <Figure 4b>, and the average pH was 7.28, 6.52, 6.27, and 7.15, respectively.

The pH of the sample <Figure 4c> decreased sharply with increasing storage time at 25 and 35°C compared to 7 and 57°C. The initial pH of 7.2 dropped to 5.7 at 35°C and 6.0 at 25°C for 48 h and then maintained pH 6.0 for 96 h (temperature effect P<0.001, time effect P<0.05). On the other hand, the saline concentration was stable at 0.05% under different storage times and temperatures, not showing a significant difference <Figure 4d>.

The pH of the fried rice samples ranged from 6.61 to 8.09, depending on the ingredients, showing an average of 7.13 (Chang *et al.* 2011). Another study suggested that the Aw of the cooked rice samples ranged from 0.97 to 0.99, but there was no correlation between Aw and toxin production (Finlay *et al.* 2002)

4. Production of cereulide of *B. cereus* under different time and temperature conditions

The levels of *B. cereus* in the samples exposed to 4 h at 7, 25, 35, and 57°C were 4.22, 4.78, 6.04, and 4.18 log cfu/g, respectively, and levels of cereulide production were 150.41, 1680.70, 2652.65, and 77.83 μg/mL, respectively. The samples exposed to 7 and 57°C showed a significant decrease in cereulide production. The samples exposed at 25 and 35°C showed a considerably higher level of cereulide production, even though the dilution factors increased with the incubated time.

McElroy *et al.*(2000) suggested that the emetic toxin is produced at a *B. cereus* level of 5 log cfu/g at 26.7°C. Another study pointed out that the emetic toxin was not produced below 12°C. Finlay *et al.* (2002), however, detected more cereulide at low temperatures than at room temperature, and no toxin was detected at the levels of <4 log CFU/g at 15°C. In contrast, the quantity of toxin increased to a 60 titer of cereulide when the mean viable count was 6.8 log cfu/g at 15°C for 48 hr, whereas a 27 titer was observed at 7.2 log cfu/g stored at 30°C. This result is consistent with the present study, even though lower cereulide levels were detected at 7°C than at 25 and 35°C.

In summary, emetic *B. cereus* was not detected in the 43 starch foods examined, but there is still a possibility of risk on generic *B. cereus* contamination during delivery to consumers. Therefore, caution regarding *B. cereus* is still needed. This study identified the prevalence of the emetic *B. cereus* and the concentration of the cereulide using a simulation experiment, depending on the storage temperatures and times. This study had some limitations. The study used convenient samples from a selling place near the laboratory and a franchise outlet having standard operation guidelines. Therefore, the prevalence of general or emetic *B. cereus* could be higher in the real industry, considering the small scale of retailers or food service facilities. In addition, a one-time experiment under different storage scenarios was used to calculate the amount of cereulide. Thus, a larger database should be instituted with the quantitative analysis of cereulide depending on the time and temperature, and selling places. Despite these limitations, this study conveys valuable information.

IV. Summary and Conclusion

Fried rice samples showed higher levels of *B. cereus* among starch-, vegetable- and protein-based foods. Moreover, *gimbap* could be a major carrier of *B. cereus*. Under a mean viable level of 3 log cfu/g, there was no detection of the emetic cereulide toxin. In addition, fried rice contaminated with emetic *B. cereus* could contain cereulide at the exposed condition of 7 and 57°C in the inoculation experiment, despite lower concentrations of emetic *B. cereus* cells being detected. This means that although the growth of *B. cereus* was stopped, the toxin could remain in the samples if they are contaminated with *B. cereus* at more than 4 log cfu/g. Through the study, the blocking pathways of emetic *B.*

cereus contamination could be suggested as the most critical control points: use of rice not contaminated with *B. cereus*, thorough cooking of rice, prevention of cross-contamination in the production process, consumption without holding the cooked foods at room temperature.

A future study, with more and varied sample types and small-scale food service facilities, will be needed to determine the prevalence of general and emetic *B. cereus* in samples and develop databases to generalize the results. In addition, the optimal production conditions for preventing the production of cereulide in higher risk *B. cereus* dishes should be identified to control the risk of emetic *B. cereus* foodborne illnesses. A future study is also needed to identify treatment methods for the inactivation of emetic *B. cereus* and cereulide.

Conflict of Interests

No potential conflict of interest relevant this article was reported.

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