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Effect of Temperatures on the Enterotoxin Production of *Bacillus cereus* in Cereal Grains

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Abstract Effect of various temperatures on enterotoxin production of *Bacillus cereus* 4 different cereal grains (brown rice, glutinous rice, barley, and Job's tear) was studied. When *B. cereus* was inoculated to 4 grains, no toxin was detected within 24 hr at 20 and 25°C although the population reached approximately 8-10 log CFU/g. However, enterotoxin was detected in all samples above 30°C. When the temperature was increased to 35°C, toxin production was observed in the range of 6.11 and 6.26 log CFU/g on brown rice and glutinous rice, respectively. At 40°C, toxin production was detected after 6 hr with the lowest bacterial population of 5.32 and 5.04 log CFU/g, whereas enterotoxin was produced in the range of 6.86 and 7.77 log CFU/g on barley and Job's tear at 40°C. Different types of food affected enterotoxin production of *B. cereus*. These results suggest that enterotoxin production was more significantly regulated in incubation temperatures than the number of *B. cereus*.

Key words: *Bacillus cereus*, enterotoxin production, temperature, cereal grain

Introduction

Bacillus cereus is a Gram-positive, spore forming, facultative aerobic bacteria. It is a common food contaminant and is etiological agent of two distinct form of illness, i.e., emetic and diarrheal. Onset of symptoms occurs after 6-12 hr for diarrheal illness, while 0.5-5 hr for emetic illness after consumption of food contaminated with *B. cereus*. Contamination of a food product by *B. cereus* does not necessarily cause deterioration of the organoleptic properties, which explains possible consumption without suspicion on the part of the consumer preference (1). The presence of large number of *B. cereus* in food is indicative of active growth and proliferation of the organism; and is consistent with potential hazard to health. The diarrheal type of food poisoning are thought to occur from ingestion of large numbers of spores and/or vegetative cells (>10^5 log cells or spores/g) in contaminated food, which subsequently produce enterotoxin in the host small intestine (2). All individuals might be susceptible to *B. cereus* food poisoning, although severity of infections has been associated with infants, young, the elderly and the immuno-suppressed adults (3,4).

As known, *B. cereus* is ubiquitous in nature; almost all kind of foods have been implicated. Majority of reported outbreaks were linked to consumption of milk and milk products, meat and meat products, spices and cereals, rice and eggs (5-8). The diarrheal type is more widely associated with a variety of food. Most often implicated food in the diarrheal syndrome includes poultry, cooked meats, soups, desserts, and occasionally fluid and dry milk products (9-11). A survey conducted in Hong Kong from 16 rice samples showed that most of the isolates were able to produce diarrheal toxin, but none was able to produce the emetic toxin (12). Surprising was that cooked rice is normally associated with the emetic disease (6,13). Incidence of *B. cereus* in cereal grains have been pointed out by Fang *et al.* (5,14). Our preliminary study also showed the incidence of 25% of *B. cereus* isolates from 293 cereal rice grains (data not shown). The concept of preformed enterotoxin in food has been reported (15,16). McKillip (7) mentioned the possibility of food poisoning arising from a combination of preformed toxin and high cells/spores counts in certain foods. Food held or stored at inadequate temperature condition are the main cause of *B. cereus* food poisoning or outbreaks.

While limited studies are available on the production of enterotoxin as a function of temperature on cereal rice grain, most of the studies dealing with this issue are about the broth, skimmed milk, pasteurized milk, cooked rice, and baby food (15,17-19). As known that foodborne illness is associated with number of cells/spores present in the food consumed, study on the effect of temperature on the bacterial population along with the toxin production at that number of bacterial population seems to be novel concept to investigate. Thus, the main objective of this study was to investigate the effect of temperature on growth and enterotoxin production of *B. cereus* contaminated on various cereal grains. Furthermore, the study was carried out to determine the critical temperature limits for enterotoxin production.

Materials and Methods

Test organisms A pool of 3 strains of *B. cereus* cocktail used in the study was ATCC 12480, ATCC 13061, and ATCC14579. Stock culture of the each strain of *B. cereus* was stored at −70°C in tryptic soy broth (TSB, Difco,

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Detroit, MI, USA) with 0.6% yeast extract (Difco) containing 20% glycerol. Strains were activated by transferring 0.1 mL of the stock culture into 10 mL of TSB and incubated at 30°C for 24 hr. Following 2 successive transfer, the cells were harvested from the culture by centrifugation at 1,500 ×g for 10 min, and re-suspended into 0.01 M phosphate buffer solution after the supernatants were discarded. Cell pellets were washed twice in 0.01 M phosphate buffer solution and resuspended. After this, the 3 strains of *B. cereus* were combined in a cocktail with approximately equal number in the final population.

Inoculation of *B. cereus* on cereal grains Cereal grains (brown rice, barley rice, glutinous rice, and Job's tear rice) were purchased from a local supermarket in Chuncheon, Korea. Three-hundred g of each cereal grain was soaked in the cocktail suspension of *B. cereus* vegetative cell with gentle agitation in order to facilitate homogeneous distribution. After 10 min, the inoculum was decanted and the grains were placed on a sterile perforated tray lined with 4 layers of cheesecloth and air-dried in a laminar flow-hood at room temperature (25±1°C) for 15 min. This procedure resulted in initial inocula levels of approximately 3.0 to 3.5 log CFU/g of each grain. Then, a 10 g set of inoculated 4 cereal samples was packed in Whirl-Pak (Nasco, Janesville, WI, USA) and incubated at 20-40°C for 24 hr.

Water activity and moisture content Water activity was determined using AquaLab Lite water activity meter (Decagon, Pullman, WA, USA). The Decagon units used electric humidity sensors, where water activity (Aw)= equilibrium relative humidity/100. Water activity values were completed within 5 min per sample (5 to 10 g) for the Decagon units. Water activity measurements were then made in triplicate in using calibration solutions of 6 M NaCl, with Aw=0.76 (Decagon). Also, moisture contents of each cereal grain were analyzed in duplicate for percent moisture (20).

Microbiological analysis At appropriate time intervals, samples (10 g) were mixed in a stomacher bag (Nasco Whirl-Pak) with 90 mL of 0.1% sterile peptone water, and homogenized for 2 min in a stomacher (Lab-blender 400; Seward, London, UK). This mixture was serially diluted and surface plated (0.1 mL) in duplicates onto the MYP agar plates. After incubation at 30°C for 24 hr, colonies were enumerated and results were expressed as log CFU/g. *B. cereus* was analyzed for the presence of enterotoxin using BCET-RPLA detection kit (Oxoid Ltd, Basingstoke, Hampshire, UK). Assays were performed according to the manufacturer's instruction provided on the kit.

Statistical analysis The experiment was replicated 2 times and each sample was plated in duplicate, resulting in 3 observations per mean. Reported plate count data and toxin production represented the mean±standard deviation (SD) obtained from 3 observations. All data were subjected to analysis of variance and the Tukey test was then conducted with least square means used to determine significant differences ($p<0.05$).

Results and Discussion

The water activity (Aw) and water content of cereal grains used in this study were measured to determine the dipping effect before and after inoculation of *B. cereus*. The Aw of barley, Job's tear, brown rice, and glutinous rice before inoculation of *B. cereus* were 0.41, 0.46, 0.49, and 0.48, respectively, whereas the Aw of cereal grains were significantly increased with 0.97, 0.95, 0.93, and 0.93 after inoculation due to the absorption of water into cereal grains during dipping treatments. Also, the water contents of barley and Job's tear, brown rice, and glutinous rice before inoculation were 15.47, 13.26, 13.48, and 14.57%, respectively, whereas the water content of cereal grains were significantly increased with 23.32, 25.56, 17.48, and 17.32% after inoculation (data not shown). Thus, the growth of *B. cereus* and toxin production on the artificially inoculated into cereal grains can be much overestimated due to the increased Aw and water contents, compared to cereal grains before inoculation, which contains low Aw and low water content.

The effect of temperatures on the growth of *B. cereus* and its enterotoxin production on the artificially inoculated into barley and Job's tear are shown in Table 1 and 2. Rapid growth was observed after 3 hr of incubation at all storage temperatures. Although the population increased approximately 3 fold (approximately 9.49 log CFU/g) after 24 hr of storage, enterotoxin was not detected in both barley and Job's tear stored at 25°C. Similar findings were documented by Jaquette and Beuchat (19), where no enterotoxin was detected below 30°C for 24 hr of storage in cereal reconstituted with milk. The population of 8.7 log CFU/g showed toxin production on barley at 30°C, but no toxin was detected at 30°C at this level of bacterial population on Job's tear. At 35, 7.81, and 8.08 log CFU/g were sufficient for toxin production for barley and Job's tear, respectively. When the temperature was increased to 40°C, *B. cereus* produced enterotoxin at the level of population of 6.86 and 7.77 log CFU/g for barley and Job's tear, respectively. In comparison to Job's tear, lesser population of bacterial cell observed on barley showed toxin production. This result is in agreement with the findings of Agata *et al.* (21) and Finlay *et al.* (18), where estimated minimal *B. cereus* population were 1 log CFU/g lesser for emetic toxin produced in skimmed milk medium (SMM) than in rice cultures at 30°C. Similar trends of *B. cereus* growth were observed when artificially *B. cereus* inoculated into brown rice and glutinous rice were stored at temperature range of 20-40°C (Table 3 and 4). Even though approximately 9.36 log CFU/g of *B. cereus* was detected after 24 hr at 25°C, toxin was not detected, which is consistent with the data presented in barley and Job's tear. However at 30, 7.76, and 8.61 log CFU/g of bacterial cell showed toxin production in brown rice and glutinous rice, respectively. The result is supported by the findings of Agata *et al.* (21) where emetic toxin produced at 30°C in SMM became detectable when *B. cereus* population reached greater than 6 log CFU/g. As temperature increased to 35 and 40°C, toxin were produced at 5.05-6.26 log CFU/g on brown rice and glutinous rice, respectively. This result

Table 1. Effect of temperatures on the growth and enterotoxin production of *Bacillus cereus* inoculated into barley[1)]

Time (hr)	Barley									
	20°C		25°C		30°C		35°C		40°C	
	G[2)]	T[3)]	G	T	G	T	G	T	G	T
0	3.00±0.16	-	3.02±0.25	-	3.02±0.11	-	3.01±0.21	-	3.00±0.19	-
1	3.02±0.23	-	3.02±0.09	-	3.02±0.24	-	3.03±0.18	-	3.00±0.22	-
3	3.30±0.18	-	3.30±0.16	-	3.70±0.15	-	3.96±0.13	-	3.70±0.34	-
6	4.81±0.29	-	4.62±0.33	-	5.51±0.19	-	5.41±0.16	-	5.03±0.16	-
9	5.08±0.31	-	4.95±0.18	-	6.01±0.31	-	6.01±0.21	-	5.92±0.27	-
12	5.71±0.14	-	5.85±0.24	-	6.94±0.27	-	6.96±0.06	-	6.86±0.31	+
15	6.20±0.08	-	6.47±0.19	-	7.86±0.22	-	7.81±0.17	+	7.94±0.25	+
18	6.93±0.24	-	7.74±0.21	-	8.70±0.13	+	8.81±0.28	+	8.89±0.29	+
21	7.18±0.35	-	8.96±0.17	-	9.04±0.18	+	9.51±0.24	+	9.79±0.18	+
24	7.95±0.17	-	9.49±0.26	-	9.83±0.25	+	9.93±0.23	+	9.98±0.15	+

[1)]Means with different letters in the same row are significantly different at 5% level.
[2)]G denotes population growth (log CFU/g).
[3)]T denotes toxin; (+),toxin detected; (-), toxin not detected.

Table 2. Effect of temperatures on the growth and enterotoxin production of *Bacillus cereus* inoculated into Job's tear[1)]

Time (hr)	Job's tear									
	20°C		25°C		30°C		35°C		40°C	
	G[2)]	T[3)]	G	T	G	T	G	T	G	T
0	3.48±0.15	-	3.48±0.08	-	3.48±0.24	-	3.48±0.13	-	3.48±0.32	-
1	3.48±0.21	-	3.48±0.16	-	3.30±0.21	-	3.48±0.21	-	3.60±0.16	-
3	3.70±0.26	-	3.60±0.27	-	3.90±0.08	-	4.04±0.15	-	3.96±0.08	-
6	4.98±0.17	-	4.89±0.31	-	5.74±0.16	-	5.72±0.09	-	5.90±0.24	-
9	5.72±0.32	-	5.78±0.28	-	6.60±0.11	-	6.34±0.11	-	6.78±0.23	-
12	6.32±0.25	-	6.79±0.15	-	7.78±0.23	-	7.56±0.24	-	7.77±0.14	+
15	6.94±0.16	-	7.51±0.24	-	8.79±0.27	-	8.08±0.31	+	8.85±0.19	+
18	7.89±0.11	-	8.04±0.35	-	9.04±0.17	+	8.98±0.26	+	9.11±0.25	+
21	8.40±0.27	-	9.44±0.21	-	9.75±0.06	+	9.75±0.22	+	9.75±0.19	+
24	8.83±0.09	-	9.98±0.14	-	9.98±0.19	+	9.89±0.17	+	9.98±0.28	+

[1)]Means with different letters in the same row are significantly different at 5% level.
[2)]G denotes population growth (log CFU/g).
[3)]T denotes toxin; (+), toxin detected; (-), toxin not detected.

is in contrast to the statement that toxin is generally not produced until *B. cereus* population reaches above 10^7 (19) or 10^6-10^7 CFU/g (22,23,25). Remarkable toxin production was observed at optimal temperature irrespective of the higher population of *B. cereus* growth. Finlay *et al.* (18) also reached same conclusion that the toxin production by *B. cereus* was achieved faster for strains incubated at higher temperature in cooked rice. It can be explained in terms of near-optimal condition for growth and toxin production as described by Fermanian *et al.* (17).

Overall from the study, in order to prevent enterotoxin production from *B. cereus* contaminated cereal grains, storage at temperature below 25°C could be recommendable. It can be inferred that the production of enterotoxin on cereal grains can be considered implicitly as the function of storage temperature. Similar results of temperature regulated toxin production were reported by Finlay *et al.* (18). It is transparent from the study that incubation for 24 hr at temperature higher than 30°C will allow production of enterotoxin. This may be of value in preparing diarrheal toxin for purification studies. The temperature used by other groups for emetic toxin production was 30°C as reported by Finley *et al.* (18) and 32°C for diarrheal toxin (7,17,25). The temperature range of 30-40°C was optimal for strains of *B. cereus* to produce enterotoxin and is considered as critical temperature for toxin production on cereal grains. However, the optimal temperature for enterotoxin production might differ with food system and is advised not to simulate the temperature as a standard for enterotoxin production in other food matrix, even though the Aw and water contents of cereal grains were much higher than natural cereal grains. Conclusively, temperature hurdle that can be complementary to food safety should be recommended to prevent enterotoxin production on cereal grains and adequate cooking, heating, chilling, or storage will be warranted to control possible *B. cereus* contamination on cereal grains.

Table 3. Effect of temperatures on the growth and enterotoxin production of *Bacillus cereus* inoculated into brown rice[1)]

Time (hr)	Brown rice									
	20°C		25°C		30°C		35°C		40°C	
	G[2)]	T[3)]	G	T	G	T	G	T	G	T
0	3.30±0.08	-	3.30±0.19	-	3.30±0.05	-	3.30±0.12	-	3.30±0.13	-
1	3.34±0.09	-	3.30±0.10	-	3.34±0.23	-	3.38±0.17	-	3.30±0.20	-
3	3.60±0.06	-	3.60±0.05	-	3.70±0.08	-	3.95±0.03	-	3.78±0.17	-
6	4.90±0.26	-	4.72±0.06	-	4.65±0.12	-	5.51±0.39	-	5.32±0.36	+
9	5.32±0.05	-	5.04±0.11	-	6.41±0.11	-	6.11±0.14	+	6.08±0.14	+
12	5.98±0.19	-	5.89±0.17	-	6.96±0.05	-	7.32±0.12	+	6.97±0.09	+
15	6.71±0.20	-	6.96±0.15	-	7.76±0.07	+	7.86±0.12	+	8.04±0.25	+
18	7.26±0.18	-	7.91±0.08	-	8.83±0.21	+	8.83±0.08	+	8.96±0.15	+
21	7.65±0.17	-	8.72±0.16	-	9.36±0.20	+	9.40±0.09	+	9.41±0.07	+
24	8.04±0.07	-	9.36±0.07	-	9.91±0.08	+	9.83±0.23	+	10.04±0.09	+

[1)]Means with different letters in the same row are significantly different at 5% level.
[2)]G denotes population growth (log CFU/g).
[3)]T denotes toxin; (+), toxin detected; (-), toxin not detected.

Table 4. Effect of temperatures on the growth and enterotoxin production of *Bacillus cereus* inoculated into glutinous rice[1)]

Time (hr)	Glutinous rice									
	20°C		25°C		30°C		35°C		40°C	
	G[2)]	T[3)]	G	T	G	T	G	T	G	T
0	3.30±0.05	-	3.30±0.07	-	3.30±0.15	-	3.30±0.05	-	3.30±0.08	-
1	3.30±0.26	-	3.30±0.09	-	3.30±0.12	-	3.31±0.13	-	3.30±0.07	-
3	3.48±0.55	-	3.78±0.09	-	3.78±0.11	-	4.01±0.06	-	3.96±0.20	-
6	4.94±0.17	-	5.79±0.33	-	5.79±0.04	-	5.49±0.10	-	5.04±0.15	+
9	5.45±0.09	-	6.70±0.25	-	6.70±0.15	-	6.26±0.08	+	6.58±0.18	+
12	6.08±0.09	-	7.08±0.08	-	7.08±0.36	-	7.32±0.25	+	7.72±0.03	+
15	6.90±0.12	-	8.61±0.12	-	8.61±0.10	+	7.98±0.15	+	8.79±0.07	+
18	7.84±0.11	-	9.32±0.15	-	9.32±0.20	+	8.90±0.12	+	9.08±0.12	+
21	8.20±0.14	-	9.81±0.07	-	9.81±0.11	+	9.18±0.09	+	9.83±0.05	+
24	8.84±0.06	-	10.08±0.07	-	10.08±0.04	+	9.81±0.10	+	10.08±0.26	+

[1)]Means with different letters in the same row are significantly different at 5% level.
[2)]G denotes population growth (log CFU/g).
[3)]T denotes toxin; (+), toxin detected; (-), toxin not detected.

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