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Research Note

Inactivation of *Bacillus cereus* ATCC 14579 Spore on Garlic with Combination Treatments of Germinant Compounds and Superheated Steam

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ABSTRACT

Garlic is one of the most popular spices in the food industry because of its unique flavor, aroma, and health benefits. However, garlic is easily contaminated by spore-forming *Bacillus cereus* from the soil. We studied inactivation of *B. cereus* spores using superheated steam (SHS) and germinant compounds such as L-alanine, inosine, and disodium 5′-inosinate. Treatment with SHS and germinant compounds (50 mM L-alanine plus 5 mM disodium inosine 5′-monophosphate) on *B. cereus* spores was more effective than SHS treatment alone. The inactivation trends were analyzed using the Weibull model, and a time required to achieve a 3-log reduction was determined. These values at 120°C after SHS and germinant compounds plus SHS were 2.14 and 1.26 min, respectively. In addition, SHS and germinant compounds plus SHS treatments inactivated *B. cereus* ATCC 14579 spores effectively without causing sublethal injury. Levels of inactivation of *B. cereus* spores enumerated on mannitol–egg yolk–polymyxin and overlaid with Brilliance Bacillus Cereus were not significantly different for all treatment conditions. Therefore, germinant compounds plus SHS treatment can be used effectively to control *B. cereus* ATCC 14579 spores on garlic.

HIGHLIGHTS

- Germinant compounds were combined with SHS to inactivate *B. cereus* spores.
- *B. cereus* spores were inactivated effectively by SHS after Ala+IMP treatment.
- Inactivation trend was analyzed by the Weibull model, and t_3d values were determined.
- Sublethal injury was not observed by SHS after Ala+IMP treatment.

Key words: *Bacillus cereus* spore; Combination treatment; Garlic; Germinant compounds; Pathogen inactivation; Superheated steam

The consumption and demand for ready-to-eat foods are increasing as the numbers of single and working households increase. Accordingly, many food companies are producing various types of ready-to-eat foods. Garlic (*Allium sativum* L.) is one of the representative spices in ready-to-eat products. Garlic has a unique flavor and aroma that are produced by diallyl thiosulfinate (allicin), which has been reported to be good for health *(5)*. However, garlic is easily contaminated by spore-forming *Bacillus cereus,* which has high thermal resistance *(9, 25, 26)*. In this regard, controlling *B. cereus* spores on garlic is of great importance in the food industry, because they can cause infection and toxin-based foodborne illnesses when they germinate *(10)*.

Thermal processing has been widely used as a cooking and sterilization method. Even though thermal processing is more efficient at inactivating bacterial spores than nonthermal processing, excessive heat treatment is needed to decontaminate all bacterial spores *(6)*. Because the excessive heat treatment inevitably causes damage to food quality, such as taste, appearance, and nutrition, it is important to apply new thermal processing methods to ensure both microbiological safety and food quality. Superheated steam (SHS) is a novel thermal technology that enables rapid heating, and several researchers pointed out that SHS can be used effectively to inactivate bacterial pathogens in food *(3, 18)*. Many researchers have reported methods to control spores *(1, 7, 23)* while minimizing the quality deterioration of food. However, to our knowledge,

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there are limited studies about the inactivation of *B. cereus* spores on garlic by SHS treatment.

Germinant compound, a germination enhancer, has been used to germinate spores before sterilization *(17),* because heat resistance of germinated spores is less than that of nongerminated spores *(21)*. According to advanced studies of the genome, there are seven putative germination operons in *B. cereus* ATCC 14579, and they react to amino acids or purine ribosides *(14, 15)*. In this regard, germinant compounds, including amino acids and other molecules (e.g., cholesterol-based compounds, organic acids, nucleosides, and peptidoglycan fragments), have been used to induce spore germination *(4)*. Although L-alanine (Ala) can act as a sole germinant, the presence of a cogerminant, such as inosine (Ino), can dramatically lower the concentration of Ala required to trigger germination. Even though Ala is not a compound that is generally recognized as safe, it is widely applied in the food and pharmaceutical industries. Ino is a well-known component of germinants, but it has not been permitted for use as a food additive by the Codex Alimentarius Commission of the International Food Standards Committee. Conversely, disodium 5′-inosinate (IMP, disodium inosine 5′-monophosphate), which is the disodium salt of inosinic acid, is allowed to be used in foods as a food additive. In this regard, Ala was combined with Ino or IMP, and germination efficacy of the combination treatment was compared with individual treatments. Furthermore, the most effective germinant compounds were combined with SHS for *B. cereus* inactivation in the present study.

Even though SHS has been widely used for inactivation of foodborne pathogens in the food industry, studies about the inactivation of *B. cereus* spores on garlic by SHS have been limited. Therefore, we observed not only the effect of SHS on the inactivation of *B. cereus* spores on garlic but also the combination effect of SHS and germinant compounds in the present study.

MATERIALS AND METHODS

***B. cereus* spore preparation.** *B. cereus* KCTC 3624 (ATCC 14579) used in this research was obtained from the Korean Collection for Type Cultures (KCTC). *B. cereus* was precultured in 5 mL of tryptic soy broth (Difco, BD, Franklin Lakes, NJ) at 30°C for 24 h. After incubation, 0.2 mL of the culture was spread onto Difco sporulation medium agar and incubated at 30°C for 5 days to produce a bacterial lawn. Spores were gradually formed for 5 days and observed through an optical microscope (×1,000). Spores were collected by scraping the surface of the Difco sporulation medium agar with 10 mL of sterile distilled water and were washed three times by centrifugation (10,000 × *g,* 15 min, 4°C) for *B. cereus* spore purification. The pellet was resuspended in 5 mL of distilled water and 5 mL of ethanol, and the suspension was held at 4°C for 12 h to reduce the number of vegetative cells *(8, 19, 20)*. In the preliminary experiments, we confirmed that a more than 6-log reduction of *B. cereus* vegetative cells was achieved by 50% ethanol. The obtained suspension was washed three times by centrifugation under the same conditions, and the final pellet was resuspended in 10 mL of 0.2% (w/v) buffered peptone water (Difco), because the spore inoculum corresponded to approximately 10^9 CFU/mL. The spores were stored at 4°C for 12 to 14 h before treatment.

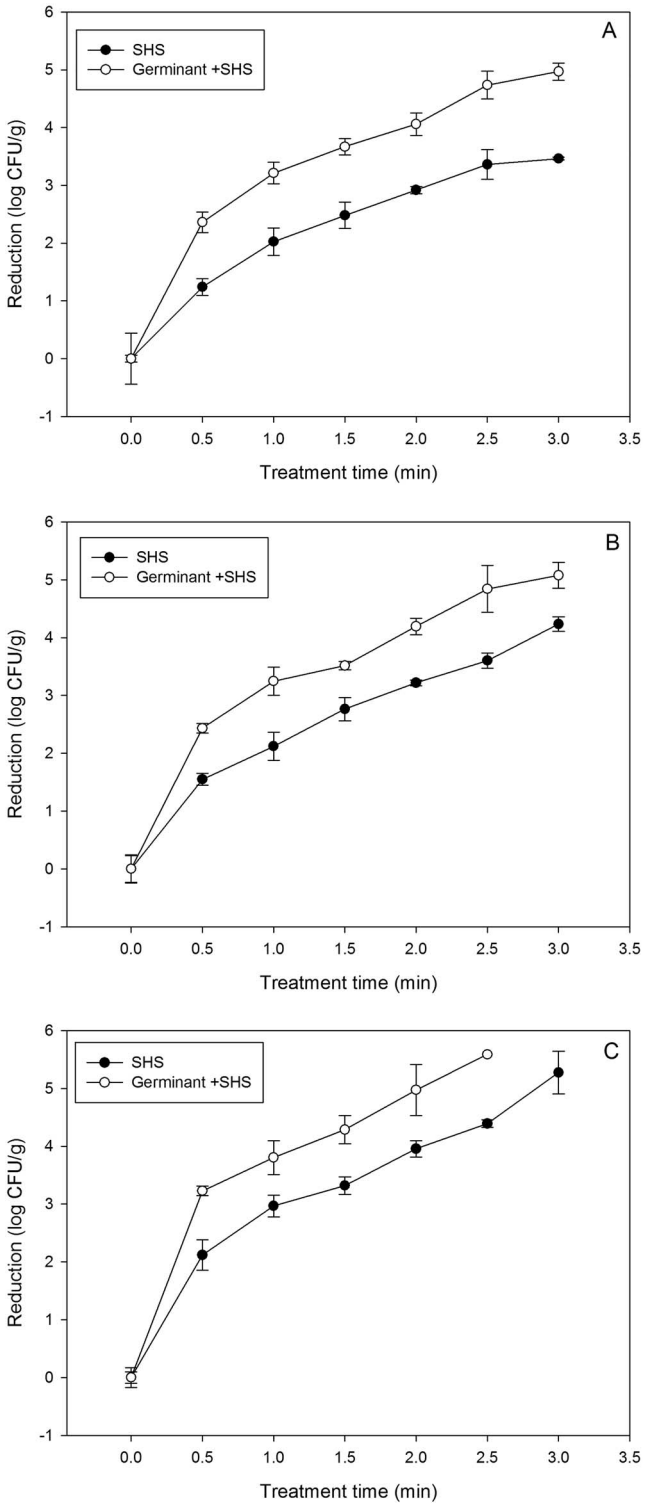

FIGURE 1. *Reduction levels (log CFU per gram) of* B. cereus *spores on garlic cloves subjected to superheated steam (SHS, ●) and SHS after* L*-alanine plus disodium 5′-inosinate spraying (germinant plus SHS, ○). Treatment temperatures of SHS were adjusted to 120°C (A), 150°C (B), and 180°C (C), and mannitol–egg yolk–polymyxin agar was used as a selective medium.*

Sample preparation and inoculation. Garlic (*Allium sativum,* Hapcheon-gun, Gyeongsangnam-do, Korea) for this study was purchased at a local market (Seoul, Korea). The garlic cloves were washed with running water and then dried. Samples

TABLE 1. *Germination rate of* B. cereus *spores with various treatments*[a]

Treatment[b]	Germination rate (%)
Water	48.9
Ala	69.5
Ino	79.8
IMP	95.4
Ala+Ino	97.4
Ala+IMP	99.6

[a] Initial level of *B. cereus* spores was 9.02 log CFU/mL.
[b] Water, sterile water; Ala, L-alanine; Ino, inosine; IMP, disodium 5′-inosinate.

were inoculated with a *B. cereus* spore inoculum using a spot-inoculation method. Inoculated garlic was dried at 4°C to prevent spore germination. Subsequently, samples were placed in a desiccator, which had been precooled at 4°C for 12 h.

Germinant treatment. Untreated (control), sterile water (water), 50 mM Ala, 5 mM Ino, 5 mM IMP, 50 mM Ala plus 5 mM Ino (Ala+Ino), and 50 mM Ala plus 5 mM IMP (Ala+IMP) were used as germinants *(12)*. The effect of germinants on *B. cereus* spore germination was identified in the liquid solutions. The germination solution was made by adding each germinant (Ala, Ino, IMP, Ala+Ino, and Ala+IMP) to distilled water. Purified spore inoculum (1 mL) was placed into a microtube and centrifuged, and the supernatant was discarded. Subsequently, 1 mL of the germination solution was filled in the microtube and mixed well with a pipette. Each microtube was allowed to equilibrate at room temperature for a set time; then, the germination solution was removed by centrifugation. After washing three times with distilled water, 0.5 mL of distilled water and 0.5 mL of ethanol were added and left at 4°C for 12 h. Germinated spores were eliminated by 50% ethanol, and the remaining spores were counted.

SHS treatment. The custom-made SHS machine was used for treatment of SHS on garlic *(2)*. The SHS equipment in this study consisted of a water reservoir, a 15-kW steam boiler (model DWE-15, Dae-Woo Steam Boiler, Daegu, Korea), a 5-kW superheater, a reacting chamber, and a power controller. The whole garlic cloves inoculated with *B. cereus* spores were treated with 120, 150, and 180°C of SHS for 0.5, 1, 1.5, 2, and 2.5 min. SHS-treated samples were then immediately transferred to a sterile stomacher bag containing peptone water. For SHS treatment after germination, 1 mL of the 50 mM Ala solution and 1 mL of the 5 mM IMP solution mixture (2 mL total) was sprayed on approximately 100 g of garlic cloves and left for 15 min before treatment. The germinant compounds were not applied in the untreated sample (control).

Bacterial enumeration. The number of *B. cereus* spore cells was enumerated by spreading on mannitol–egg yolk–polymyxin (MYP) agar (Oxoid, Basingstoke, UK). The population of germinated spores was calculated by subtracting the number of survivors after purification with 50% ethanol from the initial population of spores *(13, 24)*:

$$\begin{aligned}&\text{Germination rate (\%)}\\&\quad=(\text{Initial number of spores [CFU/mL]}\\&\qquad-\text{Spore survivors [CFU/mL]})\\&\qquad\times 100/\text{Initial number of spores (CFU/mL)}\end{aligned}\tag{1}$$

The overlay method (OV) was used to recover sublethally injured spore cells of *B. cereus*. After spore cells were resuscitated on tryptic soy agar (Difco) at 37°C for 2 h, plates were overlaid with 7 to 8 mL of Brilliance Bacillus Cereus (OV-BBC) agar (Oxoid). Injured and then resuscitated *B. cereus* spore cells were calculated by subtracting the populations enumerated on the selective medium (MYP) from those of enumerated on the resuscitation medium (OV-BBC).

Kinetic parameters of *B. cereus* inactivation. Survival curves were fitted by the Weibull model using GinaFit *(11)* to determine the kinetics of *B. cereus* spores. The inactivation pattern of spores was analyzed to determine the survival kinetics of *B. cereus* spores with the Weibull model. The fitting equation of the Weibull model was as follows:

$$\log(N)=\log(N_0)-x\cdot\left(\frac{t}{t_xd}\right)^p\tag{2}$$

where N (CFU/g) is the population of the microorganisms, N_0 is the initial population, t (min) is the treatment time, p is the parameter related to the scale and shape of the survival curve, x is the number of decimal reductions, and t_xd is the time required to achieve an x-log reduction *(22)*. The Weibull distribution corresponds to a concave downward survival curve if $p > 1$ and upward survival curve if $p < 1$.

Statistical analysis. Biologically independent spore batches were used to evaluate the effect of biological variation. All experiments were conducted in triplicate, and one-way analysis of variance was performed using IBM SPSS 21.1 (SPSS Inc., Chicago, IL). Duncan's multiple comparison test was used to investigate significant differences ($P < 0.05$) of the results.

TABLE 2. *Parameters of Weibull models for inactivation of* B. cereus *on garlic cloves*[a]

Temp (°C)	Treatment	δ (min) ± SE[b]	p ± SE[b]	R^2	t_3d (min)
120	SHS	0.28 ± 0.06	0.54 ± 0.04	0.99	2.14
	Germinant + SHS	0.14 ± 0.06	0.50 ± 0.07	0.99	1.26
150	SHS	0.27 ± 0.04	0.58 ± 0.04	0.99	1.79
	Germinant + SHS	0.07 ± 0.03	0.43 ± 0.04	0.99	0.90
180	SHS	0.13 ± 0.05	0.52 ± 0.06	0.99	1.08
	Germinant + SHS	0.02 ± 0.01	0.34 ± 0.04	0.99	0.51

[a] Garlic cloves were subjected to superheated steam (SHS) and SHS after L-alanine plus disodium 5′-inosinate spraying (germinant plus SHS).
[b] Delta and p values were calculated using GinaFit *(11)*. The relationship of the delta value (δ), p, and t_3d is $t_3d = \delta \times (3)^{\frac{1}{p}}$.

TABLE 3. *Survival of* B. cereus *on garlic cloves after SHS treatment and enumeration by MYP or OV-BBC*[a]

Treatment time (min)	Survival (log CFU/g)					
	120°C		150°C		180°C	
	MYP	OV-BBC	MYP	OV-BBC	MYP	OV-BBC
0.5	5.14 ± 0.15 A	5.23 ± 0.12 A	4.83 ± 0.10 A	5.04 ± 0.32 A	4.42 ± 0.26 A	4.65 ± 0.27 A
1	4.36 ± 0.24 A	4.71 ± 0.24 A	4.26 ± 0.25 A	4.38 ± 0.25 A	3.57 ± 0.19 A	3.96 ± 0.03 A
1.5	3.90 ± 0.23 A	4.12 ± 0.16 A	3.62 ± 0.20 A	3.82 ± 0.24 A	3.21 ± 0.15 A	3.40 ± 0.14 A
2	3.46 ± 0.06 A	3.50 ± 0.04 A	3.16 ± 0.05 A	3.21 ± 0.05 A	2.58 ± 0.14 A	2.90 ± 0.04 A
2.5	3.02 ± 0.25 A	3.29 ± 0.03 A	2.78 ± 0.13 A	3.04 ± 0.03 A	2.14 ± 0.06 A	2.53 ± 0.10 A
3	2.92 ± 0.02 A	3.15 ± 0.04 A	2.15 ± 0.12 A	2.97 ± 0.02 B	1.26 ± 0.37 A	2.03 ± 0.04 A

[a] Average initial spore cell counts were 6.43 ± 0.19 log CFU/g. Values are means ± standard deviations. The values with different letters in a same row for each treatment temperature are significantly different ($P < 0.05$).

RESULTS AND DISCUSSION

Before the SHS inactivation experiments of *B. cereus* spores on garlic, we determined the germination ability of Ala, Ino, IMP, Ala+Ino, and Ala+IMP to choose germinants to combine with SHS. The levels of spore germination were 48.9, 69.5, 79.8, 95.4, 97.4, and 99.6% for water, Ala, Ino, IMP, Ala+Ino, and Ala+IMP, respectively (Table 1). This result indicates that combination treatments of Ala and Ino or IMP have a greater germination effect than individual treatments. Because Ala+IMP was the most effective method to germinate *B. cereus* and IMP is allowed for use in foods as a food additive, it was applied before SHS treatment for inactivation of *B. cereus* spores on garlic.

Germination induction, followed by bactericidal treatment, is an effective way to inactivate bacterial spores *(16)*. We also identified that Ala+IMP combined SHS treatment was more effective for inactivation of *B. cereus* than individual SHS treatments, regardless of treatment temperature (Fig. 1). For example, reduction levels of *B. cereus* subjected to SHS and germinant compounds plus SHS at 120°C for 3 min were 3.46 and 4.97 log CFU/g, respectively, from the initial spore cell counts of 6.43 and 6.58, respectively. Inactivation levels of *B. cereus* increased as treatment time and temperature increased, and reduction levels of *B. cereus* subjected to SHS and germinant compounds plus SHS at 180°C for 3 min, which is the maximum treatment in this study, were 5.27 and more than 5.59 log CFU/g, respectively. Because inactivation trends were not linear to treatment time, the graphs were analyzed by the Weibull model, and the time required to achieve a 3-log reduction t_3d was determined (Table 2). Regardless of treatment temperature, t_3d values of SHS were shortened by combination with germinant compounds. For example, t_3d values at 120°C after SHS and germinant compounds plus SHS were 2.14 and 1.26 min, respectively. This result indicated that quality deterioration by SHS can be minimized by combining with germinant compounds, even though further study about the quality change of garlic by SHS is needed. Based on the results in the present study, it is recommended to apply Ala+IMP before SHS to control *B. cereus* spores on garlic.

Bacterial pathogens sublethally injured by a preservative method can be resuscitated under favorable conditions, and these resuscitated pathogens are more resistant to other stresses via cross-protection *(27)*. Therefore, we identified the sublethal injury of *B. cereus* by comparing the population enumerated on MYP and OV-BBC (Tables 3 and 4). Even though populations of *B. cereus* enumerated on OV-BBC were higher than those enumerated on MYP, significant differences were not observed, regardless of treatment type (SHS or germinant compounds plus SHS), treatment temperature (120, 150, or 180°C), and treatment time (0 to 3 min). This result indicated that SHS or germinant compounds plus SHS treatment would inactivate

TABLE 4. *Survival of* B. cereus *on garlic cloves subjected to SHS treatment after* L*-alanine plus disodium 5′-inosinate spraying and enumeration by MYP or OV-BBC*[a]

Treatment time (min)	Survival (log CFU/g)					
	120°C		150°C		180°C	
	MYP	OV-BBC	MYP	OV-BBC	MYP	OV-BBC
0.5	4.41 ± 0.18 A	5.54 ± 0.81 A	3.96 ± 0.08 A	4.74 ± 0.13 A	3.36 ± 0.08 A	3.67 ± 0.11 A
1	3.56 ± 0.19 A	3.74 ± 0.43 A	3.15 ± 0.24 A	3.96 ± 0.08 A	2.78 ± 0.29 A	3.27 ± 0.06 A
1.5	3.10 ± 0.14 A	3.38 ± 0.35 A	2.88 ± 0.07 A	3.19 ± 0.21 A	2.30 ± 0.25 A	2.99 ± 0.17 A
2	2.71 ± 0.19 A	3.17 ± 0.49 A	2.20 ± 0.14 A	2.82 ± 0.16 A	1.62 ± 0.44 A	2.71 ± 0.19 A
2.5	2.03 ± 0.24 A	2.71 ± 0.10 A	1.55 ± 0.40 A	2.40 ± 0.38 A	<1.00 A	1.93 ± 0.18 B
3	1.80 ± 0.15 A	2.43 ± 0.15 A	0.98 ± 0.70 A	1.90 ± 0.27 A	<1.00 A	1.52 ± 0.06 B

[a] Average initial spore cell counts were 6.58 ± 0.33 log CFU/g. Values are means ± standard deviations. The values with different letters in a same row for each treatment temperature are significantly different ($P < 0.05$).

B. cereus ATCC 14579 spores effectively without causing sublethal injury. Sensory evaluation of the germinant compounds plus SHS–treated sample is needed for further study.

In conclusion, *B. cereus* ATCC 14579 spores can be inactivated more efficiently when the germinant compounds are used before SHS treatment. Ala+IMP were the most effective germinant compounds for *B. cereus* among the germinants used in the present study, and the inactivation efficacy of SHS after Ala+IMP treatment was significantly higher than that of SHS treatment. Moreover, sublethal injury was not observed by SHS or germinant compounds plus SHS treatment. Therefore, SHS combined with germinant compounds could be used effectively to reduce the processing time of products in which garlic is used as a raw material.

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