

## The high thermal resistance of *Bacillus cereus* spores inoculated in rice flour

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### ABSTRACT

Numerous studies highlight the increased thermal resistance of bacteria in low-moisture food, mainly due to *Salmonella* outbreaks reported in products such as milk powder and peanut butter. However, most research focuses on bacterial vegetative forms. This study assessed the thermal resistance of *Bacillus cereus* spores isolated from U.S. pre-launch spacecraft, known to withstand multiple cleaning protocols. *B. cereus* spores were inoculated in rice flour with an initial water activity ( $a_w$ ) of 0.48 and treated at temperatures of 90°, 95°, and 100°C. The  $a_w$  change was evaluated at specific temperature intervals using an Aqualab Vapor Sorption Analyzer and relative humidity sensors. The D-values at the selected temperatures were determined using aluminum thermal death test cells. The  $a_w$  value increased ( $P < 0.05$ ) from 0.48 to  $0.85 \pm 0.02$ ,  $0.86 \pm 0.01$ , and  $0.89 \pm 0.02$  at 90°, 95°, and 100°C. The D-values from the spores ranged from 20.33 to 27.8 h and 30.8–40.7 h for the selected *B. cereus* strains. A high thermal resistance was observed in the spores, primarily due to the harsh environments where these bacteria samples were collected. This work demonstrates the spores' ability to survive thermal treatment and the necessity to develop alternative methods for inactivating these bacteria in low-moisture foods.

### Glossary

**Acid tolerance response (ATR)** A bacterial mechanism that allows survival and adaptation in low pH (acidic) environments, which may involve changes in metabolism, protein synthesis, and pH regulation.

**Autoclaving** A sterilization process using high-pressure saturated steam at 121°C to eliminate microbial contamination.

***Bacillus cereus*** A spore-forming bacterium that can cause foodborne illness, often found in cereals, rice, and other grains.

**Bacterial spores** Resistant bacterial form capable of surviving extreme conditions such as heat, dehydration, and radiation.

**Biofilm** Community of microorganisms attached to a surface, protected by an extracellular matrix that makes them difficult to eliminate.

**Buffer Peptone Water (BPW)** A nutrient-rich solution used to maintain bacterial viability during testing.

**Centrifugation** A technique that uses centrifugal force to separate components of a liquid sample based on density.

**Cognate response regulator** A protein in a two-component system

that interacts with the histidine kinase and mediates changes in gene expression in response to external stimuli.

**Colony-Forming Unit (CFU)** A unit used to estimate the number of viable bacteria or fungal cells in a sample.

**Come-up time** The time it takes for the temperature of a product to reach the target temperature during thermal processing.

**Cross-protection mechanisms** Mechanisms where exposure to one stress, like heat, can increase resistance to another stress, such as desiccation or acid.

**cspA gene signatures** The genetic markers associated with cold shock proteins (CSPs), which are involved in a bacterial cell's response to cold stress. These genes play a role in adapting to different temperatures and are also involved in other stress responses.

**Cytoplasmic pH homeostasis** The process by which a bacterial cell maintains a stable internal pH, even when the external environment is acidic, helping it survive under stressful conditions.

**Dehydration damage** Damage to cells and microbial spores caused by the loss of water content, which can affect their survival and

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heat resistance.

- Desiccation** The process of drying or removing water from an organism, which can cause stress to microorganisms, including spores.
- D-values** the necessary time to reduce the microbial population by 90 % at a specific temperature
- Dynamic Vapor Sorption (DVS)** A technique used to measure how materials absorb or release moisture under controlled humidity and temperature conditions.
- Electrolytic balance** The balance of ions (like sodium, potassium, and calcium) inside and outside of cells, which can be disrupted by heat treatments.
- Heat shock proteins (HSPs)** Proteins produced in response to heat stress that help protect cells from damage, repair proteins, and maintain cellular integrity.
- High-Temperature Cell (HTC)** A specialized chamber designed to measure water activity and relative humidity at elevated temperatures.
- Histidine kinase (HK)** A type of enzyme that is part of the two-component system. It detects environmental signals and phosphorylates itself, which in turn activates the response regulator to adjust the bacterial response.
- Hygroscopic** The ability of a material to absorb moisture from the surrounding environment, important for the survival of spores.
- Inoculation** The deliberate introduction of microorganisms into a medium or material for study or experimentation.
- Log reduction** The logarithmic reduction in the number of viable microorganisms, commonly used to measure the effectiveness of sterilization.
- Low-moisture food (LMF)** Food products with a water activity ( $a_w$ ) below 0.85, typically under 0.6, which limits microbial growth but allows certain pathogens to survive for extended periods.
- Microbial inactivation** The process of reducing or eliminating the activity of microorganisms, usually through methods like heat, radiation, or chemicals.
- Moisture content** The amount of water present in a product, which can affect microbial growth and the effectiveness of thermal treatments.
- Oxidative damage** Damage caused to cells and molecules by reactive oxygen species (ROS) during exposure to heat or other stress factors.
- Precipitate Zone** A visible reaction in agar-based media indicating microbial enzyme activity or toxin production.
- Radiofrequency technology** A method used for food pasteurization, where radio waves are applied to kill microorganisms by heating.
- Relative Humidity (RH)** The percentage of water vapor present in the air compared to the maximum amount the air can hold at a given temperature.
- Rotational correlation times** A measure of how fast molecules (such as proteins) rotate in a solution. In spores, low rotational correlation times suggest that proteins are highly immobilized, which contributes to stability and resistance to heat.
- Shaking Incubator** A device that maintains controlled temperature and agitation to promote microbial growth.
- Sigma factor** A protein that is essential for initiating the process of transcription in bacteria. It helps the RNA polymerase recognize the correct DNA promoter sequences for gene expression, particularly under stress conditions.
- σB (Sigma factor B)** A specific sigma factor involved in the stress response in *B. cereus*. It activates genes related to stress adaptation, including those that help with heat resistance and resistance to hydrogen peroxide.

**Small acid-soluble spore proteins (SASP)** Proteins found in spores that help protect genetic material from damage during stress conditions, such as heat.

- Spore attachment** The process by which spores adhere to surfaces, which can influence their survival and inactivation during treatments.
- Spore Suspension** A liquid medium containing bacterial spores used for inoculation in microbiological studies.
- Sporulation** The process by which bacteria form spores to survive in unfavorable environmental conditions.
- Spread Plating** A microbiological technique where a liquid sample is evenly distributed on an agar plate for bacterial enumeration.
- Stomaching** A sample preparation technique that homogenizes food or biological samples using mechanical agitation.
- Supernatant** The clear liquid portion remaining after centrifugation or sedimentation of a sample.
- Swelling power** The ability of a substance, typically starch, to absorb water and expand, a factor that can affect microbial survival during heat treatments.
- Thermal Death Time (TDT) Cell** A metal chamber used to determine the time required to achieve microbial inactivation at a specific temperature.
- Thermal Inactivation** The process of reducing or eliminating microbial viability through heat exposure.
- Thermal resistance** The ability of a microorganism to withstand high temperatures without being inactivated.
- Vegetative cells** Metabolically active bacterial cells that are sensitive to heat and chemical treatments.
- Water activity ( $a_w$ )** The ratio of the vapor pressure of water in a substance to the vapor pressure of pure water at the same temperature. It indicates the availability of water for microbial growth and chemical reactions

## 2. Introduction

Numerous outbreaks have been reported in recent years (2001–2019) involving foods traditionally considered microbiologically safe (Palmieri et al., 2016). These outbreaks have affected a wide range of products including, food powders such as flour, cake mix, and chia powder; different kinds of nuts, including pistachios, almonds, and peanuts; spices; and more complex foods such as peanut butter and German chocolate (Reyes et al., 2021). All of these products are similarly identified as low-moisture foods, which are defined by a water activity ( $a_w$ ) level below 0.85  $a_w$ , though they typically have levels lower than 0.6  $a_w$ . These  $a_w$  levels have traditionally been considered safe since it was understood that bacterial growth is inhibited under such conditions (Tadapaneni et al., 2017a). However, various studies have shown that bacteria can survive for long periods in low moisture conditions (Podolak and Black, 2017; Syamaladevi et al., 2016b; Wason et al., 2021).

The most common pathogens in these outbreaks are *Salmonella* spp, responsible for 80 % of illnesses related to low-moisture food, followed by *Escherichia coli* at 16 %, and *Listeria* spp. at 4 % (Wason et al., 2021). Several studies have been conducted to determine the thermal inactivation conditions of these bacteria, indicating that *Salmonella* spp. has higher thermal resistance in low-moisture food compared to liquid or high-moisture food (Syamaladevi et al., 2016a; Syamaladevi et al., 2016b; Villa-Rojas et al., 2017; Xu et al., 2019). For instance, the D-values (the required time to reduce the microbial population by 90 % at a specific temperature) for *Salmonella* at 58.3°C is around 1 min in liquid egg whites. In contrast, in egg white powder at 80°C, the D-Values range from 5.3 to 25.9 minutes, demonstrating the higher thermal resistance of the bacteria in low moisture and low  $a_w$  conditions (Reyes et al., 2021).

Research efforts have determined thermal inactivation conditions for these bacteria in different kinds of low-moisture foods, including wheat

flour, peanut butter, chocolate, milk powder, almonds, and spices (Liu et al., 2022; Syamaladevi et al., 2016a; Villa-Rojas et al., 2017; Xu et al., 2019). Most of these bacteria are vegetative cells, that are physiologically active, capable of producing toxins and form biofilms, but generally sensitive to heat and/or chemical treatments (Gauvry et al., 2019). In contrast, bacteria spores are a much more resistant life form, and although metabolically inactive, they can be resistant to heat, chemicals, UV-radiation, and high hydrostatic pressure treatments applied in the food industry (Carlin, 2016). Spores are oval structures formed through cytoplasmic dehydration in response to stress and are 40° to 45°C more heat resistant than their vegetative cell form. (Erkmen, 2021). Despite their dormancy, spores contain a complete enzymatic system and can maintain all their essential functions, allowing bacteria survival under adverse conditions. When the environment becomes favorable, spores can germinate and resume metabolic activity, posing a continued risk to food safety (Wohlgemuth and Kämpfer, 2014; Zhou and Li, 2015).

Currently, there is limited research regarding spore inactivation in low-moisture foods. Only gram-positive bacteria form spores, and within this group is the genus *Bacillus* which has been identified as one of the leading causes of food poisoning. About one million foodborne illness cases are caused by *Bacillus* toxins yearly around the world (Lamba et al., 2022; Zhou and Li, 2015). Within this genus, the *B. cereus* species has been identified and described as a motile, aerobic or facultatively anaerobic, rod-shaped, bacterium (Buckley and Grotticelli, 2022; Markland and Hoover, 2016). *B. cereus* is commonly found in soil and is easily found in plants as well as the intestinal tract of animals. *B. cereus* is ubiquitous in the environment, which is directly related to its ability to form spores that endure harsh conditions. This organism has been associated with different foods including rice, nuts, spices, potatoes, vegetables, milk, and dairy products (Marrollo, 2016; Mohammadi et al., 2022, 2023).

Each year, over 63,000 cases of *B. Cereus*-related illness are reported in the United States. These foodborne illnesses result from bacterial toxins that can cause gastrointestinal diseases, such as diarrhea and emesis. Some of these toxins are heat stable and can remain after cooking or heating, which makes *B. cereus* a significant health risk (Markland and Hoover, 2016). Additionally, it is a matter of concern that *B. cereus* can easily contaminate low-moisture food. Due to its hydrophobic properties, *B. cereus* spores can easily adhere to various surfaces, including food processing equipment, and resist disinfectants and heat (S. Choi et al., 2014; Lamba et al., 2022). Furthermore, *B. cereus* forms biofilms that are hard to remove regardless of the surface type. Stainless steel, one of the primary materials used in the food industry, is particularly susceptible to contamination (W. Choi and Kim, 2020; Markland and Hoover, 2016).

The survivability of *B. cereus* spores has been demonstrated after being isolated from pre-launch spacecraft after undergoing bioburden reduction measures. These findings are of concern for astronaut health since foodborne disease treatments would be limited during space travel (Mohammadi et al., 2023; Venkateswaran et al., 2017). Furthermore, the *B. cereus* spores survived multiple cleaning protocols, including exposure to antimicrobial agents such as ethanol, as well as air filtering, dry conditions, and strict temperature and humidity control. In an effort to maintain a sterile environment, staff working in the cleanroom adhered to specific access protocols to prevent further contamination and used special suits and sterile tools (Moissl-Eichinger, 2017).

Studies conducted on different spacecraft and spacecraft assembly facilities, including Odyssey, the Japanese Experiment Module, the Russian Segment Zvezda Module, Kennedy Space Center, and the Mars Science Laboratory, identified *B. cereus* as one of the main contaminating pathogens (Smith et al., 2017; Venkateswaran et al., 2017). Once isolated, experiments showed that the microorganism could grow on potential substrates, such as sulfate, found on Mars. Further, it was resistant to desiccation and ultraviolet radiation (UVC) (Smith et al., 2017). Additionally, Leguerinel et al., 2005 demonstrated that harsh environmental conditions, such as low pH, high temperature, and low

$a_w$ , can increase thermal resistance of *B. cereus* spores. When combined with microgravity and radiation, these conditions further compound the risk of increased antibiotic resistance (Madrigal et al., 2022; Mohammadi et al., 2023).

*B. cereus* spores are also able to easily contaminate low-moisture foods, which are commonly used in space travel. Moreover, spores with higher thermal and antibiotic resistance can contaminate commercial products, making this a potential outbreak risk to public health (Pandith et al., 2023).

Their ability to survive in low-moisture food is related to their dormant nature. During sporulation, their protoplasm dehydrates, enhancing longevity by reducing metabolism and lowering energetic demand. Additionally, spores are resistant to dehydration damage due to small acid-soluble spore proteins (SASP), which bind to DNA, protecting it from oxidative damage. As a result, low-moisture food provides an ideal environment for their survival (Marquis, 1998).

In this study, we selected *B. cereus* strains 104.1.1 and 227.1.2 and inoculated them into rice flour. These strains possess genes for enterotoxin and ceruleide toxin production and were originally isolated from pre-launch spacecraft in the U.S., highlighting their high resistance, as they survive multiple cleaning protocols (Smith et al., 2017).

*B. cereus* frequently contaminates rice products due to their close contact with soil during cultivation. For instance, *B. cereus* populations as high as  $10^6$  CFU/g have been observed in soil, and the bacterium's ability to form spores allows it to persist in rice grains for extended periods (Rodrigo et al., 2021). Given this, we selected rice flour as the low-moisture food for our study. This common product made from finely ground or polished milled rice, can be easily contaminated with *B. cereus* spores. Additionally, rice flour is a key ingredient in various foods, such as rice noodles, rice cakes, and breads, meaning that these products could also be at risk of contamination (Qian and Zhang, 2013).

The objective of this study was to determine thermal inactivation parameters and simulate a worst-case scenario involving rice flour contaminated with highly resistant *B. cereus* spores. By doing so, we aimed to establish an effective thermal treatment to ensure the safety of rice flour and its derived products

### 3. Material and methods

#### 3.1. Materials

Rice flour from the brand Bob's Red Mill (Oregon, USA) was used for this study. This brand was selected for its use of the dried stone milling method and its initial  $a_w$  of 0.48 at room temperature. This method involves two horizontal stones placed upon the other, with a small gap between them. The upper stone turns while the lower remains fixed, breaking down the rice kernels and separating the endosperm from the bran and germ. (Cappelli et al., 2020). The milling method is important because it affects the properties of rice flour, such as color and particle size, which can vary depending on the production method. Typically, in the dry milling process, the average particle size is  $\sim D_{t100}$   $\mu\text{m}$ , which is adequate for various products, including rice noodles, at risk of contamination with *B. cereus* (Mohammadi et al., 2024; Qian and Zhang, 2013; Wang et al., 2023).

The stock cultures of *B. cereus* strains 104.1.1 and 227.1.2 were provided by Dr. Stephanie Smith's laboratory at Washington State University. This collection was obtained from pre-launch spacecraft assembled at different spacecraft assembly facilities, including the Kennedy Space Center, and the Jet Propulsion Laboratory (Mohammadi et al., 2023). The cultures were stored at  $-80^\circ\text{C}$  in TSB supplemented with 20 % (vol/vol) glycerol. Working cultures were prepared by streaking each isolate onto TSA plates and incubating them 24–48 h at  $37^\circ\text{C}$ .

Both *B. Cereus* strains were selected based on the evidence presented by Mohammadi et al. (2023) which showed that out of 41 strains, these two were the only ones carrying enterotoxin genes (hIbC, nheA, and

entFM) and emetic toxin genes (CER), making them a significant health risk. Additionally, these strains withstood multiple cleaning protocols, indicating their high resilience (Smith et al., 2017). For this study, we used temperatures of 90, 95 and 100°C for thermal treatments, as spores are generally 40–45°C more resistant than vegetative cells (Wohlgemuth and Kämpfer, 2014). Several authors have also reported that long exposure times at temperatures ranging from 80 to 100°C are required to achieve thermal inactivation of *B. cereus* spores in high moisture foods (Gaillard et al., 1998; Luu-Thi et al., 2014; Penna and Moraes, 2002; Rajkowski and Mikolajcik, 1987). Moreover, it is well known that microorganisms increase their thermal resistance in low moisture foods, further justifying the selection of these temperatures (Jin et al., 2020; Liu et al., 2018; Pérez-Reyes et al., 2021; Syamaladevi et al., 2016b, 2016a; Villa-Rojas et al., 2013; Xu et al., 2019)

### 3.2. Water activity measurement

Testing of the  $a_w$  change in rice flour from 20°C to 60°C was performed using an AQUALAB Vapor Sorption Analyser (VSA, Addium, Pullman, WA) following the dynamic vapor sorption (DVS) method (Reyes et al., 2021).

To evaluate the  $a_w$  change at different thermal treatment temperatures, we followed a method that is based on using high-temperature cells (HTC) developed by Tadapaneni et al. (2017b). The main component within an HTC is a sensor module (HYT939 Innovative Sensor Technology, Ebnat-Kappel, Switzerland), which is designed to monitor temperature and relative humidity (Rh) levels within the headspace above the sample.

For this experimental procedure, approximately four grams of each sample were hermetically sealed within an HTC. Subsequently, the HTCs were sequentially heated inside an oven (Memmert HCP50, Schwabach, Germany) at 25°, 90°, 95°, and 100°C, each followed by a one-hour holding period. Temperature and Rh data from each HTC were logged automatically at one-minute intervals using a computer. The equilibrium Rh values were determined once both the temperature and Rh curves were stable, and the corresponding  $a_w$  values of the samples at the specified temperatures were recorded (Yang et al., 2020).

Each HTC was calibrated against standard lithium chloride solutions at all designated temperatures. This calibration method was adapted from Tadapaneni et al. (2017b). The standard  $a_w$  values of the lithium chloride solutions at the target temperatures were computed using an empirical equation documented by Yang et al. (2020). This method has been successfully applied in previous studies to assess  $a_w$  changes at elevated temperatures in various low-moisture foods, such as sugar, honey, almonds, peanut butter, milk and egg powder (Pérez-Reyes et al., 2021; Syamaladevi et al., 2016b, 2016a; Tadapaneni et al., 2017; Villa-Rojas et al., 2017, 2013). Accurately capturing  $a_w$  changes during heating is essential for understanding the thermal behavior of spores and for designing more effective thermal treatment (Syamaladevi et al., 2016b).

### 3.3. Spore suspension and rice powder inoculation

*B. cereus* strains 104.1.1 and 227.1.2 were selected for this study. Both produce enterotoxins and express emetic genes, as confirmed by Mohammadi et al. (2023).

*B. cereus*' presence in rice flour samples was evaluated following the Bacteriological Analytical Manual's (BAM) procedure (et al., 1998). Fifty grams of a rice flour sample was diluted in 450 ml of buffer peptone (BP) water and homogenized with a stomacher (Stomacher 400 Circulator, Seward, England). Serial dilutions were prepared from  $10^{-1}$  to  $10^{-3}$  by transferring 10 ml of the prepared sample (1:10) to 90 ml of buffer peptone water until the desired solution was reached. Then 0.1 ml from each dilution was spread plated on MYP agar plates in duplicate. The plates were incubated at 32°C for 18–24 hours and observed for a precipitate zone and pink colonies that indicate the presence of *B. cereus*.

When background flora was confirmed, the rice flour samples were autoclaved in a thin layer inside autoclave bags to avoid further contamination for 20 min at 121°C and dried for 10 minutes at the same temperature. Subsequently, the absence of *B. cereus* was confirmed by repeating the same procedure.

The spore suspension was prepared using the following procedure: vegetative cells from each *B. cereus* strain were plated and incubated on tryptic soy agar (TSA) plates at 32°C for 24 hours. The vegetative cell colonies were transferred to 10 ml of tryptic soy broth (TSB) in a shaking incubator for 48 hours at 32°C. The cell suspension sample was spread plated on a sporulation medium composed of peptone (5 g/l), meat extract (20 g/l), agar (1 g/l), KCl (1 g/l), and MgSO<sub>4</sub>·7H<sub>2</sub>O (0.12 g/l). After autoclaving, 1 ml of each of the following filter-sterilized solutions were added to the medium: 1 M Ca(NO<sub>3</sub>)<sub>2</sub>, 0.1 M MnCl<sub>2</sub>, and 1 mM FeSO<sub>4</sub> (Stoeckel et al., 2013, 2014; Xu et al., 2021).

The sporulation media plates were incubated for 5 days at 37°C until they reached a 95 % sporulation, as confirmed by microscope observation. After incubation, the spores were harvested from the media plates by washing with 0.1 % (w/v) buffer peptone water and centrifuged at 5900 g for 15 min at 4 °C. The supernatant was removed, and the pellet was resuspended in 3 ml of 0.1 (w/v) buffer peptone water and heat shocked at 80°C for 20 minutes to eliminate the remaining vegetative cells. Finally, the spore solution was cooled on ice and stored at –20°C before usage. The final concentration of the spore solution was approximately  $10^{10}$  CFU/ml. A 90 % grade of sporulation was confirmed under microscopic observation (Stoeckel et al., 2013; Xu et al., 2021).

Three batches of rice flour were inoculated by hand mixing 10 g of the flour with 1 ml of the *B. cereus* spore solution. Mixing continued until all the pellets were removed, and no clumps were visible. Then, the 10 g inoculated sample was added to a 90 g rice flour sample and mixed to complete the inoculation process. Finally, the inoculated samples were placed in a sterile petri dish at room temperature for 24 hours and then stored in a container with a supersaturated solution of MgNO<sub>3</sub>, until they reached their original  $a_w$  of 0.48. The appropriate distribution of the inoculum was confirmed by randomly sampling ten 1 g portions from each inoculated rice flour sample, followed by enumeration on TSA as detailed below (Pérez-Reyes et al., 2021a; Xu et al., 2019).

### 3.4. Thermal inactivation of *B. cereus* spores in rice flour

The thermal inactivation studies were conducted using an improved design of the thermal death time test (TDT) cells designed by Dr. Juming Tang's lab at Washington State University. The TDT cell consists of an aluminum body with a height of 1.30 mm and a diameter of 31.18 mm. A schematic representation of the cell is shown in Fig. 1. A gram of the inoculated samples was loaded into the TDT cells. Then, the cells were sealed and immersed in an oil bath preheated to the selected temperature (90°, 95°, and 100°C). Once the TDT cells reached the objective temperature in about 40 s, they were removed at predetermined periods and cooled in ice water to stop thermal inactivation completely.

The TDT cells are designed to be hermetically sealed in a way that controls the moisture content of the sample while still allowing changes in  $a_w$ , thereby simulating real-case scenarios in which  $a_w$  varies with temperature. This feature makes them adequate for studying the thermal resistance of microorganisms in low-moisture foods. TDT cells have been previously employed for thermal inactivation studies of *Salmonella* spp. and *Listeria monocytogenes* (Jin et al., 2020; Jin and Tang, 2019; Pérez-Reyes et al., 2021a; Xu et al., 2018).

Following thermal inactivation, the treated samples were scraped from the TDT cells into 9 ml of 0.1 % (w/v) peptone water to obtain a 10-fold dilution. The solution was stomached for 2 min at 260 rpm with a Seward Stomacher (Seward, UK). The samples were 10-fold serially diluted in 0.1 % (w/v) peptone water, spread-plated onto TSA agar plates, and incubated at 37°C for 48 hours to determine the population (CFU/g) of survivors. This procedure was verified by performing the experiment in triplicate and from the samples taken from each batch to

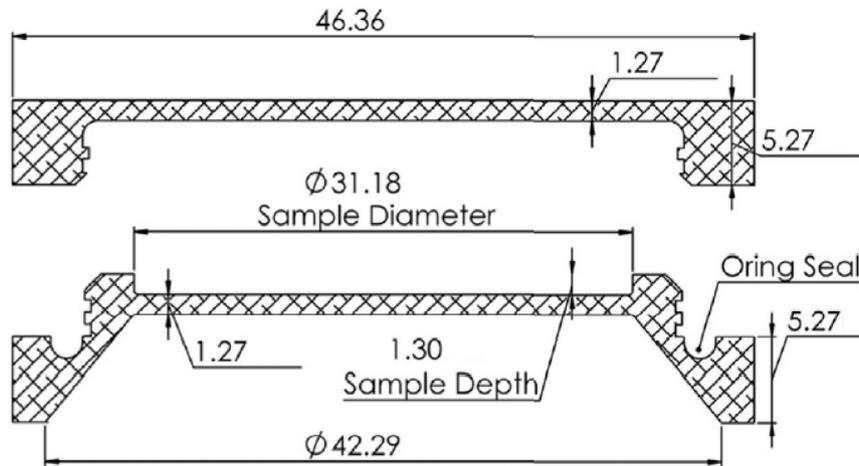


Fig. 1. Schematic representation of the thermal death time (TDT) cell improved design (Jin, 2019). The dimensions are in mm.

evaluate the predetermined time intervals (Technical replicates) (Liu et al., 2018a; Liu et al., 2018b; Pérez-Reyes et al., 2021a; Xu et al., 2019).

### 3.5. Inactivation kinetics

The linear regression model was used to fit the *B. cereus* spores inactivation data in rice flour by the following equation:

$$\log\left(\frac{N}{N_0}\right) = \frac{-t}{D - \text{value}} \quad (1)$$

where  $N$  and  $N_0$  (CFU/g) are the surviving bacteria population at times  $t$  (min) and 0 (following the come-up time of 40 s, which is the time required to reach 0.5 °C within the objective temperature), respectively. The D-value is defined as the necessary time (min) to inactivate the bacteria population by 90 % at the selected temperature (90°, 95°, and 100°C) (Pérez-Reyes et al., 2021a; Villa-Rojas et al., 2013).

The inactivation data at each selected temperature were fit to the linear model to obtain the thermal inactivation curves using Excel (Microsoft Corporation, Redmond, WA, USA). The regression coefficient ( $R^2$ ) and the root mean square error (RMSE) were used to evaluate the goodness of fit (Eqs. 2, 3).

$$R^2 = 1 - \frac{\left(\sum_{i=1}^n \left(\log\left(\frac{N}{N_0}\right)_{i,obs} - \log\left(\frac{N}{N_0}\right)_{i,model}\right)^2\right)}{\left(\sum_{i=1}^n \left(\log\left(\frac{N}{N_0}\right)_{i,obs} - \overline{\log\left(\frac{N}{N_0}\right)}\right)^2\right)} \quad (2)$$

$$RMSE = \sqrt{\frac{\sum_{i=1}^n \left(\log\left(\frac{N}{N_0}\right)_{i,model} - \log\left(\frac{N}{N_0}\right)_{i,obs}\right)^2}{n - p}} \quad (3)$$

where  $\log\left(\frac{N}{N_0}\right)_{i,obs}$  is the determined log reduction,  $\log\left(\frac{N}{N_0}\right)_{i,model}$  is the predicted log reduction from the model,  $\overline{\log\left(\frac{N}{N_0}\right)}$  is the mean of the log reduction from the linear regression model,  $n$  is the total number of observations, and  $p$  ( $= 2$ ) is the number of parameters.

Inactivation curves were determined by plotting the logarithm of the surviving microorganisms versus the heating times. The D-values of both strains were calculated from the slope of their corresponding inactivation curves at the selected temperatures. The mean D-values and the standard deviation for each strain were based on three independent replicates.

Since the D-values of both *B. cereus* strains were determined at different temperatures, the Z-value (required increase in temperature to cause 90 % reduction in D-value) in rice was determined by Eq. 4:

$$Z = \frac{T_2 - T_1}{\log\left(\frac{D_1}{D_2}\right)} \quad (4)$$

where  $D_1$  and  $D_2$  are the required time to reduce 90 % of the bacterial population at temperatures  $T_1$  and  $T_2$ , respectively (Liu et al., 2018b; Pérez-Reyes et al., 2021a). Differences between D-values among samples ( $P < 0.05$ ) were evaluated using ANOVA in Minitab 17. (Minitab Inc, State College, PA).

## 4. Results and discussion

### 4.1. Rice Flour's water activity at elevated temperatures

Numerous studies have confirmed that, in most cases, the  $a_w$  of food increases as temperature rises (Syamaladevi et al., 2016a; Syamaladevi et al., 2016b; Tadapaneni et al., 2017a). Fig. 2. illustrates the increase in  $a_w$  from 0.48 at 22°C to 0.64 at 60°C. When the temperature was further increased to 90°C, the  $a_w$  value rose significantly to 0.85. Between 90° and 95°C, the  $a_w$  increased by 0.03. The rise of rice flour's  $a_w$  was related to particle size since a fine particle size presents a high swelling power compared to a larger particle size, the smaller particles have greater water strength retention (Qian and Zhang, 2013). Since the rice flour was produced using the dried stone milling process, the particle size was relatively bigger compared to other methods that can produce particles between 2 and 61 μm. The particle size of the rice flour used (100 μm) affected its capacity to retain water and, therefore, its  $a_w$  value is elevated compared to finer food powders, which have a higher capacity to absorb and retain moisture (Chiang and Yeh, 2002; Qian and Zhang, 2013). Additionally, as temperature increases, the mobility of water molecules is affected, causing rice flour to become less hygroscopic. This phenomenon leads to the separation of water molecules from the binding sites within the food matrix, explaining the significant increase in  $a_w$  observed in our rice flour samples. This trend has also been observed in other high-starch flours, suggesting a common response to temperature increases (Al-Muhtaseb et al., 2002; Durakova and Menkov, 2004; Jin et al., 2020).

### 4.2. Thermal resistance of *Bacillus cereus* strains

#### 4.2.1. Thermal inactivation kinetics of *B. cereus* strain 104.1.1

The inactivation kinetics of *B. cereus* strain 104.1.1, inoculated in

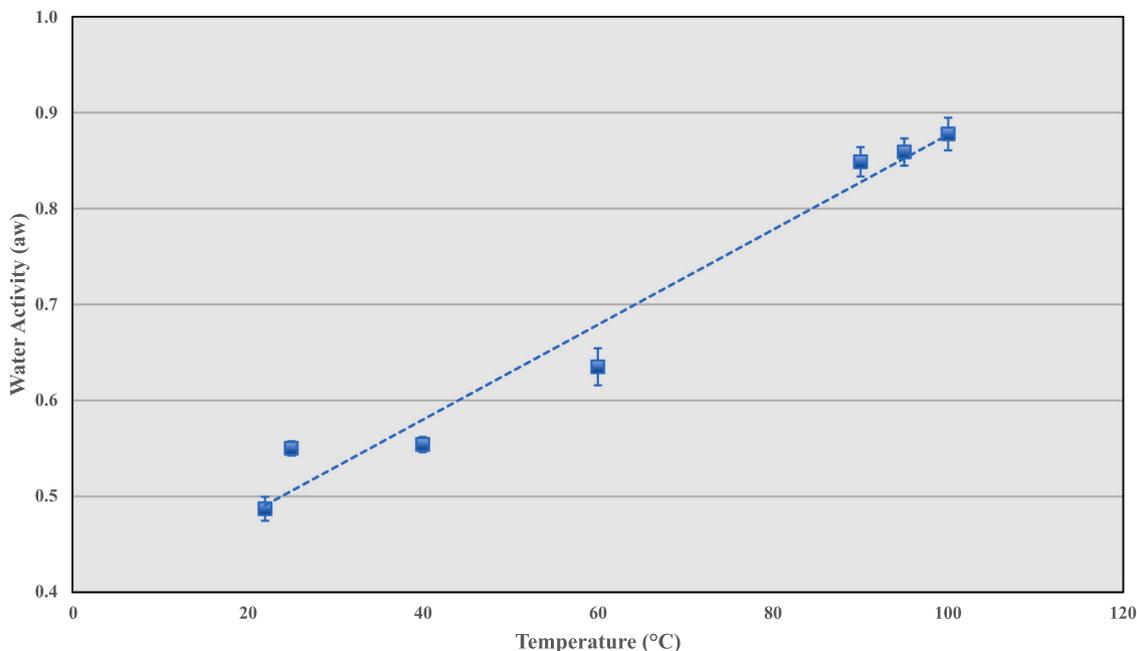


Fig. 2. Water activity ( $a_w$ ) of rice flour at different temperatures. The data points are the means of triplicate measurements with standard deviations ( $\pm$  SD).

rice flour and evaluated at 90, 95 and 100°C, as shown in Fig. 3, were well described by the first-order model. This is evidenced in high  $R^2$  values and low RMSE, as detailed in Table 1. To negate any interference with the calculation of the slopes, time zero was taken as the come-up time (the time required to reach the objective temperature  $\sim$  40 s) to reach within 0.5°C from the objective temperature (Jin et al., 2020; Xu et al., 2019). For the three thermal treatment temperatures, it took approximately 90 hours to obtain a 3-log reduction (Fig. 3) indicating a very high thermal resistance. Table 1 presents the D-Values of the different *B. cereus* strains inoculated in rice flour at different temperatures. For *B. cereus* strain 104.1.1, D-Value was 40.19 hours at 90°C with a final  $a_w$  of 0.85. Increasing the temperature to 100°C and the  $a_w$  to 0.89 reduced the D-Value by approximately 10 hours. The high thermal resistance of the *B. cereus* 104.1.1 spore compromises rice flour food safety since a prolonged thermal treatment would be required to achieve a significant spore reduction.

The thermal resistance of *B. cereus* spore strain 104.1.1 obtained

from this study was significantly higher than observed in other strains in both high and low moisture foods. For instance, Byrne et al. (2006) reported that *B. cereus* spores from the strain DSM 4313 had D-values of 29.5, 10.1 and 2.0 min at 85°, 90°, 95°C, respectively, when inoculated into pork luncheon rolls with an  $a_w$  around 0.9–0.8. Additionally, another study reported D-values of 30.5 and 2.8 min for *B. cereus* spores from the strain CNRZ 110 when inoculated in broth with a controlled  $a_w$  of 0.98 at 85° and 95°C, respectively. In the case of rice products, *B. cereus* spores inoculated in rice water and phosphate buffer showed D-values of 1.90 and 5.6 h at 82°C (Penna and Moraes, 2002; Rajkowski and Mikolajcik, 1987). Similarly, D-values of 21.7 minutes in buffer and 39.7 min in uncooked rice were reported for *B. cereus* strain ATCC 14579 and a spore cocktail at 95°C (Rajkowski and Mikolajcik, 1987). Luu-Thi et al. (2014) reported a D-value of 96.6 min for *B. cereus* spores of the INRA PA strain in a spore suspension. In whole milk *B. cereus* spores from strain B63 showed a D-value of 24.31 min at 90°C, allowing survival through the spray drying process. Therefore, spores could be found in

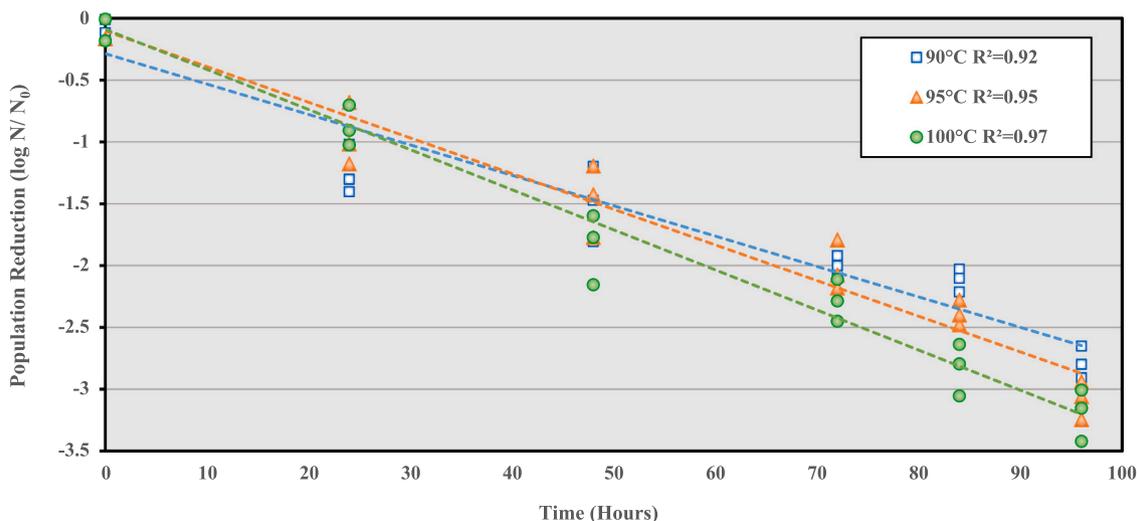


Fig. 3. Thermal inactivation kinetics curves of *B. Cereus* strain 104.1.1 in rice flour with an initial  $a_w$  of 0.48 at different temperatures: 90°C (□), 95°C (Δ) and 100°C (○). Inactivation was conducted independently at least three times.

**Table 1**D-Values and Z-Values of *Bacillus Cereus* individual strains in rice flour at different temperatures with initial  $a_w$  of 0.48.

Strain	Temperature (°C)	$a_w$ Treatment Temperature	D-Values (hours)	R <sup>2</sup>	RMSE (log CFU/g)	Z - Value (°C)
104.1.1	90	0.85 ± 0.02	40.19 ± 0.82 <sup>a</sup>	0.93	0.25	86.95
	95	0.86 ± 0.01	34.67 ± 0.65 <sup>b</sup>	0.95	0.29	
	100	0.89 ± 0.02	30.84 ± 0.19 <sup>c</sup>	0.97	0.28	
227.1.2	90	0.85 ± 0.02	27.75 ± 0.28 <sup>d</sup>	0.97	0.21	72.46
	95	0.86 ± 0.01	24.05 ± 0.25 <sup>e</sup>	0.96	0.29	
	100	0.89 ± 0.02	20.20 ± 0.59 <sup>f</sup>	0.97	0.27	

Mean ± SD, n = 3. R<sup>2</sup>: coefficient of determination; RMSE root mean square error. D-values with different letters are significantly different ( $P \leq 0.05$ ).

milk powder, which is a low-moisture food (Alvarenga et al., 2018). Additional studies in buffer solutions with controlled  $a_w$  ranging from 0.8 to 1 and at pH levels of 4.5, 5.5, and 6.5, conducted at 85° to 105°C, found D-values ranging from 0.15 to 102.8 min (Gaillard et al., 1998).

Most reported D-values for *B. cereus* spores have been determined in liquid media. However, they are relatively high compared to those of other bacteria, such as *Salmonella*, which has a D-value of 0.32 min at 70 °C in chicken products. This highlights that *B. cereus* spores are potentially more dangerous than vegetative forms of other bacteria due to their elevated thermal resistance (Murphy et al., 2002). Our study reveals that the thermal resistance of *B. cereus* spores is significantly higher in low-moisture foods. We obtained D-values as high as 40.19 hours (Table 1) when spores from the strain 104.1.1 were inoculated into rice flour with an initial  $a_w$  of 0.45 and a final  $a_w$  of 0.85 at 90°C. This behavior aligns with the observations in *Salmonella* spp. and *Bacillus subtilis* in low-moisture foods, where thermal resistance increases as initial  $a_w$  decreases (Hauck-Tiburski et al., 2019; Liu et al., 2018a; Pérez-Reyes et al., 2021a; Syamaladevi et al., 2016a; Tadapaneni et al., 2017a; Xu et al., 2019).

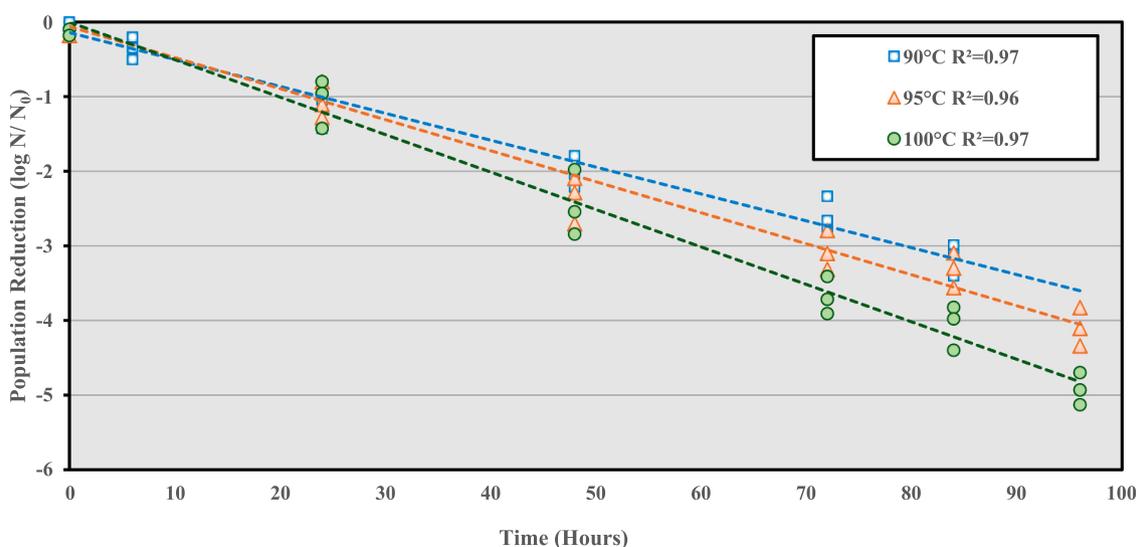
There have been few studies of thermal inactivation of *B. cereus* spores in low-moisture food. However, the D-values obtained in those studies are as high as the ones we report in this study. Recent research has shown that radiofrequency technology achieved approximately a 4-log reduction of *B. cereus* spores of the strain ATCC 14579 in 59 min at 70°C in red pepper powder with an initial  $a_w$  of 0.6 (Jiao et al., 2019). In another case, *B. cereus* spores naturally present in mesquite flour had a D-value of 0.63 hours at 100°C (Fan et al., 2021). Dry heat inactivation studies on rice, conducted by Houška et al. (2007), demonstrated that achieving a 2-log reduction of *B. cereus* required 2 hours of exposure at 120°C, which was the highest D-value observed at the highest temperature at the highest temperature tested.

It is important to note that the strains used in the study had previously been exposed to harsh conditions, including multiple cleaning protocols by the spacecraft assembly team, involving disinfectants with varying pH levels (Smith et al., 2017; Venkateswaran et al., 2017). Moreover, exposure to extreme conditions, such as prior heat exposure and certain antibiotics, can enhance the thermal resistance of *B. cereus* spores (Alvarenga et al., 2018). Cleaning solutions may also disrupt the cell's electrolytic balance, alter membrane composition, and protect macromolecules, further enhancing spore resistance (Alvarenga et al., 2018; Juneja et al., 2020). Furthermore, cross-adaptation between different stressors has been observed; for instance, salt stress and exposure to low temperatures have been shown to enhance the thermal resistance of *B. cereus* spores (Carlin et al., 2010).

#### 4.2.2. Thermal inactivation kinetics of *B. cereus* strain 227.1.2

The thermal inactivation kinetics of *B. cereus* strain 227.1.2 are shown in Fig. 4. This strain exhibited high thermal resistance, requiring approximately 60–90 hours for a ~ 3-log reduction- Table 1. presents the D-values for both strains, showing a significant difference ( $P \leq 0.05$ ). For example, strain 104.1.1 had a D-value of 40.19 ± 0.82 hours at 90°C while strain 227.1.2 has a D-value of 27.75 ± 0.28 hours, both with a final  $a_w$  of 0.85. The highest D-value for strain 227.1.2 was 27.75 ± 0.28 at 90°C while the lowest was 20.20 ± 0.59 at 100°C.

As with strain 104.1.1, the thermal inactivation kinetics of the strain 227.1. were well described by the first order-model as indicated by high R<sup>2</sup> and low RMSE values at each selected temperature (Table 1). Although the D-values for strain 227.1.2 were lower than those of strain 104.1.1, it still poses a significant food safety risk, since a prolonged thermal treatment would be required to achieve a significant log reduction, potentially compromising the food quality. As mentioned



**Fig. 4.** Thermal inactivation kinetics curves of *B. Cereus* strain 227.1.2 in rice flour with an initial  $a_w$  of 0.48 at different temperatures: 90°C (□), 95°C (Δ) and 100°C (○). Inactivation was conducted independently at least three times.

earlier, the increased thermal resistance of this spore may be attributed to its isolation from spacecraft facilities (Smith et al., 2017).

#### 4.2.3. Variability in thermal inactivation Among *B. cereus* strains

Variations in D-values at the same temperature between *B. cereus* strains have been reported in other studies (Alvarenga et al., 2018; Penna and Moraes, 2002; Rajkowski and Mikolajcik, 1987). This variability in thermal resistance may be influenced by prior exposure of *B. cereus* to environmental stressors such as heat, acidity or cold, that can induce the development of mechanisms to prevent DNA damage. These adaptive responses, in turn, influences the thermal resistance of each strain after germination (W. Choi and Kim, 2020; Juneja et al., 2020; Le Marc et al., 2022). These mechanisms are often associated with *cspA* gene signatures, which reflect strain-specific strategies for environmental adaptation. For instance, it is known that toxin-producing strains tend to be more heat tolerant (Carlin et al., 2010; Francis et al., 1998). Some of these adaptations are also linked to genomic variations among strains, with genome size differences being correlated with stress responses, further increasing strain variability (Carlin et al., 2010). In other *Bacillus* species, stress conditions have shown to induce changes in spore composition and structure, specifically in the spore coat, cortex and the SASP that bind to DNA. These structural differences help explain the variation in D-values observed among strains in this and other studies (Carlin et al., 2010; Nicholson et al., 2000).

Additionally, it has been observed that the attachment of spores to the heating medium can result in significant differences in D-values for *B. cereus* spores. Moreover, differences in the composition of the medium from which *B. cereus* cells were originally isolated may also affect the resulting D-values between strains, as higher levels of fat, carbohydrates, and proteins can increase thermal resistance. Furthermore, low  $a_w$  and pH levels in heated medium have been reported to enhance thermal resistance as well. (Byrne et al., 2006). This phenomenon might be related to the remarkable adaptability of *B. cereus* to diverse natural environments, including various soil types, food matrices and even the human gastrointestinal tract (Schoeni and Wong, 2005; Vilain et al., 2006). Sigma factor expression plays a fundamental role in these adaptive responses. For example, sigma factor  $\sigma^B$  is activated under stress conditions and induces structural changes in the spores, modifies germination dynamics and enhances resistance to heat and hydrogen peroxide (De Vries et al., 2005). Two-component systems (TCS) also contribute to environmental adaptability by mediating signal transduction. TCS typically consist of a membrane-bound histidine kinase (HK) that detects environmental stimuli and activates a cognate response regulator, thereby modulating gene expression. TCS are essential for various survival functions in *B. cereus* strains including biofilm formation, antibiotic resistance, thermal resistance, adaptation to low redox potential, and enterotoxin production (Aguilar, 2001; Schumann, 2009). In addition, *B. cereus* adapts to low pH environments through the acid tolerance response (ATR), which involves mechanism such as cytoplasmic pH homeostasis, metabolic modifications, and the synthesis of stress protein that protect or degrade macromolecules (Cotter and Hill, 2003; Mols and Abe, 2008). It has been reported that all these adaptative mechanisms can contribute to cross-protection, thereby influencing the thermal resistance of *B. cereus* spores. Therefore, the presence or absence of these mechanisms may explain the variability in D-values observed in this and other studies (Carlin et al., 2010).

The data corresponding to the increase in  $a_w$  with increasing thermal treatment temperatures are included in Table 1. Higher  $a_w$  values corresponded to a higher temperature ( $0.89 \pm 0.02$  at  $100^\circ\text{C}$ ). As expected, the lowest D-values for both *B. cereus* strains were obtained at the highest temperatures and  $a_w$  levels. For example, the D-value obtained for *B. cereus* strain 227.1.2 with  $a_w$  of 0.89 at  $100^\circ\text{C}$  was  $20.20 \pm 0.59$  hours, which is  $\sim 7$  hours lower than the D-value obtained at  $90^\circ\text{C}$ . Although the differences between the  $a_w$  values at the treatment temperatures were relatively small, the effect of  $a_w$  in the thermal

inactivation of *B. cereus* spores was still observed. This effect has also been observed in the thermal inactivation of other pathogen, including *Salmonella* and *Listeria* (Fan et al., 2021; Liu et al., 2018b; Pérez-Reyes et al., 2021a). At lower  $a_w$  values, the loss of water leads to more stable protein structures, hindering thermal denaturation and allowing spores to better withstand heat. In other spore-forming bacteria, such as *B. subtilis*, studies have reported low water rotational correlation times ( $50 \times 10^{-11}$  s) within the spore core, indicating that most proteins are rotationally immobilized. This immobilization, along with dehydration-induced conformational changes in key enzymes. Therefore, contributing to the increased heat resistance observed under low-moisture conditions. Similar mechanisms might explain the increased thermal resistance of *B. cereus* spores under low  $a_w$  conditions (Liu et al., 2018; Sunde et al., 2009). However, we are only analyzing the increase in  $a_w$  of rice flour with the thermal treatment temperature. Moreover, follow-up studies with multiple  $a_w$  values, temperatures, pH conditions and other food matrices are needed to better understand the impact of this property on *B. cereus* thermal inactivation.

The relationship between the D-values of the spores from the two *B. cereus* strains, the treatment temperature, and the corresponding  $a_w$  of the rice flour is shown in Fig. 5. For both strains, D-values decrease while temperature and  $a_w$  increase. This graph includes the power trend line for each strain and presents their corresponding  $R^2$  values, which have a high value indicating a good fit.

The Z-value, defined as the temperature increase required to reduce the D-value of the *B. cereus* spores in rice flour by 90 % is also indicated in Table 1 for each strain. Although limited studies have addressed spore Z-value in low moisture food, previous research has reported Z-values of multiple *B. cereus* strains in liquid media, typically ranging from  $8.3^\circ$  to  $12.8^\circ\text{C}$ , with some significant deviations of  $4.2^\circ$  and  $19^\circ\text{C}$  (Afchain et al., 2008; Luu-Thi et al., 2014; van Asselt and Zwietering, 2006). Similar Z-values have been observed for other *Bacillus* species when inactivated within food matrices. For instance, *Bacillus stearothermophilus* spores exhibit Z-values ranging from  $10.7$  to  $15.6^\circ\text{C}$  when treated in rehydrated dry pasta, with lower Z-values observed as the pasta's moisture content increased (Cunningham et al., 2007). On the other hand, the thermal inactivation of vegetative bacteria in low-moisture foods has been extensively studied. For example, *Salmonella* Weltevreden inoculated in wheat flour yielded Z-values ranging from  $15.2^\circ$  to  $53.9^\circ\text{C}$  (Podolak and Black, 2017), while *Salmonella* Enteritidis PT30 in almond kernel flour showed Z-values from  $7.2^\circ$  to  $10.4^\circ\text{C}$  (Villa-Rojas et al., 2013). In comparison, the Z-values for *B. cereus* strains spores in our study are significantly higher:  $87.59^\circ\text{C}$  for strain 104.1.1 and  $72.46^\circ\text{C}$  for strain 227.1.2. Comparable results were reported by Jiao et al. (2019), who documented a Z-value of  $90^\circ\text{C}$  for *B. cereus* spores in red pepper. These elevated Z-values suggest that the spores produced by these *B. cereus* strains exhibit a very high thermal resistance, meaning that a temperature increase of  $72.46^\circ\text{C}$  is required to reduce the D-Value by 90 % for strain 227.1.2.

Given extremely high D- and Z-values obtained for the *B. cereus* spores in this study, developing an effective thermal treatment to inactivate them is challenging. Traditional low-moisture pasteurization processes, such as baking, roasting and drying, allow *Salmonella* survival, whose thermal resistance is lower than that of *B. cereus* spores; therefore, *B. cereus* would likely survive these processes as well (Anderson, 2019). Additionally, prolonged thermal treatments may negatively affect the quality and the appearance of the final product. As previously discussed, the high thermal resistance observed in spores from these particular *B. cereus* strains may be attributed to disinfection protocols used in U.S. spacecraft assembly facilities (Alvarenga et al., 2018; Juneja et al., 2020; Smith et al., 2017; Venkateswaran et al., 2017). These findings suggest that the effects of harsh environments on *B. cereus* thermal resistance should be further studied. Given the extremely large D-values at even  $100^\circ\text{C}$ , it will be extremely difficult to develop a thermal process to inactivate the studied strains of *B. cereus* in a commercial setting. Yet, as long as the spores remain dormant, they

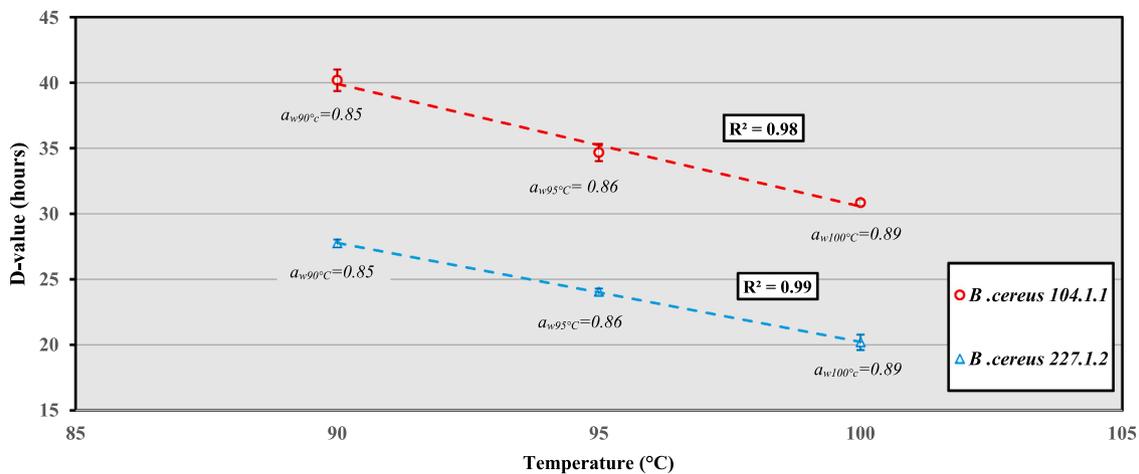


Fig. 5. D-values of *B. Cereus* strains: 104.1.1 (●) and 227.1.2 (▲) at their respective water activities ( $a_w$ ) at the selected temperature, along with their power trend line: experiments were conducted in triplicate.

may not cause harm to humans. Thus, in addition to taking proper measures to reduce the initial load, care should be taken on post-cooking cooling: the cooked products should be immediately cooled to below 30–40 °C to avoid germination and growth of germinated cells.

## 5. Conclusions

Our findings indicate that spores from *B. cereus* strains 104.1.1 and 227.1.2 exhibit a remarkably high thermal resistance, as evidenced by their high D-values. This resistance likely results from the harsh conditions—such as exposure to antimicrobial agents, air filtering, dry conditions, and rigorous temperature control—present at the U.S. spacecraft assembly facilities where these strains were isolated. Such stressors may have promoted the development of adaptive mechanisms. Including expression of *cspA* genes, sigma factors, TCS and SASP, all of which are known to enhance thermal resistance of spores. Consequently, conventional thermal treatments are insufficient for effectively inactivating these spores, and prolonged heating may compromise the quality and appearance of the final food product.

Therefore, future research should explore alternative treatments, such as modifying the  $a_w$  and employing hurdle technologies like infrared heating or radiation. Additionally, non-thermal treatments, such as essential oils, and fumigation by ethylene oxide and propylene oxide, should also be considered for further studies. Additionally, further studies are needed to understand the specific conditions that contribute to enhancing the thermal resistance of *B. cereus* spores in such environments. Given their ability to easily survive thermal processing and contaminate food products, these spores represent a significant challenge to food safety and public health.

## CRedit authorship contribution statement

**Barakatullah Mohammadi:** Writing – review & editing, Software, Methodology, Investigation, Conceptualization. **Ren Yang:** Writing – review & editing, Software, Resources, Investigation, Data curation. **Sathish Yerrapati:** Writing – review & editing, Methodology, Investigation, Data curation. **Zachary Cartwright:** Writing – review & editing, Software, Resources, Methodology, Investigation, Formal analysis. **Pérez Reyes Marco Esteban:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Juming Tang:** Writing – review & editing, Resources, Methodology, Investigation, Funding acquisition. **Stephanie Smith:** Writing – review & editing, Visualization, Validation, Resources, Investigation, Funding acquisition, Conceptualization.

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## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data Availability

Data will be made available on request.

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