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Dry-inoculation method for thermal inactivation studies in wheat flour using freeze-dried *Enterococcus faecium* NRRL B-2354

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ABSTRACT

Liquid cultures are commonly used to inoculate low-moisture foods. However, liquid-inoculation may generate varied results due to differences in preparation methods, and the introduction of liquid into a low-moisture matrix may cause stickiness and clumping. The objectives of this study were to develop a dry-inoculation method using freeze-dried *Enterococcus faecium* NBRL-2354, and to evaluate the stability of freeze-dried inoculum over time (35 days at −20 °C) in terms of survival and heat resistance. Two protectants, freeze-drying buffer and skim milk, mixed separately with liquid *E. faecium* were freeze-dried and their preservative effects were compared. Heat resistance of freeze-dried inoculum was determined by decimal reduction time at 80 °C (D_{80}) in wheat flour (water activity 0.45 at room temperature). Freeze-drying buffer and skim milk formed slimy layers around bacterial cells and yielded freeze-dried inoculum with high survivability. D_{80}-values of freeze-dried *E. faecium* protected by freeze-drying buffer or skim milk were 6.67 ± 0.22 min and 5.92 ± 0.39 min, respectively. Freeze-dried inoculum was stable with a high population concentration (> 10.0 log CFU/g), while skim milk-protected inoculum showed stable heat resistance throughout storage. In summary, freeze-dried *E. faecium* protected by skim milk was the optimum dried inoculum and can be used as a substitute for liquid-inoculum in low-moisture foods.

1. Introduction

Decimal reduction time (D-value) is a commonly used parameter to compare heat resistance among different microorganisms (Lopez, 1987; Smelt & Brul, 2014). D-value of microorganisms is influenced by external factors such as water activity (a_w), pH and osmotic pressure, and intrinsic factors related to the microorganisms, such as strain, growth conditions and growth stages (Algie, 1984; Finn, Condell, McClure, Amezquita, & Fanning, 2013; Minh, Perrier-Cornet, & Gervais, 2008; Riondet et al., 2000; Syamaladevi et al., 2016). Sparse information is available about preparation of appropriate inoculum for certain food categories such as low-moisture foods (Francois, Devlieghere, Uyttendaele, & Debevere, 2006; NACMCF., 2010). Liquid culture inoculum is widely used to inoculate low-moisture substrates, but due to differences in preparation methods of the inoculum, D-values can vary (Tyann Blessington, Mitcham, & Harris, 2012; Podolak, Enache, Stone, Black, & Elliott, 2010; Smith, 2014; van Boekel, 2002). For example, D-values of *Salmonella* Enteritidis PT30 at 80 °C in wheat flour varied from 3.8 to 8.4 min as observed in 5 artificially prepared inoculants generated by different procedures (Hildebrandt et al., 2016; Villa-Rojas, 2015). Moreover, the introduction of liquid inoculum may influence moisture content and other physical properties of the inoculated low-moisture foods, e.g., caking and clumping (Aguilera, del Valle, & Karel, 1995; Chuy & Labuza, 1994; Kimber, Kaur, Wang, Danyluk, & Harris, 2012; Palipane & Driscoll, 1993). To generate an adequate inoculum for low-moisture foods, chalk powder and talc have been used as dry carriers of *Salmonella* to inoculate pecans (Beuchat & Mann, 2011) and peanut paste (Enache et al., 2015). Dry inoculum in powdered form is a desirable inoculation method for low-moisture foods since it provides a more uniform distribution of the microorganism in the sample and has a minimal impact on properties of mixtures (Hoffmans & Fung, 1992).

Freeze-drying is currently an industrial standard for preservation of microorganisms and a convenient method for transportation of vast culture collections (Bjerketorp, Håkansson, Belkin, & Jansson, 2006;

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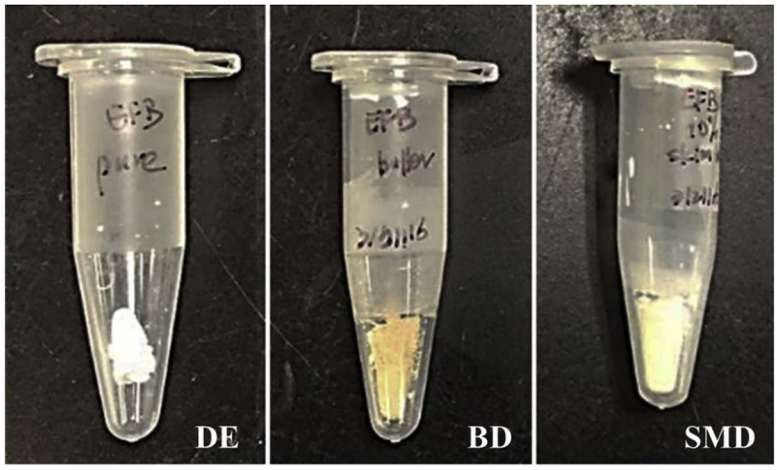

Fig. 1. *E. faecium* NRRL B-2354 after freeze-drying process. DE: freeze-dried *E. faecium* without protectant, BD: freeze-drying buffer protected freeze-dried *E. faecium*, SMD: skim milk protected freeze-dried *E. faecium*. The residual mass in DE was from 250 μL of bacterial suspension before freeze-drying. The residual mass in BD or SMD was from 250 μL bacterial suspension mixed with an equal volume of freeze-drying buffer or 10% skim milk solution, respectively.

Morgan, Herman, White, & Vesey, 2006). Systematic studies on the potential of freeze-dried bacteria as a dry inoculum for low-moisture foods has not been reported in the literature. Due to the added water when using liquid inoculum, inoculated foods require 4–5 days of exposure to a low relative humidity environment to re-equilibrate to its original low a_w condition, while freeze-dried inoculum wouldn't need re-equilibration since it can equilibrate with its surroundings within seconds (Syamaladevi, Tang, & Zhong, 2016) and could enable an inactivation study to be conducted immediately after inoculation. Moreover, freeze-dried bacteria are easy to prepare and transport, and the usage of freeze-dried inoculum can dramatically simplify culture preparation and enable cross-lab comparison without limitations of test times, locations or variations due to discrepancies in preparation (Enache et al., 2015).

Freeze-drying processing and subsequent storage result in death and injury of bacterial cells (Peiren, Hellemans, & De Vos, 2016; Ray, Jezeski, & Busta, 1971b). Protective agents are highly recommended additions to bacterial suspension prior to freeze-drying, to exert a protection effect by minimizing molecular mobility and plasma membrane changes of bacteria (Dhewa, Pant, & Mishra, 2014; Lee, Kim, & Park, 2016; Louis, Trüper, & Galinski, 1994). Selecting appropriate protectants and understanding the properties of freeze-dried bacteria in terms of survival and heat resistance during extended storage are imperative before applying freeze-dried inoculum in thermal inactivation studies of low-moisture foods.

The goal of this study was to develop a dry-inoculation method using freeze-dried *E. faecium* NBRL-2354 as a surrogate of *Salmonella* for thermal inactivation in low-moisture foods. The specific objectives were to: i) evaluate preservative effects of two protectants and develop a preparation protocol for freeze-dried inoculum; ii) determine and compare heat resistance of liquid- and freeze-dried- inoculants by performing thermal inactivation tests at 80 °C in wheat flour (a_w 0.45); and iii) evaluate the stability of freeze-dried inoculum over time in terms of survival and heat resistance.

2. Materials and methods

2.1. Culture preparation

Stock culture of *E. faecium* NRRL B-2354 (ATCC® 8459™) was acquired from Dr. Linda Harris at the University of California, Davis and stored in 20% glycerol (v: v) at −80 °C until use. A loop (10 μL) of thawed culture stock was inoculated into 9 mL of trypticase soy broth (TSB, Difco, Detroit, MI, USA) and incubated, then 3 mL was transferred into 30 mL of TSB and incubated. Four mL was further transferred into 400 mL TSB and incubated with constant shaking at 200 rpm to optimize bacterial growth. All cultures were incubated to stationary growth phase at 37 °C for 24 h.

2.2. Inoculum preparation

One liquid and three freeze-dried inoculants were prepared by centrifuging the overnight activated culture at 6,000 × *g* for 15 min at 4 °C and washing the pellets with sterile double-deionized water (dd H_2O). The obtained bacterial pellet was re-suspended in 2 mL of sterile ddH_2O and used as liquid *E. faecium* inoculum (LE). For preparing freeze-dried inoculum, 250 μL of LE was distributed into 1.5 mL Snaplock Microtubes (model MCT-150-X, Axygen, Union City, CA, USA) to prepare freeze-dried *E. faecium* (DE) without protectant, or mixed with an equal amount of freeze-drying buffer (MFDB 500-06, OPS Diagnostics, Lebanon, New Jersey), or 10% skim milk solution (Difco, Detroit, MI, USA) to prepare freeze-drying buffer- or skim milk-protected freeze-dried inoculum (BD and SMD), respectively. Bio-samples in microtubes were pre-frozen immediately in liquid nitrogen and desiccated in a FreeZone plus 4.5 L cascade freeze dry system (Labconco Corporation, Kansas City, MO, USA) for 48 h (−90 °C and 45 torr). After freeze-drying, microtubes with bacteria were vacuum-sealed in 4 oz Whirl-Pak bags (Nasco, Modesto, CA, USA) and stored at −20 °C.

2.3. Microstructure analysis and viability test

LE was transferred to fixative (2% paraformaldehyde/2% glutaraldehyde/0.1 M phosphate buffer) and held overnight at 4 °C. Bacterial cells were centrifuged and rinsed with 0.1 M phosphate buffer. Post-fixation was achieved in 1% osmium tetroxide overnight at 4 °C and rinsed with 0.1 M phosphate buffer. Dehydration was performed with a graded ethanol series (30%, 50%, 70%, 95%, 100%) and Hexamethyldisilazane for 10 min. Dehydrated LE and freeze-dried inoculants were thinly spread onto double coated carbon conductive tabs (Ted Pella Inc., Redding, CA, USA) and gold coated to a thickness of 6 nm in a vacuum-evaporator (Technics Hummer V Sputter Coater, Technics, San Jose, CA, USA). The microstructure of the four inoculants (LE, DE, BD, and SMD) was examined using a Quanta 200F environmental field emission gun scanning electron microscope (SEM, FEI company, Hillsboro, OR, USA). Images were captured by a digital camera (Quartz Imaging Corporation, Vancouver, British Columbia, Canada).

To determine the viability and population of bacteria immediately after freeze drying, 0.1% (w/v) peptone water (0.05 mM, PH 7) was added to the microtubes to rehydrate freeze-dried bacteria and reconstitute them to the original volume. Rehydrated suspensions were 10-fold serially diluted in 0.1% (w/v) peptone water and then plated onto trypticase soy agar (TSA, Difco, Detroit, MI, USA) and incubated at 37 °C for 24 h to enumerate viable cells. The viability test was evaluated

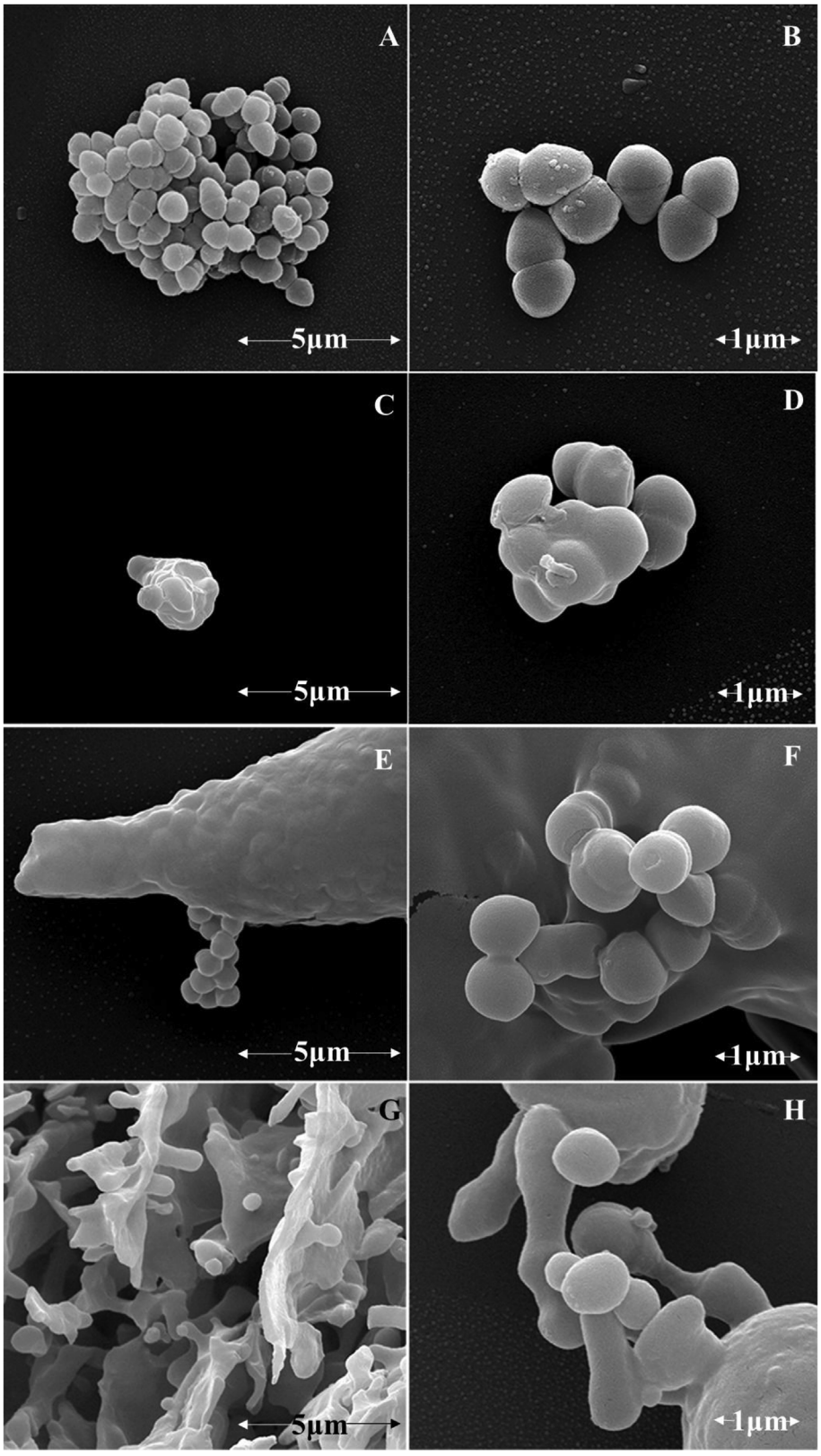

Fig. 2. Scanning Electron Microscopy images of *E. faecium* NRRL B-2354 cells. A, B liquid suspension of *E. faecium*; **C, D** freeze-dried *E. faecium* without protectant; **E, F** freeze-drying buffer protected freeze-dried *E. faecium*; **G, H** skim milk protected freeze-dried *E. faecium*. Magnification × 20,000 and scale bar indicates 5 μm for A, C, E, G; Magnification × 50,000 and scale bar indicates 1 μm for B, D, F, H.

on freeze-dried bacteria produced from three independent batches (three replicates for each batch) and the average results were regarded as the population counts (CFU/g) before inoculation.

2.4. Inoculated wheat flour preparation and equilibration

Organic soft wheat flour (Eden Foods, Clinton, MI, USA) was used as the model food due to its association with recent foodborne outbreaks (McCallum et al., 2013). A_w of flour was measured with a a_w meter (Model 3TE, Decagon Devices, Pullman, WA, USA) at room

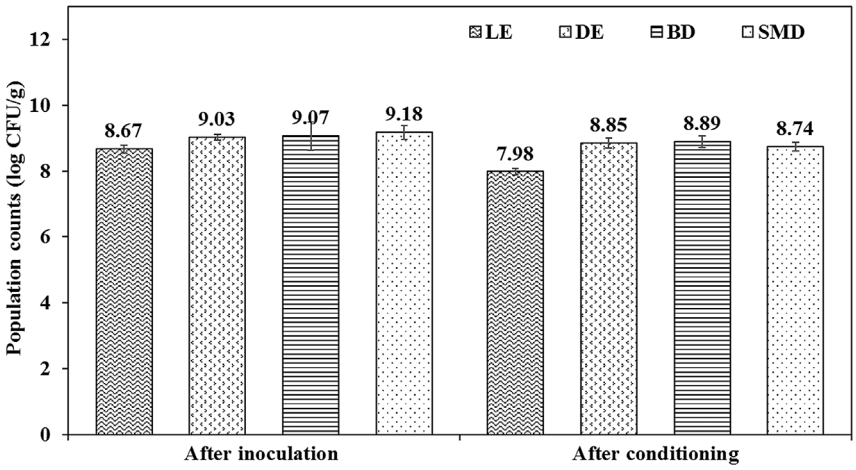

Fig. 3. Population counts of *E. faecium* NRRL B-2354 at different stages of inoculation process. LE: liquid suspension of *E. faecium*, DE: freeze-dried *E. faecium* without protectant, BD: freeze-drying buffer protected freeze-dried *E. faecium*, SMD: skim milk protected freeze-dried *E. faecium*. Population counts of inoculum were assayed before inoculation (after preparation of inoculum), after inoculation (immediately after inoculation with wheat flour), and after conditioning (when inoculated samples were conditioned to water activity 0.45). Each *column* represents the average population counts of bacteria prepared from three independent batches (three replicates for each batch). The *error bars* indicate the standard deviation.

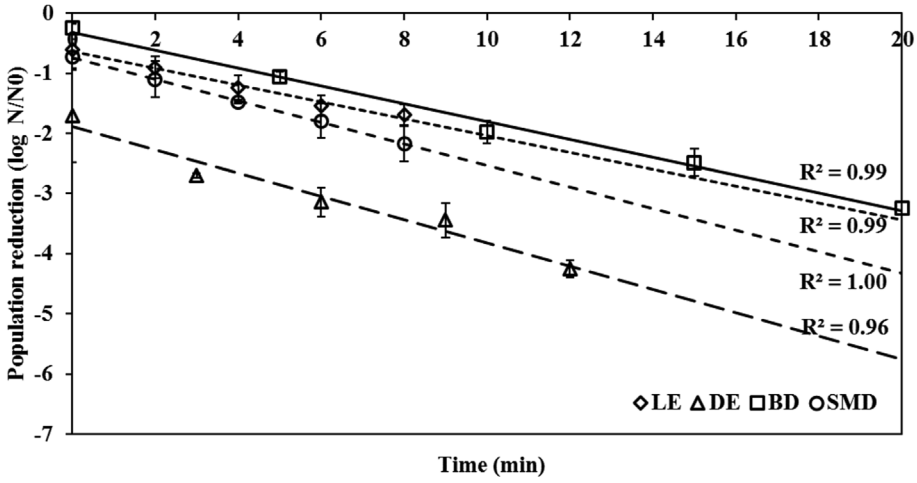

Fig. 4. Thermal inactivation curves of *E. faecium* NRRL B-2354 inoculants in wheat flour (water activity 0.45). LE: liquid *E. faecium*, DE: freeze-dried *E. faecium* without protectant, BD: freeze-drying buffer protected freeze-dried *E. faecium*, SMD: skim milk protected freeze-dried *E. faecium*. Population reduction (log N/N_0) of inoculum in wheat flour (water activity 0.45) was determined at different holding time intervals at 80 °C. *N* is the population count (CFU/g) at time t (min), N_0 is the initial population (CFU/g) of bacteria. *Diamond* LE, *triangle* DE, *square* BD, *circle* SMD. The inactivation curves were generated from the first-order kinetic model with R^2 coefficient as a goodness of fit index. The *error bars* indicate the standard deviations of triplicate measurements.

Table 1
D_{80}-values of *E. faecium* NRRL B-2354 in wheat flour (water activity 0.45). LE: liquid suspension of *E. faecium*, DE: freeze-dried *E. faecium* without protectant, BD: freeze-drying buffer protected freeze-dried *E. faecium*, SMD: skim milk protected freeze-dried *E. faecium*.

Inoculum	D_{80}-values (min)
LE	6.48 ± 1.00[a]
DE	4.90 ± 0.30[b]
BD	6.67 ± 0.22[a]
SMD	5.92 ± 0.39[ab]

[ab] D-values with different letters indicate significantly differences ($P < 0.05$) via one-way analysis of variance and subsequent least significant difference pairwise comparison test. The data shown are the mean values from three independent replicates plus the standard deviations.

temperature. For the liquid-inoculation method, inoculated samples were prepared by mixing 100 μL of LE into 10 g wheat flour and conditioned for approximately 4–5 days at room temperature to reach a_w 0.45 in a small glove box (EW-34788-00, Cole Parmer, Vernon Hills, IL, USA) with a humidity control system custom designed at Michigan State University. For the dry-inoculation method, one microtube (~10.0 mg) of lyophilized bacteria (DE, BD and SMD) was mixed directly with 10 g wheat flour (conditioned to a_w 0.45 in advance). To ensure the inoculum was evenly distributed, inoculated wheat flour was mixed by hand for at least 5 min until all clumps were unobservable. Three, one g inoculated but untreated samples were randomly selected, diluted in 9 mL of 0.1% (w/v) peptone water and stomached for 3 min at 230 rpm (Stomacher® 400 Circulator, Seward Laboratory Systems Inc, Bohemia, NY). Each stomached sample was then 10-fold serially diluted, spread-plated on TSA and incubated at 37 °C for 24 h. The number of CFU/g of inoculum after inoculation and after conditioning (prior to thermal treatments) were determined based on the average results of three replicates. Background microbial flora before inoculation was also determined from five random 1 g samples, and each sample was diluted and plated as previously described.

2.5. Thermal inactivation of freeze-dried E. faecium in wheat flour

Thermal death time (TDT) cells as described by Chung (Chung, Birla, & Tang, 2008) were filled with 0.7 g inoculated samples, sealed and immersed in a hot oil bath (Isotemp 5150 H11, Fisher 180 Scientific, Inc., Pittsburgh, PA, USA) preheated to 80 °C. TDT cells were removed at predetermined time intervals and cooled in an ice-water bath. The come-up time (CUT) for reaching 79.5 °C in the center of the TDT cells was 2.2 min, and regarded as the time zero (Villa-Rojas et al., 2017). Treated samples were scraped into 6.3 mL of 0.1% (w/v) peptone water to constitute a 10-fold dilution and stomached for 3 min at 230 rpm. Appropriate 10-fold serial dilutions were enumerated on TSA plates to determine the population counts (CFU/g) of survivors as previously described. Three biological replicates (batches inoculated with independently grown inoculum) and two technical replicates (samples from the same batch) were evaluated at each time interval.

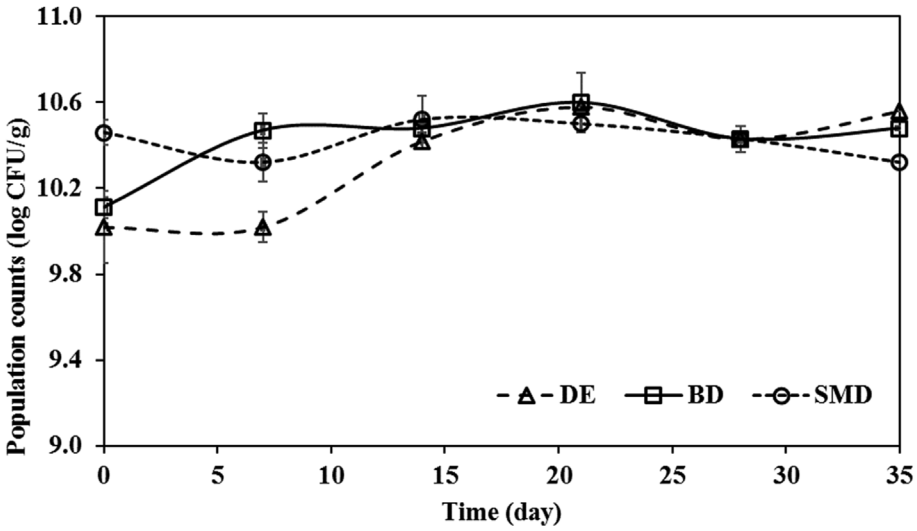

Fig. 5. Population counts of freeze-dried *E. faecium* NRRL B-2354 when vacuum-packed and stored at -20°C. DE: freeze-dried *E. faecium* without protectant, BD: freeze-drying buffer protected freeze-dried *E. faecium*, SMD: skim milk protected freeze-dried *E. faecium.* Numbers of viable cells were determined at 7-day intervals during storage for up to 35 days. *Triangle* DE, *square* BD, *circle* SMD. The data shown are the mean values from three independent replicates and the *error bars* indicate the standard deviations. The lines are generated by using scatter with smooth lines as a chart type in Excel.

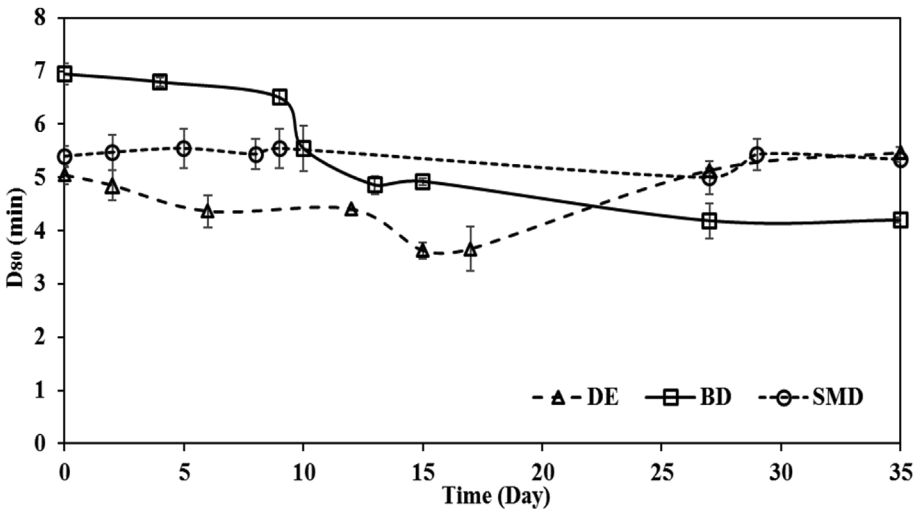

Fig. 6. D_{80}-values of freeze-dried *E. faecium* NRRL B-2354 during storage. DE: freeze-dried *E. faecium* without protectant, BD: freeze-drying buffer protected freeze-dried *E. faecium*, SMD: skim milk protected freeze-dried *E. faecium*. D_{80}-values were obtained from thermal inactivation studies in wheat flour (water activity 0.45) after different storage periods. Thermal inactivation was conducted during storage (beginning on day 0 after inoculation). *Triangle* DE, *square* BD, *circle* SMD. The data shown are the mean values from two replicates and the *error bars* indicate the standard deviations. The lines are generated by using scatter with smooth lines as a chart type in Excel.

2.6. Stability test of freeze-dried inoculum

Freeze-dried bacteria (DE, BD, and SMD) were vacuum-packed in a 4 oz sterile Whirl-Pak bag and stored at −20 °C for up to 35 days. They were sampled for changes in population counts (CFU/g) every 7 days. To determine the stability of heat resistance, freeze-dried bacteria at each storage time were subjected to thermal inactivation in wheat flour as described previously to determine D_{80}-values. The stability of survival was based on three time-independent replications and stability of heat resistance was conducted in duplicate.

2.7. Data analysis

Thermal inactivation data were fitted using the first-order kinetic model (Peleg, 2006) by the following equation:

$$\mathrm{Log}\left(\frac{N}{N_0}\right) = -\frac{t}{D}$$

Where N is the population count (CFU/g) at time t (min), N_0 is the initial population (CFU/g), t is the heating time (min), and D (min) is the time required to kill 1 log cycle of bacteria at a pre-determined temperature (80 °C). The left side of the equation refers to the population reduction of bacteria in logarithmic form.

Thermal inactivation curves were obtained by plotting population reduction of bacteria versus heating time, and the goodness of fit was evaluated using the R^2 coefficient. D_{80}-values of bacteria were calculated by the slope of the corresponding inactivation curve. The differences of population counts before and after inoculation and D_{80}-values of bacteria were compared for statistical significance via one-way analysis of variance and subsequent least significant difference pairwise comparison test with $\alpha = 0.05$ (Peleg, 2006).

3. Results

3.1. Properties of freeze-dried inoculum

The properties of freeze-dried inoculum were evaluated by their residual mass (visual observation), microstructure under SEM, and survivor counts. After freeze-drying, bacteria were dehydrated into a powder form (a final $a_w < 0.04$ measured at room temperature) and with abundant residual mass remaining in the microtubes (Fig. 1). The addition of protectants (BD and SMD) visually increased the residual mass of the final bacterial products by increasing the solids content of the suspension. After freeze-drying, DE became a fine bright white powder, BD formed porous sandy colored flakes, and SMD formed a powdery residue of bright but light yellow color. *E. faecium,* as a gram-positive bacterium, has a thick peptidoglycan layer along with teichoic and lipoteichoic acids. SEM images of *E. faecium* cells in liquid suspension showed spherical shaped cocci occurring in pairs or chains (Fig. 2 A, B). After freeze-drying, *E. faecium* (DE) cells formed slimy

layers and tended to clump with secreted aggregation substances (Fig. 2 C, D). *E. faecium* cells freeze-dried with protectants were completely embedded with protective materials, and typical isolated cells were hard to identify (Fig. 2 E–H). Freeze-drying buffer yielded clumps of different shapes with a smooth surface (Fig. 2 E, F), while skim milk revealed a porous fractured structure (Fig. 2 G, H).

3.2. Thermal inactivation kinetics of freeze-dried inoculum in wheat flour at 80 °C

The background mesophilic flora count for uninoculated wheat flour was 2.20 ± 0.45 log CFU/g, which was low and wouldn't interfere with the inactivation counts (Villa-Rojas, 2015). Fig. 3 shows the population levels of freeze-dried bacteria after inoculation, and after 4–5 days of conditioning. After freeze-drying, the population of *E. faecium* was reduced from 11.55 ± 1.30 log CFU/g (LE) to 9.82 ± 0.98 log CFU/g (DE). Less but not significant population reduction was observed for BD (10.17 ± 1.14 log CFU/g) and SMD (10.12 ± 1.01 log CFU/g). After inoculation, the population concentrations (log CFU/g) of different inoculants in wheat flour were 8.67 ± 0.12 (LE), 9.03 ± 0.09 (DE), 9.07 ± 0.43 (BD), and 9.18 ± 0.21 (SMD) (Fig. 3, after inoculation). Inoculated flours were uniformly distributed with standard deviations in population counts of less than 0.5 log CFU/g. Based on statistical analysis, no significant difference of population concentration was observed for freeze-dried inoculum. The populations of LE declined < 1 log CFU/g, while freeze-dried inoculants declined by less than 0.5 log CFU/g after conditioning (Fig. 3). No significant difference in population was observed for all inocula after conditioning.

To examine the heat resistance of freeze-dried inoculum, D-values at 80 °C (D_{80}) of DE, BD, and SMD in wheat flour were compared with that of LE. The thermal inactivation curves of inoculants showed a linear trend; the first-order kinetic model gave good correlation coefficients (> 0.96) for all inoculants (Fig. 4). The logarithmic decline of survivors during the CUT interval was higher for DE (1.71 log CFU/g) compared to those of LE (0.60 log CFU/g), BD (0.25 log CFU/g), and SMD (0.69 log CFU/g). Freeze-dried inoculants with protectants showed higher thermal resistance ($D_{80,\ BD}$: 6.67 ± 0.22 min, $D_{80,\ SMD}$: 5.92 ± 0.39 min) compared to that of DE ($D_{80,\ DE}$ 4.90 ± 0.30 min), but there was no significant difference (P < 0.05) compared with that of LE ($D_{80,\ LE}$ 6.48 ± 1.00 min) (Table 1).

3.3. Survival and heat resistance stability of freeze-dried inoculum

The population density of freeze-dried inoculum (vacuum packed) remained stable throughout 35 days storage at −20 °C (DE: 10.02–10.58 log CFU/g, BD: 10.11–10.60 log CFU/g, and SMD: 10.32–10.52 log CFU/g) (Fig. 5). However, the stability of heat resistance (as reflected by D_{80}-values) varied depending on samples (Fig. 6). D_{80}-values of DE were the lowest among the three inoculants with a slight decrease during the first period of storage (0–17 day), but gradually increased with longer storage time (after 17 days). D_{80}-values of BD were consistent during the first 9 days, but decreased continuously after that. SMD inoculum showed the best stability in heat resistance during storage with a D_{80}-value variation of less than 0.55 min, which was lower than that of DE (1.84 min) or BD (2.76 min). Based on the consistency in both population counts and heat resistance during storage, SMD was considered the best dried inoculum among the groups, and can be chosen as a potential substitute for liquid-inoculation methods in thermal inactivation studies for low-moisture foods.

4. Discussion

The addition of protectants helped produce a larger residual mass of freeze-dried bacteria, with higher survival counts after freeze-drying than the control (freeze-dried *E. faecium* without protectant). In our study, freeze-drying buffer was chosen because of its commercial availability and evolved formulation from Reagent 18, a proven solution for microbial freeze-drying (American Type Culture Collection 2012). Skim milk solution was chosen because of its good performance as a bacterial cell preservation during freeze-drying processing (Dhewa et al., 2014; Hubalek, 2003; Lu, Huang, Yang, Lv, & Lu, 2017; Nahr et al., 2015). Freeze-drying buffer and skim milk formed protective layers that aggregated into larger bacterial clusters and facilitated better survival probability of cells with higher survival rates. A similar protective effect of skim milk on *Lactobacillus plantarum* JH287 during freeze-drying has been observed (Lee et al., 2016; Wang, Yu, Xu, Aguilar, & Wei, 2016). Many studies have also found that protectants enable better survival of bacterial cells by maintaining cellular structures and preventing denaturing or loss in function (Billi & Potts, 2002; Potts, 1994).

Freeze-drying buffer and skim milk-protected *E. faecium* had similar thermal inactivation compared with liquid *E. faecium* suspension, and exhibited higher heat resistance than the control. In contrast, liquid *E. faecium* suspension (the precursor to freeze-dried inoculum) showed significantly higher (P < 0.05) heat resistance than the control. Studies reported that desiccated stress caused by low-moisture may activate the thermal stress network of vegetative cells resulting in an increased heat resistance (Gruzdev, Pinto, & Sela, 2011). In this case, liquid *E. faecium* suspension seemed more adapted to the low-moisture environment since it was conditioned for 4–5 days before thermal inactivation, while the control was immediately treated with heat after inoculation. The reasons for thermally treating freeze-dried bacteria without lengthy equilibration are: 1) freeze-dried inoculum can quickly condition with the water activity of surrounding foods in seconds (Syamaladevi et al., 2016); 2) freeze-dried inoculum is sensitive to oxygen and moisture, and extended exposure time during equilibration may cause unexpected cell death (Keith, 1913); 3) accelerating thermal inactivation procedures by omitting equilibration time is practical and time efficient for commercial applications. The decreased heat resistance in the control group was probably due to negative impacts of cellular sub-lethal injury caused by freeze-drying (Ray, Jezeski, & Busta, 1971a; Ray et al., 1971b). The increased D_{80}-values of freeze-drying buffer- and skim milk-protected *E. faecium* may be the result of proteins and/or sugar components present in protectants. Previous studies observed that heat resistance of microorganisms was enhanced in carbohydrate- or protein-rich systems (Gibson, 1973; Goepfert, Iskander, & Amundson, 1970; Moats, Dabbah, & Edwards, 1971).

An inoculum with a long shelf-life, and consistent population and heat resistance is more ideal for research/validation studies. In this investigation, freeze-drying buffer- and skim milk-prepared freeze-dried bacteria showed consistency in survival during storage. Survival stability of dry inoculum was also found in ten cultures inoculated with chalk (six months) and dry-inoculated *Salmonella* on almonds and walnuts (98 days) (Blessington, Theofel, & Harris, 2013; Hoffmans & Fung, 1992). The heat resistance of dry inoculum of *E. faecium* in talc stored for at least 30 days at room temperature was also consistent (Enache et al., 2015). However, freeze-dried inoculum performed differently in terms of heat resistance over time in the present study. Few research investigations have reported on the influence of protectants on heat resistance of freeze-dried bacteria over time, the varied stability of freeze-drying buffer- and skim milk-protected *E. faecium* may be caused by differences in the composition of protectants. Moreover, environmental factors (such as exposure to oxygen, moisture, or temperature) influence the viability and thermal inactivation of freeze-dried bacteria; the proper protectant selection and storage conditions are essential for producing stable freeze-dried inoculum for practical usage (Louis et al., 1994; Peiren et al., 2015).

E. faecium NRRL B-2354 has been identified as a surrogate for *S.* Enteritidis PT 30 and is widely used in validation processes for almonds (Bingol et al., 2011), dairy products (Annous & Kozempel, 1998), juice (Piyasena, McKellar, & Bartlett, 2003), and meat (Ma, Kornacki, Zhang,

Lin, & Doyle, 2007). The dry-inoculation of a low-moisture food using freeze-dried *E. faecium* developed in this study provides a useful option for evaluating the effectiveness of thermal inactivation. Compared to liquid inoculum, freeze-dried bacteria made into a fine powder would be easier to mix with fine particulate foods (Hoffmans & Fung, 1992). But freeze-dried inoculum may not be suitable for other solid foods, such as chocolate or dry dog food kibbles, since it may not be easily imbedded in or attached to the surface of those products. Compared with dry inoculum using dried carriers, freeze-dried inoculum can be prepared and preserved with a high concentration and stability. Such a stable, easier, and faster method is imperative for academic/industrial application in designing standard protocols for inactivation studies especially for low-moisture foods. More systematic collection of data associated with other low-moisture foods should be conducted and specific limitations should also be addressed before applying freeze-dried inoculum to commercial processes.

Abbreviations

LE: liquid suspension of *E. faecium* NRRL B-2354; DE: freeze-dried *E. faecium* NRRL B-2354 without protectant; BD: freeze-drying buffer protected freeze-dried *E. faecium* NRRL B-2354; SMD: skim milk protected freeze-dried *E. faecium* NRRL B-2354; CUT: come up time.

Competing interests

The authors declare that they have no conflict of interest.

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