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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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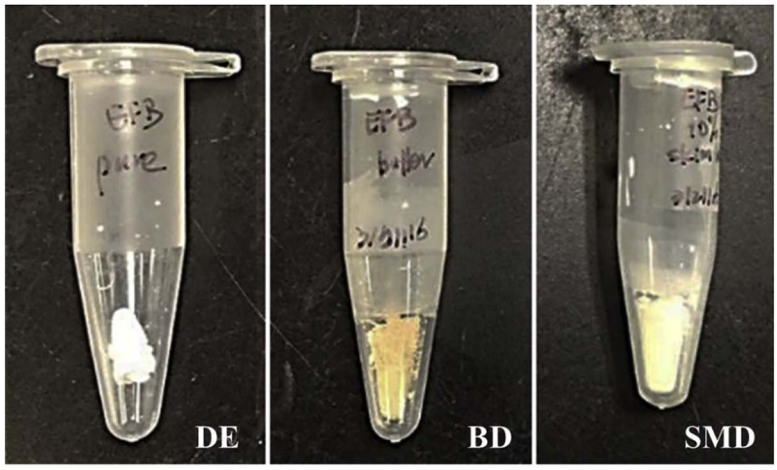

Fig. 1. *E. faecium* NRRL B-2354 after freeze-drying process. DE: freeze-dried *E. faecium* without protectant, BD: freeze-drying buffer protected freeze-dried *E. faecium*, SMD: skim milk protected freeze-dried *E. faecium*. The residual mass in DE was from 250 μL of bacterial suspension before freeze-drying. 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