Short communication: Effects of vacuum freeze-drying on inactivation of *Cronobacter sakazakii* ATCC29544 in liquid media with different initial inoculum levels

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ABSTRACT

Vacuum freeze-drying is an important food-processing technology for valid retention of nutrients and bioactive compounds. *Cronobacter sakazakii* has been reported to be associated with severe infections in neonates through consumption of contaminated powdered infant formula. In this study, effects of vacuum freeze-drying treatment for 12, 24, and 36 h on inactivation of *C. sakazakii* with different initial inoculum levels in sterile water, tryptic soy broth (TSB), skim milk, and whole milk were determined. Results indicated that the lethality rate of *C. sakazakii* in each sample increased with the extension of vacuum freeze-drying time. With initial inoculum levels of 10^2 and 10^3 cfu/mL, the survival of *C. sakazakii* in different liquid media was significantly affected by vacuum freeze-drying for 12, 24, and 36 h. In addition, the lethality rates of *C. sakazakii* in whole milk, skim milk, and TSB was significantly reduced compared with those in sterile water. Furthermore, whole milk showed the strongest protective role for *C. sakazakii* cells, followed by skim milk and TSB medium. Using the scanning electron microscope, the intracellular damage and obvious distortion of *C. sakazakii* cells were observed after vacuum freeze-drying for 24 and 36 h compared with the untreated sample, and the injured cells increased with the extension of vacuum-drying time. We concluded that inactivation of vacuum freeze-drying on *C. sakazakii* cells is related to the food matrix, and a combination with other methods for inactivating *C. sakazakii* is required for ensuring microbial safety of powdered infant formula.

Key words: *Cronobacter sakazakii*, vacuum freeze-drying, liquid media

Short Communication

Cronobacter sakazakii is considered an opportunistic food-borne pathogen involved in meningitis, septicemia, and necrotizing enterocolitis in neonates (Ye et al., 2014). Epidemic surveys showed that powdered infant formula might play an important role in *Cronobacter* infections as the main transmission vehicle (Biering et al., 1989; van Acker et al., 2001). However, the true source of *Cronobacter* spp. in powdered infant formula is largely unknown (Ye et al., 2014).

In China, *Cronobacter* isolates in dry food samples have been described (Li et al., 2014; Xu et al., 2014a,b), and *C. sakazakii* is the most frequently isolated species within the genus in powdered formula (Xu et al., 2014). Presence of *Cronobacter* strains in dry food samples showed unusual abilities to survive dry stress (Breeuwer et al., 2003; Osaili and Forsythe, 2009; Kuo et al., 2013). Consequently, effects of drying technologies on inactivation of *C. sakazakii* is of importance for understanding the risk of contamination in powdered infant formula.

Freeze-drying is considered a promising technology for preservation of microorganisms (Miyamoto-Shinohara et al., 2006). Freeze-drying also can inactivate food-borne pathogens through dehydration of food samples. Vacuum freeze-drying is a complex process consisting of a freezing component as well as a vacuum-drying component (Claussen et al., 2007; Shi et al., 2012). However, scant research has focused on the effects of the vacuum freeze-drying on inactivation of *C. sakazakii* in liquid media.

The present study aimed to determine the effects of vacuum freeze-drying for 12, 24, and 36 h on survival of *C. sakazakii* in whole milk, skim milk, tryptic soy

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brother (TSB), and sterile water at different initial levels (10^3 , 10^2 , and 10^1 cfu/mL). Furthermore, scanning electron microscopy was used to evaluate the morphological changes of *C. sakazakii* cell after vacuum freeze-drying treatment.

Cronobacter sakazakii ATCC 29544 was inoculated into TSB (Luqiao, Beijing, China) for incubation at 37°C for 16 to 18 h. *Cronobacter sakazakii* suspensions at different concentrations were prepared through 10-fold dilutions, and then *C. sakazakii* was inoculated under sterile conditions, at 10^3 , 10^2 , and 10^1 cfu/mL into different liquid media (whole milk, skim milk, TSB, and water). Thereafter, the liquid media was frozen at -30°C for 12 h, and was further transferred to a vacuum freeze-dryer (FD-1-50, Boyikang, Beijing, China) for 12, 24, and 36 h. The samples frozen at -30°C for 12 h were used as control samples.

After vacuum freeze-drying, whole milk, skim milk, TSB, and water samples (10 mL) were diluted using sterile 0.85% NaCl (90 mL). The appropriate dilution was then spread onto the tryptic soy agar (Luqiao) and left to sit for 24 h at 37°C. Plates containing 30 to 300 colonies were selected to determine the survival of *C. sakazakii* in different liquid media after vacuum freeze-drying for 12, 24, and 36 h. In addition, the lethality rate was regarded as the decreasing percentage of colonies between the control (untreated) and treated samples. Each experiment was replicated 3 times.

To determine morphological changes of *C. sakazakii* cells, TSB samples mixed with *C. sakazakii* cells after vacuum freeze-drying for 12, 24, and 36 h were reconstituted. The treated and untreated samples were then centrifuged at $3,000 \times g$ for 10 min at room temperature and the pellets were further washed using 0.1 mol/L phosphate buffer (pH = 7.4). Thereafter, subsequent steps were completed as described in Wang et al. (2013). Finally, the morphological changes of *C. sakazakii* after vacuum freeze-drying were determined through scanning electron microscopy (Hitachi S4700, Tokyo, Japan).

From Figure 1A, the survival of *C. sakazakii* in different liquid media at 10^3 cfu/mL was significantly affected ($P < 0.05$) by vacuum freeze-drying for 12, 24, and 36 h compared with untreated samples. With extension of treatment time, the number of colonies significantly decreased. Furthermore, compared with sterile water, whole milk had the highest protective role in inhibiting inactivation of *C. sakazakii*, followed by skim milk and TSB medium. A similar result was observed in liquid media with 10^2 cfu/mL, as shown in Figure 1B. The survival of *C. sakazakii* in sterile water with 10^1 cfu/mL was affected significantly by vacuum freeze-drying for 12, 24, and 36 h, whereas no significant changes of *C. sakazakii* in whole milk, skim milk, and TSB were

Figure 1. Treatment of vacuum freeze-drying on *Cronobacter sakazakii* cells in different media with different concentrations. TSB = tryptic soy broth. Data are means $\pm$ SD; asterisks represent a difference compared with time zero at * $P < 0.05$ and ** $P < 0.01$.

Figure 2. Scanning electron micrographs of *Cronobacter sakazakii* cells after vacuum freeze-drying treatment for 0, 12, 24, and 36 h. Arrows indicate intracellular damage and distortion of *C. sakazakii* after vacuum freeze-drying compared with the untreated sample.

observed (Figure 1C). Initial concentrations of cells for freeze-drying were related to the protective medium and growth phase in order to obtain the highest recovery (Costa et al., 2000; Corcoran et al., 2004). Survival rate of 10 species (*Saccharomyces cerevisiae*, *Brevibacterium flavum*, *Brevibacterium lactofermentum*, *Corynebacterium acetoacidophilum*, *Corynebacterium glutamicum*, *Streptococcus mutans*, *Escherichia coli*, *Pseudomonas putida*, *Serratia marcescens*, and *Alcaligenes faecalis*) after freeze-drying and long-term storage was evaluated. The results indicated that survival rates of yeast as well as gram-positive and gram-negative organisms were 10, 80, and 50%, respectively, after 10 yr of storage under a vacuum at 5°C. In addition, the survival rates of positive bacteria were higher than those of negative cells (Miyamoto-Shinohara et al., 2000). Those authors further inferred that the excellent survival of each species after freeze-drying might be attributed to the high level of desiccation and to sealing under vacuum. The survival of microorganisms such as *Escherichia* (n = 1), *Pseudomonas* (n = 4), and *Lactobacillus* (n = 1) in dry conditions without a vacuum for 4 yr was not detected (Antheunisse et al., 1981). *Pseudomonas* sealed in ampoules at 6.7 Pa and stored in the dark at room

temperature for 5 to 6 yr did not survive (Antheunisse, 1973), whereas *Pseudomonas* was able to be recovered after 10 yr of storage under a vacuum at 5°C (Miyamoto-Shinohara et al., 2006). In Table 1, the mean lethality rates of *C. sakazakii* in whole milk with different initial inoculum levels after vacuum freeze-drying for 12, 24, and 36 h were 0 to 8.22, 12.5 to 59.69, and 25 to 83.36% respectively; similarly, mean lethality rates were 6.82 to 13.61, 20.45 to 63.15, and 29.54 to 84.65% in skim milk, 10.36 to 21.62, 20.45 to 68.73, and 29.54 to 85.16% in TSB, and 91.28 to 100, 99.38 to 100, and 99.82 to 100% in sterile water, respectively. The differences of lethality rates ($P < 0.01$) were significant between nutrients in the food matrix (whole milk, skim milk, and TSB) and oligotrophic medium (sterile water), but survival rates of *C. sakazakii* after vacuum freeze-drying in whole milk and skim milk were not significantly different ($P > 0.05$). Results indicated that the food matrix could play a protective role in inhibiting inactivation of *C. sakazakii* for vacuum freeze-drying. *Pantoea agglomerans* showed the highest viability in 10% nonfat skim milk after freeze-drying (Costa et al., 2000). In another paper, trehalose was found to be an effective protectant for freeze-dried and vacuum-dried samples (Conrad et

Table 1. Lethality rates of vacuum freeze-drying treatment on *Cronobacter sakazakii* in different liquid media

Initial levels (cfu/mL)	Treatment time (h)	Lethality rates of <i>C. sakazakii</i> (%; mean $\pm$ SD)			
		Whole milk	Skim milk	Tryptic soy broth	Sterile water
10 ³	12	5.02 $\pm$ 3.89 ^{A,a}	9.54 $\pm$ 2.25 ^{AB,a}	21.62 $\pm$ 3.09 ^{B,b}	94.97 $\pm$ 0.80 ^{C,c}
	24	59.69 $\pm$ 3.80 ^{A,a}	63.15 $\pm$ 3.08 ^{AB,ab}	68.73 $\pm$ 1.90 ^{B,b}	99.38 $\pm$ 0.10 ^{C,c}
	36	83.36 $\pm$ 1.60 ^{A,a}	84.56 $\pm$ 0.98 ^{AB,ab}	85.16 $\pm$ 3.39 ^{B,b}	99.82 $\pm$ 0.04 ^{C,c}
10 ²	12	8.22 $\pm$ 2.55 ^{A,a}	13.61 $\pm$ 6.11 ^{AB,ab}	18.24 $\pm$ 1.73 ^{B,b}	91.28 $\pm$ 2.13 ^{C,c}
	24	45.04 $\pm$ 7.99 ^{A,a}	47.78 $\pm$ 6.94 ^{A,a}	54.72 $\pm$ 4.71 ^{A,a}	100
	36	72.80 $\pm$ 2.55 ^{A,a}	71.94 $\pm$ 0.69 ^{A,a}	75.16 $\pm$ 2.04 ^{A,a}	100
10 ¹	12	0 $\pm$ 6.0 ^{A,a}	6.82 $\pm$ 19.32 ^{A,a}	11.36 $\pm$ 7.91 ^{A,a}	100
	24	12.5 $\pm$ 6.25 ^{A,a}	20.45 $\pm$ 12.5 ^{A,a}	20.45 $\pm$ 10.23 ^{A,a}	100
	36	25 $\pm$ 5.0 ^{A,a}	29.54 $\pm$ 14.76 ^{A,a}	29.54 $\pm$ 12.5 ^{A,a}	100

^{a-c}Different lowercase superscripts in the same row indicate significant differences ($P < 0.05$).

^{A-C}Different uppercase superscripts in the same row indicate significant differences ($P < 0.01$).

al., 2000). Carvalho et al. (2004) reported that fructose and sorbitol could provide better protection than carbohydrate glucose in *Lactobacillus*. Using a mixture of skim milk and carbohydrate sugars, recoveries of *Candida sake* cell ranged from 45 to 85% (Abadias et al., 2001). Consequently, effects of the food matrix on the viability of microorganisms may be species-dependent.

In Figure 2, scanning electron microscopy images show intracellular damage and distortion of *C. sakazakii* after vacuum freeze-drying for 24 and 36 h compared with the untreated sample. Furthermore, the injured cells and distortion of cells increased with the extension of vacuum freeze-drying time. During vacuum freeze-drying, the formation of ice crystals might have contributed to injury of cells and subsequent release of cellular contents, resulting in irreversible damages (Orndorff and MacKenzie, 1973). Although the freeze-drying is an alternative method for preservation of microorganisms, freezing is also detrimental to the viability of bacterial cells after drying (Morgan et al., 2006).

In summary, as a potential food-engineering technology for the production of powdered milk, vacuum freeze-drying can cause loss of viability of bacterial cells but cannot thoroughly inactivate *C. sakazakii* within 36 h in the food matrix. Scanning electron microscopy further indicated that the inactivation of *C. sakazakii* after vacuum freeze-drying was involved in injured cell membranes, further release of cellular contents, and irreversible damage of partial cells. The findings here contribute to the understanding and evaluation of the risks or hazards in production of powdered food using vacuum freeze-drying technology.

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