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ORIGINAL RESEARCH

Viability of *Bifidobacterium Pseudocatenulatum* G4 after Spray-Drying and Freeze-Drying

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Abstract: Viability of *Bifidobacterium pseudocatenulatum* G4 following spray-drying and freeze-drying in skim milk was evaluated. After spray-drying, the strain experienced over 99% loss in viability regardless of the air outlet temperature (75 and 85 °C) and the heat-adaptation temperature (45 and 65 °C, 30 min). The use of heat-adaptation treatment to improve the thermotolerance of this strain was ineffective. On the other hand, the strain showed a superior survival at 71.65%–82.07% after freeze-drying. Viable populations of 9.319–9.487 $\log_{10}$ cfu/g were obtained when different combinations of skim milk and sugar were used as cryoprotectant. However, the addition of sugars did not result in increased survival during the freeze-drying process. Hence, 10% (w/v) skim milk alone is recommended as a suitable protectant and drying medium for this strain. The residual moisture content obtained was 4.41% ± 0.44%.

Keywords: bifidobacteria, freeze-drying, cryoprotectant, spray-drying, survival, viability

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Introduction

Bifidobacteria, a predominant genus in the stools of breast-fed infants has come a long way.[1] It is widely used as probiotic dietary adjuncts with health benefiting properties. Bifidobacteria are extensively incorporated into yogurts, cultured milk drinks, cheese or as dietary supplements in the form of dried product.[2]

For probiotic to exert health benefits to the host, viability of the strain is of utmost importance.[3,4] The recommended minimum count is of 10^6 live organisms per g of food at the point of consumption.[3,4] The conventional technique of using stock cultures with multiple subculturing steps for the preparation of bulk starters are time consuming with higher risk of contamination. With advances in cell mass production technology, the development of concentrated starter cultures in freeze-dried and spray-dried forms for direct product vat inoculation has proved to be a better alternative.[2]

Dehydration is a common practice to preserve biological materials so that they are stable in the long run.[5] Among the many cell preservation methods, spray-drying is widely used in the industry because it is economical, especially on a large-scale.[6] Despite the fact that spray-drying is more cost-effective, many microorganisms cannot tolerate the drying process due to the high heat involved. Some of the many factors affecting survival during spray-drying are the strain, growth phase, protective medium used, outlet temperature of spray-drier and pre-adaptation treatment of the culture.[7–10] Heat-adaptation, also known as heat-shock treatment, has been reported to increase thermotolerance of bacterial cells during spray-drying.[8,11] Thus, the application of heat-adaptation technique prior to spray-drying has gained popularity.

Freeze-drying, the sublimation of ice from frozen preparations is a popular method for the preservation of lactic acid bacteria (LAB).[5] Although freeze-drying is commonly used, microorganisms are also susceptible to various stresses (such as freezing and osmotic stress) and lead to cell injury (such as membrane and cell wall damage. Therefore, cryoprotectants are commonly added to minimize cell damage. Skim milk is a popular drying medium and protectant because it contains protein that prevents cellular injury[12] and facilitates rehydration by creating a porous structure in the freeze-dried powder.[13] Many other compounds have also been tested to improve survival of LAB during freeze-drying such as disaccharides, polyols, vitamins and proteins.[12]

This research was conducted to identify a suitable preservation method for *B. pseudocatenulatum* G4 which shows potential probiotic activities.[14] In order to improve bacterial survival, different milk-based cryoprotectant and heat-adaptation treatments at various air outlet temperatures were carried out during freeze-drying and spray-drying.

Materials and Methods

Microorganism and preparation of culture

B. pseudocatenulatum G4 was obtained from Probiotic Laboratory, Faculty of Food Science and Technology, Universiti Putra Malaysia. The strain was previously isolated from breast-fed infants.[15,16] The strain was stored at –20 °C in Trypticase PhytoneYeast extract broth:glycerol (20:80) as stock culture. For experiment, the purified strain was cultured in TPY broth (Scharlau-Chemie, Spain) at 37 °C overnight, under anaerobic condition. Cells in the early stage of stationary phase were harvested by centrifugation (Sigma Sartorious 3–18 K with rotor 12171) at 3000 rpm for 15 min at 4 °C.

Heat-adaptation of culture prior spray-drying

Harvested cell pellets were resuspended in glass bottles (SCHOTT DURAN®, Mainz, Germany) containing 1000 ml volume of 10% (w/v) sterile skim milk (NZMP medium heat skim milk powder, New Zealand). The samples in glass bottles were transferred to temperature controlled water baths set at 45 °C and 60 °C. The bottles were submerged such that the water level was at the neck of the bottles and were held here for 30 min. Samples which were not subjected to heat adaptation were considered as control.

Spray-Drying at different air outlet temperature

The samples were spray-dried in a laboratory-scale spray-drier (Anhydro, Denmark), with centrifugal atomization. Hot drying air was introduced into the chamber at a constant air inlet temperature of 160 °C. This hot air was mixed continuously with the mist of atomized liquid followed by instantaneous evaporation. The flow rate of the feed solution was regulated to obtain air outlet temperature of 75 ± 2 °C and 85 ± 2 °C. Spray dried powders were collected

and were mixed thoroughly with a sterile spatula. The dried powders were stored in tightly sealed sterile glass bottles.

Addition of different protective medium prior freeze-drying

Harvested cells were resuspended in sterile protective medium consisting of sterile skim milk (10% or 18%, w/v) supplemented with different sugar solutions (glucose 5%, lactose 5% or sucrose 5%, w/v). Distilled water was used as control. The protective media were sterilized by autoclaving at 121 °C for 15 min. The treatment combinations are described in Table 1.

Freeze-drying condition

The cultures in different cryoprotectants were first placed in different sterile metal trays and evenly spread to obtain approximately 1 cm thickness. The cultures were then frozen at −80 °C and subsequently lyophilized in a freeze-drier (Christ® Epsilon 1–80, Germany) at 35 °C for 24 h at a pressure of 1.65 mbar. During the final drying stage, the pressure was dropped to 0.01 mbar to ensure that all the moisture and the gas were completely removed. After freeze-drying, the dried powders were evenly mixed using a sterile spatula and finally stored in tightly sealed sterile glass bottles.

Rehydration and enumeration of *B. pseudocatenulatum* G4

1 g of dried powder was resuspended in 9 ml skim milk (10%, w/v) to rehydrate the samples. The samples were maintained at room temperature for 10 min as reported earlier by Valdez et al.[17] Serial dilutions of each sample were made using 0.1% (w/v) sterile peptone water and plated on TPY agar in duplicate. This was followed by anaerobic incubation at 37 °C for 48 h. Viable cells were enumerated before drying (initial count) and immediately after the drying process.

Percent survival was calculated according to method of Hyun and Shin:[10]

$$\text{Percent Survival (\%)} = N/N_o \times 100 \quad (1)$$

$$N_o = \text{Viable count (c.f.u. mL}^{-1}) \times (\text{Volume before FD or SD/Weight after FD or SD}) \quad (2)$$

where N represents the viable count after FD or SD (cfug^{-1})

N_o represents the initial viable count before FD or SD (cfug^{-1})

FD represents freeze-drying

SD represents spray-drying.

Table 1. Different protective medium used prior freeze drying of *B. pseudocatenulatum* G4.

Treatment	Protective medium
1	Distilled water
2	10% skim milk
3	18% skim milk
4	10% skim milk + 5% glucose
5	10% skim milk + 5% sucrose
6	10% skim milk + 5% lactose
7	18% skim milk + 5% glucose
8	18% skim milk + 5% sucrose
9	18% skim milk + 5% lactose

Moisture content analysis

The moisture content of spray-or freeze-dried powders was determined by oven drying at 102 °C.[18] Moisture content was analyzed by determination of the difference in weight before and after drying, expressed as a percentage of the initial powder weight.

Statistical analysis

Statistical analysis was performed using MINITAB version 13 (Minitab Inc., United States). The experiments were repeated twice. Significance differences between values are at $P < 0.05$ levels using T-test.

Results and Discussion

Spray-drying

Table 2 shows the viability, percentage survival and moisture content of spray-dried *B. pseudocatenulatum* G4 treated at different spray-drier air outlet temperature and heat-adaptation temperature. The initial viable cell count prior to spray-drying was 10^9 cfu/g. After spray-drying, 2.454–3.346 log reduction in population was recorded, with surviving bacterial population of 5.797–6.871 $\log_{10}$ cfu/g. The high mortality rates were also reflected in terms of the percentage survival, where all treatments yielded low survival of 0.05%–0.36%. Hence, the heat-adaptation treatment applied at both outlet temperatures was considered as ineffective.

The survival of organisms during spray drying depends on their heat resistance and the applied temperature.[19] Therefore, spray-drier air outlet temperature is an important factor that affects the viability of the organism. Various range of air outlet temperatures have been used by researchers: 95–105 °C;[20] 85–90 °C[21] 75–95 °C[6] and 70–100 °C.[22] Since temperature above 90 °C resulted in cell death due to protein denaturation and destruction of DNA,[19] outlet temperature of 75 ± 2 °C and 85 ± 2 °C were used in this study.

At outlet temperature of 75 ± 2 °C, the spray-dried powder had residual moisture content ranging between 8.62%–9.27%. Significantly lower moisture content between 5.25%–6.31% were obtained when it was spray-dried at higher outlet temperature (85 ± 2 °C). This was in accordance with study by Ananta et al where the residual moisture content was inversely proportional to outlet temperature.[23] Since the moisture content of dried culture is a crucial factor in determining the stability and shelf life of the culture, it is important to obtain a proper ratio between the outlet temperature and the residual moisture content.[21]

Bacteria constantly face adverse environmental changes and they have to adapt to the new conditions. It has been hypothesized that bacteria respond to these changes through metabolic reprogramming that may subsequently increase their resistance towards environmental stress.[22] Their resistance develops upon exposure to sublethal stress, which induces greater tolerance against the same stress later on.[24] The enhanced thermotolerance of many organisms have been linked to the production of heat shock protein (Hsps) upon exposure to elevated temperature condition.[25] Heat shock proteins act as molecular chaperones in protecting cells against heat damage by binding to cellular proteins in such a way that maintains their original conformation and reduce denaturation.[9] This has led to the intensified research on heat-adaptation to increase survival of bacteria cells during spray-drying.

The use of heat-adaptation technique had been successfully applied on spray-drying of *B. longum* BB 536, in which high viability of 8.300 $\log_{10}$ cfug^{-1} was obtained when the culture was heat-adapted at 45 °C for 30 min at outlet temperature 75 °C.[11] In a similar work on *Lactobacillus paracasei,* a 300-fold greater thermotolerance was reported when the bacteria was heat-adapted at 52 °C for 15 min.[8] In this study, the application of heat-adaptation was not successful, as the final viable count was very low with over 99% loss in viability. Therefore, it was concluded that *B. pseudocatenulatum* G4 was particularly sensitive to the high heat involved during spray-drying and the application of heat shock treatment to enhance thermotolerance was not suitable for this strain.

Freeze-drying

In comparison, freeze-dried cells exhibited higher viability than spray-dried cells regardless of the protective media used (Table 3). The initial viable cell count prior freeze-drying was 10^9 cfu/g. After freeze-drying, viability remained in the same log unit, meeting the recommended minimum count of 10^6 live organism per g of probiotic product.[3,4] However, for the control treatment, substantial drop in viable count was observed when distilled water was used as the protectant.

Table 2. Viability, percent survival and moisture content of spray-dried *B. pseudocatenulatum* G4.

Treatment	Outlet temperature (°C)	Heat-adaptation temperature (°C)	Viability before spray-drying ($\log_{10}$ c.f.u. g^{-1})	Viability after spray-drying ($\log_{10}$ c.f.u. g^{-1})	Survival (%)	Moisture content (%)
A	75 ± 2	–	9.38 ± 0.02^{ad}	6.79 ± 0.01^{ac}	0.26 ± 0.01^{ac}	9.17 ± 0.24^{a}
B	85 ± 2	–	9.14 ± 0.03^{b}	5.80 ± 0.01^{b}	0.05 ± 0.01^{b}	6.24 ± 0.30^{b}
C	75 ± 2	45	9.42 ± 0.02^{a}	6.84 ± 0.04^{a}	0.26 ± 0.04^{ac}	9.27 ± 0.49^{a}
D	85 ± 2	45	9.29 ± 0.08^{abd}	6.84 ± 0.01^{a}	0.36 ± 0.08^{a}	5.25 ± 0.35^{b}
E	75 ± 2	60	9.60 ± 0.01^{c}	6.87 ± 0.04^{a}	0.19 ± 0.02^{cb}	8.62 ± 0.51^{a}
F	85 ± 2	60	9.24 ± 0.05^{bd}	6.74 ± 0.01^{c}	0.32 ± 0.04^{ac}	6.31 ± 0.27^{b}

Results are shown as mean ± S.D. of 2 experiments.
Values in the same column with different superscripts differ significantly ($P < 0.05$).

In terms of percentage survival, high survival rates of 71.65%–82.07% were obtained when different combinations of skim milk and sugar were used as protectant. However, the values were not significantly different from one another. Therefore, the addition of sugar did not contribute to higher cellular protection against freeze-drying. Hence, we recommend the use of 10% skim milk as the cryoprotectant for this strain. Generally, the freeze-drying performance of this strain was considered satisfactory as high percentage survival was obtained after the drying-process. In comparison, lower percent survival of freeze-dried bifidobacteria (43.1%–51.9%) had been reported by Wang et al in fermented soymilk.[26] In another study on *Lactobacillus salivarius*, 83%–85% percent survival was obtained when the organism was dried in skim milk protectant with trehalose and sucrose.[14]

In terms of moisture content, freeze-dried powder had lower percentage values ranging between 3.72%–5.10% (Table 3). Although microorganisms generally show higher survival rate under low moisture condition, excessive drying may be harmful to the survival of organisms. Extreme drying causes removal of three types of cellular water; free, intermediate and structured water, which risks damage to cellular proteins.[27] A minimum amount of water must remain in the dried cultures for a higher rate of survival, which in turn depends on the drying medium and freeze-drying condition. Zayed and Roos[28] reported that the optimum moisture content for freeze-dried *L. salivarius* ranged from 2.80%–5.60%. Moisture above 5.60% resulted in loss of cell viability. Therefore, due to the lack of information on the optimum moisture content for preservation of bifidobacteria, the range of 2.80%–5.60% recommended moisture content for *L. salivarius* was opted as reference. Hence, 10% skim milk, which yielded moisture content of 4.41%, is placed within the acceptable range.

It is recognized that organisms preserved by drying techniques undergo various cellular stresses that may lead to cell injury or even cell death. The rate of survival is strain-specific and depends on the drying method used. Our finding was in accordance with study by Wang et al in which they reported that *B. longum* and *B. infantis* that were subjected to freeze-drying exhibited higher survival rate when compared to spray-drying.[26] Johnson and Etzel also made a similar observation, where freeze-dried *Lactobacillus helveticus* CNRZ-32 survived better than spray-dried cells.[6] This shows that *Lactobacillus helveticus* CNRZ-32 cannot tolerate the high temperature involved during spray-drying but endure better the freeze-drying process. It must be noted that freeze-drying may cause losses in cell viability due to factors such as formation of intracellular ice crystal, membrane damage caused by high concentrations of internal solutes and denaturation of sensitive proteins.[29] According to Fonseca et al[30] with higher cell surface area, the risk of membrane damage is higher due to the formation of extracellular ice crystal.

Table 3. Viability, percent survival and moisture content of freeze-dried *B. pseudocatenulatum* G4.

Treatment	Protective medium	Viability before freeze-drying (log_{10} c.f.u. g^{-1})	Viability after freeze-drying (log_{10} c.f.u. g^{-1})	Survival (%)	Moisture content (%)
1	Distilled water	9.50 ± 0.01^{ab}	8.587 ± 0.16^{a}	12.68 ± 4.67^{a}	0.87 ± 0.05^{a}
2	10% skim milk	9.45 ± 0.02^{a}	9.364 ± 0.02^{b}	82.07 ± 1.54^{b}	4.41 ± 0.44^{bcde}
3	18% skim milk	9.51 ± 0.01^{ab}	9.415 ± 0.01^{b}	80.35 ± 0.74^{b}	3.72 ± 0.11^{b}
4	10% skim milk + 5% glucose	9.43 ± 0.03^{a}	9.335 ± 0.01^{b}	80.06 ± 3.06^{b}	4.20 ± 0.03^{bce}
5	10% skim milk + 5% sucrose	9.56 ± 0.03^{b}	9.445 ± 0.04^{b}	77.06 ± 1.73^{b}	4.64 ± 0.18^{cde}
6	10% skim milk + 5% lactose	9.56 ± 0.03^{b}	9.472 ± 0.02^{b}	81.08 ± 0.78^{b}	5.10 ± 0.16^{d}
7	18% skim milk + 5% glucose	9.55 ± 0.03^{bc}	9.430 ± 0.01^{b}	76.41 ± 6.89^{b}	4.06 ± 0.23^{bc}
8	18% skim milk + 5% sucrose	9.46 ± 0.02^{ac}	9.319 ± 0.004^{b}	71.65 ± 3.67^{b}	4.92 ± 0.26^{de}
9	18% skim milk + 5% lactose	9.59 ± 0.01^{b}	9.487 ± 0.06^{b}	80.08 ± 9.97^{b}	4.21 ± 0.13^{bc}

Results are shown as mean ± S.D. of 2 experiments.
Values in the same column with different superscripts differ significantly ($P < 0.05$).

Therefore, small spherical cells are more resistant to freeze-drying than rods, as in the case of bifidobacteria and lactobacilli. Hence, various protectants have been tested to improve the stability of microorganisms during freeze-drying.[12]

The addition of sugars as cryoprotectant for the inhibition of free radical production is highly recommended by many researchers.[31,32] This is because sugars are food-grade, easily available and cost effective. Sugars increase the stability of cellular protein by forming hydrogen bonds with them, thus reducing the risk of exposure to the environment.[12] They should be present on both sides of the membrane to be able to maintain the cell protein structure in the dry state.[33] Meanwhile, Carvalho et al demonstrated that the cryoprotective effect of sugars in the drying medium depends on the sugar previously found in the growth medium.[34] However in this case, no significant differences in percent survival were observed when different combinations of skim milk and sugars were used. This may be due to the presence of naturally occurring protein and lactose in milk, which offer the necessary protection to this strain. Therefore, the inclusion of additional sugars in skim milk did not result in increased survival during freeze-drying. King and Su[35] also reported a similar finding, where no significant difference in the protective effect of 10% nonfat dry milk solids added with sucrose and other compounds on freeze-dried *Lactobacillus acidophilus.* Hence, the effectiveness of a cryoprotectant is strain-dependent.

Conclusions

In order to be successfully incorporated into functional food applications, the culture should be able to withstand the harsh conditions during cell preservation. This study demonstrated that *B. pseudocatenulatum* G4 was more susceptible to the high heat involved in the spray-drying process, experiencing over 99% loss in viability. According to the superior survival of this strain after lyophilization (~10^9 cfu/g). Thus freeze-drying was recommended as a better method for preservation of this organism using skim milk as the protective medium. Addition of different sugars did not increase the survival during the freeze-drying process. The use of 10% skim milk as the protective medium during freeze-drying seemed to be optimum. Using the above-mentioned condition, satisfactory survival rate and residual moisture content of 4.41% ± 0.44% was obtained.

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Disclosures

The authors report no conflicts of interest.

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