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Optimization of Spray-Drying Workflow as a Method for Preparing Concentrated Cultures of *Lactobacillus fermentum*

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Abstract: This research is to optimize preparation of *L. fermentum* and its survival rate by method of microcapsulation using a spray drying technique. After spray drying, the effect of protective agents and spray drying characteristics on the survival rate were studied after which the survival change over storage time was determined. Researchers concluded that the number of surviving encapsulated *L. fermentum* was 2.20×10^9 CFU g^{-1} after spray drying when the wall materials were 20% skim milk power and the initial concentration of *L. fermentum* (2.60×10^9 CFU mL^{-1}). When the air temperature at the inlet was 130°C, the survival rate was not affected by the outlet air temperature and the bacteria survival rate was improved from 14.63-29.13% by increasing drying air volume. It was found that all kinds of carbohydrates have a beneficial effect on the survival rate and the bacteria survival rate reached 60.44% when lactose was added. After 30 days of storage at 4 and 20°C, the survival rates were >20% and <5%, respectively. The results suggest the possibility of achieving a good formulation system for *L. fermentum* with a high number of viable cells to be used for the industrial development of probiotics for livestock.

Key words: *Lactobacillus fermentum*, spray drying, protective agent, characteristic, storage

INTRODUCTION

Probiotic bacteria are defined as a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance (Fuller, 1989) or as live microorganisms that when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2006). Probiotics can be used both for animals as well as for human beings (Fuller, 1989). Recently, some lactic acid bacteria have been tested as probiotics for livestock. For example, Pollmann *et al.* (1980) observed that administration of *L. acidophilus* was an effective way to promote BW gain and feed conversion in piglets.

Freezing is to date, the best process known to dry bacteria while keeping their viability (Chavez and Ledeboer, 2007). However, commercial scale freeze dryers are expensive and production output is low. Moreover, subzero storage temperatures and bulkiness increase the final cost of the frozen cultures (To and Etzel, 1997a). So, freeze drying costs have hindered their use in large-scale processes. Spray drying is the most popular and widely studied alternative to freeze drying because it is cost effective, readily available, easy to operate and can be implemented for large-scale throughputs. It has been estimated that the cost of spray drying is six times lower per kilogram of water removed than the cost of freeze drying (Knorr, 1998). Successful spray drying of Lactobacilli has previously been reported for a number of different strains including *L. paracasei* (Gardiner *et al.*, 2000; Desmond *et al.*, 2002), *Lactobacillus curvatus* (Mauriello *et al.*, 1999), *L. acidophilus* (Prajapati *et al.*, 1987) and *L. rhamnosus* (Corcoran *et al.*, 2004). Meanwhile, spray-drying has been shown to be as suitable as freeze-drying for preserving LAB strains during a 2 month storage period (Zamora *et al.*, 2006).

It is now a days acknowledged that the survival rate of the culture during spray drying and subsequent storage depends upon a number of factors including the species and strain of the culture, the drying conditions and the use of protective agents (Teixeira *et al.*, 1995a; Bielecka and Majkowska, 2000; Gardiner *et al.*, 2000; Desmond *et al.*, 2001; Conrad *et al.*, 2000). Therefore, the objective of this research is to identify protective carrier materials and to optimize spray drying conditions in order to enhance the survival of probiotics during drying. Another objective is to evaluate the storage stability by determining the percentage of cells remaining viable after a 30 days storage period at 20 and 4°C.

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MATERIALS AND METHODS

***Lactobacillus fermentum*:** The *L. fermentum* used in the current study was isolated from the gastrointestinal mucosa of healthy weaning piglets and identified by the Institute of Microbiology, Chinese Academy of Sciences (Beijing, China).

Culturing and inoculation of *Lactobacillus fermentum*: A pre-culture of *Lactobacillus fermentum* in 10 mL of MRS broth was incubated first for 18 h at 37°C then incubated in 3 L of an industry culture (Gao *et al*., 2009) at 37°C until the stationary phase was reached (around 8 h). The cultured broth was centrifuged at 4000 g for 10 min. The resulting cells were resuspended in Maximum Recovery Diluent (MRD, 8.5 g L^{-1} NaCl and 1 g L^{-1} peptone) for washing, centrifuged again and resuspended in carrier dispersion before spray drying.

Determination of the rate of protective agent to bacteria: Two carrier concentrations, 10 and 20% skim milk powder (skim milk powder, a commercial product from Yili company, China) were used and two bacteria concentrations, 2.6×10^9 and 5.15×10^9 CFU mL^{-1} were used by resuspending in 3.0 L of 20% SMP dispersion on original volume or concentrated one time by resuspending in 1.5 L of 20% SMP dispersion. Before mixing bacteria, all the carrier materials were pasteurised (90°C for 30 min). After adding bacteria into the dispersion, all samples were homogenized at 600 r sec^{-1} for 15~30 min with a mixer (HLJ100, Jiangsu, China) for a homogenous blend. The process parameters are as follows: inlet temperature 170°C; outlet temperature 70°C; feed rate 40 mL min^{-1}, drying air volume 275 m^3 h^{-1}. Experiments were carried out in triplicate.

Optimization of spray drying characteristics: Samples were spray-dried by using a LPG-8 Spray Dryer (Changzhou Le Er equipment Limited company, Jiangsu, China). Two inlet-air temperatures (170 and 130°C), two outlet-air temperatures (76 and 70°C, 67 and 57°C) and two drying air volumes (275 and 367 m^3 h^{-1}) were used. When the inlet temperature was 170°C, the outlet temperature was adjusted at 76 and 70°C by manually adjusting the feed flow rate.

Similarly when the inlet-air temperature was 130°C, the outlet temperature was adjusted at 67 and 57°C by manually adjusting the feed flow rate. The study of different spray drying configurations was done using SMP as a carrier material. Powder was collected in a single cyclone separator. Experiments were carried out in triplicate.

Optimization of spray drying protective agents: Four kinds of carbohydrates were selected. *Lactobacillus fermentum* were suspended in water dispersions with the following carrier materials: 20% SMP+5% AG (Arabic gum from acacia tree, Sigma-Aldrich), 20% SMP+5% Suc (Sucrose, analytical grade), 20% SMP+5% Tre (D-trehalose dehydrate, analytical grade) and 20% SMP + 5% Lac (Lactose, analytical grade). Before mixing bacteria, all the carrier materials were pasteurised (90°C for 30 min). After adding bacteria into the dispersion, all samples were homogenized at 600 r sec^{-1} for 15~30 min with a mixer (HLJ100, Jiangsu, China) for a homogenous blend. The study of different carrier materials was done with the process parameters only being as follows: inlet temperature 130°C; outlet temperature 67°C; feed rate 40 mL min^{-1} and drying air volume 367 m^3 h^{-1}. Experiments were carried out in triplicate.

Enumeration of viable bacteria: Viable bacteria were counted in carrier solutions prior to spray drying (from 1 mL) and also in 1 g of powder after spray drying and during storage. After dehydration, spray-dried samples were rehydrated using MRD. All samples were homogenized for 1 min with a Vortex mixer and incubated at 25°C for 1 h for complete rehydration.

Samples were serially diluted in duplicate from 10^{-1} to 10^{-7} in MRD and duplicate samples were removed from 10^{-5}, 10^{-6}, 10^{-7} in MRD and pour-plated on MRS agar. A viable cells count was determined after incubation for 48 h at 37°C. The resulting viability of encapsulated *L. fermentum* was expressed as a colony forming unit, CFU g^{-1} which equaled the colony number in multiples of 10^5, 10^6 or 10^7 and in percentage of survivors with respect to initial counts before drying.

Determination of moisture content of dried samples: The moisture content of spray dried powders was determined by oven drying at 102°C which involved determination of the difference in weight before and after drying and was expressed as a percentage of the initial powder weight.

Storage test: Dried samples were stored for 30 days at 4 and at 25°C in polyethylene sample bags. Storage experiments were performed in two replicate trials. The stability of dehydrated cultures was established from the percentage of cells remaining viable during storage (N/N_0). In this case, N refers to the bacterial count at the end of a particular storage period while N_0 refers to the bacterial count at the beginning of storage.

Statistical analysis: The data were analysed with the statistical software SAS. Analyses of Variance (ANOVA)

using the General Linear Model (GLM) procedure and t-test were applied to the results. The significance level for all tests was a = 0.05.

RESULTS AND DISCUSSION

The effect of different carrier and cell concentrations on the spray drying of *Lactobacillus fermentum*: During spray drying, cellular injury can occur from dehydration and exposure to high temperatures in the atomizer and from droplet drying (Fu and Etzel, 1995) thus the cells simultaneously experience both thermal and dehydration stresses. One of the most susceptible sites in bacterial cells is the cytoplasmic membrane as indicated by the increased sensitivity of the sub-lethally injured bacteria to NaCl (Teixeira *et al*., 1997). Loss of viability can be related to the destruction of the cell components including the cell membrane, cell wall and DNA (Teixeira *et al*., 1995b). A number of researchers have improved the performance of probiotics in food systems by the addition of protectants to the media prior to spray drying. A protectant of 20% Non-Fat Skimmed Milk (NFSM) has been used to protect bacteria cells during spray drying in many researches (Ananta *et al*., 2005; Chavez and Ledeboer, 2007).

Generally, the higher ratio of the carrier to the bacteria cell provided a protective effect on the cells and resulted in less damage to the cells during spray drying. Other than protecting the cell better, a higher concentration of carrier reduces the energy cost due to a lower quantity of water needing to be evaporated. Reddy *et al*. (2009) reported that there was no significant difference in viability of *L. plantarum* and *L. salivarius* at all the three concentrations of cells used which is not consistent with the results (Table 1). The results in the present study showed that the number of survival encapsulated *L. fermentum* was improved from 2.20×10^{9}-3.25×10^{9} CFU g^{-1} after spray drying with increasing the bacteria concentration from 2.60×10^{9} to 5.15×10^{9} CFU mL^{-1} however, the survival rate was reduced from 16.92-12.62%. Meanwhile, the results in the present study show that the number of surviving encapsulated *L. fermentum* were reduced from 2.20×10^{9} to 1.68×10^{9} CFU g^{-1} after spray drying when reducing the protective agent concentration from 20-10% and the survival was reduced from 16.92-6.20%.

Table 1: The effect of different carrier and cell concentrations on the spray drying of *L. fermentum*

Concentration of protective agent (g mL^{-1}%)	Bacteria (CFU mL^{-1})	Live bacteria (CFU g^{-1})	Survival rate (%)
20	2.60×10^{9}	2.20×10^{9}	16.92^{a}
10	2.71×10^{9}	1.68×10^{9}	6.20^{b}
20	5.15×10^{9}	3.25×10^{9}	12.62^{a}

Means within a column with different superscripts differ ($p<0.05$)

The effects of processing parameters on the survival rate during spray drying: Survival rate of lactic acid bacteria after spray drying was highly dependent on the type of bacteria (To and Etzel, 1997b). For example, it is apparent that *L. paracasei* NFBC 338 survived better than *L. salivarius* during spray drying (Gardiner *et al*., 2000) so optimization of spray drying conditions needs improvement for each type of bacterium. Although, the time of exposure to heat is very short during spray drying, inlet and outlet temperatures are obviously the most important spray drying characteristics causing bacterial damage and mortality. Gardiner *et al*. (2000) reported that the survival rate for *L. paracasei* NFBC 338 during spray drying with 20% SMP as the protective agent ranged from 97% at an outlet temperature of 70-75°C to about 50% at 85-90°C. The percent surviving decreased linearly with increasing outlet-air temperature and was halved for every 5°C increase in the outlet-air temperature using a 4.5 L drying chamber (To and Etzel, 1997b). The effects of spray drying characteristics on the survival rate after spray drying are shown in Table 2. The results in the present study showed that for given inlet temperatures of 170°C, the number of surviving encapsulated *L. fermentum* after spray drying improved from 1.13×10^{9} to 2.20×10^{9} CFU g^{-1} with reducing the outlet temperature from 76-70°C by increasing feed flow and the survival improved from 8.80-16.92%. However, the results in the present study showed that for a given inlet temperature of 130°C, the survival rate of *L. fermentum* after spray drying with the outlet temperature of 67 and 57°C was 14.63 and 17.73% with corresponding viable counts of 1.88×10^{9} and 2.28×10^{9} CFU g^{-1}.

The average retention time of drying particles was changed by adjusting the drying air volume of the spray dryer used in this study. The results in the present study show that shortening retention time by increasing drying air volume did not influence the survival rate of *L. fermentum* after spray drying with an inlet temperature of 170°C. This result was unexpected because a short retention time would shorten the period during which

Table 2: The effect of spray drying characteristics on the survival rate after spray drying

Inlet T (°C)	Feed rate (mL min^{-1})	Drying air volume (m^3 h^{-1})	Outlet T (°C)	Live bacteria (CFU g^{-1})	Survival rate (%)	Moisture (%)
170	30	275	76±1	1.13×10^{9}	8.80^{B}	4.02^{C}
170	60	367	76±1	1.08×10^{9}	8.41^{B}	5.81^{A}
170	40	275	70±1	2.20×10^{9}	16.92^{A}	4.81^{B}
130	20	275	67±1	1.88×10^{9}	14.63^{b}	4.11^{c}
130	40	367	67±1	3.75×10^{9}	29.13^{a}	5.57^{a}
130	40	275	57±1	2.28×10^{9}	17.73^{b}	4.97^{b}

Means within a column with different superscripts differ, $p<0.05$; Means within a column with different superscripts differ ($p<0.05$)

high temperature damage to the cells would occur. Yet, the results showed that shortening the retention time by increasing drying air volume improved the number of surviving dried *L. fermentum* from 1.88×10^9 to 3.75×10^9 CFU g^{-1} after spray drying with an inlet temperature of 130°C and the survival rate of *L. fermentum* improved from 14.63-29.13%, correspondingly.

The effects of incorporating different kinds of carbohydrate in the carrier medium on the survival rate during spray drying: Sugars are known to protect dehydrated biomaterials and it has been suggested that they act as water substitutes and replace water molecules around proteins and polar residues of membrane phospholipids (Rokka and Rantamaki, 2010). Sugars are also able to form hydrogen bonds with the proteins when water is removed and prevent protein denaturation (Ananta *et al*., 2005).

Protective roles of trehalose and sucrose on both membranes and proteins in intact bacteria during drying were also reported (Leslie *et al*., 1995). A number of studies have shown that the incorporation of thermoprotectants such as trehalose (Conrad *et al*., 2000), non-fat milk solids (Selmer-Olsen *et al*., 1999) and gum acacia (Desmond *et al*., 2002) can improve culture viability during drying and storage. The effects of the protective agents on the survival rate after spray drying are shown in Table 3. The results of the present study showed that incorporation of any kind of carbohydrate into a milk based medium prior to spray drying provoked an increase in viability after spray drying. These advantages are attributed to factors such as the presence of carbohydrates (lactose, trehalose, sucrose and gum acacia) that would act to replace water molecule bonds, avoiding protein denaturation and through the formation of vitreous structures and microencapsulated molecules that surround the bacteria, generate a protecting effect during to the dehydration process.

The effects of storage temperature on the survival rate during storage: Typical survival rate in the spray-drying process is in the range of 70~85%. Although, this survival rate may be acceptable, the prolonged storage stability of the product is often low (Rokka and Rantamaki, 2010).

Table 3: The effect of the protective agents on the survival rate after spray drying

Protective agents	Outlet T (°C)	Live bacteria (CFU g^{-1})	Survival rate (%)	Moisture (%)
20% SMP+5% Lac	67±1	6.23×10^9	60.44^a	5.54
20% SMP+5% Tre	67±1	5.57×10^9	54.13^b	5.61
20% SMP+5% AG	67±1	6.00×10^9	58.25^{ab}	5.71
20% SMP+5% Suc	67±1	5.10×10^9	48.51^c	5.65

Means within a column with different superscripts differ ($p<0.05$)

This is associated with stress that is induced by temperature changes and drying, a combination of which tend to damage cell membranes and proteins (Teixeira *et al*., 1995a, b; Gardiner *et al*., 2000; Ross *et al*., 2005).

Previous studies have also shown that temperature is critical for microbial survival during storage and higher survival rates have been obtained at lower storage temperatures (Teixeira *et al*., 1995b; Gardiner *et al*., 2000). In this study, powders of the probiotic cultures produced by spray-drying at a constant air inlet and outlet temperatures of 130 and 67±1°C and with 20% SMP+5% Lac as carrier materials were stored at 4°C or 25°C. The effects of storage temperature on the survival rate during storage are shown in Fig. 1.

The results showed that the survival of spray drying power stored at 4°C decreased slowly compared to 20°C. The residual survival rate remained >50% after 7 days storage at 4°C and the residual survival rate remained >20% after 30 days storage at 4°C. However, the residual survival rate decreased <5% after 7 days of storage at 20°C. This result agrees with the majority of the research performed with microorganisms and principally demonstrated that an increase in storage temperature provokes a decrease in bacterial viability.

Moisture content has an influence on storage stability. Gardiner *et al*. (2000) reported that spray drying bacteria power with moisture content <4% showed stability when stored at 4°C and Chavez and Ledeboer (2007) suggested that moisture content <5% are safe conditions for long-term storage. The present study showed that the survival of spray-dried *Lactobacillus fermentum* with the moisture content at about 5.54% were acceptable when stored in the presence of oxygen and moisture at 4°C for 30 days. However, Riveros *et al*. (2009) reported that dried *Lactobacillus acidophilus* product with content <10% maintains its viability for

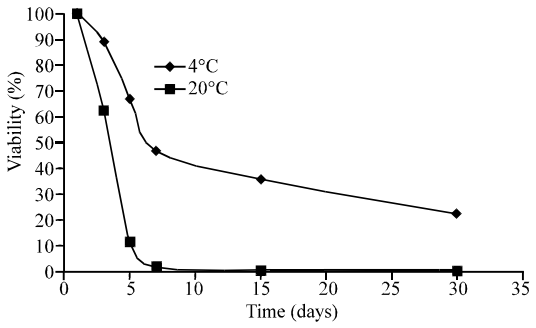

Fig. 1: Viability of dried *L. fermentum* during storage at 4 or 20°C (with 20% SMP+5% Lac as carrier material)

2 months when stored at 4°C although, its viability drops 1 order of magnitude when stored at 25°C for 1 month. Storage stability also improved when the spray-dried samples were maintained at 4°C in anaerobic jars containing desiccant (To and Etzel, 1997b). Therefore, compared to the moisture content of the dried product, the storage temperature may be a more important factor on storage stability.

CONCLUSION

When spray drying characteristics were optimized, their effect on bacterial survival might influence each other. Incorporation of a kind of carbohydrate into a SMP-based medium provoked an increase in viability after spray drying. The survival of spray drying power stored at 4°C decreased slowly compared to 20°C and the residual survival rate remained >20% after 30 days storage at 4°C.

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