Spray drying as a method for preparing concentrated cultures of *Lactobacillus bulgaricus*

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P. TEIXEIRA, H. CASTRO AND R. KIRBY. 1995. Spray drying and freeze drying as methods for concentration of *Lactobacillus bulgaricus* starter cultures were compared in terms of viability, lag phase until onset of pH decrease and total acid production. For the experimental conditions used, no significant differences were detected between the methods.

The effect of spray drying on the cell membrane of *Lactobacillus bulgaricus* was studied. Five separate methods were used to study the theory that spray drying causes cell membrane damage; three relating to leakage of intracellular components from the cell into the surrounding environment (260 and 280 nm absorbing materials, potassium ions and proteins); and two relating to increased cell permeability (increased sensitivity to NaCl and increased permeability to *o*-nitrophenyl-β-D-galactopyranoside (ONPG). Partial loss of some cytoplasmic material from the damaged cells was observed. The dried cells also became sensitive to NaCl and permeable to ONPG. Heat shock increased the survival of exponential cells as compared to controls but did not result in normal levels found with unshocked stationary phase cells. Heat shock had no effect on stationary phase cells. Different rehydration methods and media were investigated: slow rehydration increased survival.

INTRODUCTION

Several investigators have considered the possibility of drying large quantities of bacterial cultures, with the idea of using it as a method to replace the usual liquid bulk starter in the production of fermented dairy products (Rogers 1914; Mamaeva 1956; Porubcan and Sellars 1975; Gillil-and 1976). For this purpose spray drying would be preferable to freeze drying because of the lower cost (Foster 1962). The spray drying of micro-organisms dates back to 1914 to the work of Rogers (1914) with dried milk cultures of lactic acid bacteria. Since then much research has been performed on the use of spray drying of bacteria in order to overcome the work involved in maintaining liquid stock cultures and to facilitate the dispatch of dried cultures by post without any loss in their activity (Prajapati *et al.* 1987). Although this process proved promising, it has not been developed commercially for use with lactic acid bacteria. Reasons for this are mainly low survival rates during drying of the cultures, low stability under storage and the difficulty in rehydrating the product. Each of the above areas has received much attention in the literature. For reviews in spray drying conditions see Espina and Packard (1979) and Kim and Bhowmik (1990), low stability under storage see Mamaeva (1956) and Porubcan and Sellars (1975), and rehydration conditions see Richardson (1960). There is little information available regarding the mechanism(s) of cell inactivation.

The objective of this study was to compare the survival of *Lactobacillus bulgaricus* during spray drying and freeze drying. Damage during spray drying is unlikely to be repaired during the subsequent phase of storage or rehydration. This damage and the inability of the cells to repair it would be expected to prejudice the survival of the bacteria during these phases, the totality of damage caused being the likely cause of the increased lag phase commonly reported for these bacteria.

In order to fully examine the survival of *Lact. bulgaricus* during spray drying and to try to define optimal process parameters in relation to the bacteria themselves the following studies were performed:

- Membrane damage: has been reported as a site of damage common to different type of stresses: freeze drying (Morichi *et al.* 1967), freeze and vacuum drying (Brennan *et al.* 1986) and heat treatments (Hurst and Hughes 1975; Hoover and Gray 1977).

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• Growth phase: it is well known that cells at exponential phase of growth are more sensitive to treatments such as heating (Griffiths and Haigh 1973; Hurst *et al.* 1974; Teixeira *et al.* 1994).
• Heat shock: it has been reported that heat shock can increase the resistance of different bacteria to heat treatments (Hogg 1989; Whitaker and Batt 1991; Teixeira *et al.* 1994).
• Rehydration: the rehydration of dried bacterial cells is a critical process which sometimes affects the cell viability (Morichi *et al.* 1967); however, the rehydration methods actually used by earlier workers have not always been described. The reasons for using determined conditions by some workers have, in general, not been supported by comparisons with alternative procedures.

MATERIALS AND METHODS

Organism

Lactobacillus delbrueckii subsp. *bulgaricus* NCFB 1489 was used. Cultures were maintained as in Teixeira *et al.* (1994).

Media

All media were prepared using deionized water and sterilized by autoclaving for 15 min at 121°C.

Diluent: AM buffer was prepared by mixing 0·01 mol l^{-1} K_2HPO_4 and 0·01 mol l^{-1} KH_2PO_4 (Merck), both dissolved in a solution of 0·15 mol l^{-1} NaCl (Merck), adjusted to pH 7 $\pm$ 0·1 and sterilized.

The complex media used for batch growth of *Lact. bulgaricus* was MRS broth prepared and sterilized according to the manufacturer's instructions.

Preparation of cultures

MRS broth was inoculated from the MRSA slopes and incubated for 24 h at 42°C. This broth was then used to inoculate a second MRS broth (1% v/v). The cultures were incubated at 42°C for 6 h (exponential phase cells) or 16 h (stationary phase cells) in a shaken water bath. Cells were harvested by centrifugation at 10 000 *g* for 10 min at 4°C and washed with sterile AM buffer.

Heat shock treatment

Cell pellet was resuspended in 50 ml of skim milk (11% solids) previously equilibrated at 50°C (test cells) and 50 ml at 37°C (control cells) and left for 30 min under agitation in a shaken water bath. Heat-shocked cell suspension and control cells were then spray dried.

Spray drying

Forty per cent maltodextrin (Glucidex 12, Roquette, Lille, France) in deionized water or skim milk (11% solids) was inoculated with *Lact. bulgaricus* cultures. This suspension was incubated for 30 min at 37°C to allow for cell adaptation, constantly agitated and then spray dried. Samples were spray dried in a laboratory scale spray drier (Niro Atomizer). Moisture in spray droplets produced by the atomization of the feed liquid into a spray by the use of a vaned wheel rotating at high speed was evaporated in a vertical, cocurrent drying chamber (0·8 m diam. and 0·6 m height). The air temperatures at the inlet and outlet of the drier were adjusted respectively at 200°C and 80°C. Powder was collected in a single cyclone separator. Each sample of spray dried bacteria was rehydrated to original volume with water, phosphate buffer, MRS and skim milk at 4°, 20°, 37° and 50°C. The cells were allowed to rehydrate for 2 min under vigorous shaking (shaking method) or by soaking for 30 min (soaking method).

Lyophilization

Skim milk (11% solids) was inoculated with *Lact. bulgaricus* cultures previously grown to stationary phase in MRS broth. Samples were frozen at − 80°C in a deep freezer and then desiccated under vacuum (50 mTorr) in a Christ Alpha 1-4 freeze drier. After the lyophilization step, dried samples were rehydrated to the original volume with deionized water at 20°C by the shaking method.

Enumeration of micro-organisms

Survivors before and after treatment were enumerated on MRSA and/or MRSA + NaCl(0·8%) by the drop count technique (Miles and Misra 1938). Plates were examined after incubation at 37°C for 48 h.

pH evolution in skim milk

The post-stress recovery was evaluated by following changes in the pH of the growth medium. Changes in the pH were determined using standard pH electrodes linked to a data acquisition and automated data logging system as previously described by Lievense (1991).

β-Galactosidase activity

Cells were grown in 2% lactose MRS broth (prepared from individual ingredients and replacing glucose with 2% lactose) for 16 h at 42°C in a shaking water bath. The cells were harvested, washed, spray dried and rehydrated using the procedure described above.

Normal and dried-rehydrated samples (original concentration about 1×10^9 cells ml^{-1}) were centrifuged at 5000 *g* for 10 min at 4°C. The supernatant fluids (after filter sterilization) and cell pellets (resuspended in 20 ml of AM buffer) were used to measure β-galactosidase activity by the method described by Citti *et al.* (1965).

The specific activity of β-galactosidase is expressed as μmol of *o*-nitrophenol liberated from *o*-nitrophenyl-β-D-galactopyranoside (ONPG) per mg of cell dry weight per min.

Total activity of each sample was determined by permeabilizing the cells with toluene (Citti *et al.* 1965) before the β-galactosidase assay. Total activity was considered as 100%.

Loss of 260 nm and 280 nm absorbing materials from the cells

Normal and spray dried-rehydrated samples (original concentration about 1×10^9 cells ml^{-1}) were centrifuged at 5000 *g* for 10 min at 4°C and the supernatant fluids were used to read the optical density at 260 and 280 nm, after filter sterilization, using deionized water as a blank, in a Shimadzu UV-265 spectrophotometer. Supernatant fluids were diluted with deionized water to give an optical density reading below 1·0.

Protein determination

The cells were harvested, washed, spray dried and rehydrated using the procedure described above. Normal and dried-rehydrated samples (original concentration about 1×10^9 cells ml^{-1}) were centrifuged at 5000 *g* for 10 min at 4°C and the supernatant fluids, after filter sterilization, were used to determine protein concentration (mg ml^{-1}) by the method of Lowry *et al.* (1951).

Potassium determination

The cells were harvested, washed with deionized water, spray dried and rehydrated using the procedure described above. Normal and dried-rehydrated samples (original concentration about 1×10^9 cells ml^{-1}) were centrifuged at 5000 *g* for 10 min at 4°C and potassium concentration in the supernatant fluids, after filter sterilization, was determined using a Jenway PFP7 flame photometer. Blanks consisted of deionized water.

Statistical analysis

Assuming a normal distribution, data were analysed using a Student's *t*-test. Significance was declared at $P < 0{\cdot}05$ (Box *et al.* 1978).

RESULTS

The evolution of pH in skim milk following spray drying and freeze drying was determined. No significant differences were obtained between the two samples either in terms of survival (Table 1) or in terms of rate of acid production (Figs 1 and 2). Results did, however, show that both treatments affected survival, acid production and lag phase as compared to controls.

Results for the determination of membrane permeability all demonstrated that it had increased. The first three methods used were based on leakage of intracellular constituents into the surrounding environment while the last two methods were based on the altered permeability of the cytoplasmic membrane to NaCl and ONPG (Table 2).

The effect of age on the survival of *Lact. bulgaricus* during spray drying is illustrated in Table 3. Cells at stationary phase are significantly ($P < 0{\cdot}05$) more resistant than cells at exponential phase.

Table 1 Survival of *Lactobacillus bulgaricus* at stationary phase in the production of dried cell concentrates by spray drying and lyophilization in milk

Spray dried cells log cfu ml^{-1}		Lyophilized cells log cfu ml^{-1}	
B	A	B	A
8·74	8·16	9·83	9·21
	Log reduction		
0·58		0·62	

B, Before drying; A, after drying.
Rehydration was performed by the shaking method.

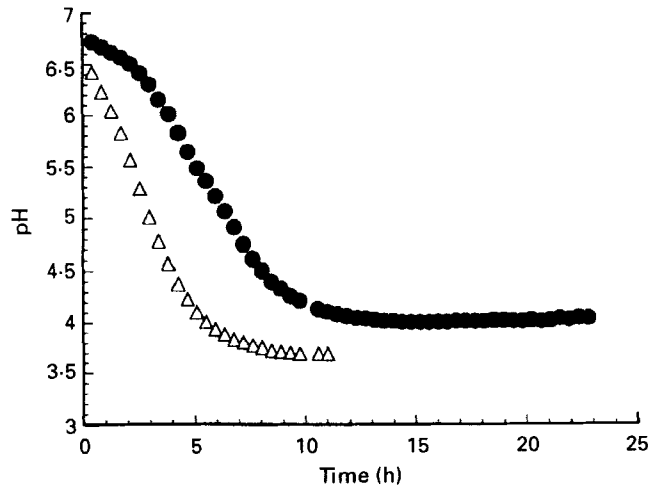

Fig. 1 pH evolution in milk. △, Before spray drying; ●, after spray drying

Table 2 Evidence of membrane damage as indicated by developed sensitivity to NaCl, increased β-galactosidase activity and leakage of cellular materials in spray dried *Lactobacillus bulgaricus* at stationary phase having maltodextrin as suspending liquid

Manifestations*	Normal cell	Spray-dried cell
Absorbancy of supernatant fluid at:		
260 nm	[a]2·876	[b]3·832
280 nm	[a]1·942	[b]2·280
Protein (mg ml^{-1}) in supernatant fluid	[a]0·382	[a]0·460
K (ppm) in supernatant fluid	[a]20·2	[b]45·13
% β-galactosidase activity in:		
Cell suspension	[a]33·5	[b]83·5
Cell supernatant fluid	[a]0	[b]1·04
Permeabilized cell	100	—
% Sensitivity to NaCl	[a]21·11	[b]72·50

* Average of four experiments.
[a,b] Means with different superscripts in the same row differ ($P < 0·05$).

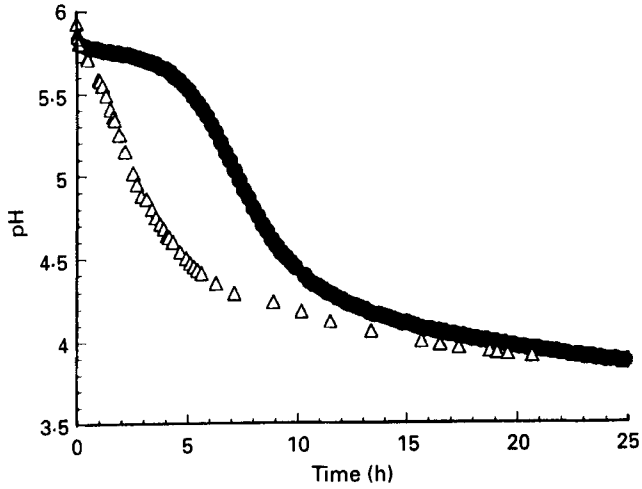

Fig. 2 pH evolution in milk. △, Before lyophilization; ●, after lyophilization

The effect of heat shock on the resistance to spray drying was evaluated as a function of the age of the cells (Table 4). When cells are heat shocked at stationary phase there is no increase in the resistance to the subsequent drying. Heat

Table 3 Effects of age on survival of *Lactobacillus bulgaricus* during spray drying in milk

	Log cfu ml^{-1}*	
Phase of growth	Before drying	After drying
Stationary	[a]8·87	[a]7·45
Exponential	[a]8·54	[b]5·80

* Average of three experiments.
[a,b] Means with different superscripts in the same column differ ($P < 0·05$).
Rehydration was performed by the shaking method.

Table 4 Effect of heat shock on the survival of *Lactobacillus bulgaricus* at different stages of growth during spray drying in milk

	Heat shocked cells log cfu ml^{-1}		Control cells* log cfu ml^{-1}	
Phase of growth	B	A	B	A
Stationary	8·72	7·14	8·59	7·37
Exponential	8·26	[a]6·05	8·53	[a]5·87

[a] Means with superscripts in the same row differ ($P < 0·05$).
B, Before drying; A, after drying.
* Average of three experiments.
Rehydration was performed by the shaking method.

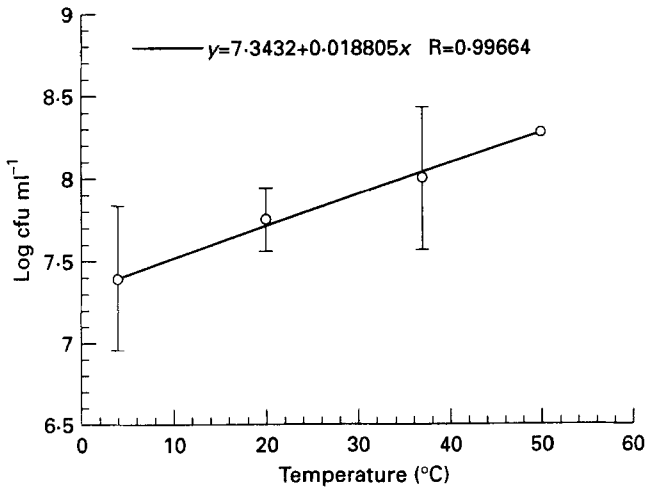

Fig. 3 Influence of rehydration temperature (shaking method) on the recovery of spray dried stationary phase cells. Vertical bars indicate overall standard errors of the mean

Table 5 Influence of different rehydration media, at 20°C, on the survival of spray dried *Lactobacillus bulgaricus* at stationary phase having milk as carrier suspension

Rehydration media	Skim milk	Phosphate buffer	MRS broth	H_2O
Log cfu ml^{-1}*	7·40	7·21	7·00	7·30

* Average of three experiments.
Rehydration was performed by the shaking method.

shocked exponential phase cells significantly ($P < 0·05$) increase their resistance to the process as compared to control cells.

The influence of rehydration temperature on the recovery of dried cells was investigated. Survival rate increases linearly with temperature between 4°C and 50°C (Fig. 3).

Recovery of *Lact. bulgaricus* was investigated as a function of the rehydration media. No significant differences ($P < 0·05$) were detected when skim milk, MRS broth, deionized water or phosphate buffer were used (Table 5).

Two different methods of rehydration were compared. Higher recovery was obtained when cells were slowly rehydrated (Table 6).

DISCUSSION

Lyophilized and spray dried cells showed similar survival and acid production following treatment. It was therefore decided to investigate the reasons for loss of viability in spray dried cells. In order to study membrane damage, a model system using maltodextrin was used as the suspending liquid. Survival in both maltodextrin and skim milk was similar indicating that mechanisms of damage in the two systems are the same.

Previous workers have reported cytoplasmic membrane damage for a wide range of stress conditions including Allwood and Russell (1968), Strange and Cox (1976), Brennan *et al.* (1986) and Ray (1993).

All of the methods used here, 260 and 280 nm absorbing material, K^+ and β-galactosidase leakage, all strongly indicate that membrane damage in stationary phase cells occurs during spray drying. Increased sensitivity to NaCl further reinforces this conclusion although universal agreement on the effect of this compound after entering into the cytoplasm has not been reached (Morichi and Irie 1973; Hurst *et al.* 1973; Hurst and Hughes 1981). The above methods for leakage, however, do not allow for the distinction of leaked material from sublethally damaged or lysed (dead) cells. It is important to stress that the method of Brennan *et al.* (1986) allows for this differentiation with the penetration of ONPG being facilitated in damaged cells.

Table 6 Influence of rehydration method, at 20°C, on the survival of *Lactobacillus bulgaricus* at stationary phase following spray drying in milk

Rehydration method	Log cfu ml^{-1}*
Soaking	[a]7·95
Shaking	[b]7·28

* Average of three experiments.
[a,b] Means with different superscripts differ ($P < 0·05$).

Cells at stationary phase are significantly more resistant to spray drying than cells at exponential phase. Similar results were obtained heating of *Lact. bulgaricus* in skim milk at different temperatures (Teixeira *et al.* 1994). The increased resistance of stationary phase cells to spray drying could be explained in terms of physiology such as the reduced amount of replicating DNA (Mirhabibollahai 1988) or in terms of acid-shock (Farber and Pagotto 1992). Kirby (1990) showed that dehydrated cells of *Salmonella typhimurium* were more resistant to heating at 135°C if subjected to amino acid starvation when compared to the unstarved control cells. Heat shock increased the survival of exponential cells as compared to controls but did not result in normal levels found with unshocked stationary phase cells. Heat shock had no effect on stationary phase cells. The same fact was observed with the same organism during heating in skim milk (Teixeira *et al.* 1994). It was therefore concluded that the differences in recovery were not of sufficient magnitude to merit further investigation.

The effect of the rehydration temperature on the recovery of spray dried *Lact. bulgaricus* was investigated. Survival rate increased linearly as the temperature of rehydration increased, with the difference between 4°C and 50°C being less than a log cycle. Speck and Myers (1946) also concluded that 50°C was the temperature allowing higher recovery after spray drying. Studying the effect of rehydration temperature on the survival of freeze dried lactic acid bacteria, de Valdez *et al.* (1985) determined 20°C as the optimum rehydration temperature. Similar results were obtained by Speck and Myers(1946). These results indicate that physiological differences between freeze dried and spray dried cells of *Lact. bulgaricus* may exist.

Rehydration media was evaluated on the recovery of spray dried *Lact. bulgaricus*. Although milk and MRS broth might supply a large variety of nutrients no significant differences were found when rehydration was carried on phosphate buffer or deionized water. Ray *et al.* (1971) found similar results during rehydration of freeze dried *Salmonella anatum* and proposed that milk may have supplied all necessary nutrients to the injured cells and thus masked any effects of various added nutrients (MRS broth). Effects of nutrient deficiency (water and phosphate buffer) may also be masked by the same fact.

Significant differences were obtained when cells were rapidly or slowly rehydrated. This is in agreement with results obtained by other investigators (van Schothorst *et al.* 1979; Ray *et al.* 1971). van Schothorst *et al.* (1979) proposed that the soaking procedure limited the amount of osmotic shock leading to higher recovery. Ray *et al.* (1971) proposed that some structure(s) necessary for the cell integrity may be damaged in spray drying. By controlling the rate of rehydration, the disruption of the cells may be reduced. Results do suggest that post-stress lethality as a result from osmotic shock during rehydration may play a role in reducing viable counts.

By comparison of the two methods, spray drying and freeze drying, in terms of survival, acid production and extent of the lag phase, no significant differences were obtained. For this reason, the authors think that it is possible and advantageous, at least with *Lact. bulgaricus*, to concentrate starter cultures by spray drying. In the scope of these results and other of previous investigators, in order to obtain a high number of viable cells of *Lact. bulgaricus* in a spray dried concentrate, it is recommended to dry the cells at the stationary phase of growth and to use slow rehydration procedures. Further investigation is necessary to try to protect the membrane from injury.

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