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RESEARCH ARTICLE

Production, survival, and storage study of freeze and spray dried *Lactococcus lactis* using whey as protectant

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Abstract: The present research was hypothesized that preservation of bacterial cell with higher viability by drying methodologies is a challenging process as *L. lactis ssp. lactis*, is heat sensitive bacteria. Thus, drying conditions must be mild enough to avoid damaging them but sufficiently efficient to yield a powder with moisture content below 4%, which is desirable for storage stability. To overcome the lack of moisture sweet whey was used as protective medium for freeze and spray drying of *L. lactis ssp. lactis.* The present study was designed to evaluate the survival and storage stability of freeze and spray dried *Lactococcus lactis ssp. lactis*, using sweet whey as protective medium up to 90 days at -20°C storage temperature. The percent survivability for freeze and spray dried powders of *Lactococcus lactis ssp. lactis* was found 60% and 36% respectively. A significant decrease of more than one log count for freeze dried culture and two log count decrease in case of spray dried culture powder was observed after 90 days of storage period. The moisture content and storage temperature played a crucial role in storage stability of both the powders, which was observed within the acceptable limit. the present study culminates that storage temperature and moisture content of bacterial powders are the key factors influencing its viability. In addition, higher survivability and storage stability of freeze-dried powder (60%) as compared to spray dried bacterial cells (36%) develops new technological route to improve cell survivability by spray drying and further during storage.

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Introduction

Drying is the most proficient technique for long term preservation of bacterial cultures in food and dairy industry. Along with a stable and extended shelf life of bacteria, drying ensures ease of storage, handling, transport, and their subsequent use in functional food applications. Among different drying methods, freeze drying (FD) is the best process known for preservation of bacteria instead of damaging their viability and highly viable cells for long-term storage period, although it's a laborious and comparatively expensive process. (Broeckx et al. 2016, Huang et al. 2013, Kupletskaya and Netrusov, 2011, Morgan et al. 2006). Sublimation is the strategic factor, ensues in three phases containing a freezing stage subsequently two step drying processes under high vacuum (Alonso, 2004). However, due to higher cost of freeze drying have limited use in large-scale processes. Spray drying (SD) has advantages over other in relative ease in operation, shorter process time, large scale production and relatively cheaper production cost (Huang et al. 2016, Schuck et al. 2013). Moreover, lower bacterial survivability of bacterial culture due to exposure to high temperature (upto 200°C) which damage the cell integrity, is the main limiting condition of spray drying in food and dairy industry (Peighambardoust et al. 2011). In relation to that lactic acid bacteria are heat sensitive, therefore drying conditions must be adjusted to avoid any kind of cellular damage although suitably competent to yield a powder with lower moisture to improve its storage stability. To overcome this challenge extensive research has been carried out which improve culture viability and subsequent storage during drying processes. Various factors have been reported which induce bacterial tolerance and protect bacteria against spray drying, such as pretreatment of bacteria with sub-lethal doses of stress, using a protective matrix as a drying medium and modification in drying conditions (Desmond et al. 2001; De Castro-Cislaghi et al. 2012; Perdana et al. 2014 and Schuck et al. 2013). Freeze drying mainly cause cellular damages due to the formation of crystals and osmotic stresses, thus to protect cells against such damages a wide range of cryoprotectants such as skim milk, sugars can be added to the drying media prior to

freeze-drying. Dairy-based ingredients have often been reported to act as efficient protective agents during drying because of their compatibility with bacterial cell and the possibility to promote their efficiency (Lee and Marco, 2015). Whey is a by-product of cheese manufacturing has found to be an excellent protective agent (Huang et al. 2016, 2017; Maciel et al. 2014). A potentially good powder quality like solubility, flowability, and dispersibility is obtained using whey as it contains good amount of lactose and whey proteins in sweet whey, obtained which makes it an ideal medium for growing dairy bacteria (Lavari et al. 2014; Sadek et al. 2013). Moreover, these components have protective effects on bacterial cell against adverse stresses during spray drying process conditions, re-suspension and storage (Huan et al. 2016; Huang et al. 2014; Yonekura, Sun, Soukoulis, & Fisk, 2014; Huang and Chen, 2013; Paéz et al. 2012; Rajam et al. 2012; Mattila-Sandholm et al. 2002; Picot and Lacroix, 2004). Among the non-reducing disaccharides trehalose is the most investigated which accumulate in bacterial cell during drying process. The protective mechanism of trehalose is due to the stabilizing effect on membranes and proteins of cell by replacing the water around polar residues within these macromolecular structures and thus decreasing the membrane phase transition temperature (Morgan et al. 2006).

During storage several factors affect survival and fermentation activity, such as temperature, presence of light, oxygen and moisture content (Morgan et al. 2006). Higher survival is reported at lower storage temperatures as well as oxidation of the fatty acids of membrane lipids which is the most likely cause of death of microbial cells due to the increased lipid oxidation during storage (Bozaet al. 2004; Corcoran et al. 2004; Desmond et al. 2002; Silva et al. 2002). In dairy industry most of the research has been carried out on the drying of *Lactobacillus, Lactococcus* and various *Bifidobacteria* species. Different bacterial species, or even strains, show variable level of resistance towards various types of stress encounter during spray drying process. *Lactococcus lactis* which is a common starter in cheese industry has found to be better survival after spray-drying due to greater tolerance to heat and oxygen (Dijkstraet al. 2014; Lavariet al. 2015). In order to perform fermentation dried powder products should meet the criteria of more than 10^6 colony-forming units (CFU/g) (Kurmann, 1992). Limited reports are available on the aspect of freeze and spray dried *mesophilic Lactococcus lactis* ssp. *lactis* and its survivability during storage conditions. Therefore, keeping this in view the present study was designed to evaluate the survivability of freeze and spray dried *Lactococcus lactis* ssp. *lactis* NCDC97 culture using whey as protectant during drying conditions.

Materials and Methods

Bacterial culture and biomass production

Lactococcus lactis sp. *lactis* NCDC97 was obtained from National Collection of Dairy Cultures (NCDC), ICAR-National Dairy Research Institute, Karnal-132001, Haryana, INDIA, subsequently, maintained and sub-cultured overnight in M17 broth (Hi Media, Mumbai, India; pH 6.8) at 30ºC under static conditions. The fresh overnight grown culture (8 $\log_{10}$ CFU/mL) was transferred into 1L M17 broth under the same conditions. The preparation of starter culture biomass, 5L sterilized M17 broth was transferred into the 14 L bio-reactor (BioFlo® 320, Eppendorf Pvt. Ltd. Germany). The previously freshly grown 1L starter culture was inoculated into the 5L sterilized M17 media followed by incubated statically at 30°C for 18 h with constant agitation of 70 rpm. Overnight grown cells were harvested by pre-cooled (4°C) centrifugation (SIGMA 3-18K, Germany) at 5000 RPM for 10 min at 4°C followed by re-suspended in drying medium and cell count were adjusted to 10^{10} cells/mL. Drying medium was prepared by rehydrating sweet whey powder (AMUL, Anand, India) and trehalose (PubChem CID: 7427) (99.8% purity, Sigma–Aldrich, Australia) in deionized water (PubChem CID: 962) @ 5:1 adjusting final total solid up to 30%. The drying medium was also sterilized under recommended conditions (121°C for 10 min).

Freeze drying

To determine the effects of dehydration on viability of the bacteria, cell suspensions of *Lactococcus lactis* sp. *lactis* NCDC97 mixed with drying medium were frozen overnight at -20°C followed by freeze drying (Lyodel 0555, DELVAC Company Pvt. Ltd., Chennai) at -40°C under vacuum of 0.2 mbar.

Spray drying

Spray drying was carried out at laboratory-scale using Laboratory spray dryer (LSD-48 Spraymate, JISL Pvt. Ltd, India) with water evaporation capacity of 1L/ h. The *Lactococcus lactis* sp. *lactis* NCDC97 bacterial suspension was pneumatically atomized using a two-fluid nozzle with an orifice diameter of 0.7 mm. The inlet and outlet air temperature were at 150 ± 1°C, and 60 ± 3°C with a feed rate of 10 mL/ min, respectively.

Storage and their survivability study

The bacterial freeze-dried powder and spray dried powder were collected through single cyclone separator and stored at -20°C for 90 days in dark cryo-vials. The storage duration was chosen based on the typical duration used in previous shelf-life studies (Gandhi et al. 2013; Fonseca et al. 2000) and in many industrial settings. Total lactic count in terms of viable cells were expressed as colony forming unit (CFU). As per the method of Gardiner et al. 2000 briefly, powder samples were re-constituted by dissolving 0.1 g in 9.9 mL sterile peptone water (1% w/v, pH = 7.0 ± 0.1). Each diluted sample of *L. lactis* sp. *lactis* NCDC97 was poured onto M17 agar, mixed and incubated at 30°C for 48 h (aerobic conditions). Survival (%) of bacteria was expressed as a percentage of the live bacterial cells immediately after drying

(freeze and spray) was enumerated (before the powders were stored = zero storage time) using the following equation:

$$\% \text{ Survival} = \frac{Nt}{N0} X\ 100$$

Where, N_t (CFU/g) refer to the bacteria population after both method of drying, *viz.,* spray drying and freeze drying, and N_0 to the initial population before drying treatments.

Analysis of Moisture content and water activity (a_w)

Moisture content of dried powders was determined gravimetrically by oven drying at 102°C for 2h (AOAC, 1990). The water activity of powders was determined using an a_w-meter (Aqua Lab, Decagon Devices, WA, USA) at 25°C.

Powder morphology

The dried bacterial cultures (freeze and spray) morphology was observed under scanning electron microscopy ZEISS EVO SEM (Zeiss, Cambridge, U.K.) as protocol described by Fu et al. (2013). Briefly, dried culture powders were fixed on carbon tape and then sputter-coated with gold palladium. These powder samples were examined followed by micrographs were taken under the scanning electron microscopy ZEISS EVO SEM.

Statistical analysis

All the experiments were performed in triplicate and all data are reported as mean ± SEM. The p value of <0.05 was considered to be statistically significant. Data were subjected to analysis of variance (ANOVA) with Tukey's multiple comparisons. Statistical analysis was performed with Graph Pad Prism 6.0 for Windows software.

Results and Discussion

Total Lactic Count of dried cultures

The initial count of freeze and spray powders of *L. lactis* sp. *lactis* NCDC97 were 11.46 and 11.18 log CFU/g, respectively. Total lactic count of freeze-dried powder was 11.03 log CFU/g up to 45 days and significantly decreased to 10.16 log CFU/g after 90 days, whereas in case of spray dried powder, lactic counts were reduced from 11.18 to 10.71 after 30 days and further 9.47 log CFU/g after 90 days with a significant reduction (Table 1). The survival of freeze dried and spray dried culture (as per the mentioned equation) was around 60% and 36% respectively (Figure 1). Similar results were obtained by Gandhi et al. 2013.

Zayed and Roos, 2004, reported the 45% survival of *L. salivarius* when combination of skim milk and sugar was used as freeze drying medium and remained stable upto 50 days. Correspondingly, slight loss of viability occurred in spray-dried *L. rhamnosus* GG (reduction of 0.25 log unit) using skim milk as drying medium upto 6 weeks (Ananta et al. 2005). Loss in viability was higher in case of SD bacterial powder as compared to FD bacterial powder due to use of high temperatures during spray drying process, which can damage bacterial cells and subsequently reduce their viability (Ananta et al. 2005). Our study reported that the presence of high amounts of protein and phosphate salts in whey may have provided a supplementary defensive coating for the cells during drying and storage.

Moisture content and water activity (a_w)

In this study we observed minimal moisture content of spray dried powder of *L. lactis* sp. *lactis* NCDC97 was 3.7% under optimized conditions (inlet 155°C, outlet 60°C and feed rate 35 mL/ min) whereas, in case of freeze-dried powder it was obtained 2.6%, which was considered for optimum survival of bacteria during storage. The water activity (a_w) of freeze dried and spray dried powders were obtained 0.35 and 0.36 respectively which, is in considerable range.

Higher moisture content (4.1-8.6%) could result into reduced stability during storage (Champagne and gardner,2001; King et al. 1998) however, Bielecka and Majkowska, (2000) reported that excessive moisture content (10%) of spray dried powder was produced when the outlet air temperature was less than 60°C. Moreover, the moisture content of spray dried powder is significantly influenced by inlet-outlet drying air temperature, composition, and feed solution concentration (Anandharamakrishnan et al. 2007). The whey proteins used as cryoprotectant in drying medium led to formation of film around the particle surface, which favors the removal of moisture more rapidly, hence controlled the diffusion of water vapour from the interior of droplet to the surface during spray drying (Adhikari et al. 2009).

Table 1: Total lactic count (log CFU/g) in dried powder of L. lactis ssp. lactis NCDC 97culture during storage at -20±1oC for 90 days

Dried culture	0 day	15th day	30th day	45th day	60th day	75th day	90th day
Freeze dried	11.46 ± 0.19^{aA}	11.29 ± 0.11^{bA}	11.19 ± 0.21^{cA}	$11.03\pm0.27d^{A}$	$10.80\pm0.18e^{A}$	10.62 ± 0.22^{fA}	10.16 ± 0.15^{gA}
Spray dried	11.18 ± 0.20^{aA}	11.11 ± 0.19^{bA}	10.72 ± 0.21^{cB}	10.46 ± 0.30^{dB}	10.02 ± 0.13^{eB}	9.72 ± 0.13^{fB}	9.47 ± 0.28^{gB}

$^{A, B}$ Mean (±SE) values with different superscript with in a column differ signiûcantly ($p<0.05$)
^{a,b,c,d,e,f,g} Mean (±SE) values with different superscript with in a row differ signiûcantly ($p<0.05$)

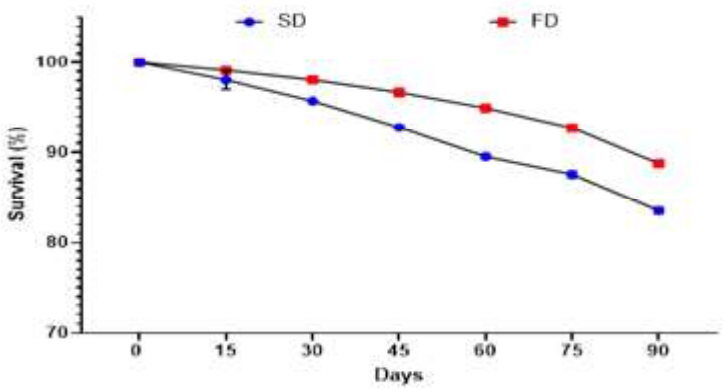

Fig. 1 Total lactic count (CFU/g) of freeze and spray dried *Lactococcus lactis* sp. *lactis* NCDC 97. (Mean ± SEM; FD= Freeze dried; SD= Spray dried)

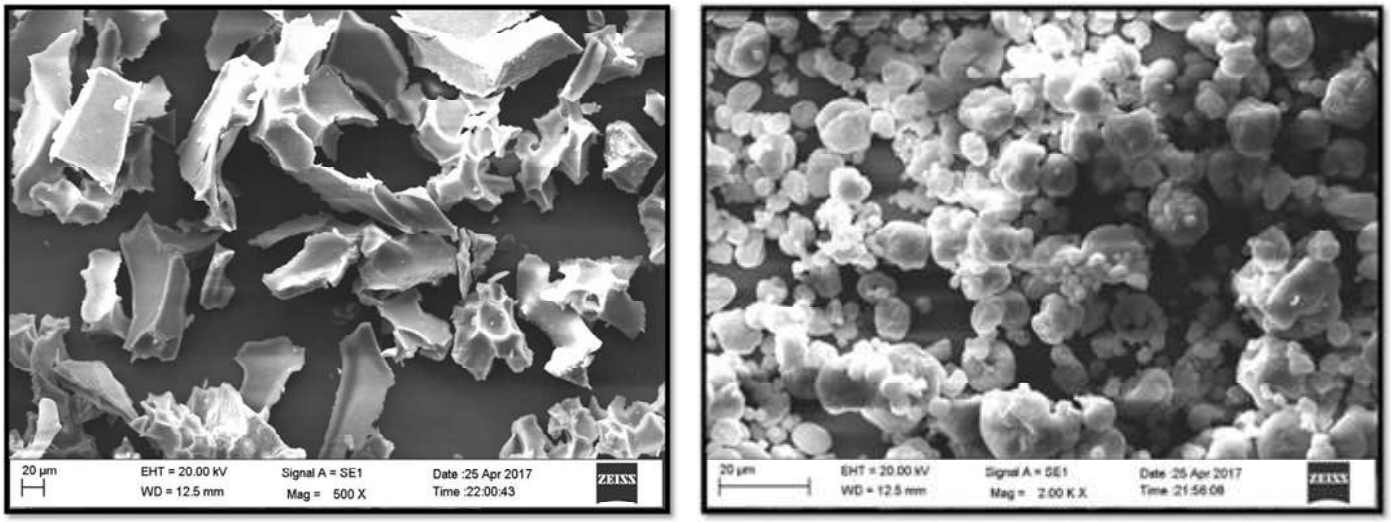

Fig. 2 SEM micrographs of freeze dried (A) and spray dried (B) powder of *Lactococcus lactis* sp. *lactis* NCDC 97

The a_w and residual water contents in dried powders are affected by hygroscopicity and water binding ability of the drying matrix components (Barbosa-Canovas *et al*, 2005). Poddar et al. (2014) observed that the a_w of spray-dried *Lb. paracasei CRL* 431 was lower than 0.33. In order to reduce detrimental effects on bacterial cells, the drying medium can be supplemented with an antioxidant such as ascorbic acid and monosodium glutamate in spray-drying medium to improved culture viability during powder storage (Sunny-Roberts and Knorr, 2009).

Powder morphology

Images obtained by scanning electron microscopy of the prepared powders of *L. lactis* sp. *lactis* NCDC97 by freeze and spray drying is shown in figure 2 (A & B), respectively. The SEM images of freeze-dried bacterial cells shows irregular shape of particles with variations in range. The freeze-dried material retained its solid amorphous form and the structure resembled as broken glass or flake-like structure. The bacterial cells can be seen as randomly distributed throughout the wall matrix.

The SEM image of spray dried powder clearly depicts that the whey protein, skim milk and sugars used in drying medium formed a protective coating around the bacterial cells which helped them to survive in drying conditions (Gaiani et al. 2007 and Sadek et al. 2014). During spray drying whey proteins encounters high temperature which causes their denaturation and results in wrinkled rough surface after spray drying (Holt et al. 1999). No bacteria were observed on the surface of micro-particles and no visible surface fissures or cracks confirmed good structural integrity and low gas permeability e.g., oxygen, water vapour that provides better protection to bacterial cells (Fritzen-Freire et al. 2012). The micro structural aspects of spray dried products are significantly affected by parameters such as the drying rates, composition, viscosity of the drying carrier aliquots and the atomization (Kim et al. 2009).This structure of freeze dried powder is due to the direct sublimation of ice into water vapor during freeze-drying operation (Ezhilarasi et al. 2013, Rajam et al.2015).

Conclusion

The results obtained in the present study concludes that storage temperature and powder moisture content of bacterial powders

are the key factors influencing its viability. In addition, higher survivability and storage stability of freeze-dried powder (60%) as compared to spray dried bacterial cells (36%) develops new technological route to improve cell survivability by spray drying and further during storage. At a molecular level the clear mechanism of bacterial cell adaptation and its interaction with whey protein during spray drying and storage could be investigated. Moreover, as the drying processes were done at laboratory scale further its feasibility should be explored on an industrial scale.

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Conflict of interests

The authors declare that there are no conflicts of interest.

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