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Exploration of *Lactobacillus fermentum* MTCC 8711 Microencapsulation by spray drying for their use as probiotic

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Abstract

Microencapsulation can carried out by many techniques but spray drying is promising, reliable, low cost and quick technique compare to other techniques. In many functional foods *L. fermentum* MTCC 8711 is used as a probiotic microorganism. Spray drying microencapsulated powder of probiotic organism increase survival rate of organism during storage; increase the shelf-life of product and micro particle optimal size increase dispersion of probiotic bacteria in final product. In this paper, we have used spray drying technique with three encapsulated material viz. 11% non-fat skimmed milk solution (SKNF 11%), 20 % non-fat skimmed milk solution (SKNF 20%) and soy milk plus 10 % maltodextrin solution (SMMD 10 %). During spray drying process inlet air temperature was 110°C, outlet air temperature was 70 °C and aspiration flow rate 70 Nm^3/h and feed pump flow rate 1ml/min were maintained. Parameter like particle size analysis (PSA) and scanning electron microscopy (SEM), moister content, water activity, acid tolerance and bile salt tolerance of *L. fermentum* MTCC 8711 encapsulated powder and free cell without encapsulated (control) were perform. Viable count of same organism were studied at before and after spray drying process, as well as time interval of 30 days and 60 days.

Keywords: Microencapsulation, spray drying, soy milk, *L. fermentum*

Shelf life and viability of probiotic bacteria in food can be increase by drying with protective agents. Generally, lyophilisation or freeze drying can be use for microencapsulation, but spray drying is more important technique compare to freeze drying because it's economically advantages, lower energy consumption and large microencapsulation forming capacity in define time (Sadikoglu., 2010). Shelf life and viability of probiotic based food has high levels (~ 10^7-10^8 CFU/g) of live probiotic bacteria which are suggested for probiotic products (Karimi *et al*., 2011). Production of an instant microencapsulated powder would provide advantage in shelf-life extension of probiotic bacteria during storage in probiotic food.

Spray drying technique and its application were developed to defend probiotic bacteria from injury caused by exterior factors such as drying, packaging and storage conditions (which include parameter i.e. level of O_2, time, temperature, moisture content) and the depletion of probiotic bacteria in the digestive tract, especially due to extreme pH (2.5 to 3.5) of gastric juices in stomach and bile secretion (0.3%) in duodenum (Shah *et al*., 2011).

Types of protective agent used for spray drying like carbohydrates (agar, sodium alginate, carrageenan, gum arabic, chitosan, starch, maltodextrin, corn syrup, sucrose), protein (reconstituted skim milk, gelatine, Gluten, whey protein, albumin) and lipid (waxes, paraffin, diglycerides, mono-glycerides, fats, stearic acid) are added in to the media before the spray drying has been reported successfully to increase the shelf-life and viability during the spray drying and storage (Serna-Cock *et al*., 2013).

The aim of the present study is to produce skimmed milk and soya milk powder containing *Lactobacillus fermentum* MTCC 8711 by spray drying encapsulation technique. Parameter like PSA and SEM were studied after encapsulation of *L. fermentum* MTCC 8711 while various parameters like viable count before and after encapsulation, moisture content, acid and bile tolerance and water activity were studied at the time interval of 30 days and 60 days during storage at different temperature i.e. 4°C and 37°C.

Materials and Methods

Preparation of soymilk from raw soybean

Soymilk was prepared according to the procedures described by Wang *et al.* (2003). 50 gm raw soy bean seeds were soaked in 150 mL sterile water (1:3 w/v) for 18-20h at room temperature. After soaking, soaked water was discarded and the seeds were washed with fresh water. 50 gm of soaked soybean seeds per 500 ml of water was used for grinding for 5 min i.e. 1:10 (w/v). The soybean seed extracted was filtered through a muslin cloth (Masoodi *et al.*, 2014). The filtrate obtained was boiled in water bath at 80°C for 10 min. The flow chart for the preparation of soymilk is shown in the Fig. 1.

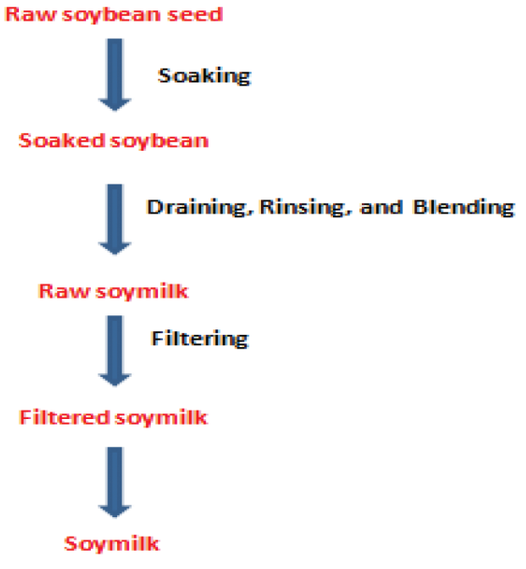


Fig. 1: Preparation of soya milk from raw soybean seed

Strain and culture conditions

Lyophilized culture (-40 °C) of *L. fermentum* MTCC 8711was cultured in MRS broth (at 37°C for 24 h. An aliquot of 1% of the propagated culture was transferred and re-grown under the same conditions. A cell density of approximately 10^9 colony forming units (CFU) per ml was obtained at the end of incubation. The culture was centrifuged at 10,000 rpm for 15 min. The cell pellet was washed twice with 0.97 % NaCl and resuspended in distilled water. *L. fermentum* MTCC 8711 culture stock were kept in 25 % glycerol and lyophilized vial at - 40 °C.

Preparation of feed solutions for spray drying (SD)

For spray drying, overnight culture of *L. fermentum* MTCC 8711 was inoculated into MRS broth and incubated at 37°C for 24h to 48 h. After centrifugation at 10000 rpm for 15 min at 4 °C, three types of feed solutions were prepared. In the first type 3 % (w/v) cell were resuspended in sterile 11% non-fat skimmed milk solution (SKNF 11%) (Silva *et al.*, 2002). Similarly, in the second type, after centrifugation 3% (w/v) cells were resuspended in sterile 20 % non-fat skimmed milk solution (SKNF 20%). In third type, 3% (w/v) cells were resuspended in sterile soy milk and 10% maltodextrin solution (SMMD 10%) (Bamrungna, 2009). These feed solutions were directly used for spray dried application (SKNF 11%, SKNF 20% and SMMD 10%).

Spray drying (SD)

A Lab scale spray dryer SPD-P-111 (Technosearch instruments, Thane, India) was used for spray drying of *L. fermentum* MTCC 8711. The equipment consisted of a spray system include blower, air heater, scrubber, feed pump, main drying chamber, cone, collection bottle and two cyclone (Primary and secondary). The inlet air, heated to 110 °C by an electrical heater, flowed concurrently with the spray into a 1 mL/min drying chamber with an outlet temperature of 70 °C. Feed solution was delivered by a peristaltic pump into a fluid stainless steel atomizer. The spray dried powder was collected at the bottom of a cyclone. The drying regimes implemented including inlet and

outlet temperatures and other parameter are as per Table 1. Spray drying of *L. fermentum* MTCC 8711 was carried out by using above three type of feed solution.

Table 1: Spray drying system parameter

Sl. No.	Parameter	
1	Inlet temperature (°C)	110
2	Outlet temperature (°C)	70
3	Plate temperature (°C)	30
4	Cooling temperature (°C)	75
5	Aspiration flow rate (Nm^3/h)	70
6	Feed pump flow rate (ml/min)	1
7	Stirrer speed (rpm)	15
8	Cycle time (min)	425

Scanning electron microscopic analysis of spray dried powder

Scanning electron microscopic observation was carried out to observe the size and surface morphology of three powder samples (SKNF 11 %, SKNF 20 % and SMMD 10 %). For this process small amount (~2 mg) of powder were used, gold coated over the sample using vacuum sputtering machine EMITECH SC 7620 sputter coater at 500 kv for 4 min and pressure currant was 10mA. Individual powder sample was put in sample holder (aluminium stub) for the SEM analysis. Microscopic observation of individual powder samples was performed using ZEISS EVO-18 scanning electron microscope having acceleration voltage of 15 kW. An individual powder sample was fixed on aluminium stub with double-sided adhesive tape. SEM image data of powder was collected over a selected area of the powder sample and 2D image was visualized that display properties include size, shape and texture of powder samples (Rosenberg and Young., 1993).

Particle Size analysis of spray dried powder

Dry powder measurements were performed using the Microtrac S3500 with the Turbotrac accessory. Powder sample was transported to the optical section through aspiration tube, applied air and a vacuum, in the optical section, the aerosolized powder interacts with the laser beam where upon diffraction detection occurs. Turbotrac measurement was automatic and self-cleaning as well as activates automatic blank measurement followed by measurement under the direction of a prescribed SOP. Method parameters for measuring powder samples were:

(a) Air pressure = 30psi

(b) Refractive index (required for Mie scattering calculations) = 1.36

The particle size distribution of the sample was represented by span factor, and it is defined as (Tonon, Grosso & Hubinger, 2011):

$$\text{Span d} = \frac{d[v,90] - d[v,10]}{d[v,50]}$$

Where, d [v,10], d[v,50], d[v,90] correspond to the diameters at which the cumulative sample volumes were under 10%, 50% and 90% respectively.

Purity of spray dried powder

The purity and probiotic property confirm using Gram morphology reaction, motility test and catalase test.

Moisture content and water activity of spray-dried powder

The moisture content of spray-dried powders (SKNF 11%, SKNF 20% and SMMD 10%) were determined by oven drying at 102 °C for 2h. Moisture content was analyzed by determination of the difference in weight before and after drying, expressed as a percentage of the initial powder weight (IDF, 1993).

Percentage of Moisture (%) = (Before drying weight – After drying weight) × 100

Water activity of the powders SKNF 11%, SKNF 20% and SMMD 10% after the spray drying was measured in duplicate using an water activity meter (novasina, Lab swift aw, Switzerland)

Probiotic properties of spray dried bacteria

Acid tolerance

Preliminary selection of acid tolerant lactobacilli using rapid method was determined according to slightly modified methods as described by Pelinescu *et al.* (2009). 100 µL overnight cultures of the *L. fermentum* MTCC 8711 were inoculated into 10 mL MRS broth with pH-3, and pH-7 as a control. The inoculated broths were then incubated in anaerobic condition for 12 h at 37°C. After incubation in anaerobic condition, 0.1mL culture was spread on MRS agar plate for checkout acid tolerance capacity, bacterial isolates were grow in plate which consider as a acid tolerance.

Bile salt tolerance

The tolerance of lactic acid bacteria to bile salts was performed in MRS supplemented with bile salts using a modified method described by Dora and Glenn (2002).

L. fermentum MTCC 8711 were tested for their bile salt tolerance by determining their growth in MRS broth containing levels (0.3% w/w) of bile salts (ox-gall, Hi Media-India) and normal MRS medium taken as a control. Freshly prepared 0.1 mL cultures were inoculated into 10 mL MRS broth which contain respective amount of ox-gall and incubated at 37 °C for 12 h under anaerobic condition. After incubation in anaerobic condition 0.1 mL culture was spread on MRS agar plate for checkout bile tolerance capacity, bacterial isolates are grow in plate which consider as a bile tolerance.

Determination of cell viability and storage of spray dried powder

For the enumeration of viable counts, 1 mL aliquots of SKNF 11%, SKNF 20 % and SMMD 10 % were added to 9 mL of saline sterile water and the appropriate serial dilutions were prepared before spreading on to the MRS agar plate (Rajam *et al.,* 2012). For the recovery of viable cells from the spray-dried powders, 0.1 g of the manufactured powder was rehydrated in 9.9 mL saline sterile water for 1 h at room temperature prior to spreading on to the MRS agar. As a describe above the viable count of SKNF 11%, SKNF 20 % and SMMD 10 % powders were carried out at the time intervals of 30 days and 60 days. Viable cell count was determined and calculated as colony forming units (CFU) per millilitre.

The survival rate Doherty *et al.* (2010) was calculated as follow:

$$\text{Survival rate (\%)} = [\log N/\log N_0] \times 100$$

Where N_0 is the viability of *L. fermentum* MTCC 8711 before spray drying and N is the viability after spray drying (Semyonov *et al.,* 2010).

Cell viability during storage, the plot of the logarithmic value of the relative cell viability (log Nt/Ni) versus storage time (t, day) were describe by equation

$$\text{Log} = Nt / Ni$$

Where Nt is the number of viable bacteria at a particular storage period (in CFU/g), Ni represent the number of viable cells at the beginning of storage (in CFU/g) and t is the storage time (in days).

Spray-dried powder (SKNF 11%, SKNF 20% and SMMD 10 %) were packed in nitrogen packing by using multilayer pouch of polyethylene terephthalate and kept at 4 °C and 37 °C for the time interval of 30 days and 60 days. Parameter like PSA and SEM were studied while various parameters like viable count before and after encapsulation, moisture content and water activity, acid and bile tolerance were studied at the time interval of 30 days and 60 days during storage at different temperature i.e. 4°C and 37°C. The viable cell count and moisture content of the *L. fermentum* MTCC 8711 were determined for their stability at above specific time intervals.

Results and Discussion

Scanning electron microscopy of spray dried powder

Scanning Electron Microscopy (SEM) has been applied for the micro structure evaluation of three different encapsulated materials with desire microorganism

(Fig. 2 a). Microcapsules having a spherical shape in case of SKNF 20% and SMMD 10% (Fig. 2 b&c) while in case of SKNF 11% spherical shape with concavities (Fig. 2 a).

Scanning electron microscopic image shown the absence of observable fractures or crack on the surface of the microcapsule which indicate the less weakness and good mechanical force in wall system for all their material. Furthermore, it can reduce the air permeability that provides better protection of probiotic microorganisms and increase viability during storage.

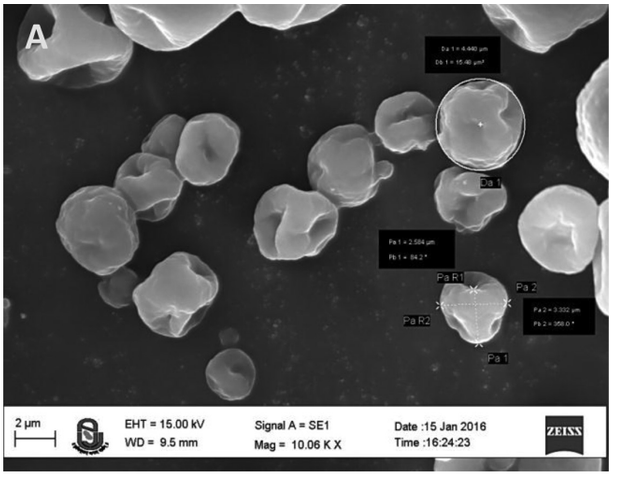


Fig. 2a

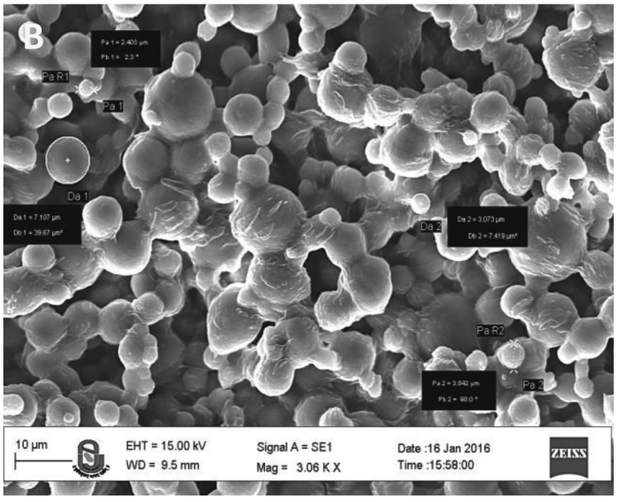


Fig. 2b

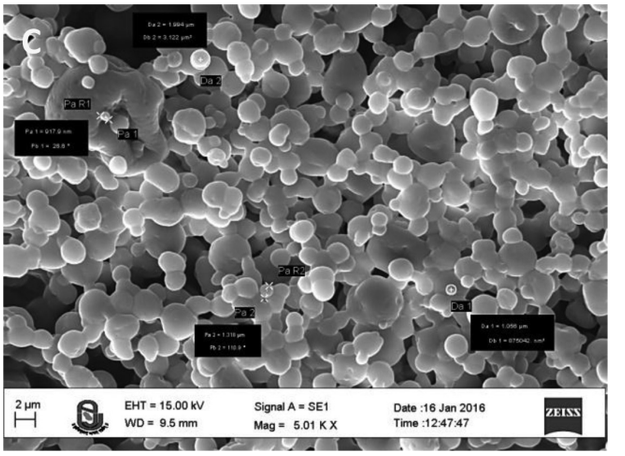


Fig. 2c

Fig. 2: SEM micrographs of spray-dried micro-particles containing *L. fermentum* MTCC 8711. Spray-drying was conducted with 11 % of non-fat skimmed milk (A), 20 % of non-fat skimmed milk (B), Soymilk plus 10 % maltodextrin (C).

It is noticed that some microcapsules display dented surfaces. This phenomenon is attributed to drying and atomization mechanisms involved in spray drying. Immediate evaporation and cooling of droplets cause shrinkage of particles and produce zones where "holes" in the structure take place (Pedroza-Islas *et al.*, 1999).

Particle size analysis of spray dried powder

Distribution and size of particle are very important physical characterisation which directly affect to consumption of microencapsulation in to food formulation. Size of microencapsulation exhibit varying from 12.09µm – 276.9µm in case of SKNF 11%, While in SKNF 20%, it is exhibit varying from 15.59µm – 339.7µm and in case of SMMD 10% particle size exhibit from 66.17µm – 390.1µm which shows in Fig. 3 and Table 2.

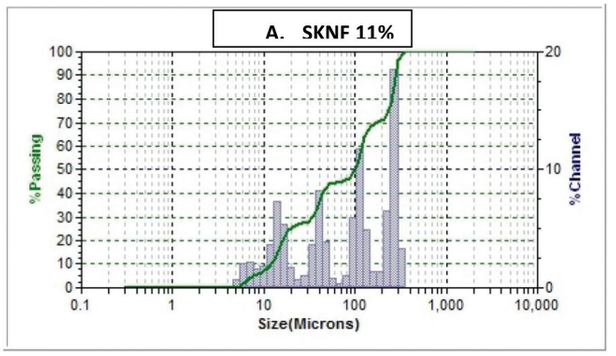
Generally the larger spray dried particle provide more protection to probiotic bacteria compare to smaller particle because low concentration of shell material in small particle compare to larger microencapsulated particle. If the size of microencapsulated particle is higher than the shelf stability of bacteria in food will be longer.

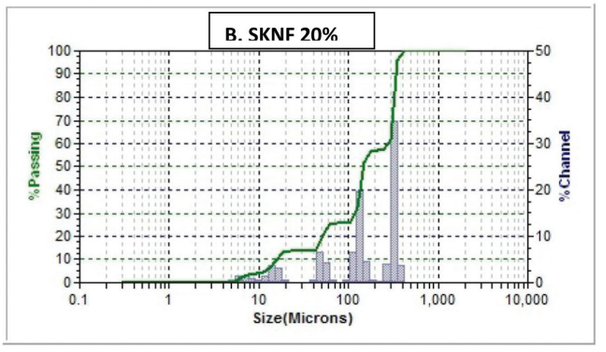
Purity of bacteria in spray dried powder

Different tests were performed of *L. fermentum* MTCC 8711 during operation and its conditions to determine whether some type of contaminants was into the powder of skimmed milk or soy milk powder. Three tests were performed to identify the contaminants during operation and storage periods.

When observing the glass slide with Gram staining, it could be appreciated that the procedure did not generate contamination in the final products. The (negative) result of the catalase testagrees with the results found for the strain when not submitted to a drying process.

When performing typical biochemical tests include for lactic bacteria, these coincide with the results for non-dried bacteria, suggested that the strain did not suffer changes in its macroscopic behaviour. This result, and the Gram stain and catalase test, assures that the strain did not suffer contamination during process.





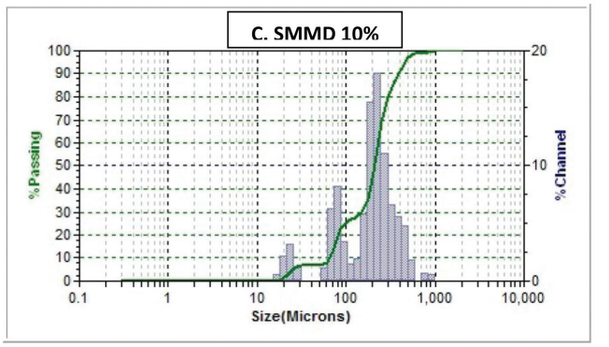


Fig. 3: The size distribution and diameter of micro-particle using different encapsulation material 11% of non-fat skimmed milk (A), 20% of non-fat skimmed milk (B), Soymilk plus 10% maltodextrin (C).

Moisture content and water activity of spray dried powder

Moisture content of the spray dried microcapsules between 1 to 4.1% which showed in Table 3 and it was considerably affected by parameter such as inlet and outlet drying air temperature, droplet formation mechanism, composition and concentration of feed solution (Anandharamakrishnan *et al.,* 2007). In present study outlet air temperature was 70 °C and inlet temperature was 110 °C. Generally, the desired level of water content in the product is achieved in between 1 and 3% after spray drying (Vega and Roos, 2006). Moisture content in dried product is important parameter to influence the viability of the microorganism during storage (de Valdez *et al*., 1985). Initially the moisture content of encapsulated material SKNF 20% is 4%, while in case of SKNF 11 % and SMMD 10% are 1% and 2% respectively. As the time gradually passed out, the moisture content of three encapsulated powder with probiotic organism were increase.

Thus our result of SKNF 11 % and SMMD 10 % of moisture content are as par with Vega *et al.* P. Wanchaitanawong and P. Bamrungna (2009) reported After 6 months of storage, the moisture contents in soy milk supplemented with maltodextrin was increased to 3.95, 4.02 and 3.64%, respectively of *L. acidophilus* TISTR 1338, *L. casei subsp. Tolerans* TISTR 1341 and *L. plantarum* P49.

The value determines of water activity after spray drying powder were SMMD 10%, SKNF 11% and SKNF 20% were 0.242, 0.248 and 0.253 respectively. Thus our result was better by using this encapsulated material.

Grosso *et al.* perform same experiment using spray drying and its water activity was between 0.204 to 0.230, our result was affirmation to that.

Probiotic properties of spray dried bacteria

The spray-dried powders of *L. fermentum* MTCC 8711 were evaluated for the acid and bile salt tolerance. The viable cell count of spraydried powder of *L. fermentum* MTCC 8711 was between 1.2×10^7 CFU/gm to 5.2×10^7CFU/gm.

Acid tolerance

After 12h exposure to acid at pH-3 (Fig. 4), the spray dried *L. fermentum* MTCC 8711 of spray dried encapsulated and without encapsulated free cell (control) shows that free cell without encapsulated (control) viable count was much more decrease compare to spray dried encapsulated (Table 4).

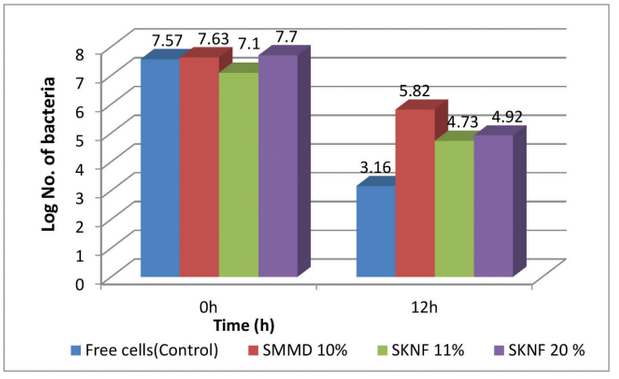


Fig. 4: Viable cell of spray dried *L. fermentum* MTCC 8711 during exposure to pH-3

We have used three encapsulated material i.e. SMMD 10%, SKNF 11% and SKNF 20%, out of these SMMD 10% shows 23.72%, SKNF 11% shows 33.38 and SKNF 20% shows 36.10% reduction in count of probiotic bacteria. Similar result obtained by Michida *et al.* (2006).They found that the viable cell of L. *plantarum* decreased dramatically from 7.24 to 1.92 log CFU/mL when exposure to acid. Our result was at par with them.

Bile salt tolerance

After 12h exposure to bile salt (Ox-gall) at 0.3% (Fig. 5) the spray dried *L. fermentum* MTCC 8711 of spray dried encapsulated and free (control) organism shows that free cell (control) viable count much more decrease compare to spray dried encapsulated (Table 4). We have used three encapsulated material i.e. SMMD 10%, SKNF 11% and SKNF 20%, out of these SMMD 10% shows 18.74%, SKNF 11% shows 22.33% and SKNF 20% shows 23.37% reduction in count of probiotic bacteria.

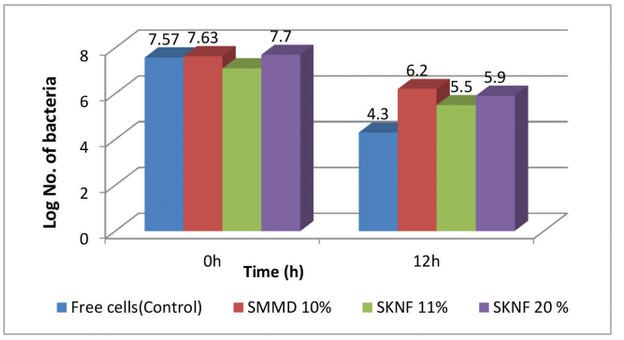


Fig. 5: Viable cell of spray dried *L. fermentum* MTCC 8711 during exposure to 0.3% ox-gall bile salt

Krasaekoopt *et al.* (2004) concluded that the survival of *L. acidophilus, B. bifidum,* and *L. casei* decreased proportionately with the time of exposure to bile salt solutions. Our result of bile salt tolerance was affirmation too this.

It was clearly observed that *L. fermentum* MTCC 8711 encapsulated with SMMD 10 %by spray drying technique was more tolerance to acid and bile salt than unprotected probiotic cell.

Determination of cell viability and storage stability of spray dried powder

The viability of *L. fermentum* MTCC 8711 encapsulated with three different encapsulated materials i.e. SKNF 11%, SKNF 20% and SMMD 10%. The viability

of encapsulated *L. fermentum* MTCC 8711 after encapsulation the live bacterial number (LBN) was decreased about 10 times than before encapsulation. The viable count of bacteria after spray drying in all encapsulation material was greater than 10^7CFU/gm.

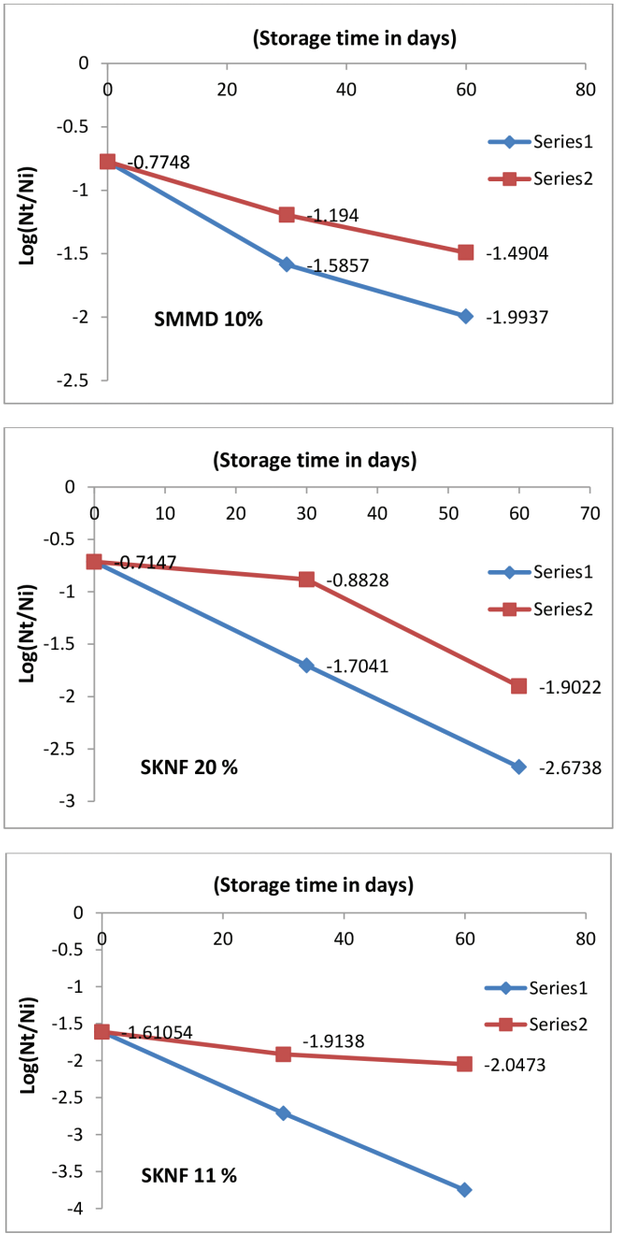


Fig. 6: Viability loss of spray dried *L. fermentum* MTCC 8711 express as function of storage time at 4 °C and 37 °C. Series 1 indicate = 4 °C and series 2 = 37 °C.

We have used encapsulated material SMMD 10 % and obtained viable count after the spray drying was 8.9×10^7. Than we have stored spray dried powder at 4° C and 37°C for 30 days and 60 days in nitrogen packing. After 30 days the viable count at 4° C was 3.3×10^7 and at 37°C, it was 1.3×10^7. While in the case of 60 days storage at 4° C the viable count was 1.7×10^7 and at 37°C, it was 5.3×10^6.

Table 2: Shown span value and D_{50} value for spray dried powder

Sl. No	Microencapsulated material	Span value of particle size	D_{50} value
1	SKNF 11%	2.64	100.2
2	SKNF 20%	2.23	144.8
3	SMMD 10%	1.56	207.4

Table 3: Moisture content of spray-dried *L. fermentum* MTCC 8711 using encapsulated material SKNF 20 %, SKNF 11 % and SMMD 10%

	Storage temperature	Encapsulation material	Percentage of Moisture
After spray drying		SKNF 20 %	4
		SKNF 11 %	1
		SMMD 10%	2
After 30 days	4 °C	SKNF 20 %	4.1
		SKNF 11 %	1.5
		SMMD 10%	2.6
	37 °C	SKNF 20 %	3
		SKNF 11 %	1.5
		SMMD 10 %	1
After 60 days	4 °C	SKNF 20 %	3.9
		SKNF 11 %	1.7
		SMMD 10%	2.1
	37 °C	SKNF 20 %	2.3
		SKNF 11 %	1.9
		SMMD 10 %	1.6

In case of second encapsulated material i.e. SKNF 10% and obtained viable count after the spray drying was 1.52×10^7. Than we have stored spray dried

powder at 4° C and 37°C for 30 days and 60 days in nitrogen packing. After 30 days the viable count at 4° C was 7.56×10^6 and at 37°C, it was 1.2×10^6. While in the case of 60 days storage at 4° C the viable count was 5.56×10^6 and at 37°C, it was 1.1×10^5.

Table 4: Viable count of *L .fermentum* MTCC 8711 free cell and microencapsulation cell

	Tolerance	Free cells (Control)	SMMD 10%	SKNF 11%	SKNF 20 %
Before (0h)		3.8×10^7	4.2×10^7	1.2×10^7	5.2×10^7
After 12h Incubation	**Acid (pH-3)**	1.4×10^3	6.6×10^5	5.3×10^4	8.3×10^4
After 12h Incubation	**Bile salt (0.3%)**	2.0×10^4	1.6×10^6	3.2×10^5	7.9×10^5

In another encapsulated material i.e. SKNF 20% and obtained viable count after the spray drying was 8.1×10^7. Than we have stored spray dried powder at 4°C and 37°C for 30 days and 60 days in nitrogen packing. After 30 days the viable count at 4°C was 5.5×10^7 and at 37°C, it was 8.3×10^6. While in the case of 60 days storage at 4°C the viable count was 5.26×10^6 and at 37°C, it was 8.9×10^5. All data represented in Table 5 and Fig. 6 shows relative viability after 30 days up to 60 days.

After spray drying process it concluded that SMMD 10% shows better result compare to SKNF 11% and SKNF 20%. At the storage condition of 30 day and 60 day at 4° C and 37 °C here once again SMMD 10 % shows better result compare to other encapsulated material. We have used three microencapsulation material out of them SKNF 11% and SKNF 20% shows decreased in viability count after 60 days at 37°C compare to initial viable count of encapsulation material SMMD 10%.

Conclusion

The present study has shown that *L. fermentum* MTCC 8711 was spray dried encapsulated and after 60 days of storage at 4 °C and 37 °C. At the storage of 37°C viability count of encapsulated bacteria was decrease compare to storage at 4°C. It means that temperature of storage was a critical parameter affecting the survival rate of encapsulated bacterial powder. In general comparison, SMMD 10% microcapsules had higher encapsulation efficiency and better storage stability and protection in acidic tolerance as well as bile salt tolerance compare SKNF 11% and SKNF 20%. Hence, microencapsulation of *L. fermentum* MTCC 8711 using SMMD 10% prove to be better for food formulation.

Table 5: The viable count of *L. fermentum* MTCC 8711 before, after, at 30 days and 60 days

Encapsulation material	Colony count before spray drying	Colony count after spray drying	Colony count after 30 days storage at 4 °C	Colony count after 30 days storage at 37 °C	Colony count after 60 days storage at 4 °C	Colony count after 60 days storage at 37 °C
SMMD 10%	5.3×10^8	8.9×10^7	3.3×10^7	1.3×10^7	1.7×10^7	5.3×10^6
SKNF 11%	6.2×10^8	1.52×10^7	7.56×10^6	1.2×10^6	5.56×10^6	1.1×10^5
SKNF 20 %	4.2×10^8	8.1×10^7	5.5×10^7	8.3×10^6	5.26×10^6	8.9×10^5

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