Stress Tolerance and Physicochemical Properties of Encapsulation Processes for *Lactobacillus rhamnosus* in Pomegranate (*Punica granatum* L.) Fruit Juice

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Abstract Encapsulation of *Lactobacillus rhamnosus* was performed using spray and freeze-drying. Maltodextrin and gum arabic were used in different combinations for spray-drying. Values of 50% maltodextrin and 40% gum arabic gave best results. Spray-drying was done at temperatures ranging from 110 to 150°C. Survivability, acid tolerance, antibiotic sensitivity testing, and total anthocyanin content and physical properties of moisture content, water activity, color analysis, bulk density, and tap density were analyzed. The moisture content of encapsulated powders ranged from 6.51 to 7.72% (wet basis) and bulk density and tap density values ranged from 0.334 to 0.308 g/cm³ and 0.350 to 0.330 g/cm³, respectively. Total anthocyanin contents were 19.28 and 7.264 mg/100 mL, respectively, for freeze and spray-dried powders. Freeze-dried probiotic pomegranate juice powder yielded best results with high survivability of *Lactobacillus rhamnosus*, a high total anthocyanin content, and other properties.

Keywords: encapsulation, *Lactobacillus rhamnosus*, pomegranate juice, spray drying, freeze drying

Introduction

In general gastrointestinal tract present in human maintain a rich microbiota comprising of about 1,800 genera (1). Microbial colonization in humans and animals occur in the colon and the majority of gut microbes are obligate anaerobes (2). Gut microbes lend to the wellness of human being as they have significant roles in the physiology of the host digestive process and assimilation of nutrients (3). Of all the human gut microbes studied so far, *Lactobacillus* spp., which belongs to a group of bacteria collectively known as lactic acid bacteria (LAB). These bacteria are omnipresent and natural dwellers of many corners, including milk, meat, plants, grains, and the gastrointestinal tracts of vertebrates (4). It has been contended that the human endogenous intestinal microbiota is actually an organ of the gastrointestinal tract that is involved in nourishment, epithelial cell development, and regulation of innate immunity (5).

The gut microbiota may be suppressed or the growth pattern may be suppressed due to physiological imbalance, including diseases, severe infections, prolonged use of medications, heavy drug use, antibiotics, constant stress, and malnutrition. Disturbance of the microbiota in the gut leads to improper digestion, an immune suppressive response, and several other physiological imbalances. Microbes belonging to the microbiota when taken with food materials can lead to re-establishment of gut microflora and normal intestinal physiology.

Lactic acid bacteria have a wide variety of use as starter cultures in food and beverage fermentations from ancient times (6). Bioactive ingredients, bioactive fatty acids, and organic acids are mostly present in the functional foods (7). This concept has become big within the food industry in late years and is now a global marketing device due to increased customer awareness of the added value of these foods. Probiotics are live microorganisms that maintain health benefits (6). As fruit juices are rich in nutrients and do not contain starter cultures that emulate with probiotics for nutrients, they may be used as an alternative conveyor for probiotics incorporation. In addition, juices are often added on with oxygen scavenging ingredients, such as ascorbic acid, thus promoting anaerobic conditions (8). Fruit juices comprising large amounts of sugar that enhances probiotic growth (9). Pomegranates (*Punica granatum*, Punicaceae) are seasonal fruits. Along with other nutritional and health advantages, its juice has an anti-atherogenic effect in healthy humans and atherosclerotic effects in mice (10).

To express a therapeutic effect to a host, probiotics must be alive at the time of processing and production of food and must retain viability after reaching the gastrointestinal tract. This is mainly dependent upon various factors, including strain, culture conditions, growth parameters, fermentation medium, drying conditions, and selection of a delivery vehicle to the gastrointestinal tract. During processing, production and gastric transit, probiotic bacterial cells face stresses such as heat and cold vulnerability during processing,

high hydrostatic pressure during packaging, oxidative and osmotic stress on production, and acid exposure to digestive enzymes during gastric transit. Thus, the quality of being able to remain viable during these steps is an important criterion for strain selection.

Protection of probiotics using microencapsulation is a method for improvement of viability in functional foods. Maltodextrin, gum arabic, xanthan gum, and whey protein concentrates are often used as encapsulation materials due to non-toxicity and ready availability. The microencapsulation agent acts as a prebiotic as well as a protects the probiotic microorganism during subsequent spray drying (9). The objective of this study was to encapsulate *Lactobacillus rhamnosus* for use with pomegranate juice using spray-drying and freeze-drying, and to study the effects of drying methods on survivability, stress tolerance, and physicochemical properties.

Materials and Methods

Test organism The probiotic bacterium *Lactobacillus rhamnosus* (MTCC-1408) was obtained from the Institute of Microbial Technology (IMTECH), Chandigarh, India as lyophilized bacterial granules were seeded into Sterile Man, Rogosa, and Sharpe Agar (MRS) broth and incubated in incubator for 72 h at 37°C. The homogeneous bacterial suspension thus formed was used as a starter culture and was further subcultured up to two times under the same culture conditions to obtain a bacterial suspension of actively growing organisms.

For study of growth kinetics, a volume of seed culture grown overnight in MRS broth was added to fresh sterile MRS broth to obtain an initial optical density of bacterial culture at 600 nm measured using a spectrophotometer. The optical density was measured every h after inoculation. A blank uninoculated and sterile medium of culture tube was kept as a reference. For optimization of pH, two culture tubes with pH values of 2 and 4 were used to check the pH and the optical density was recorded after inoculation and incubation of the organisms by shaker incubator.

Fruit juice preparation Ripened pomegranate fruits were purchased from a local fruit market in Potheri Local Market, Chennai, India in September of 2014, and fruits were washed, air dried by hot water and surfaced sterilized by using 70% ethanol. Fruits were then peeled and mashed in a blender. The fruit sap was filtered using sterile muslin cloth and Whatmann filter paper No.1. The fruit juice was passed through a syringe filter (0.22 µm) to eliminate microbes before use.

Encapsulation of *Lactobacillus rhamnosus* *L. rhamnosus* was freshly cultivated twice in MRS broth. The bacterial suspension was transferred to 6 centrifuge tubes and centrifuged at 4,500×g for 15 min. The supernatant was removed and bacterial cells were washed with sterile distilled water with addition of water to pellets, resulting in resuspension of pellets, followed by centrifugation by

centrifuge equipment at 4,500×g for 15 min, then the supernatant was removed. Bacterial cells were again resuspended in sterile distilled water, then incorporated into 100 mL of pomegranate juice at a final concentration of 1% (v/v), mixed thoroughly by the mixing of bacterial culture, and incubated in shaker incubator at 37°C for 24 h.

Preparation of encapsulation materials Maltodextrin and gum arabic were used as a carrier material for encapsulation at concentrations ranging from 10 to 50%. A free flowing powder was obtained using 50% maltodextrin and 40% gum arabic. Wall materials were mixed with 100 mL of water and kept at 50°C for 10 min on a hot plate, then inoculated fruit juice was added and homogenized by mixing of encapsulated material before spray drying.

Encapsulation using spray and freeze-drying Powder was obtained using a laboratory tall type spray dryer (SMST; S.M. Scientech, Kolkatta, India). The spray dryer had a spray nozzle and 2 fluid atomizers with a 0.7 mm diameter. Experiments were conducted at temperatures ranging 110 to 150°C and the outlet air temperature was kept constant at 80°C. Inoculated pomegranate juice with maltodextrin and gum arabic was fed into the drying chamber using a peristaltic pump. The product obtained was sealed by sealers in polythene bags and stored in refrigerator at 4°C. Encapsulation was also performed using a freeze dryer at −40°C.

Survivability testing For analysis of the viability of *L. rhamnosus* in probiotic powder obtained using spray and freeze-drying methods, powder was separately added to culture tubes containing sterile MRS broth and incubated for 48 h at 37°C in shaker incubator at 120 rpm. Growth was recorded in spectro-photometer and based on optical density values at 600 nm with reference to sterile MRS broth (11).

Acid tolerance testing One mL of fresh culture was added to conical flasks containing 100 mL of MRS broth at pH values of 2.0 and 4.0, followed by incubation in incubator at 37°C. Every hour, a broth sample was taken and the optical density was recorded at 600 nm. The growth pattern for 24 h was recorded for development of a growth curve under acidic conditions (12).

Antibiotic sensitivity testing The agar disk diffusion method was used for assessment of the antibiotic sensitivity of *L. rhamnosus*. An MRS agar plate was spread with 100 µL of active log phase culture and 4 antibiotic disks were placed on the plate along with a sterile disk as a negative control using a sterile forceps. Antibiotics used were 30 µg of amikacin, 30 µg of tetracycline, 30 µg of ofloxacin, and 30 µg of azithromycin. Plates were incubated in incubator and zones of inhibition were measured after 48 h (13).

Analysis of physicochemical properties

Moisture content: The moisture content was determined following

the Association of Official Analytical Communities (AOAC) method (14). Five g of encapsulated powder was kept at 105°C for 3 h in an oven. Powder samples were removed from the oven, cooled in a desiccator, and weighed in weighing machine; weight loss was calculated with respect to the initial powder sample weight.

Water activity (a_w): The water activity was determined using a water activity meter (Aqualab series 3TE; Device Co., Christchurch, New Zealand).

Color analysis: The color of encapsulated *L. rhamnosus*-pomegranate powder was measured using a Hunter Lab Color quest XE-Hunter color meter based on L^* , a^* , and b^* values (15).

Bulk density and tap density: The bulk density was determined based on division of the powder weight by the volume occupied. Tap density was determined based on division of the weight of the powder by the volume occupied after tapping the cylinder 20 times (15).

The total anthocyanin content (TAC): The total anthocyanin content of pomegranate juice was determined using the pH differential method with 2 buffer systems following the method outlined by Zarei *et al.* (16). A potassium chloride buffer had a pH of 1.0 (25 mM) and a sodium acetate buffer had pH of 4.5 (0.4 M). The pH was attained after addition of hydrochloric acid. Two g of powder was dissolved in 20 mL of distilled water and centrifuged at 4,000×g for 15 min. The supernatant was used for measurement of the total anthocyanin content. Powder samples of 1 g were mixed with 24 mL of corresponding buffers like potassium chloride, sodium acetate and measure the reading by spectrophotometer against water as a blank at 510 and 700 nm. The absorbance (A) value was calculated using the following equation with absorbance at 510 nm as A_{510} and absorbance at 700 nm as A_{700} :

$$A = (A_{510} - A_{700})pH_{1.0} - (A_{510} - A_{700})pH_{4.5}$$

The total anthocyanin content (TAC) of each powder sample (mg of cyaniding-3-glucoside/100 mL) was determined as:

$$TAC = \frac{A \times MW \times DF \times 100}{MA}$$

where A is absorbance calculated, MW is the molecular weight of cyaniding-3-glucoside (449.2), DF is the dilution factor (25), and MA is the molar extinction coefficient of cyanide-3 glucoside (26,900) (17).

Statistical analysis All tests were carried out at least in 3 replications and physicochemical property data were analyzed using an analysis of variance (ANOVA) with SPSS 19.0 software (SPSS Inc, Chicago, IL, USA).

Results and Discussion

Survivability testing Survivability of *L. rhamnosus* under acidic

Fig. 1. Optical density values showing survivability of probiotic bacteria after encapsulation using different methods.

conditions showed a clear pattern of rate of survival based on treatment of bacteria during the encapsulation process. Samples treated at −40°C had more regenerative power than samples treated at a high temperature. The viability of probiotic bacteria after encapsulation using different drying methods is shown in Fig. 1. Powders encapsulated and dried at a high temperature (110 to 150°C) by using a spray-drier had lower survival rate than freeze-dried powder sample with a lower rate of survivability for samples treated at 150°C than for samples treated at 110°C during drying after encapsulation. The survival rate of probiotic bacteria decreased when the spray-drying method was used (18). To and Etzel (19) reported that the survival rate of spray-dried bacteria was much lower than for freeze-dried LAB. Kim and Bhowmik (20) reported that high temperatures reduced the viability of lactic acid bacteria probiotics during spray-drying of plain yogurt.

Acid tolerance *L. rhamnosus* was resistant to acidic conditions. Treatment with freeze-drying at −40°C was superior providing more bacterial resistance, compared with encapsulation using spray-drying at 110 to 150°C. Spray-dried powders exhibited a slower growth pattern under acid stress conditions (Fig. 2). *L. rhamnosus* was resistant and tolerated low pH conditions, which indicated probable survival in the gastric juice of the stomach and survival to pass through the stomach and colonize the intestine. Hassanzadazar *et al.* (12) reported that bacterial growth increased at a low pH of 2.

Antibiotic sensitivity The zone of inhibition of *L. rhamnosus* is shown in Fig. 3. The bacterium was screened against 4 antibiotics and was found to be sensitive to 21.0 mm amikacin, 24.0 mm ofloxacin, and 31 mm azithromycine, and less sensitive to 8.5 mm tetracycline. *L. rhamnosus* is a drug sensitive bacterium, the growth of which was checked in the presence of several antibiotics (21). *L. rhamnosus* was resistant towards tetracycline and could survive against this drug during mutual delivery of drugs and probiotics.

Physicochemical properties

Moisture content and water activity: Physicochemical properties of spray and freeze-dried pomegranate powders are shown in Table 1.

Fig. 2. Optical density values showing growth patterns of bacteria at different pH values under different temperatures.

Spray-drying temperatures of 110, 120, 130, 140, and 150°C were used, leading to variations in the moisture content of 7.72, 7.60, 7.49, 7.10, and 6.51% (wet basis, w.b), respectively. As the temperature increased, there was a decrease in the moisture content. Under high temperatures, the amount of heat and the rate at which heat is transferred to particles were reported to both higher, producing a large impact for moisture evaporation (22). In the process of freeze-drying, the temperature used was −40°C and the moisture content of the product obtained was 9.37%. The moisture content of spray-dried pomegranate powder was lower than for freeze-dried powder. Thus, there was a higher degree of moisture absorption in freeze-dried powders.

The water activity of powders is shown in Table 1. Water activity values of spray-dried powders at 110, 120, 130, 140, and 150°C were 0.431, 0.412, 0.385, 0.379, and 0.369, respectively. The water activity of freeze-dried powder was 0.571. Freeze-dried powder had a higher water activity than spray-dried powder (17).

Bulk density and tap density: Bulk density and tap density values of spray and freeze-dried powders are shown in Table 1. Bulk density ranged from 0.334 to 0.308 g/cm³ for spray-dried pomegranate juice powder. Turchiuli *et al.* (23) reported bulk density values of 0.320–0.340 g/cm³ for encapsulated vegetable oil using spray-drying. The bulk density of freeze-dried powder was 0.267 g/cm³. The bulk density decreased with an increase in the spray-drying inlet air temperature, similar to a result reported by Souza *et al.* (24) and Goula and Adamopoulos (25).

The tap density ranged from 0.350 to 0.330 g/cm³ for spray-dried and 0.277 g/cm³ for freeze-dried encapsulated powder. Knowledge

Fig. 3. Zone of inhibition of *L. rhamnosus* against amikacin (1), tetracycline (2), ofloxacin (3), azithromycin (4), and a sterile disk (5).

of bulk density and tap density is important during packaging and transport of powdered materials (26). Similar results were reported by Finney *et al.* (26) and Bae and Lee (27) as 0.25–0.28 g/mL^{−1} and 0.48–0.65 g/mL^{−1} for avocado oil and orange essential oil, respectively. An increase in the drying inlet temperature lowered the tap density value, in agreement with results reported by Cai and Corke (28) and Chegini and Ghobadian (29).

Color analysis and total anthocyanin content (TAC): Color analytical results and TAC values of encapsulated powders are shown in Table 2. Spray dried pomegranate powder obtained at 110 to 150°C had L* values in the range of 74.44–83.49%. There was a visible difference in color between powder samples dried at 110 to 150°C. A similar trend was observed by Tze *et al.* (15) with spray-dried pitaya. An increased inlet temperature affected the color of the dried product due to non-enzymatic browning reactions (30). The main parameter observed in this study was the L* value. Lower the L* value, darker will be the product. The L* value of freeze-dried powder was 66.106%, indicating a better retention of color than for freeze-drying. Powder degradation in spray-drying resulted in higher L* values. The L* value was higher for powder samples dried at 150°C than for samples dried at 110°C.

The anthocyanin content also affected the powder color. The highest TAC value of 19.287 mg/100 mL was observed for freeze-dried powder. The lowest TAC value of 7.264 mg/100 mL was observed in powder spray-dried at 150°C. Powders dried at 110°C exhibited values of 15.028 mg/100 mL and showed better retention of TAC values than powders dried at 150°C with values of 7.264 mg/100 mL. The color of pomegranate juice powder changed from light pink to brown due to the anthocyanin pigment content. Anthocyanin retention was affected by temperature. Thus, variation in the

Table 1. Physicochemical properties of pomegranate juice powder

Drying Condition				
Spray drying (°C)	Moisture content (% w.b)	Water activity (a _w)	Bulk density (g/cm ³)	Tap density (g/cm ³)
110	7.720±0.060	0.431±0.038	0.334±0.015	0.350±0.006
120	7.600±0.020	0.412±0.012	0.323±0.003	0.340±0.030
130	7.490±0.390	0.385±0.042	0.318±0.008	0.330±0.004
140	7.100±0.000	0.379±0.011	0.316±0.005	0.330±0.003
150	6.510±0.150	0.369±0.027	0.308±0.001	0.314±0.002
Freeze-drying	9.370±0.010	0.571±0.002	0.269±0.004	0.277±0.000

Table 2. Color attributes and total anthocyanin contents (TAC) of pomegranate powders

Drying Condition	Color attribute			Total anthocyanin content (mg/100 mL)
	L*	a*	b*	
Spray-drying (°C)				
110	74.440±0.000	6.330±0.012	0.976±0.015	15.028
120	76.380±0.000	5.623±0.006	1.280±0.003	13.609
130	77.760±0.000	4.723±0.012	1.356±0.008	11.522
140	82.620±0.033	3.990±0.005	1.236±0.008	8.349
150	83.480±0.100	4.040±0.000	1.236±0.003	7.264
Freeze-drying	66.106±0.082	4.180±0.011	3.960±0.005	19.287

powder anthocyanin content might have been related to differences in the temperature of spray-drying and freeze-drying for powder production. Yousefi et al. (10) stated that, residues of spray dried product were collected from connecting tubes with a greater air flow, since the stickiness of pomegranate juice might be the cause of the low total anthocyanin content.

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