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Method for the preparation of stabile microencapsulated lactic acid bacteria

H.S. Kim, B.J. Kamara, I.C. Good and G.L. Enders, Jr.

Miles Inc., Elkhart, IN, U.S.A.

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SUMMARY

A method to produce viable and stabile dry microorganisms for food and agricultural purposes was developed. Spray-dried, freeze-dried or liquid culture concentrates of lactic acid-producing bacteria were mixed with various bulking agents to form a homogeneous wet granulation having a water content of 35–60% (w/w). The wet granulation was extruded through a dye onto a spinning plate (350–500 rpm) of a spheronizing device which resulted in the formation of discrete spherical particles. After forming spheres, the aggregate cell particles, both coated and uncoated, were dried to a moisture level of 5–10% using a temperature below the microorganism's optimum growth temperature. The coated and uncoated products were stored at different temperatures and periodically sampled to determine stability. Uncoated cell particles were more stabile at 4°C than at 22°C for 76 days. While both coated (with sodium alginate or carboxymethylcellulose) and uncoated particles showed similar stability at 4°C, at higher storage temperatures the applied coating improved the storage stability of the culture particles.

INTRODUCTION

Dried cultures have been employed as an ingredient by food and agricultural industries and the retention of the dry culture viability in marketed products has been a great concern. Lactic acid bacteria have been the primary microorganisms of interest in these industries and several approaches have been investigated to enhance storage stability of the dried cultures. The use of stabilizers has been reported to reduce the loss of cell viability during freeze-drying [2,3,9]. In order to minimize viability losses incurred during the drying process, several investigators have evaluated various drying methods [1,7,10,11]. In addition to drying, particle microencapsulation could have the potential to: (1) increase shelf-life of cultures by slowing down the rate of viability loss at room temperature; and (2) increase the durability of the cells by minimizing the effects of atmospheric moisture and oxygen, which can be detrimental to the dried cells.

A process for accelerating the aging process of cheese [8] by the use of a microencapsulated enzyme

Correspondence: G.L. Enders, Jr., Miles Inc., 1127 Myrtle St., P.O. Box 932, Elkhart, IN 46515, U.S.A.

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or an enzyme-containing microorganism was developed by means of multiple-phase emulsion techniques which maintained enzyme activity. Several other studies reported in the patent literature based on microencapsulation techniques dealt mostly with the preparation of enzyme spheres or aggregates and coating thereof. Recently a patent application [4] described a method for the production of spherically shaped bacterial cell aggregates. This method involves introducing a flocculating agent plus cross-linking agent into an aqueous medium, thereby forming a cell/cross-linked polyamine aggregate which can be extruded and spheronized. The primary objective of this methodology was to form spheres of immobilized active intracellular enzyme rather than to maintain viable cells of the microorganisms.

The objective of this study was to develop a method for the preparation of viable and stabile dry microorganism-containing particles for food and agricultural applications. Protection of the lactic acid-producing cultures was shown by employing coating technology to improve storage stability. Several factors influencing the viability of the cultures include: (1) residual moisture content of the cell particles [5,7]; (2) storage temperature [6]; (3) chemical scavenger effects of additives; (4) inherent culture resistance to different environmental factors; (5) atmosphere under which particles are stored; and (6) the amount of exposed surface area of the particles [12]. In this investigation the design was intended to maintain the following parameters at a constant level: (1) residual moisture content (5–10% w/w); (2) air atmosphere; and (3) surface area by controlled particle size range (-10 to $+50$ mesh sieve).

METHODS AND MATERIALS

Cultures

Lactobacillus plantarum, Pediococcus acidilactici, and *Streptococcus cremoris* were grown in appropriate fermentation media to yield 1–5 $\times$ 10^9 viable cells per milliliter and harvested by continuous flow centrifugation. The cell concentrates or dry culture powders, prepared from the cell concentrates by lyophilization, were then used to prepare the dry particles by marumerization (the process of extruding a material as filaments or noodles which are rolled by a rotating plate into spheres).

Marumerization

Preparation with dry culture powder. Fifty parts by weight of spray-dried, viable *L. plantarum* containing 2–4% by weight of nonfat milk solids and having a moisture content of 8.8% (w/w) was mixed to homogeneity with 50 parts by weight of Solka Floc BW300 (a powdered food grade cellulose from Berlin-Gorham Group of Hackensack, NJ). One part by weight of glycerol was mixed with 54 parts by weight of distilled water and this mixture was slowly added in droplets to the homogeneous powder mixture at 22°C and mixed very slowly with a Hobart mechanical mixer. This procedure resulted in the formation of a homogeneous granulation (37% moisture) which was fed at 22°C into a Wenger XBD extruder modified to use a peripheral screen having orifices of 1.2 mm with the screw operating at 66 rpm. The extruded filaments were then placed on the 9 inch diameter friction plate of a spheronizing device to make spheres having diameters of about 1.2 mm by rotating the plate at 350 rpm.

Preparation with liquid culture concentrate. A preparation containing viable cells of *P. acidilactici, S. cremoris,* or *L. plantarum* liquid concentrate was prepared as follows:

One part by weight of glycerol was mixed with 20–24 parts by weight of the liquid concentrate. Solka Floc BW300 (12–14 parts by weight) was then added to the liquid concentrate/glycerol mixture to provide a homogeneous wet granulation. Spherical particles were obtained by the method described for dry culture powder except that a 0.7 mm screen was used on the extruder.

Drying and sizing

The spheres were dried to a final moisture content of 10% (w/w) in a Uni Glatt fluidized bed dryer from Glatt Air Techniques, Inc. (Ramsey, NJ). Inlet temperatures were controlled at 25–30°C and

resulted in outlet temperatures of 20–25°C. The dried spheres were mechanically sized by screening using appropriate molecular sieves.

Coating

Five hundred grams of uniform size spheres (sized by mesh sieving, -10 to $+50$ range) were spray-coated in the Uni Glatt fluidized bed dryer using a Wurster insert. The coatings used included: (1) 0.2% (w/w) aqueous sodium alginate (final 0.04% (w/w) coating); (2) 0.2% (w/w) aqueous carboxymethylcellulose (CMC) (final 0.04% (w/w) coating); (3) 50% (w/v) aqueous polyethylene glycol 3000 (PEG) (final 10% (w/w) coating); and (4) 200 ml of aqueous polyvinyl acetate phthalate (PVAP) solution prepared as follows. Thirty grams of PVAP was added slowly over 30 min to 200 ml of distilled water at room temperature (20–24°C) with constant mixing. High shear mixing was needed to ensure proper dispersion. After 30 min, 1.2 ml of a 30% United States Pharmacopeia grade purity solution of ammonium hydroxide was added with stirring. The resulting suspension was passed through a 40 mesh screen before spraying.

Storage conditions and viability assay

The coated and uncoated spheres were stored at various temperatures and sampled periodically to determine stability of the preparations.

For testing purposes, samples of 0.1 g were transferred into 9.9 ml sterile peptone water (0.1%) in tubes at room temperature. The dissolved samples were vortexed at high speed to break up particles and several dilutions were made in sterile 0.1% peptone water. Dilutions were plated to bracket 1×10^{10} colony forming units (cfu) per g of sample and 0.1 ml portions of the dilutions were plated, in duplicate, onto APT (All Purpose Tween medium from Difco) agar plates. The plates were incubated anaerobically for 48 h at 37°C and the cfu/g were determined by the use of a Quebec automatic colony counter. Means of duplicate plates are reported in the tables.

Enteric coated and uncoated *L. plantarum* spheres were exposed for various periods of time to simulated intestinal fluids (U.S. Pharmacopeia formulation) at pH 2, followed by a quantitative assay for viability.

Table 1

Uncoated *S. cremoris* spheres: viability during storage

Days	Viability (cfu/g)	
	4°C	22°C
0	3.0×10^9	3.0×10^9
10	3.0×10^9	3.0×10^9
42	5.6×10^9	1.6×10^8
76	3.2×10^9	1.0×10^6

RESULTS AND DISCUSSION

The dry microencapsulated culture particles were evaluated under various storage conditions for improved recovery of viability. The uncoated marumerized culture particles of *S. cremoris* (Table 1) retained viability for 76 days when stored at 4°C. Similarly *L. plantarum* (Table 2) and *P. acidilactici* (Table 3) also were stabile when stored at 4°C. Collectively, this indicates that the extrusion, formation of spheres, spheronization and drying methods employed were not detrimental to the viability of the culture. However, data for each of these uncoated culture particles stored at temperatures of 22°C or higher (Tables 1–3) showed that significant loss of viability occurred as the storage temperature increased. *P. acidilactici,* which is the most thermoduric culture of those tested, did not show the decreased viability until the particles were stored for more than 15 days at 32°C

Cell morphology has often been considered to influence the survival rates of different cultures. A comparison of the cultures tested at 22°C showed that both *S. cremoris* (Table 1) and *P. acidilactici* (Table 3), which are cocci, demonstrated higher viable cell recoveries than the rod-shaped *L. plantarum* (Table 2) after 1 month storage.

Stability of uncoated and coated (CMC and so-

Table 2

Stability of *L. plantarum* marums uncoated and coated with CMC and sodium alginate

Days	Viability (cfu/g)					
	4°C			22°C		
	uncoated	0.04% w/w CMC	0.04% w/w sodium alginate	uncoated	0.04% w/w CMC	0.04% w/w sodium alginate
1	1.9×10^{10}	1.9×10^{10}	1.9×10^{10}	1.9×10^{10}	1.9×10^{10}	1.9×10^{10}
8	3.0×10^{10}	2.1×10^{10}	1.2×10^{10}	9.1×10^{8}	1.1×10^{10}	1.0×10^{10}
15	5.6×10^{10}	2.3×10^{10}	1.6×10^{10}	7.7×10^{7}	3.2×10^{9}	5.5×10^{9}
23	–	–	–	1.3×10^{6}	5.8×10^{7}	–
51	1.4×10^{10}	3.4×10^{10}	2.5×10^{10}	–	–	–
Days	32°C			37°C		
	uncoated	0.04% w/w CMC	0.04% w/w sodium alginate	uncoated	0.04% w/w CMC	0.04% w/w sodium alginate
1	1.9×10^{10}	1.9×10^{10}	1.9×10^{10}	1.9×10^{10}	1.9×10^{10}	1.9×10^{10}
2	2.9×10^{9}	1.2×10^{10}	5.9×10^{9}	4.4×10^{7}	1.9×10^{8}	4.2×10^{7}
8	3.0×10^{4}	1.5×10^{8}	1.8×10^{8}	–	–	–
14	1.3×10^{3}	9.5×10^{4}	–	2.3×10^{4}	6.5×10^{4}	3.4×10^{4}

dium alginate) *L. plantarum* particles during storage at various temperatures is shown in Table 2. Both coated and uncoated particles, stored at 4°C, had comparable stability. At 22°C, the application of either coating improved the viable cell count more than 40-fold compared to uncoated particles after 15 days storage. Under increased storage temperatures (32°C and 37°C) both coated particles were more stabile than uncoated particles. In addition the CMC coating was found to provide a greater degree of protection against elevated temperature stress. These data agree with the report by Espina and Packard [1] which indicated that microorganisms, blended with carrier and formed into

Table 3

Viability during storage of *P. acidilactici* spheres coated with PEG

Days	Viability (cfu/g)					
	4°C		22°C		32°C	
	uncoated	10% w/w PEG	uncoated	10% w/w PEG	uncoated	10% w/w PEG
0	2.0×10^{11}	2.0×10^{11}	2.0×10^{11}	2.0×10^{11}	2.0×10^{11}	2.0×10^{11}
7	2.5×10^{11}	1.9×10^{11}	2.6×10^{11}	1.9×10^{11}	2.6×10^{11}	1.9×10^{11}
15	1.9×10^{11}	2.5×10^{11}	3.7×10^{11}	4.2×10^{11}	1.3×10^{11}	4.4×10^{11}
35	2.6×10^{11}	2.0×10^{11}	4.9×10^{11}	3.9×10^{11}	1.0×10^{9}	2.2×10^{10}

Table 4

Viability of uncoated and PVAP-coated *L. plantarum* particles upon exposure to simulated gastric fluids

Hours in gastric fluid	Viability (cfu/g)	
	PVAP-coated spheres	uncoated spheres
0.0	4.6×10^{10}	5.0×10^{8}
0.5	3.8×10^{10}	1.3×10^{8}
1.0	4.4×10^{10}	–
2.0	4.9×10^{10}	9.1×10^{7}
4.0	6.0×10^{10}	5.1×10^{4}
6.0	4.4×10^{10}	1.0×10^{2}

particles which are subjected to heat transfer effects, show varied culture survival rates depending on the physical and chemical characteristics of the carrier or, in this case, the coating.

In contrast to *L. plantarum, P. acidilactici* particles showed essentially no loss of viability at storage temperatures up to 22°C for 1 month whether uncoated or PEG-coated (Table 3). However, at 32°C both coated and uncoated *P. acidilactici* particles were stabile for only 2 weeks, while the addition of PEG enhanced the stability 20-fold after 1 month storage. These observations demonstrate not only the better inherent resistance of this culture but also the potential of the protective coating to maximize viable cell recovery under extended storage or adverse conditions.

A method of applying coatings to enhance culture storage in air at various stress temperatures has been described. Cultures used in food and agricultural applications, however, also are often exposed to other environmental extremes. One example of this type of stress is the passage of cells through the gastrointestinal tract and specifically the need to successfully pass through the low pH environment of the stomach. Table 4 illustrates the significant degree of viability loss encountered when uncoated *L. plantarum* particles are exposed to simulated gastric fluid. Coating with PVAP protected the particles during at least 6 exposure to this low pH stress.

In summary, a method for the preparation of spherical culture particles and their drying with maximum retention of viability was demonstrated. The additional coating of the spherical particles with a material which microencapsulates the culture particles and thus provides specific protection while allowing it to carry out its desired function also was shown.

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