Journal of Bioscience and Bioengineering
VOL. 120 No. 6, 650–656, 2015

ELSEVIER

www.elsevier.com/locate/jbiosc

Protection of *Lactobacillus acidophilus* NRRL-B 4495 under *in vitro* gastrointestinal conditions with whey protein/pullulan microcapsules

Burcu Çabuk and Şebnem Tellioğlu Harsa*

Department of Food Engineering, Faculty of Engineering, Izmir Institute of Technology, 35430 Gülbahçe Köyü, Urla, Izmir, Turkey

Received 3 March 2015; accepted 20 April 2015
Available online 19 June 2015

In this research, whey protein/pullulan (WP/pullulan) microcapsules were developed in order to assess its protective effect on the viability of *Lactobacillus acidophilus* NRRL-B 4495 under *in vitro* gastrointestinal conditions. Results demonstrated that WP/pullulan microencapsulated cells exhibited significantly ($p \leq 0.05$) higher resistance to simulated gastric acid and bile salt. Pullulan incorporation into protein wall matrix resulted in improved survival as compared to free cells after 3 h incubation in simulated gastric solution. Moreover WP/pullulan microcapsules were found to release over 70% of encapsulated *L. acidophilus* NRRL-B 4495 cells within 1 h. The effect of encapsulation during refrigerated storage was also studied. Free bacteria exhibited 3.96 log reduction while, WP/pullulan encapsulated bacteria showed 1.64 log reduction after 4 weeks of storage.

[**Key words:** Encapsulation; Pullulan; Whey protein; *Lactobacillus acidophilus*; Probiotic]

Viability losses during storage and gastrointestinal transit due to detrimental conditions such as harsh acidic environment, oxygen stress and enzymatic reactions reduce the functionality of probiotics to exert health benefits (1,2). Thus, microencapsulation of probiotics is considered as an effective approach for their efficient survival under gastrointestinal conditions and to improve the viability during shelf life to maintain their health promoting effects.

Additionally, mechanical protection of probiotic cells during incorporation process into food product is another advantage of microencapsulation (3,4). Despite wide applications of several microencapsulation materials for probiotics, it is a challenge to produce microcapsules for preservation of desired bacterial culture with high viability which does not cause non desirable texture in the final product. Proteins and polysaccharides are widely used materials for the microencapsulation of bioactive ingredients (5–8). Whey protein is one of the most widely used polymer for microencapsulation processes. It is proved to be an effective encapsulating agent in microencapsulation of bioactive ingredients because of their ability to form microcapsules easily under mild conditions using different techniques (9,10).

Pullulan is an extracellular polysaccharide produced by *Aureobasidium pullulans*. It is especially used as a coating material in the food industry, paper industry and pharmaceutical area because of its non-toxic, water-soluble, colorless, tasteless, odorless and heat stable characteristics. Many different industrial wastes such as potato peel, grape skin and olive oil wastes found to be used as a carbon source for the synthesis of pullulan (11,12). More recently, combination of proteins, especially whey proteins, with polysaccharides has been studied for microencapsulation of bioactive substances. Whey protein and pullulan mixtures have been studied to form edible films to reduce moisture loss and increase the shelf life in food applications (13,14).

The present study aimed at microencapsulation of probiotic *Lactobacillus acidophilus* NRRL-B 4495 within whey protein/pullulan biopolymer blend and evaluating the survivability of encapsulated cells under *in vitro* gastrointestinal conditions. We also attempted to examine protective effect of polymer matrix during storage.

MATERIALS AND METHODS

Materials Commercial strain of *L. acidophilus* NRRL-B 4495 was obtained from the ARS Culture Collection (NRRL, USA). Ox-bile and de Man, Rogosa and Sharpe (MRS) media were purchased from Fluka (Buchs, Switzerland). Trypsin (from bovine) was purchased from Merck (Darmstadt, Germany) and pepsin (from porcine stomach mucosa) from Sigma (St. Louis, MO, USA). Sunflower oil was obtained from a local store. Pullulan was a gift from Hayashibara Co. (Japan). Whey protein isolate (WP) was obtained from BiPro, Danisco. All other chemicals were obtained from Sigma.

Bacterial strain and culture preparations *L. acidophilus* NRRL-B 4495 cells were inoculated into 5 ml of MRS broth in 0.1% ratio and incubated at 37°C for 24 h under anaerobic conditions. The cultures were then subcultured into 20 ml of MRS broth and incubated under same conditions for 12 h. The cells were harvested by centrifugation at 15,000 rpm for 10 min at 4°C from 20 ml of a 12 h culture at the initial stationary phase. The supernatant was decanted, and the cells were re-suspended in 100 ml of pullulan-WP solution obtaining a cell load of about 9.0 log CFU ml^{-1}.

Formation of WP/pullulan wall matrix Whey protein isolate-pullulan microcapsules were prepared according to the method of Wood (15) with some modifications. Briefly, whey protein isolate (WP, 9% w/v) was dispersed by mixing the protein powder in sterile distilled water at ambient temperature. Protein solution was then stirred for approximately 3 h using a magnetic stirrer to ensure proper dissolution under 4°C and after hydration, protein solution was denatured

* Corresponding author. Tel.: +90 232 750 6291; fax: +90 232 750 6196.
E-mail addresses: burcucabuk@iyte.edu.tr (B. Çabuk), sebnemharsa@iyte.edu.tr (Ş. Tellioğlu Harsa).

1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2015.04.014

at 80°C for 30 min. Denatured protein solution was cooled to room temperature in ice bath.

Pullulan were dissolved in distilled water at ambient temperature and stirred for approximately 3 h using a magnetic stirrer to ensure proper dissolution. In order to form WP/pullulan polymer blend as a wall material, pullulan solution was then sterilized by 0.45 μm filter and mixed with the denatured WP solution (9.0%, w/v) at a final concentration of 2.0% (w/v).

Preparation of microcapsules Microcapsules were prepared by emulsification/cold gelation technique performed as described by Chen and Subirade (16) with some modifications. In the first step, primary water-in-oil emulsions (W_1/O) were formed by emulsifying an inner aqueous phase (W_1) made up by WP/pullulan polymer blend containing bacteria into an oil phase (O) containing 1% Polyglycerol polyricinoleate (PGPR) as an emulsifier. W_1/O emulsion was prepared by an Ultra Turrax homogenizer (Ultra Turrax, model T25, IKA Labortechnik, Staufen, Germany) at 3000 rpm for 15 min. This emulsion was then added with gentle mixing to $CaCl_2$ solution (100 mM). After formation of microcapsules, this slurry was orbitally shaked at 160 rpm for 30 min to harden the microcapsules. The hardened microcapsules were separated from the solution and oil phase by two sets of homogenization at 1000 rpm for 1 h. It is important to state that in order to avoid the destructive heat generation possibly formed during homogenization; microencapsulation process was conducted inside the ice bath.

Bacterial enumeration Viable counts of non-encapsulated *L. acidophilus* NRRL-B 4495 were determined by a pour plate method using MRS agar after serial dilutions in peptone water. The plates were incubated anaerobically at 37°C for 72 h and colony forming units were counted.

For enumeration of microencapsulated bacteria in microcapsules, 10 g of microcapsules were suspended in peptone water. This peptone water containing microcapsules was homogenized at 11,000 rpm for 5 min. Under these conditions, microcapsules were broken and samples of 1 ml of the peptone water were diluted to an appropriate dilution and plated by the pour plate technique using MRS agar. Colonies were counted after 72 h of anaerobic incubation at 37°C. Viable cell number was express as CFU per gram of microcapsule (CFU/g) and the efficiency was determined as following Eq. 1:

$$\text{Encapsulation yield } (\%) = 100 \times (N/N_0) \quad (1)$$

where N is the total viable count of *L. acidophilus* after microencapsulation and N_0 is the total viable count of *L. acidophilus* before microencapsulation.

Survival in simulated gastrointestinal conditions Simulated gastric juice (SGJ) was prepared according to the method described by Guo et al. (17) with some modifications. Saline solution (0.85%) pH was adjusted to 2.0 using 0.1 N HCl and sterilized by autoclaving at 121°C for 15 min. Pepsin was dissolved in sterile deionized water and filtered through 0.22 μm sterile membrane filter, then suspended in sterile saline to a final concentration of 3.0 g/L. To prepare bile salt solution, MRS media was supplemented with 0.6% ox-bile (18).

For resistance in simulated gastrointestinal conditions, 1.0 ml of free or 1.0 g of microencapsulated *L. acidophilus* NRRL-B 4495 cells were inoculated into 9.0 ml of sterile SGJ/bile salt solution and incubated at 37°C under orbital shaking at 160 rpm for 3 h/24 h. After the incubation, samples were removed from solutions and survival rate (%) was calculated by Eq. 2.

$$\text{Survival rate } \% = (\log \text{CFU } N_1 / \log \text{CFU } N_0) \times 100\% \quad (2)$$

where N_1 is the is the number of viable cells in microcapsules after treatment by SGJ/bile salt solution and N_0 is the is the number of viable cells in microcapsules before treatment.

Release into simulated intestinal juice Saline solution (0.85%) pH was adjusted to the 8.0 using 0.5 M NaOH and sterilized by autoclaving at 121°C for 15 min. Trypsin was dissolved in sterile deionized water and filtered through 0.45 μm sterile membrane filter, then suspended in sterile saline solution to a final concentration of 1 g/L (17). 1.0 g of microencapsulated bacteria were transferred into the 9.0 ml of simulated intestinal juice and incubated at 37°C under orbital shaking at 160 rpm for 24 h. After the incubation, samples were taken from supernatant and viable bacteria released in SIJ were enumerated. Released rate (%) was calculated according to Eq. 3:

$$\text{Release rate } \% = (\log \text{CFU } N_1 / \log \text{CFU } N_0) \times 100\% \quad (3)$$

where N_1 is the number of viable cells released from microcapsules in SIJ and N_0 is the number of viable cells in microcapsules added to SIJ.

Morphology and particle diameter distribution of microcapsules The morphology of microcapsules was examined by scanning electron microscopy (SEM). Prior to SEM, microcapsules were freeze dried and then placed on strips of double side carbon tape attached to aluminum SEM stubs and photographs were taken under low vacuum using an electron acceleration voltage of 10.0 kv with SEM (Quanta 250, FEI).

For light microscopy images, 1.0 g of microcapsules was placed on a glass microscope slide with a cover slide. Microscopic pictures were taken using an Olympus CX31 Microscope, fitted with an Olympus DP25 Camera and diameter analysis was done with software (Olympus DP2-BSW).

TABLE 1. Encapsulation efficiency and particle mean diameter of WP and WP/pullulan microcapsules loaded with *L. acidophilus* NRRL-B 4495.

	Microbial load (log CFU/g)	Encapsulation efficiency (%)	Mean particle size (μm)
WP microcapsules	9.20 ± 0.18	93.57 ± 0.21	65.12 ± 1.12[a]
WP/pullulan microcapsules	9.33 ± 0.21	93.72 ± 0.10	76.40 ± 1.54[b]

[a,b]Means ± standard deviation (n = 3) with different superscript letters in the same column indicate significant differences ($p \leq 0.05$) among the studied samples.

Color measurement Konica Minolta colorimeter (model CR 410, Konica Minolta, Tokyo, Japan) was used for color measurements of microcapsules. The CIE Lab system, defined in rectangular coordinates L*, a*, b*, where L* represents lightness, a* represents red-green and b* represents yellow-blue.

Moisture content and water activity Water activity of the microcapsules was determined using a Hygrolab C1 water activity meter (Hygrolab C1, Rotronic, Bassersdorf, Switzerland) (19). The moisture content of the microcapsules was determined gravimetrically by oven-drying at 105°C for 24 h to reach weight equilibrium (20). The mean moisture content (MC) was estimated by the following Eq. 4:

$$\text{MC } (\%) = \left[\left(W_{wet} - W_{dry}\right)/W_{wet}\right] * 100 \quad (4)$$

where W_{wet} is the weight of the wet microcapsules and W_{dry} is the weight of fully dry microcapsules.

Storage stability test In order to examine the storage stability, both free bacteria and the microcapsules were placed at 4°C in glass bottles for 4 weeks with compressed N_2 for 4 weeks. The number of viable cell counts was determined weekly and all the samples were analyzed in triplicate.

Statistical analysis Experiments were performed with three different batches of drying, and each batch was tested in triplicate. Results were expressed as means ± standard deviation. Data analysis was carried out using Minitab 14.0 software (Minitab Inc., State College, PA, USA). Significance of differences between formulations was performed by analysis of variance (ANOVA) test followed by Tukey's test (95% confidence interval).

RESULTS AND DISCUSSION

Microencapsulation Table 1 shows that existence of pullulan resulted in a bigger mean diameter size of microcapsules providing to a microcapsule size of 76.40 μm, whereas WP microcapsules obtained mean particle size of 65.12 μm. This can be attributed to the increased polymer concentration of wall matrix due to pullulan incorporation. By the increase in polymer concentration, the viscosity of the inner phase of the primary emulsion might be increased causing resistance to break into smaller droplets and resulting in larger microcapsule sizes (21–24). Besides, increased particle mean diameter, results revealed that no significant ($p > 0.05$) difference was calculated between encapsulation efficiencies of pullulan containing and non pullulan containing microcapsules. Encapsulation efficiency is one of the most important parameter indicating the effect of encapsulation process and selected wall matrix. Cell loading achieved with WP/pullulan was calculated to be 93.72%, while microencapsulation efficiency of 93.57% was achieved with WP microcapsules. The average count of *L. acidophilus* NRRL-B 4495 in WP/pullulan microcapsules was 9.33 ± 0.21 log CFU/g.

Survival under simulated gastric juice and simulated bile salt solution One of the main objectives of microencapsulation is providing protection of probiotic cells during exposure to low pH gastric environment. Fig. 1A shows the number of survived cell counts of free and microencapsulated *L. acidophilus* NRRL B-4495 under *simulated* gastric juice. After an hour of incubation, *L. acidophilus* NRRL B-4495 was reduced by 0.95 log units when added as free cells and followed nearly a linear reduction in cell numbers. At the end of incubation, viable cell numbers of free cells decreased to 6.67 log CFU/ml possessing the survival rate of 73.19%. On the other hand, microencapsulation into WP and WP/