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Encapsulation of *Lactobacillus casei* ATCC 393 cells and evaluation of their survival after freeze-drying, storage and under gastrointestinal conditions

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ABSTRACT

The aim of this work was to investigate the encapsulation of *Lactobacillus casei* ATCC 393 cells with a pea protein isolate-alginate hydrogel matrix and to study the protective effects of such matrix on the bacteria during freeze-drying, storage and under harsh gastrointestinal conditions. The encapsulation of *L. casei* achieved a high yield of 85.69% ± 4.82 which indicated that the matrix and the encapsulation technique are compatible with the probiotic strain. During the freeze-drying process, the matrix did not show any protective effect as compared to the non-encapsulated cells. The dried capsules have been taken into subsequent storage tests at three temperatures (+22, +4 and −15 °C). After 84 days of storage, the encapsulated *L. casei* stored at −15 °C showed the highest survival rate among all samples (59.9% ± 17.4). After 84 days of storage, the capsules stored at −15 °C were submitted to further survival and release tests in simulated gastrointestinal fluids. These dried and stored capsules displayed a weaker buffering effect against acidic gastric conditions as compared to the fresh capsules which were tested right after the encapsulation. However, both stored and fresh capsules showed similar release profiles of *L. casei* in simulated intestinal fluid.

1. Introduction

Probiotics are microorganisms which, when consumed in adequate amounts, confer a health benefit on the host (Burgain et al., 2011). Due to the rising interest of consumers in food products containing probiotics, the volume of research related to the subject has been expanding rapidly since the year of 2000 (Jankovic et al., 2010). The most important probiotic microorganisms in the food industry are lactic acid bacteria (LAB) (Burgain et al., 2011). They are widely used for the production of fermented dairy products such as cheese, yogurt and ice cream. Dry products such as capsules, cereal products and beverage powders containing probiotics have also been developed (Heidebach et al., 2010). Recent studies on LAB confirmed their health benefits on the human gut and immune system (Shah, 2007). Among the LAB, *Lactobacillus casei* strains are widely used in the production of fermented food (Kourkoutas et al., 2006). It has been reported that *L. casei* strains can reduce the cholesterol level (Lye et al., 2010) and can be used against cancer cell proliferation (Choi et al., 2006). However, their probiotic benefits strongly depend on their viability. It has been suggested that probiotic-based products should contain at least 7 log cfu/g of viable cells at the time of consumption to provide probiotic benefits (World Health Organization, 2006). However, it has been reported that the survival rate of probiotics is relatively low in traditional dairy products (de Vos et al., 2010). The poor survival rate of probiotics during processing and storage is attributed to environmental stressors such as the variations in pH and the toxicity of oxygen and UV light. After ingestion, adequate amount of probiotic cells have to survive through the upper digestive tract and reach the intestine of the host (Anal and Singh, 2007). The harsh gastric environment combined with a variety of digestive enzymes can lead to the loss of viable probiotic cells. The detrimental effects of simulated gastric environment on several strains of *L. casei* have been reported (Mishra and Prasad, 2005).

The entrapment of probiotic cells by encapsulation provides a physical barrier against environmental stressors (Burgain et al., 2011) and therefore reduce the unavoidable loss of viability of probiotic cells during processing, storage and digestion (Heidebach et al., 2012). The selection of a wall material for encapsulation is always a challenge. The material should be food-grade and capable

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of entrapping and protecting probiotics. Moreover, the material and the encapsulation process should be compatible with the probiotics (de Vos et al., 2010).

Food-grade natural polymers are excellent materials for encapsulation due to their non-toxicity and good biocompatibility as well as their ability to form gels. Legume proteins have been used as substrate in the production of fermented product with *L. casei* ATCC 393 (Parra et al., 2013). Moreover, capsules based on legume protein isolates and alginate have been reported (Khan et al., 2013). These capsules have been used to encapsulate *Bifidobacterium adolescentis*, an acid sensitive probiotic. These protein-polysaccharide based capsules showed an excellent protective effect against simulated gastric conditions. The dense gel structure formed by legume proteins and alginate served as a barrier between the probiotics and the environment.

Among legume proteins, pea proteins are increasingly attracting interest due to their high nutritional value, digestibility, bioavailability and long term health benefits (Yang et al., 2012). Pea protein isolate (PPI) is a food-grade material that has been used as additive to enrich the protein content in food industry (Shi and Dumont, 2013). To the best of our knowledge, no research has investigated the encapsulation of *L. casei* with pea protein isolate-alginate (PPI-alginate) based hydrogels and the survival of *L. casei* in freeze-dried PPI-alginate hydrogels during long-term storage.

The overall objective of this work was to encapsulate *L. casei* ATCC 393 cells with PPI-alginate hydrogel capsules beads via extrusion. The effects of freeze-drying and subsequent storage at different temperatures on the viability of *L. casei* ATCC 393 cells were studied. Moreover, the capsules were tested in simulated gastrointestinal conditions just after their preparation and after a period of 84 days (12 weeks) of storage. The protective effect and the release profile of the capsules before and after storage were compared to examine their stability.

2. Materials and method

2.1. Materials

L. casei ATCC® 393™ was purchased from the American Type Culture Collection (ATCC, Manassas, VA). Pea protein isolate (PPI) (Propulse N™, 81.73% protein, <10.3% sugars, <0.7% starch, 3.40% moisture, <0.5% fat and <4.0% ash) was obtained from Nutri-Pea Ltd. (Portage la Prairie, MB, Canada). Alginic acid sodium salt (alginate) with low viscosity (1% aq. solution: <300 cps) was purchased from MP Biomedicals, LLC (Solon, OH). Calcium chloride dihydrate ($CaCl_2{\cdot}2H_2O$), sodium chloride (NaCl) and hydrochloric acid (HCl) were purchased from Fisher Scientific (Fair Lawn, NJ). Sodium hydroxide (NaOH) was purchased from EMD (Damstadt, Germany). Agar and deMan, Rogosa Sharpe (MRS) broth, ammonium sulfate ($(NH_4)_2SO_4$), potassium phosphate monobasic (KH_2PO_4), potassium phosphate dibasic trihydrate ($K_2HPO_4{\cdot}3H_2O$), trisodium citrate ($HOC(COONa)(CH_2COONa)_2{\cdot}_2H_2O$), magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$), pepsin from porcine gastric mucosa (powder, ⩾250 units/mg solid) and pancreatin from porcine pancreas (4 × USP specifications) were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Preparation of the bacteria cells for encapsulation

L. casei ATCC 393 dry pellet was rehydrated with 5 mL of MRS broth and incubated for 24 h (37 °C, 200 × rpm) in an incubator (INFORS AG CH 4103 Bottingen, Switzerland) to revive the bacteria. The liquid culture was then used to inoculate MRS agar plates via t-streak. Single colonies of *L. casei* were obtained after 48 h of incubation at 37 °C under an anaerobic environment created with a jar and anaerobic atmosphere generation bags (Sigma–Aldrich, Oakville, Canada). MRS broth (100 mL) was inoculated with one single colony and incubated for 24 h at 37 °C under constant agitation (200 × rpm). Thereafter, this liquid culture was mixed with equal amount of sterilized 50% glycerol solution and stored at −80 °C to be used as stock culture.

Prior to encapsulation, single colonies obtained from streaking stock cultures on MRS plates, were grown in 100 mL of MRS broth for 24 h at 37 °C under constant agitation (200 × rpm). Cells were harvested at early stationary stage by centrifugation at 8000 rpm for 10 min at 20 °C (Sigma centrifuge, 3-16PK, Germany). The cells with a final concentration of 9 log cfu/mL were resuspended in 20 mL of sterile modified phosphate buffer ($(NH_4)_2SO_4$ 0.2%, $K_2HPO_4{\cdot}3H_2O$ 1.83%, KH_2PO_4 0.6%, $HOC(COONa)(CH_2COONa)_2{\cdot}2H_2O$ 0.1% and $MgSO_4{\cdot}7H_2O$ 0.02%).

2.3. Encapsulation

The *L. casei* loaded PPI-alginate capsules were prepared via extrusion technology (Khan et al., 2013). Sterilized distilled water and glassware were used for the encapsulation process. The 3.6% (w/v) protein solution was prepared by dissolving PPI powder in 0.05 M NaOH solution. The solution was heated to 80 °C to denature and dissolve the proteins. After 30 min, the solution was cooled to room temperature in a cold water bath and neutralized to pH 7 with 1 M HCl. The solution was reheated to 80 °C and the alginate powder was added to produce a final concentration of 0.9% (w/v). Complete dissolution of the alginate powder was achieved at 80 °C under magnetic stirring for 30 min. Thereafter, the solution was cooled to room temperature. The bacterial suspension was subsequently added to the PPI-alginate solution at a bacteria-to-polymer ratio of 1:10 (v/v). The capsules loaded with bacteria were formed via extrusion of the bacteria-polymer solution through a 26G needle into a 0.05 M $CaCl_2$ solution. The resulting capsules were allowed to harden in the $CaCl_2$ solution for 30 min. Thereafter, the capsules were collected and rinsed with distilled water. The capsules were separated into two portions. One portion was immediately used for the survival and release tests in simulated gastrointestinal conditions. These capsules will be referred as 'fresh capsules' throughout the text. The remaining capsules were freeze-dried for the storage test. The encapsulation and freeze-drying steps were quadruplicated.

2.4. Encapsulation yield

In order to investigate the survival of bacteria after encapsulation, 1 g of fresh capsules was immersed in 9 g of modified phosphate buffer. The capsule suspension was incubated at room temperature under constant agitation (250 × rpm) for one hour to completely dissolve the capsules. Preliminary tests confirmed that no significant change occurred on the numbers of *L. casei* viable cells after one hour of incubation in the modified phosphate buffer at room temperature. Viable cells (colony forming units) enumeration was conducted by plating 10-fold serial dilutions of dissolved capsules and free *L. casei* cell suspension onto MRS agar plates. The plating was incubated at 37 °C for 48 h in an anaerobic environment before colony enumeration. Plating was done in triplicate. The encapsulation yield was calculated by applying Eq. (1):

$$\text{Encapsulation yield} = \frac{\text{total viable cells after encapsulation}}{\text{total viable cells before en capsulation}} = \frac{N_E \times M_E}{N_0 \times V} \quad (1)$$