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Improving survivability of *Lactobacillus plantarum* in alginate-chitosan beads reinforced by Na-tripolyphosphate dual cross-linking

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ABSTRACT

In the present study, *Lactobacillus plantarum* PTCC 1058 was microencapsulated in Alginate-chitosan blended beads based on ionically dual cross-linking by Na-tripolyphosphate (Na-TPP). The homogenous solution of bacteria and alginate was dripped into the gelling bath of calcium chloride. After 30 min hardening, filtrate beads were transferred into the solution of chitosan. Dual cross-linked gel beads were prepared by employing Na-TPP solution to the chitosan coated beads. Morphology, particle size, alginate-chitosan interaction, characterizing the structure of single and dual cross-linked beads were determined by using scanning electron microscopy SEM, FTIR and XRD analysis. FTIR analysis demonstrated fabrication of the Na-tripolyphosphate dual cross-linked microcapsules. X-ray diffractograms revealed less amorphous nature of Ag/Cs/TPP microcapsules compared to Ag/Cs ones. Survivability of probiotics and swelling behavior of microcapsules were also evaluated under simulated gastrointestinal conditions. Based on the results, the highest viability corresponds to the dual cross-linked beads (by only 1.58 ± 0.03 log reduction) compared to single cross-linked alginate (2.26 ± 0.09 log) and chitosan-coated alginate beads (1.78 ± 0.1 log). Thus, the reinforced microcapsules could be suitable for improving the capsule stability in site-specific delivery applications.

1. Introduction

Probiotics are defined as “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (Hill et al., 2014). Interest in using probiotic bacteria as food cultures has been increased continuously. However the viability of probiotics is reduced significantly when incorporated into foods and during the passage through harsh environment of the gastric and intestinal tract (Chandramoulia, Kailasapathy, Peirisb, & Jones, 2004). Alginate hydrogel microspheres are one of the most attractive delivery systems widely for entrapment of bioactive components and delivery applications due to excellent biocompatibility, biodegradability, mild gelation conditions in the present of multivalent ions, generally recognized as safe, low cost and low toxicity (Cook, Charalampopoulos, & Khutoryanskiy, 2014). Major limitations in using pure alginate beads such as permeability and mechanical properties can be improved by coating with a polycation such as chitosan. Ionic interaction between amino groups of chitosan and carboxyl residues of alginate reduces the porosity of alginate beads (Vandenberg, Drolet, Scott, & Noüe, 2001; Wong, Chan, Kho, & Sia Heng, 2002; Xu, Zhan, Fan, Wang, & Zheng, 2007).

Many researchers have been widely studied on the efficient application of alginate-chitosan polyelectrolyte complex (Caetano, Almeida, & Gonçalves, 2016; Gåserød, Smidsrød, & Skjåk-Bræk, 1998; Lee, Cha, & Park, 2004; Zhao et al., 2007). The preparation of beads is mainly categorized into two methods: one step method consists of the dispersion of cells and sodium alginate in which it is dropped in a mixture solution of calcium chloride and chitosan. The second one is the two-step method in which the dispersion of cells and sodium alginate is dropped into calcium chloride and then the resultant beads were transferred into a chitosan solution (Xu et al., 2007). In both methods which are mainly focused on single cross-linking via calcium ions, dealing with the problems of bio adhesion, pH sensitivities, stability and in vitro release are a great concern.

Dual cross-linking can enhance the stability of microparticles and prevent the adhesion of them. $CuCl_2$ and glutaraldehyde were used for chemically dual cross-linking of alginate-chitosan blend beads and resulted in quick sorption of metal ions in wastewater (Gotoh, Matsushima, & Kikuchi, 2004). Physical cross-linking agents like sulfate, citrate, and tripolyphosphate are considered more biocompatible and much less toxic than chemical cross-linkers and can easily interact ionically with chitosan. The effect of TPP cross-linked Ag-Cs microspheres was investigated and resulted in slower release (20–30% release during 4 h) of ampicillin compared to single layer Ag-Cs beads

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(70% release during 4 h) (Anal & Stevens, 2005). The alginate-chitosan gel beads reinforced by a natural cross-linker, genipin, prepared for the purpose of drug delivery (Mi, Sung, & Shyu, 2002). Based on the results of this study, the stability of beads increased and the release rate was limited by increasing the degree of cross-linking. Blended alginate-chitosan beads prepared based on dual ionic cross-linking by sodium sulfate, had the potential for drug delivery system (Xu et al., 2007). Alginate-chitosan microcapsules were synthesized for enzyme immobilization and cured by placing them into a 3% (w/v) Na-TPP solution for 90 min (Taqieddin & Amiji, 2004). Na-tripolyphosphate cross-linking of alginate-chitosan beads resulted in the phosphate ions extracting Ca^{2+} and liquefying the system. In the case of Ba^{2+} alginate, solid core microcapsules were formed as could not be extracted by Na-TPP.

The main purpose of this study was to entrap *Lactobacillus plantarum* PTCC 1058 in the stable alginate-chitosan microbeads based on a dual ionic cross-linking agent, Na-tripolyphosphate (Na-TPP) and to investigate the effect of Na-TPP on the survivability of bacteria in the simulated gastrointestinal tract in comparison to single cross-linked beads.

2. Materials and methods

2.1. Materials

Medium molecular weight chitosan with 85% degree of deacetylation and sodium alginate (MW = 80–120 kDa with a viscosity ~ 2000 cp of for a 2% aqueous solution at 25 °C) were applied as matrix or coating polymers (Sigma Aldrich, USA). Calcium chloride dehydrate (Merck, Germany) and penta sodium tripolyphosphate (Sigma Aldrich, USA) were employed as cross-linking agents. *Lactobacillus plantarum* PTCC 1058 as probiotic bacteria strain (Bagher Hashemi & Mahmoodi, 2017; Soltan Dallal, Zamaniahari, Davoodabadi, Hosseini, & Rajabi, 2017; Shafiei, Razavilar, Mirzaei, & Javadi, 2012; Soltan) supplied from Iranian Research Organization for Science and Technology (IROST, Iran). Bile salts, pepsin (0.7 FIP-U/mg), pancreatin (350 FIP-U/g protease, 6000 FIP-U/g lipase, 7500 FIP-U/g amylase), sodium hydroxide, hydrochloric acid (HCl), KH_2PO_4 were used for preparing simulated gastric and intestinal juices and were provided either by Merck or Sigma Aldrich companies. All others such as MRS (de Man, Rogosa, Sharpe), agar-agar, sodium chloride (NaCl), sodium citrate were of analytical grade and purchased from Merck.

2.2. Preparation of bacterial cells and solutions

Lactobacillus Plantarum was cultured in MRS broth at 37 °C under aerobic conditions. Cells were harvested after 24 h incubation by centrifugation at 4000 × g and 4 °C for 10 min and washed twice with sterile physiological saline and enumeration was done by pour plate method in MRS agar in order to determine the initial cells used for encapsulation. Chitosan solution was prepared by dissolving 0.5 g of powder in 0.3 ml acetic acid and adjusting the pH to 5.0–5.1 by adding 0.1 mol/l NaOH. The final volume was adjusted to 100 ml and stirred overnight for complete dissolution. Other solutions were prepared by adding a certain amount of powder into distilled water. All solutions used were autoclaved at 121 °C for 20 min and the procedure was performed in a laminar air-flow biological hood under sterile conditions. Each assay was repeated in triplicate.

2.3. Encapsulation of L. plantarum PTCC 1058

Microcapsules prepared by extrusion method based on the previous reports (Krasaekoopt, Bhandari, & Deeth, 2004; Xu et al., 2007). In this method, 10 ml of 3%w/v sodium alginate blended with centrifuged cells were dripped through a 21. g syringe needle with a constant flow rate of 1 ml/min adjusted by a syringe pump (HPM 1000 SP, Fnm Co., Iran) into 5% w/v $CaCl_2$ as a first cross-linker agent. After 30 min hardening in the gelling bath of $CaCl_2$, smooth and spherical beads were formed immediately. Beads were filtered by vacuum filtration (ZSA-4D 8CFM, Zensen Co., Japan) and were washed with distilled water. Chitosan-coated beads were prepared by transferring calcium-alginate beads into 0.5% w/v chitosan solution and were cured for 40 min under gentle agitation. Resultant beads were washed with distilled water, followed by 2% w/v Na-TPP solution as a second cross-linker agent in order to prevent adhesion of beads by hardening the chitosan coat. Alginate-chitosan and alginate-chitosan-TPP beads were freeze-dried by a freeze drier (LP plus4-2 ALPHA, Crist Co., Germany) for further analysis or used directly to evaluate the survivability of cells in simulated gastric and intestinal juices.

2.4. Morphology observation

Surface morphology and diameter of freeze-dried beads were characterized by using scanning electron microscopy (ALS 2010; Seron technologies Inc., Korea). For this purpose, a layer of gold was coated on the samples under vacuum before observation.

2.5. Encapsulation efficiency (EE)

Encapsulation yield was defined as the number of the viable bacterial cells entrapped inside the bead divided by the amount of the initial cells used for encapsulation. The number of viable cells was determined by diluting 1 ml of sample into 9 ml of sterile saline serially and plated 0.1 ml of each diluted sample on MRS agar by pour plating method in triplicate. After 48 h incubation at 37 °C under aerobic conditions, colony forming units (CFU) were determined. In order to enumerate the viable cells entrapped in beads, 1 gr freshly beads were mixed with 9 ml of 3%w/v sodium citrate solution by gently stirring until the beads were released completely. The encapsulation efficiency was measured according to the equation below:

$$EE\ (\%) = (N/N_0) \times 100 \quad (1)$$

Where N is a cell log after microencapsulation and N_0 is before microencapsulation.

2.6. FT-IR spectroscopy analysis

Fourier transform infrared spectroscopy was performed using an FTIR spectrophotometer (Spectrum RX I, Perkin Elmer, USA) to characterize the interaction between alginate, chitosan, and TPP in the microcapsules. Peaks adsorptions were recorded in the range of 400–4000 cm^{-1}.

2.7. X-ray diffraction analysis

The X-ray diffraction data were obtained with an X-ray diffractometer (X'Pert PRO MPD, Panalytical, Netherlands) equipped with Ni-filtered CuKα radiation. Scan speed at 0.02°/s was operated at 40 kv/30 mA and the 2θ angle was in the range of 10°-40°.

2.8. Swelling analysis

The capacity of water adsorption in single and dual cross-linked alginate-chitosan beads at dry state was calculated. A certain amount of dried beads was incubated in simulated gastrointestinal tract at 37 °C under 150 rpm shaking. First, beads were swollen in a solution of 0.2% NaCl at pH 1.2 for 2 h. Then the medium was replaced by sodium phosphate buffer at pH 7.5. Every 30 min, samples were taken out and blotted by a paper towel and weighted immediately. The ratio of swelling was calculated as follows: