Carbohydrate Polymers 96 (2013) 181–189

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Microencapsulated probiotics using emulsification technique coupled with internal or external gelation process

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ARTICLE INFO

Article history:
Received 17 November 2012
Received in revised form 28 February 2013
Accepted 24 March 2013
Available online 29 March 2013

Keywords:
Alginate–chitosan microcapsule
Emulsification/external gelation
Emulsification/internal gelation
Probiotics

ABSTRACT

Alginate–chitosan microcapsules containing probiotics (Yeast, Y235) were prepared by emulsification/external gelation and emulsification/internal gelation techniques respectively. The gel beads by external gelation showed asymmetrical structure, but those by internal gelation showed symmetrical structure in morphology. The cell viability was approximately 80% for these two techniques. However, during cell culture process, emulsification/internal gelation microcapsules showed higher cell growth and lower cell leakage. Moreover, the survival rate of entrapped low density cells with culture (ELDCwc) increased obviously than that directly entrapped high density cells (dEHDC) and free cells when keeping in simulated gastrointestinal conditions. It indicated the growth process of cells in microcapsule was important and beneficial to keep enough active probiotics under harmful environment stress. Therefore, the emulsification/internal gelation technique was the preferred method for application in food or biotechnological industries.

1. Introduction

Probiotics are defined as live microbial supplements, which beneficially affect the host by improving its intestinal microbial balance (FAO/WHO, 2002). Probiotics can relieve lactose intolerance, promote the immune system, reduce serum cholesterol and control gastrointestinal infections (Delcenserie et al., 2008; Roberfroid, 2000; Zhou, Pillidge, Gopal, & Gill, 2005). However, the number of active bacteria must be sufficient to be potential probiotics. The International Dairy Federation (IDF) recommends that the number of active bacteria for therapeutic effects should be 10^7 colony-forming units (CFU)/g (Ouwehand & Salminen, 1998). As a result, different approaches have been proposed to improve the viability of cells in food during shelf life, to endure the acidic conditions of stomach, and to protect from degradation by hydrolytic enzymes and bile salts in the small intestine (Doleyres, Fliss, & Lacroix, 2004; Latha, Babu, Thompkinson, & Kapila, 2010).

To date, microencapsulation techniques have been widely applied to provide probiotics with a physical barrier against adverse environmental conditions in commercial products and intestinal tract (Anal & Singh, 2007; Kailasapathy, 2002). Several studies have shown successful microencapsulation and coating of bacteria using various encapsulating materials and methods (Arup, Kyoung-Sik, & Harjinder, 2011; Géraldine et al., 2010; Maria & Sofia, 2009).

The most common materials used for probiotics encapsulation are food grade biopolymers, such as alginate or chitosan, gellan and xanthan gum, gelatin and whey proteins. The gelation mechanism of protein-based materials was based on protein denaturation and temperature-triggered instant gelation effect. For example, milk-proteins containing probiotic cells were incubated with rennet solution at 5 °C to perform the enzymatic cleavage of the κ-casein, which leads to an aggregation of the casein micelles. Followed initiation with $CaCl_2$ solution, above cold-renneted mixture was dispersed in cold vegetable oil to form water-in-oil (W/O) emulsion. Subsequently the temperature was quickly raised to 40 °C to make the emulsified droplets into gel particles instantly (Heidebach, Först, & Kulozik, 2009). Gellan–xanthan-based microcapsules are also formed by temperature-triggered process. However, the heating process and the multivalent metal ions necessary for gelation reaction have harmful effect on cell viability.

Alginate is a linear unbranched polysaccharide composed of varying proportion of 1,4-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues, which has the ability of mildly forming a gel when it encounters some divalent cations such as Ca^{2+} (preferably used due to biocompatibility) and Ba^{2+} (except Mg^{2+}) (Liu et al., 2002; Mørch, Donati, Strand, & Skjåk-Bræk, 2006). It is a generally regarded as safe (GRAS) material by FDA (George & Abraham, 2006),

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http://dx.doi.org/10.1016/j.carbpol.2013.03.068

and the acceptable daily intake (ADI) is "not specified", which is the highest possible classification for food additives. Therefore, alginate has been widely used as matrix for probiotics microencapsulation for several decades. Moreover, the mechanical and physicochemical properties of alginate gel can be improved using polymers such as chitosan (Yu et al., 2010) and poly-L-lysine (Cui, Cao, Choi, Lee, & Lee, 2006) through forming the complex microcapsule membrane by electrostatic interaction.

Several methods of alginate microencapsulation of probiotics have been reported including spray-drying, spray-coating, extrusion, emulsification, and coacervation (Champagne & Fustier, 2007; Gouin, 2004). Considering the simplicity, low cost, and gentle formulation conditions for high retention of cell viability, extrusion and emulsification techniques are extensively used (Eng-Seng et al., 2011; Homayouni, Ehsani, Azizi, Yarmand, & Razavi, 2007). Extrusion technique is widely used at lab-scale for scientific research because of low yield, while emulsification technique has a potential for large-scale production of the beads in shorter time (Takei, Yoshida, Hatate, Shiomori, & Kiyoyama, 2009), which is essential for commercial application. For emulsification/external gelation technique, polysaccharide aqueous solution is dispersed in oil phase to form W/O emulsion, and calcium chloride solution is then added at stirring for gelation and encapsulation of probiotics (Homayouni, Azizi, Ehsani, Yarmand, & Razavi, 2008; Mokarram, Mortazavi, Najafi, & Shahidi, 2009; Wan, Heng, & Chan, 1992). However, the disruption of the emulsion system equilibrium may cause a significant clumping of microcapsules (Poncelet, Babak, Dulieu, & Picot, 1999) before properly hardening. For emulsification/internal gelation technique, an insoluble calcium salt is added to alginate solution and the mixture added into oil to form emulsion. The latter is acidified to release Ca^{2+} from the insoluble salt for cross-linking with alginate (Poncelet et al., 1992).

Although external and internal gelation techniques have been used to prepare alginate microcapsules as drug carriers of DNA, BSA, and acetaminophen (Chan, Lee, & Heng, 2006; Quong, Neufeld, Skjåk-Bræk, & Poncelet, 1998; Vandenberg & De La Noüe, 2001), the external gelation was based on extrusion technique instead of emulsification technique. To our knowledge, there are only four research reports about using emulsification/internal gelation technique to prepare alginate microcapsules for cell immobilization (Corinne et al., 2011; Larisch, Poncelet, Champagne, & Neufeld, 1994; Yu, Lin, Liu, & Ma, 2009; Zou et al., 2011). In accordance with common sense, different formation methods could result in different structure of alginate microcapsules and suggest different properties. Moreover, the different encapsulated substances are also thought to affect the structure and properties of microcapsules.

In this study, emulsification/external gelation and emulsification/internal gelation techniques were used to prepare microcapsules with the natural carbohydrate polymers of alginate and chitosan, which were used to encapsulate probiotics. The structure, size and size distribution, cell viability, cultural characteristics of microencapsulated cells will be investigated with the purpose of producing enough active bacteria for potential application in food or biotechnological industries.

2. Materials and methods

2.1. Cells and materials

Probiotics yeast cells (Y235) were obtained from the Institute of Applied Ecology, Chinese Academy of Science (Shenyang, China). The strain was maintained in YPD medium (20 g of glucose, 10 g of polypepton, 10 g of yeast extract, in 1 L distilled water at 30 °C). Sodium alginate was purchased from the Chemical Reagent Corp (Qingdao, China), whose viscosity was over 0.02 Pa s when dissolved to form a 1.5% (w/v) aqueous solution at 20 °C. The compositions of the alginate were characterized by ^{1}H NMR with G/M ratio of 34/66, and the molecular weight (M_w) was 430 kDa. The deacetylation degree (DD) of chitosan samples was 96%, and M_w was 60 kDa, which was degraded from raw chitosan (Yuhuan Ocean Biomaterials Corporation, China) with gamma (γ) rays irradiation by Key Laboratory of Nuclear Analysis Techniques, Chinese Academy of Sciences. All other reagents and solvents were of reagent grade and were used without further purification.

2.2. Preparation of calcium alginate beads entrapping yeast cells by emulsification/external gelation technique

Yeast cells Y235, in late exponential phase, were centrifuged and suspended in sodium alginate solution. Sodium alginate was dissolved in 0.9% (w/v) NaCl solution to form concentration of 1.5% (w/v). After being filtered through a 0.22 μm membrane filter, the solution was stored overnight before use to facilitate deaeration. 10 mL alginate-cell mixture was subsequently emulsified in 50 mL liquid paraffin containing 0.5% (v/v) Span 85 under magnetic agitation at 200 rpm for 30 min. By addition of 10 mL 0.05 M calcium chloride ($CaCl_2$) solution into emulsion, the gelation was initiated and the phase separation of W/O emulsion occurred. The system was stirred for another 1 h in order to allow for encapsulation. After a gentle centrifugation at 1000 rpm for 5 min, the top layer of oil phase was drained by aspiration, and the beads were collected by the same centrifugation conditions, and stored at 4 °C.

2.3. Preparation of calcium alginate beads entrapping yeast cells by emulsification/internal gelation technique

The alginate beads by emulsification/internal gelation were produced according to Liu, Yu, Lin, Ma, and Yuan (2007). The Sodium alginate solution containing yeast cells as-mentioned above, was mixed with micro-crystalline $CaCO_3$ powder to form finely dispersed suspension. Then, 10 mL alginate–calcium salt-cell mixture was dispersed in 50 mL liquid paraffin containing 0.5% (v/v) Span 85 to form emulsion by stirring at 200 rpm for 30 min. After emulsification for 30 min, glacial acetic acid was slowly added into the emulsion to liberate Ca^{2+} for gelation. The calcium alginate beads were collected and successively rinsed with 1% (v/v) Tween 80 solution and distilled water, and stored in water at 4 °C.

2.4. Preparation of alginate–chitosan microcapsules entrapping yeast cells

The calcium alginate beads produced by both techniques were immersed in 0.5% (w/v) chitosan solution dissolved in 0.1 mol/L sodium acetate-acetic buffer at the ratio of 1:5 (beads/solution) to form alginate–chitosan (AC) microcapsules, followed by rinsing with 0.9% (w/v) NaCl solution. After being liquified by 0.055 mol/L sodium citrate and rinsed three times with 0.9% (w/v) NaCl solution, AC microcapsules entrapping yeast cells were formed.

2.5. Characterization of the morphology of calcium alginate beads and AC microcapsules entrapping yeast cells during cell culture process

The morphology of calcium alginate beads entrapping yeast cells was observed with a Nikon Eclipse TE2000 Inverted Research Microscope (Nikon Corp., Japan). While AC microencapsulated yeast cells were firstly cultured for 24 h and then observed at the same way.

Fluorochrome fluoresceinamine labeled alginate was used to prepare gel beads and microcapsules according to the method mentioned above. Then the alginate distributions in beads were