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Improved probiotic viability in stress environments with post-culture of alginate–chitosan microencapsulated low density cells

Huiyi Song[a,c], Weiting Yu[a,*], Xiudong Liu[b], Xiaojun Ma[a,*]

[a] Laboratory of Biomedical Materials Engineering, Dalian Institute of Chemical Physics(DICP), Chinese Academy of Sciences(CAS), Dalian 116023, PR China
[b] College of Environment and Chemical Engineering, Dalian University, Dalian Economic Technological Development Zone, Dalian 116622, PR China
[c] University of Chinese Academy of Sciences, Beijing 100049, China

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ABSTRACT

In this study, probiotics (*Saccharomyces cerevisiae* Y235) were entrapped in alginate–chitosan microcapsules by emulsification/internal gelation technique. Two different encapsulation patterns were established as directly entrapped high density cells (*d*EHDC) and entrapped low density cells with culture (ELDC*wc*). The performance of microencapsulated cells, with free cells (FC) as control, was investigated against sequential stress environments of freeze-drying, storage, and simulated gastrointestinal fluids. After being freeze-dried without cryoprotectant, the survival rate of ELDC*wc* (14.33%) was significantly higher than 10.00% of *d*EHDC, and 0.05% of FC. The lower temperature (−20 °C) and ELDC*wc* pattern were beneficial for keeping viable cells at 7.00 log CFU g^{-1} after 6 months. Furthermore, the ELDC*wc* microcapsule maintained viable cells of 6.29 log CFU g^{-1} after incubation in SGF and SIF. These studies demonstrated that the pattern of entrapped low density cells with culture was an effective and superior technique of resisting harmful stress environments.

1. Introduction

Probiotics are live microbial strains that beneficially affect the host by improving intestinal microbial balance when consumed in adequate doses (FAO/WHO, 2002), and therefore have been widely utilized as additives to produce functional foods. However, the considerable loss of probiotic viability during fermentation, separation, storage and eating processes is inevitably reducing the actual efficacy of functional foods. To enhance the efficacy of probiotics, microencapsulation has been introduced by entrapping cells into polymer matrix (Dianawati, Mishra, & Shah, 2013; Dong et al., 2013). Microencapsulation offers probiotics good protection against all kinds of above stress environments by isolating microbial cells from outside, which becomes potential solution to maintain the viability as high as possible (Anal & Singh, 2007).

Sodium alginate is a generally regarded as safe (GRAS) material certified by FDA (George & Abraham, 2006), and has the ability of forming hydrogel under mild ionotropy effect with the existence of some divalent cations such as Ca^{2+} (Liu et al., 2002). Chitosan is a polycation in acidic solution with superior properties such as biocompatibility and biodegradability (Muzzarelli & Muzzarelli, 2006; Muzzarelli, Stanic, Gobbi, Tosi, & Muzzarelli, 2004). It can complex with above hydrogel to form alginate–chitosan microcapsules for probiotic encapsulation (Ortakci & Sert, 2012; Pimentel-González, Campos-Montiel, Lobato-Calleros, & Vernon-Carter, 2009).

Generally, probiotics are directly entrapped with high density cells in alginate-based microcapsules (hereafter abbreviated as *d*EHDC), which are thought as products without further culture. Because the microencapsulated probiotics have to undergo stress environments in drying, storage and edible stages, it is technologically and economically essential to maximize the viability of probiotics (Carvalho et al., 2004; Kanmani et al., 2011; Shi et al., 2013). Many reports illustrated that the drying temperature, osmotic pressure, storage temperature, pH and water activity of the *d*EHDC products still had adverse effect on the viability of the cells (Borges et al., 2012; De Castro-Cislaghi, Silva, Fritzen-Freire, Lorenz, & Sant'Anna, 2012). Meantime, some of the published reports have investigated different cryoprotectants (Li et al., 2011), the water activity (Kearney et al., 2009), pH of the products (Mohammadi, Mortazavian, Khosrokhavar, & da Cruz, 2011), and rehydration (Selmer-Olsen, Birkeland, & Sorhaug, 1999) on the cell viability improvement during the drying and storage process. For example,

Abbreviations: FC, free cells; *d*EHDC, directly entrapped high density cells; ELDC*wc*, entrapped low density cells with culture.

* Corresponding authors at: Laboratory of Biomedical Materials Engineering, Dalian Institute of Chemical Physics (DICP), Chinese Academy of Sciences (CAS), 457 Zhongshan Road, Dalian 116023, PR China.
E-mail addresses: yuwt@dicp.ac.cn (W. Yu), maxj@dicp.ac.cn (X. Ma).

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the cryoprotectants could significantly improve the cell viability to a variable extent during freeze-drying and storage (Reddy, Awasthi, Madhu, & Prapulla, 2009).

In our previous research, probiotics are entrapped with low density cells and followed culture (abbreviated as ELDC*wc*) to produce high density cells, which is different to traditional *d*EHDC. It was found that the survival rate of freshly made ELDC*wc* increased obviously than that of *d*EHDC and free cells when keeping in simulated gastrointestinal conditions (Song, Yu, Gao, Liu, & Ma, 2013). This finding suggested that ELDC*wc* technique would be a novel way for production of microencapsulated probiotics with higher cell viability.

However, the cell viability of ELDC*wc* microcapsules to stress environments during sequential drying, storage and edible stages is still unknown in the view of product development and application. Therefore, both low density cells (yeast cell Y235) and high density cells were separately entrapped in alginate–chitosan (AC) microcapsules by emulsification/internal gelation technique. The entrapped low density cells were then cultured (ELDC*wc*) to reach as high cell density as that of *d*EHDC. Subsequently, ELDC*wc* and *d*EHDC groups, together with free cells (FC group) as control, were subjected to the sequential processes of freeze-drying and storage for the evaluation of stress resistance behavior and cell viability. Moreover, the cell viability of dried microencapsulated probiotics was also determined after the treatment under simulated gastrointestinal tract conditions.

2. Materials and methods

2.1. Cells and materials

Probiotics yeast cells *Saccharomyces cerevisiae* Y235 (*S. cerevisiae* Y235) were obtained from the Institute of Applied Ecology, Chinese Academy of Sciences (Shenyang, China). This strain was maintained in YPD medium (20 g of glucose, 10 g of polypeptone, 10 g of yeast extract, in 1 L distilled water). Sodium alginate was purchased from the Chemical Reagent Corp. (Qingdao, China). The molecular weight (M_w) and G/M ratio of alginate were determined as 430 kDa and 34/66, respectively. Chitosan 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, which gives M_w of 60 kDa and deacetylation degree (DD) of 96%. All other reagents and solvents were of reagent grade and were used without further purification.

2.2. Preparation of alginate–chitosan (AC) microencapsulated probiotic cells

2.2.1. Preparation of alginate beads by emulsification/internal gelation technique

Based on our report (Liu, Yu, Lin, Ma, & Yuan, 2007), the calcium alginate beads were produced by emulsification/internal gelation technique. Sodium alginate solution of 1.5% (w/v) was obtained by being dissolved in 0.9% (w/v) NaCl solution and filtered through a 0.22 μm membrane filter. Yeast cells (*S. cerevisiae* Y235) were cultured to late exponential phase and harvested by centrifugation (3000 rpm, 10 min). The cells and micro-crystalline $CaCO_3$ powder were finely dispersed in sterile sodium alginate solution. Then, the alginate–calcium salt-cell suspension was added in 1.5 L liquid paraffin containing 0.5% (v/v) Span 85 in a turbine reactor under stirring at 200 rpm for 30 min. After emulsification for 30 min, glacial acetic acid was added into the emulsion for gelation. Then 1 L deionized water was added into the above emulsion under stirring for 30 min at a speed of 200 rpm. The cell entrapped calcium alginate beads were rinsed with 1% (v/v) Tween 80 solution and distilled water successively, and then stored in water at 4 °C.

2.2.2. Preparation of alginate–chitosan (AC) microcapsules

In general, the cell entrapped calcium alginate beads were put in 0.5% (w/v) chitosan solution dissolved in 0.1 mol/L acetate buffer at the beads/solution ratio of 1:5 (v/v). After being rinsed and liquefied for 6 min using 0.055 mol/L sodium citrate, the cell entrapped alginate–chitosan (AC) microcapsules were formed. As for the above-mentioned two kinds of cell-loaded AC microcapsules, the detailed preparation methods were separately displayed below.

Directly entrapped high density cells (*d*EHDC) group: high density cells were directly entrapped into AC microcapsules according to the above method. The entrapment yield of yeast cells was determined to reach 10^9 CFU mL^{-1} microcapsules, which was thought as the initial cell loading value of *d*EHDC group.

Entrapped low density cells with culture (ELDC*wc*) group: the initial inoculum cells entrapped into AC microcapsules was 5.00×10^6 CFU mL^{-1} microcapsules. Then AC microencapsulated cells were cultured for 48 h in shaking incubator at 30 °C and 170 rpm. The final cell density was about 10^9 CFU mL^{-1} microcapsules, which illustrated the high cell density could be achieved by microencapsulated cell culture.

2.3. Freeze-drying method

The freeze drying process was performed as described by Anwar and Kunz (2011) with a little modification. The samples were placed into aluminum plates and frozen at −70 °C for 1 h, and freeze dried for 24 h by A Christ Alpha 2-4 LSC freeze dryer. During the drying process, the temperature of ice condenser was set at lower than −50 °C, and the pressure was around 0.12 mbar. The dried samples were tightly packed and stored for further analysis.

2.4. Morphology and size of microencapsulated probiotic cells

The morphology of *d*EHDC and ELDC*wc* microcapsules before freeze-drying was observed under inverted optical microscope (Olympus CK-40, Olympus Corp., Japan). After freeze-drying, *d*EHDC and ELDC*wc* microcapsules was recorded with digital photo camera (Coolpix 995, NIKON, Japan) and observed under stereo microscope (SMZ 800, NIKON, Japan).

The size and size distribution of the dried microcapsules were determined with laser diffraction particle analyzer (LS 100 Q, Beckman-Coulter Corp., USA).

2.5. Survival rate of microencapsulated cells after freeze-drying and storage

To determine viable cell counts, the entrapped cells after freeze-drying were released from microcapsules according to the method proposed by our lab (Xue, Yu, Liu, Wang, & Ma, 2004). One hundred milligram dried samples of *d*EHDC or ELDC*wc* microcapsules were re-suspended and homogenized in 10 mL microcapsule-broken solution. Then the cell suspension was serially diluted with normal saline and plated on YPD agar. The viable cells were counted and expressed as log colony forming unit per gram (log CFU g^{-1}). Free cells were also freeze dried and the viable cells were counted as control.

The survival rate of cells after the freeze-drying was calculated as follows:

$$\text{Survival rate}_{\text{dry}} = \frac{N_d}{N_0} \times 100\% \quad (1)$$