



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

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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.

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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 (Dalcenserie 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, Thompson, & 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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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 ^1H 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

observed by confocal laser scanning microscope (CLSM) (Leica, TCS-SP2, Germany), equipped with laser sources blue (Ar 488 nm/5 mW) and an inverted microscope (Leica, DMIRE2, Germany).

2.6. Characterization of the size and size distribution of calcium alginate beads

The size and size distribution of the beads produced by the two techniques were determined with laser diffraction particle analyzer (LS 100 Q, Beckman-Coulter Corp., USA). The SD and CV represent the standard deviation and coefficient variation, respectively.

The size distribution was estimated with the SPAN (size distribution) factor (El-Mahdy et al., 1998), which is defined as Eq. (1):

$$\text{SPAN} = \frac{D_{90\%} - D_{10\%}}{D_{50\%}} \quad (1)$$

where $D_{90\%}$, $D_{50\%}$, and $D_{10\%}$ were the mean diameters at which 90%, 50% and 10% (vol%) of the particles were counted and calculated. A high SPAN indicates a wide distribution in size, whereas a low value indicates a narrow size distribution.

2.7. Evaluation of the viability of cells in the beads

For viability evaluation of the cells in the beads after preparation, 0.1 mL beads were collected and inoculated in 0.055 mol/L sodium citrate medium to release the entrapped cells. Cell viability was assessed by counts of yeast in the Neubauer Chamber. To identify a viable from nonviable yeast, the cell suspension was previously stained with methylene blue staining method (Vairo, 1961).

2.8. Characterization of cell growth in AC microcapsules during cell culture process

About 0.8 mL AC microencapsulated yeast cells were inoculated in 9.2 mL YPD medium, and the inoculum cell density was about 1×10^7 /mL microcapsule. Then AC microencapsulated cells were cultured in a shaking incubator at 30 °C and 170 rpm for 48 h. After being breaking up of AC microcapsules with a chemical method (Xue, Yu, Liu, Wang, & Ma, 2004), yeast cells were counted through the Neubauer Chamber every 4 h.

To determine the degree of the yeast cells that released from the microcapsules (leakage), the culture medium outside the microcapsules were collected and counted as a function of time. And the concentration of yeast cells in the medium was measured as above. All cell experiments were carried out in triplicate samples.

2.9. Survival rate in simulated gastrointestinal conditions of free cells (FC), directly entrapped high density cells (dEHDC) and entrapped low density cells with culture (ELDCwc)

Yeast cells for this experiment were propagated in 500 mL YPD broth at 30 °C and 170 rpm for 24 h. Cells were harvested by centrifugation at 3000 rpm for 15 min, and washed twice with phosphate buffer saline (pH 7.0). Then two kinds of cell-loaded AC microcapsules were prepared as follows to obtain the same cell density at different condition for comparing survival rate.

Directly entrapped high density cells (dEHDC) group: high density of yeast cells was directly entrapped into AC microcapsules according to Section 2.3 and 2.4. The entrapment yield of yeast cells was determined to reach 10^9 cfu/mL microcapsules.

Entrapped low density cells with culture (ELDCwc) group: The inoculum cell density entrapped into AC microcapsules was as low as 5×10^6 cfu/mL 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 microcapsules.

Free cells (FC) group: 10^9 cfu/mL free cells were used as control.

Previously described methods (Erkkila & Petaja, 2000) were applied to test the survival rate of above three cell groups. The microencapsulated cells (dEHDC group and ELDCwc group) and free cells (FC group) were individually added to YPD broth that had been adjusted to pH 1.0 with 5 M HCl in 10 mL aliquots (simulated gastric fluids, SGF), followed by incubation at 37 °C for 2 h. Then the microencapsulated cells and free cells were transferred into simulated intestinal fluids and incubated at 37 °C for 12 h. 0.05 M sodium phosphate buffer (pH 7.4) was used as simulated intestinal fluids (SIF). Finally, the survival rate of cells in FC, dEHDC, and ELDCwc groups were determined by spread plate count on YPD agar respectively.

2.10. Statistical analysis

All presented data are mean values from triplicate beads or microcapsules. Data were subjected to one-way analysis of variance (ANOVA) and statistical significance was set at $p < 0.05$. All analyses were performed using SPSS version 16.0 for Windows (SPSS, Chicago, USA).

3. Results and discussion

3.1. Morphology of the beads prepared by emulsification/external gelation and emulsification/internal gelation techniques

The empty and cell-loaded calcium alginate gel beads were prepared as described in Sections 2.2 and 2.3, and the experiments were repeated at least for three times. The freshly made beads by both techniques were treated and observed with optical microscope at the same way. The empty beads prepared by emulsification/external gelation were of less spherical in shape and the sizes were obviously not uniform (Fig. 1a). This is typical bead image with poor shape, wide distribution and debris even with repeatable and parameter optimized experiments. The random disruption of W/O emulsion, the merge of disrupted emulsion droplet, the nonuniform distribution and diffusion of added Ca^{2+} were considered to cause a random and nonuniform gelation before properly hardening. The similar morphology and size distribution can be seen for cell-loaded beads (Fig. 1b). On the contrary, the beads prepared by emulsification/internal gelation appeared much better morphology with spherical shape and smooth-surface. They were also well dispersed in the solution with relatively uniform size (Fig. 1c). Because CaCO_3 powder was finely dispersed in alginate solution of W/O emulsion, the dissociated Ca^{2+} by added acetic acid can *in situ* gelate without disrupting the emulsion droplet so as to keep the spherical shape. Moreover, the inclusion of yeast cells within the alginate beads does not affect their integrity and shape (Fig. 1d). Because of the high initial seeding density, the cells took up almost all inner space of the beads, which results in the appearance of dark beads.

3.2. Size and size distribution of the beads prepared by both techniques

It has been known that the size and size distribution of the beads could be influenced by the ratio of oil to water (v/v), the concentration of surfactant, and the stirring speed (Lin, Yu, Xu, Liu, & Ma, 2007). In this study, the beads were prepared to ensure the comparability between both techniques at given conditions, that is, the same batch of alginate solution, stirring speed of 200 rpm, ratio of oil to water of 5:1 and surfactant concentration of 0.5% (v/v) span 85. Table 1 and Fig. 2 indicated that the beads produced by the two techniques exhibited nearly unimodal and normal

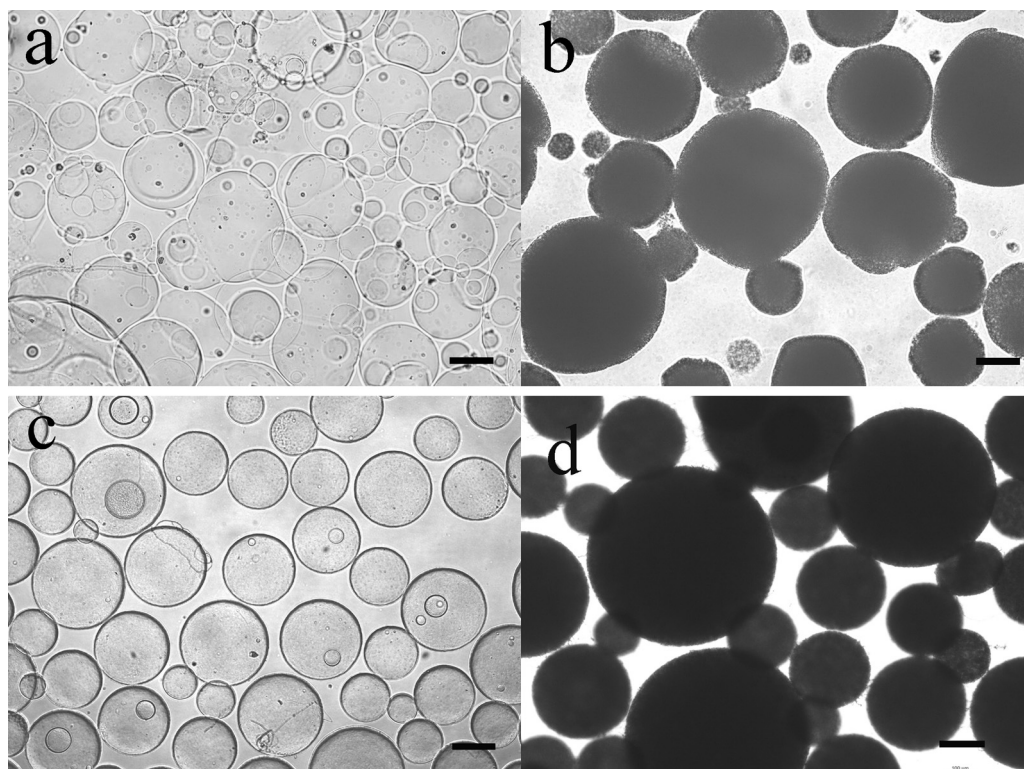


Fig. 1. Optical images of empty beads (a) and containing Y235 beads (b) produced by emulsification/external gelation technique, empty beads (c) and containing Y235 beads (d) produced by emulsification/internal gelation technique (bar = 100 μm).

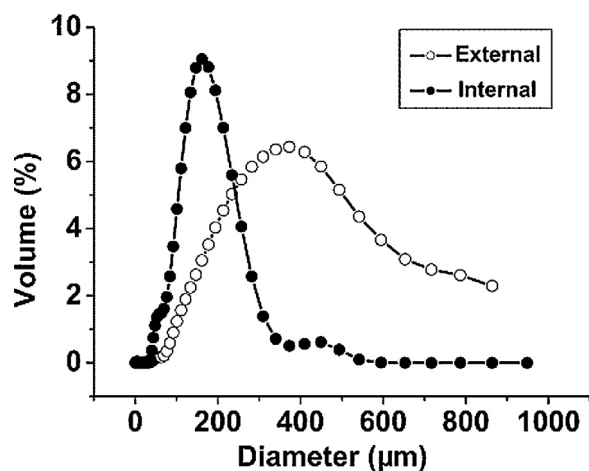


Fig. 2. Size and size distribution of the beads containing Y235 made by emulsification/external gelation and emulsification/internal gelation techniques.

particle-size distribution. The beads by emulsification/external gelation showed a broader size distribution ranging from 35 to 863 μm with average diameter of 325.4 μm , and rather large standard deviation ($\pm 201.5 \mu\text{m}$). While the beads by emulsification/internal gelation showed relatively narrow size distribution ranging from 35 to 373 μm with average diameter of 151.1 μm and standard deviation ($\pm 77.2 \mu\text{m}$). Moreover, the SPAN values

were calculated as 1.59 (emulsification/external gelation) and 1.11 (emulsification/internal gelation) from data by the software of the particle analyzer, which also indicated a narrower size distribution of beads by emulsification/internal gelation technique.

The droplet size distribution obtained from external and internal gelation was different. For a fixed rate of energy dissipation during emulsification, the final droplet size distribution is determined by the time taken for the interface to be covered with emulsifier, or the probability that an emulsifier molecule adsorbed to the surface of a droplet, or the optimum amount that the emulsifier reduces the interfacial tension, or the effectiveness of the emulsifier membrane in protecting the droplets against coalescence (Dickinson, 2009; Homayouni et al., 2007). When the emulsifier adsorbs too slowly, or is present at too low concentration, most of the individual droplets formed during the intense energy dissipation of emulsification are not retained in the final emulsion. This may be due to breakage of the thin film between colliding droplets (coalescence). When using the emulsification/external gelation technique, the larger mean size was attributed to the collision of the dispersed alginate and calcium chloride droplets. The collision could involve two or more droplets. The gradual diffusion of CaCl_2 into the core of the particles would eventually congeal the liquid core into rigid microcapsules (Silva, Ribeiro, Figueiredo, Goncalves, & Veiga, 2006). Size distribution of beads obtained by emulsification/internal gelation depends on the size of the emulsion droplets. The latter is determined by a balance between the dispersive and the surface tension forces. The former tends to disperse the emulsion and the latter causes coalescence (Chan, Lee, & Heng, 2002).

Table 1

Size and size distribution of the beads containing Y235 made by emulsification/external gelation and emulsification/internal gelation techniques.

Emulsification technology	Mean (μm)	Mode (μm)	S.D. (μm)	C.V. (μm)	$D_{10\%}$ (μm)	$D_{50\%}$ (μm)	$D_{90\%}$ (μm)	SPAN
Emulsification/external	325.4	390.9	201.5	54.4	147.8	340.0	688.2	1.59
Emulsification/Internal	151.1	168.9	77.18	45.3	83.06	160.4	262.7	1.11

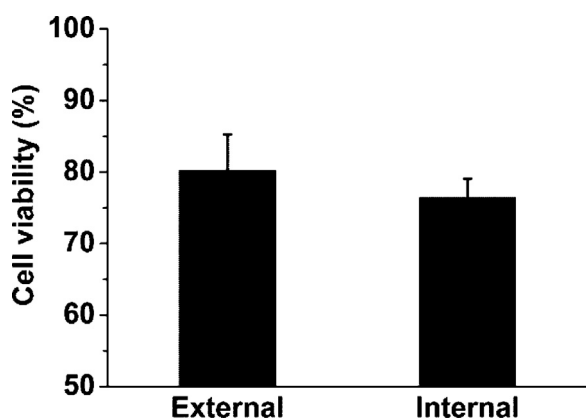


Fig. 3. Cell viability in the beads made by emulsification/external gelation and emulsification/internal gelation techniques.

Microcapsule size distributions with a very wide range have been reported by several researchers and the optimum microcapsule size is a compromise between the effectiveness of encapsulation and the sensory properties (Arup et al., 2011). A minimum diameter of 100 μm has been suggested to offer better protection in gastric juice, and an optimum range of 100–200 μm has been proposed (McMaster, Kokott, & Slatter, 2005). The mean diameter of our emulsification/internal gelation beads was just within this limit.

3.3. The cell viability during bead production by two techniques

In the process of both emulsification techniques, some organic reagents and a series of operation steps were used, which was thought to have adverse effect on cell viability. Therefore the survival rates of cells in the beads by two techniques were investigated as shown in Fig. 3. Cell viability was determined of 77% at the end of the emulsification/internal gelation process, and 80% at the end of the emulsification/external gelation process. It demonstrated that cell viability was affected by both techniques, while there was no significant difference.

The maintenance of cell viability is also an important factor for the selection of alginate beads production process. In regard to the emulsification/internal gelation technique, the most harmful factor to cell viability may be the use of acid. Other process parameters, such as mixer rotational speed, concentration of emulsifier, and W/O ratio, did not brought significant effect on cell viability. According to our previous studies, the concentration of acetic acid (HAC) has been optimized not only to provide sufficient HAC for initiating the gelation reaction, but also to decrease the damage to cells (Yu et al., 2009).

For production purposes, the high cell viability was enormously appealing. There was no significant difference in cell viability of the two emulsification techniques used, indicating that the emulsification method was gentle and did not put severe lethal physical stress on the cells. Combination with the high encapsulation efficiency, the emulsification/internal gelation technique was more suitable for cell encapsulation or immobilization in large-scale production.

3.4. Structure of calcium alginate gel beads prepared by emulsification/external gelation and emulsification/internal gelation techniques

Fig. 4 showed the gelation preparation principle of calcium alginate gel beads by emulsification/external and emulsification/internal gelation techniques respectively, and CLSM images of fluoresceinamine labeled alginate beads by both techniques were

given to demonstrate the principle and interpret the structure difference. For emulsification/external gelation technique, both alginate solution and CaCl_2 solution need to be dispersed into oil phase for gelation. However, the collision of the dispersed alginate and CaCl_2 droplets happened stochastically and included several kinds of hypotheses of Fig. 4a–d. When the bigger CaCl_2 droplet swallowed small sodium alginate droplet, Ca^{2+} could diffuse into the core of particles from every direction simultaneously (Fig. 4a). The green fluorescence image (upper) and stable intensity curve (lower) across radial direction of bead in Fig. 4a showed the symmetrical gel structure correspondingly. However, most of alginate droplets were bigger due to high viscosity than CaCl_2 droplets, which meant the beads with symmetrical structure only occupied a very small percentage. In fact, the collision between bigger alginate droplets and smaller CaCl_2 droplets followed the hypotheses in Fig. 4b–d. The nonuniform distribution and diffusion of added Ca^{2+} resulted into asymmetrical gel structure, which was clearly seen by accordingly fluorescence images and asymmetrical fluorescence intensity curve through the bead surface to the core in Fig. 4b–d. It implied the contact between alginate and calcium droplets usually occurred on the surface to form gel layer immediately, which resulted in resistance layer for further Ca^{2+} diffusion and unbalanced forces around the bead. Therefore, the external gelation beads displayed irregular shape and asymmetrical structure.

On the contrary, CaCO_3 powder was homogeneously dispersed into alginate droplet beforehand in emulsification/internal gelation technique. Then *in situ* gelation of alginate happened by initiating the release of Ca^{2+} when the diffusion of oil-soluble acetic acid through the oil-water interface (Fig. 4e). The acid dispersed in water phase quickly whatever contact point on the surface of alginate droplet, and Ca^{2+} released simultaneously *in situ* and initiated gelation reaction by the lower pH. Therefore, the structure of calcium alginate gel beads showed homogeneous and symmetrical in all direction. The green fluorescence image (left) and stable intensity curve (right) across radial direction of bead in Fig. 4e also confirmed the symmetrical gel structure correspondingly.

Irrespective of the droplet size, the internal gelation method produced generally spherical beads while the external gelation method produced beads of irregular shape. In the production of emulsification/external gelation beads, calcium chloride solution was added in the final stage of microencapsulation. This will cause disruption of the emulsion equilibrium that easily leads to beads of irregular shape. Moreover, the beads may easily aggregate due to the poor encapsulation efficiency and low mechanical strength of the gel (Magdy, Mohamed, Abou, & Elsayed, 2010), and result in the formation of hard aggregates after drying. While in internal gelation method, calcium salts are homogeneously distributed in the alginate solution initially, thus diffusion of protons into the droplets would induce gelation, giving rise to homogenous droplets (Liu et al., 2004). In the meantime, the shearing force caused by stirring provided a rounding action to the progressively densified clusters, leading to the formation of spherical beads. The spherical shape could be better for the maintenance of the textural quality of food so as to provide protection for the encapsulated probiotics.

3.5. Characterization of AC microcapsules entrapping yeast cells during cell culture process

After the formation of calcium alginate gel beads by emulsification/external gelation and emulsification/internal gelation techniques, AC microcapsules entrapping yeast cells were prepared by immersing alginate beads in chitosan solution. The membrane of AC microcapsules by emulsification/external gelation was uneven and discontinuous due to the asymmetrical and non-spherical structure of gel beads, which caused some broken microcapsules

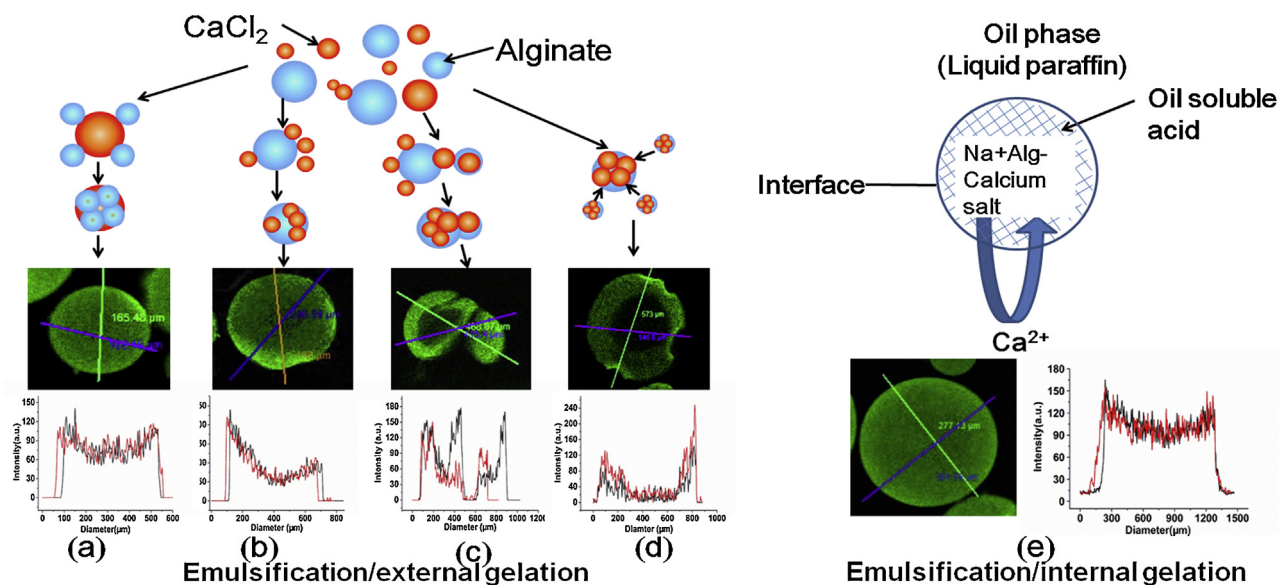


Fig. 4. Schematic diagram of the gelation preparation principle, CLSM images and corresponding fluorescence intensities of emulsification/external gelation (a–d) and emulsification/internal gelation (e) gel beads.

after liquification (Fig. 5a). After cultured for 48 h, the cell-loaded microcapsules broke severely, and the membrane could not keep intact, which resulted in the obvious cell leakage (Fig. 5b). On the contrary, it can be seen that the membrane of AC microcapsules by emulsification/internal gelation was intact with smooth surface. Yeast cells uniformly scattered in microcapsules after preparation (Fig. 5c). After cultured for 48 h, there was no breakage or apparent change in spherical structure of the cell-loaded microcapsules, and the dark inner space demonstrated obvious cell growth (Fig. 5d).

Furthermore, the cell density in AC microcapsules by emulsification/external gelation appeared no significant growth, which remained at about 5.2×10^6 cfu/mL microcapsule. The cell leakage was time dependent because the cell number found in the medium increased with time due to the nonuniform distribution of the alginate–chitosan membrane. The maximum cell leakage number was 8.9×10^7 cfu/mL after cultured for 20 h and remained stable in the following culture time. In comparison with the inoculum cell density of 5.2×10^6 /mL microcapsule, yeast

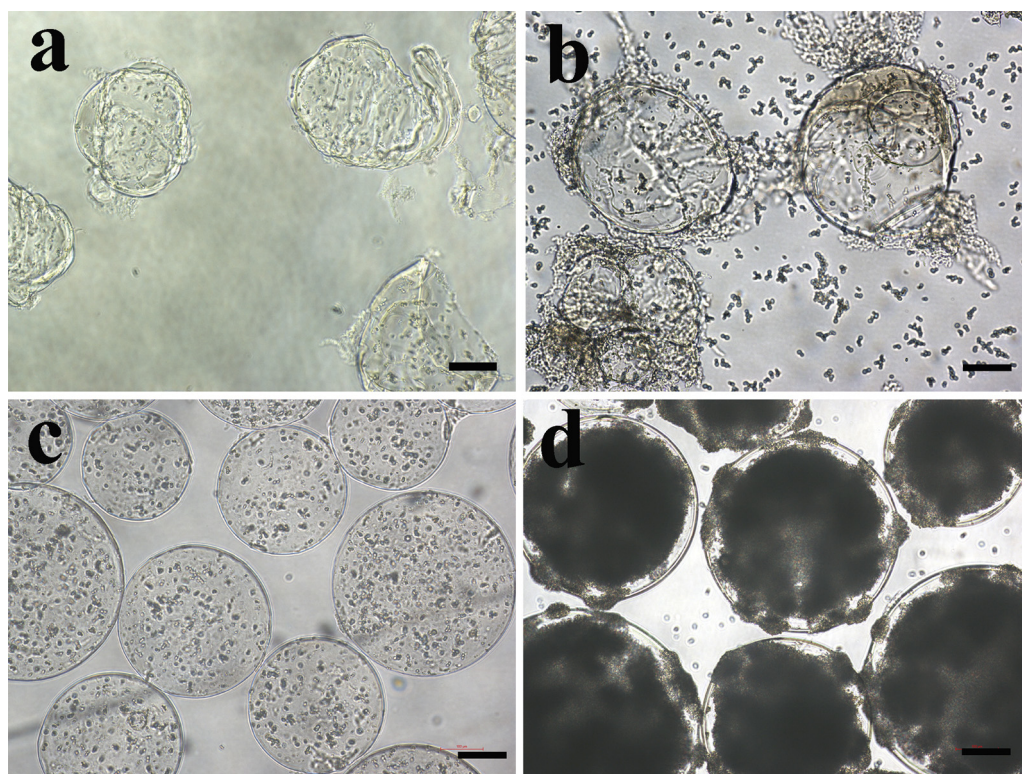


Fig. 5. Optical images of AC microencapsulated cells made by emulsification/external gelation (a, b) and emulsification/internal gelation (c, d) techniques before culture (a, c) and after culture for 48 h (b, d) (bar = 100 μm).

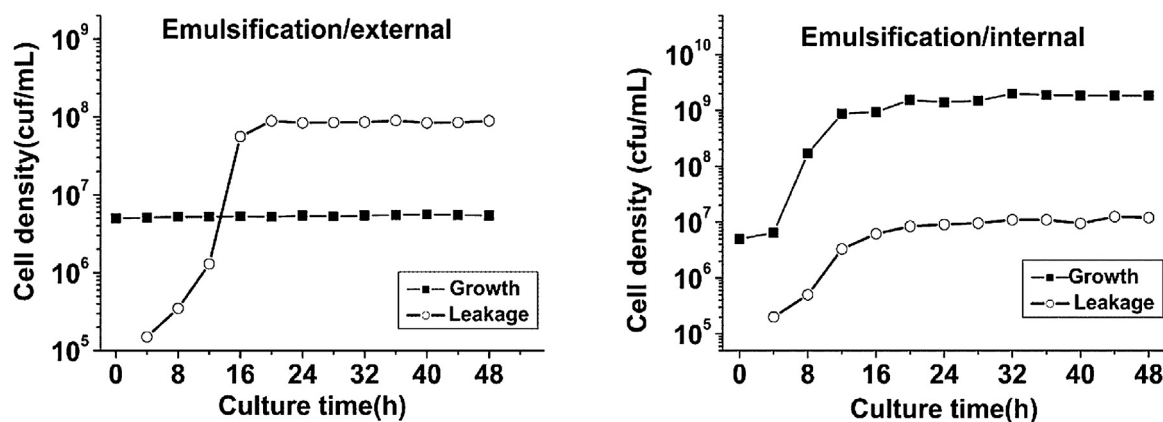


Fig. 6. Cell densities in AC microcapsules made by emulsification/external gelation and emulsification/internal gelation techniques and the leakage during cultured for 48 h.

cells in AC microcapsules by emulsification/internal gelation grew remarkably with cell density over 1.7×10^8 /mL microcapsule after cultured for 8 h (the exponential phase), and over 2.0×10^9 /mL microcapsule after cultured for 32 h (the stable phase). However, the leakage of cells was only 1.0×10^7 cfu/mL after cultured for 20 h and remained stable in the following culture time (Fig. 6).

Both the growth and leakage of cells demonstrated that the microcapsule membrane clearly have effect on the microcapsule stability. During the microcapsule membrane forming process in emulsification/external gelation technique, the membrane thickness varies with the distribution of alginate, and the liquefied procedure also caused a damage to the membrane with sever disfiguration. When sodium alginate encounters divalent cations such as Ca^{2+} , the ionotropy effect occurs between the divalent cations and Na^+ resulting in the formation of an elastic hydrogel with mechanical strength under biologically benign conditions (room temperature and neutral pH). It is the G residues that bind with Ca^{2+} . It has been speculated that there should be four complexation bonds formed between one Ca^{2+} and two GG blocks in the egg-box structure, that is, 5-COO⁻ and 2-OH of G unit taking part in the bond formation. (Xue et al., 2004). However, the pK_{a1} , pK_{a2} and pK_{a3} of sodium citrate are 2.14, 4.09 and 4.75 respectively. According to the Pearson hard and soft acid-base (HSAB) theory, Ca^{2+} is the hard acid, and sodium citrate is the more harder base. As a result, the sodium citrate is more easily to chelate Ca^{2+} . Then, alginate 'egg-box' structure was depolymerized to result in the conversion to liquid with the trend of volume expansion that is called 'liquefaction'. (Shoichet, Li, White, & Winn, 1996; Xue et al., 2004). Apparently, the emulsification/external gelation microcapsule has weak anti-swelling ability while being liquefied due to the irregular and non-uniform shape. However, the droplet-shape

beads produced by emulsification/internal gelation results in clear and symmetrical membrane with strong anti-swelling ability (Yu et al., 2009; Yu et al., 2011).

Combining with the anti-swelling ability and microcapsule leaking caused by the osmotic pressure, it could be deduced that emulsification/internal gelation technique was also superior to emulsification/external gelation technique for microencapsulate cell culture.

Moreover, the directly entrapped high density cells (dEHDC) and entrapped low density cells with culture (ELDCwc) by emulsification/internal gelation technique showed different environmental stress reaction when keeping at 10^9 cfu/mL microcapsules. The resistance of cells to simulated gastrointestinal conditions in ELDCwc group was obvious higher than that in dEHDC group as shown in Fig. 7. Compared to FC group, the viable cells in dEHDC group and ELDCwc group increased 250-fold and 300-fold after 2 h incubation in SGF, respectively. After incubated in SIF for 12 h, the final viable cells in FC group, dEHDC group and ELDCwc group were 4.6×10^3 , 2.5×10^6 and 2.0×10^7 cfu/mL, respectively. And only the ELDCwc group reached the recommended viable cell number by IDF. The survival rates of cells in ELDCwc group were significantly higher than cells in dEHDC group ($p < 0.01$) and FC group ($p < 0.01$), which suggested that the technique of entrapped low density cells with culture could protect probiotics better from the extreme acidity in the human stomach (Fig. 7 right). Moreover, the probiotic cells in ELDCwc group showed higher stress resistance ability whatever in freeze-drying and different storage environments (data not shown).

Nowadays, the microencapsulated probiotics are usually produced by directly high density encapsulation with emulsification/external gelation technique due to gentle preparative

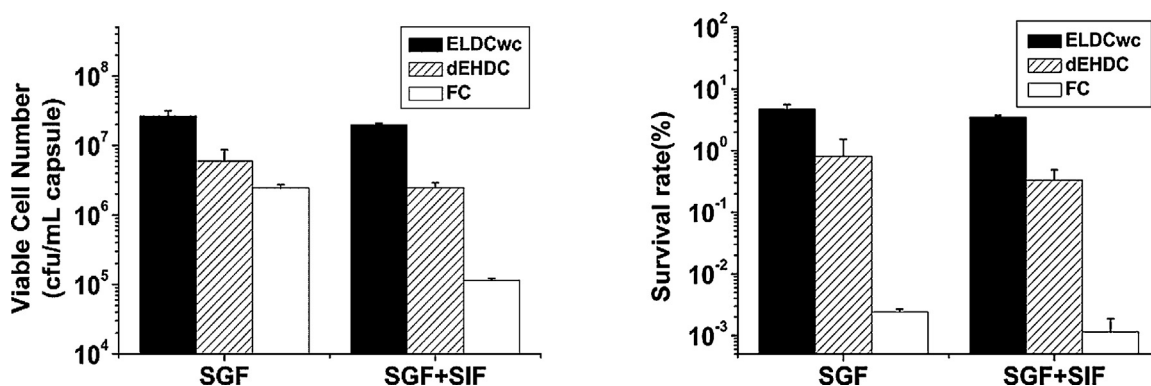


Fig. 7. Viable cell numbers (left) and survival rates (right) of microencapsulation and free cells of Y235 in simulated gastrointestinal conditions.

conditions, potential for large-scale production, and lower cost. However, the structure and membrane of microcapsules by emulsification/external gelation technique couldn't endure the culture and application environment resulting in the loss of cell viability. The results in this paper showed that the emulsification/internal gelation technique and the culture process after microencapsulation improved the stress resistance ability of probiotic cells for IDF standard. Therefore, the emulsification/internal gelation technique provided an available way for microencapsulated probiotic product with high density, high viability, and large-scale production.

4. Conclusion

Emulsification/external gelation and emulsification/internal gelation techniques have been reported to produce calcium alginate gel beads and microcapsules for probiotics entrapment. In the study, the properties of microencapsulated cells were evaluated including the morphology, size and size distribution, cell viability, cultural characteristics and survival rates in simulated gastrointestinal conditions. Except that there was no significant difference of cell viability during preparation between these two techniques, the morphology, size and size distribution of microcapsules by emulsification/internal gelation were superior to those of emulsification/external gelation technique. Moreover, high cell growth and low cell leakage of microcapsules by emulsification/internal gelation demonstrated potential microencapsulated probiotics application. The survival rate of cells in simulated gastrointestinal conditions also proved that ELDCwc microcapsules produced by emulsification/internal gelation technique could produce enough active probiotics for potential application in food or biotechnological industries.

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