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

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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.

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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:

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

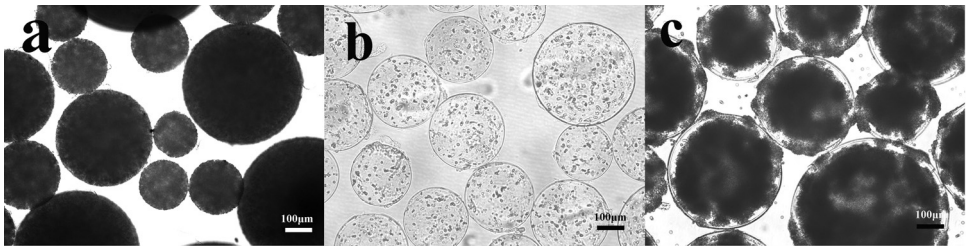

Fig. 1. Optical images of (a) directly entrapped high density cells (*d*EHDC) microcapsules, (b) entrapped low density cells with culture (ELDC*wc*) microcapsules before culture, and (c) ELDC*wc* microcapsules after cultured for 48 h (bar = 100 μm).

where N_0 and N_d represent the counted viable cells before and after drying, respectively.

Then the freeze-drying samples were stored at different temperature and time, which was designed to determine the effect of storage condition on the viability of *d*EHDC, ELDC*wc* and free cells (FC).

The influence of four storage temperatures on the viability of dried *d*EHDC, ELDC*wc* and FC samples was studied through 6 months. For storage at standard atmosphere, the dried samples were placed in sterile plastic containers at −20 °C (typical freezing temperature), 4 °C (typical refrigerating temperature), 25 °C (proper room temperature storage) and 37 °C (abused storage condition) respectively. Then the viable cells were counted according to the above method. And the survival rate of cells during storage was calculated as follows:

$$\text{Survival rate}_{\text{storage}} = \frac{N_t^*}{N_d} \times 100\% \quad (2)$$

where N_d and N_t^* represent the counted viable cells after drying and storage at different time and temperature, respectively.

2.6. Survival rate of dried microencapsulated cells in simulated gastrointestinal fluids

To evaluate the protection of microcapsules to probiotics, 100 mg freeze-drying samples of *d*EHDC and ELDC*wc* were placed separately into conical flask containing 10 mL simulated gastric fluids (SGF, pH 1.0 HCl solution). Samples were incubated at 37 °C for 2 h in shaker bath. Then the samples were transferred into simulated intestinal fluids (SIF, pH 7.4 PBS solution) and incubated at 37 °C for 12 h. After incubation in SGF and SIF, the viable cells were counted according to the above method, and 30 mg freeze-drying FC sample was used as control. The survival rate of samples was calculated as follows:

$$\text{Survival rate}_{\text{SGF}} = \frac{N_G}{N_d} \times 100\% \quad (3)$$

$$\text{Survival rate}_{\text{SGF+SIF}} = \frac{N_{G+I}}{N_d} \times 100\% \quad (4)$$

where N_d represents the counted viable cells after freeze-drying, N_G and N_{G+I} represent the counted viable cells after incubation in SGF or incubation in SGF and SIF, respectively.

2.7. Statistical analysis

All experiments were performed at least in triplicates, and the results were expressed as mean ± standard deviation (SD). The significance of the difference between groups was tested using *t*-test analysis. A probability level ($p < 0.05$) was considered to be statistically significant, and the statistic analysis was performed by one-way ANOVA text in Originpro75 software.

3. Results and discussion

3.1. Preparation of AC microcapsules entrapping probiotic cells by emulsification/internal gelation technique

AC microcapsules entrapping probiotic cells were prepared by emulsification/internal gelation technique in this study. High density cells were directly entrapped in AC microcapsules, which was called *d*EHDC group. Meantime, low density cells were firstly entrapped in AC microcapsules and then cultured for 48 h to reach as high cell density as that of *d*EHDC, which was called ELDC*wc* group.

Fig. 1 shows the representative microscopic images of freshly prepared *d*EHDC microcapsules, and ELDC*wc* microcapsules before culture and after cultured for 48 h respectively. It can be seen that all microcapsules were spherical and intact with smooth surface, and well dispersed in solution. The cells took up almost all the space of *d*EHDC microcapsules, which appeared dark due to the high initial seeding density of 10.58 log CFU g^{-1} microcapsules (Fig. 1a). However, in the group of ELDC*wc* microcapsules before culture, the probiotic cells scattered sparsely with lower initial seeding density of 8.22 log CFU g^{-1} microcapsules (Fig. 1b). After cultured for 48 h, the cells filled the inner space of microcapsules with the cell density of 10.58 log CFU g^{-1}, which demonstrated obvious cell growth in microcapsules (Fig. 1c).

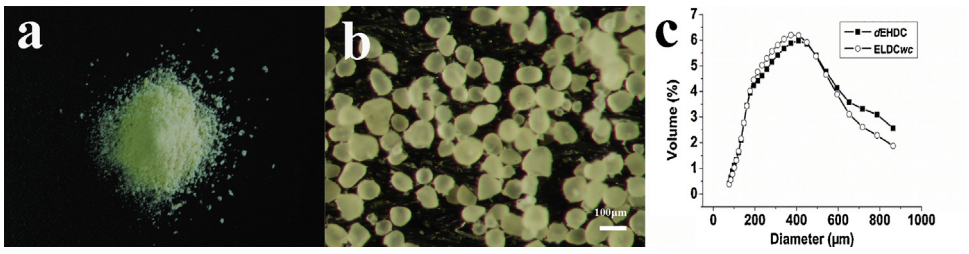

Fig. 2. The morphology of ELDC*wc* microcapsules after freeze-drying, (a) large quantity of uniform dried microcapsule under digital photo camera, (b) magnified microcapsules at 60× under stereo microscope and (c) the size distribution of dried *d*EHDC and ELDC*wc* microcapsules.

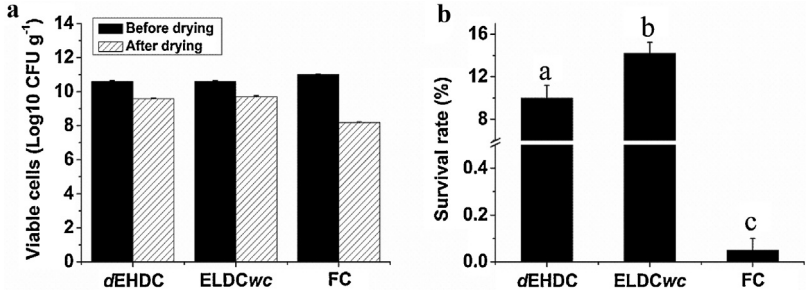

Fig. 3. Effect of freeze-drying on viability (a) and survival rate (b) of FC, *d*EHDC and ELDC*wc* groups. Different letters denote significant differences within each group ($p < 0.05$).

3.2. Survival rate of FC, dEHDC and ELDCwc after freeze-drying

In the view of convenience for both manufacturers and consumers, it is better to dry probiotics into solid forms for a longer shelf life (Otero, Espeche, & Nader-Macías, 2007). Among the different drying technologies, freeze-drying is preferred because it can minimize the damage to biological materials and sensitive foods. In this study, *d*EHDC and ELDC*wc* microcapsules were freeze-dried as described in Section 2.3. They showed the same morphology, and here only the images of dried ELDC*wc* microcapsules were provided in Fig. 2. It can be seen that the dried microcapsules were dispersed and uniform powders (Fig. 2a), shrank obviously due to the loss of water, but still kept spherical and intact morphology (Fig. 2b). Meantime, the mean sizes of dried *d*EHDC and ELDC*wc* microcapsules were also similar as 378.9 μm and 364.7 μm, respectively (Fig. 2c). These results demonstrated that AC microcapsules were strong enough to resist the low temperature stress from freeze-drying process, which was independent of different encapsulation patterns.

The cell viabilities of FC, *d*EHDC and ELDC*wc* in AC microcapsules were all analyzed after freeze-drying without using cryoprotectant. It showed that the freeze-drying process was harmful for the maintenance of cell viability (Fig. 3). The viable cells decreased from initial 11.52 to 8.19 log CFU g^{-1} in FC group, from 10.58 to 9.58 log CFU g^{-1} in *d*EHDC group, and from 10.58 to 9.73 log CFU g^{-1} in ELDC*wc* group, respectively (Fig. 3a). Accordingly, the calculated survival rate of probiotics was 0.05% in FC group, 10.00% in *d*EHDC group and 14.33% in ELDC*wc* group (Fig. 3b). This suggested that the encapsulation technique can significantly improved the survival of probiotics (over 200-fold) compared to that of FC group ($p < 0.01$). The higher survival rate of encapsulated cells could be explained by a possible protection against an osmotic shock caused by a delayed rehydration due to the microenvironment of the capsule (Capela, Hay, & Shah, 2006; Heidebach, Först, & Kulozik, 2010). Moreover, the survival rate of ELDC*wc* group was also significantly higher than that of *d*EHDC group ($p < 0.01$), which demonstrated the superior endurance ability of ELDC*wc* to freeze-drying condition. According to the different encapsulation techniques, cells in *d*EHDC group were physically aggregated, while cells in ELDC*wc* group experienced physiological growth to reach high density. Therefore, it indicated that the cell growth in microcapsules can affect probiotic cells behavior and give cells better ability of stress resistance.

3.3. Survival rate of dried FC, dEHDC and ELDCwc after storage

One of the most considerable prerequisites for the use of probiotics is that they must survive throughout the production process and shelf life. Therefore, dried AC microencapsulated *d*EHDC and ELDC*wc*, together with dried FC group were stored at temperatures −20 °C, 4 °C, 25 °C and 37 °C over 6 months to investigate the cell viability. As shown in Fig. 4a–c, the cell viability of dried FC, *d*EHDC

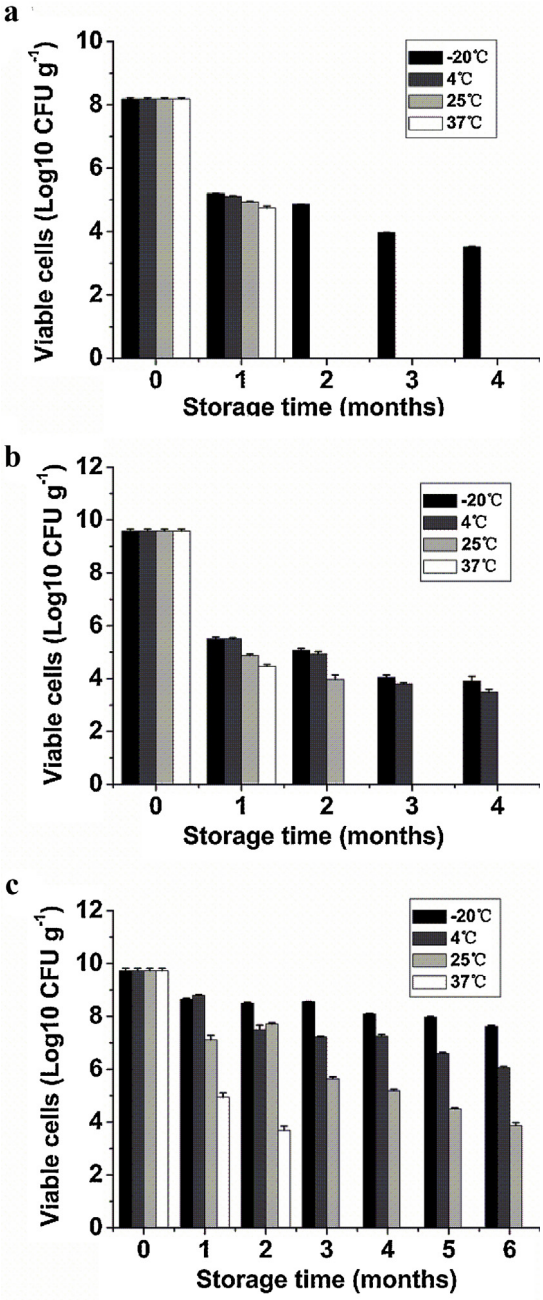

Fig. 4. Effect of storage time and temperature on the viability of (a) FC, (b) *d*EHDC microcapsule and (c) ELDC*wc* microcapsule during storage.

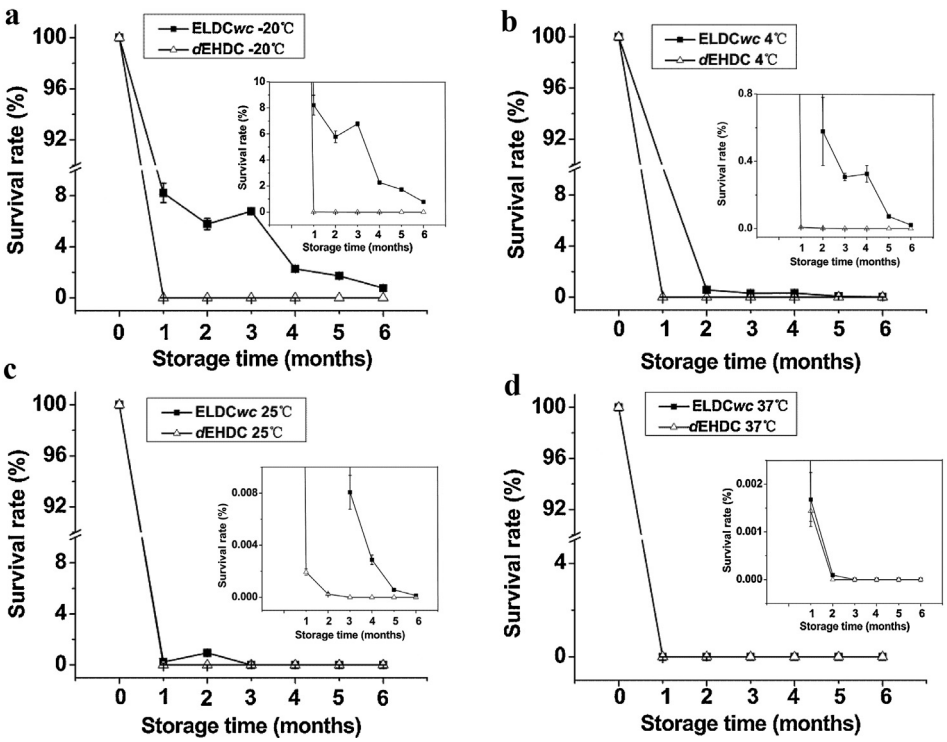

Fig. 5. Effect of different microencapsulation processes on survival rate of *d*EHDC and ELDC*wc* groups at (a) −20 °C, (b) 4 °C, (c) 25 °C and (d) 37 °C.

and ELDC*wc* groups displayed the same decrease trend with the increase of storage temperature and time.

In FC group (Fig. 4a), the amount of viable cells dropped from 8.19 to 3.52 log CFU g^{-1} after storage for 4 months, and to an undetectable level after 5 months at −20 °C. Meantime, the amount of viable cells could only keep 5.09 log CFU g^{-1}, 4.94 log CFU g^{-1} and 4.74 log CFU g^{-1} after storage for 1 month at 4 °C, 25 °C and 37 °C respectively. Then the viable cells could not be detected after 2 months at above three temperatures.

In *d*EHDC group (Fig. 4b), the amount of viable cells similarly declined with the storage time till 4 months. At storage temperature of −20 °C and 4 °C, the viable cells remained above 8.00 log CFU g^{-1} after storage for 2 weeks, and then decreased to 3.90 log CFU g^{-1} and 3.49 log CFU g^{-1} at the end of 4 months, respectively. However, a larger inactivation effect was observed at higher storage temperature of 25 °C and 37 °C. The viable cells dropped from 9.58 log CFU g^{-1} to an undetectable level in 3 months at 25 °C and 2 months at 37 °C respectively.

In ELDC*wc* group (Fig. 4c), a minor loss of cell viability was shown in comparison with *d*EHDC and FC groups. The amount of viable cells remained above 8.00 log CFU g^{-1} after storage for over 3 months at −20 °C, and there was no significant difference ($p = 0.6$) at different time point. The viable cells could keep at 7.00 log CFU g^{-1} after 6 months of storage, which suggested that the low temperature led to a considerable maintenance of cell viability. In the refrigerator at 4 °C, the viable cells remained at 8.82 log CFU g^{-1} after 1 month, and decreased to 6.03 log CFU g^{-1} after 6 months. At 25 °C and 37 °C, the cell viability was dramatically reduced from 9.73 to 7.11 log CFU g^{-1}, and from 9.73 to 4.96 log CFU g^{-1} after 1 month storage, respectively. The final viable cells were 3.85 log CFU g^{-1} at 25 °C after 6 months storage, but undetectable at 37 °C after 2 months storage.

From the above results, all the tested temperatures obviously led to the decrease on cell viability of FC, *d*EHDC, ELDC*wc* groups. Microencapsulation process (*d*EHDC and ELDC*wc* groups), however, was helpful to protect cells against storage environment. Moreover, the lower temperature was beneficial for the maintenance of cell viability, which was in agreement with previous report that higher inactivation rates *k* were found under storage at 25 °C compared to 4 °C (Heidebach et al., 2010). It was explained that lower temperature could reduce the rates of detrimental chemical reactions, such as fatty acid oxidation (Gardiner et al., 2000; Goldberg & Eschar, 1977; Higl et al., 2007). Therefore, it demonstrated the storage temperature was significant factor to affect cell viability, and the cold storage condition is advantageous for probiotic powders to achieve higher viable cells. Meantime, it should notice that the storage period had negative effect on cell viability at each temperature, but the decrease was not sharp after 1 month storage.

In order to compare the effect of different microencapsulation processes on cell viability during storage, the survival rates of *d*EHDC and ELDC*wc* groups at different storage temperatures were calculated and shown in Fig. 5. At −20 °C (Fig. 5a), the survival rate of *d*EHDC group sharply decreased to almost zero (0.008%) after 1 month storage, and then kept the steady state till 6 months. While the survival rate of ELDC*wc* group remained at 8.22% after 1 month storage, slowly decreased to 2.27% after 4 months, and reached 0.78% after 6 months. At 4 °C and 25 °C (Fig. 5b and c), the survival rates of *d*EHDC and ELDC*wc* groups displayed similarly sharp decrease to almost zero with the storage time, but the embedded figures still demonstrated significant higher survival rate of ELDC*wc* than that of *d*EHDC ($p < 0.01$). At 37 °C, the survival rates of *d*EHDC and ELDC*wc* decreased sharply to zero ($p = 0.44$) due to the higher storage temperature (Fig. 5d).

These differences clearly showed that the growth process of cells in microcapsule (ELDC*wc*) compared to *d*EHDC was important and beneficial to keep higher active probiotics during storage in dry state. According to our previous report (Yu et al., 2011), the growth of entrapped cells clearly has effect on the membrane characteristics of AC microcapsules. It was found that the secreted macromolecular metabolites such as proteins by entrapped cells increased the membrane thickness and subsequently decreased the

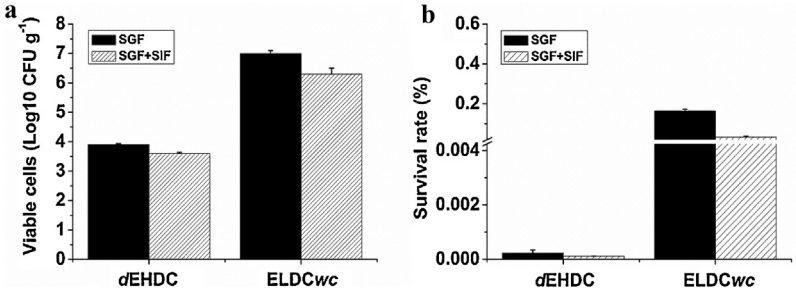

Fig. 6. Cell viability (a) and survival rate (b) of FC, *d*EHDC and ELDC*wc* microcapsules after exposure to simulated gastric fluids (SGF) for 2 h and simulated intestinal fluids (SIF) for 12 h.

substance permeability, which might improve the resistance ability of cells to freeze-drying and storage environments.

3.4. Survival rate of dried FC, dEHDC and ELDCwc in simulated gastric and intestinal fluids

Probiotics must be in adequate viable dose following oral administration to exert their beneficial effects. Therefore, the dried FC, *d*EHDC and ELDC*wc* were separately exposed to simulated gastric fluids (SGF) and simulated intestinal fluids (SIF) to test survival ability. This experimental design was based on the report that there was only a slight difference between *in vitro* and *in vivo* test on the acidity resistance (Berrada, Lemeland, Laroche, Thouvenot, & Piaia, 1991).

As shown in Fig. 6a, *d*EHDC decreased from 9.58 log CFU g^{-1} to 3.92 log CFU g^{-1}, while ELDC*wc* showed viability maintenance as high as 6.99 log CFU g^{-1} after 2 h of incubation in SGF (pH 1.0) at 37 °C. Meantime, FC group (non-encapsulated Y235 cells) was completely inactivated. The results clearly demonstrated that non-encapsulated cells cannot endure the harsh SGF environment (pH 1.0), while the microencapsulation provided significant protection for probiotics ($p < 0.01$). After being rinsed and transferred into SIF (pH 7.4) up to 12 h, the cell viabilities of *d*EHDC and ELDC*wc* were also determined (Fig. 6a). Both *d*EHDC and ELDC*wc* displayed slight decrease from 3.92 log CFU g^{-1} to 3.62 log CFU g^{-1}, and 6.99 log CFU g^{-1} to 6.29 log CFU g^{-1} respectively. Accordingly, the survival rate of *d*EHDC group decreased to almost zero but the survival rate of ELDC*wc* group kept at 0.2% after incubation in SGF (Fig. 6b). These results showed that the harsh SGF condition was against the viability maintenance while SIF was mild and had negligible adverse effect on both microencapsulated cells.

Moreover, it should be noted that ELDC*wc* group demonstrated significantly higher viability (three orders of magnitudes) than *d*EHDC group after incubation in SGF and SIF. The viable cells also reached the international recommended value in application. As discussed before, the decreased substance permeability caused by cell growth and metabolism of ELDC*wc* group (Yu et al., 2011), and the affected cell aggregation pattern by the changes of cell membrane composition (Reimann et al., 2011) might contribute to the resistance improvement of cultured cells under harsh SGF condition.

4. Conclusion

Microencapsulation technology has been utilized to protect probiotics against harmful stress environments such as drying, storage and oral administration in gastrointestinal tract. The traditional pattern is to directly entrap high density cells (*d*EHDC) into alginate microcapsules as final products. In this study, an alternative pattern of entrapping low density cells with subsequent culture (ELDC*wc*) was introduced by emulsification/internal gelation technique, and the performance of two kinds of microencapsulated probiotics was concerned when being subjected to different stress environments.

After the treatment of freeze-drying, microcapsules and free cells were stored at different temperature and timescale, and incubated in SGF and SIF. The viability of free cells, *d*EHDC and ELDC*wc* displayed obvious decrease trend in each stress environment. However, it clearly showed that microencapsulation technique significantly improved probiotics survival in orders of magnification compared to FC. Moreover, the new pattern of microencapsulation with culture (ELDC*wc*) also demonstrated higher resistance ability to different stress environments than *d*EHDC pattern. The viable cells of ELDC*wc* microcapsules could keep at 7.00 log CFU g^{-1} after 6 months storage at −20 °C, and maintained 6.29 log CFU g^{-1} after incubation in SGF for 2 h and subsequently in SIF for 12 h, which reached the recommended international standard of 10^6–10^7 CFU g^{-1}. Therefore, it suggested that the pattern of entrapping low density cells with subsequent culture (ELDC*wc*) might be a potential technique in application of functional foods and pharmaceutical industry.

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