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Soy Protein Isolate-Alginate Microspheres for Encapsulation of *Enterococcus faecalis* HZNU P2

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ABSTRACT

In this work, the mixture of alginate and soy protein isolate used as a wall material was developed to encapsulate Enterococcus faecalis *HZNU P2 (*E. faecalis *HZNU P2). The survival ability in the simulated gastric fluid (SGF) and bile salt solution, storage stability at different temperatures and release properties in the simulated intestinal fluid (SIF) of encapsulated cells were assessed. The results showed that encapsulation could offer sufficient protection to* E. faecalis *HZNU P2. The viability of encapsulated* E. faecalis *HZNU P2 did not decrease in SGF at pH 2.5 or 2.0 after 2 h incubation, while free cells were reduced from 11 to 9.85 log CFU/mL in SGF (pH 2.5) at the same exposure time. Only minor viability of encapsulated* E. faecalis *HZNU P2 lost in 1.0 or 2.0% bile salt solution for 1 or 2 h exposure, compared with no survival of free* E. faecalis *HZNU P2 under the same conditions. Encapsulated* E. faecalis *HZNU P2 was completely released from the microspheres in SIF within 1 h. The viability of encapsulated* E. faecalis *HZNU P2 stored for two weeks at 4°C was fully retained. Viabilities of encapsulated* E. faecalis *HZNU P2, 9.6 and 9.0 Log CFU/g were obtained at 25 and 37°C after 21 days storage, respectively. However, around 1.0 log CFU/mL of free cells was reduced after two weeks storage at 4°C. Encapsulated* E. faecalis *HZNU P2 using soy protein isolate and alginate as wall materials could play an important role in food applications.*

Key words: *Enterococcus faecalis*, Encapsulation, Soy protein isolate, Alginate, Stability, Viability

INTRODUCTION

Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (Sohail et al. 2012; Dong et al. 2013; Pan et al. 2013; Shi et al. 2013a). It can provide various health benefits, including preventing diarrhoea, balancing intestinal microflora, stimulating the immune system, improving lactose intolerance, etc. (Rajam et al. 2012; Dong et al. 2013; Khan et al. 2013; Pan et al. 2013; Shi et al. 2013a, b; Chen et al. 2014). However, it has been reported that many factors, such as low pH, hydrogen peroxide, dissolved oxygen, and storage temperature can affect the viability of probiotics (Champagne et al. 2011; Rajam et al. 2012; Dong et al. 2013; Chen et al. 2014). Many ways, such as appropriate selection of acid and bile resistant strains, two-step fermentation, stress adaptation, incorporation of micronutrients and encapsulation, have been proposed to increase the viability of probiotics against the adverse environmental conditions (Anal and Singh 2007; Rajam et al. 2012; Dong et al. 2013; Shi et al. 2013a, b). Among them, encapsulation is one of effective methods that

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improve the viability and stability of probiotics and also provide the release of probiotics in the gastrointestinal tract (Krasaekoopt et al. 2003; Dolly et al. 2011; Rajam et al. 2012; Dong et al. 2013; Pan et al. 2013; Shi et al. 2013a, b; Chen et al. 2014). It has many different applications in the food, biomedical, pharmaceutical and cosmetic industries as well as in agriculture and catalysis (Dubey et al. 2009; Dong et al. 2013; Nesterenko et al. 2013).

Alginate, a natural polysaccharide, has been widely used as a wall material for encapsulation. However, encapsulation of the probiotics only using alginate as a wall material can not protect the probiotics effectively from low pH environment (Krasaekoopt et al. 2003; Rajam et al. 2012; Pan et al. 2013; Shi et al. 2013a, b; Chen et al. 2014). Soy protein isolate can be used as an encapsulation material due to its interesting physico-chemical properties, in particular gel-forming and emulsifying properties (Gu et al. 2009; Dong et al. 2013; Nesterenko et al. 2013). Many works have indicated that relative to sodium caseinate or whey protein, the encapsulated products with soy protein isolate as a wall material exhibit comparable or even better encapsulation efficiency, and higher stability against oxidation (Charve and Reineccius 2009; Rascon et al. 2011; Nesterenko et al. 2013). Generally, soy protein isolate is not only used as an individual coating material, but also can be mixed with polysaccharides (Augustin et al. 2006; Rusli et al. 2006; Nesterenko et al. 2013). However, the application of soy protein isolate in probiotics encapsulation is still very limited.

Enterococci belong to lactic acid bacteria (LAB), which play an important role in the development of the sensory characteristics of fermentation foods, such as sausages and cheeses (Sánchez et al. 2007; Zheng et al. 2015). Some enterococcal strains have been successfully used as preservatives to inhibit the growth of food spoilage microorganisms (Zheng et al. 2015). A new strain, *Enterococcus faecalis* HZNU P2 from peacock fecal samples was isolated, which showed good tolerance to NaCl and simulated intestinal juice, good adhesion ability to the intestinal cell, as well as good antimicrobial activity against selected pathogens. However, it did not show good stability in SGF and bile salt solution. Therefore, in present study, it was encapsulated in soy protein isolate-alginate microspheres. The tolerance ability to low pH and bile solution, as well as release characteristic in SIF was investigated. In addition, storage viability of the cells was also determined.

MATERIAL AND METHODS

The bacterial strain

The strain *E. faecalis* HZNU P2 was isolated from the fresh peacock fecal samples, which was collected in China Center for Type Culture Collection (CCTCC). The preservation number is CCTCC M 2014197. The culture was stored at -80℃ in MRS broth (Oxoid), supplemented with 25% glycerol. Stock culture was propagated in MRS broth at 37°C for 24 h and cell biomass was harvested after centrifugation at 3000 g, 4°C for 10 min, washed by sterile water, and then re-suspended in saline solution (0.85%). The cell suspension was used for the following work.

Encapsulation of *E. faecalis* HZNU P2 in soy protein isolate-alginate microspheres

Soy protein isolate (Yunxin Cooperation Inc., Shandong, China; protein concentration >90%, fat concentration <1.0%, moisture content < 7.0%) and sodium alginate (low viscosity, Sigma Aldrich, Shanghai) were sterilized at 110 and 121°C for 15 min, respectively. Soy protein isolate-alginate microspheres were prepared according to previous works (Pan et al. 2013; Shi et al. 2013a, b; Chen et al. 2014; Tang et al. 2013, 2015). Briefly, *E. faecalis* HZNU P2 was mixed with sodium alginate and soy protein isolate. The mixture (alginate/soy protein isolate=1/3, v/v, alginate concentration 1.0%) was injected through an Inotech Encapsulator IER-50 (Inotech Biosystems Intl. Inc., Reppischhof, Switzerland) into 100 mM $CaCl_2$ at gently stirring (100 rpm) using 450 μm nozzle. The microspheres were allowed to stand for 30 min for gelification, then washed by distilled water, and subsequently kept in sterile conical tubes (High Clarity Polypropylene, Fisher Scientific Inc., Shanghai, China).

The encapsulation yield of *E. faecalis* HZNU P2 was calculated as follows: encapsulate yield (%) = (quantity of *E. faecalis* HZNU P2 released from the broken microspheres/quantity of *E. faecalis* HZNU P2 initially taken to prepare the microspheres) × 100. The size of microspheres was measured using an optical microscope (Carl Zeiss, Germany). Fifty microspheres were randomly picked to determine the size. The size of

each sample was presented as the mean size ± standard deviation (SD).

Bacterial enumeration

The microspheres containing *E. faecalis* HZNU P2 (0.50 g) were broken in 4.5 mL, 50 mM sterile sodium citrate solution by gently shaking at room temperature. Appropriate dilutions were made in 0.85% saline solution. Diluted solutions (100 μL) were plated onto MRS agar. Colonies of *E. faecalis* HZNU P2 were enumerated after the incubation at 37°C for 24 h. Free *E. faecalis* HZNU P2 was 10 times serially diluted with saline solution and 100 μl aliquots were plated on MRS agar. Colonies of *E. faecalis* HZNU P2 were enumerated according to the method used for enumeration of encapsulated *E. faecalis* HZNU P2.

Resistance ability of free and encapsulated *E. faecalis* HZNU P2 to SGF

The tolerance of free and encapsulated *E. faecalis* HZNU P2 to the simulated gastric fluid (SGF, 0.20 % NaCl) was determined as described previously (Pan et al. 2013; Shi et al. 2013a, b; Tang et al. 2013, 2015; Chen et al. 2014). SGF was adjusted to pH 2.0 and 2.5 with concentrated HCl. A 0.50 mL cell suspension or 0.50 g encapsulated *E. faecalis* HZNU P2 was mixed with 4.5 mL SGF and incubated at 37°C for 10, 30, 60, 90 and 120 min. After the specified time intervals, samples were harvested and immediately used for the enumeration of viable cells according to the method described above.

Survival of free and encapsulated *E. faecalis* HZNU P2 in bile salt solution

Tolerance of the free and encapsulated *E. faecalis* HZNU P2 to bile salt solutions with various concentrations were carried out as described previously (Pan et al. 2013; Shi et al. 2013a, b; Chen et al. 2014; Tang et al. 2013, 2015). Briefly, 0.50 g microspheres or 0.50 mL cell suspension was transferred in test tubes containing 4.5 mL, 1.0 or 2.0% bile salt solution (Sigma-Aldrich, Shanghai) and incubated at 37°C for 1 or 2 h. The enumeration of viable cells was carried out according to the method described above.

Release of encapsulated *E. faecalis* HZNU P2 in SIF

The release of encapsulated *E. faecalis* HZNU P2 in SIF (pH 6.8, 50 mM KH_2PO_4) was tested using the method described previously (Pan et al. 2013; Shi et al. 2013a, b; Chen et al. 2014; Tang et al. 2013, 2015). Microspheres of 0.50 g were added to 4.5 mL SIF and the mixture was incubated at 37°C. A 100 μL the supernatant was taken at time intervals of 0, 10, 30, 60, 90 and 120 min and then serially diluted in 0.85 % saline solution. The same volume of the fresh medium was added to replace the volume of the withdrawn samples. The enumeration of viable cells was carried out according to the method described above.

Shelf lives of free and encapsulated *E. faecalis* HZNU P2

The survival of free and encapsulated *E. faecalis* HZNU P2 were tested at 4, 25 and 37°C. The samples were taken at time intervals of storage and the number of viable cells was determined as the method described above.

Statistics analysis

All the experiments were conducted in triplicate and the results were expressed as mean value ± standard deviation (SD). The data was analyzed using Origin 8.0 for Windows. The significant differences were compared using Student's t test. The statistical significance was accepted at the level of $P < 0.05$.

RESULTS AND DISCUSSION

One of necessary pre-requisites for encapsulation method is high encapsulation yield (Chen et al. 2014). The initial numbers in *E. faecalis* HZNU P2 suspension were about 11 log CFU/mL. The numbers in microspheres were 10.98 log CFU/g. High encapsulation rate (≈ 100 %) was achieved. Therefore, soy protein isolate-alginate matrices had a good compatibility with *E. faecalis* HZNU P2.

The diameters of soy protein isolate-alginate microspheres obtained in this work were around 800 ± 10 μm (data not shown).The size of the microspheres is an important factor for the application in food industry. Large microspheres can adversely affect the texture of the foods; small microspheres may not provide sufficient protection to probiotics (Shi et al. 2013a, b; Chen et al. 2014). In this study, soy protein isolate-alginate microspheres were below 1.0 mm. It would not produce enough detrimental effect on the texture of foods.

Stability of free and encapsulated *E. faecalis* HZNU P2 in SGF

One of major problems for the probiotic foods is the low survival of the cells in the gastric pH (Pan et al. 2013; Shi et al. 2013a, b; Chen et al. 2014). Free and encapsulated *E. faecalis* HZNU P2 were tested for the survival ability in SGF (pH 2.0 and 2.5) (Fig. 1). Evidently encapsulation improved the stability of *E. faecalis* HZNU P2 in SGF. The numbers of encapsulated *E. faecalis* HZNU P2 did not decrease after 2.0 h incubation in SGF (pH 2.5 and 2.0). However, the viability of free *E. faecalis* HZNU P2 decreased from around 11 Log CFU/mL to 9.85 and 9.45 Log CFU/mL for 2.0 h exposure at SGF pH 2.5 and 2.0, respectively. Many studies have indicated that encapsulation can help in improving the survival of the cells in SGF (Chandramouli et al. 2004; Krasaekoopt et al. 2004; Iyer and Kailasapathy 2005; Annan et al. 2008; Chavarri et al. 2010; Pan et al. 2013; Shi et al. 2013a, b; Chen et al. 2014; Sathyabama et al. 2014). To date, the use of plant protein isolate as a wall material for probiotic encapsulation, particularly soy protein isolate, is extremely limited. Klemmer et al. (2011) reported that the viable cell numbers in pea protein isolate-alginate capsules containing *B. adolescentis* were reduced 1.0 Log CFU/g over a 2 h exposure in SGF at pH 2.0 and 37°C. Kotikalapudi et al. (2010) found that encapsulated *Lactobacillus acidophilus* in pea protein isolate-alginate capsules showed high resistance to SGF at pH 2.0.

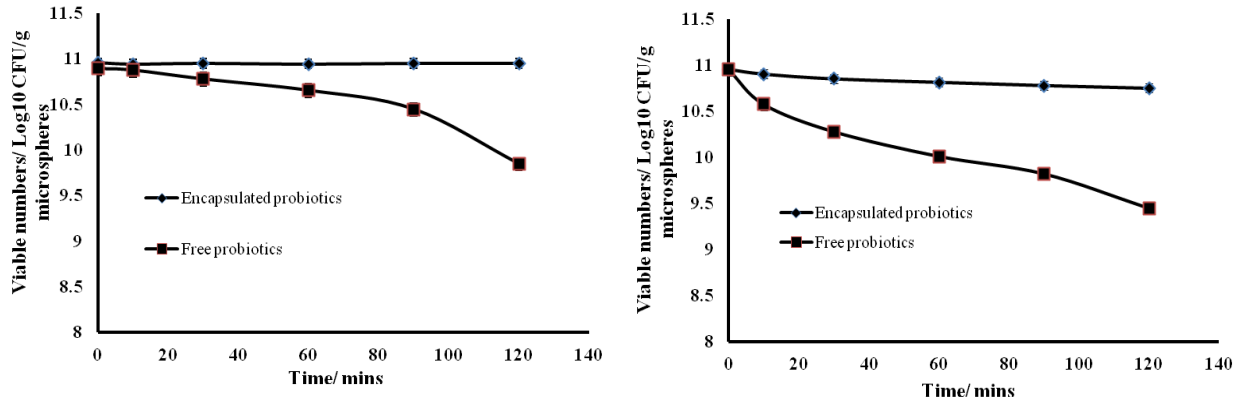

Figure 1 - pH stability of free and encapsulated *E. faecalis* HZNU P2 in simulated gastric fluid (SGF) pH 2.0 and 2.5. Fig. 1-A The stability of free and encapsulated *E. faecalis* HZNU P2 in SGF pH 2.5. Fig. 1-B The stability of free and encapsulated *E. faecalis* HZNU P2 in SGF pH 2.0.

Stability of free and encapsulated *E. faecalis* HZNU P2 in bile salt solution

The stability results of free and encapsulated *E. faecalis* HZNU P2 subjected to bile salt solutions (1.0 and 2.0%) are shown in Table 1. No survival of free *E. faecalis* HZNU P2 cells was found after 1 h exposure in 1.0 or 2.0 % bile salt solution. The sensitivity of many strains of probiotics to bile salt solutions has been reported by many researchers (Amor et al. 2002; Picot and Lacroix 2004; Pan et al. 2013; Shi et al. 2013a, b; Chen et al. 2014; Sathyabama et al. 2014). Table 1 shows slight reductions of encapsulated *E. faecalis* HZNU P2 after 2 h incubation in 1.0 or 2.0 % bile salt solutions. Many studies have shown that encapsulation can improve the survival numbers of probiotics in bile salt conditions (Krasaekoopt et al. 2003; Chandramouli et al. 2004; Iyer and Kailasapathy 2005; Mandal et al. 2006; Pan et al. 2013; Shi et al. 2013a, b; Chen et al. 2014; Sathyabama et al. 2014). However, in contrast to the present results, Trindade and Grosso (2000) observed that encapsulation of *Bifidobacterium bifidum* and *L. acidophilus* in alginate beads was not effective in protecting the cells from 2.0 to 4.0% bile salt solutions. In the present study, high survival numbers of *E. faecalis* HZNU P2 in soy protein isolate-alginate microspheres after bile salt solution treatment was pre-requisite for providing a beneficial health effect to the host.

Table 1 - Survival of free and encapsulated *E. faecalis* HZNU P2 after treatment in bile salt solutions of 1.0 and 2.0 % for 1 and 2 h (Log CFU/mL or g microspheres)

Incubation time (h)	Sample	Bile concentration (%)		
		0	1	2
	Free	10.944± 0.12	0	0
1	Encapsulated	10.854± 0.07	10.645± 0.09	10.402± 0.13
	Free	10.954± 0.11	0	0
2	Encapsulated	10.944± 0.09	10.406± 0.12	10.277± 0.14

Release of encapsulated *E. faecalis* HZNU P2 in SIF

The release of probiotics from the microspheres in intestinal tract is essential for the growth and colonization of probiotics (Pan et al. 2013; Shi et al. 2013a, b; Chen et al. 2014). Figure 2 shows the release of encapsulated *E. faecalis* HZNU P2 in SIF. When encapsulated *E. faecalis* HZNU P2 was placed into SIF, the microspheres began to swell and eventually disintegrated. *E. faecalis* HZNU P2 could be released from the soy protein isolate-alginate microspheres in SIF within 1 h. Analogous probiotic release behaviour was reported by Klemmer et al. (2011) for encapsulated *B. adolescentis* in pea protein isolate–alginate. It is suggested that the developed encapsulated formulations have potential for the targeted delivery of probiotics to the lower gastrointestinal tract. Chen et al. (2014) found that 70% of encapsulated *L. bulgaricus* in whey protein isolate-alginate microspheres could be released in SIF within 1 h. Tang et al. (2015) clearly demonstrated that encapsulated phage K in whey protein–alginate microspheres had a burst release in the first 1 h, followed by a sustained release for up to 2 h.

Storage stability of free and encapsulated *E. faecalis* HZNU P2

Storage stability of the free and encapsulated *E. faecalis* HZNU P2 at 4, 25 and 37°C is shown in Figure 3. The viability of encapsulated *E. faecalis* HZNU P2 showed higher storage stability compared to that of free *E. faecalis* HZNU P2. Viability of the free *E. faecalis* HZNU P2 was reduced from 10.94 to 10.76 log CFU/mL after one week, and around 9.80 log CFU/mL after two weeks (Fig. 3A). However, the numbers of encapsulated *E. faecalis* HZNU P2 were only slightly reduced (Fig. 3A). The survival numbers of encapsulated cells were reduced from around 11.0 Log CFU/g to 9.6 and 9.0 Log CFU/g after 21 days storage at 25 and 37 °C, respectively (Fig. 3B). Therefore, soy protein isolate-alginate microspheres could improve the storage stability of *E. faecalis* HZNU P2. Several studies have indicated that encapsulated probiotics in alginate-based microspheres had better storage ability than free cells (Krasaekoopt et al. 2003; Yew et al. 2011; Pan et al. 2013; Shi et al. 2013a, b; Chen et al. 2014).

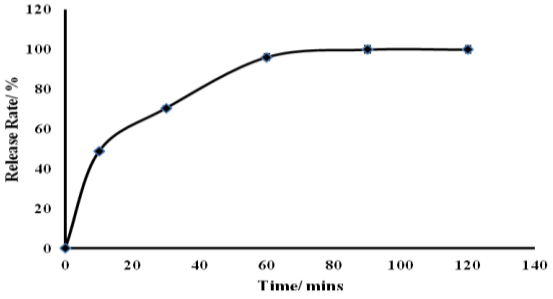

Figure 2 - Release of encapsulated *E. faecalis* HZNU P2 in simulated intestine fluid (SIF).

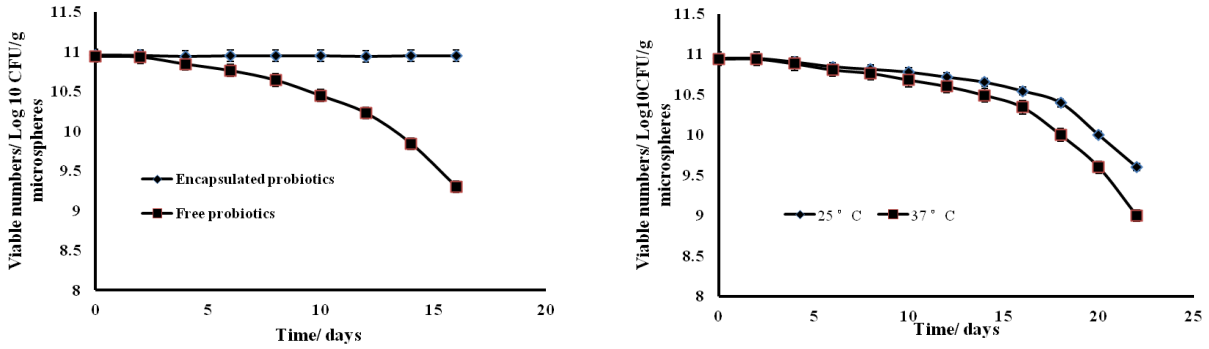

Figure 3 - Storage stability of free and encapsulated *E. faecalis* HZNU P2 at 4, 25 and 37°C. Fig. 3-A Storage stability of free and encapsulated *E. faecalis* HZNU P2 at 4°C. Fig. 3-B Storage stability of encapsulated *E. faecalis* HZNU P2 at 25 and 37°C.

CONCLUSIONS

This study demonstrated that *E. faecalis* HZNU P2 could be encapsulated in soy protein isolate-alginate microspheres. Microspheres provided good protection to *E. faecalis* HZNU P2 against adverse influences of gastric and bile salt conditions. The encapsulated cells showed a rapid release in SIF. The results suggested that soy protein isolate-alginate microspheres could be a potential candidate for oral delivery of *E. faecalis* HZNU P2 and could pave the way for utilizing the market-friendly plant-based protein for producing controlled delivery systems for the probiotic industry.

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