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Enhancement of Probiotic Survival in Low pH and Bile salt Condition using Alginate–Hi–maize Starch Encapsulation

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Abstract

There has been an increased interest in foods-containing probiotics over the past few decades. To provide health benefits to consumers, probiotics must survive in food during storage and during transit through the gastrointestinal tract. In this study, the encapsulated probiotic bacteria were tested in comparison with free cells for their acid and bile tolerance. Two probiotic strains, i.e. *Lactobacillus casei* TISTR 1463 (LC 1463) and *Lactobacillus acidophilus* TISTR 1338 (LA 1338) were separately encapsulated with calcium alginate and co-encapsulated with 0-3% Hi-maize starch using extrusion method. The acid tolerance of probiotics was examined in acidified MRS broth at pH 2, 4 and 6.5 over a 3-h incubation period. Bile tolerance was tested using 0.5 or 1% bile salt in MRS broth over a 3-h of incubation. The results showed that co-encapsulation with 2% Hi-maize starch enhanced the viability of both probiotic strains in acid. For LC 1463, viability of co-encapsulated bacteria decreased by 1.62 log CFU/ml after 3-h of incubation at pH 2, while 3.9 log CFU/ml and 4.5 log CFU/ml were lost in the encapsulated and free cells, respectively. For LA 1338, there was a 2.06 log CFU/ml reduction in viability of co-encapsulated bacteria after 3-h exposure to pH 2, compared with the decrease by 2.6 log CFU/ml and 4.2 log CFU/ml in encapsulated and free cells, respectively. However, at pH 4 and 6.5 no significant difference in the reduction of viable count was found between free and encapsulated cells. Similar results were obtained in bile salt tolerance test. Co-encapsulated LC 1463 survived better than other samples, showing only 1.4 log CFU/ml reduction after exposure to 1% bile salt for 3 h. Under the same condition, the viability reduction of free and encapsulated cells was 3.7-4.7 log CFU/ml. For LA 1338, co-encapsulated bacteria survived with a loss of 2.2 log CFU/ml, compared to 4.75-4.84 log CFU/ml loss with free and encapsulated cells. Overall, encapsulation with alginate-starch could be a useful method for maintaining the viability of probiotics in acidic foods and for improving the bacterial delivery to the human gut.

Keywords: Hi-maize starch, Probiotics, Survival, Co-encapsulation

1. Introduction

Probiotics are live microorganisms which confer a wide range of health benefit to humans by improving intestinal microbial balance and thus inhibiting pathogens and toxin producing bacteria (1). Lactobacillus is one of the most commonly studied genus of probiotic bacteria. These bacteria have been found to prevent infectious diarrhea in children, lower serum cholesterol levels, boost immune system, alleviate symptoms of lactose indigestion and reduce a risk of certain cancers (2-3). To exert its benefits, there should be a minimum level of 10^7 CFU probiotic bacteria per ml or g food at the time of consumption (4). Therefore, these organisms must survive the adverse conditions, e.g. the presence of acid, oxygen exposure and high temperature during manufacturing, packaging and storage of food. Additionally, probiotic bacteria must be viable and maintained their activity in the gastric acidity, bile salts and enzymes conditions of the human upper gastrointestinal tract (5).

Encapsulation is a process of enclosing solid or gas microparticles in an inert matrix, which protects them from the external environment. This technique is currently accepted within the food, pharmaceutical, chemical and cosmetic industries. For the food industry, encapsulation has been employed to protect nutrients, biologically active ingredients or bacterial cells from stressful environment and chemical interaction (6). Among the various developed methods, the encapsulation extrusion method has been frequently used for the protection of probiotic bacteria (7). By this technique, the choice of ingredients for the protective matrix is an essential factor in maintaining probiotic bacteria stability. The most widely used gel matrix for encapsulation is alginate because of easy handling, non-toxicity to the cells, and cheapness (8-11). However, alginate gels are susceptible to disintegration in the presence of excess monovalent ions, Ca^{2+} chelating agents, and harsh chemical environments. The application of coating alginate beads with polyelectrolytes such as chitosan and polylysine, as well as incorporation of resistant starch (prebiotic) as co-encapsulant have been reported to improve the effectiveness of encapsulation (11-12).

The aim of this study was to investigate the survivals of free, alginate encapsulated and co-encapsulated lactobacilli in in vitro acidic and bile salt conditions. Several parameters, i.e. the degree of acidity, the concentration of bile salt and the time of exposure were determine in order to know whether co-encapsulation technique could improve the availability of probiotics against adverse gastrointestinal conditions.

2. Materials and Methods

2.1 Probiotic strains and growth condition

Freeze-dried probiotic cultures, i.e. *Lactobacillus casei* TISTR 1463 (LC 1463) and *Lactobacillus acidophilus* TISTR 1338 (LA 1338) were obtained from Thailand Institute of Scientific and Technological Research. Pure probiotic cultures were separately activated by inoculating in a 250 ml de Man-Rogosa-Sharpe (MRS) broth at 37°C for 16-18 h on an orbital shaker at 100 rpm. Each culture was stored at 4°C and routinely cultured in MRS broth.

2.2 Preparation of probiotic cell suspension

Stock culture was streaked into plates prepared from MRS agar. A single colony of each probiotic culture from a plate was transferred into a 10 ml MRS broth, which was then used to inoculate 250 ml of MRS broth. The 250 ml broth was incubated at 37°C for16-18 h until reaching early log phase of cellular growth. Subsequently, the cells of each probiotic strain were harvested by centrifugation at 3,500 rpm for 15 min at 4°C. The free cells were washed twice with sterile 0.1% peptone water. The washed cells were resuspended in the minimum volume of sterile peptone water and the cell suspension (2.2×10^9 CFU/ml)

was submitted to encapsulation and co-encapsulation as described below.

2.3 Encapsulation of probiotic bacteria

Encapsulation of probiotic bacteria with alginate as the supporting matrix was performed using the extrusion technique as described by Krasaekoopt et al. (9), Sheu and Marshall (13). Briefly, a 2% sodium alginate solution was sterilized at 121°C for 15 min and cooled to 38-40°C. To prepare the alginate-cell mixture, 15 ml of probiotic cell suspension was mixed with 100 ml of sterile alginate solution by using magnetic stirrer for 5 min. Subsequently, the alginate-cell mixture was placed in a sterile syringe and injected through a 0.5 mm needle into a 500 ml sterile 0.1 M calcium chloride ($CaCl_2$) solution containing 0.1% Tween 80 under constant stirring at 100 rpm. The calcium-alginate beads were allowed to settle at the bottom for 30 min. The $CaCl_2$ solution was then drained and the culture beads were rinsed and kept in sterile distilled water at 4°C for 10 h to allow the beads to fully harden. The diameter of the beads was approximately 0.3-0.5 mm.

2.4 Co-encapsulation of probiotic bacteria

Probiotic cultures were co-encapsulated using methods described by Krasaekoopt et al. (9), Sheu and Marshall (13), and Sultana et al. (11). In brief, sterile 2% sodium alginate solutions containing 1%, 2% or 3% Hi-maize starch (Sigma-aldrich, USA) were prepared. A 100 ml of alginate-starch solution was then mixed with 15 ml of cell suspension to yield a final viable cell of 3.8-6.3 x 10^8 CFU/ml. To form beads, the alginate-starch solution was transferred to a sterile syringe which was connected to a 0.5 mm needle. The beads were extruded into a 500 ml bath of sterile 0.1 M $CaCl_2$ containing 0.1% Tween 80 and were left to polymerize for 30 min at room temperature. The $CaCl_2$ solution was removed, and the beads were washed using sterile distilled water.

2.5 Cell survival in acid condition

The acid tolerance of encapsulated and co-encapsulated probiotic bacteria was examined at various pH values over a 3-h incubation period. Free probiotic organisms were used as a control. Sterile MRS broth with pH adjusted to 2.0, 4.0 and 6.5 with 5M hydrochloric acid was prepared according to Ding and Shah (5). One gram of beads with entrapped probiotic bacteria (LC 1463 or LA 1338) or 1 ml of cell suspensions, was mixed in 10 ml of acidified MRS broth and incubated at 37°C for 0, 1, 2 and 3 h with constant agitation at 100 rpm.

2.6 Cell survival in bile salt condition

The survivals of free, single-encapsulated cells, and co-encapsulated probiotic bacteria in bile salts conditions were studied as described below. Sterile MRS broth containing 0, 0.5 or 1% bile salt was prepared and adjusted the pH to 7.0 with 5M hydrochloric acid. To evaluate the tolerance to the action of bile salt, 1 g of single-encapsulated and co-encapsulated cells or 1 ml of free cell suspension was added to 10 ml of prepared MRS broth. Then, the samples were incubated in a temperature-controlled orbital shaker at 37°C with agitation at 100 rpm. Samples were tested for viable counts at different time intervals (0, 1, 2 and 3 h).

2.7 Enumeration of probiotic bacteria

The viable count of probiotic bacteria was enumerated on MRS agar using the pour plate technique. For the enumeration of encapsulated and co-encapsulated probiotic organisms, the entrapped bacteria were released from the capsules by sequestering calcium ions with a pH 7.0 phosphate buffer solution under gentle agitation for 30 min. Viable cell counts were determined after 72 h of incubation at 37°C under aerobic condition.

2.8 Statistical analyses

Each experiment was individually repeated at least 2 times. Enumerations of probiotic bacteria were done in

duplicate. All data were statistically analyzed using SPSS 16.0 software (SPSS Inc., Chicago, USA). One way ANOVA analysis was employed to evaluate the significant differences between sample means at a significance level of 0.05. Significant differences between the means of probiotic counts were determined using Duncan's multiple range tests.

3. Results and Discussion

3.1 Cell survival in acidic condition

The results showing cell survival of LC 1463 and LA 1338 in acidic condition were presented in Figures 1 and 2. Overall, there was no significant difference (P>0.05) in the viability of free, alginate encapsulated and co-encapsulated cells of both Lactobacillus strains in acidified MRS broths with pH 4.0 and pH 6.5 over a 3-h of incubation. However, co-encapsulation with Hi-maize showed positive influence on cell viability in MRS broth with pH 2.0 compared to the control (free cell) at 95% confidence level.

Figure 1(a) showed the survivals of free, encapsulated and co-encapsulated LA 1338 at the pH 2.0. The cell reductions of 4.2 and 2.6 log CFU/ml were achieved for free and encapsulated LA 1338, respectively after 3-h of incubation. For co-encapsulation with 1, 2 and 3% Hi-maize starch, the viability losses of probiotics were 2.5, 2.1, and 2.13 log CFU/ml, respectively.

A similar result was observed for LC 1463. As shown in Figure 2(a), there was a decreased viability of free and encapsulated LC 1463 cells by 4.6 and 3.9 log CFU/ml, respectively when they were exposed to MRS broth with pH 2.0 for 3 h. Under the same condition, the viable cells of co-encapsulated bacteria with 1, 2 and 3% Hi-maize starch decreased approximately 2.3, 1.6, and 1.1 log CFU/ml, respectively.

These results indicated that the co-encapsulation of probiotics with Hi-maize resistant starch provided high ability in protecting probiotic bacteria to acid. Adding 2 or 3% of Hi-maize starch into alginate solution showed higher survival number of probiotic cells than those of 1% Hi-maize concentration.

3.2 Cell survival in bile salt condition

The survivals of two Lactobacillus strains in bile salt tolerance test were presented in Figures 3 and 4. Overall, co-encapsulation could significantly enhance the viability of probiotic cells in bile salt conditions as compared to non-encapsulation and alginate encapsulation. In addition, the effect in protecting the co-encapsulated bacteria was increased with increasing Hi-maize concentration from 1 to 3 %.

In MRS broth without bile salt addition, no significant changes (P>0.05) in the viability of free, encapsulated and co-encapsulated cells were observed over a 3-h incubation period as shown in Figures 3(a) and 4 (a). This confirmed that MRS broth had no adversely effect on the viability of probiotics. On the other hand, there was a statistically significant difference in reduction of viable count among free, encapsulated and co-encapsulated cells at 0.5 and 1% bile salts ($P \leq 0.05$).

For LA 1338, small reduction in viability occurred in co-encapsulated cells with 2 and 3% Hi-maize starch after 3-h of the exposure period to 1% bile salt (1.28 and 1.12 log CFU/ml, respectively). Under the same condition, the losses of free and alginate encapsulated cells were about 4.75 and 4.84 log CFU/ml, respectively. Cell of co-encapsulated sample with 1% Hi-maize declined at a similar rate as its free cells (Figure 3c).

In Figure 4(c), after 3-h of exposure to 1% bile salt, co-encapsulated LC 1463 with 1, 2 and 3% Hi-maize starch had high survival rate, showing approximately 2.2, 1.4 and 1.3 log CFU/ml reduction, respectively. Alginate encapsulated and free cell samples were a poor survivor. The viability of those two samples was declined by 3.7 and 4.7 log CFU/ml, respectively.

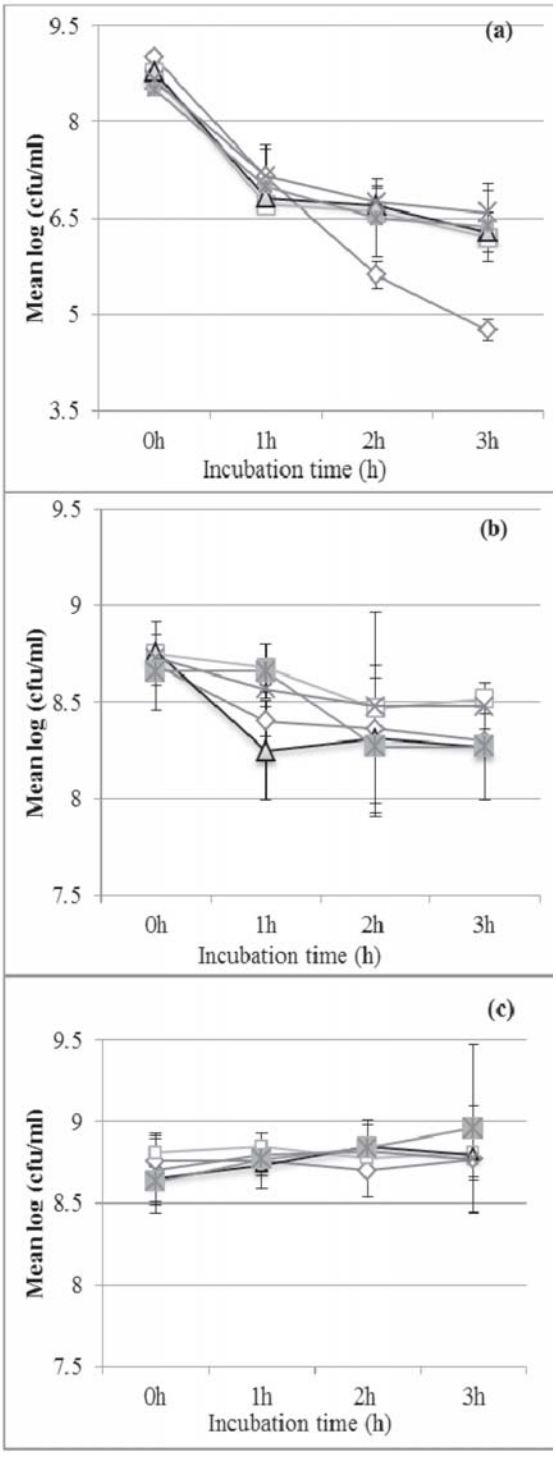

Figure 1. Survival of LA 1338 in acidified MRS broth pH 2 (a), pH 4 (b) and pH 6.5 (c). ◊ = Free, □ = Encapsulated, △ = Co-encapsulated+1% Hi-maize, ╳ = Co-encapsulated + 2% Hi-maize, ✳ = Co-encapsulated + 3% Hi-maize

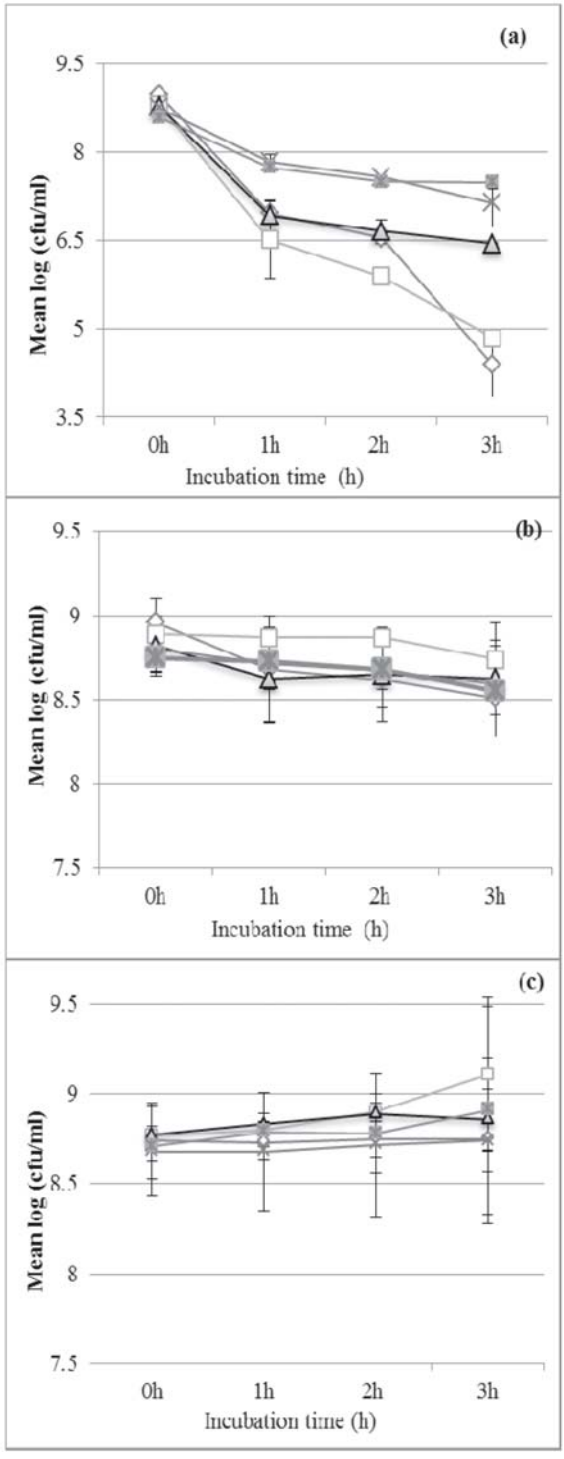

Figure 2. Survival of LC 1446 in acidified MRS broth pH 2 (a), pH 4 (b) and pH 6.5 (c). ◊ = Free, □ = Encapsulated, △ = Co-encapsulated+ 1% Hi-maize, ╳ = Co-encapsulated + 2% Hi-maize, ✳ = Co-encapsulated + 3% Hi-maize

3.3 Discussion

To exert the health benefits, probiotic bacteria must survive gastric acid presented in the stomach and bile in the duodenum, and reach the colon in a high number of cells. Although alginate hydrogels are preferred for encapsulating probiotic cells, alginate beads are very porous and sensitive to the acidic condition. Therefore, in this study, a type II resistant starch derived from Hi-maize was applied as co-encapsulating material with alginate to protect probiotic bacteria from low pH and high bile salt conditions. Hi-maize starch that consists of > 70% amylose content and 32.5% total dietary fiber has been chosen due to high resistivity against gastric juice and enzymatic digestion (14-15). Moreover, Hi-maize starch could beneficially be substrates for microbial fermentation if reached the human colon.

The results of the study indicated that the incorporation of Hi-maize starch into the alginate matrix provided better protection to probiotic cells than alginate encapsulation and without encapsulation in both conditions. Viability of co-encapsulated probiotic cells also depended on the concentration of Hi-maize starch. It was found that the addition of starch at the rate 3% (w/v) showed the greatest protection. Co-encapsulation with 2% starch had a similar, but lesser, effect than that of 3% addition.

When subjected to acidified MRS broths with pH 6.5, the viability of free, encapsulated and co-encapsulated LA 1338 and LC 1463 maintained fairly constant over a 2-h incubation period as shown in Figures 1(c) and 2(c). This could be because the probioitc bacteria did not injured under low acid condition. However, the co-encapsulation with 3% Hi-maize starch resulted in a slight, although insignificant, increase (P>0.05) in the cell concentration of both probiotics at 3 h of exposure. Possibly, Hi-Maize starch might have provided additional nutrients or modified a negative environmental impact, which therefore stimulated the growth of probiotics.

The effect of encapsulation on the survival of probiotic bacteria under adverse conditions was previously reported with contradicting results. Some studies indicated that encapsulation did not effectively protect probiotic bacteria from strong acidic condition (14). Sultana et al. (11) reported similarly that the survival of encapsulated probiotics with alginate-Hi-maize starch did not enhance after exposure to acidic and bile salt solutions. On the other hand, Iyer and Kailasapathy (8) found that co-encapsulated probiotic bacteria with Hi-maize starch survived better in gastric juice than those of alginate beads. The present study suggested that an extrusion encapsulation with alginate-Hi-maize starch was proved to protect both panels of probiotic strain from harmful environmental conditions. This could be explained that mixing alginate with starch has led to the modification of alginate hydrogel structure. Perhaps, the pore size and porosity of the obtained beads were both decreased (16), thus providing additional protection and preventing the diffusion of acidic and bile salt solutions into the bead.

4. Conclusion

The concept of encapsulation offers benefits to protect bacterial cell from harsh environments. In the present study, the inclusion of Hi-maize starch (prebiotic) as co-encapsulant into alginate beads at the rate 2 and 3% has been proved to enhance the survivals of LC TISTR 1463 and LA TISTR 1338 in acid and bile salt conditions as compared with encapsulated and free cells. Further research is needed to determine whether co-encapsulation with Hi-maize starch is an effective technique in ensuring the viability of encapsulated bacteria in food models.

5. Acknowledgement

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