Viability of lactic acid bacteria coated as synbiotic during storage and gastro-intestinal simulation

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Abstract. Lactic acid bacteria (LAB) has been added to various food products as a probiotic agent because it has been known to provide beneficial health effects in humans. In the application of LAB, cell viability often decreased as influenced by environment stresses. Encapsulation technique is one of the cell protection techniques using a coating material. Effective coating material is required to produce maximum protection of LAB cells. In this study, candidate of probiotic LAB (isolate US7) was encapsulated with alginate-mung bean flour and alginate-gram flour with inulin prebiotic by extrusion technique. Viability of encapsulated LAB cells were able to survive by up to $10^8$ CFU g$^{-1}$ after 4 weeks of storage at 4 °C. Beads were incubated in simulated liquid gastric acid (pH=2) for 2 hrs and simulated intestinal fluid (pH=6) for 3 hrs at 37 °C. The results showed that encapsulated LAB cells maintained the survival rate of 97% with the number of cells at 9.07 Log CFU g$^{-1}$ in the simulated liquid gastric acid and then followed by releasing cells in simulated intestinal fluid. In general, this study indicates that encapsulation with alginate-mung bean flour and alginate-gram flour with inulin successfully protect probiotic bacteria against simulated human gastro-intestinal conditions.

Keywords: Alginate-mung bean, encapsulation, lactic acid bacteria, probiotic

1. Introduction

Probiotics have been studied for its health promoting effects such as: lowering cholesterol, improving lactose tolerance, nutritional enhancement and preventing some cancers and antibiotic associated diarrhea [1]. The benefit of using probiotic is due to their nature on production of acid and bacteriocins, competition with pathogens rendering their adhesion to the intestine and enhancement of the immune system [2]. Probiotics are commonly lactic acid bacteria (LAB) with common examples like Lactobacillus and Bifidobacterium which are extensively investigated for their beneficial effects when incorporated in fermented foods [3,4]. Therefore, LAB have been considered as Generally Recognized as Safe or GRAS [5].

Due to the perceived health benefits of probiotics, there has been an increased use of probiotics in different health based products. Probiotics have been incorporated into a variety of food products. Probiotics have gained global popularity but several studies showed some generala weakness in terms of their cells viability in food products (especially fermented ones). When administered into physiological body, e.g. gastrointestinal conditions may become the barrier for their existence in
human. These have encouraged researchers to innovate different methods on delivering probiotics. Encapsulation of the probiotic cells is one of the newest and highly efficient methods, which is now under the special attention and is being developed by various researchers. A variety of substrates have been employed as encapsulating materials for entrapping live microorganisms, including alginate, whey protein, pea protein, gelatin, methylcellulose, etc. [6,7]. Among these, alginate-gel beads have frequently been used for the immobilization of LAB [6]. In this study, Lactic acid bacteria (LAB) as a candidate of probiotic was isolated from North Sumatera freshwater fish farm and intestine of Oreochromis niloticus L. Alginate, mung bean flour, gram flour and inulin were used to encapsulate probiotic for maintaining cell viability during processing, storage and in artificial gastric juice simulation. The aim of this study was to determine the effective material ingredients of encapsulation and to determine the effects of temperature, storage duration and tolerance within simulated gastric juice to cells viability of encapsulated probiotics.

2. Methods

2.1 Preparatory of Lactic Acid Bacteria Culture

Pure cultures of isolated LAB namely AK1, AK3, EK2 and US7 were sub-cultured into MRS agar (Oxoid™, UK) medium and incubated at 37 °C for 24 hr.

2.2 Antimicrobial activity

Bacteria used as test organism in this study consisted of three pathogenic bacteria: Escherichia coli ATCC 25922, Salmonella typhimurium and Staphylococcus aureus ATCC 25922. The antimicrobial activity of LAB isolates was determined by disc diffusion method. Briefly, LABs were grown in MRS broth and incubated at 37 °C until cell density reached 10^8 CFU/ml then 20 µL of cell solution was pipette and dropped on the surface of the blank disc and waited for ±1 hr until the bacterial cell diffused into the disc. Pathogenic bacteria were streaked on Muller Hinton Agar (Merck®, USA) then discs containing LAB cells regularly placed on the surface of the test medium incubated at 37 °C for 24 hr. The antimicrobial activity was recorded as growth-free inhibition zones (clear zone) diameter.

2.3 Preparation of probiotic LAB for encapsulation

Selected probiotic LAB were subcultured three times in MRS broth at 37 °C for 8 h. Cells were harvested by centrifugation at 3000 xg for 10 min at 4 °C.

2.4 Encapsulation procedure

Beads were produced by using extrusion method with modification [8]. Bacterial cells were dissolved in 100 ml of a mixture consisting of 5% glycerol (v/v), inulin 2% (w/v), and CaCO_3 0.1% (w/v) for control treatment. For AM treatment bacterial cells were dissolved in 100 ml of a mixture consisting of mung bean flour 2% (w/v), 5% glycerol (v/v), inulin 2% (w/v), and CaCO_3 0.1% (w/v), were trapped for 45 minutes in 100 ml of alginate solution 3% (w/v). Wall material–culture mixture was dropped through a 27G syringe needle into CaCl_2. After one hour the gel was transferred in 0.85 %saline solution to obtain a compact gel structure. Gel beads formed were transferred to distilled water and stirred slowly at 50 rpm for 1 hr to remove CaCO_3to obtain rigid beads. For AF treatment, treatment is then repeated with 2% gram flour (w/v), 5% glycerol (v/v), inulin2% (w/v), and CaCO_3 0.1% (w/v). Beads were dried using hot air oven at 45 °C for 48 h.
2.5. Viability of encapsulated isolate US7 during storage
Viability test was done immediately after the encapsulation process was completed (week 0) and weekly for a month at cool 4 °C and mild temperature. Beads (1 g) were added to 9 ml of citrate buffers pH 6 homogenized by vortex for 10-15 minutes and performed serial dilutions. 0.1 ml of homogenized samples were removed, diluted to appropriate concentrations and spread into Plate Count Agar (Merck®, USA) then incubated at 37 °C for 24 h.

2.6. Viability of free cell and beads in gastrointestinal tract condition
The procedure for examination was modified from Woraharn et al. [9]. Free cell survival and cell encapsulated were added in simulated gastric juice (0.08 M HCl with 0.2% (w/v) NaCl; Raoet al.1989). Simulated gastric fluid (SGF) was prepared with pH 2 (adjusted with 0.5 N HCl). The choice of pH 2 for SGF took into account the activity of pepsin, which was maximal in a pH range of 1.7 to 3.0 [10]. Simulated intestinal fluid (SIF) was prepared with pH 6 (adjusted with 0.1 N NaOH). Beads were incubated in SGF for 120 min and subsequently transferred to SIF for 180 min at 37 °C. Control samples of free cells and beads were dissolved in phosphate buffered saline (PBS) of pH 7.4. Cell counts were determined by counting growth of colonies in Plate Count Agar. Treatment is done in duplicate.

3. Results and Discussion
The results will be discussed in 4 subsections which are Antimicrobial activity, Encapsulation of probiotic bacteria (US7), Viability of encapsulated LAB during storage, and Viability of free cells from encapsulated LAB during gastric acid simulation

3.1. Antimicrobial activity
The bacteria used as indicators in this study included Gram-positive bacteria (S. aureus) and Gram-negative bacteria (E. coli and S. typhimurium). Only two isolates, AK1 and US7 showed inhibition effect against all indicator strains (Table 1). Among them, US7 showed the higher inhibitory effect against all pathogenic bacteria. The results indicated that LAB isolates US7 could be determined as a candidate of probiotic and was later used in encapsulation experiment.

| Isolate code | Diameter of inhibition zone (mm) | Escherichia coli | Staphylococcus aureus | Salmonella typhimurium |
|--------------|---------------------------------|------------------|----------------------|-----------------------|
| AK1          | 11.88                           | 10.93            | 10.11                |
| AK3          | 8.86                            | 0                | 0                    |
| EK2          | 0                               | 0                | 0                    |
| US7          | 10.8                            | 16.32            | 13.54                |

From the results of antagonist test between LAB isolates and food pathogenic bacteria, only two isolates namely, AK1 and US7 which showed antimicrobial activity against three indicator strains. There was only one isolate (AK3) which inhibited E. coli with diameter of inhibition zone reaching 8.86 mm and one isolate (EK2) which did not inhibit any antimicrobial activity. Isolate US7 produced the largest inhibition zone measuring of 10.8 mm against E. coli, 16.32 mm to S. aureus and 13.54 mm to S. typhimurium. Diameter of inhibition zone produced by US7 isolate against pathogenic bacteria showed higher result than other LAB isolates reported in previous researches. Previous study tested antibacterial activity of some Lactobacillus strains against pathogenic bacteria such as Listeria innocua LMHAE-LI 107, Escherichia coli EC 108 LMHAESA, Escherichia coli ATCC25922, Enterococcus faecalis ATCC 25212, Streptococcus D, and Klebsiella pneumoniae CIP 53 153. Their results indicated that the average diameter of inhibition zone is greater than 6 mm on each pathogen.
using well diffusion method. Other study also reported that *L. acidophilus* showed the greatest inhibition zone against *E. coli* and *Cl. sordeli* with diameter of inhibition zone measuring 13 ± 0.8 mm and 14 ± 0.2 mm respectively using dots method.

3.2. *Encapsulation of probiotic bacteria (US7)*
In this study LAB isolate named US7 was selected for encapsulated by extrusion. The size and spherical shape of the beads is shown in Figure 1.

![Figure 1. Beads of encapsulated US7 viewed under stereo microscope: A. Control with alginate; B. Alginate + mung bean flour (AM); C. Alginate + gram flour (AF)](image)

From the figure it can be seen that control beads produced smaller sizes compared to the treatments: AM (Alginate + Mung bean flour) and AF (Alginate + Gram flour) beads. This is due to the use of only one layer (alginate) as coating material whereas the other treatments used two layers, with mung bean flour and gram flour as coating ingredients. All beads: A (Alginate), AM (Alginate + Mung bean flour) and AF (Alginate + Gram flour), have a considerably uniform spherical shape and compact structures, and a little difference in color. Beads A were transparent, beads AM were brownly and beads AF were whitish in colour. Beads have a chewy texture and diameter size ranged between 9-10 mm. The size and shape of the resulting beads were affected by distance from injection, syringe needle size, concentration of alginate and concentration of CaCl₂ solution. Size of the injection, needle syringe and distance of syringe to CaCl₂ solution were closely related to the size of beads. Distance injection in the process of making the beads is 10-15 cm from the beads collecting solution and syringe needle with size of 23G x 1 1/4 mm was used. The concentration of 3% alginate could provide sufficient size and uniform spherical shape and chewy texture that were more stable against temperature treatment. Some studies reported also use of a variety of coating materials such as whey proteins as capsule material [13,14,15], soybean oil as a capsule coating on Gum Arabic and gelatin mixture [16], and calcium chloride-to-alginate [17] capsule coating that also being used to coat probiotics in this study.

3.3. *Viability of encapsulated LAB during storage*
In this experiment, it was found that encapsulation technique could protect higher number of live cells. The viability of isolate US7 entrapped in alginate beads (A), Alginate + Mung bean flour (AM) beads and Alginate + Gram flour (AF) beads during alternating temperature of 4 °C and room temperature for 1-month storage is shown in Figure 2.
The survival of cells in A, AM and AF beads gradually declined with increasing duration of storage for both temperatures. However, beads stored at room temperature have the highest decline reaching 19%, 16% and 25% for A, AM, and AF respectively. All beads were stored for 4 weeks at 4 °C and at room temperature/ambient (26-30 °C). All treatments showed no considerable loss of viability with increasing storage period at 4 °C. Control treatment (A) decreased viability of 0.90 Log CFU.g⁻¹ (13%), (AM) treatment of 1.17 Log CFU.g⁻¹ (12.7%) and (AF) treatment of 0.68 Log CFU.g⁻¹ (7.5%). All treatments decreased the viability of probiotic during prolonged storage. The decrease of viability in room temperature was greater than in low temperature. The greatest loss on viability was found from AF treatment with 2.31 Log CFU.g⁻¹ (25%). Control treatment (A) decreased viability of 1.36 Log CFU.g⁻¹ (19%) while AM decreased until 1.47 Log CFU.g⁻¹ (16%). From this results storage temperature at 4 °C is the optimum condition for maintaining cell viability in beads. The effect of storage conditions on viability of Isolate US7 was similar to Cui et al. [18] who found that the stability of free flowing Bifidobacteria-loaded alginate poly-L-lysine microparticles was enhanced during storage at cool temperature (4 °C) when compared to Bifidobacteria cultures.

3.4. Viability of free cells from encapsulated LAB during gastric acid simulation

To evaluate the release and viability of isolate US7 beads in gastrointestinal condition, beads were tested in simulated gastric fluid (SGF) for 120 min and simulated intestinal fluid (SIF) for 180 min. The viability of encapsulated US7 under SGF and SIF is shown in Figure 3.
Three formulation of beads were disintegrated within 60 min in SGF. A viable count on free cells of US7 decreased rapidly with increasing contact time in SGF (from ~ 9.49 to 6.39 log CFU g\(^{-1}\)) and SIF (from ~ 5.17 to 9 to 0 log CFU g\(^{-1}\)). However, in entrapped state or beads, probiotic survived well in gastrointestinal conditions compared to non-entrapped free probiotic cells. The survival of cells in gel beads gradually declined with increasing contact time in SGF and SIF for (A) (from ~ 7.13 log to 5.4 log CFU g\(^{-1}\)) for (AM) (from ~ 9.48 log to 7 log CFU g\(^{-1}\)) and for (AF) (from ~ 9.32 log to 7 log CFU g\(^{-1}\)). For the viability of US7 beads in SGF and SIF, the results of our study corresponded well with other reports [17,19,20]. Other study reported that L. acidophilus CSCC 2400, Bifidobacterium longum and Bifidobacterium infantis in calcium alginate survived better when exposed to SGF [20]. Furthermore, viability of encapsulated L. acidophilus CSCC 2400 in simulated gastric conditions increased as addition to alginate gel concentration from 0.75% to 1.8% (w/v) [17]. Microscopy examination on microcapsules showed that bacteria are settled within the capsule material during SGF and were released in SIF. It seemed likely that in our experiment, gastric fluid entered the microparticles through the surface pinholes resulting in a loss of viability. Thus, the survival of US7 declined as increasing of incubation time due to the detrimental effects of low pH on their cells’ environment. However, the dense membrane was expected to create diffusion resistance through the beads, which resulted in lower diffusion of SGF and SIF. Consequently, cell survival increased with increased of alginate gel concentrations.

4. Conclusions
Encapsulation of US7 in (AM) Alginate + Mung bean flour and (AF) Alginate + Gram flour with prebiotic inulin coating resulted in better survival of cells after gastro-intestinal simulation, as compared to free cells. Therefore the results from our study might prove beneficial to apply two coating materials (mung bean and gram flour) to improve delivery of probiotic cultures for further application. Beads formulated from Alginate + Gram flour or AM provided the best protection to US7 cells and there was no survival of US7 free cell in the presence of simulated intestinal fluid due to its low acid resistance. Storage temperature at 4 °C is the optimum condition to maintain cell viability in beads.

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