The encapsulation of *Lactobacillus casei* probiotic bacteria based on sodium alginate and chitosan

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Abstract. Chitosan is a polysaccharide that can be used as a material in the encapsulation of probiotic bacteria. The process of bacterial encapsulation with chitosan polymers was carried out to protect the *Lactobacillus casei* bacteria which cannot last long in very acidic environments so that they can survive when exposed to gastric acid conditions and can live in the intestine. The purpose of this study was to determine the effect of extrusion voltage on the survivability of *L. casei* bacteria in the encapsulation process. The encapsulation process in this study was carried out by the extrusion-emulsion method using a sodium alginate of 1% (w/v) and chitosan of 0.2% (w/v) and voltage variations of 0 kV, 10 kV and 20 kV. The resulting beads were immersed in a simulated gastric fluid (SGF) (NaCl 0.2%; HCl 0.5 M with a pH of 1.5) for 0, 60 and 120 minutes at 37 °C. The number of *L. casei* cells before encapsulation was $1 \times 10^9$ cfu/mL. After encapsulation with voltage variations of 0 kV, 10 kV and 20 kV, the viability of probiotics were $2.6 \times 10^8$ cfu/g, $1 \times 10^7$ cfu/g, and $1 \times 10^3$ cfu/g, respectively. After testing the beads in SGF, the obtained results indicated that viability of *L. casei* in the sodium alginate - chitosan beads with an extrusion voltage of 0 kV were 20,300 cfu/g, 10 kV were 30 cfu/g and 20 kV were 0 cfu/g. The results of these studies indicated that survivability of *L. casei* in the sodium alginate - chitosan beads with a voltage of 0 kV indicates the highest survivability level of 51.19 % of the number of cells encapsulated after incubation in SGF and the higher voltage can kill more *L. casei*.

Keywords: Encapsulation; probiotic *L. casei*; na-alginat; chitosan; extrusion

1. Introduction

The probiotic is technically defined as live microbes which upon consume in certain numbers exert health benefits [1]. Probiotic bacteria can provide health benefits [2–6] if they present at a minimum viability of $1 \times 10^6$ cfu/g of food product [7] or $1 \times 10^7$ cfu/g at point of delivery [8] or be eaten in sufficient amounts to yield a daily consume of $1 \times 10^8$ cfu [9]. Their health effects are due to the nutritional and curative benefits, especially protection against harmful microorganisms and improvement of the immune system [10,11].

The global consumption of probiotic foods has increased considerably in recent years. Probiotic bacteria are incorporated into dairy products such as fermented milks, milk powder, ice cream, cheese and yogurt[10,12,13]. Furthermore, lactose intolerance and the cholesterol content are two major weakness related to the consumption of fermented dairy products [14]. The most important probiotic microbes in the food industry are lactic acid bacteria (LAB) [15]. Recent studies on LAB confirmed
their health benefits on the human intestine and immune system [6]. Among the LAB, *Lactobacillus casei* strains are widely used in the production of fermented dairy food [16]. It has been reported that *L. casei* strains can reduce the cholesterol content [17] and can be used against cancer cell reproduction [18]. The probiotic bacteria must survive during food processing and storage as well as gastrointestinal transition to reach alive their site of action in order to probiotic bacteria can give their health benefits. Furthermore, the probiotic bacteria should not influence the sensory characteristics of the juices all the time processing and storage of the product [19].

Encapsulation of probiotics in jello beads is an appropriate procedure to protect the bacterial cells from the disturbances caused by the external environment in food products and during gastrointestinal transition. Several studies have reported the probiotic microencapsulation by using alginate, gelatin, resistant starch, vegetable gum and chitosan to provide protection to *Bifidobacteria* and *Lactobacilli* [20–23]. Among the encapsulating materials, alginate is the most commonly used polymer for encapsulating viable cells [24–26]. Alginate is also an accepted food additive and can be safely used in foods [26-27]. It consists of L-guluronic acid and D-mannuronic acid. Although alginate is non-toxic and forms a soft beads with calcium chloride to encapsulate probiotic bacteria, it is affected to acidic environments[28]. The use of alginate is limited due to its low stability in the presence of chelating agents and in acidic conditions below pH 2.0 [29–33]. The coating of alginate beads and its effectiveness in protecting probiotic bacteria has been extensively studied. Previous researchers have reported that coating alginate microcapsules with chitosan had improved the stability of the alginate beads and thus improved the viability of the encapsulated probiotic bacteria [22]. Polycations, such as chitosan or polyamine acids can reduce the porosity of the alginate beads l [31–33]. Chitosan, a deacetylated chitin, is a natural, non-toxic and inexpensive linear polysaccharide with positive charge at low pH, which has been used for coating the alginate beads. Low concentration chitosan solution (2 - 4 g/m²) has been applied for shell-making on alginate beads [21,34,35]. The aim of this study was to determine the effect of extrusion voltage and time immersion in SGF to the survivability of *L. casei* bacteria.

2. Materials and Methods

2.1. Equipment

Petri dishes (Normax), ose needles, beaker glass (Pyrex), measuring flask (Pyrex), Erlenmeyer, test tubes, stirring rods, funnels, spatulas, volume pipettes, micro pipettes (Effendorf), watch glass, autoclaves (Tomy SX- 300), shaker incubator (CERTOMAT® BS-1)), incubator (Memmert 854 Schwabach), refrigerators, aluminum foil, plastic wrab, vortex (Thermo), analytical balance, glass preparations, microscopes, hot plates, magnetic stirrers, Laminar Air Flow (ETL), pH meter (Horiba, Japan), syringe (Therumo) 22 G (inner diameter 0.394 mm), syringe 60 mL, filter paper Whatman no. 40, Scanning Electron Microscopy (SEM) (JEOL JSM-IT300) were used in this study.

2.2. Materials

The microorganism used in this study was *L. casei* from dadih. The chemicals used were MRSA (de Man Rogosa Sharpe Agar) and MRSB (de Man Rogosa Sharpe Broth) (Merck, KGaA), Na-alginate 1 % (HIMEDIA® REF MB-114-100G), chitosan 0.2 % (Sigma Aldrich PCode: 101729402), NaCl 0.2 % pH 1.5, Na-Citrate 1 %, CaCl₂ Solution 32 g / L.

2.3. Sodium alginate solution containing bacterial suspension preparation

Preparation of sodium alginate solution was first made of alginate with a concentration of 1% (w/v) with distilled water. Then they were sterilized by autoclaving at 121 °C for 15 minutes. After sodium alginate solution had been cooled to room temperature, and then 50 mL of *L. casei* suspension was added.
2.4. Chitosan solution preparation
Preparation of chitosan solution was made with a concentration of chitosan 0.2 % (w/v). Chitosan was dissolved in 1 % (v/v) acetic acid solution, then the solution was sterilized by autoclaving at 121 °C for 15 minutes.

2.5. Encapsulation of L. casei using sodium alginate-chitosan mixture
The L. casei probiotic encapsulation method was the extrusion method and the ionic gelation method. The extrusion method was chosen because besides using a simple tool in the form of a syringe. The method was also chosen with the aim of avoiding extreme temperatures and pressures and from unfavourable environments such as low temperatures in the freeze drying method, which can result in reduced probiotic viability [36]. While the choice of ionic gelation method was due to a simple process, did not use organic solvents, and can be controlled easily. In the extrusion technique, the hydrocolloid solution was first prepared, then probiotics were added and the mixture was dripped through a syringe which will fall into CaCl₂ solution (32 g/L) [37]. While the principle of particle formation in the ionic gelation method was the occurrence of ionic interactions between the carboxylic anion (COO-) of the alginate monomer and the divalent cation (Ca²⁺). Crosslinking occurred because a calcium ion replaced two sodium ions in alginate. The crosslinking structure caused limited molecular motion and inhibited the development of polymers in a medium[38].

One mL of L. casei suspension (10⁹ cfu/mL) was directly put into 50 ml of 1 % (w/v) sodium alginate solution, homogenized using magnetic stirring for 1 minute. Then the mixture was poured into a syringe measuring 50 mL and pumped to the encapsulation system (extruder) through a needle with a diameter of 0.394 mm at a flow rate of 1 mL/min using a syringe pump. Whereas the extruded voltages were varied at 0 kV, 10 kV and 20 kV. Extrusion was carried out at room temperature during the encapsulation process. Extruded droplets were immediately collected in a 100 mL calcium chloride solution (32 g/L) while magnetic stirring for 30 minutes. The beads were filtered with Whatman™ 40 (125 mm) filter paper and rinsed with sufficient distilled water, then the beads formed were immersed in a 0.2 % (w/v) chitosan solution for 15 minutes. After that the beads were rinsed with sufficient distilled water and dried. Then the beads were collected in plastic zipper and ready to be tested for the viability of the probiotic.

2.6. Viability and survivability testing of L. casei probiotic
Simulated gastric fluid (SGF) consisted of 0.2 % Sodium chloride with a pH of 1.5 (adjusting the pH by adding 0.5 M hydrochloric acid). One gram capsule (extrusion flow rate 1 mL/minute with extruder voltage of 0 kV, 10 kV and 20 kV) was immersed in 9 mL gastric fluid simulation solution, incubated for 0 minutes, 60 minutes, and 120 minutes at temperature of 37 °C. After that, it was filtered using Whatman filter paper then immersed in 9 mL (1 g/100 mL) sterile sodium citrate solution with slow stirring at room temperature. Then sequential dilution was prepared to reach the number of cells can be calculated by pouring a suspension technique that was spread on MRS Agar media. After that, it was incubated for 48 hours at 37 °C [39]. The colony of probiotics can be calculated by the Total Plate Count (TPC) method.

3. Results and Discussions

3.1. Encapsulation probiotic L. casei
The result of encapsulation with sodium alginate : chitosan (1% : 0.2%), the alginate solution flow rate of 1 mL/ minutes and voltages of 0, 10 and 20 kV were shown in Figure 1. These were shown that the greater the extrusion voltage the smaller the size of the beads. These happened because at high voltages it also created a high electrostatic force so that the liquid in the extruder were pulled to the bottom faster and even gushes.
3.2. Test of viability and survivability of probiotic L. casei encapsulation process In sodium citrate solution

The newly encapsulated particles (1 g) were then soaked in 9 mL (1 g/100 mL) sterile sodium citrate solution with slow stirring at room temperature. The sequential dilution was prepared to reach the number of cells that can be calculated by pouring plates in MRS Agar. Figure 2 showed that the highest L. casei viability of 8.41 (2.6 × 10^8 cfu/g) was obtained for the variation of the 0 kV voltage extrusion experiment. The greater voltage can decrease viability of probiotics.

The level of survivability was shown in Figure 3. It was shown that the highest L. casei survivability of 93.5% was obtained for the variation of the 0 kV voltage extrusion experiment. It was proved that L. casei cannot withstand high voltages.

![Figure 1. Encapsulated beads: (a) 0 kV, (b) 10 kV and (c) 20 kV](image)

![Figure 2. Viability of L. casei in various voltage variations of extrusion experiments](image)
3.3. Test the viability and survivability of L. casei probiotics in SGF

3.3.1. Viability and survivability test of L. casei (free cell) in SGF

L. casei preparations which had viability of $1 \times 10^9$ cfu/mL was immersed in SGF containing 0.2 % NaCl solution (pH 1.5) at various variations of immersion time 0, 60 and 120 minutes. The result of the calculation of the Total Plate Count (TPC) of the probiotic L. casei showed the viability of 0 cfu/mL for 0, 60 and 120 minutes. It was shown that L. casei which was not encapsulated experience direct death once immersed in gastric acid pH 1.5 and thus the survival rate was 0 %.

3.3.2. Test the viability and survivability of L. casei (encapsulated) in SGF

L. casei probiotic which had been encapsulated with a mixture of alginate-chitosan matrix with an extrusion method produces a bead shape with a diameter of about 0.5 mm. The beads were then immersed in SGF containing 0.2 % NaCl solution with a pH of 1.5 and varying incubation time from 0 minutes, 60 minutes and 120 minutes.

When the beads were immersed in the liquid, they shrink in size. After the beads were immersed, they were removed and dried. The beads were then immersed in sodium citrate pH 8. As a result of immersion in the sodium citrate solution, the beads swell so the probiotics trapped in the beads can come out. Then the probiotic viability was tested using the TPC method (Figure 4). It was shown that the highest L. casei viability of 4.31 (20,300 cfu/g) was obtained for the variation of the 0 kV voltage extrusion experiment and immersion in SGF for 0 minutes. Whereas at the voltage of 20 kV, the viability value was 0 cfu/g. Also the immersion for 120 minutes showed a viability value of 0 cfu/g. The fact showed that the greater the extrusion voltage would kill L. casei more, also the longer the immersion in SGF pH 1.5 would more eliminate L. casei.

While the level of survivability was shown in Figure 5. The results showed that the highest L. casei survivability of 51.19 % was obtained for the variation of the 0 kV voltage extrusion experiment and immersion in SGF for 0 minutes. Whereas at the 20 kV voltage the viability value was 0 %. Also the immersion for 120 minutes showed a viability value of 0 %. It was shown that L. casei cannot withstand high voltages and very low SGF pH.

![Figure 3. L. casei survivability in various voltage variations in extrusion experiment](image-url)
Figure 4. *L. casei* viability in a variety of experiments

Figure 5. *L. casei* survivability in various experiment variations

4. Conclusions

This study showed the survivability of *L. casei* in the sodium alginate - chitosan beads with a voltage of 0 kV indicated the highest survivability level of 51.19% of the number of cells encapsulated after incubation in SGF. The increasing of electricity voltage and the increasing of immersion time of the beads in SGF would be decreased the survivability of *L. casei*. 
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References

[1] Information A 2012 FAO / WHO Guidelines on Probiotics : 10 Years Later 46 1–2
[2] Colbère-Garapin F, Martin-Latil S, Blondel B, Mousson L, Pelletier I, Autret A, François A, Niborski V, Grompone G, Catonnet G and van de Moer A 2007 Prevention and treatment of enteric viral infections: possible benefits of probiotic bacteria Microbes Infect. 9 1623–31
[3] de Vrese M and Offick B 2010 Probiotics and Prebiotics: Effects on Diarrhea (Elsevier Inc.)
[4] Reid G, Sanders M E, Gaskins H R, Gibson G R, Mercenier A, Rastall R, Roberfroid M, Rowland I, Cherbut C and Klaenhammer T R 2003 New scientific paradigms for probiotics and prebiotics J. Clin. Gastroenterol. 37 105–18
[5] Shah N P 2007 Functional cultures and health benefits Int. Dairy J. 17 1262–77
[6] Vaughan E E, Heilig H G H J, Ben-Amor K and De Vos W M 2005 Diversity, vitality and activities of intestinal lactic acid bacteria and bifidobacteria assessed by molecular approaches FEMS Microbiol. Rev. 29 477–90
[7] Doleyres Y and Lacroix C 2005 Technologies with free and immobilised cells for probiotic bifidobacteria production and protection Int. Dairy J. 15 973–88
[8] Lee Y K and Salminen S 1995 The coming of age of probiotics Trends Food Sci. Technol. 6 241–5
[9] Lopez-Rubio A, Gavara R and Lagaron J M 2006 Bioactive packaging: turning foods into healthier foods through biomaterials Trends Food Sci. Technol. 17 567–75
[10] Soccol C R, Vandenberghe L P de S, Spier M R, Medeiros A B P, Yamaguishi C T, De Dea Lindner J, Pandey A and Thomaz-Soccol V 2010 The potential of probiotics: A review Food Technol. Biotechnol. 48 413–34
[11] Roberfroid M 2007 Prebiotics: The Concept Revisited J. Nutr. 137S30S–837S
[12] Ranadheera R D C S, Baines S K and Adams M C 2010 Importance of food in probiotic efficacy Food Res. Int. 43 1–7
[13] Sanders M E 2003 Probiotics: Considerations for human health Nutr. Rev. 61 91–9
[14] Yoon K Y, Woodams E E and Hang Y D 2006 Production of probiotic cabbage juice by lactic acid bacteria Bioreour. Technol. 97 1427–30
[15] Burgain J, Gaiani C, Jeandel C and Scher J 2013 Encapsulation of Lactobacillus rhamnosus GG in microparticles: Influence of casein to whey protein ratio on bacterial survival during digestion Innov. Food Sci. Emerg. Technol. 19 233–42
[16] Kourkoutas Y, Bosnea L, Taboukos S, Baras C, Lambrou D and Kanellaki M 2006 Probiotic cheese production using Lactobacillus casei cells immobilized on fruit pieces J. Dairy Sci. 89 1439–51
[17] Lye H S, Rusul G and Liong M T 2010 Removal of cholesterol by lactobacilli via incorporation and conversion to coprostanol J. Dairy Sci. 93 1383–92
[18] Choi S S, Kim Y, Han K S, You S, Oh S and Kim S H 2006 Effects of Lactobacillus strains on cancer cell proliferation and oxidative stress in vitro Lett. Appl. Microbiol. 42 452–8
[19] Prado F C, Parada J L, Pandey A and Soccol C R 2008 Trends in non-dairy probiotic beverages Food Res. Int. 41 111–23
[20] Abbaszadeh S, Gandomi H, Misaghi A, Bokaei S and Noori N 2014 The effect of alginate and chitosan concentrations on some properties of chitosan-coated alginate beads and survivability of encapsulated Lactobacillus rhamnosus in simulated gastrointestinal conditions and during heat processing J. Sci. Food Agric. 94 2210–6
[21] Krasaekoot W, Bhandari B and Deeth H 2004 The influence of coating materials on some properties of alginate beads and survivability of microencapsulated probiotic bacteria 14 737–
[22] Ortakci F and Sert S 2012 Stability of free and encapsulated Lactobacillus acidophilus ATCC 4356 in yogurt and in an artificial human gastric digestion system J. Dairy Sci. 95 6918–25
[23] Journal I, Vol P, Soodbakhsh S, Gheisari H, Raminari M and Dehnavi T 2012 Department of Food Hygiene and public health, School of Veterinary Medicine, Shiraz University, Shiraz, Iran; Department of Biochemistry, School of Veterinary Medicine, Shiraz University, Shiraz, Iran; and 3 Research and development unit of Rama 7 2012
[24] Orive G, Hernández R M, Gascon A R, Calafia R, Chang T M S, De Vos P, Hortelano G, Hunkeler D, Lacik I, Shapiro A M J and Pedraz J L 2003 Cell encapsulation: Promise and progress Nat. Med. 9 104–7
[25] Krasasekoopt W, Bhandari B and Deeth H 2003 Evaluation of encapsulation techniques of probiotics for yoghurt Int. Dairy J. 13 3–13
[26] SHEU T Y and MARSHALL R T 1993 Microentrapment of Lactobacilli in Calcium Alginate Gels J. Food Sci. 58 557–61
[27] Dinakar P and Mistry V V. 1994 Growth and Viability of Bifidobacterium bifidum in Cheddar Cheese J. Dairy Sci. 77 2854–64
[28] Gouin S 2004 Microencapsulation: Industrial appraisal of existing technologies and trends Trends Food Sci. Technol. 15 330–47
[29] Adhihaki K, Mustapha A, Grün I U and Fernando L 2000 Viability of microencapsulated bifidobacteria in set yogurt during refrigerated storage J. Dairy Sci. 83 1946–51
[30] Ding W K and Shah N P 2007 Acid, bile, and heat tolerance of free and microencapsulated probiotic bacteria J. Food Sci. 72 446–50
[31] Gombotz W R and Wee S F 1998 Protein release from alginate matrices Adv. Drug Deliv. Rev. 31 267–85
[32] Hussein S A and Kebary K M K 1999 Improving viability of bifidobacteria by microentrapment and their effect on some pathogenic bacteria in stirred yoghurt Accredit. Qual. Assur. 4 113–31
[33] Sultana K, Godward G, Reynolds N, Arumugaswamy R, Peiris P and Kailasapathy K 2000 Encapsulation of probiotic bacteria with alginate-starch and evaluation of survival in simulated gastrointestinal conditions and in yoghurt Int. J. Food Microbiol. 62 47–55
[34] Sashiwa H and Aiba S I 2004 Chemically modified chitin and chitosan as biomaterials Prog. Polym. Sci. 29 887–908
[35] Zhou Y, Martins E, Groboillot A, Champagne C P and Neufeld R J 1998 Spectrophotometric quantification of lactic bacteria in alginate and control of cell release with chitosan coating J. Appl. Microbiol. 84 342–8
[36] Desmond C, Stanton C, Fitzgerald G F, Collins K and Paul Ross R 2002 Environmental adaptation of probiotic lactobacilli towards improvement of performance during spray drying Int. Dairy J. 12 183–90
[37] Silva M P, Tulini F L, Martins E, Penning M, Fávaro-trindade C S and Poncelet D 2018 LWT - Food Science and Technology Comparison of extrusion and co-extrusion encapsulation techniques to protect Lactobacillus acidophilus LA3 in simulated gastrointestinal fluids LWT - Food Sci. Technol. 89 392–9
[38] Mardliyati E, Muttaqien S El and Setyawati D R 2012 Sintesis Nanopartikel Kitosan- Trypoly Phosphate Dengan Metode Gelasi Ionik : Pengaruh Konsentrasi Dan Rasio Volume Terhadap Karakteristik Partikel Sintesis Nanopartikel Kitosan-TPP Pros. Pertem. Ilm. Ilmu Pengetah. dan Teknol. Bahan 90–3
[39] Woraharn S, Chaiyasut C and Sirithunyalug B 2010 Survival enhancement of probiotic Lactobacillus plantarum CMU-FP002 by granulation and encapsulation techniques African J. Microbiol. Res. 4 2086–93