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The Effect of Carboxymethyl Glucomannan Concentration on the Properties of Glucomannan-Chitosan Hydrogel for *Lactobacillus acidophilus* FNCC 0051 Encapsulation

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Abstract

The effect of porang (*Amorphophallus oncophyllus)* glucomannan concentration on the properties of glucomannan-chitosan hydrogel was investigated for *Lactobacillus acidophilus* FNCC 0051 encapsulation. The spherical shape with a continuous surface of the particle was self-assembly formed. The increase of glucomannan concentration from 0.3 to 0.9 % smoothly increased their small particle size from 1.08 ± 0.02 µm to 2.12 ± 0.00 µm and no significant change on the positive zeta potential values. The polydispersity indexes with the value between 0.4 to 0.5 were categorized as uniform particles. However, these values were higher compared to other studies which used konjac glucomannan-chitosan as the hydrogel materials. The encapsulation study with *Lactobacillus acidophilus* FNCC 0051 showed that the highest value was achieved when the same ratio of glucomannan and chitosan was applied (0.5 %). The viability study proved the perfect protection of hydrogel during 56 days of cold storage and pasteurization treatment with the cell viabilities of 100 % and 58.13 ± 18.5 %, respectively.

Keywords: Chitosan, Encapsulation, Glucomannan, Hydrogel, Probiotic, Properties

Introduction

Probiotics are considered to be a functional food that has beneficial effects on human health if administered in adequate amounts [1]. To achieve the minimum standard of viable cells (10^6 - 10^7) in foods [2], several challenges must be considered during the development of probiotic products. Harsh treatments in both processing and storage may lead to a decrease in their viability, such as heating, cooling, incorporation of higher acid and salts, oxygen, mechanical stress, water [3,4]. Encapsulation is an alternative effort to protect the cells from that harsh environment [5-8].

To provide sufficient protection for the cells, the encapsulation matrix must be decided appropriately. Polysaccharides have been fascinated to be promising materials because of their great usability, such as being easily found and modified, safe, biocompatible and biodegradable [9]. Recently, polysaccharide-based hydrogel became popular in the pharmaceutical, biomedical, and nutraceutical fields because of its potential as a delivery carrier of bioactive compounds [10].

Hydrogel is a cross-linked polymeric material that can absorb a lot of water. There must be more than molecules that are interacted to generate hydrogel. Glucomannan is a promising hydrogel material that could be extracted from porang (*Amorphophallus oncophyllus)* tuber, an Indonesian local tuber. It has excellent properties such as higher solubility (100 %), viscosity (74000 cPs) [11], purity (90.98 %),

and transparency (57.74 %) than those of konjac glucomannan [12]. It also has been reported for its prebiotic activity [13]. Glucomannan has been studied for its good interaction with other natural carbohydrate polymers, such as xanthan gum, alginate, gelatin, and chitosan [14-17]. Among them, chitosan was a polymer that could generate unique characters with many benefits when interacted with glucomannan. It could be self-assembled based on the electrostatic interaction, formed round-shape, pH-responsive, and be used as the encapsulant of enzyme, protein, or drug in high encapsulation efficiency [10,14,17-19]. Those characters were also needed in the carrying of live cells such as probiotics to get into the gastrointestinal tract [4,8,20-24]. The previous study reported the simple glucomannan-chitosan hydrogel characteristics and its pH-sensitive potential benefit in encapsulation of *Lactobacillus acidophilus* [24]. However, there was still no information about the optimum condition to encapsulate as many as live cells in the hydrogel that could be measured as encapsulation efficiency. Previous studies showed that the concentrations of the polymer were a factor influencing the encapsulation efficiency [17,19,25,26].

This research studied the effect of different concentrations of glucomannan in the properties of the hydrogel, like morphology, particle size, polydispersity index, zeta potential, and its encapsulation efficiency in entrapping *Lactobacillus acidophilus* FNCC 0051. The survival analyses were also done to know the role of hydrogel in the protection of cells during heat and cold storage treatments.

Materials and methods

Materials

Glucomannan was extracted from porang tuber (*Amorphophallus oncophyllus*) and obtained from the Faculty of Agricultural Technology, Universitas Gadjah Mada. Glucomannan was modified by carboxymethylation with Na-chloroacetate in the alkaline environment at 70 °C for 40 min [24]. Food-grade chitosan with 85 - 89 % degree of deacetylation was purchased from PT Biotech Surindo, Cirebon, West-Java, Indonesia.

Probiotic

Lactobacillus acidophilus FNCC 0051 cells were used as the core. They were obtained from the stock culture collection of Food and Nutrition Culture Collection (FNCC), Laboratory of Food Microbiology, Center for Food and Nutrition Studies, Universitas Gadjah Mada. Cells were reactivated from the working stocks in skim milk-glycerol suspension by growing twice successively in de Man, Rogosa, and Sharpe (MRS) Broth at 37 °C overnight. Centrifugation at 2400 g for 9 min at 4 °C was done to collect the cell biomass [27]. It was then washed twice with sterile saline solution and resuspended in saline solution before it was used in the encapsulation process.

Encapsulation of probiotic in hydrogel

The hydrogel was formed by the complex coacervation method [24]. The concentration of chitosan was 0.5 % (w/v) in acetic acid solution, while the concentration of glucomannan varied between 0.3, 0.5, 0.7 and 0.9 % (w/v). Before treatment, all the materials have been sterilized. The cells were mixed with the polymer before coacervation. The hydrogel was then analyzed for the morphology, particle size, polydispersity index, zeta potential, FTIR (Fourier-transform infrared spectroscopy) spectra, and swelling ratio as described below. The concentration of glucomannan that generated the highest encapsulation efficiency was then analyzed for its viability during heating (pasteurization) at 65 °C for 30 min and storage at 5 °C for 2 months.

Hydrogel morphology

The morphology of hydrogel was observed by optical microscope (Olympus BX51, Olympus Corp., Japan) equipped with OptiLab pro digital camera (Miconos, Indonesia). To observe the surface by scanning electron microscope/SEM (Inspect S50, EDAX-AMETEK, USA), the hydrogel and probiotic were freeze-dried and then put in a sample holder using carbon double-sided tape. The gold coating was done with a sputter coater (Emitech SC7620, UK).

FTIR spectroscopic analysis

FTIR was performed to compare the interaction between glucomannan and chitosan in different concentrations of glucomannan. The FTIR spectra were recorded on a Shimadzu 8201 PC spectrophotometer in the region between 4,000 and 400 cm^{-1}. The freeze-dried hydrogel was mixed with KBr and pressed to a plate for measurement.

Particle size, polydispersity index and zeta potential of hydrogel

The size and polydispersity index of hydrogels were measured by using a particle size analyzer (Horiba SZ-100 series, Japan). Zeta potential was measured by Zetasizer (Nano ZS Ver 6.20, Malvern Instruments Ltd, Malver, UK).

Encapsulation efficiency of hydrogel

Encapsulation efficiencies were calculated by dividing the number of cells entrapped in the hydrogel with the number of cells added in the polymer [7]. Cells in hydrogel were released with a buffer solution of pH 8 [24]. The hydrogels were then incubated for 24 h at 37 °C. They were then serially diluted in saline solution before plated on MRS agar. Encapsulation efficiencies of hydrogels in several probiotics were also determined.

Swelling ratio of hydrogel

The hydrogel was determined for its swelling ratio in different pH solutions and salt concentrations [17]. The solutions for swelling studies were buffer-produced from HCl-KCl (pH 1 and 2), citrate (pH 3), acetate (pH 4 and 5), phosphate (pH 6, 7 and 8), carbonate (pH 9). The concentrations of salt solution were 0, 0.2, 0.4, 0.6, 0.8 and 1 %. The swelling ratios were then calculated by using the formula based on Du *et al.* [17].

Survival of *L. acidophilus* FNCC 0051 during heat and storage treatment

The cell survival test was conducted to know the properties of hydrogel in protecting the cells during heat and storage treatment. The stability of cells was compared between free cells, encapsulated cells in the hydrogel of porang glucomannan-chitosan, konjac glucomannan-chitosan and Ca-alginate. In the stability test, 1 g of hydrogel was mixed with 9 mL of milk. It was then pasteurized at 65 °C for 30 min [28]. For the storage stability test, it was stored in a cold room with a temperature of 5 °C for 56 days. The cells were enumerated on the days of 1^{st}, 7^{th}, 14^{th}, 28^{th} and 56^{th}.

Before enumerating, cells in hydrogel were released by mixing 1 g of hydrogel in 9 mL of phosphate buffer of pH 8 and incubated overnight at 37 °C [24]. One mL of solution was then serially diluted in 0.85 % salt solution and pour-plated into MRS agar. Cells were enumerated after 48 h of incubation. The survival rate was calculated by dividing the number of viable cells within the hydrogel after treatment with the initial number of cells [8].

Statistical analysis

Data were reported as mean ± standard deviation (SD) and analyzed using 1-way analysis of variance (ANOVA). Multiple comparisons were performed using Duncan's multiple range tests (DMRT) at $p < 0.05$. All data were analyzed using the Statistical Package for the Social Sciences (SPSS) software (version 16.0 for Windows; SPSS Inc., Chicago, IL, USA).

Results and discussion

The hydrogel was successfully prepared by complex coacervation between the positive charge of chitosan and the negative charge of glucomannan samples. This method was chosen as it is a promising encapsulation technology for microbes with many advantages, i.e easier, cheaper, high loading capacity, and could be processed in a mild condition (lower temperature) [7,29].

The microscopy image of hydrogel demonstrated that hydrogel had a spherical shape as presented in **Figure 1(A)**. This was related to the preparation method of hydrogel, included the technique, chemically

bond, the type/modification of polymer, the pH of medium [10]. In this research, a certain degree of carboxymethylation, the concentration of glucomannan and pH medium were important factors in the formation of the hydrogel that determined the chemical interaction leading to the quick and self-assembled hydrogel formation. The quick formation of hydrogel should be combined with physical treatment like steering and dropping technique. The negation of this treatment produced an irregular shape of a hydrogel. The dropping technique is similar to the extrusion technique that allows chitosan solution to free-fall into glucomannan solution through the syringe needle. Lakkis [29]; Zuidam and Nedovic [3] proved that extrusion was an alternative technique to produce spherical beads.

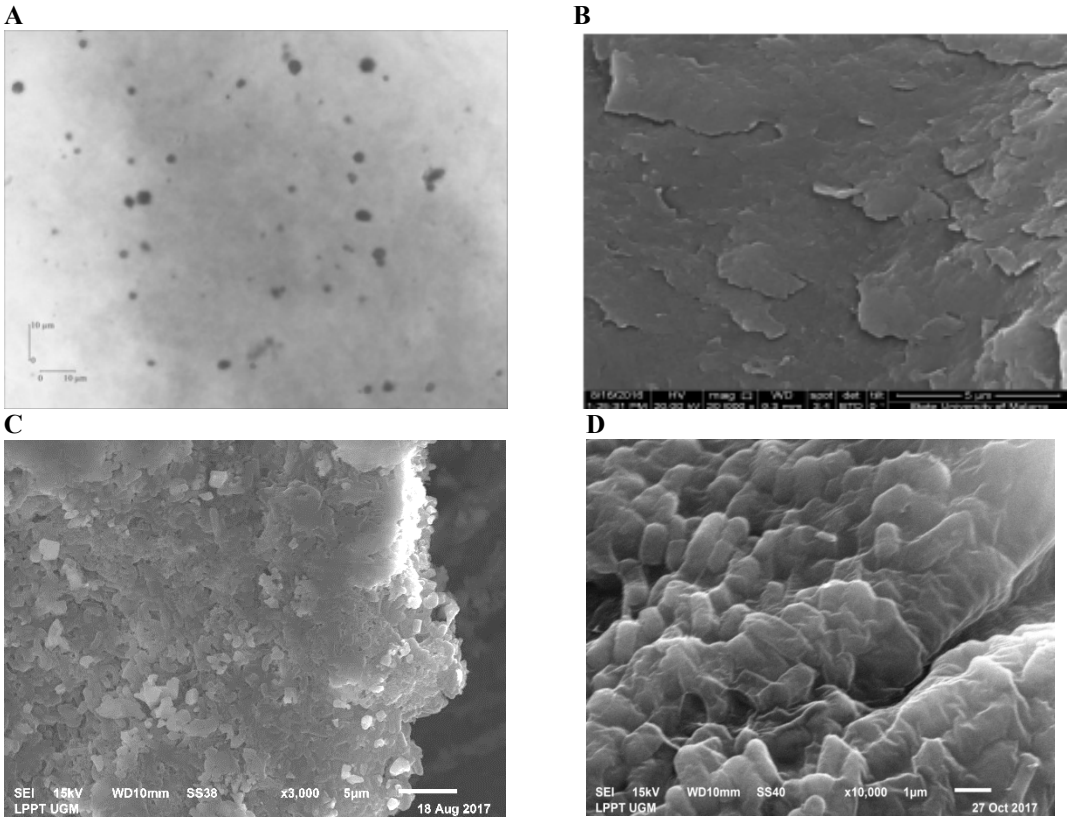

Figure 1 Optical microscope image of porang glucomannan-chitosan hydrogel (magnification 1300×) (A); scanning electron microscope (SEM) image of freeze-dried hydrogel (magnification 10000×) (B); *L. acidophilus* FNCC 0051 free cells (non-encapsulated) (magnification 3000×) (C); and cells-encapsulated freeze-dried hydrogel (magnification 10000×) (D).

Figures 1(B) and **1(D)** give the SEM features of the hydrogel. As shown, the surface of the blank hydrogel appeared layered, continuous, without disruptions (**Figure 1(B)**). The layered surface reflects an inhomogeneous surface that may be influenced by higher polymer concentration on certain areas of the hydrogel. However, this continuous surface potentially provides a stronger physical barrier for the cells against harsh environments or treatments, like the extreme pH, temperature, or during gastric and bile diffusion in the GI tract [5]. In **Figure 1(D)**, there was a rough surface similar to the cells shape (**Figure 1(C)**). This revealed that some of the cells were embedded on the wall of hydrogel and some others were visibly attached on the surface of hydrogel (**Figure 1(D)**). The cell-shape surface image of

this hydrogel was in agreement with other studies that used alginate and its combination with chitosan to encapsulate *P. acidilactici, L. plantarum*, and *L. casei* [5,6,8].

FTIR spectroscopic analysis

FTIR spectra of hydrogels in different concentrations of glucomannan were performed as shown in **Figure 2**. For the IR spectrum of chitosan, the characteristic absorptions appeared at 1597 cm^{-1} (protonated amide I), 1658 cm^{-1} (amide I, vibration from C=O and C=N) and amide III (1381 and 1419 cm^{-1}). The absorption peaks at 810 cm^{-1} (mannose), while 1627 cm^{-1} (symmetric carbonyl) and 3418 cm^{-1} (OH) for carboxylic acid was characterized for glucomannan. The interaction between glucomannan and chitosan was indicated from the stronger intensity at 2924 cm^{-1} compared to chitosan's, but it was weaker compared to glucomannans. At the peak of 2337 cm^{-1}, there was a stronger intensity compared to both polymers. Among all hydrogels, different concentrations of glucomannan gave an impact on the absorption peak between 1026 and 1087 cm^{-1}. Those peaks were attributed to the bending vibration of C-O-C groups [30] that came from glucomannan.

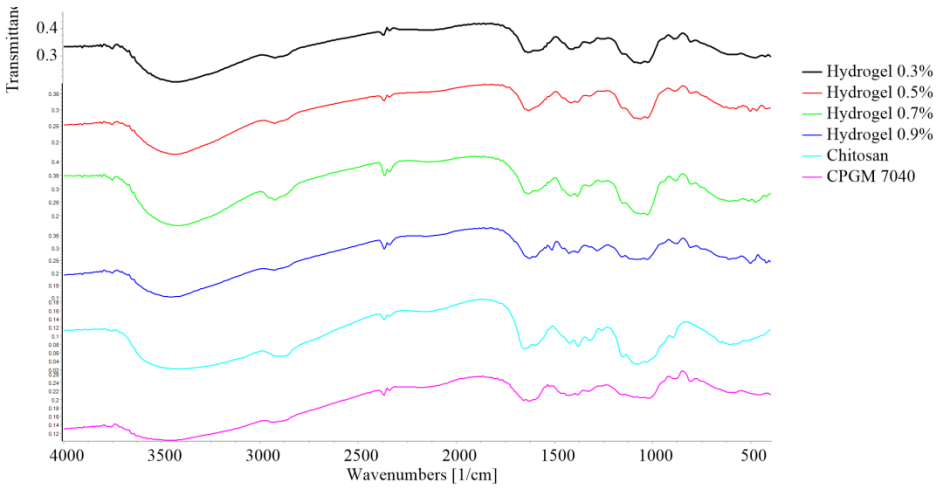

Figure 2 FTIR spectra of hydrogel formed in different concentrations of glucomannan.

Particle size, polydispersity index, and zeta potential of hydrogel

The impact of glucomannan concentration on the properties of the particle was also studied (**Figure 3**). The mean particle sizes were between 1.08 ± 0.02 to 2.12 ± 0.00 µm. Particle sizes increased smoothly in the higher concentration of glucomannan (slope 1.505). It was influenced by the increase of molecule units in solutions when a higher concentration was applied. It led to the more compound produced and required a larger area, thus the particle became bigger [30]. Other factors that were proved to give an impact on the particle size were the size of the nozzle used; the type, concentration, and temperature of polymer; the distance between the nozzle and polymer; and the condition of environment like pH and salt concentration [31-34]. This result was also confirmed by a previous study in konjac glucomannan-chitosan hydrogel. The bigger particle size was due to the increased number of molecule units at higher polymer concentrations.

Polydispersity index is a parameter to measure the uniformity of particles. As shown in **Figure 3**, the polydispersity index was almost no change in the increase of glucomannan concentration (slope was very low, 0.225). It may be due to the control of the spinning rate during the coacervation process [32]. The polydispersity indexes of hydrogel in this research were between 0.4 - 0.5 that was higher compared to other studies that used konjac glucomannan-chitosan as the hydrogel materials [30].

The increase of glucomannan concentration gave an impact on the lower value of hydrogel zeta potentials. It was shown by the negative slope value (**Figure 3**). The higher the glucomannan concentration, the lower the positive charge of the hydrogel. It may be caused by the more glucomannan proportion in particles, the more negative charge from carbonyl groups leading to lower resultant charge between glucomannan and chitosan. The Zeta potential of particles was influenced by the total charge of particles with the microbes entrapped inside them [4].

Zeta potentials were measured as the total charge in particles. The data of this study showed that all hydrogels had positive charges. It indicated the domination of positive charge in the surface of hydrogels although they were produced from the opposite charged polymers. Du *et al.* [30] explained that chitosan has a cationic charge. The cationic charge becomes higher when the deacetylation degree was increased.

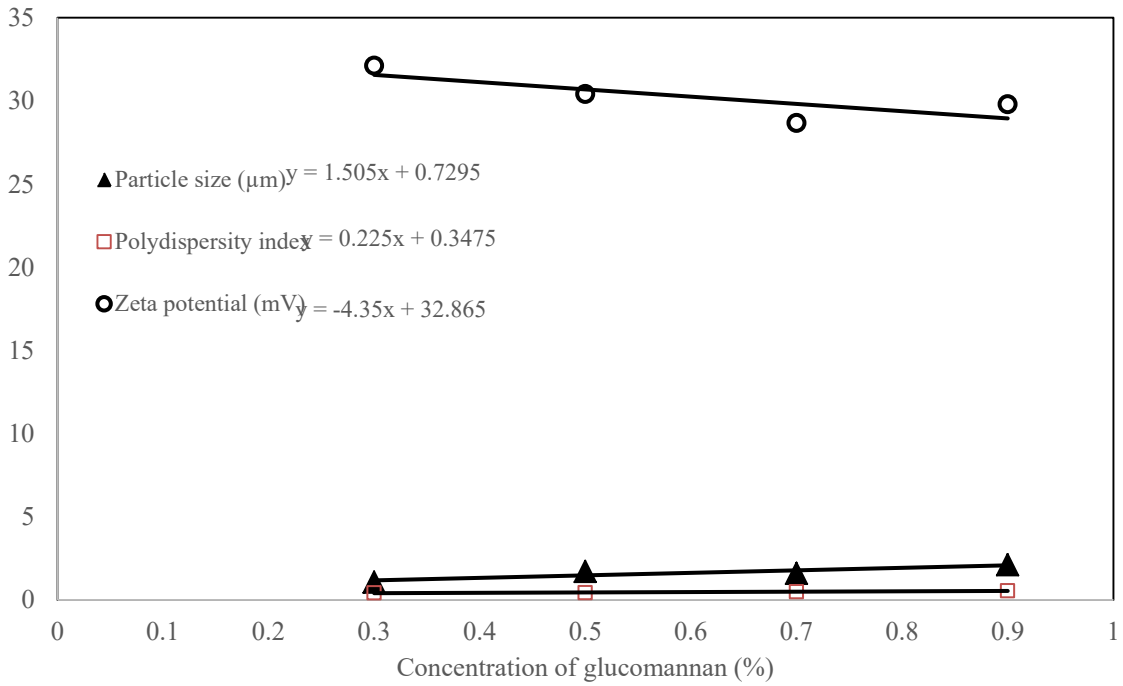

Figure 3 Effect of glucomannan concentration on the particle size, zeta potential and polydispersity index of hydrogel.

Encapsulation efficiency of hydrogel

Encapsulation efficiencies of hydrogel were almost the same when different concentrations of glucomannan were conducted, except at the concentration of 0.5 % glucomannan (**Table 1**). This was in line with a previous study that used the same polymers with L-asparaginase as the core. The same ratio concentration of glucomannan and chitosan was needed not only for the electrostatic interaction but also for chemical bonding [35]. The difference in charge between the hydrogel and the core also influenced the entrapment of cells. It served as the substrate for the adsorption of polycation as the 1st layer polymer encapsulant [4].

Table 1 Encapsulation efficiency of hydrogel in different concentrations of glucomannan.

Concentration of glucomannan (% w/v)	Encapsulation efficiency (%)
0.3	51.20 ± 5.74a
0.5	65.83 ± 1.37b
0.7	51.59 ± 3.39a
0.9	56.27 ± 4.12a

Values represent mean ± SD. Different superscript letters in the same column indicate significant different results at $p < 0.05$.

Swelling ratio of hydrogel

Figure 4 showed the swelling ratio of the hydrogel at different media (concentration of salt and pH). **Figure 4(A)** showed the increase of swelling ratio in all hydrogels with the salt concentration up to 1 M. It due to hydrogel could not resist the external ionic strength from sodium chloride solution. The higher the salt concentration, the higher the ionic strength. It disturbed the ionic interaction in the hydrogel. This condition made the water easier to enter the hydrogel, therefore increasing the swelling ratio. It was supported by Du *et al.* [31] who reported the increase of hydrogel size when salt concentration was increased. Egan *et al.* [36] also proved that salt concentration could give cationic competition and led to the release of core from microgel.

Figure 4(B) showed that the swelling ratio of hydrogel began to increase at pH up to 5. A previous study reported that this was due to the difference in interaction strength at different pH. At pH < 4.5, there was ionic interaction between both polymers which leads to the lower swelling ratio, while at pH 4.5 - 6, a positive charge from chitosan and ionic charge from glucomannan was almost the same which leads to the lower swelling ratio. At pH above 6, both polymers had the same charge; therefore, there was repulsion between polymers which yielded a higher swelling ratio [17]. The variation of glucomannan concentration added to hydrogel processing influenced the swelling ratio. When lower glucomannan concentrations (0.3 and 0.5 %) were used, swelling began at pH > 6, but they did at pH > 8 when higher glucomannan concentrations (0.7 and 0.9 %) were applicated. It was influenced by more carboxymethyl groups in higher glucomannan concentration which led to more interaction with the amine group from chitosan that made hydrogel more stable. Yu *et al.* [37] reported the same result when producing hydrogel from oxidized glucomannan and chitosan. The sensitivity of hydrogel made from the lower concentration of glucomannan may be used to control the release of the entrapped core. In the delivery of bioactive substances in the digestive tract, it may protect the bioactive in the low pH of gastric juices but it may be released in neutral pH of intestinal juice [38].

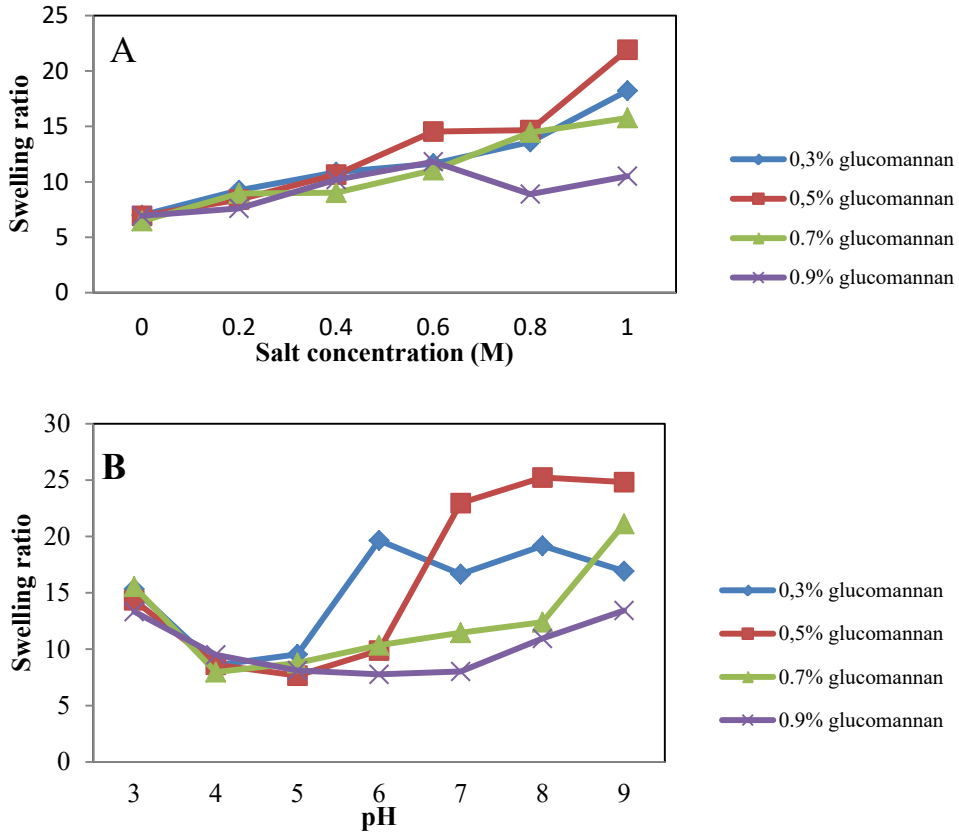

Figure 4 Swelling ratio of glucomannan-chitosan hydrogel in different salt concentrations (A) and pH medium (B).

Survival of *L. acidophilus* FNCC 0051 during heat and storage treatment

Figure 5A showed that hydrogel made from porang glucomannan-chitosan had the same ability in protecting *L. acidophilus* from heat treatment with other popular hydrogels. The viability of free cells in this study was about 58.13 ± 18.5 %, and there was statistically no difference with the cell encapsulated in other hydrogel tested. Jiang *et al.* [39] reported that it may be the attenuation of interaction in hydrogel during heating because of polymer degradation.

A study on the impact of cold storage (**Figure 5(B)**) on cell viability showed an extraordinary result due to the increase of its viability during 56 days and it was higher compared to other hydrogel used (konjac glucomannan-hydrogel and Ca-alginate hydrogel). It was also different from other studies that showed 1 log cycle reduction of cells during 20 days storage in yogurt at 5 °C [40] and 4 log cycles reduction when applied in concentrated juice at 4 °C for 21 days [41]. The elevation of cells in hydrogel also proved that even though the SEM image showed a continuous surface, there were still pores that enable the milk (media) to insert the hydrogel. It could be the source of nutrients to microbes and be used as growth media. Rathore *et al.* [42] declared that the permeability of encapsulant was needed to exchange the nutrients, gases, and metabolites; therefore, cell viability could be maintained.

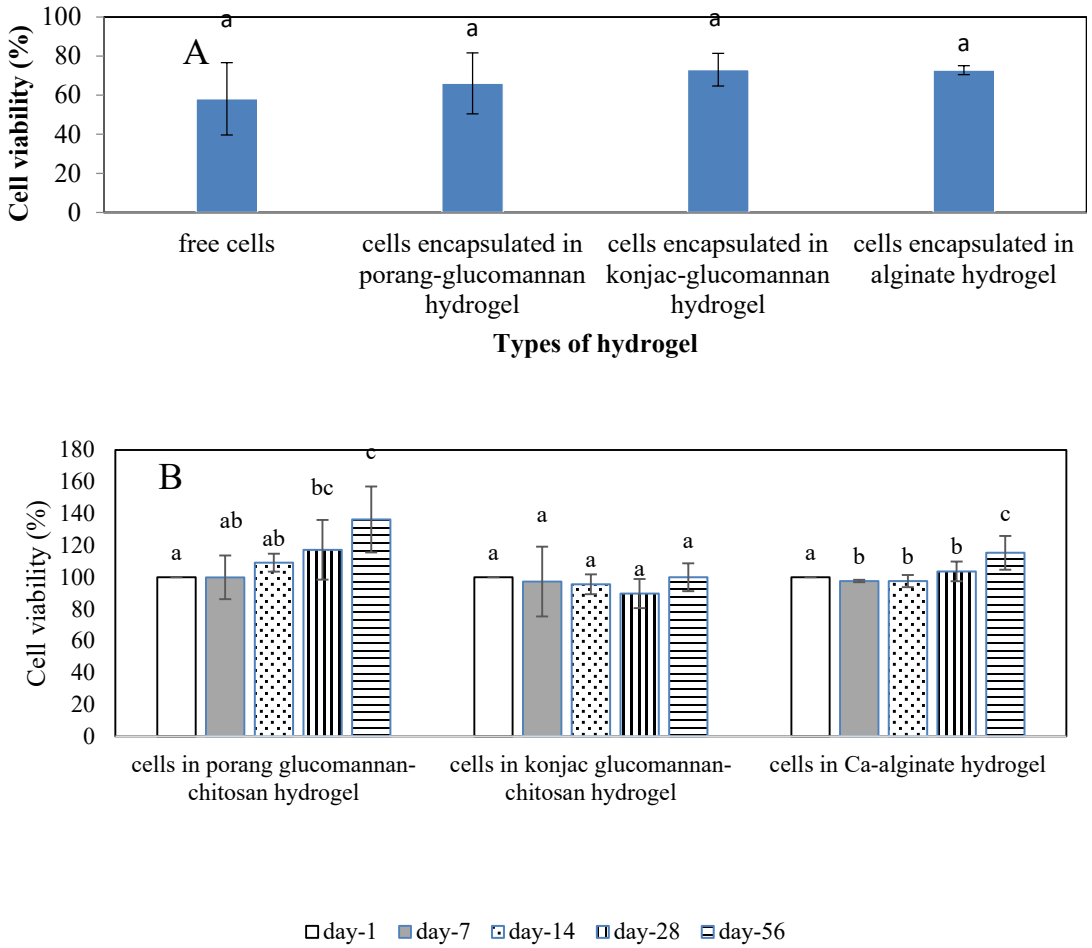

Figure 5 Survival test of *L. acidophilus* FNCC 0051 encapsulated in different types of hydrogels during heat treatment at 65 °C for 30 min (A) and 56 days of cold storage at 5 °C (B).

Conclusions

Hydrogel with a spherical shape can be efficiently prepared by combining 0.5 % porang glucomannan and 0.5 % chitosan. The increase of glucomannan concentration from 0.3 to 0.9 % had an impact on the smoothly increasing of particle sizes, zeta potential, and polydispersity indexes. These were shown by the small values of slopes, i.e. 1.505 - 4.35 and 0.225. The hydrogels were also sensitive in different pH environments. The swelling ratio began to increase when the pH of the media was up to 5 and 1 M of salt solution treated. It is potential to use the hydrogel in GI tracts that allow the hydrogel to de-swell when it reached the stomach and swell in the intestinal colon. This characteristic is beneficial for hydrogel to encapsulate and release the cells in the desired area. The encapsulation efficiency achieved 65.83 % when *L. acidophilus* FNCC 0051 was applied. The cells were also well protected during heat treatment and cold storage with 58.13 ± 18.5 and 100 % of cells viabilities. The good permeability of hydrogel can function as the exchange surface of the nutrients, gases, and metabolites.

Acknowledgements

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