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Research Article
Encapsulation of *Lactobacillus acidophilus* FNCC 0051 in Hydrogel Using a Complex Coacervation of Glucomannan and Chitosan

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Abstract

Background and Objective: Probiotic is functional food gave good effect to human body if it is consumed in adequate amount. Its viability becomes lower during processing, storing and delivering to the body. Therefore, it needs to be protected. Hydrogel formed from interaction between glucomannan and chitosan may protect it. The aims of this study were to evaluate the characteristics of hydrogel made from glucomannan and chitosan and its efficiency in encapsulating *Lactobacillus acidophilus* FNCC 0051. **Materials and Methods:** Hydrogel was prepared by extruding 0.5% w/v chitosan in 1% acetic acid to 0.5% w/v carboxymethyl glucomannan. Properties of hydrogel such as particle size, morphology Fourier Transform Infra Red (FTIR) spectra and swelling ratio were measured. Encapsulation efficiencies were evaluated by enumerating encapsulated and unencapsulated cells. Data were analyzed using one way-ANOVA, then continued with Duncan's Multiple Range Test (DMRT). **Results:** The result of the study showed that hydrogel formed using complex coacervation between glucomannan and chitosan had the spherical shape with the particle sizes around 1.09-2.31 μm. The new peak spectra at 1589 cm^{-1} confirmed that *L. acidophilus* was encapsulated in hydrogel matrix. The hydrogel respectively showed minimum and maximum swelling ratio at pH values of 5 and 8. Encapsulation efficiency was 66.1±3.0% when the cells were released in pH 8. It was not significantly different to the efficiency in pH 7. Enumerating unencapsulated cells provided lower encapsulation efficiency. **Conclusions:** *L. acidophilus* can be encapsulated in hydrogel using complex coacervation of glucomannan and chitosan. The low swelling ratio at lower pH provide better protection for the cell in the stomach, while the high swelling ratio at higher pH may be used to release the cell in small intestine. Further researches may be conducted to increase the encapsulation efficiency.

Key words: Hydrogel, glucomannan, encapsulation, *Lactobacillus acidophilus*, chitosan, swelling ratio, complex coacervation, encapsulation efficiency

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Probiotics, recently increase widespreadly because of their health benefits on the hosts' body. They gave good effect to human body, if they are consumed in adequate amounts[1], that is about 10^6-10^7 CFU g^{-1} [2,3]. To fulfill that amount, probiotics should be saved in products during processing, storaging and delivering to the body. However, the viability of probiotics in the product may be lower because the existence of lactic and acetic acid leads to decrease of pH, hydrogen peroxide and oxygen. It causes high oxidative stress condition, the addition of food additives such as salt, sugar, artificial flavor, coloring agents and also processing parameters like heating, cooling and freezing treatments[4-6].

Lactobacillus acidophilus FNCC 0051 is one of probiotics model isolated from human gastrointestinal tract. It was commercially used in fermented food products[7]. Generally, *lactobacillus* can grow in the environment of 10-45°C and concentration of NaCl 6.5%. However, it has low survival in the body, especially when it passes through gastrointestinal tract to harsh environment[8,9].

To maintain the survival of probiotics, encapsulation may be the recommended method. Hydrogel is a three dimensional matrix that may protect the probiotics. It usually uses biopolymer as the material, because of its advantages like lower toxicity, high compatibility and biodegradability. Many biopolymers were proved to increase the survival of probiotics when it applied as hydrogel, such as alginate, cellulose, carrageenan, locust bean gum and chitosan[8,10-12]. Chitosan combined with glucomannan could form hydrogel quickly[13] and it may have many good properties such as; spherical shape, semi-permeable, low release in gastric juice and high encapsulation efficiency[14,15]. However, the application of this hydrogel in encapsulation of probiotics is still limited.

In this paper, the characteristics of hydrogel made from carboxymethyl glucomannan and chitosan was studied and the encapsulation efficiency of hydrogel in encapsulating *Lactobacillus acidophilus* FNCC 0051 was also measured.

MATERIALS AND METHODS

Materials: The study was carried out on December, 2016-March, 2017. Glucomannan was extracted from Porang (*Amorphophallus oncophyllus*). It was carboxymethylated with Na-chloroacetate under alkaline at 70°C during 40 min[16]. Food-grade chitosan with degree of deacetylation 85-89% was purchased from PT Biotech Surindo.

Microorganism: *L. acidophilus* FNCC 0051 was provided from Food and Nutrition Culture Collection (FNCC), Laboratory of Food Microbiology, Center for Food and Nutrition Studies, Universitas Gadjah Mada. The working stocks of cells were stored in 20% skim milk-glycerol suspension frozen at -20°C. *L. acidophilus* was reactivated by growing twice successively in de Man, Rogosa and Sharpe (MRS) broth at 37°C overnight. The cell biomass were harvested by centrifugation at 2400 g for 9 min at 4°C[17], washed twice with sterile saline solution and re-suspended in saline solution before it was mixed with glucomannan.

Encapsulation of *L. acidophilus* in hydrogel: Encapsulation process was performed by complex coacervation method based on previous study[18] with different kind and amount of the core. *L. acidophilus* cells (10^8-10^9 CFU mL^{-1}) were used as the core. They were encapsulated by mixing one part of culture concentrated with four parts of polymer solution. Hydrogel in suspension was then analyzed for its particle size and morphology, swelling ratio, FTIR spectroscopic analysis and encapsulation efficiency.

Particle size and morphology of hydrogel: The size and morphology of hydrogel in suspension were measured with optical microscope (Olympus BX51, Olympus Corp., Japan) equipped with optilab pro digital camera (Miconos, Indonesia). All particles in one field of view were calculated and presented as the mean size. The morphology of hydrogel was observed after dyeing with iodine solution and the *L. acidophilus* in hydrogel were stained by using Gram's method.

Fourier Transform Infra-Red (FTIR) spectroscopic analysis: FTIR was analyzed to ensure that *L. acidophilus* was encapsulated in hydrogel. It used Shimadzu 8201 PC spectrophotometer in the region between 4,000 and 400 cm^{-1}. Freeze-dried hydrogel was mixed with KBr and pressed to a plate for measurement[19].

Swelling ratio of hydrogel in various pH: The swelling ratio of hydrogel was determined by Du *et al.*[18] with different pH media. The media for swelling studies were buffer HCl-KCl buffer (pH 1 and 2), citrate buffer (pH 3), acetate buffer (pH 4 and 5), phosphate buffer (pH 6, 7 and 8) and carbonate buffer (pH 9). The swelling ratios were then calculated by using Eq. 1[18]:

$$\text{Swelling ratio} = \frac{(W - W_0)}{W_0} \quad (1)$$

Where:
W = Weight of hydrogel at equilibrium state (g)
W_0 = Weight of initial/dried hydrogel (g)

Encapsulation efficiency of hydrogel: Encapsulation efficiencies were calculated by enumerating encapsulated and unencapsulated cells.

Encapsulated cells were determined by releasing *L. acidophilus* cells from hydrogel with buffer solution of pH 7 and 8. The hydrogel was incubated for 24 h at 37°C. Hydrogel in buffer was then serially diluted in saline solution. Encapsulation efficiency was calculated by using Eq. 2[20]:

$$\text{Encapsulation efficiency}(\%) = \left(\frac{\log N}{\log N_0}\right) \times 100\% \quad (2)$$

Where:
N = Number of *L. acidophilus* encapsulated in hydrogel and could be released with buffer solution (CFU)
N_0 = Number of *L. acidophilus* added to glucomannan (CFU)

Unencapsulated cells were determined by enumerating cells outside the hydrogel, those were from the total of supernatant and its rinse suspensions. To calculate encapsulation efficiency, the cells encapsulated in hydrogel were enumerated by subtracting number of cells outside the hydrogel from the total cells added. The supernatant of hydrogel was obtained from the hydrogel suspension after centrifugation at 500 g for 10 min. The residue was then rinsed with buffer acetate pH 4 for twice and each of rinsed suspension was labeled as first rinsed and second rinsed suspension. One mL of each suspensions were serially diluted in saline solution, then plated on MRS agar. Free *L. acidophilus* (N1) was the total of supernatant, 1st and 2nd rinse suspensions. Encapsulation efficiency determination (Eq. 3) was modified from the Eq. 2[20] as follow:

$$\text{Encapsulation efficiency}(\%) = \frac{(\log N_0 - \log N_1)}{\log N_0} \times 100\% \quad (3)$$

Where:
N_0 = Number of *L. acidophilus* added to glucomannan (CFU)
N_1 = Number of *L. acidophilus* outside the hydrogel (CFU)

Statistical analysis: Statistical studies used Statistical Package for the Social Sciences (SPSS) software (version 16.0 for Windows; SPSS Inc., Chicago, IL, USA)[21]. Data were reported as Mean±Standard Deviation (SD) and analyzed using one-way analysis of variance (ANOVA). Means were then compared using Duncan's Multiple Range Test (DMRT) at $p<0.05$.

RESULTS

Particle size and morphology of hydrogel: As shown in Fig. 1a, almost hydrogel particles generated from the interaction between glucomannan and chitosan had spherical shape with the diameter between 1.09-2.31 µm. The mean of diameter size of hydrogel was 2.05±0.40 µm. Fig. 1b also showed that *L. acidophilus* was encapsulated in hydrogel.

FTIR spectroscopic analysis: To confirm the encapsulation of *L. acidophilus* in hydrogel, FTIR spectra data were analyzed. As illustrated in Fig. 2, the interaction between C=O of glucomannan and C=N of chitosan was characterized from

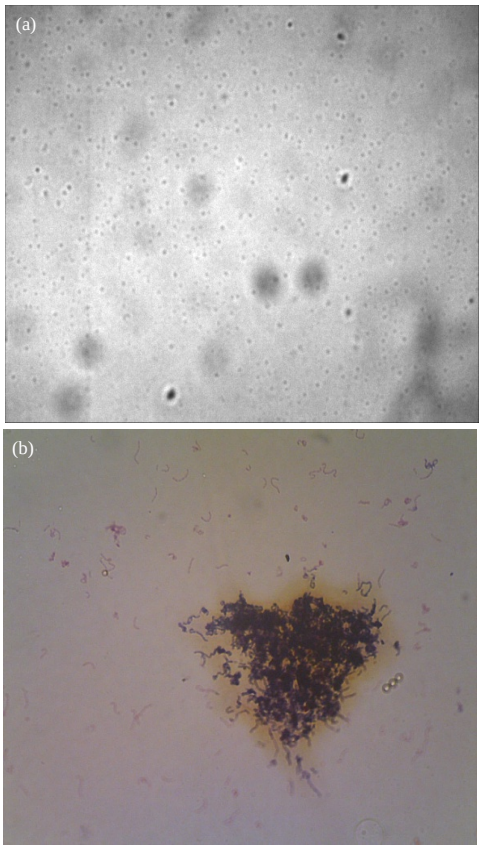

Fig. 1(a-b): (a) Shape of hydrogel carrying *L. acidophilus* from combination of carboymethyl glucomannan with chitosan and (b) Gram's staining of *L. acidophilus* in hydrogel

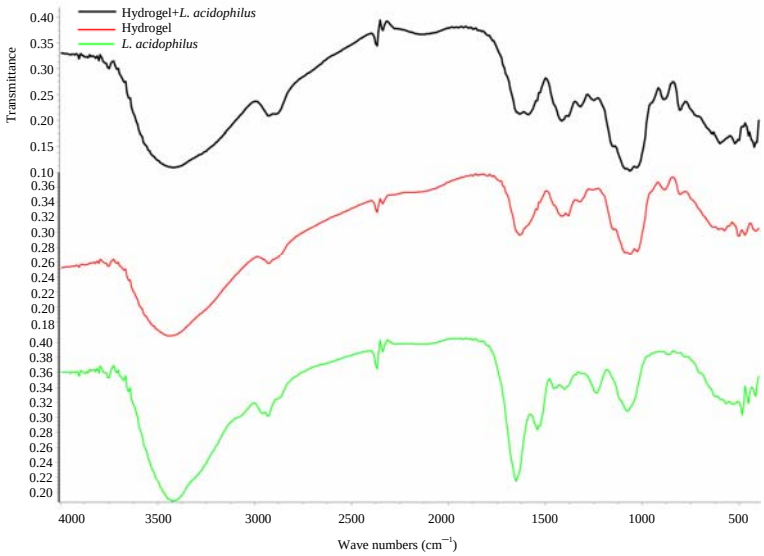

Fig. 2: FTIR spectra of *L. acidophilus* in hydrogel

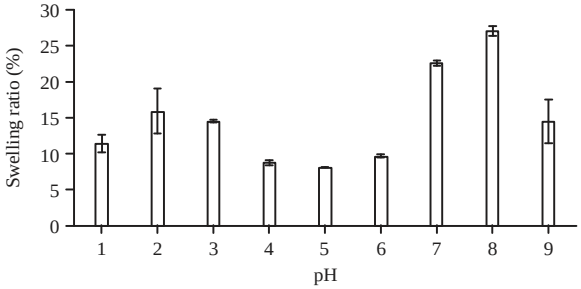

Fig. 3: Swelling ratio of hydrogel formed by complex coacervation between carboxymethyl glucomannan and chitosan in different pH medium. Bars represent mean values±standard deviation in triplicate

Table 1: Encapsulation efficiency of *L. acidophilus* FNCC 0051 in hydrogel formed by complex coacervation between glucomannan and chitosan

Techniques	Encapsulation efficiency (%)
Enumeration cells encapsulated in hydrogel	
Releasing cells with buffer pH 7	58.49±2.07[a]
Releasing cells with buffer pH 8	66.12±3.00[a]
Enumeration cells unencapsulated in hydrogel	
Counting cells in supernatant of hydrogel and rinsed suspension	40.72±3.87[b]

Values represent Mean±SD. Different superscript letters in the same coloumn indicate significant different results at p<0.05

the peak of 1635 cm^{-1}. In hydrogel carrying cells, the peaks at 1651 and 1543 cm^{-1} from *L. acidophilus* cannot be seen. In the other hand, there is a new peak at 1589 cm^{-1} that may be attributed from the encapsulation of cells in hydrogel. Based on this research, some of *L. acidophilus* might be encapsulated in hydrogel chemically.

Swelling ratio of hydrogel in various pH: The swelling ratio of hydrogel was studied in pH 1-9 media. Generally, swelling ratios of hydrogel were lower at pH <6. It began to increase at pH 7-8. This research showed that hydrogel was responsive in pH environment (Fig. 3).

Encapsulation efficiency of hydrogel: As shown in Table 1, encapsulation efficiency determined by enumeration of encapsulated cells in hydrogel was higher than the efficiency in unencapsulated cells. To confirm that the amount of cells outside the hydrogel did not adhere on the surface of hydrogel during centrifugation, the enumeration of cells in suspension containing hydrogel was also done. The data

showed that the amount of cells in supernatant (hydrogel has been discarded from suspension) and suspensions were almost similar. Those were 3.78×10^5 and 1.67×10^5 CFU mL^{-1}, respectively.

Table 1 also showed no differences of encapsulation efficiency determined by releasing cells from hydrogel, either with buffer pH 7 or 8 ($p>0.05$). It may be the amount of cells released was similar in both of pH solution, although the swelling ratio of hydrogel was higher in pH 8 than that was in pH 7, as shown in Fig. 3.

DISCUSSION

The particles produced from the complex coacervation between glucomannan and chitosan was spherical in shape (Fig. 1a). This results was similar to L-asparaginase[14] and and Bovine Serum Albumin (BSA)[18] used as the cores. It could be known that the differences in core carried by hydrogel did not change the shape of hydrogel, because it processed with the same technique. In complex coacervation, two or more charged polymers were interacted. This interaction may be repulsive or attractive leading to phase separation or coacervation reactions[22]. This research used negative charged of glucomannan (modified as carboxymethyl glucomannan) and positively charged of chitosan.

Hydrogel was in micron size diameter between 1.09-2.31 μm. Therefore, the particles were included in microgel. This particle size was different when it carried in different core. Previous studies reported that diameter of hydrogel from the interaction between glucomannan and chitosan with L-aspariganase and BSA as the cores were 0.3-3 and 0.048-1.19 μm, respectively[14,18]. There were limited study reported the effect of core on the size of hydrogel. Several studies showed that factors affecting the size of hydrogel were usually the size of nozzle, viscosity and flow rate of the polymeric solution, the distance between nozzle and solution, concentration and temperature of polymer solution and the environment like pH and salt[19,22-24]. Comparing this result with other different core in encapsulation process, it may be assumed that beside the differences in the properties of polymers, the properties of the core may influence the compactness of hydrogel, leading to the differences in size of hydrogel. The shear rate during encapsulation process also influenced the size of particle[22].

Figure 1b showed that *L. acidophilus* was encapsulated in hydrogel. The encapsulation was proved by the discovery of new peak at 1589 cm^{-1} in hydrogel carrying cells (Fig. 2). It presumed that some of the cells were adsorb in the matrix of hydrogel. This study showed the absorption peak at 1635 cm^{-1} in hydrogel spectra. It indicated interaction between C=O of glucomannan and C=N of chitosan[25]. There were also the peak spectra at 810 cm^{-1} from mannose of glucomannan and at 1381 and 1319 cm^{-1} from amide 3 of chitosan also existed.

The effect of pH on the swelling ratio was shown in Fig. 3. The pH may cause the dissociation of weak acid or base groups on the polyelectrolyte chains, therefore influence on swelling ratio of hydrogel. At lower pH ($\leq$6), swelling ratio of hydrogel were lower but it was high when exposed to medium of pH 7 and 8. It related to pKa of chitosan and glucomannan, that reported by previous study. It was 6.48 and 2.69, respectively[18].

The different pKa value led to different charge of polymer. At the pH between 2.69-6.48, chitosan was in positive charge, while glucomannan was in negative charged. It resulted higher ionic interaction between glucomannan and chitosan and allowed more cross-linking in hydrogel. The more cross-linking in hydrogel, the more stable of hydrogel[26]. This condition may avoid the release of cells and allow better protection of cells inside the hydrogel.

At higher pH (>6), chitosan became negative and glucomannan did too. Both polymers repulsed each others. The repulsion of polymers had the impact on the weakness of cross-linking and was resulted in swelling and unstable hydrogel (Fig. 3). This condition let releasing of the cells. This research was in accordance with previous study reporting the swelling-deswelling transition let on the encapsulation or releasing of the core from hydrogel[27].

The ability of hydrogel to swell and de-swell in different pH may be the advantage of using glucomannan-chitosan as encapsulant of probiotics. Other polymer that is usually used to encapsulate probiotics, like alginate did not have this behavior. In lower pH, matrix formed by the cross-linking between alginate and Ca^{2+} would broken resulting in cell release[4].

Determination of encapsulation efficiency by enumeration cells encapsulated in hydrogel was higher than the efficiency in unencapsulated cells (Table 1). Firstly, it was assumed that the unencapsulated cells settled when centrifugation, whereas the aim of centrifugation of this research was to precipitate the hydrogel from suspension only. The separation of unencapsulated cells from hydrogel was based on differential centrifugation principle. Particles of different densities or sizes in suspension will precipitate at different rates[28]. The precipitation of cells resulted in lower cell amount in supernatant and its rinse suspensions, so that the encapsulation efficiency becomes lower (Table 1). The second reason to answer why the encapsulation efficiency becomes

lower was that the possibility of cells to adhere in the outside surface of hydrogel. It also influenced the lower encapsulation efficiency value.

The encapsulation efficiencies were not different when the encapsulated cells were released in different pH solution ($p>0.05$) (Table 1). The amount of released cells may be similar in both pH solution, although the swelling ratio of hydrogel was higher in pH 8 than that in pH 7. Theoretically, the higher the swelling ratio, the more cells could release from the hydrogel. It because when swelling ratio achieved the higher value, there were also higher electrostatic repulsion between glucomannan and chitosan. Its repulsion was influenced by zeta potential of carboxymethyl glucomannan and chitosan. In basic solution, both polymers had negative zeta potential[18]. The higher swelling ratio led to the larger pore in the matrix, therefore it may release more number of *L. acidophilus* from hydrogel. However, in this research, the difference swelling ratio of hydrogel, either in pH 7 and 8 may lead to the same wide of pore, so there were similar amount of released cells. The study in releasing Bovine Serum Albumin (BSA) from hydrogel formed by interaction between carboxymethylated konjac glucomannan with chitosan also showed the similar encapsulation efficiency. It could achieve 73% when the hydrogel was exposed to pH 7.4 during 3 h.

This study found the potency of glucomannan-chitosan hydrogel as encapsulant of probiotics in higher survival. Its survival can be supported by its sensitivity to different pH medium. In the acidic condition, the hydrogel become stable allowing better protection of the cells. On the other hand, the hydrogel let the release of the cells on alkali to neutral condition. The hydrogel may be applied in food product that was expected to deliver probiotic safely in small intestine. However, the study of hydrogel stability in gastrointestinal fluid in vitro is needed for the representative condition. The optimation of encapsulation efficiency is also important to be studied.

CONCLUSION AND FUTURE RECOMMENDATION

Hydrogel from combination between glucomannan and chitosan is an effective encapsulant for *L. acidophilus.* This was confirmed from the FTIR spectra. The complex coacervation technique yielded sphere to micron-size particles. This study also discovered that hydrogel was sensitive to pH. Swelling ratio of hydrogel was minimum in acidic medium, while in the high pH (7-8) swelling ratio was high. This condition supports the survival of probiotic in the stomach. Encapsulation efficiency in glucomannan-chitosan hydrogel was also better determined by direct enumeration of the encapsulated cells. The future studies may be important to optimize its encapsulation efficiency.

SIGNIFICANCE STATEMENT

This study discovered the method to encapsulate bacterial cells in glucomannan-chitosan hydrogel and prepared using coacervation technique, effectively. The hydrogel possesses different sensitivity to different pH that can be beneficial to protect cells in gastrointestinal tract and delivered to large intestine safely. This study will help the researcher to improve the method of delivering probiotic to expected target.

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