Original Research Article

Microencapsulated *Lactobacillus acidophilus* FNCC 0051 Dan *Streptococcus thermophilus* FNCC 0040 Technique Emulsion Using Gelatin and Na Alginate

ABSTRACT

The research was held from September 2021 until January 2022 in Laboratory Livestock Product Technology Universitas Brawijaya, Malang, Indonesia. Microencapsulation is a technique used to protect bacteria from harmful (extreme) environmental factors such as heating, freezing and low pH through a coating process or coating a core substance in this case LAB with a polymer wall layer. The purpose of this study was to obtain a combination of the use of gelatin and Na alginate as a coating material in the encapsulation of *Lactobacillus acidophilus* FNCC 0051 and *Streptococcus thermophilus* FNCC 0040 using the emulsion technique based on its physical and microbiological properties. The research method used is a laboratory experiment using a completely randomized design (CRD) pattern with 3 treatments and 3 replications. The treatment using a combination of gelatin and Na alginate consisted of P1 (1:1); P2 (1:2); P3 (1:3). Data analysis used Analysis of Variance (ANOVA); if the results obtained are significant, the analysis will continue with Duncan's Multiple Range Test (DMRT). The results of the analysis show that encapsulation using a combination of gelatin and Na alginate gives a very significant difference ($P<0.01$) to the value of encapsulation efficiency and does not give a significant difference ($P>0.05$) on microcapsule particle size and microcapsule particle size distribution, with percentages T1 97.43±0.31%, T2 98.50±0.48%, P3 99.00±0.44 %; T1 1.08±0.07 µm; T2 1.18±0.11 µm; T3 0.95±0.11µm; and T1 4.79±1.04; T2 2.53±2.16; T3 4.15±3.13 and microcapsules using SEM showed the microcapsules were round and smooth. The combination of gelatin and Na alginate P3 (1:3) as a microcapsule material is a good alternative to protect lactic acid bacteria so that it can be applied in food products functional.

Keywords: probiotic, microencapsulation, emulsion technique, gelatin, Na alginate

1. INTRODUCTION

Currently functional food products have played an important role in the food industry sector due to the increasing public awareness of the importance of eating healthier and higher nutritional value foods. The functional food sector has represented one of the most dynamic and innovative categories in the food industry. These foods not only contribute to basic nutrition but also

contain active physiological components, which is one of the main segments represented by probiotic-based food products.

Lactobacillus acidophilus and *Streptococcus thermophilus* are a group of probiotic bacteria known to provide health benefits for humans. Probiotic bacteria will be able to work optimally if they contain living cells that can survive during the heating, food processing and storage processes. The number of live microbes must be sufficient to have a positive effect on health and be able to colonize so that they can reach the required number for a certain time. The minimum concentration of probiotics that must be met is around 10^6-10^7 CFU/mL at the end of the product shelf life [1]. Meanwhile, to lead to health benefits, the recommended amount is 10^8-10^9/day [2]. Therefore, the viability of probiotics in the product needs to be considered. One of the efforts to maintain the viability of probiotics is to apply microencapsulation techniques.

Microencapsulation by emulsion technique is an appropriate method for producing water particles in oil emulsions [3]. One of the advantages of this method is that the resulting particles are smaller (less than 100 m) and do not change the sensory properties of the product. This method does not require special equipment and sophisticated techniques, the formulation is simple and low cost, this method has high cell viability and porous particles [4]. Materials commonly used as encapsulants are organic or inorganic polymers either derived from natural or artificial materials.

Na alginate is a natural polymer that is widely used in the microencapsulation process because it is biocompatible, biodegradable, soluble in water, non-toxic and has a relatively low price. Na alginate can form a gel with two valence cations, including Ca^{2+} ions, so in this study a $CaCl_2$ as a cross-linking material, but alginate has a drawback that the microcapsules produced are too porous, so they are not optimal in protecting probiotics from environmental factors. The porous structure will also result in low probiotic trapping ability [5]. Therefore, other polymers are needed to minimize these deficiencies.

Gelatin is a polymer that is biodegradable, biocompatible, stable over a wide pH range, non-toxic, inexpensive, has good extensibility, is water-soluble, can form films, can act as an emulsifier and can undergo cross-linking such as Na alginate [6].
The size of the microparticles plays an important role in the process of the bacterial release mechanism. The lack of ability to predict and control the size distribution of microparticles can be a problem that leads to the inability to accurately predict the chemical properties of the particles and the level of material availability. Therefore, physical protection against probiotics is very necessary. The purpose of this study was to determine the concentration of addition of gelatin and Na alginate using emulsion technique on microencapsulation of *Lactobacillus acidophilus* FNCC 0051 and *Streptococcus thermophilus* FNCC 0040 based on the value of encapsulation efficiency, microcapsule particle size, microcapsule particle size distribution and microstructure using Scanning Electron Microscopy (SEM).

2. MATERIALS AND METHODS

2.1 Materials

The materials used in this study include biomass *Lactobacillus acidophilus* FNCC 0051 and *Streptococcus thermophilus* FNCC 0040, MRS agar (merck), MRS broth (oxoid), gelatin, sodium alginate, paraffin oil, span 80, NaCl 0.9% (braun), NaCl 0.85 % (braun), $CaCl_2$, 70% alcohol, aluminum foil, and aquades. The equipment used in this study included an autoclave (Hirayama), light microscope (Novex Holland), Laminar Air Flow (LAM), Particle size Analyzer (PSA) (microtrac), Scanning Electron Microscopy (SEM) (Hitachi TM 3000) analytical balance, centrifugator (jouan), vortex (labinco L 46), petri dish (duran), beaker glass (pyrex), erlenmeyer (pyrex), measuring cup (iwaki), magnetic stirrer (SH-2),

incubator (memmert), needle ose, 5 mL syringe (sakamed), water bath (GFL), test tube (pyrex), test tube rack, glass funnel (pyrex), measuring flask (duran), micro pipette, blue tip, white tip, bunsen, bottle (duran), centrifuge tube (sakamed), dropper, portable stove, refrigerator (LG), whatman filter paper no. 1, thermometer, gloves, cover glass, object glass, tweezers and stirrer.

2.2 Methods

The research method used is a Laboratory Experiment by using a Completely Randomized Design (CRD) pattern with 3 treatments and 3 replications. The treatment that was tried was the use of a combination of gelatin and sodium alginate T1 (1:1); T2 (1:2); T3 (1:3). Data were analyzed using Analysis of Variety (ANOVA), and if there were significant differences, it would be further testing with Duncan's Multiple Range Test (DMRT). Parameters observed were encapsulation efficiency [7], particle size and particle size distribution using Particle Size Analyzer each based on [8] [9], microcapsule microstructure using Scanning Electron Microscopy [10].

2.2.1 Probiotic Culture Preparation

Procedure for the propagation of *Lactobacillus acidophilus* FNCC 0051 and *Streptococcus thermophilus* FNCC 0040 in this study refers to Lestari, et al. [11] with modifications, namely the length of the incubation period and the number of LAB inoculated. Cultures *Lactobacillus acidophilus* FNCC 0051 and *Streptococcus thermophilus* FNCC 0040 in ampoules were taken as many as 3 oses were inoculated into 10 ml of MRSB, incubated at 37°C for 40 hours. The cell suspension was then centrifuged at 4.500 rpm for 10 minutes at 4°C to obtain cell biomass. The cell biomass obtained was washed twice with sterile NaCl (0.85% w/v).

2.2.2 Encapsulation *Lactobacillus acidophilus* FNCC 0051 and *Streptococcus thermophilus* FNCC 0040

Lactobacillus acidophilus FNCC 0051 and *Streptococcus thermophilus* FNCC 0040 were encapsulated with gelatin and Na alginate in this study referring to Lestari, et al. [11] with several modifications including the type of LAB, encapsulation material (gelatin and Na alginate) and stirring time. First, gelatin solution (5% w/v) was dissolved in distilled water heated at 50-55 °C and Na alginate (1.5% w/v) was dissolved in distilled water and sterilized at 121°C for 15 minutes. Then, 7 mL of a suspension of *Lactobacillus acidophilus* FNCC 0051 and *Streptococcus thermophilus* FNCC 0040 homogenized with a solution of gelatin and Na alginate in a ratio (1:1); (1:2); (1:3) respectively as treatment P1, P2 and P3. It was continuously inserted with a sterile syringe into 50 ml of sterile paraffin oil containing 0.5% span 80, then 10 ml sterile 0.1 M CaCl2 and homogenized using a magnetic stirrer at a speed of 450 rpm for 30 minutes. After the sedimentation of the microcapsules was formed, the microcapsules were harvested by centrifugation at a speed of 3.500 rpm for 15 minutes. The microcapsules were washed twice with sterile distilled water to separate the microcapsules from the oil. The microcapsules were filtered using filter paper and then transferred into sterile petri dishes.

3. RESULTS AND DISCUSSION

3.1 Encapsulation Efficiency

The results of the analysis of variance showed that the microencapsulated *Lactobacillus acidophilus* FNCC 0051 and *Streptococcus thermophilus* FNCC 0040 using a combination of gelatin and Na alginate through the emulsion technique gave a very

significant difference (P<0.01) on the average encapsulation efficiency. The higher the concentration of the use of encapsulation material, the higher the efficiency of the resulting encapsulation. The average value of encapsulation efficiency at T1 was 97.43±0.31%, T2 98.50±0.48% and T3 99.00±0.44% (Table 1).

The average encapsulation efficiency increased along with the increase in the use of Na alginate. The use of a combination of gelatin and Na alginate (1:3) as T3 showed the percentage of the best efficiency value of 99.00±0.44% compared to the use of a combination of gelatin and Na alginate at T1 (1:1) and T2 (1:2), respectively 97.43±0.31% and 98.50±0.48%. This indicates that the amount of LAB released from T3 is less than that of P1 and T2. The bacteria in T3 is thought to have not been completely released because the gelatin composition in P3 can close the pores formed from the use of the Na alginate matrix, thereby increasing the density of the matrix. [12] explained that Na alginate with a negative charge tends to gel faster due to the presence of Ca^{2+} to form a three-dimensional "egg-box" structure, whereas gelatin will form a three-dimensional "honeycomb" network structure under the double action of cooling after cooling. heating and ion induction. Gelatin can also assist in increasing the strength and rigidity of the working structure of the network through stability and electrostatic interactions. Hydrogen bonds are formed intermolecular carboxylic groups (-COO-) in Na alginate and cationic amine groups ($-NH^{3+)}$ in gelatin. This interaction is intended to create a safe environment so that the probiotics embedded in the microcapsules can be protected from the external environment. The gelatin solution at 40°C will be more dilute. When the gelatin is cross-linked with CaCl2at room temperature, the gelatin hardens rapidly, resulting in small particles [6].

The use of a combination of gelatin and Na alginate on P1 and P2 resulted in a lower encapsulation efficiency value than P3 because the gelatin filled the pores of the Na alginate imperfectly, thus forming a less closed network around the capsule, it is likely that LAB was not trapped completely during encapsulation process so that the encapsulation efficiency is lower. According to [13] Na alginate is the lyophilized component (host polyelectrolyte), while gelatin acts as an inhibitory component (guest polyelectrolyte). Na alginate cannot form an "egg-box" so that the particle size of the formula with Na alginate composition is larger than that of gelatin. In addition, gelatin is thermally reversible. Most lactic acid bacteria not only grow more slow at low pH, but possible suffer damage and loss of viability when the cell is at a low pH. Acidic conditions can cause damage membranes and the release of intracellular components which can cause death. Bacteria acid-resistant ones have good resistance greater resistance to membrane damage due to decrease in extracellular pH compared to with acid-resistant bacteria [14].

The interaction between gelatin and Na alginate molecules causes a lower number of polar groups on the surface of the mixture. These interactions result from the strong intermolecular hydrogen bonds formed within and between the biopolymer chains involving their carbonyl hydroxyl, and amino groups, as well as electrostatic interactions. The gelatin in the composition of the microcapsules in this study showed that the presence of gelatin increased the chemical stability of the microcapsules [15]. The encapsulation efficiency level test was conducted to measure the effectiveness of the encapsulation process. A high-efficiency value indicates a high number of encapsulated bacteria. The high value of encapsulation efficiency indicates that the encapsulation process is working optimally [16]. Encapsulation efficiency is influenced by the nature of the encapsulation material used. It has been observed that Na alginate provides effective cross-linking and mechanical support for protection against probiotics. Probiotic bacteria coated with Na alginate showed the highest level of efficiency because it is a

good wall material and has good compatibility [17]. The emulsion method is one of the methods used to increase encapsulation efficiency which has low water solubility by producing microcapsules that can improve particle size control and distribution [18].

3.2 Particle Size

The results of the analysis of variance showed that the microencapsulated namely LAB microcapsules with a combination of gelatin : Na alginate (1:2). Microcapsule particle size is an important characteristic in microencapsulated products because it is related to the release and absorption of particles by the mucosa. Changes in microparticle process parameters can affect the properties and performance of microparticles such as mechanical strength, ease of filtration and brittleness [19].

The particle size of the encapsulated live bacterial cells is an important factor in the design of industrial applications to produce the required uniformity and precision level of microcapsules [20]. Smaller microcapsules showed poor encapsulation efficiency, unwanted release of core material or migration from the injection site. Larger microparticles may also exhibit poor encapsulation efficiency due to prolonged release time and undesirable size [21]. Therefore, the size of the gel beads should be within the appropriate range, neither too large nor too small [22]. The LAB microcapsule measurement results obtained at T1, T2 and T3 have reached the expected target, the microcapsules formed fall into the microcapsules size range of 0.2 µm - 5000 µm. According to [23] the size of the microencapsulation is divided into three sizes, namely macro (>5000 µm), micro (0.2-5.000 µm) and nano (>0.2 µm). Capsules with the emulsion technique have a smaller diameter so that the resulting pore size is also smaller. This small pore size makes the transfer of fluid from outside the capsule into the capsule more limited [24]. The size of the microcapsules produced in this study was able to increase the encapsulation efficiency value up to 99.00%.

Lactobacillus acidophilus FNCC 0051 and *Streptococcus thermophilus* FNCC 0040 using a combination of gelatin and Na alginate through the emulsion technique did not give a significant difference (P>0.05) on the particle size of the microcapsules. The mean size of the T1, T2 and T3 microcapsules were 1.08±0.07 µm, 1.18±0.11 µm, and 0.95±0.11 µm, respectively (Table 1). The smallest particle size was found in

3.3 Particle Size Distribution

The results of the analysis of variance showed that the microencapsulation of Lactobacillus acidophilus FNCC 0051 and Streptococcus thermophilus FNCC 0040 using a combination of gelatin and Na alginate through emulsion technique did not give a significant difference (P>0.05) to the mean particle size distribution of microcapsules. The mean particle size distribution of microcapsules ranged from 2.53±2.16 to 4.79±1.04 (Table 1). The smallest particle distribution average was found at T2 2.53±2.16, namely probiotic microcapsules with a combination matrix of gelatin:Na alginate (1:2) and the largest particle distribution average was at T1 which was 4.79±1.04 namely probiotic microcapsules with a combination gelatin:Na alginate (1:1).

The size and size distribution of the microcapsules have a great impact on the process mechanism and the release rate of the encapsulated core material. The particle size distribution shows that the higher the polymer ratio used, the larger the particle size produced. The various sizes of microcapsules are influenced by several factors, including the distance between the syringe and the microcapsules-forming solution, the polymer concentration, the pressure difference when forming the microcapsules through the *syringe* and the high and low position of the *syringe* when dropping the microcapsules. The agitation speed from 300 rpm to 2.000 rpm had a significant effect on the size distribution. The microparticles prepared with high agitation speed were much smaller than those prepared with low agitation speed. The high agitation rate helps the

large droplets to be broken down into smaller droplets [25].

The different particle size distribution of microcapsules at P1, P2 and P3 were indicated to occur due to differences in the stability of the emulsifier due to the unstable surface tension of the oil and water phases. [26] states that the smaller the water phase particles in the water-in-oil emulsion, the smaller the diameter of the resulting capsule will also be. For this reason, an emulsifier is used to reduce the surface tension of the oil and water phases so that the resulting droplet size becomes smaller. The particle size distribution containing anticancer drugs ranges from 1 to 30 µm and by mixing smaller and larger microparticles, a release profile with an intermediate release rate can be achieved [21]. The large and variable size of the capsule can cause uneven distribution of cells in the capsule. In addition, capsules with a diameter of 1,000-3,000 µm can have an unpleasant effect on the sensory assessment of the product [24].

Table I. Average Encapsulation Efficiency, Particle Size and Distribution Particle Size of *Lactobacillus acidophilus* FNCC 0051 and *Streptococcus thermophilus* FNCC 0040 encapsulation using a combination of gelatin and Na alginate through emulsion technique

Treatment	Analysis		
	Encapsulation Efficiency	**Particle Size**	**Distribution Particle Size**
T1	97.43±0.31[a]	1.08±0.07	4.79±1.04
T2	98.50±0.48[ab]	1.18±0.11	2.53±2.16
T3	99.00±0.44[b]	0.95±0.11	4.15±3.13

3.4 Microstructur Microcapsules

Examination of microcapsules using a combination of gelatin and Na alginate at different concentrations through the emulsion technique was carried out through microscopic analysis using *Scanning Electron Microscopy* (SEM) aiming to determine the surface structure in protecting bacterial cells. The cross-section of the microcapsule (2.000x magnification) shows the integrity of the bacterial cells trapped inside the capsule (Figure 1). The presence of cells confirms that this technique is effective for the microencapsulation of bacteria. Image scans using SEM of T1 and T2 microcapsules prepared using a combination of gelatin and Na alginate (1:1) and (1:2) respectively showed that the capsules were round, rough and thick although some were found to be irregular (Figure 1). Observation of the spherical shape of the microcapsules was carried out using a light microscope as shown in Figure 2.

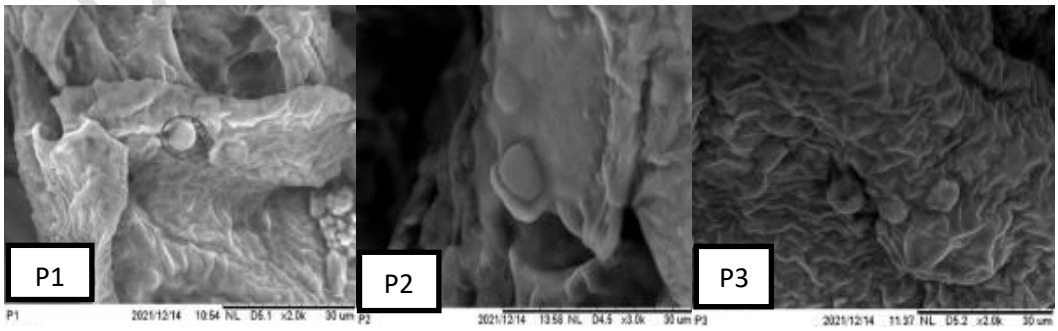

Figure 1. Microstructure of encapsulated LAB microcapsules using Scanning Electron Microscopy (SEM) with a magnification of 2,000x. P1 microcapsules using a

combination of gelatin:Na alginate (1:1); P2 microcapsules using a combination of gelatin:Na alginate (1:2); P3 microcapsules using a combination of gelatin:Na alginate (1:3).

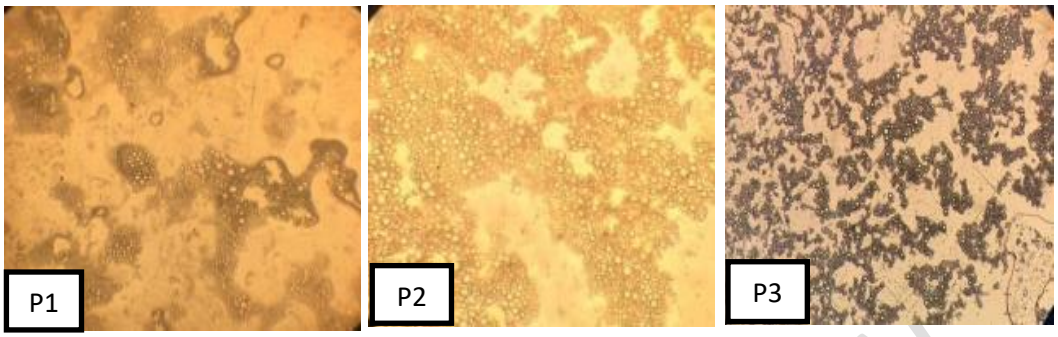

Figure 2. The shape of the encapsulated LAB microcapsules observed using a light microscope with a magnification of 10x. P1 microcapsules using a combination of gelatin:Na alginate (1:1); P2 microcapsules using a combination of gelatin:Na alginate (1:2); P3 microcapsules using a combination of gelatin:Na alginate (1:3)

The Na alginate material used in the encapsulation was able to produce small round beads on the surface in such a way as to protect bacterial cells [27]. The emulsion method used can protect bacterial cells from an unfavourable environment to increase cell survival. Small microcapsules can trap cells better when compared to larger ones. The results of the experiment on T3 showed that the capsule was spherical, had a smooth surface gel structure and shrunk (Figure 1). Further observations using a microscope showed that the microcapsules were spherical to protect bacteria from environmental conditions as shown in Figure 2. The rough surface of the microcapsules indicated the presence of probiotics in the capsules [28]. Generally, wrinkling is the result of mechanical stress caused by the non-uniform drying of various parts of the liquid droplet in the early stages of drying. The high molecular weight polymer dries quickly to prevent internal vapour release, resulting in increased bubble formation in the material wall matrix, expanding the internal space of the microcapsule, and creating more hollows. Gelatinization kinetics can create a capsule-like structure on the alginate particles, determined by the concentration and rate of penetration of $Ca^{2+,}$ the structure and concentration of alginate, and the presence of Na^{+} that prevent alginate gelatinization. The presence of Ca^{2+} and Na^{+} also encourages the formation of a homogeneous gel. Inside the microcapsule consists of a semi-porous network. Alginate porosity is important in keeping bacteria alive as they pass through the digestive tract [4].

4. CONCLUSION

Based on the results of the research conducted, it can be concluded that the microcapsules of *Lactobacillus acidophilus* FNCC 0051 and *Streptococcus thermophilus* FNCC 0040 were produced through the emulsion technique using gelatin and sodium alginate coatings as microcapsules. The best treatment was obtained at T3 (1:3) with an encapsulation efficiency value of 99.00%, resulting in good particle size, particle distribution and morphological structure.

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COMPETING INTERESTS

Aouthors have declaret that no competing interests exist.

AUTHORS' CONTRIBUTION

Author, RDN wrote the draft of manuscript and performed the statistical analysis. Author MES and AM designed the study, managed the analysis study and the literature searches. All authors read and approved the final manuscript.

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