Carrageenan : the difference between PNG and KCL gel precipitation method as *Lactobacillus acidophilus* encapsulation material

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**Abstract.** The study on the effects of using materials and methods in the preparation of the microcapsules *Lactobacillus acidophilus* towards the viability has been done. The research method used is experimental laboratory design. Variable research was kind of material (A) as the first factor with sub factor (A1 = *Eucheuma cottonii*) (A2 = *Eucheuma spinosum*) (A3 = mixture of *Eucheuma cottonii* and *Eucheuma spinosum* 1:1 ratio), while the second factor is a method of extraction to produce caragenan (B) with sub factor (B1 = Philippine Natural Grade modification) (B2 = KCl gel Press Precipitation). Analysis of different influences uses Analysis Of Varians followed by Fisher's test. Analysis of data uses Mini tab 16. The results shows that the kind of extraction factors and methods gave significantly different effects on the viability of *Lactobacillus acidophilus*. The highest mean of Viablity obtained in the treatment of materials with a mixture of *Eucheuma cottonii* and *Eucheuma spinosum* and used KCl Gel Press method is equal to 7.14 log (CFU / mL). It is suggested using of kappa-iota carrageenan mixture as encapsulation material with KCl Gel Press method on *Lactobacillus acidophilus* microencapsulation process because it treatment gave the highest average of *Lactobacillus acidophilus* viability.

1. Introduction

Microencapsulation is one of the means commonly used to protect probiotic bacteria from the pressure of external conditions such as temperatures and pH. *Lactobacillus acidophilus* is a probiotic bacterium that gives health benefits. Microencapsulation process is influenced by core material, shell material and method. One of the groups for encapsulation materials originating from hydrocolloid is carrageenan [1]. Carrageenan is an extraction of red hydrocolloid such as *Eucheuma* sp., *Chondrus* sp., *Gigartina* sp. and Fucellaran. *Eucheuma* sp. commonly found in Indonesia is *Eucheuma cottonii* and *Eucheuma spinosum*. *Eucheuma cottonii* produces kappa carrageenan, while *Eucheuma spinosum* produces iota carrageenan in different extraction condition. Extraction condition difference uses chemical substances and the methods. Several extraction methods commonly used in producing carrageenan are the Philippine Natural Grade (PNG) and KCl gel Press methods producing Semi Refine Carrageenan (SRC), whereas alcohol precipitation method produced carrageenan in the form of Refine carrageenan (RC). The differences in the use of methods in the production of carrageenan result in different qualities in terms of gel strength and
viscosity. Gel strength and viscosity can affect the formation of gel beads and stability in the production of microencapsulation. Moreover, both types of materials have different properties. *Eucheuma spinosum* produces iota carrageenan with soft gel properties and elastic. Meanwhile, the gel properties of kappa carrageenan are strong, sturdy, but easy syneresis. The mixture of Kappa carrageenan and *Locust Bean Gum* will provide cohesive elastic gel texture. These properties are similar to the mixture of kappa and iota carrageenan [2].

Carrageenan used as the encapsulation material using emulsification method produces microcapsules in a semi-solid form [3, 4]. Probiotics encapsulated by carrageenan and xanthan gum are similar to alginate, having the ability to protect the viability of probiotics compared to no encapsulation on a condition of pH and a high concentration of bile salts [5, 6]. In another research, K-carrageenan material was used to encapsulate probiotic through emulsification methods [3,7] and the application of the product cheddar cheese [7]. However, probiotic encapsulation using biopolymer materials such as calcium alginate, k-carrageenan, and gellan gum directly is still applicable in a laboratory scale [8]. The k-carrageenan microencapsulation technique using emulsification method is easier in determining the scale-up for larger productions [9]. The use of probiotic of *Lactobacillus bulgaricus* using K-carrageenan concentration of 3% and soybean oil as the continuous phase does not need special treatments and can be applied for mass production used 2% k-carrageenan in the process of microencapsulation *Bifidobacterium longum* with vegetable oil as the continuous phase, given no special treatment, and applied to set yogurt [10].

Meanwhile, the microencapsulation of bacterium *Bifidobacterium longum* uses k-carrageenan as the material with concentration of 2%, vegetable oil as a continuous phase, given no special treatment, while further applications were on stirred yogurt [4]. Moreover, research on the use of encapsulation materials such as *Eucheuma cottonii* and *Eucheuma spinosum* as well as the mixture of *Eucheuma cottonii* and *Eucheuma spinosum* in the forms of kappa, iota and kappa-iota carrageenan mixture from the methods of different extraction towards the viability of *Lactobacillus acidophilus* has not been done yet.

2. Methodology

2.1. Research materials

*Eucheuma cottonii, Eucheuma spinosum*, water, KOH, Ca(OH)$_2$, KCL, plastics, rubber, distilled water, filter cloth), culture of *Lactobacillus acidophilus*, MRS Agar, MRS broth, saline, alcohol, NaFis, KCl3M, NaCl, distilled water, corn oil, tween 80. Autoclave, water bath, incubator, vacuum dryer, homogenizer, centrifuge, microbiology testing tools

2.2. Stages of Research are an experimental laboratory study

The stages of the research are as follows:

1. The making of carrageenan using PNG method with *Eucheuma cottonii* and *Eucheuma spinosum* as materials was done through the following steps: seaweed *Eucheuma cottonii* and *Eucheuma spinosum* were weighed, and then washed, and extracted using an alkaline solution KOH 6% for *Eucheuma cottonii* and Ca(OH)$_2$ for *Eucheuma spinosum*. After 2 hours, then, they were neutralized to pH 6-8, after that, cut, dried, and pulverized to 80 mesh.

2. The making of carrageenan using KCL Gel Press Precipitation method with *Eucheuma cottonii* and *Eucheuma spinosum* as materials was conducted through the following steps: seaweed *Eucheuma cottonii* and *Eucheuma spinosum* were weighed, washed and put in the extraction solution using KOH 6% for *Eucheuma cottonii* and Ca(OH)$_2$ for *Eucheuma spinosum* at a temperature of 85-90 °C for 30 minutes. After that, the materials were pulverized using a blender. Later, hot water was added with 1:10 composition (comparison of seaweed and hot water). HCl was then added to neutralize the pH. Besides, 1.5% KCl was also added into the filtrate. After 2 hours of extraction, the paste was filtered using a filter cloth. Then, it was frozen, dried and finally made into flour.
3. The making of microcapsules of *Lactobacillus acidophilus* was done using the emulsification method which Adhikari [4] has been modified Setijawati [11] through the preparatory steps of the carrageenan materials, kappa, iota, a mixture of kappa-iota (1: 1) at a concentration of 3 % (w / v). Then, the materials were heated at a temperature of 95 °C for 5-6 minutes and thoroughly mixed until homogeneous. The culture of *Lactobacillus acidophilus* were prepared at $10^9$ CFU / mL of 10 ml. After that, the temperature of the carrageenan in the sol condition was lowered to 42 °C. After reaching the temperature, 10 ml culture of *Lactobacillus acidophilus* was mixed with sol carrageenan. The second step was to prepare 50 mL vegetable oil as emulsifier, heated on a hotplate stirrer to a temperature of 40 °C for 2-3 minutes. Then, the mixture of cells and carrageenan was added to the oil, stirred for 10 minutes using a speed of 250 rpm, later added a solution of KCl 3.9 M. After the oil phase was removed from the mixture, the microcapsules were harvested centrifugally. Furthermore, the microcapsules were washed using a solution of KCl 3.9M twice. The microcapsules then were stored at a temperature of 4 °C in semi-solid form. After that, the viability of *Lactobacillus acidophilus* microcapsules was analyzed.

2.3. Variables of Treatment

A1 = *Eucheuma cottonii*; B1 = the making process of carrageenan using PNG method
A2 = *Eucheuma spinosum*; B2 = the making process of carrageenan using Gel KCl Press
A3 = mixture of *Eucheuma cottonii* and *Eucheuma spinosum*

2.4. Data Analysis

To measure the quality of carrageenan, some tests were conducted, such as the test of gel strength, viscosity, water content, FTIR, gelling point, and melting point. Meanwhile, the viability of *Lactobacillus acidophilus* was tested using the pour method (pour plate) with MRSA media. The effect of treatment used ANOVA factorial, and the difference test used Fisher's test. Moreover, the analysis of data used Minitab software 16.

3. Result and Discussion

3.1. The materials characteristics of functional groups kappa, iota carrageenan using FTIR

One of the ways to identify the quality of carrageenan is through differences in the characteristics of functional groups. The characteristics of functional groups of carrageenan *Eucheuma Cottonii* (*Kappaphyzus zalvarezi*) producing kappa caragenan and *Eucheuma spinosum* producing iota caragenan using FTIR can be seen in figure 1 and figure 2.
Figure 1. The characteristics of functional groups of Carrageenan species *Eucheuma cottonii* using FTIR.

Figure 1 shows that the functional groups results of the analysis respectively sulfate ester functional groups at the wave number of 1243 cm\(^{-1}\), glycosidic bond at 977-1000.47 cm\(^{-1}\), anhydrogalaktosa at 960 cm\(^{-1}\), galactose sulfate at 848 cm\(^{-1}\), and galactose 2 sulfate at 800-805 cm\(^{-1}\). The absorption intensity indicated by sulfate ester glycosidic bond and galactose is very strong. In the fingerprint region, the absorption is sharp and broad at a wavenumber of 1210-1260 cm\(^{-1}\). It is also showed by other functional groups.

Figure 2. The characteristics the functional groups of carrageenan species *Eucheuma spinosum* producing iota caragenan using FTIR.
Figure 2 shows that the functional groups results of the analysis respectively sulfate ester functional groups at the wave number of 1180-1241.11 cm\(^{-1}\) glycosidic bond at 1033.77 - 1072.23 cm\(^{-1}\) anhydrogalaktosa at 930.9 - 970.13 cm\(^{-1}\) galactose sulfate at 846.56 cm\(^{-1}\) and galactose 2 sulfate at 804.25 - 849.56 cm\(^{-1}\). The absorption intensity indicated by sulfate ester glycosidic bond and galactose is very strong. This is supported by the results of testing IR on functional groups and testing on *Eucheuma cottonii* producing kappa carrageenan and *Eucheuma spinosum* producing iota caragenan standards using Sigma product. Sulfate ester functional group is at the wave number of 1210-1260 cm\(^{-1}\) glycosidic bond at 1010-1080 cm\(^{-1}\) anhydrogalaktosa at 928-933 cm\(^{-1}\) galactose sulfate at 840-850 cm\(^{-1}\) and galactose 2 sulfate at 800-805 cm\(^{-1}\). The absorption intensity indicated by sulfate ester glycosidic bond and galactose is very strong. In the fingerprint region, the absorption is sharp and broad at a wavenumber of 1210-1260 cm\(^{-1}\).

Similarly, it is showed by other functional groups. The functional groups using standard caragenan using Sigma products are shown in Table 2.

Table 1. The wave number of carrageenan polysaccharide functional group in spectroscopy FTIR 8108 Shimadzu.

| Sample               | Ester sulfate | Glycoside bond | 3,6 anhydride D-galactan | D-galactan -4 SO4 | D-galactan -2 SO4 | D-gal-6S | 3,6-anhydro galactan-2SO4 |
|----------------------|---------------|----------------|--------------------------|-------------------|-----------------|---------|--------------------------|
| Kappa Carrageenan-   | 1261.8        | 1068.7         | 929.8                    | 844.9             | -               | -       | 802.5                    |
| (standard)*          |               |                |                          |                   |                 |         |                          |
| Iota Carrageenan     | 1260          | 1072.6         | 931.7                    | 848.8             | -               | -       | 804.4                    |
| (standard)*          |               |                |                          |                   |                 |         |                          |
| Kappa caragenan      | 1258          | 1070           | 937.7                    | 847.7             | -               | -       | -                        |
| *                    |               |                |                          |                   |                 |         |                          |
| Iota caragenan**     | 1241.1-1262.32| 1010-1080      | 930.59                   | 930.59            | -               | -       | 804.26                   |

: *[12]**Research Result

One of the ways to demonstrate the differences of carrageenan and its type is by infrared spectroscopy, which will show caragenan functional groups and the intensity of absorption at certain wavenumbers. The absorption intensity of glycosidic bonds and ester sulfate is very strong in all three types of carrageenan. The absorption intensity shown by 3,6-anhydrogalactose is very strong on carrageenan kappa type, while carrageenan iota type is very weak, and the type of carrageenan lambda is completely none. In addition, the importance of determining these types relates to the use of carrageenan in various industries. Carrageenan with different types provides different physical properties, especially viscosity and gel strength, which in turn affect the applications in food, beverages and other industries [13].
3.2. The physicochemical quality of encapsulation material
The results of the analysis of the physicochemical quality identification in carrageenan-making process using PNG and Gel KCl Press methods of seaweed species *Eucheuma cottonii* and *Eucheuma spinosum* can be seen in Table 2.

Table 2. The physicochemical quality of kappa, iota, kappa-iota carrageenan with different methods of extraction.

| Quality       | Eucheuma cottonii PNG | Eucheuma cottonii KCL Gel Press | Eucheuma spinosum PNG | Eucheuma spinosum KCL Gel Press | Mixture of Eucheuma cottonii and Eucheuma spinosum (1:1) PNG | Mixture of Eucheuma cottonii and Eucheuma spinosum (1:1) KCL Gel Press |
|---------------|-----------------------|---------------------------------|-----------------------|---------------------------------|---------------------------------------------------------------|---------------------------------------------------------------------|
| Water content (%) | 10.64                 | 9.61                            | 11.72                | 11.04                           | 10.67                                                         | 10.54                                                               |
| Ash (%)       | 1.27                  | 1.37                            | 1.33                 | 1.6                             | 1.4                                                           | 1.2                                                                |
| Sulfate (%)   | 24.2                  | 21.71                           | 28                   | 25.77                           | 24.5                                                          | 24.2                                                               |
| Gel strength (N) | 1.06                  | 1.18                            | 0.6                  | 0.9                             | 0.94                                                          | 0.98                                                               |
| Gelling point (°C) | 25.2                  | 30.21                           | 23.8                 | 25.5                            | 25.5                                                          | 32.0                                                               |
| Melting point (°C) | 80.2                  | 76.35                           | 80.3                 | 80                              | 76                                                           | 79                                                                |
| Viscosity (mPas) | 225                   | 245                             | 180                  | 210                             | 224                                                          | 189                                                               |

3.3. The different effect of the treatments to the encapsulation materials and methods of extraction on the viability of *Lactobacillus acidophilus*
The result of analysis of variance shows that the treatment of materials, methods of extraction and their interactions significantly give different effects on the viability (P ≤ 0.05) with R-Sq = 98.23% R-Sq (adj) = 97.49%.

![Figure 3. The effects of the treatments to the materials and methods of extraction toward the *Lactobacillus acidophilus* viability.](image-url)
Figure 3 shows that the treatments of encapsulation materials with *Eucheuma cottonii* producing kappa-carrageenan and *Eucheuma spinosum* producing iota-carrageenan are different but the treatments of encapsulation materials with the mixture of *Eucheuma cottonii* and *Eucheuma spinosum* producing kappa-iota-carrageenan are similar. The treatments using the mixture of *Eucheuma cottonii* and *Eucheuma spinosum* evenly give *L. acidophilus* higher viability than *Eucheuma cottonii* and *Eucheuma spinosum*. Meanwhile, PNG and Gel KCl Press precipitation process are different on the viability of *L. acidophilus*. The treatments using KCl gel Press precipitation process evenly give *L. acidophilus* higher viability than the PNG processing method. The effect of treatments of the interaction between the materials and methods of extraction provides significantly different effects on the average viability, as can be seen in figure 4.

Figure 3 and 4 show that the encapsulation material of kappa caragelan-iotacaragelan using KCl Gel Press precipitation process gives the highest viability. This is due to the mixture of kappa-iota using KCl Gel Press precipitation, a mixture of materials having elastic, cohesive and not syneresis properties. Therefore, it can affect the formation of microcapsules matrix to improve the viability. *Eucheuma cottonii*, on average, has a higher gel strength, a lower sulphate content, and a higher gelling point than *Eucheuma spinosum*. The species of seaweed and carrageenan methods of extraction process differences are factors that affect the quality observed from the strength of the gel, and the sulfate content of the gelling point or melting point. They are also related to the use of temperature and type of cations as extraction materials. KCl Gel Press precipitation process method produces a higher gel strength, a lower sulphate content, and a higher gelling point than PNG process of extraction method. This is related to the content of 3.6-AG and sulphate content. PNG extraction process uses the extraction process at temperatures of 80°C to 82°C, whereas KCL Gel Press extraction process uses the extraction process at temperatures of 85°C to 90°C. Therefore, the closer the optimum process with the temperature, the more perfect the termination of sulfate ester group into cluster 3.6 -AG will be. The existence of group 3.6 - AG can affect the strength of the gel. The gel strength of Carrageenan is noted as *breaking force* defined as the maximum load required for breaking the polymer matrix in the burdened areas. Meanwhile, carrageenan gel formation is a process which involves the deposition of ionic bonds between metal cation with negative charge from sulfate ester.
If the sulfate ester group is a lot more than the sulfate, the sulfate will bind with water. A higher sulfuric content in carrageenan causing three-dimensional structures formed absorbs more water. Thus, it reduces the gelation strength. Ester sulfate is "hydrophilic" while 3.6 AG is "hydrophobic". Besides, the species *Eucheuma cottonii* and *Eucheuma spinosum* have differences on the formation of double helix and single helix structure. So, it affects the formation of aggregation. The aggregation formation of kappa carrageenan leads to the formation of the matrix layer thicker than iota carrageenan. It is also supported by Chandrasekaran [14] that describes double helix structure and single helix structure on kappa and iota carrageenan through X-ray diffraction, as shown in figure 5.

Figure 5 shows that kappa carrageenan molecule projection shows a disorganized and denser molecular structure compared to the structure of iota carrageenan molecules. Kappa carrageenan has molecular bonds that are fragile and easily broken [14]. The presence of K⁺ that is needed by kappa caragenan in breaking sulfate ester group into cluster 3.6 AG generates gel properties that are strong, rigid, and syneresis. On the other hand, the presence of Ca²⁺ needed by iota carrageenan produces gel properties that are weak, elastic and not syneresis. So, the mixture of *Eucheuma cottonii* and *Eucheuma spinosum* producing kappa-iota carageenan have such gel properties as strong, elastic and no syneresis. In line with the description from Phillip [2] and Chandrasekaratan [14], it is presumably that the differences on the gel properties can affect the viability of *Lactobacillus acidophilus*, because the structure of microcapsules matrix formation and stability of matrix are also affected by the gel properties. The structure of microcapsules matrix formation and stability of matrix will protect the *L. acidophilus* from external conditions so that the viability is improved. It is supported by Setijawati [11] that the mixture of kappa caragenan and iota caragenan from *Eucheuma cottonii* and Eucheuma spinosum gave the highest viability. Species and concentration factors gave different influences toward *Lactobacillus acidophilus* viability through simulation of GI Tract pH condition. The best result was mix kappa-iota *carrageenan* (75:25), 6 % concentration with the highest viability average 6.1097 cfu/ml (log).

4. Conclusion
The encapsulation materials with process extraction methods give different effects on the viability of *L. acidophilus*. The highest average *L. acidophilus* viability was obtained from the treatment of the mixture of *Eucheuma cottonii* and *Eucheuma spinosum* (1:1) producing kappa-iota caragenan with KCl Gel Press precipitation process method of 7.14 log (CFU/mL).
5. References

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