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Carrageenan from *Eucheuma sp* and concentration difference as encapsulation material toward *Lactobacillus acidophilus* viability at simulation *GI Tract* pH condition

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ABSTRACT

Eucheuma cottonii species produce kappa and *Eucheuma spinosum* species produce *iota carrageenan*. Kappa gave strong, rigid, syneresis, but Iota soft, elastic and no syneresis gel properties. Mixture both of them material improved gel properties. So usage of them as encapsulation material influenced *Lactobacillus acidophilus* viability. Aimed of this research was study of influence and find the best treatment from *carrageenan* as encapsulation material with species and concentration difference toward *Lactobacillus acidophilus* viability in simulation *GI Tract* pH condition. The method was experimental laboratory design. Material was: 1) *carrageenan* from species *Eucheuma cottonii* and *Eucheuma spinosum* harvested by Lombok Island in 42 days; 2) *Lactobacillus acidophilus* microcapsule, RAL factorial design, data analysed by Minitab 16 software. Treatment was A1=kappa; A2=iota; A3 = kappa: iota (75:25); A4 = kappa: iota (50:50); B1=4,5%; B2=6%. Enumeration viability used MRS agar pour plate method. The result was species and concentration gave different influence toward *Lactobacillus acidophilus* viability through simulation *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). The standard of benefit value for health was 10^7 cfu/ml viability. Suggestion usage mix kappa-iota *carrageenan* as encapsulation material and improved *Lactobacillus acidophilus* viability, but must to increased it's concentration to fulfill the standard of benefit value for health.

KEYWORDS: *Eucheuma cottonii, Eucheuma spinosum*, microencapsulation, emulsification

INTRODUCTION

Polysaccharides derived from the ocean as *Eucheuma sp* has been used extensively in the food industry and medicine, because it is biocompatibility and low toxicity properties [1][2]. *Eucheuma sp* extraction using a particular method is called with *carrageenan*. *Carrageenan* is one hydrocolloid which can be used by an encapsulation material in the process of microencapsulation [3]. Probiotic microencapsulation technology needed encapsulation material in order to protect the probiotic bacteria from the pressure of external conditions, especially gastrointestinal pH conditions. Immobilization of probiotic bacteria using microencapsulation process has been shown to increase the resistance of microorganisms on the product during processing, storage, and passage pH conditions of the gastrointestinal tract or *GI tract* pH [4][5][6][7][8][9]. Encapsulation materials testing in protecting the ability of probiotics as it reaches the colon or large intestine is a condition in its use. Viability testing can be done by in vitro and in vivo. Encapsulation materials testing performed in vitro requires the use of a simulated solution with pH corresponding to the conditions of the gastrointestinal tract or *GI tract* called the pH simulation. According to Kos *et al.* [10] states that the solution of this simulation is a solution that has a pH 2 and pH 7 with the addition of pepsin and bile salts. *Lactobacillus acidophilus* is very rapid loss of viability, when through the pH *GI Tract* and bile salt conditions [11][12][13][14][15]. The type of encapsulation material and concentration are important factors in microencapsulation process. Encapsulation material properties will determine its ability as a protective *Lactobacillus acidophilus* [16]. Study of the use encapsulation probiotics using emulsification method has been carried out on kappa-*carrageenan* material [17][18][19]. The use of emulsification method based on the ability to form gels and solids and viscosity of encapsulation materials, and temperature resistance of *Lactobacillus acidophilus.*

Carrageenan as encapsulation material from the species of *Eucheuma spinosum* and *Eucheuma cottonii* make a difference of gel properties like gel strength and viscosity. *Eucheuma cottonii* produce *carrageenan* kappa-type, whereas *Eucheuma spinosum* producing iota-type *carrageenan*. Kappa-*carrageenan* gel is strong, stiff and easily cracked, high viscosity, and need potassium supporting to improve the gel properties. While Iota-*carrageenan* gel is soft and elastic, low viscosity, and need Calcium supporting to improve the gel properties [20]. Klein and Vorlop [21] states that the Kappa-*carrageenan* is a neutral polysaccharide with a concentration of 2 to 5% that require high temperature 60°C to 90^o C for solubility, if used in the microencapsulation process. According to Audet *et al.* [22]; Arnauld [23] states that a mixture of kappa-*carrageenan* and *Locust Bean Gum* been widely used as encapsulation in probiotic fermentation

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products. Gel formation in a mixture of kappa-*carrageenan* and *Locust Bean Gum* dependent on calcium ions that will directly influence on the viability of *Bifidobacterium spp* [24][25][26] reported that the ratio between *Locust Bean Gum* and *carrageenan* ratio 1:2 gave a strong gel for microencapsulation of product. *Locust bean gum* has the characteristic gel properties similar to iota- *carrageenan* which produced *Eucheuma spinosum* [27]. The use of *Eucheuma cottonii* in the form of kappa-*carrageenan* with a concentration of 3% can be used as *Lactobacillus sp* encapsulation [18]. Ding and Shah [28] reported that several kinds of encapsulation material, especially xanthan gum and *carrageenan* can provide more effective protection to the probiotic compared by alginate from extreme environmental conditions. For extreme environmental conditions can affect the viability of *Lactobacillus acidophilus. Lactobacillus acidophilus* needs potassium to supporting of their viability. Differences and improvements gel properties can affect the formation and structure of microcapsules in order to maintain viability of *Lactobacillus acidophilus*. Expected mixture of kappa and iota-*carrageenan* can improve the gel properties, which can affect the formation of the microcapsule matrix and can maintain the viability of *Lactobacillus acidophilus* after passage through the *GI tract* pH simulation condition. Indicator of success in the microencapsulation process is to maintain the viability of probiotic bacteria in accordance with value benefit for health standards in the range 10^7 to 10^8 cfu / ml after passage through the *GI tract* pH simulation (International Dairy Federation, 1988). Meanwhile, according to Kaillasapathy [29] stated that the viability of probiotics in a product is an important factor in view the value of benefits. Consumption of probiotics in food products that provide benefits equivalent to the value 10^6 to 10^7 cfu/ml.

From the above description can be said that the use of kappa-*carrageenan* as an encapsulation material in the process of probiotics microencapsulation, especially *Lactobacillus acidophilus* has been done, while the use of iota-*carrageenan* and a mixture of kappa-and iota-*carrageenan* as encapsulation material in microencapsulation process has not been done. So that research the use of a mixture of kappa and iota *carrageenan* as an encapsulation material in the process of *Lactobacillus acidophilus* microencapsulation is needed. The purpose of this research was to study and analyse difference factor of *Eucheuma cottonii*, *Eucheuma spinosum*, mixture of *Eucheuma cottonii* and *Eucheuma spinosum* as encapsulation material and it's concentration toward *Lactobacillus acidophilus* viability after passage through the pH of the *GI tract* simulation. The last purpose determined the best treatment result.

MATERIAL AND METHOD

Experimental design

The research is experimental laboratory design. The study was compiled by using Complete Randomized Design (CRD) factorial, the data were analysed using ANOVA to determine the different influence, followed by the F test to see differences between treatments. All data analysed by Minitab16 software.

CRD factorial experimental design

Experimental design based on proceeded commencement of the trials. This treatment were : 1) the first factor is *carrageenan* as the encapsulation material with difference species as the sub factor (A) with 4 levels of treatment are: *Eucheuma cottonii* (A1); *Eucheuma spinosum* (A2); mixture of *Eucheuma cottonii and Eucheuma spinosum* in 75:25 ratio (A3); mixture of *Eucheuma cottonii* and *Eucheuma spinosum* in 50:50 ratio (A4). The second factor is the concentration of encapsulation material (B) with two levels of treatment, namely: the concentration of 4.5% (B1); concentration of 6% (B2), all treatments with three replicates.

Microencapsulation of *Lactobacillus acidophilus* According to Experimental Design

Research material was *carrageenan* Gel Press method [30] using of species of seaweed *Eucheuma cottonii* and *Eucheuma spinosum* harvested from the Lombok Island and 42 days of age. *Culture conditions.* A pure culture of *Lactobacillus acidophilus* (strain ATCC 4356) was purchased from the Culture Collection and Research Centre (University of Gajah Mada, Indonesia). *Deman Rogosa and Sharp Agar* (MRSA) were used as the medium for *Lactobacillus acidophilus* (Merck,). Preparation of *Lactobacillus acidophilus* cultures [10] according the steps of dried starter cultures were grown in 10 ml sterile MRS broth medium. Incubated at 30^oC for 24 hours, then be stretched on MRS skew media sterile. Incubated at 30^oC for 24 hours, then grown in 10 ml sterile medium MRS broth. Incubated at 30^oC for 24 hours, calculated density of the number of bacteria by OD at λ= 620 nm, using spectrophotometry. Bacterial culture is ready for use.

Lactobacillus acidophilus microencapsulation

Lactobacillus acidophilus microcapsule, through the following procedure: 1) Preparation *carrageenan* of *Eucheuma cottonii* and *Eucheuma spinosum* species with Gel Press method [30] which modified by the steps: species of seaweed *Eucheuma spinosum* and *Eucheuma cottonii* dry, cleaned and washed. Then boiled in a $CaOH_2$ or KOH solution depend on kind of seaweed. Extraction result was filtered and the pH extraction is

lowered by the addition of 0.2 N HCl, 15 minutes before end of the extraction, added by 1.5% KCl solution. The extraction results freeze, then dried, grinded, powder form. Then analyzed of functional group; 2) Preparation of *Lactobacillus acidophilus* microcapsules with emulsification method [19] which modified by the following steps: *carrageenan* of *Eucheuma cottonii* and *Eucheuma spinosum* species, dissolved in water with a concentration in accordance the treatment, then heated at a temperature of 96^{o}C for 5-6 minutes, after which the position of the temperature up to 42^{o}C derived sol was added 10 ml of *Lactobacillus acidophilus* cell suspension at a density of 10^{9} cfu / ml, the mixture is called a Phase Dispersion (DP), the next step was prepared 100 ml of vegetable oil and emulsifier (tween 80), stirred on hotplate with stirrer, heated at a temperature of 42^{o}C, the mixture called a Phase Continue (CP), DP mixture is poured into the CP, then stirred for 10 minutes at 250 rpm, added 75 ml of KCl (3.5 M), the oil phase is removed from the mixture, the microcapsules harvested by centrifugation for 10 minutes, and then the microcapsules washed by 3 M KCl with 2 times repetition, the microcapsules ready to analysed; 4) *Lactobacillus acidophilus* microcapsules encapsulation by *carrageenan* from *Eucheuma cottonii* and *Eucheuma spinosum* species each treatment soaked in the pH 2 *GI tract* simulated solution for 2 h and a of pH 7 *GI tract* simulated solution for 4 hours, pH 2 and pH 7 *GI tract* simulated solution for 6 hours. Effect treatments were analysed by the *Lactobacillus acidophilus* viability and sought the best treatment.

RESULT AND DISCUSSION

The use of infrared spectroscopy (IR) aimed to identifying the presence of functional groups and to distinguishing each *carrageenan* type. Functional groups characteristic of *carrageenan* from *Eucheuma Cottonii* (*kappaphyzuz alvarezii*) and *Eucheuma spinosum* species and mixture of *Eucheuma Cottonii* (*kappaphyzuz alvarezii*) and *Eucheuma spinosum* analysed by FTIR, can be seen in Figure 1, Figure 2 and Figure 3.

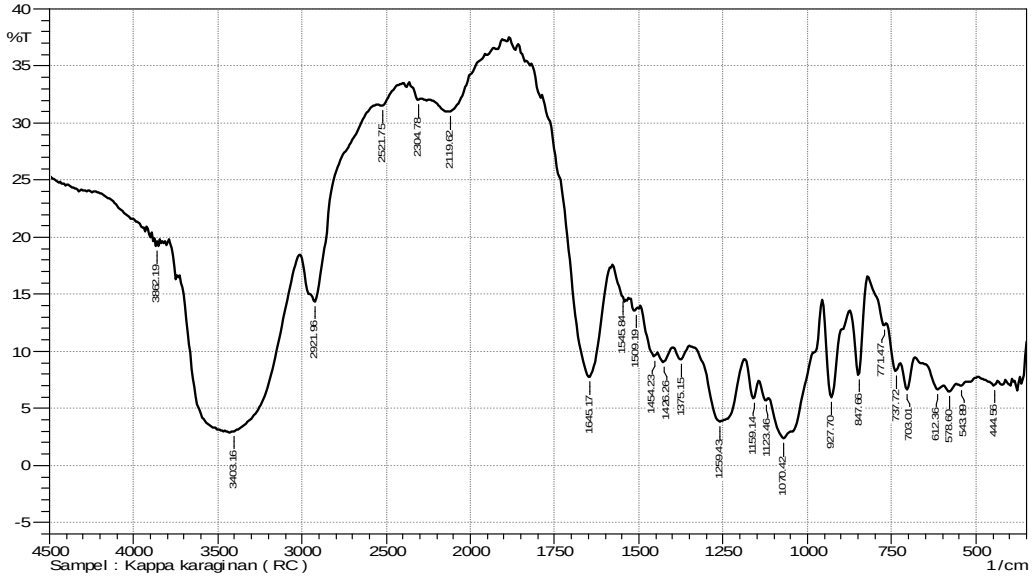

Figure 1 Characteristic functional groups *Carrageenan* of *Eucheuma cottonii* species using FTIR

Figure 1, show that functional group analysis is a sulphate ester functional groups at the absorption wavenumber 1258 cm^{-1}, a glycoside bond at 1070 cm^{-1}, anhydro-galactose (AG) at 937.7 cm^{-1}, galactose sulphate at 847.7 cm^{-1}. Absorption intensity shown by the ester sulphate and galactose glycoside bond is very strong. In the fingerprint region are sharp and broad absorption at wave number 1210-1260cm^{-1}. It is similar as the other functional groups.

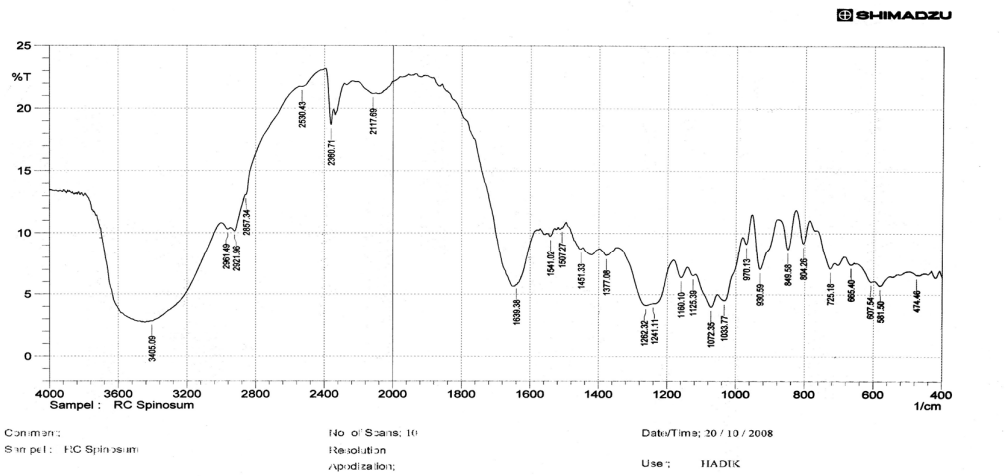

Figure 2 Characteristic functional groups *Carrageenan* of *Eucheuma spinosum* species using FTIR

Figure 2 of the analysis on the functional groups of *Eucheuma spinosum* species results is sulphate ester functional groups at the absorption wave number 1241.11 to 1262.32 cm^{-1}, a glycoside bond at 1033.77 to 1072.23 cm^{-1}, anhydro-*galactose* (AG) at 930.59 cm^{-1}, galactose sulphate at 930.59 cm^{-1}, and 2 galactose sulphate at 804.26 cm^{-1}. Absorption intensity shown by the ester sulphate, galactose, and glycoside bond is very strong. *Eucheuma cottonii* contain kappa *carrageenan* composed of (1.3)-D-galactose-4-sulphate and (1.4) -3.6-Anhydro-D-galactose. While *Eucheuma spinosum* containing iota-*carrageenan* composed of D-galactose-4-sulphate and3.6-anhydro-D_galactosa-2-sulphate.

This is supported by the results of testing of IR on *carrageenan* species *Eucheuma spinosum* and *Eucheuma cottonii* standards using Sigma products. Sulphate ester functional groups at the wavenumber 1210-1260 cm^{-1}, a glycoside bond at 1010-1080 cm^{-1}, Anhydro-*galactose* (AG) in the 928-933 cm^{-1}, galactose sulphate at 840-850 cm^{-1}, and sulphate at galactose 2 in 800-805 cm^{-1}. Absorption intensity shown by the ester sulphate, galactose and glycoside bond is very strong. Functional groups using standard *carrageenan* Sigma products are shown in Table 1[31].

Table 1 Wavenumber of functional groups on the polysaccharide *carrageenan* standards using FTIR spectroscopy Shimadzu 8108

	wave number of Functional group (cm-1)						
Sample	**Sulphate Ester**	**Glycoside bond**	**3,6 Anhydro-galactose**	**D-galactose -4-**	**D-galactose-2 Sulphate**	**D-galactiose-6S**	**3,6-Anhydro-galactose -2 Sulphate**
***Carrageenan*-Kappa (standard)**	1261,8	1068,7	929,8	844,9	-	-	802,5
***Carrageenan* – iota (standard)**	1260	1072,6	931,7	848,8	-	-	804,4

Sources: Rahmat [31]

IR analysis of the functional groups will differentiate *carrageenan* types. The results observations of functional groups using IR and compared by standard products *carrageenan* Sigma stated that the materials used as research was kappa-and iota-*carrageenan*. *Carrageenan* functional groups relate gel properties.

Matsuhasi [32] stated that the characteristics of functional groups are characterized by a content of 3.6 Anhydro Galactose (3.6 AG) to *carrageenan* will make a difference in the quality of gel strength. While the presence of AG molecules showed increased flexibility in the polymer bonds, which would provide a greater contraction in the random coil structure [33]. This shows the double helix structure is important in the formation of three-dimensional network that will affect of gel properties. AG that it contains will result in the formation of gel properties with higher gel strength as it does in agar and *carrageenan* [34].

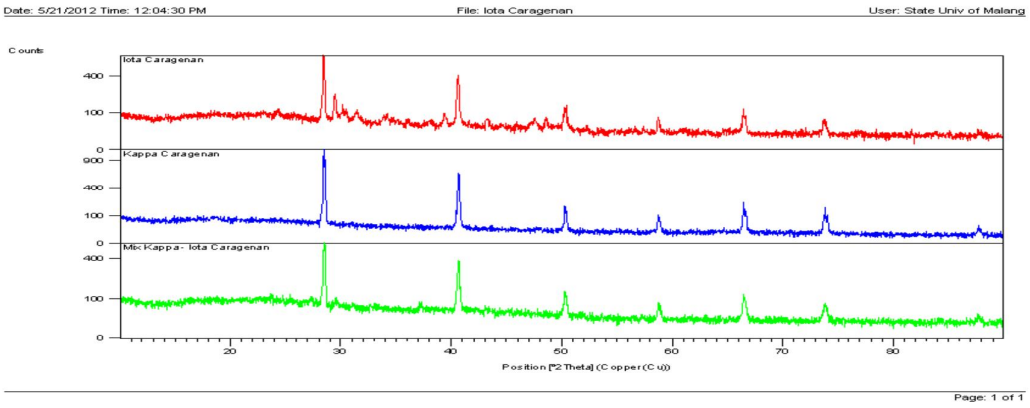

Figure 3 the test results are mixed *carrageenan Eucheuma spinosum* and *Eucheuma cottonii* using X-ray Diffraction

The results are shown as in Figure 3 that obtained from multiple observations of existing peaks; there is a common model of peaks showing the crystal structure of the molecule. Peaks reading that related by the cohesive properties of the two materials is shown by the emergence of peaks at the kappa-iota different from kappa and iota alone. In principle, the peaks show a tendency to resemble a large peak at kappa, although not as high as it. While the small peaks resemble some are owned by the iota-*carrageenan*, though not entirely the same. So the approach to the resulting of peaks which not exactly the same as the kappa, so that kappa-iota blend is said to be cohesive. The observation and analysis using X-ray diffracts amplified by using FTIR and analysed by looking at the frame structure and the similarity of existing functional groups.

Testing the viability of *Lactobacillus acidophilus* using Completely Randomized Factorial Design

Results of analysis using ANOVA are all treatment gave significan different influence, to see the different effects on all treatments are shown in Table 2.

Table 2.Effect of encapsulation materials and concentration at difference pH conditions treatment toward *Lactobacillus acidophilus* viability.

Treatment		**pH 2**	**pH7**	**pH 2 - pH 7**
Encapsulate material	Concentration (%)	mean ± St. Dev.	mean± St. dev.	mean± St. dev.
A1	B1	5.4781 ± 0.0707 ^{d}	4.4526 ± 0.1130 ^{cd}	4.5815 ± 0.0511 ^{cd}
A1	B2	5.7055 ± 0.1262 ^{cd}	5.7940 ± 0.0314 ^{d}	5.5743 ± 0.0463 ^{cd}
A2	B1	4.2590 ± 0.0750 ^{c}	3.8060 ± 0.4647 ^{c}	4.1467 ± 0.2102 ^{c}
A2	B2	4.9224 ± 0.6082 ^{c}	4.6968 ± 0.6382 ^{c}	4.9914 ± 0.6565^{c}
A3	B1	6.7044 ± 0.0201^{b}	6.3433 ± 0.5023 ^{ab}	6.0238 ± 0.3601^{ab}
A3	B2	7.0946 ± 0.5796 ^{ab}	6.8236 ± 0.0790 ^{ab}	6.5994 ± 0.0597^{ab}
A4	B1	5.6058 ± 0.1159^{a}	6.6032 ± 0.0897 ^{a}	6.1097 ± 0.1879^{a}
A4	B2	7.3343 ± 0.6160^{a}	7.2057 ± 0.5894^{a}	6.9958 ± 0.1563^{a}

Notification: * treatment with no common superscript differ ($p \leq 0,05$)
** S = 0,290038 R-Sq = 93, 90% R-Sq(adj) = 91,22%

From the analysis shown in Table 2 show that the effect of encapsulation material and concentration in different pH conditions treatment. The average viability of *Lactobacillus acdophilus* tends increases with increasing concentration in all the different pH conditions. While encapsulation materials containing *Eucheuma cottonii* gave viability of *Lactobacillus acidophilus* higher than encapsulation materials containing *Eucheuma spinosum*. Use of a mixture of *Eucheuma cottonii* and *Eucheuma spinosum* at the ratio 50:50 as encapsulation material at various pH conditions gave the highest *Lactobacillus acidophilus* viability.

Eucheuma cottonii differenced by *Eucheuma spinosum* in the formation of double-helical structure and the single helix, thereby affecting the formation of aggregation. Formation of the kappa-*carrageenan* aggregation is resulting in the formation matrix layer thicker than the iota-*carrageenan*. The existence k+ of the required kappa-*carrageenan* sulphate ester groups in the termination of a group nature of the gel. 3.6 AG produces strong, stiff, rigid or easily cracked and syneresis. While the presence of Ca^{2+} iota *carrageenan* necessary produce weak, elastic and no syneresis gel properties.

Chandrasekaran [35] describes that the structure of the double helix and single helix at kappa and iota *carrageenan* through X-ray diffraction showed that kappa-*carrageenan* molecular projection showing the molecular structure irregularity and more dense than on the molecular composition of iota-*carrageenan*. However, kappa-*carrageenan* have molecular bonds are fragile and easily broken.

The higher the concentration of encapsulation material will the resulting microcapsule wall thickness, so that the greater the ability of the microcapsules in maintaining the viability of *Lactobacillus acidophilus* from the pressure of the external pH. While an elastic gel has a lower gel strength values, so that it causes the gel so hard to maintain its shape when exposed to pressure. *Carrageenan* gel properties on *Eucheuma cottonii* has a higher gel strength than *Eucheuma spinosum*, which along with increased concentration. Nature of the gel on *Eucheuma cottoni*i is "strong gel", while *Eucheuma spinosum* "weak gel. Mixture of *Eucheuma spinosum* and *Eucheuma cottonii* can improve gel properties. In studying the framework of the network structure formed in a mixture of *carrageenan* species of *Eucheuma cottonii* (kappa-*carrageenan*) and *Eucheuma spinosum* (iota-*carrageenan*) using FTIR, showed that the analysis can provide an overview of the framework crosslinking structure is formed.

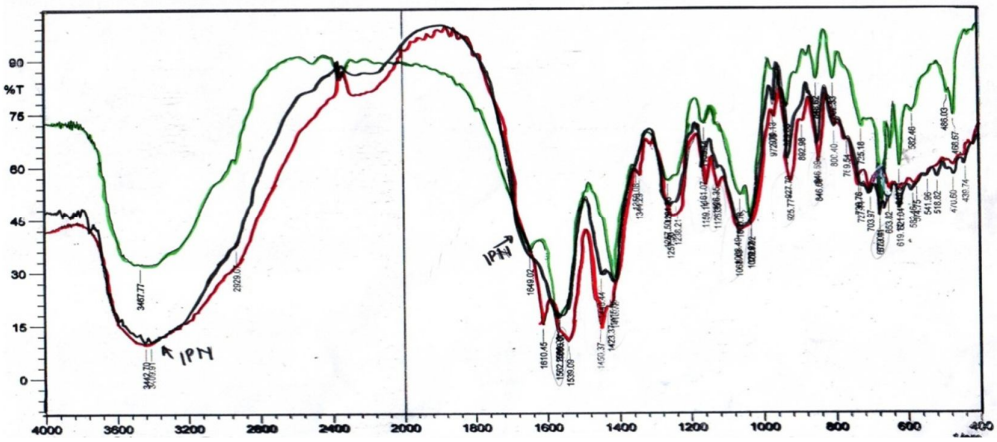

Figure 4 kappa, iota and mixture kappa-iota caragenan functional group using FTIR

Figure 4 indicate that usage mixture of kappa-iota *carrageenan* from *Eucheuma cottonii* and *Eucheuma spinosum* could make a crosslinking connecting; forming Interpenetrating Network (IPN), where kappa and iota make interpenetrate connecting between mixture of kappa-iota to entanglement formed making chain interlocking. The model entanglement each other like that inhibit auto-hydrolysis and syneresis when face of lower pH external. Increasing concentration of encapsulation material will increase of the closest IPN connection. The closest connection of IPN influences microcapsules beads matrix porosity.

Schlicher *et al*., [36] stated that increasing concentration encapsulation material will gave bigger gel strength and viscosity until certain limit than it is not. Micro particle porosity influence *Lactobacillus acidophilus* viability when passage external pH condition.

Encapsulated cell is influenced by species and concentration encapsulation material, beads diameter and bacterial species [37]. Many kinds of encapsulation material including *carrageenan* is showing good bacterial protection from *GI Tract* pH condition.

CONCLUSION

Carrageenan made of *Eucheuma cottonii* and *Eucheuma spinosum* species can be used as *Lactobacillus acidophilus* encapsulation material along with increasing it's concentration toward the viability. The best treatment was mixture of *Eucheuma cottonii* and *Eucheuma spinosum* at 6% concentration in 50:50 ratio with 6.9958 cfu/ml (log) viability after passage through pH2 followed pH7 *GI Tract* simulation solution.

SUGGESTION

It is recommended to use *carrageenan* a mixture of species between *Eucheuma cottonii* and *Eucheuma spinosum* at 50:50 ratio of 6% concentration although not yet to fulfil the standard of value benefit for health 10^7 Cfu / ml or 7 Cfu / ml (log), so that increasing concentration is further advice in this study.

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