Analysis of probiotic characteristics and LAB viability of seaweed hydrolysis (*Eucheuma cottonii*)

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**Abstract.** *Eucheuma cottonii* is one of the most utilised marine commodities for carrageenan production. Carrageenan can also be used for probiotics production. This study aimed to obtain bacterial growth media with the highest probiotic activity before and after drying. The study consisted of several stages, include: pre-hydrolysis, hydrolysis with inactive enzymes, chemical and microbiological characterisation, drying with spray drying, and lactic acid bacteria (LAB) viability test. The results show that the untreated sample had the best results compared to that of the filtering and precipitation samples. The untreated sample had reducing sugar content of 0.35%, total sugar 2.81%, pH 5.37, total acid 0.147%, and total LAB of 8.2x10⁸ CFU/mL. After drying with spray drying, the LAB viability reduced to 9.7x10⁶ CFU/mL.

1. Introduction
Seaweed in Indonesia is mainly exported in raw form, while the rest is processed into various foods such as agar, carrageenan, and alginate. *Eucheuma cottonii* is one of the most utilised seaweed for carrageenan production [1]. *Eucheuma cottonii* contains galactose, sulphate, and several carbohydrates, such as xylose, glucose, and uronic acid [2]. Oligosaccharides are carbohydrates, usually obtained by extracting natural ingredients, hydrolysing polysaccharides chemically, or chemical/enzymatic synthesis of disaccharides [3]. Galacto-oligosaccharides is one of the derivative products from oligosaccharides [4]. Galacto-oligosaccharides are classified as prebiotics because they stimulate the development of good bacteria in the intestine so that it is beneficial for health. Probiotic production from *E. cottonii* seaweed was carried out by hydrolysis using a live *Vibrio* inactive culture [5].

According to the previous research by Meliawati [4] and Zhu [6], carrageenan can be hydrolysed to GOS using the kappa-collagenase enzyme isolated from the microbe of *Vibrio alginolyticus*. The former study showed that enzymatic hydrolysis produces hydrolysates with a thick texture [5]. Therefore, this study used a combination of 3% sulfuric acid and inactive *Vibrio* culture. Inactive cultures have a low polymerisation degree because the sugar is easier to utilize for lactic acid bacteria (LAB) compared to active cultures [7].

The hydrolysed seaweed was in powder form to extend the shelf life and facilitate application. The drying process was performed by the spray drying method. Spray drying application is not limited for
food ingredients but also for bacterial cells to form capsules for cell protection from the extreme conditions [8]. The aim of the study was to obtain the hydrolysate with the best probiotic activity and to assess the ability of the dried-seaweed hydrolysate as a growth medium for probiotic bacteria.

2. Materials and Method

2.1. Materials
Dried *E. cottonii* was obtained from one of small and medium enterprises in Bogor. Pure culture of Lactic acid bacteria (LAB) was inoculated on to the broth and incubated at 37°C for 48 hours. The sediment cells were washed three times with 1% buffer peptone and used immediately in the experiment. Culture of *V. alginolyticus* was inoculated on to the broth and incubated at 37°C for 48 hours, harvested by centrifugation at 2500 rpm for 15 minutes, then sonicated at 4°C for 5 minutes to get an inactive culture.

2.2. Preparation and pre hydrolysis
Preliminary research consisted of raw materials preparation and the production of inactive cultures for the hydrolysis process. Fresh seaweed was soaked, rinsed, dried, chopped, and analysed. Analysis of chemical components consisted of water content, ash content, total fat, crude fibre, protein, and carbohydrates. *V. alginolyticus* marine microbes were utilized in the production of inactive cultures. *V. alginolyticus* was grown on carrageenan solid-media, inoculated into liquid starter media, centrifuged, and sonicated at 4°C [5].

2.3. Hydrolysis with acids and inactive cultures
Hydrolysis is aimed to convert cellulose into reducing sugars through the combination of 3% sulfuric acid and 10% inactive culture (v/v). The solution was then placed on a water bath at 30°C for 48 hours [5].

2.4. Chemical and microbiological characterisation
Hydrolysates from three types of treatment (filtration, precipitation, and without treatment). Filtration obtained from filtering seaweed hydrolysate with filter cloth. Precipitation obtained by precipitating hydrolysate with isopropyl alcohol, while the third treatment is given no treatment. All three treatments were tested for the hydrolysate content, including reducing sugars, total sugar, pH, and total acid. Microbiology examination was performed by calculating the total LAB. All tests were carried out before and after the fermentation. Furthermore, galactose content was analysed using HPLC. The best two treatments were selected based on their chemical and microbiological analysis, to be further analysed [4].

2.5. LAB viability test
The cell viability test of lactic acid bacteria was carried out after the spray drying process. This test was carried out on MRS agar media with a plate count method using several series of dilutions. About 1 mL of culture before drying and 0.1 g of dried culture were mixed, then diluted until 10-9 dilutions, 1 ml of the dilution was planted into sterile Petri dishes and poured with MRS media, mixed evenly, and then incubated at 37°C for 48 hours [7].

3. Results and Discussion

3.1. Comparison of the characteristics of hydrolysates as a result of further treatment before and after fermentation
The purpose of filtration and precipitation as further treatment was to obtain a dissolved phase and purified carrageenan and GOS. The following graph represents the results of reducing sugars, total sugar, pH, total acid, and total LAB of the three treatments (Figure 1-5).
3.1.1. Reducing sugar
Figure 2 shows there is a decrease in the reducing sugars due to the detection of simple sugars in the hydrolysis. This finding follows the statement of the former study that sugar levels tend to decrease during fermentation because the sugar contained in the media is utilised as a carbon source to synthesise energy [9]. Reducing sugar is utilised by probiotic bacteria as an energy source in the fermentation process. High reducing sugar is suspected due to the hydrolysing of complex sugars into simple sugars during fermentation.

Of the three types of treatment, hydrolysate treated with filtration and precipitation had higher reducing sugar. Since both treatments can separate glucose and other suspended materials (non-glucose organic matter), higher pure sugar can be obtained. During filtration, bacteria consume sugar mostly for the metabolic process, thus non-glucose material, and other suspended materials are higher than glucose. Consequently, the LAB is not maximised in the metabolic process. As for filtration, the fermented phase is dissolved, which contains higher sugar.

![Reducing Sugar Content](image)

**Figure 1.** Reducing sugar content before and after fermentation for 48 hours

3.1.2. Total sugar
Figure 2 shows the total sugar from the 48 hours fermentation of the three treatments, which resulted in a different reaction. In contrast to filtration treatment, precipitation treatment experienced an increase, referring to increase the total sugar. This finding proves that hydrolysis for 48 hours of a mixture of acids and inactive enzymes produces oligosaccharides. The conversion of complex sugars to simple sugars is effective. In addition to the filtration, non-water-soluble carbohydrate has been released, which decrease the total sugar.

Increased sugar content during the fermentation process is due to *B. longum* activity in degrading polysaccharides into simple sugars. *Bifidobacterium sp.* is a type of bacteria that produces the hydrolase glycoside enzyme with the ability to degrade polysaccharides and oligosaccharides into simple sugars, useful in fermentation [10]. Increased sugar levels indicate the effectiveness of the hydrolysis process in extracting *E. cottonii*. This complex sugar content is utilised by the LAB for metabolic processes during fermentation. Bacteria activity in producing lactic acid can increase along with the high availability of sugar in the growth medium.
3.1.3. pH
Deposition treatment has the highest pH due to the addition of acidic isopropanol. The pH after fermentation ranges from 4.5 to 6.5, indicating that the bacteria found in hydrolysate E. cottonii are a group of lactic acid bacteria. It is following the results of the former study, stating that the optimum acidity for lactic acid bacteria ranges from 3.8 to 8.0 [8].

Similarly, this finding is conveyed by previous research in which during the fermentation process, lactose is broken down into glucose and galactose by LAB to be converted into lactic acid and several other organic acids [11]. At 48 hours fermentation (apart from the increase in total LAB), the pH tends to decrease, and acidity tends to increase insignificantly. The breakdown of sugar in LAB cells will produce energy for probiotic bacteria activity in producing lactic acid. The formation of lactic acid will reduce the pH and resulting in a sour taste in the product. Statistical analysis confirmed that screening treatment was significantly different from deposition.

![Figure 2. Total sugar before and after fermentation for 48 hours](image)

3.1.4. Total acid
Figure 4 illustrated the finding of this study also demonstrates that total acid value increases along with the fermentation process. In other words, the total acid is inversely proportional to the pH, which decreases during fermentation. The total acid formed is dominated by lactic acid as a result of added LAB metabolite. Although the pH decrease is not too significant, the total acid is still formed.

![Figure 3. pH content before and after fermentation for 48 hours](image)
According to SNI 2981-2009, about the quality requirements of yogurt, yogurt has a total acid value of 0.5% - 2.0%, while the total acid in seaweed hydrolysate is only around 0.12 to 0.15 or below. *B. longum* is a heterofermentative LAB group, which during fermentation does not produce lactic acid as a single product, but in other products such as organic acids, ethanol, and several carbon dioxide (CO$_2$) [12]. Therefore, the total amount of acid in the seaweed hydrolysate is low.

![Figure 4. Total Acid before and after fermentation for 48 hours](image)

3.1.5. Total LAB

Based on Figure 5, it is apparent that the three different treatments experienced an increase in the number of bacteria because *B. longum* growth increases the cell number. Microbes utilize nutrients (carbohydrates), which have been broken down into simple sugars, to accommodate growth activities. The total increase in LAB during fermentation is caused by bacteria with an optimal pH to produce lactic acid from sugars and proteins into simpler components, such as organic acids, lactic acid, CO$_2$, H$_2$O, and energy [13].

After incubating for 72 hours, a higher LAB was found in the third sample (without filtration and deposition), due to the highest nutrient content compared to other samples. It is estimated that nutrients in the form of simple sugars from seaweed hydrolysate was partly settled during the deposition and filtration process. Whereas, in the third sample, the nutrients remained intact. Therefore, bacteria were more actively grown during incubation. Based on statistical analysis, the treatment of filtration and precipitation significantly demonstrate the expected result.
The viability of probiotic generally decreases while in the food and during digestion [14]. Powder hydrolysate is one effort to maintain the viability of probiotics. The drying process was performed by the spray-drying method. Spray drying is not limited for food ingredients but also for bacterial cells to form capsules for cell protection from extreme conditions [8]. This technique is widely applied in the food industry to prolong the shelf life, thereby reducing the risk related to microbes [15, 16].

The viability of the dried hydrolysate was tested by storing in the cold room (4ºC) for four weeks. The total LAB was calculated at weeks 0, 2, and 4 [13]. The viability of the LAB viability is presented in Table 1.

| Week | Total LAB (CFU/g) |
|------|------------------|
| 0    | 9.7 x 10^6       |
| 2    | 3 x 10^6         |
| 4    | 1.3 x 10^6       |

Based on Table 1, the total decrease of LAB on cold storage for four weeks is 8.4 x 10^6 CFU/g. The decrease occurs because cold temperatures damage cells. The total sugar in the hydrolysate also functions as a cryoprotectant agent or a substance that acts as a protective barrier for bacterial cell walls during the cooling process. To provide a health benefit, the viability of probiotics in products is generally around 10^8 CFU/g [18], while the US FDA recommends the minimum amount of 10^6 CFU/g [18].

**4. Conclusions**

Untreated hydrolysate had the best results compared to the other two treatments, with the reducing sugar content of 0.35%, total sugar of 2.81%, pH of 5.37, total acid of 0.25%, total LAB of 8.2x10^8 CFU/mL, galactose of 0.14%, and GOS levels of 1.18% respectively. After the spray drying and LAB viability test, the number of the LAB still meets the requirements of probiotics by the US FDA, which is equal to 10^6 CFU/g.
Acknowledgement
This research is fully supported by the Surfactant and Bioenergy Research Center, Bogor Agricultural University, Bogor Indonesia.

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