Preliminary study on seaweed fermentation for lactic acid production

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Abstract. Indonesia is a maritime country that is rich in seaweed. However, seaweed fermentation into lactic acid is not yet usually. Seaweed fermentation has outstanding potential because it has the most abundant polysaccharides compared to other sources. This research aims to synthesize lactic acid by fermentation using a single culture of Lactobacillus plantarum (L. plantarum) and two substrates, namely seaweed flour and refined salt Kappa-Carrageenan (RKC). Lactic acid was analyzed by fourier-transform infrared (FT-IR) spectroscopy and its concentration was determined by gas chromatography-mass spectrometry (GC-MS). The proximate analysis showed that crude Fiber and starch levels in seaweed are 25.37% and 14.66% (w/w) and also in RKC are 16.45% and 1.07% (w/w), respectively. The highest reducing sugar was attained at H2SO4 2% (w/w), which were 51,184 mg/L in RKC and 24,824 mg/L in seaweed flour. Based on FT-IR data, lactic acid characteristic signals were found at broadband approximately 3000 – 3500 cm⁻¹, which indicated the presence of OH band, a band at 1656 cm⁻¹ revealed C=O stretching of carbonyl groups, and a band at 1118 cm⁻¹ for C-O stretching of alcohol. Based on GC-MS data, the highest lactic acid production was 42,267 mg/L in RKC and 37,130 mg/L in seaweed flour. In this study, we can conclude that the efficiency of hydrolysis and fermentation of RKC was better than seaweed flour. However, the substrate concentration for optimum lactic acid production was unknown, so a more in-depth study was needed.

Keywords: Carrageenan; fermentation; lactic acid; Lactobacillus plantarum; seaweed.

1. Introduction

Indonesia is the largest archipelagic country in the world which has a water area of ± 5,877,879 km² or 2/3 of the total area [1]. With such wide waters, Indonesia is the largest seaweed producer globally. But on the other hand, the application of seaweed in Indonesia is still very limited, so seaweed is only exported in dry form with low economic value [2,3].

Seaweed is a group of algae and is classified into three main classes based on the pigments it contains, namely green (Chlorophyceae), brown (Phaeophyceae), and red (Rhodophyceae) seaweed [4–6]. This group has the main content of polysaccharides (50-76%) and protein (44-50%) as well as a small amount of fat (<5%), vitamins, and minerals [4,6,7]. As renewable biomass sources, seaweed polysaccharides such as carrageenan, agar, alginate, fucoidan, and laminarin are key sources of hydrocolloids. Therefore,
they are widely used in industries such as the food industry, sugar, textiles, pharmaceuticals, dairy industry, paper, gelling agents, stabilizing agents, manure, etc. [4,6,8]. So far, the application of seaweed has tremendous potential because it has several advantages such as low prices; does not require fertilizer; abundant productive land; and can maintain the cost of food commodities. In addition, seaweed contains the most abundant polysaccharides compared to other sources.

Studies on lactic acid production have recently been carried out due to its applications in the food, medical, material, and cosmetic industries [9,10]. One of the most developed uses of lactic acid is Polylactic Acid (PLA) through the lactic acid polymerization process. This polymer is very interesting because it is biodegradable [10].

Lactic acid can be produced through chemical synthesis processes or microbial fermentation. In the fermentation process, a pure product (L)-lactic acid is produced, while in the chemical synthesis process, two isomers of lactic acid are produced, namely (D)-lactic Acid and (L)-lactic acid [11]. This is a distinct advantage for the microbial fermentation process. In addition, the microbial fermentation process has other benefits, namely a relatively fast process, cheap raw materials, low byproducts, and high yield [12]. Given the promising potential, lactic acid fermentation is increasingly being carried out lately.

Studies of seaweed fermentation to produce lactic acid have been conducted with starchy biomass and agricultural residues for glucose sources such as sugar beet, molasses, whey, barley whey, cassava bagasse, and others [9,10]. The seaweed fermentation utilizes the dominant content in carrageenan or agar, which is a source of galactose.

This research is a preliminary test of the potential of seaweed as a substrate in lactic acid production. The substrate consisted of Refined Kappa-Carrageenan (RKC) and seaweed flour. The microorganisms used in single cultures of *L. plantarum*.

2. Materials and Methods

2.1. Materials

MRS Broth and MRS agar were purchased from Himedia (Mumbai, India). Sodium hydroxide (NaOH), sulfuric acid (H$_2$SO$_4$) p.a, Ammonium sulfate ((NH$_4$)$_2$SO$_4$), ethanol p.a, and lactic acid standard were obtained from Merck (Darmstadt, Germany).

The raw materials consist of Refined Kappa-Carrageenan (RKC) and seaweed flour from the *Euchema cotonii* species purchased from the local market in Bandung, West Java, Indonesia. Samples were proximately analyzed, including moisture, ash, crude fiber, and starch content. The gravimetric method carried the moisture, crude fiber, and ash content, while the starch was carried out by the Luff Schoorl [13]. The lactic acid bacteria was a pure culture of *L. plantarum* purchased from the local laboratory, namely "Labideal" in Bandung, West Java, Indonesia.

2.2. Hydrolysis of refined kappa carrageenan (RKC) and seaweed flour

Hydrolysis of RKC and seaweed flour with H$_2$SO$_4$ catalyst used a concentration of 15% (w/v). The samples were added with H$_2$SO$_4$ in various concentrations, i.e. 1%, 2%, and 3% (v/v). Then, samples were put into an autoclave and hydrolyzed at a temperature of 121 °C, a pressure of 1 atm for 45 minutes. The hydrolysate is cooled, neutralized with 20% NaOH (v/v), and filtered [14]. The reducing sugar concentration was determined by the Nelson-Somogyi method [15].

2.3. Inoculum Preparation

*Lactobacillus Plantarum* was maintained on Man, Rogosa, and Sharpe (MRS) agar growth media. After incubation at 37 °C for 48 h, the fully grown slopes were stored at 4 °C. Stock cultures were transferred to fresh MRS slanted agar every month. Seed cultures were prepared as follows: a circle of cells from fresh culture fully grown slopes was inoculated into 100 mL sterile MRS Broth in a 250 ml Erlenmeyer flask and incubated for 48 h at 37 °C with shaking at 120 rpm on a rotary shaker.
2.4. Fermentation of lactic acid
According to a previous report [16], the lactic acid fermentation was accomplished with some modifications. Briefly, 1% seed culture containing 92 × 10⁹ CFU/mL was inoculated in a 250 mL Erlenmeyer flask containing 50 mL of fermentation medium (hydrolysate solution and (NH₄)₂SO₄ 0.5% (w/v)) and then incubated on a rotary shaker for 48 h (37 °C; 120 rpm). Lactic acid fermentation was done in three times replication.

2.5. Lactic acid analysis with FT-IR and GC-MS
The lactic acid fermented products were analyzed using FT-IR spectroscopy (Thermo Scientific Nicolet iS5, Madison, U.S.A.) to determine the functional groups of lactic acid. The sample measurement technique was Attenuated Total Reflectance (ATR). It was accomplished according to a standard operating procedure (SOP) of FT-IR spectroscopy tools. Each sample was placed on an ATR prism crystal and then clamped. Measurements are carried out by the reflection method, where infrared rays are reflected to the position of each sample. And then, the spectrum was recorded by the detector. Each spectrum was scanned from 4,000 to 400 cm⁻¹. The FT-IR spectra were acquired for each treatment at room temperature. OriginPro 2019b software (Copyright 1991-2019 OriginLab Corporation) was used for data analysis.

GC-MS analysis was performed to determine lactic acid quantitatively. The fermented sample was evaporated to remove the solvent and then dissolved into 15 mL of ethanol (analytical grade). Samples measured by GCMS were diluted 100 times. Lactic acid standards were made with 50, 100, 200, and 300 mg/L concentrations.

GCMS-QP2010 ultra was used in this study. The basic parameters are given:
- **GC condition.** An Rtx-5MS 30 m × 0.25mm (ID) × 0.25 μm quartz capillary column was used, helium served as the carrier gas, injector temperature was 230 °C, and sample injection was 1.0 µL, splitless. The GC temperature programming is as follow: 4 min at 70 °C, 1 min at 70-80 °C, 3 min at 80 °C, 2 min 80-90 °C.
- **MS condition.** The ionization mode was electron ionization (EI), electron bombarding energy was 70 eV, charging multiplier tube voltage at 500 V, scan range from m/z 40 to 300 at 3 scan/s was used, solvent delay at 2 min.

3. Results and Discussion

3.1. Proximate analysis
The proximates composition of RKC and seaweed flour are shown in Table 1. The RKC contains moisture, crude fiber, starch, and ash of 1.59%, 16.45%, 1.07%, and 29.90%, respectively. In comparison, the seaweed samples contained 1.14% moisture, 25.37% crude fiber, 14.66% starch, and 27.51% ash.

Based on Table 1, the crude fiber of seaweed flour was higher than RKC. Crude fiber is part of insoluble dietary fiber, which has the main composition of cellulose, hemicellulose, and lignin. Crude fiber couldn't be hydrolysed by sulfuric acid and sodium hydroxide solution, so residues were obtained after undergoing a series of acid-base treatments [17–19].

Starch is a mixture of amylose and amylopectin, which are homopolysaccharides that develop glucose units [20]. Amylose has a linear chain structure consisting of 500-2,000 glucose units, while amylopectin has a highly branched-chain structure consisting of more than 1 million glucose units [21]. In this study, the starch content of seaweed flour was higher than RKC. In other words, the potential source of glucose from seaweed flour was higher than RKC. However, the purity of Kappa-Carrageenan in RKC is higher than in seaweed flour, so the source of galactose in RKC will also be higher than in seaweed flour.

Carrageenan is a linear sulfated galactan polysaccharide consisting of D-galactose groups bonded at -1.4 and -1.3 positions. In the industry, three kinds of carrageenan that are widely exploited are Kappa, Iota, and Lambda, which are distinguished by the presence of one, two, and three ester sulfate in the unit.
disaccharide group repeatedly [4]. The widely used carrageenan source comes from one species of red seaweed *Kappaphycus sp.*, better known as *Eucheuma cottonii* (about 65.75% w/w) [22]. Other sources of carrageenan can also be found in the genera *Ahnfeltia, Anatheca, Furcellaria, Gigartina, Gymnogongrus, Hypnea, Iridaea, Meristotheca,* and *Phyllophora* [6].

| Parameter | Unit | Carrageenan | Seaweed |
|-----------|------|-------------|---------|
| Moisture  | % w/w| 1.59        | 1.14    |
| Crude Fiber | % w/w | 16.45      | 25.37   |
| Starch    | % w/w| 1.07        | 14.66   |
| Ash       | % w/w| 29.90       | 27.51   |

### 3.2. Effect of sulfuric acid concentration on the seaweed hydrolysis

The seaweed needs to be hydrolyzed before the fermentation to break down polysaccharides into monosaccharides, thus the fermentation could be more easily. The hydrolysis of polysaccharides can be carried out with a chemical reaction using an acid/base catalyst or/and through an enzymatic process [23]. However, chemical hydrolysis is the preferred process in the industrial scales due to higher productivity and cost-effectiveness. According to several studies, hydrolysis with H₂SO₄ produces a higher yield of reducing sugars than other acids [24].

This study carried out the hydrolysis process using a dilute H₂SO₄ catalyst. As shown in Figure 1, various concentrations of H₂SO₄ (1, 2, and 3 % (v/v)) were used for hydrolyzing 15 % (w/v) of RKC and seaweed flour. The reducing sugar contents of the RKC and seaweed flour hydrolysate using H₂SO₄ 2 % were increased from 44,259 to 51,184 and 19,940 to 24,824 mg/L, respectively. However, the reducing sugar content was decreased from 51,184 to 50,158 mg/L for RKC and 24,824 to 22,981 mg/L for seaweed flour when using H₂SO₄ 3 % (v/v). The reducing sugar of RKC was about two times higher than seaweed flour because the crude fiber content in seaweed flour was higher than RKC. Therefore, the highest reduction sugar concentration was obtained using H₂SO₄ 2 % (v/v) catalyst, 51,184 mg/L and 24,824 mg/L for RKC and seaweed flour, respectively.

![Figure 1](image-url)  
**Figure 1.** Reducing sugar concentration obtained from hydrolysis of 15% (w/v) refined Kappa-carrageenan and seaweed flours using sulphuric acid catalyst.
There are some reports on polysaccharide hydrolysis methods, either chemically (acid/base), enzymatically, or combining the two methods. Lakshmikandan et al. (2021) hydrolyzed green seaweed *Valoniopsis pachynema* with (1- 5%) H$_2$SO$_4$ to produce reducing sugars and got the highest amount of total sugar content was 38.5 mg/g recorded at 3% sulfuric acid [24]. Wang et al. (2015) hydrolyzed wheat straw (50 g/L) with alkaline and enzymatic to produce reducing sugars of 13.82 g/L. Nancib et al. (2017) carried out acid hydrolysis followed by enzymatic hydrolysis of sawdust to produce glucose of 8.68 g/L[11].

3.3. Fermentation of lactic acid

3.3.1. FT-IR analysis

The samples of seaweeds fermented were analyzed by FT-IR spectroscopy to determine and evaluate the functional groups of lactic acid. The FT-IR spectrum (Figure 2) shows the molecular presence of samples and the lactic acid standard has high similarity. Spectral bands are read at wavenumbers 4000 to 500 cm$^{-1}$. Lactic acid characteristic signals were found at broadband approximately 3000 – 3500 cm$^{-1}$ which indicated the presence of OH band, a band at 1656 cm$^{-1}$ which revealed C=O stretching of carbonyl groups [25,26], and a band at 1118 cm$^{-1}$ which indicated C-O stretching of alcohol [26]. On the lactic acid standard, a band at 1118 cm$^{-1}$ was almost not detected; this meant that the fermented carrageenan and seaweed flour are not pure lactic acid yet.

![Figure 2. FT-IR spectrum of lactic acid.](image)

Notes: C is carrageenan flour; S is seaweed flour, and Std. L.A is lactic or acid standard

3.3.2. GC-MS analysis

The analysis of GC-MS only focused on the lactic acid product, even though this fermentation also produced other compounds as a byproduct. Based on previous reports, not all lactic acid bacteria can ferment galactose [11]. However, Lin et al. (2020) research revealed that *L. plantarum* could ferment galactose. In this study, the types of substrates with different characteristics and amounts of reducing sugar from the hydrolysis process were compared.
A standard lactic acid solution was plotted with the peak area on the GC-MS tool to determine the correlation between lactic acid concentration and peak area. The results (Figure 3) showed that the lactic acid concentration and area had a very good correlation ($R^2 = 0.9887$). In the GC spectrum, the peak of lactic acid in the sample and standard appears in retention time about 2.62 – 2.83 minutes. Recognizing lactic acid compounds in MS was compared with NIST 147 as the library, and it had a similarity level of 91 - 94%.

![Figure 3. Lactic acid standard curve for GC-MS analysis.](image)

Based on Table 2, the highest lactic acid content in RKC was 42,267 mg/L produced by hydrolysis with 1% sulfuric acid. As for the seaweed flour sample, the highest lactic acid was yielded by 3% sulfuric acid hydrolysis (37,130 mg/L). The high reducing sugar in the substrate (2% sulfuric acid), as depicted in Figure 1, is not directly proportional to the increased lactic acid production because it can inhibit bacterial's activities in the fermentation process [27]. However, lactic acid production in RKC and seaweed flour were not significantly different, even though the substrate concentration of RKC was much higher. Therefore, this study reports that RKC indicates more potential as biomass for lactic acid production than seaweed flour. However, the substrate content to produce optimum lactic acid has not been obtained, so further research is still needed.

| Conc. H2SO4 (%) | Lactobacillus plantarum count (CFU/mL) | Lactic acid content (mg/L) |
|-----------------|---------------------------------------|---------------------------|
| RKC             | 1 (92 × 10^7)                         | 42,267                    |
|                 | 2 (92 × 10^7)                         | 30,078                    |
|                 | 3 (92 × 10^7)                         | 14,411                    |
| Seaweed flour   | 1 (92 × 10^7)                         | 26,199                    |
|                 | 2 (92 × 10^7)                         | 36,646                    |
|                 | 3 (92 × 10^7)                         | 37,130                    |

4. Conclusion
Lactic acid production using the fermentation method on seaweed flour and kappa-carrageenan refined with a single L. plantarum culture have been successfully carried out. The highest lactic acid production of RKC (42,267 mg/L) and seaweed flour (37,130 mg/L) was reached when hydrolysis using 1% and 3% H2SO4, respectively. The high reducing sugar contained in the substrates can inhibit bacterial's
activities in the fermentation process. The sulfuric acid concentration needed to obtain the optimum reducing sugar in the hydrolysis process was 2% (v/v). The substrate concentration to produce the optimum lactic acid has not yet been achieved, so further research is still needed.

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Author contribution
Rossy Choerun Nissa, Akbar Hanif Dawam, and Dyah Marganingrum contributed equally as the main contributor to this paper. All authors read and approved the final paper.

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