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Sequential acid and enzymatic hydrolysis of carrageenan solid waste for bioethanol production: a biorefinery approach

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Abstract

The seaweed industry has developed rapidly over the last decade, and carrageenan is the leading hydrocolloid in the seaweed industry. Approximately 57,500 t of carrageenan is produced annually throughout the world. As a consequence of the increase in carrageenan production, the enormous amount of waste resulting from the carrageenan industry has also increased. This study investigated the possibility of ethanol production using carrageenan solid waste from the carrageenan extraction of *Kappaphycus alvarezii*, the principal species used in the carrageenan industry. Optimum acid hydrolysis followed by enzymatic hydrolysis enhanced the production of both galactose and glucose. Fermentation of the enzymatic hydrolysate using *Saccharomyces cerevisiae* ATCC 200062 resulted in an ethanol yield of 13.8 g L^{-1}.

Keywords Kappaphycus alvarezii · Seaweed · Bioethanol · Enzyme · Waste

Introduction

The seaweed industry which has developed rapidly over the past decade uses the commercial exploitation of seaweed as a food and as a source of three hydrocolloids: carrageenan, agar, and alginate (McHugh 2003). Of these three principle hydrocolloids, carrageenan has the highest production and sales value. The carrageenan industry supports a wide variety of products in the food, cosmetic, pharmaceutical, and dairy industries that have an estimated total annual value of US$ 518 million (Porse and Rudolph 2017).

Carrageenan is the generic name for a hydrocolloid consisting of sulfated galactan with an alternating backbone consisting of α(1-4)-anhydro-D-galactose and β(1-3)-D-galactose (Jol et al. 1999). The word "carrageenan" originates from "carrageenin," which was used for the first time in 1829 by Stanford, a British pharmacist (Pereira et al. 2009). Carrageenans can be divided into three principal types based on their chemical structure and properties: kappa, iota, and lambda. Kappa carrageenans encompass the strongest gels

of all carrageenans; hence, kappa carrageenans are important in the food, dairy, and pharmaceutical industries, where they are used as thickeners, gelling agents, and stabilizers. Most carrageenan is commercially extracted from *Kappaphycus alvarezii* ("cottonii"), the principal species producer of kappa carrageenans. Previous studies have shown that *K. alvarezii* is one of the most promising carrageenophytes for bioethanol production (Meinita et al. 2012; Khambhaty et al. 2012; Hargreaves et al. 2013). However, the utilization of *K. alvarezii* for bioethanol faces challenges due to biomass availability and other commercial uses as a carrageenan resource.

Large producers of carrageenan, such as CPKelco, FMC, and Cargill, are located in Europe and the US; small- to medium-size producers are located in Indonesia and the Philippines (Pambudi et al. 2010; Bixler and Porse 2011). World carrageenan production exceeds 57,500 t each year (Porse and Rudolph 2017). A significant increase in production has been observed in the carrageenan market. As a consequence of this increase in carrageenan production, the enormous amount of solid waste produced by the carrageenan industry has also been increasing each year. Solid waste resulting from carrageenan extraction may contain a large amount of polysaccharide materials. Management of solid waste resulted from industrial is a challenge, since these wastes are discarded directly to the environment which might cause several environmental problems (Guerrero et al. 2013; Marshall and Farahbakhsh 2013). However, the solid waste

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resulting from the carrageenan industry is not being utilized. Instead, this waste is discarded directly into the environment, which could cause several environmental problems. The utilization of carrageenan waste for bioethanol production may be a biorefinery approach that can be applied in the carrageenan industry. This study investigated the use of sequential acid hydrolysis, enzymatic hydrolysis, and fermentation of carrageenan waste from *K. alvarezii* for bioethanol production.

Materials and methods

Carrageenan waste material

Carrageenan waste materials are solid wastes resulting from the carrageenan extraction of the carrageenophyte, *Kappaphycus alvarezii*. This species is the most common raw material and is widely used in the carrageenan industry. Carrageenan waste materials collected from carrageenan extraction were freeze-dried and used for subsequent analyses.

Carrageenan extraction

The extraction of carrageenan from *K. alvarezii* was carried out by the alkaline method using 2 kg of dried carrageenan waste material. The wastes were boiled in 60% KOH solution and maintained at 80 °C in a water bath for 3 h. The extracts were then frozen overnight. After thawing, the extracts were washed with fresh water to remove any residue and dried in an oven (Ohno et al. 1994). Carrageenan waste was collected and used for the next step.

Carrageenan yield was determined by the following equation:

$$\text{Carrageenan yield} = \frac{\text{Carrageenan dry weight (g)}}{\text{Seaweed dry weight (g)}} \times 100$$

Acid hydrolysis

Acid hydrolysis was performed in a 250-mL flask by optimizing the H_2SO_4 concentration (0–1 M), carrageenan waste concentration (0–12%), hydrolysis time (0–45 min), and hydrolysis temperature (0–130 °C). The optimum acid hydrolysis conditions were then used for the next step.

Enzymatic hydrolysis

The carrageenan waste pretreated with acid hydrolysis was subsequently used for enzymatic hydrolysis. Optimization of the enzymatic hydrolysis of carrageenan waste was carried out for enzyme type, enzyme dosage, and incubation period. Four commercial enzymes—Celluclast, Viscozyme, Cellic C Tec II, and Cellic H Tec II—and five mixed enzymes—Celluclast + Viscozyme (1:1), Cellic C tec II + Cellic H tec II (1:1), Viscozyme + Cellic H tec II (1:1), Celluclast + Cellic H Tec II, and Cellic C Tec II + Viscozyme + Cellic H Tec II (1:1:0.1)—were purchased from Sigma (Novozymes, Denmark) and added to the carrageenan waste. Different concentrations of enzyme (0, 5, 10, 20, 30, and 40%) were applied to the acid hydrolysate of carrageenan waste. Optimization of the incubation period was performed by incubating carrageenan waste hydrolysate and measuring glucose and galactose production at 0, 3, 6, 9, 12, 24, 36, and 72 h. The pH of pretreated carrageenan waste was adjusted to 5 prior to enzymatic hydrolysis. Enzymatic hydrolysis was carried out at 50 °C in a water bath shaker at 130 rpm for 72 h. Enzymatic hydrolysates were evaluated by measuring the sugar content by high-performance liquid chromatography (HPLC) and used for the subsequent steps.

Microorganisms and medium

Prior to fermentation, hydrolysate samples were neutralized using 10 N NaOH to reach a pH = 5. *Saccharomyces cerevisiae* ATCC 200062 was used for fermentation. The inoculum culture was prepared in yeast medium containing 10 g yeast extract, 6.4 g urea, and 20 g glucose per liter. The yeast suspension was incubated at 30 °C with shaking at 130 rpm in an incubator. Fermentation was performed in triplicate in 100-mL Erlenmeyer flasks with a working volume of 50 mL. Samplings were conducted periodically to measure sugar and bioethanol production (Meinita et al. 2012, 2015).

Sugar, by-product, and ethanol determination

Monosaccharides (galactose and glucose) were measured by HPLC on an Alltech IOA 1000 organic acid column (300 × 7.8 mm) equipped with a refractive index detector. The column temperature was maintained at 60 °C. Ethanol production was measured using a Gas Chromatography (GC) Agilent model 6890N Series with a 2B-WAX column (Agilent Technologies, USA). The injection volume was 2 μL with an inlet split ratio of 30:1. The initial oven temperature was set at 35 °C, which can reach a maximum temperature of 250 °C. Bioethanol yield was calculated based on the following equation:

$$Y_{p/s} = \frac{[\text{EtOH}]_{max}}{[\text{Sugar}]_{ini}},$$

where $Y_{P/S}$ is ethanol yield (g g^{-1}), $[\text{EtOH}]_{max}$ is the highest concentration of bioethanol obtained during fermentation (g L^{-1}), and $[\text{Sugar}]_{ini}$ is the total initial sugar concentration at the onset of fermentation (g L^{-1}). The percent theoretical yield was calculated based on the following equation:

$$Y\% = \frac{Y_{p/s}}{0.51} \times 100,$$

where $Y\%$ is the percent theoretical yield (%) and 0.51 is the maximum ethanol yield per unit of hexose sugar from glycolytic fermentation (g g^{-1}).

Results and discussion

Carrageenan is the main polysaccharide found in *K. alvarezii*. Over 50,000 t of carrageenan is produced each year throughout the world, with a higher sales volume and sales value compared to the alginate and agar industries (Table 1). In this study, carrageenan yield was $32.95 \pm 1.43\%$ (Table 2), which is comparable to that reported elsewhere for the same alga (Ohno et al. 1994; Hayashi et al. 2007; Hayashi et al. 2011; de Góes and Reis 2012; Periyasamy et al. 2014). Carrageenan yields of 25.4–35.3% and 15–28% have been reported by Hayashi et al. (2011) and Hayashi et al. (2007), whereas Ohno et al. (1994) reported yields of 27.6–58.8% in Japan and the Philippines (Ohno et al. 1994; Hayashi et al. 2011). A carrageenan yield of 29.10–31.00% has been reported for *K. alvarezii* in India, while a yield of 46.1% has been recorded for *K. alvarezii* in Brazil (de Góes and Reis 2012; Periyasamy et al. 2014). Thus, carrageenan yield may vary according to seaweed strain, environmental parameters, geographical location, and extraction method. Some environmental parameters, such as water temperature and salinity, may also influence carrageenan yield (Hayashi et al. 2011; de Góes and Reis 2012).

In this study, the amount of carrageenan waste was $30.52 \pm 0.79\%$ (Table 2). Hence, we assumed that 30.52% or 15,260 t of seaweed raw material, which could be used as raw material for bioethanol production, remains as waste produced from the carrageenan industry each year.

Acid hydrolysis

Pretreatment is an important process for converting the structural characteristics of polysaccharides contained in carrageenan waste. This process is aimed at breaking down

Table 1 Sales volume, average price, sales value, and estimated waste volume of seaweed hydrocolloid (modified from Porse and Rudolph 2017)

Seaweed hydrocolloid	Sales volume (t)	Price (US$ kg^{-1})	Sales value (million US$)
Agar	14,500	17	246
Alginates	24,644	14	345
Carrageenans	57,500	9	518

Table 2 Carrageenan yield (%) and carrageenan waste (%) resulted from agar extraction; sugars (%) resulted from sequential acid hydrolysis and enzymatic hydrolysis under the optimum condition. Values represent the mean $\pm$ SD ($n \geq 3$)

Component	Concentration (% g g^{-1})
1. Carrageenan and waste	
Carrageenan yield	32.95 ± 1.43
Carrageenan waste	30.52 ± 0.79
2. Carrageenan waste after acid pretreatment	
Galactose	15.10 ± 0.006
Glucose	0.44 ± 0.026
HMF	3.09 ± 0.010
Levulinic acid	2.11 ± 0.010
3. Carrageenan waste after enzymatic hydrolysis	
Galactose	19.84 ± 0.024
Glucose	29.76 ± 0.037
HMF	2.65 ± 0.020
Levulinic acid	2.02 ± 0.010

polysaccharides into simpler sugars and enhancing the effectiveness of enzymatic hydrolysis. The selection of a pretreatment method plays an important role, as it significantly affects the subsequent enzymatic hydrolysis and fermentability of the hydrolysate.

Dilute acid hydrolysis has been successfully applied to the pretreatment or hydrolysis of seaweed materials (Meinita et al. 2012, 2013, 2015). The benefits of using dilute sulfuric acid hydrolysis are its high reaction rates and the efficiency with which it breaks down seaweed polysaccharides. A high temperature is favorable as it produces a high yield of sugar decomposition. In this study, acid pretreatment was optimized by applying different H_2SO_4 concentrations (0–1 M), amounts of carrageenan waste (0–12%), hydrolysis times (0–45 min), and hydrolysis temperatures (0–130 °C).

Effect of H_2SO_4 concentration on glucose and galactose content

The effect of H_2SO_4 concentration on the hydrolysis of carrageenan waste was determined (Fig. 1a). Among the different H_2SO_4 concentrations tested, the highest production of galactose and glucose was obtained with 0.2 M H_2SO_4 under standard conditions of hydrolysis at 120 °C for 15 min (13.43 ± 1.3 g L^{-1} galactose and 0.30 ± 0.019 g L^{-1} glucose). At lower and higher concentrations, galactose and glucose concentrations decreased. This finding is in agreement with the effect of acid concentration on seaweed hydrolysis observed by Meinita et al. (2012, 2015). Thus, the higher acid concentration results in a lower amount of sugar released.
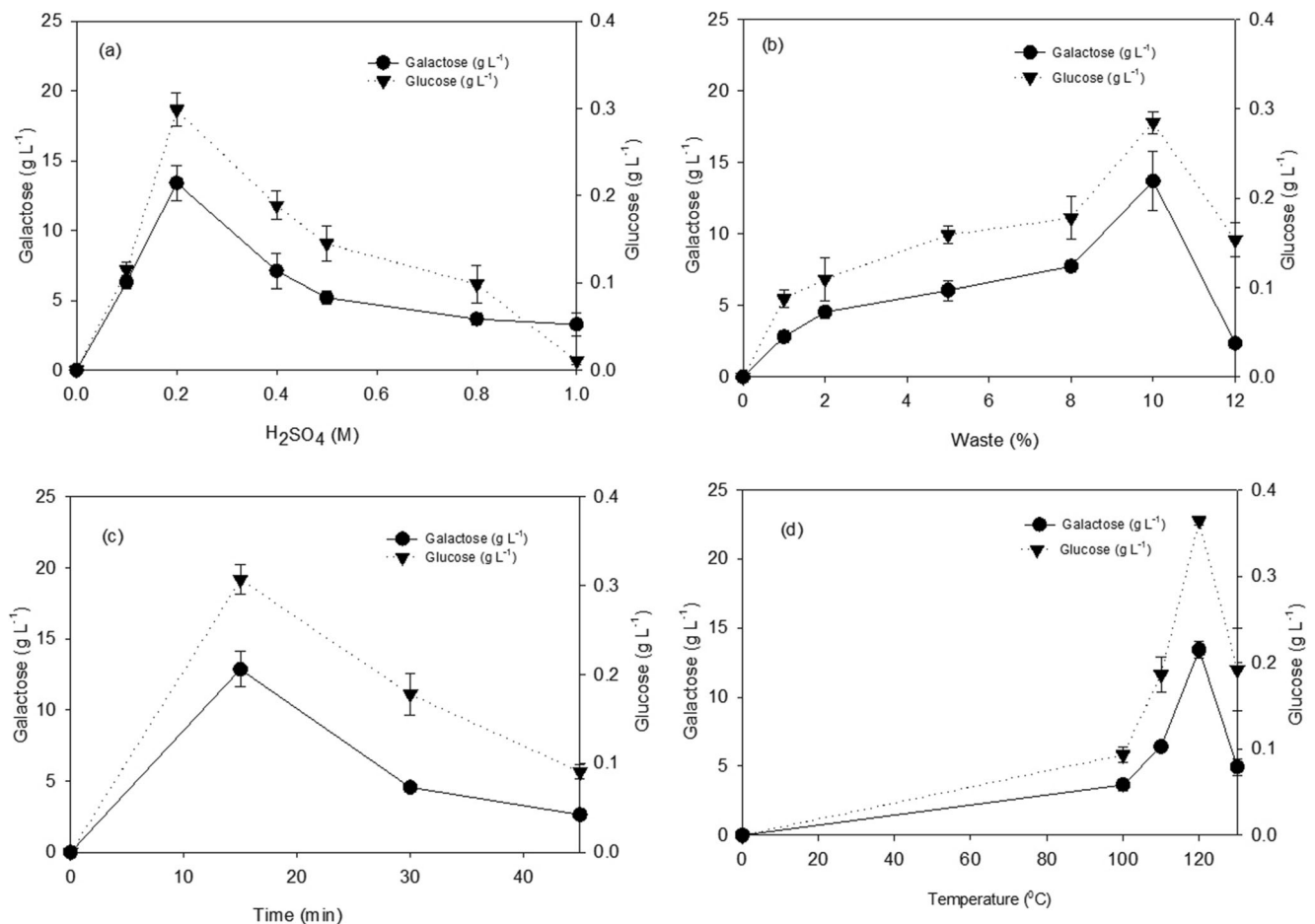
Fig. 1 Acid hydrolysis optimization of carrageenan waste by various parameters. **a** Effect of H_2SO_4 concentrations on glucose and galactose content. **b** Effect of carrageenan waste concentrations on glucose and galactose content. **c** Effect of hydrolysis time on glucose and galactose content. **d** Effect of hydrolysis temperature on glucose and galactose content. Values represent the mean ± SD ($n \geq 3$)

Effect of carrageenan waste concentration on glucose and galactose content

Various concentrations of carrageenan waste were treated under standard conditions of hydrolysis at 120 °C for 15 min with 0.2 M H_2SO_4 (Fig. 1b). The optimum amount of carrageenan waste material was determined to be 10%. When the carrageenan waste concentration was increased from 8 to 10%, the glucose obtained increased from 0.178 ± 0.02 to 0.285 ± 0.013 g L^{-1} and the galactose obtained increased from 7.73 ± 0.44 to 13.70 ± 2.07 g L^{-1}. When the concentration of carrageenan waste increased to 12%, the glucose and galactose content decreased. However, with the subsequent increase in carrageenan waste concentration, the galactose and glucose content dropped dramatically.

Effect of hydrolysis time on glucose and galactose content

The optimum hydrolysis time with 0.2 M H_2SO_4 was 15 min under standard conditions of hydrolysis at 120 °C with 10%

carrageenan waste (Fig. 1c). Increasing the hydrolysis time to longer than 15 min resulted in decreased production of galactose and glucose, suggesting that longer reaction times may degrade the sugar compounds and produce more by-product inhibitors, such as 5-hydroxy-methyl-furfural and levulinic acid (Meinita et al. 2012, 2013).

Effect of hydrolysis temperature on glucose and galactose content

The optimum galactose (13.43 ± 0.58 g L^{-1}) and glucose (0.37 ± 0.004 g L^{-1}) content was achieved at 120 °C for 15 min with 0.2 M H_2SO_4 and 10% carrageenan waste as the standard conditions. Thus, we concluded that increasing the temperature above 120 °C tends to decrease the production of both glucose and galactose.

Enzymatic hydrolysis

Enzymatic hydrolysis is an important process in bioethanol production. In this process, polysaccharides are hydrolyzed

into simpler saccharides. The factors that affect enzymatic hydrolysis include the type of enzyme, the enzyme concentration, and the incubation time. In enzymatic hydrolysis, a mild pH and temperature are applied. These mild conditions will not produce toxic and corrosive by-product compounds, as seen in acid hydrolysis (Alvira et al. 2010). However, there are some important factors that may influence enzymatic hydrolysis. In this study, we considered these factors by optimizing the type of enzyme, the enzyme concentration, and the enzyme incubation time. Enzymatic hydrolysis was performed sequentially to acid hydrolysis.

Optimization of the enzymatic hydrolysis of carrageenan waste is shown in Fig. 2. Among the different enzymes tested on carrageenan waste from the carrageenan extraction of *K. alvarezii*, the mixed enzyme of Cellic C tec II + Viscozyme + Cellic H tec II (1:1:0.1) was more effective than the single enzymes (Fig. 2a). The optimum galactose and glucose contents of carrageenan waste from the carrageenan extraction of *K. alvarezii* that saccharified using a mixed enzyme were 18.00 ± 0.50 g L^{-1} and 25.68 ± 0.01 g L^{-1}, respectively. Hence, this mixed enzyme was selected for further research to determine the optimum enzyme concentration and incubation time of carrageenan waste from the carrageenan extraction of *K. alvarezii*.

Substrate concentration is also an important variable that has a significant effect on enzymatic hydrolysis. The optimization of enzyme concentration is shown in Fig. 2b. In general, when the substrate concentration was gradually increased from 0% (w/v) to 20% of the biomass weight, the glucose yield increased gradually. This phenomenon indicates the availability of more cellulose that can be hydrolyzed to glucose. When the substrate concentration was increased from 20% (w/v) to 40% (w/v), there was a decrease in glucose and galactose yields. The optimum galactose and glucose contents of carrageenan waste from the carrageenan extraction of *K. alvarezii* at a 20% enzyme concentration were 17.50 ± 0.50 and 25.33 ± 0.01 g L^{-1}, respectively. Hence, we concluded that 20% was the optimum enzyme concentration. When the substrate concentration was increased from 20% (w/v) to 30% (w/v), there was a significant decrease in glucose yield. This optimum concentration occurs when the substrate concentration increases above its optimum value, thus becoming a limiting factor. A higher enzyme concentration may increase the viscosity of the hydrolysate, which would subsequently result in end-product inhibition and mass transfer limitations within the reaction mixture (i.e., it will produce a low glucose yield).

The effect of enzyme incubation time is shown in Fig. 2c. The time course of the enzymatic hydrolysis of carrageenan waste from the carrageenan extraction of *K. alvarezii* revealed an increase in glucose and galactose release at 24 h, which remained almost constant thereafter. However, the patterns of glucose and galactose release differed in the enzymatic
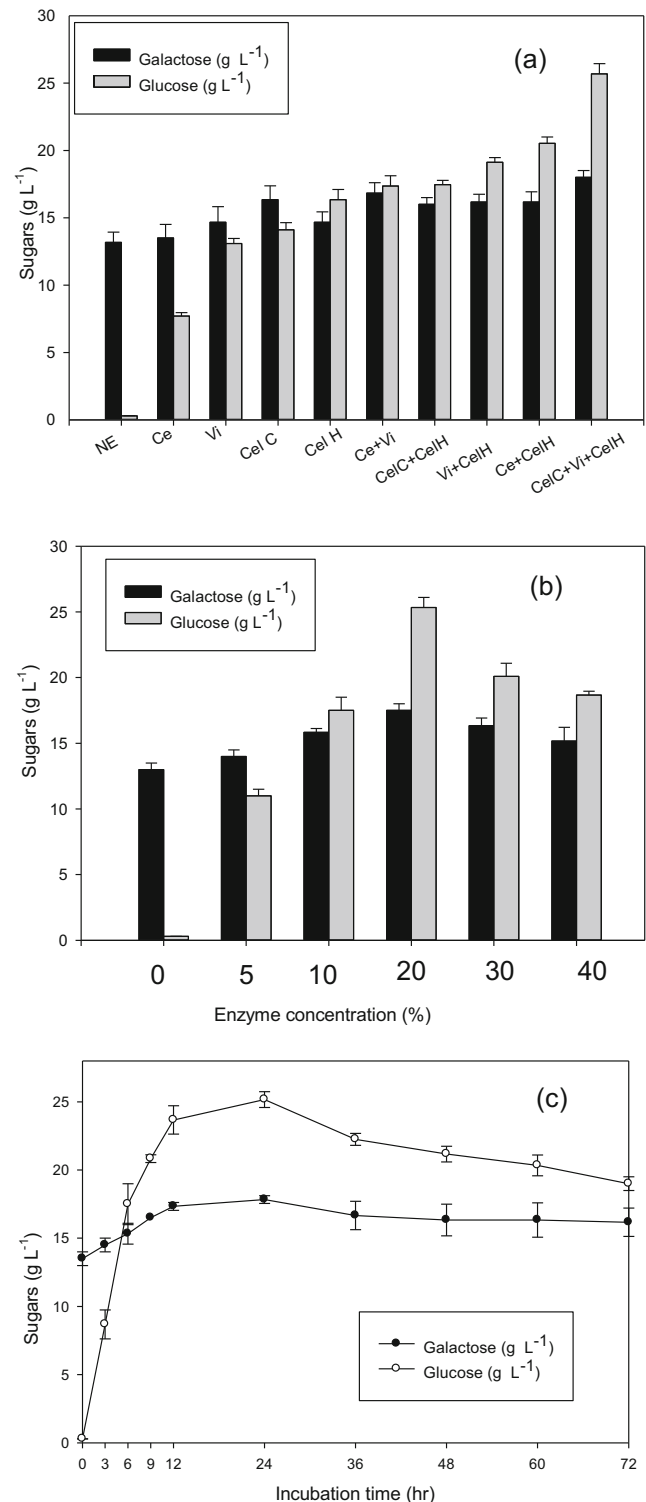

Fig. 2 Enzymatic hydrolysis optimization of carrageenan waste. **a** Effect type of enzymes on glucose and galactose content. **b** Effect of enzyme concentration on glucose and galactose content. **c** Effect of enzyme incubation time on glucose and galactose content. Values represent the mean $\pm$ SD ($n \geq 3$)

hydrolysis of carrageenan waste. Glucose increased significantly, whereas galactose remained relatively constant.

Table 3 Comparison of sugar (g L^{-1}), by-product (g L^{-1}) and ethanol (g L^{-1}) results from the present and previous studies

Material	Hydrolysis	Sugar		By-product		Ethanol	References
		Glu (g L^{-1})	Gal (g L^{-1})	HMF (g L^{-1})	LA (g L^{-1})	(g L^{-1})	
Seaweed							
Kappaphycus alvarezii	Acid	0.78	22.39	4.67	1.14	1.5	(Meinita et al. 2012)
Gelidium latifolium	Acid	2.40	34.43	5.70	2.56	NA	(Meinita et al. 2015)
Gelidium amansii	Acid	0.81	20.04	4.83	0.29	0.66	(Meinita et al. 2013)
Undaria pinnatifida	Acid	NA	NA	NA	NA	9.42	(Cho et al. 2013)
Graciaria gigas	Acid + Enzyme	9.7	20	NA	NA	3.56	(Meinita et al. 2017b)
Gracilaria fisheri	Acid	7.76	8.93	NA	NA	NA	(Nunraksa et al. 2018)
Gracilaria tenuistipitata	Acid	4.58	8.78	NA	NA	NA	(Nunraksa et al. 2018)
Seaweed waste							
Agar industrial spent	Acid + Bacterial consortia	NA	NA	NA	NA	2.34	(Sudhakar et al. 2017)
Alginate industrial spent	Acid + Bacterial consortia	NA	NA	NA	NA	2.60	(Sudhakar et al. 2017)
Agar waste (*G. verrucosa*)	Acid + Enzyme	14.3	7.3	NA	NA	5.52	(Meinita et al. 2017a)
Agar waste (*G. latifolium*)	Acid + Enzyme	16.2	18.2	NA	NA	10.83	(Meinita et al. 2017a)
Carrageenan waste	Acid + Enzyme	NA	NA	NA	NA	5.47	(Tan and Lee 2014)
Carrageenan waste (*K. alvarezii*)	Acid + Enzyme	29.76	19.84	2.30	1.75	13.80	This study

Based on this result, we conclude that enzymatic hydrolysis is more efficient at increasing glucose in carrageenan waste from the carrageenan extraction of *K. alvarezii* than is galactose. Sequential acid and enzymatic hydrolysis effectively convert polysaccharides into sugar and increase the sugar content in the carrageenan waste hydrolysate. Carrageenan waste from the carrageenan extraction of *K. alvarezii* showed a different pattern in enzymatic hydrolysis, and this may have been caused by the different compositions of the compounds; hence, the enzymes reacted differently. Thus, the combination of different types of enzyme, different enzyme concentrations, and different incubation times used in enzymatic hydrolysis greatly affects the production of sugars and the efficiency of ethanol production.

Sugars and by-products before and after enzymatic hydrolysis

The comparison of glucose and galactose production after acid hydrolysis and enzymatic hydrolysis is shown in Table 2. We found a significant increase in glucose after enzymatic hydrolysis. There was also an increase in galactose after enzymatic hydrolysis, although the increase was not significantly relative to glucose. However, one drawback of acid

Fig. 3 Time course of ethanol production, galactose, and glucose during fermentation of sequential acidic-enzymatic hydrolysate of carrageenan waste. Fermentation was performed in in 100-mL Erlenmeyer flask at 30 °C. Samples were taken periodically for measurement of sugar and bioethanol production. Values represent the mean ± SD ($n \geq 3$)
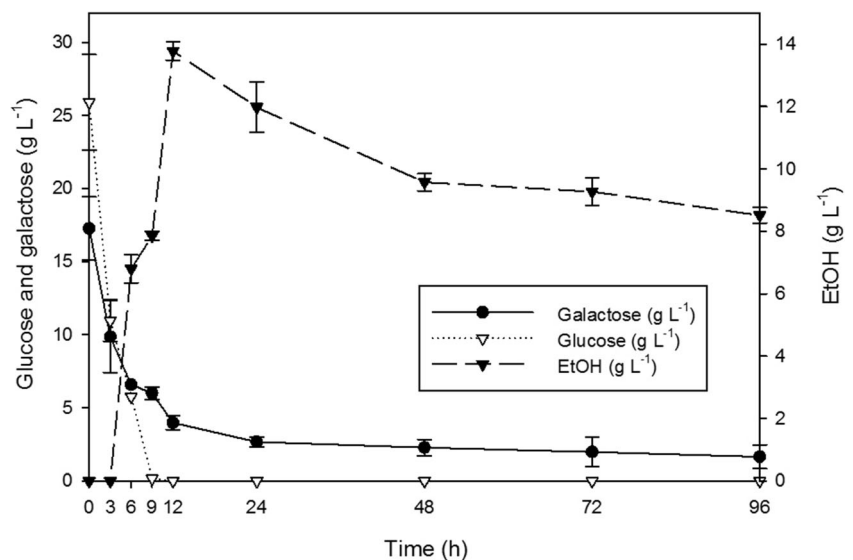
Fig. 4 Biorefinery concept of carrageenophyte *K. alvarezii* and its carrageenan waste
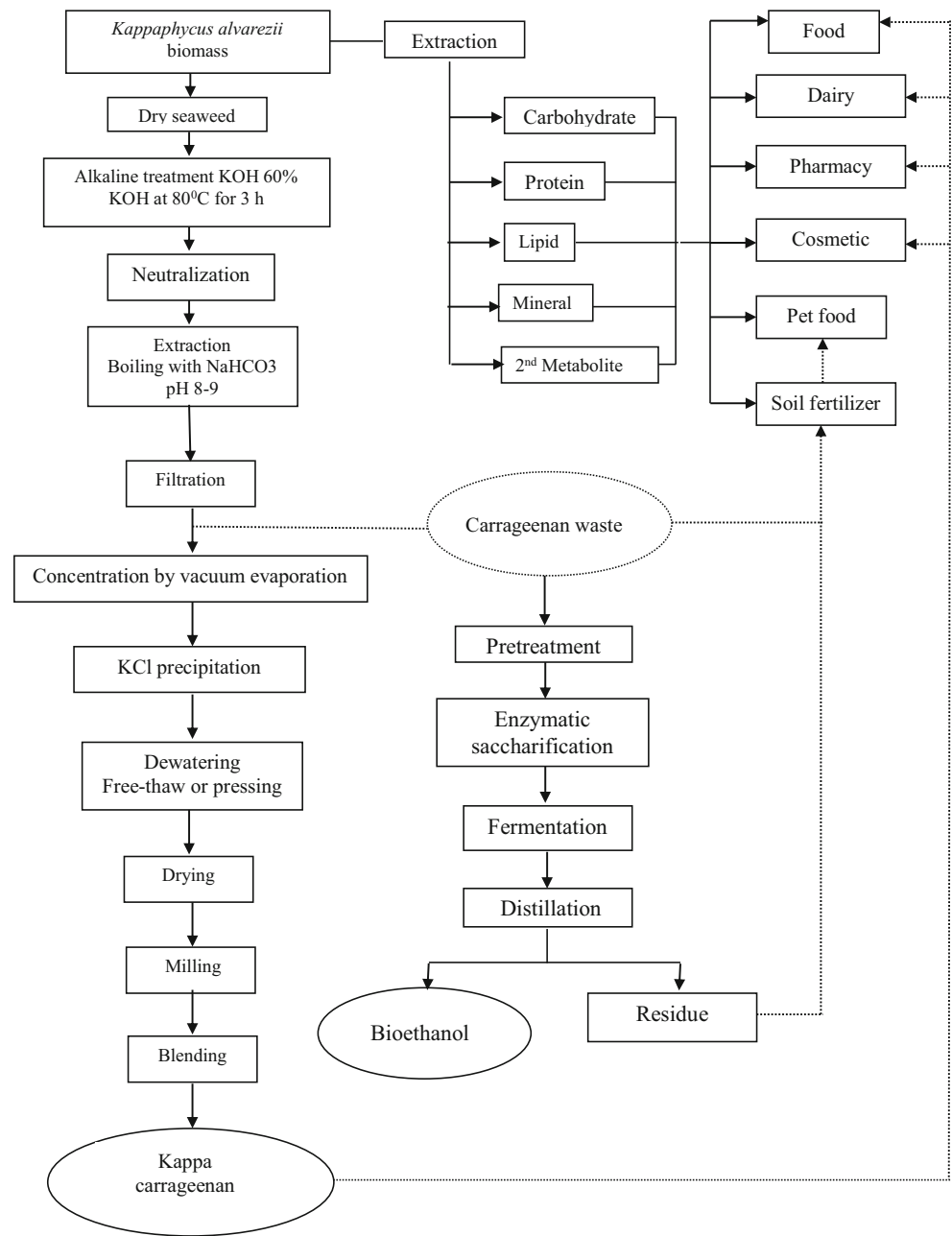

Table 4 Estimation of carrageenan waste, galactose, glucose, and ethanol yield in industrial scale

Component	*Kappaphycus alvarezii*	Reference
1. Carrageenan production (t year^{-1})	57,500	Porse and Rudolph 2017
2. Carrageenan waste (t year^{-1})	17,549	Present study
3. Galactose (% g g^{-1})	19.84	Present study
4. Glucose (% g g^{-1})	29.76	Present study
5. Estimated galactose (t year^{-1})	3482	Present study
6. Estimated glucose (t year^{-1})	5223	Present study
7. Ethanol efficiency	0.69	Present study
8. Ethanol yield estimation (t year^{-1})	3069	Present study

hydrolysis is the formation of hydroxymethylfurfural (HMF) and levulinic acid. HMF is a toxic compound that results from the degradation of hexoses, while levulinic acid is formed by the degradation of HMF. HMF and levulinic acid were formed during acid hydrolysis. After enzymatic hydrolysis, we found a decrease in both HMF and levulinic acid (Table 2). However, the amounts of HMF and levulinic detected in our study after enzymatic hydrolysis were much lower than those reported previously (Table 3). We also found that the level of glucose produced from our acid and enzymatic hydrolysis was higher than that in previous studies. Thus, we concluded that acid hydrolysis followed by enzymatic hydrolysis is a suitable method that can produce a high glucose yield and generate a small amount of by-product compounds.

Fermentation and ethanol production

In this study, we examined the time course of main bioethanol fermentation from 50 mL enzymatic hydrolysate of carrageenan waste fermented using *S. cerevisiae* ATCC 200062 (Fig. 3). Our previous study found that *S. cerevisiae* ATCC 200062 performs well in producing bioethanol from red seaweed. The hydrolysate containing 25.89 g L^{-1} glucose and 17.26–25.89 g L^{-1} galactose was fermented using *S. cerevisiae* ATCC 200062 and produced 13.78 g L^{-1} of ethanol in 12 h.

In the ethanol fermentation of carrageenan waste hydrolysate of *K. alvarezii*, the ethanol production rate increased slowly in the early phase but increased rapidly after 9 h. The ethanol production rate reached a maximum after 12 h of fermentation, and the maximum ethanol yield of 0.35 g g^{-1} sugars was also obtained after 12 h. The glucose was exhausted after 12 h, while galactose continued to decrease slowly and remained steady until 96 h of fermentation. The maximum ethanol concentration of 13.78 g L^{-1} was obtained with 69% of the theoretical yield.

The ethanol production achieved in this study was higher than that reported by previous studies on bioethanol production from seaweed (Table 3). This might be related to carrageenan content as fermentable polysaccharide in carrageenan waste, the effectiveness of hydrolysis and fermentation method. Acid and enzymatic hydrolysis play an important role in converting the structural characteristic of polysaccharide, enhancing fermentable sugar production, which will also affect ethanol production. Hence, the selection of hydrolysis is a crucial step which might determine the fermentable sugar yield and ethanol production. The major polysaccharide found in carrageenan waste hydrolysate of *K. alvarezii* is classified as kappa carrageenan. Chemically, kappa carrageenan consists of repeating D-galactose units, 3,6-anhydrogalactose and sulfated at C4 in the 1,3-linked galactose ring (Jol et al. 1999). Acid hydrolysis breakdown the complexity of kappa carrageenan which still remained in carrageenan waste to fermentable sugars and making remained kappa carrageenan more

workable to the enzyme hydrolysis. Combination of acid and enzymatic hydrolysis resulted in higher glucose. Compared to galactose, glucose is the preferred carbon source for *S. cerevisiae* ATCC 200062 to produce ethanol (Meinita et al. 2017a). Glucose was thoroughly consumed by *S. cerevisiae* ATCC 200062 while galactose was consumed partially and remained until the end of fermentation. Sequential acid and enzymatic hydrolysis appear as the best method to optimize the glucose; hence, the ethanol production in this study is higher than the previous study which mostly only uses acid or enzymatic hydrolysis.

Biorefinery approach

A biorefinery approach can be developed from *K. alvarezii* biomass and its carrageenan waste. We can extract chemicals from *K. alvarezii* for use in food, dairy, pharmaceuticals, cosmetics, pet food, soil fertilizers, and other bioproducts. While carrageenan waste can be utilized for bioethanol production, it contains rich sulfated galactans that can be converted into simple sugars by sequential acid and enzymatic hydrolysis. The by-products resulting from bioethanol production contain large amounts of organic matter and useful minerals that can be used as soil fertilizer or pet food (Fig. 4).

From the industrial point of view, it is very important to consider many aspects in the bioethanol process. The biggest challenges of large-scale bioethanol production are technology and the availability and sustainability of raw material. The present study showed that sequential acid and enzymatic hydrolysis followed by ethanol fermentation by *S. cerevisiae* ATCC 200062 can be used as an efficient technology to produce bioethanol form carrageenan waste. According to Porse and Rudolph (2017), the total carrageenan sales volume reaches approximately 57,500 t per year, with the sales value reaching US$ 518 million. Based on our study, we believe that carrageenan extraction of *K. alvarezii* will produce 30.52% of carrageenan waste. We estimate that the galactose and glucose resulting from enzymatic hydrolysis would be equivalent to 3481 t year^{-1} and 5222 t year^{-1}, respectively. The large-scale ethanol yield from carrageenan waste hydrolysate of *K. alvarezii* is estimated to be approximately 3069 t year^{-1}. The schematic of bioethanol production and estimation of ethanol production from carrageenan waste hydrolysate of *K. alvarezii* is shown in Fig. 4 and Table 4. The abundance of polysaccharides that remains in carrageenan waste can be used in bioethanol production. Based on technology, availability, and sustainability, carrageenan waste can be one of the best candidate raw materials for bioethanol production.

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