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Profiling and Prebiotics Potential of *Gracilaria* sp. Hydrolysate Obtained from Optimized Citric Acid-Assisted Hydrolysis

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ABSTRACT

Gracilaria sp. is widely recognized as a rich source of agar-type polysaccharides. These complex polysaccharides can be hydrolyzed into oligosaccharides and simpler sugar molecules, yielding a biosugar-rich hydrolysis that serves as a potential substrate for fermentation products. Acid-catalyzed hydrolysis (saccharification) using citric acid offers a simpler and more economical than enzymatic hydrolysis. The study aimed to optimize the hydrolysis process of *Gracilaria* sp. using citric acid and evaluate the prebiotic potential of resulting hydrolysate. A Box–Behnken design under the Response Surface Methodology (RSM) framework was employed using Design Expert 13 software, with three independent variables: substrate amount (1–4 g), acid concentration (0.01 N–1 N), and hydrolysis time (10, 20, and 30 minutes). The response variable measured was the reducing sugar content. The probiotic activity of the hydrolysate was evaluated based on the growth performance of the probiotic candidates *L. acidophilus* and *L. delbrueckii* spp. *bulgaricus*, along with assessments of the prebiotic effect and prebiotic index. The optimized hydrolysis conditions predicted by the quadratic model were obtained at a substrate amount of 3.209 g, a citric acid concentration of 0.117 N, and a hydrolysis time of 30 minutes. Validation experiments under these conditions produced a hydrolysate with a reducing sugar content of 57.14 mg/100 mL. Furthermore, the prebiotic effect scores of the hydrolysate ranged from 3.01 to 1.025, while its prebiotic index values (1.31 and 1.03) were higher than those of inulin. These findings confirm that citric acid hydrolysis of *Gracilaria* sp. yields a functional biosugar with promising prebiotic potential, supporting further investigation for food and health applications.

INTRODUCTION

Seaweed is considered one of the most promising marine resources as an alternative substance for both nutritional and industrial applications. Currently, it contributes approximately 0.13% to the global food energy supply. The growth rate of seaweed

production has surpassed that of many staple terrestrial crops **(van Oort *et al.*, 2023)**. Seaweed is recognized for its rich nutritional profile and significant health benefits. It also represents a high-potential biomass source for biosugar production. In recent years, seaweed has been increasingly explored as a substrate for biotransformation through fermentation processes. However, current applications are largely focused on biofuel production, with limited attention to edible fermentation products **(Reboleira *et al.*, 2021)**.

Among the various seaweed species, *Gracilaria* sp., a red seaweed, is notable for its high carbohydrate content. The carbohydrates present in *Gracilaria* sp. are primarily complex polysaccharides, such as cellulose and hemicellulose, which are resistant to complete digestion in the human body. As a result, they provide lower caloric intake and are suitable for dietary food formulations **(Kazir *et al.*, 2019; Torres *et al.*, 2019)**. Biosugars derived from seaweed serve as valuable substrates rich in nutrients for various fermentation applications.

Biosugar can be produced from seaweed by hydrolysis using an acid catalyst. Hydrolysis is a reaction between a compound and water, resulting in the cleavage or decomposition of the compound. Since the reaction between water and the substrate proceeds slowly under normal conditions, catalysts are employed to accelerate the process. Strong acids such as hydrochloric acid (HCl) and sulfuric acid (H_2SO_4) are commonly used catalysts for hydrolysis reactions **(Sunaryanto *et al.*, 2013; Mayasari & Sulaiman, 2022)**. Strong acids generally lead to the production of monosaccharides only. Hence, weak acid alternatives such as organic acids can be utilized to obtained oligosaccharides. Acetic and citric acid are among the organic acids that are considered safe for consumption (edible).

Citric acid, an important organic acid widely used in various industries is naturally present in fruits like oranges, pineapples, and pears, particularly in food and beverage production, accounting for approximately 70% of its consumption **(Show *et al.*, 2015)**. However, the application of organic acids, particularly citric acid, as catalysts for polysaccharide hydrolysis in biosugars has not been sufficiently investigated. In our previous study, hydrolysis conditions using citric acid were optimized within a limited range of Response Surface Methodology (RSM). The results obtained from the previous study indicate that the acid concentration used was still too high, leading to a dominant presence of monosaccharides and a relatively low concentration of reducing sugars, amounting to 16.34 mg/mL **(Wulansari *et al.*, 2025)**. In this study, the RSM range is expanded to encompass a broader operational window. The hydrolysis process that produces oligosaccharides has a promising potential as a source of beneficial prebiotics. Furthermore, the prebiotic potential of the hydrolysates, obtained under the optimal conditions determined by Response Surface Methodology (RSM).

Prebiotics are non-digestible oligosaccharides that cannot be absorbed in the human gastrointentional tract but can be utilized by probiotic bacteria. Probiotic bacteria provide

various health benefit to the host, including the production of beneficial metabolites such as short-chain fatty acid (SCFAs), minerals and vitamins **(Kustyawati *et al.*, 2022)**. Agaran, a polysaccharide found in *Gracilaria* sp., contains fewer and differently positioned sulfate groups compared to carrageenans. Agaran has a negative impact on supporting microbial metabolic activity, reducing both fermentation capacity and SCFA production. Therefore, agaran must first be hydrolyzed through a suitable hydrolysis mechanism **(Gotteland *et al.*, 2020)**. However, the production of oligosaccharides as prebiotic candidates via citric acid saccharification still requires further investigation.

This study addresses this gap by optimizing the citric acid hydrolysis conditions to produce biosugars that serve as fermentation substrates and potential prebiotic candidates. The functional evaluation conducted here contributes to the development of marine-based prebiotics.

MATERIALS AND METHODS

1. Materials

Two-month-old *Gracilaria* sp. algae were obtained from aquaculture pond located in Karawang Regency, West Java. Food-grade citric acid was obtained from a food additive manufacturer (Koepoe-Koepoe). Lactic acid bacteria (LAB) used in this study, namely *L. acidophilus* ATCC 4356 and *L. delbrueckii* subsp. *bulgaricus* ATCC 11842, were obtained from Agavi Lab, Bandung, West Java. Other materials used included MRSB growth media, $NaHCO_3$ (Choice Chemical), DNS reagent (Sigma-Aldrich), and CaO.

2. Methods

Seaweed pre-treatment

Seaweed was obtained from farmers in dry form. The seaweed was washed with flow water until it was cleaned of any adhering dirt, soaked with saturated CaO solution for 12 hours, and washed again with flow water until clean. Finally, the seaweed was dried in the sun in a shady place for 1-2 days to dry, with moisture content as much as 17%.

2.1 Optimization of biosugar production using enhanced RSM

Acid hydrolysis

Dry seaweed, pre-treated in amounts of 1 g, 2.5 g, and 4 g, was soaked in 100 mL of acid solution at concentrations of 0.01 N, 0.505 N, and 1 N, respectively, as determined by the Box–Behnken design. The mixtures were heated at 95 ± 2 °C with constant stirring. Hydrolysis times of 10, 20, and 30 minutes were applied for each group. The hydrolysis process was immediately stopped by immersing the mixtures in an ice water bath. The hydrolysate was then filtered using vacuum-assisted filtration. The

filtrate was neutralized with sodium bicarbonate to a pH of 6.8–7.2 and subsequently evaporated at 60 °C using a rotary evaporator (DLab, China), hereinafter referred to as HoG.

Determination of Reducing Sugar Content

Reducing sugar content was determined using the DNS (3,5-dinitrosalicylic acid) method and measured by UV-Vis spectrophotometry. Glucose was used as the standard, with concentrations of 50, 100, 150, 200, 250, and 300 ppm. Hydrolysate samples and glucose standards were reacted with DNS reagent at a ratio of 1:3 and incubated for 15 minutes. Absorbance was measured at 550 nm using a UV-Vis spectrophotometer (Kim et al., 2015). The reducing sugar content served as the response variable in the RSM model to identify the optimal hydrolysis conditions. The hydrolysate showing the best results was selected for further profiling and characterization.

2.2 Profiling and Characterization of Selected Gracilaria sp. Hydrolysate

Qualitative Analysis of Oligosaccharides by Thin-Layer Chromatography (TLC)

Oligosaccharide profiling was performed using TLC. The mobile phase consisted of n-butanol, acetic acid, and distilled water in a 2:1:1 (v/v/v) ratio. Samples were spotted onto silica gel GF 254 plates and developed for 1.5 hours. Plates were air-dried and sprayed with DAP reagent (diphenylamine-acetone-phosphoric acid), then heated at 120 °C for 15 minutes to visualize the spots. The DAP reagent was prepared by mixing 0.2 g of diphenylamine, 0.2 mL of aniline, 10 mL of acetone, and 1.5 mL of phosphoric acid **(Wijaya *et al.*, 2020)**.

Analysis of Monosaccharides by HPLC

Monosaccharides (glucose and galactose) and by-product 5-hydroxymethylfurfural (5-HMF) were analyzed using High-Performance Liquid Chromatography (HPLC). Samples were filtered through a 0.2 μm Millipore membrane filter before analysis. Separation was performed on a Coregel 87H3 column using 5 mM sulfuric acid as the mobile phase. Detection was carried out with a refractive index detector (RID). The column oven was maintained at 80 °C, with a flow rate of 0.6 mL/min, a 40-minute run time, and an injection volume of 20 μL **(Wijaya *et al.*, 2020)**.

Assessment of Prebiotic Activity

Prebiotic activity was evaluated using *Lactobacillus acidophilus* and *Lactobacillus bulgaricus* as probiotic candidates, following the method of **Huebner *et al.* (2007)**. Growth media consisted of mMRS broth supplemented with 2.5% (w/v) hydrolysate (T2), while mMRS broth without hydrolysate served as the control (T1). Both media were sterilized prior to inoculation. A 24-hour LAB culture (10^5 CFU/mL) was inoculated (2.5 mL) into 50 mL of each medium and incubated at 37 °C for 24 hours.

After incubation, serial dilutions (10^{-1} to 10^{-8}) were prepared using 0.85% NaCl. Aliquots from the 10^{-6} to 10^{-8} dilutions were plated in duplicate on MRS agar (MRSA)

and incubated at 37 °C for 48 hours in an inverted position. Colony-forming units (CFU/mL) were enumerated following ISO guidelines. The prebiotic effect was calculated as:

$$\text{Prebiotic Effect} = \log_{10}[(\text{CFU/mL})_{t2} - (\text{CFU/mL})_{t1}],$$

Where, T1 represents mMRSB without hydrolisate and T2 represents mMRSB containing 2.5% hydrolisate.

In addition, the Prebiotic Index (PI) was used to evaluate the relative growth of probiotic bacteria in the prebiotic substrate compared to a carbohydrate control (glucose):

$$\text{Prebiotic Index (PI)} = \Sigma \text{ Probiotics in Prebiotic} / \Sigma \text{ Probiotics in Carbohydrate}$$

A PI value greater than 1 indicates a positive effect of the substrate on probiotic growth, while a value close to 1 suggests low prebiotic effectiveness. Commercial inulin was used as a reference prebiotic control, while glucose served as the carbohydrate control.

3. Statistical Analysis

The study was designed using Design Expert 13.0 software with Response Surface Methodology using Box Behken modelling. Data were analyzed statistically using ANOVA with a 95% confidence interval.

RESULTS AND DISCUSSION

1. Optimization of hydrolysis condition of *Gracilaria* sp.

In the acid-catalyzed hydrolysis process, various factors can affect the hydrolysis results. This study used a combination of 3 (three) factors: amount of substrate, concentration of citric acid, and length of hydrolysis time. Seventeen experimental runs were conducted using a combination of these factors, then reducing sugar content was determined as previously described. The results of the reducing sugar content tests are presented in Table (1).

Table 1. Result of reduction sugar content using actual design of Box Behken modeling

Run	X_1 Amount of substrat (grams)	X_2 Acid citric conc. (N)	X_3 Hydrolysis time (minutes)	RDS Reducing sugar content (mg/100ml)
1	1	0.505	30	33.39
2	2.5	0.505	20	70.07
3	2.5	1	30	8.04
4	2.5	0.505	20	57.97

5	4	0.505	30	83.66
6	2.5	0.01	10	2.48
7	2.5	0.505	20	45.24
8	2.5	0.01	30	4.33
9	4	0.01	20	4.29
10	2.5	0.505	20	65.28
11	1	1	20	2.88
12	2.5	0.505	20	58.34
13	4	0.505	10	73.52
14	2.5	1	10	18.77
15	4	1	20	20.78
16	1	0.505	10	15.47
17	1	0.01	20	0.39

Note = X_1: Amount of substrat *Gracilaria* sp.; X_2: Acid citric concentration; X_3: hydrolysis time; RSD: reducing sugar content as response.

According to Table (1), the highest concentration of reducing sugar content (83.66 mg/100 mL) was obtained in assay 5 (substrate 4 g, citric acid concentration 0.505 N, and a hydrolysis time of 30 minutes). By contrast, the lowest reducing sugar content was observed in assay 17, which utilized a substrate of 1 g, a citric acid concentration of 0.01 N, and a hydrolysis duration of 20 minutes. Other studies have shown that *Gracilaria* sp. have the highest cellulose content (19.7%). This high cellulose content has the potential to produce bioethanol via hydrolysis. However, the cellulose content of each seaweed can differ depending on the type, treatment, cultivation, and place of cultivation **(Salem & Ismail, 2022)**.

Effect of acid concentration on pH and reducing sugar content

Acid catalysts have the advantage of accelerating reaction rates. In principle, complex carbohydrates can be degraded into simple saccharides through hydrolysis; however, the process can be slow. Therefore, the use of acid catalysts is beneficial to reduce hydrolysis time. Similar research reported that hydrolysis without a sulfuric acid catalyst did not yield sugar formation, as the breakdown of oil palm bunches into cellulose requires an acid catalyst and sufficient reaction time **(Ahmad *et al.*, 2022)**. The results of reducing sugar content at varying acid concentrations are presented in Table (2).

Table 2. Results of reducing sugar content with varying acid concentration

run	Time (min.)	amount of substrate (grams)	Acid conc. (N)	reducing sugar content (mg/ml)
6	10	2.5	0.01	2.48
14	10	2.5	1	8.04
11	20	1	1	2.88
17	20	1	0.01	0.39

Table (2) shows that increasing the concentrations of both acid and substrate led to higher reducing sugar content. This indicates that citric acid effectively breaks down the polysaccharides in *Gracilaria* sp. into simpler reducing sugars. In this study, a citric acid concentration of 0.01 N was sufficient to hydrolyze *Gracilaria* sp. Similarly, **Musdalifa *et al.* (2024)** reported that increasing the concentration of hydrochloric acid and the duration of heat treatment resulted in higher reducing sugar content. Higher acid concentrations accelerate the breakdown of starch molecules by releasing bound water, facilitating the formation of simpler sugar molecules.

Effect of hydrolysis time on reducing sugar content

The duration of hydrolysis can significantly affect the amount of reducing sugars produced. Shortening the reaction time of the solids generally has a positive effect on process efficiency, likely due to higher product concentrations. The hydrolysis reaction begins under hydrothermal conditions, with the release of glucose initiated early in the process **(Świątek *et al.*, 2020)**. The test results of reducing sugar content with variation of hydrolysis time are shown in Table (3).

Table 3. Results of reducing sugar content with variation of hydrolysis time

Run	Amount of substrate (grams)	Acid conc. (N)	Time (minutes)	Reducing sugar content (mg/ml)
6	2.5	0.01	10	2.48
8	2.5	0.01	30	4.33
14	2.5	1	10	8.04
3	2.5	1	30	18.77

Table (3) shows that at the same concentration of citric acid, reducing sugars have increased with the length of hydrolysis time and the amount of substrate used. The hydrolysis time period depends on the components that composed up the biomass. Compared to cellulose, hemicellulose was more rapidly hydrolyzed **(Świątek *et al.*, 2020)**.

Gracilaria sp. has no lignin in its cellular structure, which shortens the hydrolysis time compared to other cellulosic biomass (**Albuquerque *et al.*, 2021**). An extended hydrolysis duration enhances the frequency of molecular collisions among the reactants, thereby promoting a more extensive reaction and leading to an increased yield of the resulting products (**Mayasari & Sulaiman, 2022**). Previous studies have demonstrated that longer heating times can lead to an increased yield of reducing sugars. Extended heating during carbohydrate hydrolysis facilitates the breakdown of starch molecules, which contain bound water, into simpler sugar molecules (**Ardiansyah *et al.*, 2024**).

Optimum condition of *Gracilaria* sp. hydrolysis selected

Optimization of hydrolysis conditions involving three factors was analyzed using the RSM method. The analysis model suggested by RSM was Quadratic model. The involvement of the factors substrate amount, citric acid concentration, and hydrolysis time on reducing sugar content were presented in the following mathematical model equation:

$$RDS = 59.38 + 16.26X_1 + 4.87X_2 + 5.08X_3 + 3.50X_1X_2 - 1.95X_1X_3 + 2.22X_2X_3 - 4.59X_1^2 - 47.70X_2^2 - 3.28X_3^2$$

The accuracy of the mathematical model was shown through the R^2 values in Table (4). The coefficient of determination on reducing sugar production was 0.9050, indicating that 90.50% of the total variation in yield had represented in the model.

The model F-value in this study was 7.41, indicating that the model is significant. There is only a 0.75% chance that an F-value this large could occur due to random noise. *P*-values less than 0.0500 indicate that model terms are significant; in this case, X1 and X^2 were identified as significant terms. Values greater than 0.1000 indicate that model terms are not significant. These results suggest that the substrate amount and acid concentration are key determinants of process efficiency under the evaluated conditions. Validation of the recommended optimal conditions was performed in triplicate, and the results are presented in Table (5).

Table 4. ANOVA results on the test of reducing sugar content

Source	Sum of Squares	df	Mean Square	F-value	*P*-value	
Model	12550.59	9	1394.51	7.41	0.0075	significant
X_1-substrate	2115.88	1	2115.88	11.24	0.0122	
X_2-acid concentration	189.89	1	189.89	1.01	0.3487	
X_3-time of hydrolysis	206.51	1	206.51	1.10	0.3298	
X_1X_2	48.96	1	48.96	0.2600	0.6258	

X_1X_3	19.68	1	19.68	0.1045	0.7559	
X_2X_3	15.19	1	15.19	0.0807	0.7846	
X_2^2	88.85	1	88.85	0.4718	0.5143	
X_1^2	9579.60	1	9579.60	50.87	0.0002	
X_3^2	45.18	1	45.18	0.2399	0.6392	
Residual	1318.12	7	188.30			
Lack of Fit	965.92	3	321.97	3.66	0.1213	not significant
Pure Error	352.20	4	88.05			
Cor Total	13868.71	16				
R^2						0.9050

Fig. (1) shows the correlation between factors and response. The curved graph showed a significant relationship between the factors and the response. X_1X_2 graph shows the correlation between acid concentration and amount of substrate on RDS, and X_2X_3 shows a correlation between acid concentration and hydrolysis time on RDS. Thus, the concentration of citric acid played an important role in optimizing the production of biosugars, followed by number of substrates and time of hydrolysis.

Table 5. Results of validation of optimal conditions for hydrolysis of *Gracilaria* sp. with citric acid

Parameters		Value	
Optimum Condition	Amount of substrate	In range	3.209 g
	Acid concentration	In range	0.117 N
	Time of hydrolysis	In range	30 mins
Response	Reducing sugar	Maximize	
Validation	Predicted		32.117 mg/100 ml
	Actual		57.14±13.72 mg/100 ml

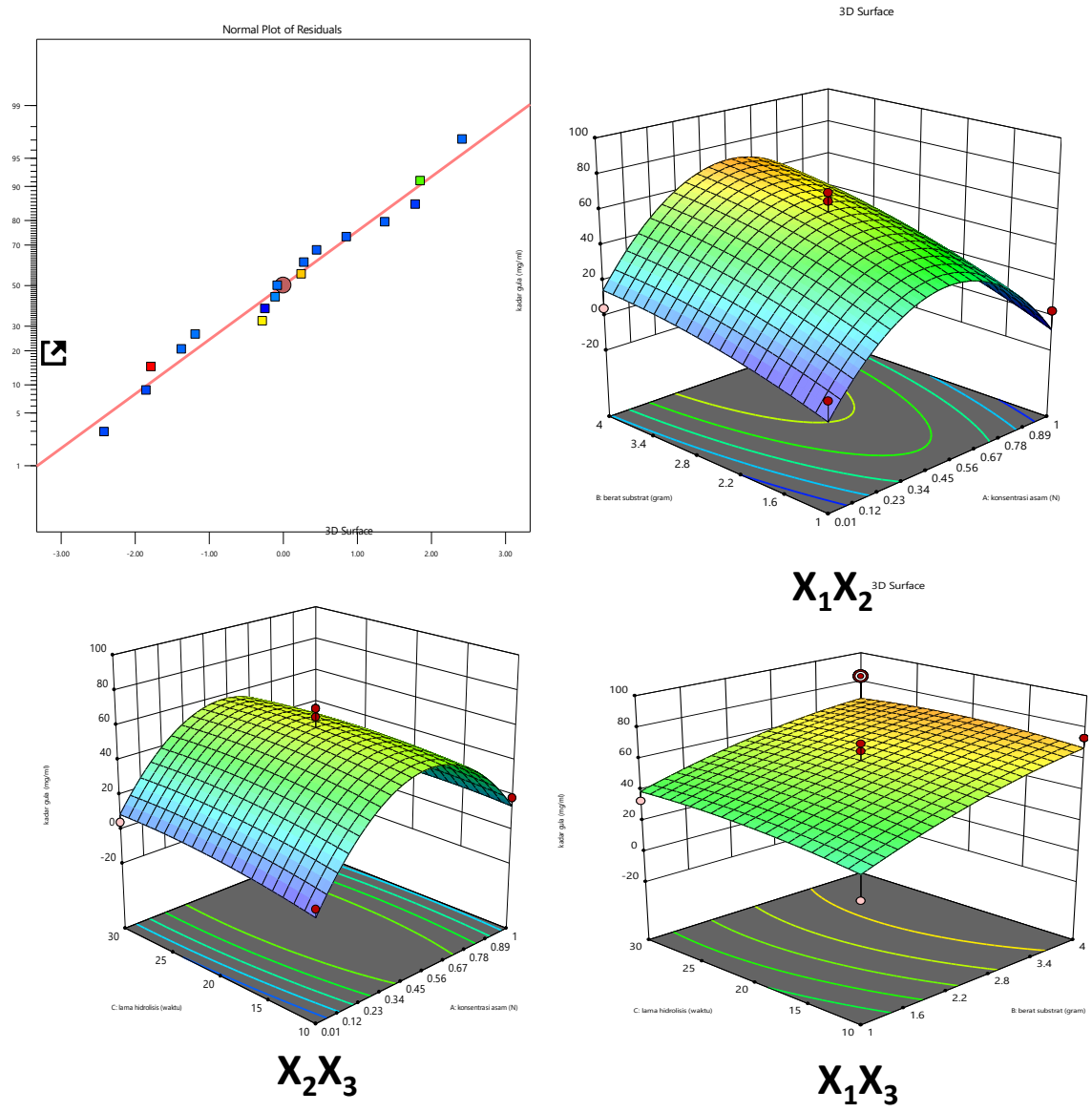


Fig. 1. Normal Plot of Residual and 3D Surface Graph of correlation between substrate amount (X_1), acid concentration (X_2), and hydrolysis time (X_3) on reducing sugar content

2. Profiling and characterization of selected *Gracilaria* sp. hydrolysate

Screening of Oligosaccharide Content

The hydrolysate obtained under the selected optimal conditions was further analyzed for saccharide content using the Thin Layer Chromatography (TLC). The presence of monosaccharides and by-products such as 5-hydroxymethylfurfural (5-HMF) was determined using High-Performance Liquid Chromatography (HPLC). As shown in Fig. (2), the HoG spot exhibited an Rf value and coloration closely resembling that of

fructooligosaccharides (FOS). For the oligosaccharide standards, the spots tended to show tailing.

The observed tailing of oligosaccharide spots in TLC analysis was likely due to strong hydrogen bonding interactions with the polar stationary phase, exacerbated by sample concentration and suboptimal mobile phase composition. In addition to its similar coloration to FOS, the HoG sample also exhibited an extended spot toward the upper region, which is presumed to be caused by the presence of monosaccharides such as glucose and galactose. These observations suggested that HoG contains both oligosaccharides and monosaccharides.

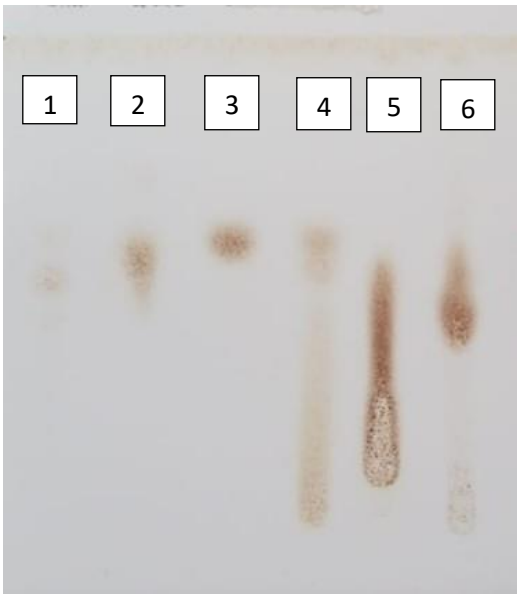


Fig. 2. Biosugar screening results using the TLC method. Spots 1 to 5 represent standards : (1) Maltose; (2) Glucose; (3) Galactose; (4) Inulin; (5) FOS, whereas spot 6 corresponds to the HoG sample

Hydrolysis products with Rf values greater than, equal to, or between those of glucose and maltose are most likely monosaccharides. In contrast, compounds with Rf values equal to or lower than that of maltose are strongly presumed to be oligosaccharides **(Song *et al.*, 2018)**. As presented in Table (6), the Rf value of HoG was 0.63, which is lower than that of maltose, indicating that oligosaccharides were the major constituents of the HoG sample. Oligosaccharides are indigestible carbohydrates in the human gastrointestinal tract but can be metabolized by gut probiotics, providing various health benefits, including immunomodulatory and antitumor activities **(Duarte *et al.*, 2017; Gao *et al.*, 2022)**.

Table 6. Retention Factor (Rf) value according to thin layer chromatogram result

Spot #	Substance	RF
1	Maltose	0.65
2	Glucose	0.68
3	Galactose	0.72
4	Inulin	0.53
5	FOS	0.5
6	HoG	0.63

Determination of Monosaccharides and by-product

Acid hydrolysis using low acid concentrations and weak acid can yield a range of saccharide molecules, from monosaccharides to oligosaccharides. As shown in Fig. (3), glucose and galactose were detected at concentrations of 0.356 mg/mL and 0.375 mg/mL respectively, along with a small amount of 5-hydroxymethylfurfural (5-HMF).

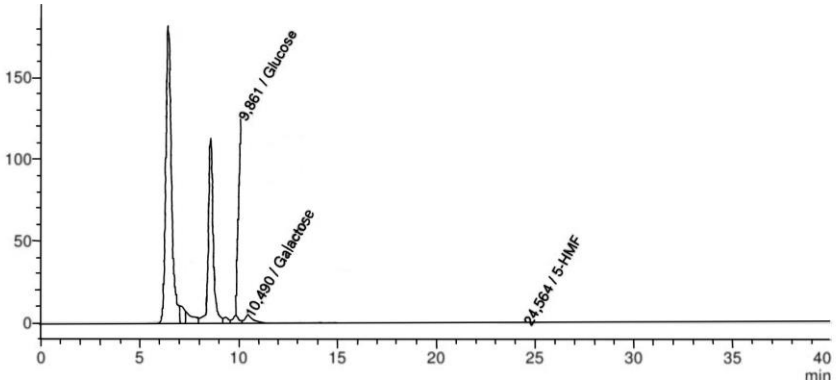


Fig. 3. Results of monosaccharide analysis chromatogram (glucose and galactose) and 5-HMF

In addition, monosaccharides were also produced. The acid hydrolysis method can also generate undesirable by-products such as 5-HMF, furfural, formic acid, and phenolic compounds. In this study, the 5-HMF content produced during hydrolysis was relatively low (0.026 mg/mL) (Table 7). Naturally, 5-HMF is found in honey and other food products. The European Food Safety Authority (EFSA) has established a maximum allowable concentration of furan as flavouring agent at 0.54 mg/day as part of quality control parameters **(Shapla *et al.*, 2018)**. The formation of 5-HMF can be minimized or prevented by controlling mild acid hydrolysis conditions. The use of weak acids, lower heating temperatures, and shorter hydrolysis durations can serve as an effective strategy to reduce the formation of 5-HMF and other by-products **(Padam *et al.*, 2023)**.

Table 7. Variations in the hydrolysis conditions of several types of seaweed on monosaccharide and HMF levels

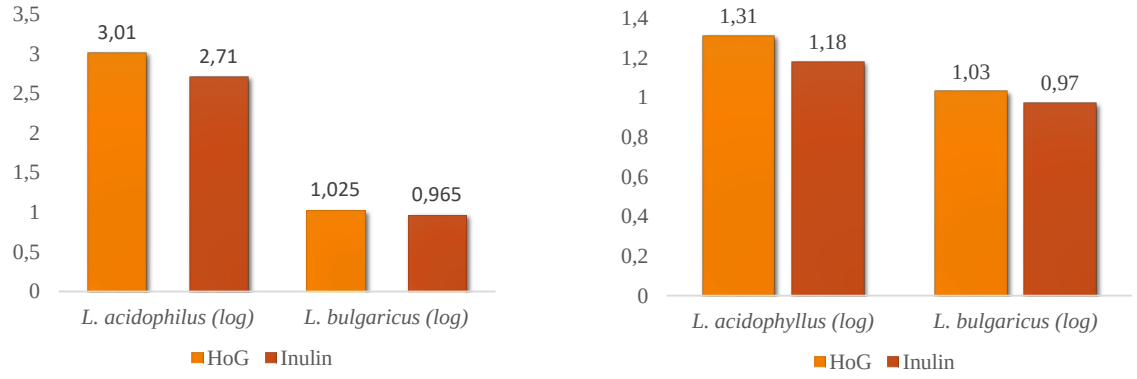
Substrate	Hydrolysis condition	Glucose (mg/mL)	Galactose (mg/mL)	HMF (mg/mL)	Reference
Gracilaria sp	Citric acid 0.117 N; 32.09g/L ; 30 min; 95^{o} C	0.356	0.375	0.026	current study
G. verrucosa	Sulphuric acid 1.92%; 66.6 g/L; 20 min; 160^{o}C	5.29	17.12	1.58	**Jeong *et al.*, 2015**
S. filiformis	Sulphuric acid 0.2 M; 50 g/L; 10 min. 121^{o} C.	2.36	8.34	0.33	**De Castro *et al.*, 2017**
E. denticulatum	Sulphuric acid 0.12 M; 21 min; 121^{o}C	NA	NA	1.11	**Padam *et al.*, 2023**

NA = Not available

5-Hydroxymethylfurfural (5-HMF) is a byproduct of the dehydration of hexose sugars under high-temperature conditions **(Mansuit *et al.*, 2015)**. Although 5-HMF is known to exhibit adverse biological effects, such as indirect mutagenicity, neoplastic transformation, and hepato-renal toxicities, it also possesses potential health benefits including antioxidant, anti-allergenic, and anti-hyperuricemic properties, as well as the ability to enhance survivability under hypobaric hypoxia **(Shapla *et al.*, 2018)**. 5-HMF is thermally unstable and can readily decompose into organic acids such as formic acid and levulinic acid at elevated temperatures. Certain by-products, including hydroxymethylfurfural (HMF) and levulinic acid, may inhibit cell growth and consequently reduce alcohol yield **(Sulfahri *et al.*, 2019)**. Therefore, critical factors, such as acid concentration and hydrolysis duration, must be carefully controlled during the hydrolysis process to minimize the formation of 5-HMF.

Determination of Prebiotic Effect and Prebiotic Index

The prebiotic effect and index are among the parameters used to determine whether oligosaccharides can be used as prebiotic, but not every oligosaccharide can function as prebiotic. Fig. (4) shows a bar chart illustrating the effects and values of the prebiotic index of HoG.



(A) (B)

Fig. 4. Bar chart showing the results of determining the prebiotic effect and the prebiotic index of HoG (A) Prebiotic effect (B) Prebiotic index

The prebiotic effect resulted in an increase in probiotic growth during 24 hours of incubation. However, this increase did not correlate with the prebiotic concentration. One of the primary characteristics of prebiotics is that they can be utilized by probiotics for their metabolic processes, similar to glucose **(Kustyawati *et al.*, 2022; Paramasivam *et al.*, 2023)**. In this study, lactic acid bacteria (LAB) strains, *Lactobacillus acidophilus* and *Lactobacillus bulgaricus* were employed. These strains are commonly used as starter cultures in yogurt production. *L. bulgaricus* is more frequently applied synergistically with *S. thermophilus*. *L. acidophilus* is also known for its role in various types of fermentation processes. Both *L. acidophilus* and *L. bulgaricus* are categorized as homofermentative lactic acid bacteria **(Setiarto *et al.*, 2017)**.

HoG supported the growth of both tested probiotics, particularly *L. acidophilus* with a growth increase of 3.01. HoG also enhanced the viability of *L. bulgaricus*, although the increase was not significant. These results indicate that both bacterial strains are capable of utilizing carbohydrates or biosugars present in HoG as energy sources.

The Prebiotic Index (PI) represents the ratio of probiotic bacterial growth in a prebiotic medium to that in a carbohydrate control medium. In this study, glucose was used as the reference carbohydrate in the growth media. According to **Huebner *et al.* (2007)**, a PI value greater than 1 indicates a positive effect on probiotic growth, whereas a value approaching 1 suggests low prebiotic effectiveness.

HoG exhibited PI values greater than 1 for both tested probiotic strains, indicating its potential as a prebiotic. This effect is attributed to the presence of complex carbohydrates in the biosugar component of HoG, which can serve as an energy source for probiotic growth. Commercial inulin was used as a reference prebiotic. However, the PI values of HoG were higher than those of inulin. the difference was not significant, less than 1 log CFU/mL. This slight difference may be related to the presence of complex saccharides in HoG, while the commercial inulin used consisted primarily of purified oligosaccharides.

However, although the result of effect and index prebiotic showed that HoG can be utilized by probiotics as a growth medium, it must be non-hydrolysable by enteric bacteria as well. A compound cannot be classified as a prebiotic if it promotes the growth of enteric bacteria. To qualify as a prebiotic, the substance must withstand acidic pH, bile salts, and digestive enzymes present in the gastrointestinal tract. Additionally, it should not be absorbed in the upper digestive system, but must reach the colon intact, where it can be selectively fermented by beneficial microbiota to support probiotic activity **(Huebner *et al.*, 2007; Kustyawati *et al.*, 2022)**. Therefore, further purification of the oligosaccharides in HoG is necessary to determine the prebiotic activity score and to ensure that HoG oligosaccharides can be reliably classified and used as a promising prebiotic.

CONCLUSION

Gracilaria sp. is recognized as a source of polysaccharides that can be converted into reducing sugars, which serve as valuable fermentation substrates. RSM modeling indicated optimal hydrolysis conditions at a biomass substrate of 3.209 g, citric acid concentration of 0.117 N, and a hydrolysis time of 30 minutes. Under these conditions, the hydrolysate contained both oligosaccharides and monosaccharides and exhibited a prebiotic index greater than 1, highlighting its potential as a fermentation substrate. This preliminary study demonstrates the feasibility of producing reducing sugars from *Gracilaria* sp., with opportunities for further improvement through oligosaccharide separation, enhanced purity, and evaluation of additional biofunctional properties.

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