



Optimization of Reducing Sugar Production from Red Algae Biomass through Acid Hydrolysis Using Response Surface Methodology

Ratu Zosa Marchanda¹, Rhafa Fathanaqi², Ni Ketut Sari^{3*}, and Silvana Dwi Nurherdiana⁴

^{1,2,3,4} Department of Chemical Engineering, Faculty of Engineering and Science, Universitas Pembangunan Nasional "Veteran" Jawa Timur, Indonesia

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CORRESPONDING AUTHOR

*E-mail: ketutsari.tk@upnjatim.ac.id

ABSTRACT

Red algae (*Gracilaria sp.*) have considerable potential as a renewable biomass resource due to their high polysaccharide content, which can be converted into reducing sugars as a feedstock for biofuel production. This study aimed to evaluate the effects of hydrolysis temperature and time on reducing sugar production and to determine the optimum hydrolysis conditions using Response Surface Methodology (RSM). The research variables consisted of hydrolysis temperatures of 100, 110, 120, 130, and 140°C and hydrolysis times of 20, 30, 40, 50, and 60 min. Prior to hydrolysis, the biomass was dried, milled, and delignified using 2% NaOH at 60°C for 3 h with stirring at 300 rpm. Acid hydrolysis was performed using 0.1 N H₂SO₄ under the specified operating conditions. Reducing sugar content was determined by the Nelson–Somogyi method using UV–Vis spectrophotometry. Data analysis was carried out using Response Surface Methodology (RSM), implemented through Design-Expert software. The results showed that both hydrolysis temperature and time significantly affected reducing sugar production. The highest reducing sugar concentration obtained experimentally was 46.57g/L at 120°C and 40 min. RSM optimization predicted an optimum condition at a hydrolysis temperature of 117.7°C and a hydrolysis time of 42.9 min, resulting in a reducing sugar content of 42.72g/L with a desirability value of 0.992. Model validation demonstrated good agreement between the predicted and experimental values, confirming the reliability of the developed model. These findings indicate that RSM is an effective tool for optimizing reducing sugar production from red algae hydrolysis.

Contribution to Sustainable Development Goals (SDGs):

SDG 7: Affordable and Clean Energy

SDG 9: Industry, Innovation and Infrastructure

SDG 12: Responsible Consumption and Production

SDG 13: Climate Action

1. INTRODUCTION

1.1. Research Background

Algae are a biomass resource with great potential for development as a feedstock for renewable energy industries [1]. As a maritime nation, Indonesia possesses abundant algae resources, particularly red algae (Rhodophyta), which are widely cultivated in various coastal regions. One type of red alga with

high potential is *Gracilaria sp.* because it contains large amounts of polysaccharides, such as cellulose and agar, which can be utilized as a source of simple sugars through hydrolysis [2]. The polysaccharide content in *Gracilaria sp.* is reported to reach 50–70% of the dry weight of the biomass, making it a potential feedstock for the production of bioethanol and other biochemicals [3].

Reducing sugars are compounds resulting from the breakdown of polysaccharides that possess free aldehyde or ketone groups, enabling them to act as reducing agents [4].



Reducing sugars, such as glucose, can be utilized as the primary substrate in the bioethanol fermentation process[5]. One of the commonly used methods for producing reducing sugars from lignocellulosic biomass is acid hydrolysis [6]. In this process, the glycosidic bonds in polysaccharides are broken using an acid catalyst, resulting in monosaccharides. Sulfuric acid (H_2SO_4) is widely used in the hydrolysis process because it is highly effective at accelerating the breakdown of polysaccharide chains into simple sugars [7].

1.2. Literature Review

Hydrolysis is a chemical process that involves the participation of water to cleave chemical bonds within a compound. This reaction is generally classified as a first-order reaction because water is present in excess, resulting in negligible changes in its concentration throughout the process [8]. In acid hydrolysis, an acidic solution is introduced to the glycosidic bonds of polysaccharides, promoting the cleavage of these bonds and converting complex carbohydrates into simpler monosaccharides, such as glucose and galactose [9]. The acid acts as a catalyst by increasing the availability of hydronium ions, which facilitate the hydrolytic breakdown of glycosidic linkages and accelerate the reaction rate [10]. Various acids are commonly employed in hydrolysis processes, including acetic acid, phosphoric acid, hydrochloric acid, and sulfuric acid. The hydrolysis rate generally increases with increasing acid concentration, resulting in more efficient polysaccharide depolymerization and sugar release [8]. Therefore, the kinetics and efficiency of the hydrolysis process are strongly influenced by both the type and concentration of the acid used

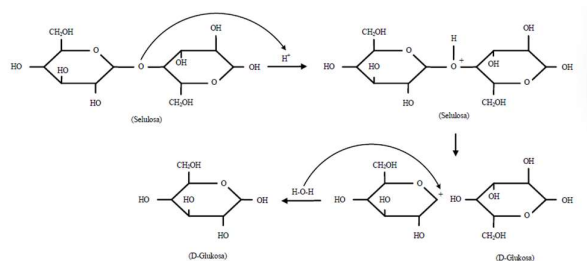


Fig 1. Acid-catalyzed hydrolysis mechanism (Source : [11])

The success of the hydrolysis process is influenced by several factors, including temperature, reaction time, acid concentration, acid type, particle size, and biomass-to-acid ratio[6]. Higher temperatures generally enhance hydrolysis by increasing molecular kinetic energy and facilitating glycosidic bond cleavage; however, excessive temperatures may lead to sugar degradation and the formation of undesirable by-products [12]. Similarly, extending the reaction time can improve polysaccharide conversion into monosaccharides, although prolonged hydrolysis may reduce sugar yield due to secondary degradation reactions [10]. Acid concentration also plays a critical role, as higher concentrations accelerate hydrolysis, but excessive levels can promote side reactions and product deterioration. The type of acid used affects hydrolysis efficiency because different acids provide varying concentrations of H^+ ions, which catalyze glycosidic bond cleavage. In addition, smaller particle sizes provide a larger surface area, improving reagent accessibility and enhancing hydrolysis efficiency. Finally, the biomass-to-acid ratio influences the extent of substrate contact with the acid solution; an appropriate ratio ensures effective

penetration of the acid into the biomass matrix and maximizes reducing sugar production [6].

However, excessively high conditions can cause glucose to degrade into other compounds such as furfural and hydroxymethylfurfural (HMF), thereby reducing the reducing sugar content [12]. Therefore, optimal operating conditions are required to ensure the hydrolysis process proceeds effectively and yields the maximum reducing sugar content. Previous studies have shown that acid hydrolysis of red algal biomass can produce a sufficiently high amount of reducing sugars. [13] reported that the hydrolysis of *Gracilaria sp.* using H_2SO_4 produced the highest glucose content at 121°C . Additionally, [14] stated that temperature and reaction time significantly influence the formation of sugars resulting from algal biomass hydrolysis. Nevertheless, research on optimizing hydrolysis conditions for red algae using variations in temperature and time still needs to be developed to achieve more effective process conditions. In addition, red algae contain higher polysaccharide levels than proteins, minerals, and lipids, making them an attractive feedstock for sugar production [3]. Previous studies reported that acid hydrolysis performance is strongly affected by acid concentration, reaction time, and biomass loading, which significantly influence glucose and galactose production[14]. Studies [13] reported that the highest glucose concentration from *Gracilaria sp.* hydrolysis was obtained at 120°C using 0.1 N H_2SO_4 , highlighting the importance of temperature optimization. Furthermore, *Gracilaria sp.* is widely cultivated and has considerable potential as a sustainable biomass resource for bioethanol production and other renewable-energy applications.

1.3. Research Objective

This study aimed to investigate the production of reducing sugars from red algae by acid hydrolysis as affected by hydrolysis temperature and reaction time, and to determine the optimum conditions for maximizing reducing sugar concentration and yield using Response Surface Methodology (RSM).

2. MATERIALS AND METHODS

2.1. Materials

The materials used in this study were red algae (*Gracilaria sp.*), H_2SO_4 0.1 N, NaOH, aquadest, Nelson reagent, arsenomolybdate reagent, and glucose standard solution. The equipment used in this study included an oven, grinder, analytical balance, autoclave, filter paper, pH meter, refractometer, and UV-Vis spectrophotometer.\

2.2. Sample Preparation

The red algae were washed using distilled water to remove impurities and dried in an oven at 105°C until constant weight was obtained. The dried algae were ground and sieved to obtain uniform particle size.

2.3. Alkaline Pretreatment

Although *Gracilaria sp.* generally contains lower lignin than terrestrial lignocellulosic biomass, alkaline pretreatment was employed to modify the biomass structure by removing alkali-soluble components and improving polysaccharide accessibility prior to acid hydrolysis[15]. The chemical composition of the

biomass, including cellulose, hemicellulose, and lignin contents, was determined before and after alkaline pretreatment using the Chesson–Datta method [16]. The compositional analysis was conducted to evaluate the effect of alkaline pretreatment on the structural composition of the biomass prior to acid hydrolysis. Alkaline treatment of the algae powder was carried out using a 2% (w/v) NaOH solution at a solid-to-liquid ratio of 1:20, aimed at improving biomass accessibility for acid hydrolysis by partially removing alkali-soluble components and enhancing polysaccharide accessibility. The alkaline pretreatment was carried out at 60°C for 3 h under continuous stirring at 300 rpm. After alkaline pretreatment, the samples were washed with distilled water until reaching neutral pH and subsequently dried in an oven.

2.4. Acid Hydrolysis Process

Acid hydrolysis was performed using 0.1 N H₂SO₄ at a solid-to-liquid ratio of 1:15, with hydrolysis temperatures ranging from 100 to 140°C and hydrolysis times ranging from 20 to 60 min. Following hydrolysis, the resulting hydrolysate was filtered and collected for reducing sugar analysis.

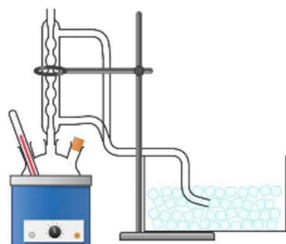


Fig 2. Acid Hydrolysis Apparatus Setup

3. RESULT AND DISCUSSION

3.1. Characteristics of Red Algae Biomass

Although *Gracilaria* sp. generally contains lower lignin than terrestrial lignocellulosic biomass [15], alkaline pretreatment was applied to modify the biomass structure, remove alkali-soluble components, and improve cellulose accessibility for subsequent acid hydrolysis. The alkaline pretreatment was performed using a 2% NaOH solution at 60°C for 3 h under continuous stirring at 300 rpm. The chemical composition of the biomass before and after alkaline pretreatment was determined using the Chesson–Datta method, As shown in Table 1.

Table 1. Raw Material Characterization Results

Delignification	Lignin (%)	Selulosa (%)	Hemisululosa (%)
Before	10.28	24.53	10.25
After	8.24	42.25	13.25

Based on Table 1, alkaline pretreatment altered the chemical composition of the biomass. The lignin content of the *Gracilaria* sp. sample analyzed in this study decreased from 10.28% to 8.24%, while the cellulose content increased from 24.53% to 42.25%. According to [15], alkaline pretreatment can modify the biomass structure by removing alkali-soluble components, increasing biomass porosity, and improving the

accessibility of structural polysaccharides during acid hydrolysis. The compositional values reported in this study represent the characteristics of the analyzed biomass as determined using the Chesson–Datta method.

Hydrolysis of red algae biomass was carried out at temperatures of 100, 110, 120, 130, and 140°C with hydrolysis times of 20, 30, 40, 50, and 60 min to evaluate their effects on reducing sugar production. The process was performed using 0.1 N H₂SO₄ solution with a solid-to-liquid ratio of 1:15 under continuous stirring at 300 rpm. The hydrolysate obtained from each experimental condition was subsequently analyzed for reducing sugar content using UV–Vis spectrophotometry to determine the influence of process variables and identify the optimum hydrolysis condition as shown in Table 2.

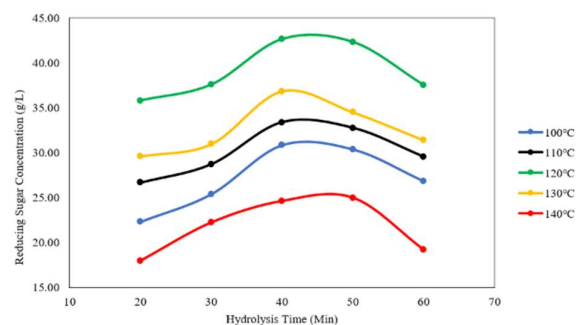


Fig 3. Graph of the Relationship Hydrolysis Time (Minutes) and Reducing Sugar Concentration (g/L) in UV-Vis Spectrophotometric Analysis

Based on Figure 3, hydrolysis time significantly affected the concentration of reducing sugars produced at each hydrolysis temperature. In general, the reducing sugar concentration increased with increasing hydrolysis time until reaching an optimum condition at 40 minutes, after which it decreased at longer reaction times. At 100°C, the reducing sugar concentration increased from 22.35 g/L at 20 minutes to 30.86 g/L at 40 minutes before slightly decreasing to 26.86 g/L at 60 minutes. A similar trend was observed at 110°C and 120°C. The most significant increase occurred at 120°C, where the reducing sugar concentration rose from 35.82 g/L at 20 minutes to 42.66 g/L at 40 minutes, which was the highest value obtained in this study. This result indicates that longer contact time between the acid catalyst and substrate promoted the depolymerization of red algae polysaccharides into reducing sugars. The highest reducing sugar concentration was obtained at 120°C and 40 minutes, reaching approximately 42.66 g/L. However, the difference between 40 and 50 minutes was relatively small, indicating that the optimum hydrolysis time was within the range of 40–50 minutes.

Beyond the optimum hydrolysis time, the reducing sugar concentration decreased at all temperatures due to the degradation of sugars into by-products such as 5-hydroxymethylfurfural (5-HMF), furfural, and other degradation compounds. This decrease was particularly evident at 140°C, where prolonged hydrolysis is consistent with degradation reactions described in the literature [17]. The results also showed that reducing sugar concentration increased with increasing hydrolysis temperature and time up to the optimum condition of 120°C and 40 minutes, reaching a maximum concentration of 42.66 g/L, before declining at more severe operating conditions. These findings are consistent with those reported by [13], who obtained an optimum reducing sugar

concentration of 57.87 g/L at 121°C and 15 min using H₂SO₄ hydrolysis combined with 15% *G. verrucosa*, likely due to the additional enzymatic hydrolysis step, which enhanced polysaccharide conversion into fermentable sugars more effectively than the acid hydrolysis process employed in the present study.

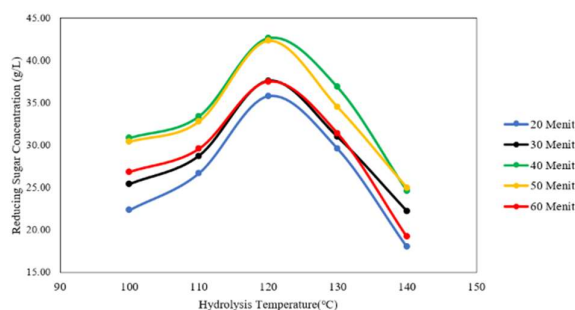


Fig 4. Graph of the Relationship Hydrolysis Temperature (°C) and Reducing Sugar Concentration (g/L) in UV-Vis Spectrophotometric Analysis

Based on Figure 4, hydrolysis temperature significantly affected the concentration of reducing sugars produced. In general, the reducing sugar concentration increased with increasing temperature from 100°C to an optimum temperature of 120°C, after which it decreased at higher temperatures. At a hydrolysis time of 20 minutes, the reducing sugar concentration increased from 22.35 g/L at 100°C to 35.82 g/L at 120°C, then decreased to 29.63 g/L at 130°C and 18.01 g/L at 140°C. A similar trend was observed at other hydrolysis times. The increase in reducing sugar concentration up to 120°C indicates that higher temperatures enhanced the cleavage of glycosidic bonds in red algae polysaccharides, thereby promoting the formation of reducing sugars. The highest reducing sugar concentration was obtained at 120°C and 40 minutes of hydrolysis, reaching 42.66 g/L, suggesting that the rate of sugar formation was still greater than the rate of degradation under these conditions.

Beyond the optimum condition, the reducing sugar concentration decreased, which may be attributed to degradation of sugars into 5-hydroxymethylfurfural (5-HMF), furfural, and other degradation compounds, as reported by [18]. Therefore, the optimum hydrolysis condition in this study was achieved at 120°C and 40 minutes. These results differ from those reported by [19], who found that red algae hydrolysis using the solid-acid catalyst Amberlyst-15 at 140°C for 150 minutes produced the highest reducing sugar concentration of 61 g/L. This discrepancy is likely attributable to differences in catalyst type and operating conditions. Amberlyst-15 possesses a higher density of active acidic sites and a larger active surface area, resulting in more effective polysaccharide depolymerization than hydrolysis using 0.1 N H₂SO₄. Furthermore, the longer reaction time allowed more extensive galactose release from red algae polysaccharides, although it also promoted the formation of degradation products such as 5-HMF and levulinic acid [19].

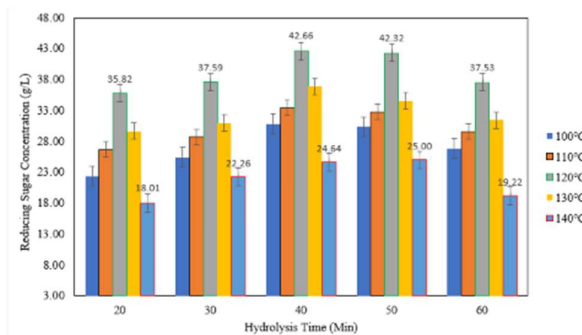


Fig 5. Graph of the Relationship Hydrolysis Time (Minutes) and Reducing Sugar Concentration (g/L) in UV-Vis Spectrophotometric Analysis with Error bar

Based on Figure 5, the concentration of reducing sugars was influenced by both hydrolysis temperature and reaction time. At all temperatures investigated, the reducing sugar concentration generally increased with increasing hydrolysis time up to 40 minutes. This trend indicates that a longer contact time between the acid catalyst and red algae polysaccharides promoted the cleavage of glycosidic bonds, resulting in a greater release of reducing sugars. The highest reducing sugar concentration was obtained at 120°C and 40 minutes, reaching 42.66 g/L. Beyond this condition, the reducing sugar concentration gradually decreased with further increases in hydrolysis time. A similar trend was observed at 100°C, 110°C, 130°C, and 140°C. The decline in reducing sugar concentration after the optimum condition suggests that some of the sugars may have undergone secondary degradation reactions producing compounds such as 5-hydroxymethylfurfural (5-HMF), furfural, levulinic acid, and other degradation products, based on mechanisms reported elsewhere [20] although not directly measured here.

The error bars presented in Figure 4, represent the variability among experimental replicates. In general, the error bars were relatively small compared to the corresponding mean values, indicating good repeatability and consistency of the experimental results. Furthermore, the relatively uniform size of the error bars across all treatments suggests that the hydrolysis and analytical procedures were performed under stable and controlled conditions. At the optimum condition of 120°C and 40 minutes, the error bar was also relatively small, indicating that the reported reducing sugar concentration is representative of the experimental observations. Therefore, the observed trends in reducing sugar concentration can be attributed primarily to the effects of hydrolysis temperature and reaction time rather than to experimental variability.

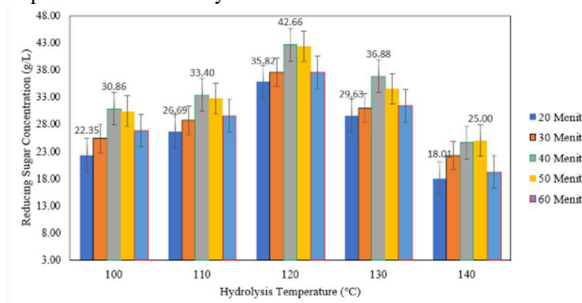


Fig 6. Graph of the Relationship Hydrolysis Temperature (°C) and Reducing Sugar Concentration (g/L) in UV-Vis Spectrophotometric Analysis with Error bar.

Based on Figure 6, hydrolysis temperature significantly affected the concentration of reducing sugars produced at different hydrolysis times. In general, the reducing sugar concentration increased as the hydrolysis temperature increased from 100°C to 120°C, followed by a decrease at higher temperatures. At a hydrolysis time of 20 minutes, the reducing sugar concentration increased from 22.35 g/L at 100°C to 35.82 g/L at 120°C before decreasing to 29.63 g/L at 130°C and 18.01 g/L at 140°C. A similar trend was observed at hydrolysis times of 30, 40, 50, and 60 minutes. The highest reducing sugar concentration was obtained at 120°C and 40 minutes, reaching 42.66 g/L. This result indicates that increasing temperature enhanced the hydrolysis of red algae polysaccharides by accelerating the cleavage of glycosidic bonds, thereby increasing the release of reducing sugars. However, temperatures above 120°C led to a decline in reducing sugar concentration, suggesting that sugar degradation reactions became more dominant under more severe hydrolysis conditions.

The error bars shown in Fig 6 represent the variability among experimental replicates. In general, the error bars were relatively small compared to the mean reducing sugar concentrations, indicating good repeatability and consistency of the experimental results. The relatively uniform magnitude of the error bars across all treatments suggests that the hydrolysis and analytical procedures were conducted under stable conditions with limited experimental variation. Furthermore, the differences in reducing sugar concentration observed between temperatures, particularly between 120°C and 140°C, were substantially greater than the corresponding error ranges, indicating that the observed trends were mainly associated with the effect of hydrolysis temperature rather than experimental variability. At the optimum condition of 120°C and 40 minutes, the error bar was also relatively small, demonstrating that the measured reducing sugar concentration was representative and reproducible.

To further evaluate the performance of the hydrolysis process, the optimum reducing sugar concentration obtained in this study was compared with those reported in previous studies using different red algal biomasses and hydrolysis methods. This comparison provides an overview of the effectiveness of the present process and highlights the influence of biomass type, hydrolysis conditions, and catalyst on reducing sugar production. The comparison is presented in Table 2.

Table 2. Comparison of Optimum Hydrolysis Conditions and Reducing Sugar Concentrations Reported in Previous Studies

No	Biomass	Parameter	Reducing Sugar	Refrence
1	Red Algae (<i>Gracilaria sp</i>)	121°C, 60 min	11.85 g/L	[21]
2	<i>Kappaphycus alvarezii</i>	110°C, 90 min	49.92 g/L	[22]
3	<i>Gracilaria verrucosa</i>	140°C, 150 min	61 g/L	[19]
4	<i>Eucheuma denticulatum</i>	160°C, 10 min	51.47 g/L	[23]
5	Red Algae (<i>Gracilaria sp</i>)	120°C, 40 min	46.57g/L	This Study

As shown in Table 2, the optimum reducing sugar concentration obtained in the present study was 46.57 g/L at a hydrolysis temperature of 120°C for 40 min, which was higher than that reported by [21](11.85 g/L) but slightly lower than those reported by [22] (49.92 g/L), [23] (51.47 g/L), and [19] (61.00

g/L). These differences are mainly attributed to variations in biomass species, hydrolysis methods, catalyst type, acid concentration, and operating conditions. Previous studies employed more intensive approaches, including acid hydrolysis combined with enzymatic hydrolysis and solid-acid catalysts, which enhanced polysaccharide conversion into fermentable sugars. In contrast, the present study used only 0.1 N H₂SO₄ and still achieved a relatively high reducing sugar concentration under moderate operating conditions. This result indicates that the hydrolysis condition of 120°C for 40 min was sufficient to maximize polysaccharide conversion while minimizing excessive degradation of reducing sugars into by-products such as 5-hydroxymethylfurfural (5-HMF), demonstrating the effectiveness of the proposed hydrolysis process

3.2. Optimization of Reducing Sugar Production Using Response Surface Methodology (RSM)

The optimization of red algae hydrolysis was conducted to determine the optimum operating conditions for maximizing reducing sugar content. Response Surface Methodology (RSM) with a Central Composite Design (CCD) was employed to evaluate the effects of independent variables, namely hydrolysis temperature (100–140°C) and hydrolysis time (20–60 min), on the response variable, namely reducing sugar concentration (g/L). Statistical analysis was performed using Design-Expert 13 software. The initial stage involved 13 experimental runs to evaluate the response across different combinations of operating conditions. The experimental data for factors and responses obtained from red algae hydrolysis are presented in Table 3.

Table 3. Experimental Design Matrix of Response Surface Methodology (RSM) with 13 Data Points

Run	Factor 1: Hydrolysis Temperature (°C)	Factor 2: Hydrolysis Time (min)	Response: Reducing Sugar Concentration (g/L)
1	100	20	22.35
2	100	40	30.86
3	100	60	26.86
4	110	20	31.65
5	120	40	42.65
6	120	50	42.32
7	120	60	37.53
8	130	20	29.62
9	130	40	36.87
10	130	60	37.53
11	140	20	18.01
12	140	40	24.6
13	140	60	19.21

The experimental data were entered into Design-Expert 13 software to evaluate the relationship between the independent variables (hydrolysis temperature and time) and the response variable (reducing sugar concentration). The data were subsequently analyzed using Analysis of Variance (ANOVA) to determine the significance of the model and process variables. Furthermore, the most appropriate regression model was selected to develop the predictive equation based on several statistical

criteria, including the Sequential p-value, Lack of Fit, and coefficient of determination (R^2). The model exhibiting the best statistical performance was chosen to accurately represent the relationship between the process variables and the response. The summary of model adequacy testing is presented in Table 4.

Table 4. Comparison of Regression Models Recommended by the Software

Source	Sequential p-value	Adjusted R^2	Predicted R^2	
Linear	0.5604	-0.0688	-0.5453	
2FI	0.8527	-0.1827	-18.428	
Quadratic	< 0.0001	0.9972	0.9950	Suggested
Cubic	0.4189	0.9977		
Quartic				<i>Aliased</i>

Based on Table 3, the quadratic model was selected as the best model to represent the relationship between process variables and reducing sugar content. This was supported by a significant sequential p-value (< 0.0001), indicating that the model was statistically significant. In addition, the model showed high predictive performance, with an Adjusted R^2 of 0.9972 and a Predicted R^2 of 0.9950, both close to 1, indicating good agreement between predicted and experimental values. The Coefficient of Variation (C.V.) was 1.41%, indicating very low variability and confirming excellent precision and reliability of the experimental data. The Adequate Precision ratio was 85.374, substantially exceeding the minimum desirable threshold of 4, indicating a strong signal-to-noise ratio and confirming that the model can be reliably used to navigate the design space. According to [24], an R^2 value close to 1 indicates a strong relationship between independent variables and the observed response. In contrast, the linear and 2FI models were not significant ($p\text{-value} > 0.05$) and showed negative Adjusted R^2 and Predicted R^2 values, indicating poor model performance. Meanwhile, the quartic model was identified as aliased and could not be applied. After establishing the predictive quadratic model, ANOVA was employed to examine the statistical significance of the regression model and the contribution of each factor to reducing sugar content. The results are presented in Table 5

Table 5. Analysis of Variance (ANOVA) for the Quadratic Model of Reducing Sugar Concentration

Source	Sum of Squares	dF	Mean Square	F-Value	P-Value	
Model	776.32	5	155.26	854.88	< 0.0001	<i>significant</i>
A-Suhu	65.49	1	65.49	360.61	< 0.0001	
B-Waktu	18.42	1	18.42	101.44	< 0.0001	
AB	3.81	1	3.81	20.95	0.0026	
A ²	508.42	1	508.42	2799.38	< 0.0001	
B ²	116.26	1	116.26	640.13	< 0.0001	
Residual	1.27	7	0.1816			
Total	777.59	12				

Based on Table 4, the quadratic model was highly significant, as indicated by an F-value of 854.88 and a p-value < 0.0001 , demonstrating that the model adequately describes the relationship between hydrolysis variables and reducing sugar content. Both hydrolysis temperature (A) and time (B)

significantly affected the response ($p < 0.0001$). The interaction between temperature and time (AB) was also significant ($p = 0.0026$), indicating that the effect of one factor depends on the level of the other factor. Furthermore, the quadratic terms of temperature (A²) and time (B²) were highly significant ($p < 0.0001$), suggesting the presence of an optimum condition within the studied range. The low residual value compared with the model sum of squares indicates a good fit between the experimental and predicted data. The quadratic regression equation generated by the software was used to describe the relationship between the independent variables and the response.

$$Y = -502.28 + 8.66X_1 + 1.64X_2 - 0.0023X_1X_2 - 0.0363X_1^2 - 0.0162X_2^2$$

Where Y represents the reducing sugar content, X_1 represents hydrolysis temperature ($^{\circ}\text{C}$), and X_2 represents hydrolysis time (min). The positive coefficients of hydrolysis temperature (X_1) and hydrolysis time (X_2) indicate a direct relationship with reducing sugar content, suggesting that increasing these variables tends to enhance sugar formation within the studied range. However, the negative quadratic coefficients of temperature (X_1^2) and time (X_2^2) imply the presence of optimum operating conditions, beyond which reducing sugar content may decrease. This decline may occur due to sugar degradation under excessive hydrolysis conditions, such as prolonged heating or high temperature. Furthermore, the relatively small negative interaction coefficient (X_1X_2) indicates that the combined effect of temperature and time on reducing sugar content is less pronounced compared to their individual effects.

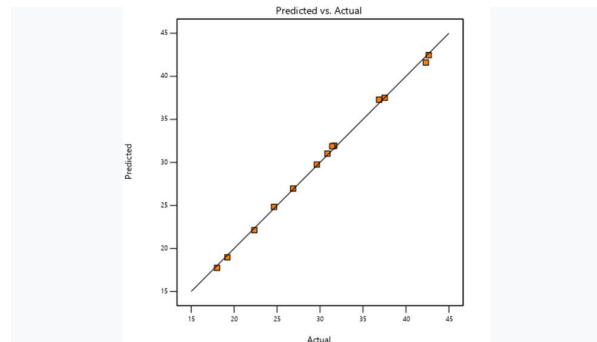


Fig 7. Comparison of Reducing Sugar Concentration Response Predicted vs. Actual

Figure 7 illustrates the correlation between the experimental and model-predicted values. A closer distribution of data points to the reference line indicates a higher degree of agreement between the observed and predicted responses, reflecting the adequacy of the developed model.

Model adequacy was further verified through residual diagnostic plots. The normal probability plot of residuals (Fig. 8) shows that the residuals were distributed approximately along a straight line, indicating that the normality assumption was satisfied and supporting the validity of the ANOVA results. Additionally, the plot of residuals versus predicted values (Fig. 9) shows a random scatter of points within the control limits (± 4.56), with no discernible funnel-shaped pattern or systematic trend. This indicates constant variance (homoscedasticity) across the range of predicted values, confirming that the quadratic model adequately captured the relationship between the process

variables and reducing sugar concentration without systematic bias.

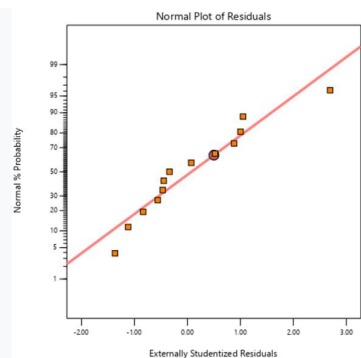


Fig 8. Normal Probability Plot of Residuals for Reducing Sugar Concentration

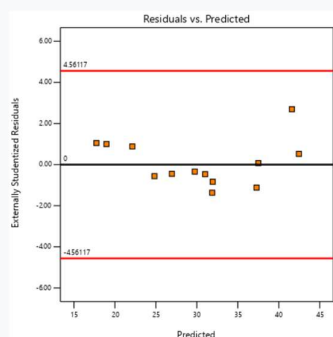


Fig 9. Plot of Residuals versus Predicted Values for Reducing Sugar Concentration

The response surface plots depicting the combined effects of the independent variables on the response are presented in Figures 10 and 11.

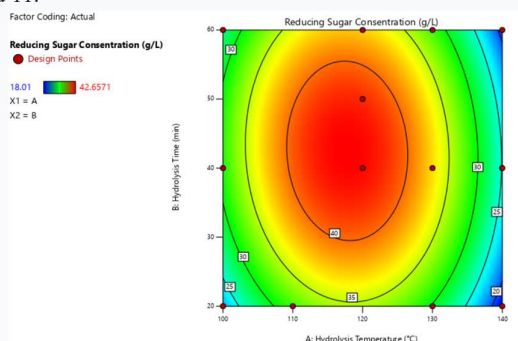


Fig 10. Contour Plot of the Effects of Hydrolysis Temperature(°C) and Hydrolysis Time (min) on Reducing Sugar Concentration (g/L)

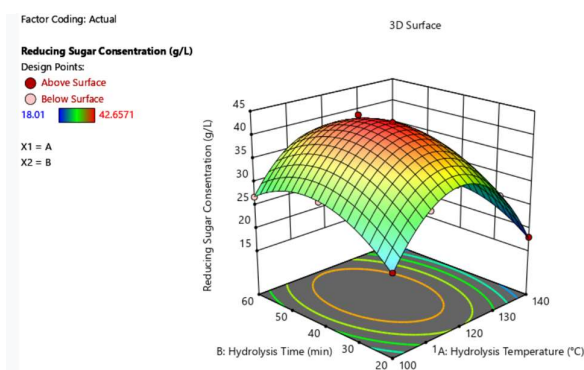


Fig 11. 3D Plot of the Effects of Hydrolysis Temperature(°C) and Hydrolysis Time (min) on Reducing Sugar Concentration (g/L)

Contour and three-dimensional (3D) response surface plots were generated to visualize the synergistic effects of hydrolysis temperature and time on reducing sugar concentration. The contour plot depicts the model-predicted response in two dimensions, while the 3D surface plot provides a more detailed understanding of the interaction between the operating variables and the response. Color variations represent the magnitude of reducing sugar concentration, with darker red shades indicating higher concentration values. As shown in Figures 8 and 9, the maximum reducing sugar concentration of 42.66 g/L was obtained at a hydrolysis temperature of 120 °C and a hydrolysis time of 40 min. The optimum operating conditions for maximizing reducing sugar concentration were determined through numerical optimization in Design-Expert software, based on the 13 experimental runs generated by the Central Composite Design (CCD). The optimization criteria and predicted optimum conditions are presented in Table 6

Table 6. Factor and Response Constraints for Optimization

Name	Goal	Lower Limit	Upper Limit
Hydrolysis Temperature (°C)	is in range	100	140
Hydrolysis Time (min)	is in range	20	60
Reducing Sugar Concentration (g/L)	maximize	18.01	42.66

Prediction intervals of the optimized model was conducted to evaluate the agreement between the model-predicted values and the actual reducing sugar concentration (g/L) obtained from the hydrolysis of red algae. This step aimed to assess the accuracy and reliability of the Response Surface Methodology (RSM) model in predicting the response. The optimum condition was determined using the desirability approach within the process variable ranges of hydrolysis temperature (100–140°C) and hydrolysis time (20–60 min). The optimal conditions for maximizing reducing sugar concentration, as determined by the optimization analysis, are presented in Table 6.

Table 7. Response Optimization Results

Hydrolysis Temperature (°C)	Hydrolysis Time (min)	Reducing Sugar Concentration (g/L)	Desirability
117.7	42.9	42.72	0.992

Based on Table 6, the optimization results indicated that the optimum condition was achieved at a hydrolysis temperature of 117.7 °C and a hydrolysis time of 42.9 min, resulting in a reducing sugar concentration of 42.72g/L. The desirability value of 0.992 indicates excellent agreement with the optimization target, confirming this condition as the optimum point for maximizing reducing sugar concentration. Furthermore, the verification results demonstrated that the RSM model was able to predict the reducing sugar concentration accurately, as shown in the Table 8

Table 8. Confidence Interval Verification

Response	95% CI Low	95% CI High	95% TI Low	95% TI High
Concentration				
Reducing Sugar (g/L)	42.12	43.23	40.26	45.09

Table 9 Prediction Interval Verification Results

Response	n	SE Pred	95% PI Low	95% PI High
Concentration				
Reducing Sugar (g/L)	1	0.489	41.46	43.77

Based on Table 9, the prediction intervals of the optimized model results showed at a 95% confidence level, the Confidence Interval (CI) ranged from 42.12% to 43.23%, indicating the estimated range of the actual mean reducing sugar concentration. Meanwhile, the wider Tolerance Interval (TI) range of 40.26% to 45.09% reflects overall data variability, while confirming that the model provided reliable predictions under optimum conditions. Based on the table 8, the 95% Prediction Interval (PI) ranged from 41.46% to 43.77%, indicating that future experimental results are expected to fall within this range at a 95% confidence level. Compared to the Confidence Interval (CI), the wider PI reflects not only the estimated mean response but also the variability of future observations. Therefore, the PI can be used to assess the reliability of the predictive model and estimate the expected response range under the optimum conditions obtained from the optimization process.

4. CONCLUSION

This study demonstrated that hydrolysis temperature and time significantly affected reducing sugar production from red algae (*Gracilaria* sp.) through acid hydrolysis using 0.1 N H₂SO₄. Increasing temperature and hydrolysis time improved polysaccharide conversion and reducing sugar formation up to an optimum condition, while excessive conditions caused sugar degradation. The highest reducing sugar concentration was obtained at 120°C for 40 min, reaching 46.57 g/L. Optimization using Response Surface Methodology (RSM) identified the optimum condition at 117.7°C and 42.9 min, with a predicted reducing sugar concentration of 42.72 g/L and a desirability value of 0.992. These findings suggest that *Gracilaria* sp. has considerable potential as a feedstock for reducing sugar production and subsequent bioethanol applications. One limitation of this study is that the optimum conditions predicted by the RSM model were not experimentally validated due to biomass availability constraints after completion of the

experimental work. Therefore, future studies should perform confirmation experiments at the predicted optimum conditions to compare the predicted and experimental responses and further validate the developed model.

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