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Sequential Hydrothermal HCl Pretreatment and Enzymatic Hydrolysis of *Saccharina japonica* Biomass

Eun-Young Park [1,†] and Jung-Kyu Park [2,*,†]

[1] Rinascita College of Liberal Arts and Sciences, Shinhan University, Uijeongbu-si 11644, Korea; eypark@shinhan.ac.kr
[2] Department of Computer Software Engineering, Changshin University, Changwon-si 51352, Korea
* Correspondence: jkpark@cs.ac.kr; Tel.: +82-55-250-1312
† These authors contributed equally to this work.

Abstract: This study investigated the production of fermentable sugars from carbohydrate-rich macroalgae *Saccharina japonica* using sequential hydrolysis (hydrothermal acid pretreatment and enzymatic hydrolysis) to determine the maximum reducing sugar yield (RS_y). The sequential hydrolysis was predicted by three independent variables (temperature, time, and HCl concentration) using response surface methodology (RSM). Enzymatic hydrolysis (8.17% $v/w_{biomass}$ Celluclast® 1.5 L, 26.4 h, 42.6 °C) was performed after hydrothermal acid pretreatment under predicted conditions (143.6 °C, 22 min, and 0.108 N HCl concentration). Using this experimental procedure, the yields of hydrothermal acid pretreatment, enzymatic hydrolysis, and sequential hydrolysis were 115.6 ± 0.4 mg/g, 117.7 ± 0.3 mg/g, and 183.5 ± 0.6 mg/g, respectively. Our results suggested that sequential hydrolysis of hydrothermal acid pretreatment and enzymatic hydrolysis was more efficient than their single treatment.

Keywords: sequential hydrolysis; hydrothermal acid pretreatment; enzymatic hydrolysis; reducing sugar yield; biomass

Citation: Park, E.-Y.; Park, J.-K. Sequential Hydrothermal HCl Pretreatment and Enzymatic Hydrolysis of *Saccharina japonica* Biomass. *Energies* **2021**, *14*, 8053. https://doi.org/10.3390/en14238053

Academic Editors: Mejdi Jeguirim and Dino Musmarra

Received: 14 October 2021
Accepted: 25 November 2021
Published: 1 December 2021

1. Introduction

Carbohydrate-rich macroalgae are a biomass of renewable feedstock for biorefineries, where the main challenges are the ability to produce fermentable sugars through the saccharification process [1–4]. Macroalgae or seaweed refers to thousands of species of macroscopic, multicellular marine algae. Eastern Pacific kelp species of fast-growing macroalgae can grow up to 10 m in length [5].

Macroalgae (e.g., brown algae, red algae, and green algae) have a high carbohydrate content and have various advantages such as a non-requirement of fertilizers, land, pesticides, or water during production [1,6].

Carbohydrates are generally stored as long polymers for energy storage [7–10] and can be directly converted into biofuel [11,12]. Brown algae do not contain lignin, and their low content of cellulose is more easily convertible than that of land plants [13].

Pretreatments typically involve standalone chemical, biological, or physical treatments, or a combination of these treatments [14]. Pretreatments used prior to enzymatic hydrolysis include mechanical [15–19], thermal [19–22], chemical [23–25], and biological treatments [26,27]. A summary of pretreatments performed on macroalgae before ethanol or methane production is presented shown in Table 1.

As aforementioned, many previous studies have reported hydrolysis methods for macroalgae. Several previous studies emphasized that the reducing sugar yield (RS_y) obtained during the combined treatment was higher than that during biological treatment.

Previous studies have reported combined treatment (sequential hydrolysis) to increase the reducing sugar yield [28]. Therefore, this study was aimed to perform sequential

hydrolysis using hydrothermal acid pretreatment followed by enzymatic hydrolysis to determine the RS_y. Parameters including temperature of hydrothermal acid pretreatment, time of hydrothermal acid pretreatment, and HCl concentration during the extraction process were predicted by response surface methodology (RSM).

Table 1. Hydrolysis methods of macroalgae.

Type of Pretreatment/ Used Enzymes or Microorganism	Macroalgae	Pretreatment	Ref.
Size reduction	*Gelidium sesquipedale*	Freshwater washed and air-dried Cutting milled then centrifugally (12,000 rpm) milled	[15]
	Laminaria spp.	Ball milled unwashed seaweed, dried at 80 °C for 24 h, Particle size: 1–2 mm	[16]
Beating	*Laminaria* spp.	Cut without washing and beaten (Hollander beater), 76 gap, 15 min	[17]
Washing	*S. muticum*	Freshwater washed, frozen (−20 °C), then blended	[18]
	Chaetomorpha linum	Freshwater washed, dried (40 °C, 48 h) milled (25 balls), 18 h, 180 rpm to <2 mm size	[19]
Microwave	*F. vesiculosus*	Cut and grounded (mortar and pestle) microwaved (700 W), 3 min	[20]
	N. zanardini	Washed, dried (40 °C, 24 h); hammer milled to <1 mm 5% seaweed, 121 °C, 0.5 h	[21]
Steam explosion	*C. linum*	Washed, dried (40 °C) and milled 1.2 kg (35% DW, 1.9 MPa), 200 °C, 5 min	[19]
	S. latissima	Defrosted, shredded into slurry steam exploded 130 °C or 160 °C, 10 min	[22]
Acidic or alkali treatment	*F. vesiculosus*	Dried, crushed, homogenised 0.2 M HCl (80 °C, 12 h)	[23]
	Ulva spp.	Fresh water rinsed, blended to slurry. 500 mL slurry, 0.01 M HCl; 0.1 M NaOH	[24]
	Ulva spp.	Washed, sun dried (1–2 weeks) 0.04 g HCl $_g^{-1}$ TS (150 °C, 0.5 h); 0.04 g NaOH $_g^{-1}$ TS (20 °C, 24 h)	[25]
Cellulase Alginate lyase Celluclast® 1.5L	*L. digitata*	Freshwater rinsed, dried (75 °C, 24 h), milled. 20% (*w*/*v*) seaweed in water with: Cellulase: 37 °C; Alginate lyase: 37 °C; or Celluclast® 1.5L: 40 °C	[26]
A. niger with β-glucosidase	*Ulva rigida*	7.5 mL A. niger filtrate to 50 mL blended seaweed (80% (*w*/*v*) in water), 50 °C, 100 rpm, 2 h Repeated with β-glucosidase	[27]

2. Materials and Methods

2.1. Materials

2.1.1. Biomass Preparation

The remaining non-commercial *Saccharina japonica* biomass after processing was obtained from the Wando Fish Market in Jeonnam, South Korea. The biomass was washed and air-dried in a clean oven (OF-22, Jeio Tech, Daejeon, Korea), subsequently milled using a grinder (HR-2870, Philips Electronics, South Korea) with a 1.25 mm diameter screen,

and then stored in a desiccator. The carbohydrate, protein, and lipid compositions of the brown algae are shown in Table 2.

Table 2. Chemical composition (i.e., Carbohydrates, protein, and lipids) of brown algae % dw.

Algae	Speices	Carbohydrate (%)	Protein (%)	Lipid (%)	Reference
Brown Algae	*Laminaria japonica*	51.9	14.8	1.8	[2]
	Laminaria japonica	59.7	9.4	2.4	[29]
	Laminaria japonica	77.4	4.0	0.7	[30]
	Saccharina japonica	66.0	10.6	1.6	[31]
	Saccharina japonica	66.2	9.6	1.8	This Study
	Mean ± SD	64.2 ± 9.4	9.7 ± 3.9	1.7 ± 0.6	

2.1.2. Chemical Reagents and Enzyme

The chemical reagents used in this study included hydrochloric acid (35%) and 3,5-dinitrosalicylic acid (DNS) of purity grade (Junsei, Tokyo, Japan). The chemical standard (glucose) was of analytical grade purity and was purchased from Asanpharm in Seoul, South Korea. Celluclast® 1.5 L was used for enzymatic hydrolysis (Novozymes Corporation, Copenhagen, Denmark).

2.2. Processing Conditions by RSM

A central composite design of RSM was used to investigate the temperature of the acid pretreatment, time of the acid pretreatment, and the HCl concentration of *Saccharina japonica* biomass. Three levels of temperature (X_1), time (X_2), and HCl concentration (X_3) were selected. A hydrolysis temperature of 150 °C, hydrolysis time of 22 min, and HCl concentration of 0.1 N were chosen as the center points. The reducing sugar yield was used as the output variable. Experiments were conducted according to the scheme shown in Figure 1. Table 3 displays the actual levels for a given coding level. The experimental data were analyzed using Design Expert (Stat-Ease, MN, USA).

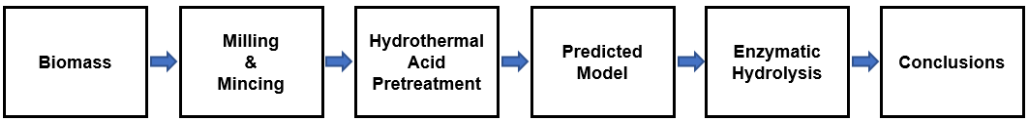

Figure 1. Steps of *Saccharina japonica* biomass processing to determine the reducing sugar yield.

Table 3. Input variables for the Central Composite Design.

Variable	Symbol	Coding Level				
		−1.682	−1	0	1	1.682
Temperature of acid pretreatment (°C)	X_1	113	128	150	172	187
Time of acid pretreatment (min)	X_2	12	16	22	28	32
HCI concentration (N)	X_3	0.0159	0.05	0.1	0.15	0.1841

2.3. Hydrothermal Acid Pretreatment

Hydrothermal acid pretreatment was carried out in a 100 mL reaction vessel (Hydrothermal Reactor, HR-8200, Hanwoul Engineering Inc., Gunpo-City, Gyeonggi-do, South Korea), into which 1 g of dried *Saccharina japonica* powder and 30 mL of 0.0159, 0.05, 0.1, 0.15, or 0.1841 N HCl acid were introduced. Hydrothermal acid pretreatment was carried out at 113, 128, 150, 172, or 187 °C for 12, 16, 22, 28, or 32 min. Independent variables obtained during the preliminary experiments were subjected to hydrothermal acid pretreatment. The hydrolysate was analyzed after centrifugation at 4500 rpm for 15 min. A schematic diagram of the hydrothermal reactor and its specifications are shown in Figure 2 and Table 4, respectively.

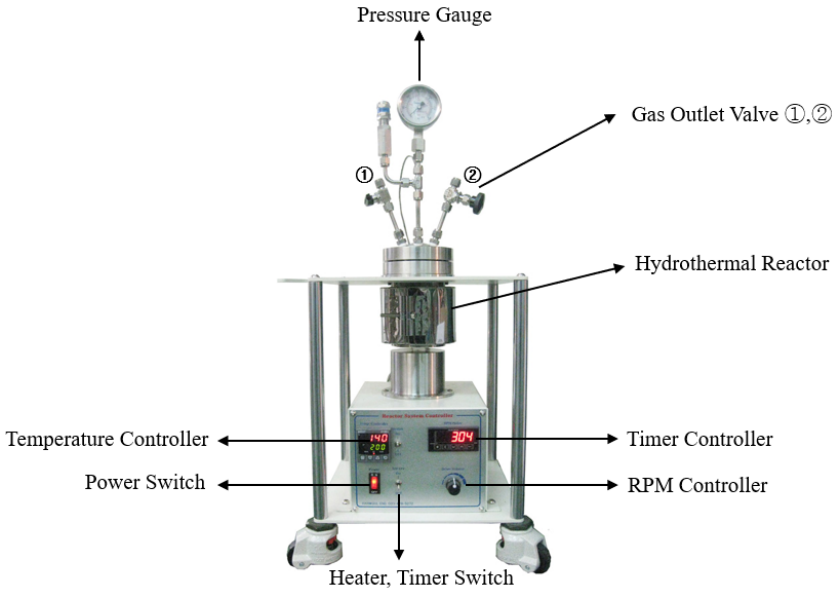

Figure 2. The hydrothermal reactor used in this study.

Table 4. Specification of the hydrothermal reactor.

Type	HR-8200 Reactor
Capacity	100∼2000 cc
Material	316SS, Monel400, Titanium, Hastelloy-C276, Inconel, etc.
Design Pressure	10∼400 bar
Design Temperature	AMB∼400 °C
Control System	Temperature Controller, RPM Controller & Indicator
Heating	Electric Band Heater or Jacket Type
Nozzles	Gas Inlet/Outlet Valve, Pressure Gauge, Pressure Safety Valve, Sampling Valve, Cooling Inlet/Outlet, etc.
Mixing Type	Magnetic Bar

2.4. Enzymatic Hydrolysis

Utilizing information obtained from previous enzymatic hydrolysis [28], Celluclast® 1.5 L (8.17% v/$w_{biomass}$), a hydrolysis time of 26.4 h, a pH of 4.1, and a temperature of 42.6 °C were selected as the predicted conditions. Enzymatic hydrolysis was conducted after the hydrothermal acid pretreatment under the predicted conditions using RSM. The pH was adjusted to approximately 4.1 using sodium hydroxide (NaOH, 0.1 N) and then sterilized at 121 °C for 15 min in an autoclave. After cooling on a clean bench, Celluclast ® 1.5 L (8.17% v/$w_{biomass}$) was added, and the hydrolysate was incubated with shaking at 42.6 °C for 26.4 h. After enzymatic saccharification, the solvent was analyzed by centrifugation.

2.5. Analytical Method

The reducing sugar yield was analyzed using the DNS method [32]. After centrifugal filtration of the hydrolysate, the solution was diluted. Next, DNS reagent (3 mL) was added to the diluted hydrolysate (1 mL). The reaction mixture was incubated at 90 °C for 5 min and diluted with 20 mL. UV–Vis absorbance was measured at 550 nm using a UV-1650 PC spectrophotometer (Shimadzu, Japan). The RS_y of samples was analyzed in a reproducible way. Measurements were performed in triplicate.

3. Results and Discussion

3.1. Hydrothermal Acid Pretreatment

As shown in Table 5, experiments were conducted to determine the influence of input factors on the results of the hydrothermal acid pretreatment. The reducing sugar yield (RS_y)

was chosen as an output variable for the efficiency of the hydrothermal acid pretreatment. The effect of the process parameters (temperature of hydrothermal acid pretreatment, time of hydrothermal acid pretreatment, and HCl concentration) on the reducing sugar yield was investigated.

Table 5. Central composite design for hydrothermal acid pretreatment of *Saccharina japonica* biomass.

No.	Temperature (°C)	Time (m)	C_{HCl} (N)	RS_y (mg/$g_{biomass}$)
1	128	16	0.05	95.43
2	172	16	0.05	18.91
3	128	28	0.05	92.73
4	172	28	0.05	20.06
5	128	16	0.15	72.88
6	172	16	0.15	45.23
7	128	28	0.15	83.16
8	172	28	0.15	24.70
9	113	22	0.1	41.47
10	187	22	0.1	22.22
11	150	12	0.1	100.21
12	150	32	0.1	101.70
13	150	22	0.0159	24.06
14	150	22	0.1841	98.35
15	150	22	0.1	115.56
16	150	22	0.1	119.54
17	150	22	0.1	118.54
18	150	22	0.1	119.46
19	150	22	0.1	120.56
20	150	22	0.1	120.37

Where X_1, X_2, and X_3 represent the temperature of hydrothermal acid pretreatment, time of hydrothermal acid pretreatment, and HCl concentration, respectively, and Y denotes the reducing sugar yield.

Analysis of variance (ANOVA) was used to determine the significance of the regression model and the corresponding model terms. The results are listed in Table 6. An F-value of 7.91 revealed that the model was significant (>99.8 %). As shown with an F-value of 29.86, temperature had a relatively greater effect than time and HCl concentration on the RS_y [33]. The square terms ${X_1}^2$ (>99.99%) and ${X_3}^2$ (>99.8%) were significant.

Table 6. Analysis of variance (ANOVA) for the regression model.

Source	Sum of Squares	DF *	Mean Square	F-Value	*p*-Value	Remark
Regression	26,604.2	9	2956.0	7.91	<0.002	Significant
X_1	5246.7	1	11,164.3	29.86	0.000	Significant
X_2	6.4	1	624.7	1.67	0.225	
X_3	1122.3	1	234.8	0.63	0.446	
${X_1}^2$	12,561.6	1	14,625.5	39.12	0.000	Significant
${X_2}^2$	424.9	1	801.3	2.14	0.4174	
${X_3}^2$	6644.6	1	6644.6	17.77	0.0002	Significant
X_1X_2	90.8	1	90.8	0.24	0.633	
X_1X_3	497.5	1	497.5	1.33	0.276	
X_2X_3	9.5	1	9.5	0.03	0.877	

* DF = The degrees of freedom of an estimate of a parameter.

As shown in Figure 3, the determination coefficient (R^2 = 0.878) indicated a good correlation between the predicted and experimental RS_y within the investigated range of variables. When $0.9 > R^2 \geq 0.8$, the model is very appropriate [34,35]. Three-dimensional response surface plots, which model synergistic effects of two variables when other variables are kept constant, are shown in Figures 4–6.

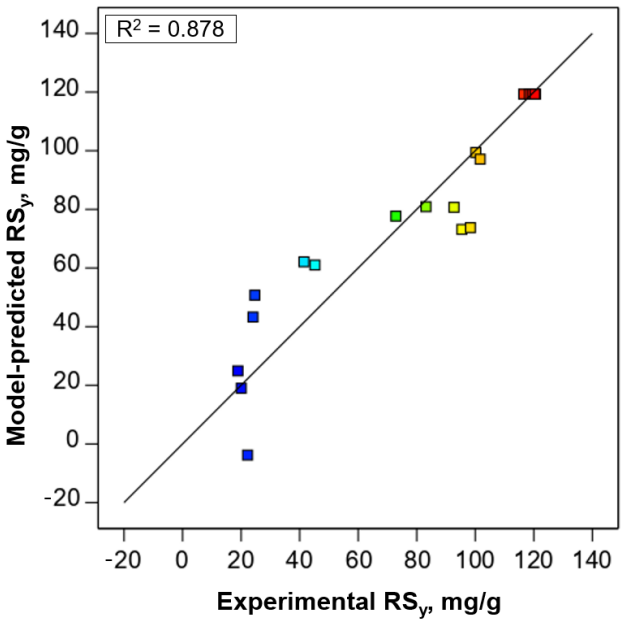

Figure 3. Parity plot for the predicted and experimental reducing sugar yield for the hydrothermal acid pretreatment.

Figure 4 displays the influence of the temperature and time of hydrothermal acid pretreatment on the reducing sugar yield (HCl concentration 0.1 N). The results indicated that the reducing sugar yield reached a maximum at 150 °C.

Figure 5 displays the effect of the temperature of hydrothermal acid pretreatment and HCl concentration on the reducing sugar yield for a constant pretreatment time over 22 min. An increase in temperature above 150 °C resulted in a decrease in reducing sugar efficiency. The highest reducing sugar yields were observed at temperatures ranging from 140–160 °C and an HCl concentration of 0.1 N.

Figure 6 displays the effect of the time of hydrothermal acid pretreatment and HCl concentration on the reducing sugar yield at a constant temperature of hydrothermal acid pretreatment of 150 °C. Under a relatively short pretreatment time, the HCl concentration had little effect on the reducing sugar yield. As shown in Figures 4–6, the hydrothermal acid pretreatment was strongly affected by temperature.

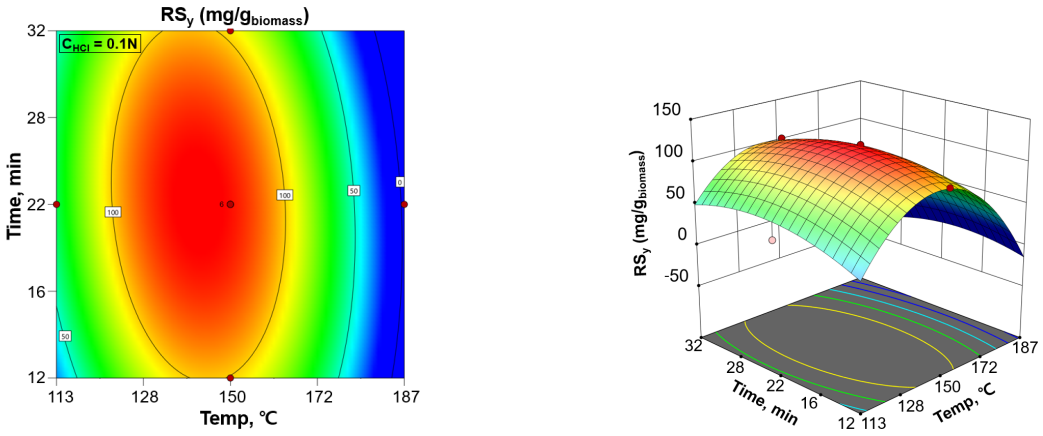

Figure 4. The contour and 3D surface diagram of the relationship between the temperature and time of hydrothermal acid pretreatment.

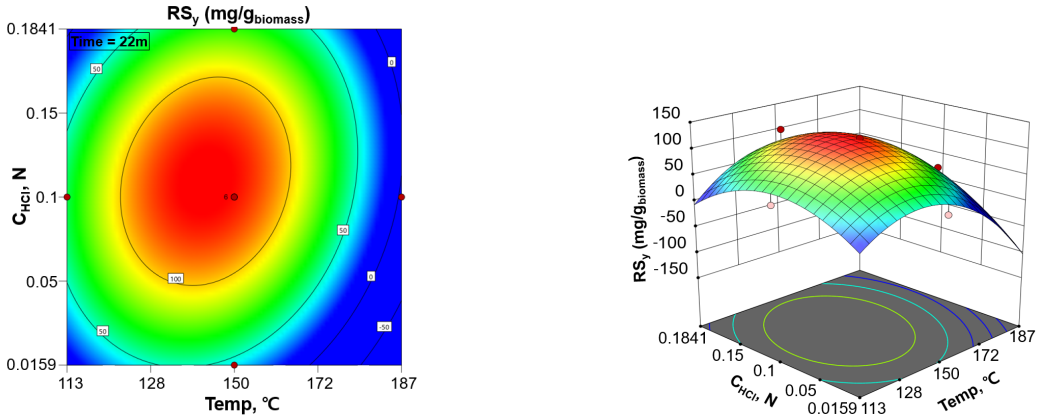

Figure 5. The contour and 3D surface diagram of the relationship between the temperature of hydrothermal acid pretreatment and HCl concentration.

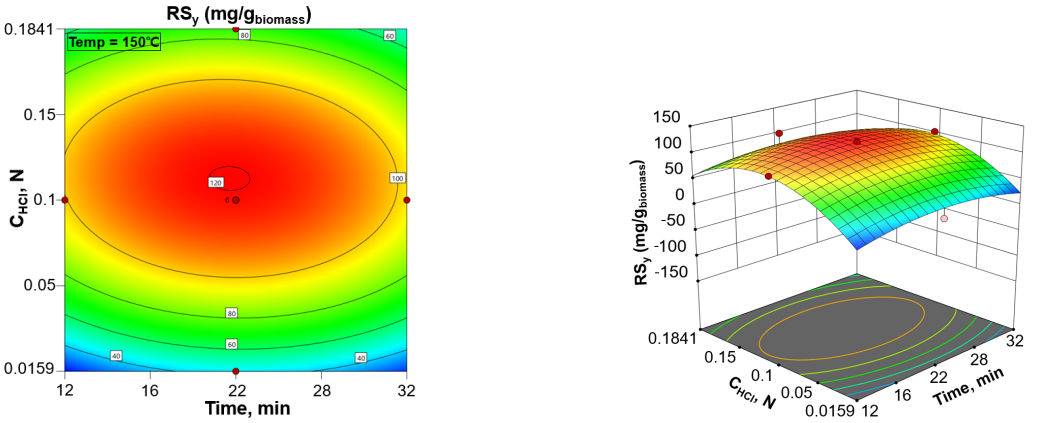

Figure 6. The contour and 3D surface diagram of the relationship between the time of hydrothermal acid pretreatment and HCl concentration.

To validate predicted conditions using the response surface model, a three-experiment setup was performed under the following conditions: 143.6 °C, 22 min, and 0.108 N HCl. The average RS_y of the three experiments was 115.6 ± 0.4 mg/g, which was found to be comparatively higher than those reported in past studies [36–38]. The comparison of saccharification efficiencies of reducing sugars reported for different brown algae biomass is shown in Table 7.

Table 7. Comparison of the reducing sugar yield from brown algae.

Brown Algae	Sqeuential Hydrolysis	Yields of Reducing Sugar Yield (% *w*/*w* Dry Biomass)	Ref.
Saccharina japonica	HCl (0.108 N, 143.6 °C, 22 min) and 700EGU Celluclast® 1.5 L (8.17 % v/$w_{biomass}$, 42.6 °C, pH 4.1, 26.4 h)	18.4%	This study
Sargassum spp.	H_2SO_4 (1% *w*/*v*, 126 °C, 30 min) and 50FPU Cellulase and 250CBU Cellobiase (50 °C, pH 4.8, 100 rpm 48 h)	8%	[36]
Sargassum spp.	H_2SO_4 (3% *w*/*v*, 121 °C, 30 min) and 53FPU Cellulase and 10U Pectinase (50 °C, pH 5, 150 rpm, 4 h)	11%	[37]
Sargassum fulvellum	Heat-treatment (121 °C, 30 min) and HCl (0.1N, 121 °C, 30 min)	11.7%	[38]
Laminaria japonica	Heat-treatment (121 °C, 30 min) and HCl (0.1N, 121 °C, 30 min)	13.1%	[38]

3.2. Enzymatic Hydrolysis

RSM was used to investigate the predicted conditions for sequential hydrolysis involving hydrothermal acid pretreatment conditions (143.6 °C, 22 min, and 0.108 N HCl) and enzymatic hydrolysis (8.17% v/$w_{biomass}$/Celluclast® 1.5 L, 26.4 h, 42.6 °C). Sequential hydrolysis resulted in the production of 183.5 ± 0.6 mg/g of reducing sugars with a yield of 18.4%. The RS_y of 183.5 ± 0.6 mg/g obtained in sequential hydrolysis was higher than the RS_y of 115.6 ± 0.4 mg/g in the hydrothermal acid pretreatment or the RS_y of 117.7 ± 0.3 mg/g in the enzymatic hydrolysis. This shows that compared to the RS_y obtained in a single treatment, the RS_y in the sequential hydrolysis was improved by 1.6 times. It has been reported that sequential hydrolysis applying two or more physical, chemical, and biological treatments can increase the RS_y [39]. Therefore, the results of our study have demonstrated that sequential hydrolysis of hydrothermal acid pretreatment or enzymatic hydrolysis was more efficient than a single treatment.

4. Conclusions

1. In sequential hydrolysis, the temperature had a relatively greater effect than time and HCl concentration on the RS_y.
2. The experimental conditions of hydrothermal acid pretreatment were: 143.6 °C, 22 min, and 0.108 N HCl. Under these conditions, the experimental yield was 115.6 ± 0.4 mg/$g_{biomass}$.
3. The experimental conditions for enzymatic hydrolysis were 8.17% v/$w_{biomass}$ Celluclast® 1.5 L, 26.4 h, and 42.6 °C. Under these conditions, the experimental yield was 117.7 ± 0.3 mg/$g_{biomass}$.
4. As a result of sequential hydrolysis, the reducing sugar yield produced from *Saccharina japonica* biomass was 183.5 ± 0.6 mg/g.

Author Contributions: Writing—review and editing, E.-Y.P. and J.-K.P. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP) (NRF-2021R1F1A1052129).

Conflicts of Interest: The authors declare no conflict of interest.

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