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Enzymatic Saccharification of *Laminaria japonica* by Cellulase for the Production of Reducing Sugars

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Abstract: Enzymatic saccharification of *Laminaria japonica* seaweed biomass was optimized by four independent factors (enzyme dose, hydrolysis time, pH, and temperature) using response surface methodology (RSM). To confirm the significance of the quadratic model, an analysis of variance (ANOVA) was performed, and the *F*-value of 8.76 showed that the regression model was highly significant ($\leq$0.1%). In the accuracy study, average recoveries were in the range of 97.00% to 98.32%. The optimum experimental conditions were an enzyme dose of 8.2%, a hydrolysis time of 26 h, a pH of 4.1, and a temperature of 43 °C. Temperature was the most important factor in the enzymatic saccharification. A relatively low temperature and short hydrolysis time were shown to improve the yield of reducing sugars.

Keywords: enzymatic hydrolysis; enzymatic saccharification; reducing-sugars yield; response surface methodology

1. Introduction

Macroalgae have been examined as a source of seaweed biomass for sustainable biofuel, and one of the main treatments is enzymatic hydrolysis of the biomass to fermentable sugars [1,2]. Seaweed biomass with high carbohydrate (i.e., laminarin, mannitol, and alginate) content has various advantages, including high growth yields with no need for pesticides, fertilizer, agricultural land, or fresh water [3,4]. In *Laminaria japonica*, laminarin (3.9–11.6% of the dry weight) is a polysaccharide containing poly β-(1 $\rightarrow$ 3)-glucan with some β-(1 $\rightarrow$ 6)-branches. Mannitol (15.0–17.4% DW) is a type of sugar alcohol corresponding to mannose that is a sugar monomer of the aldohexose series of carbohydrates [5,6]. Alginate may not be fermented to ethanol, while laminarin and mannitol may be converted to ethanol [7].

Carbohydrates (i.e., monosaccharide, disaccharides, and polysaccharides) are stored as long polymers of monosaccharide units bound together by glycosidic linkages for structural support or for energy storage [8]. Algal polysaccharides can be further converted into bioenergy (biofuels, power, heat) and other value-added products (food, feed, chemicals, materials) [9–11]. Macroalgae-based biorefinery has attracted much attention recently for functional products (stabilisers, thickeners, emulsifiers, food, feed, beverages, etc.) and in the pharmaceutical, cosmetic, and chemical industries [12,13].

Brown seaweeds lack lignin and have low cellulose contents. Thus, brown seaweeds are easily broken down biologically compared to land plants [7]. Physical, chemical, or biological pretreatment methods can be combined to obtain high yields from enzymatic saccharification [14]. In general, pretreatment methods used prior to biological treatment are γ-irradiation [15], chemical treatment (tap water [16], hydrochloric acid [17,18], sulfuric acid [19–21], sulfuric acid and hot

water [22]), and hydrothermal treatment (distilled water [23], or distilled water and cellulase saccharification [24,25]). Enzymatic saccharification of brown seaweed has been studied using enzymes such as laminarinases [26], alginate lyases [27], commercial cellulase blends [28], and commercial meicelase [29].

As mentioned above, many studies have examined hydrolysis methods of macroalgae, but only few of those studies mentioned the names of the enzymes used in the enzymatic saccharification. Moreover, most of the previous studies suggest the combined treatment due to reducing sugars yield (*RSy*) over the biological treatment since it yields higher, leading to the enhancement of macroalgal biomass hydrolysis [15,17,21].

Therefore, the present study explores bioethanol prospects from *Laminaria japonica* biomass through comparative analysis of *RSy* of combined treatment with biological treatment (enzymatic hydrolysis), which involves:

1. Experimental design and statistical analysis;
2. Validation of the analytical methodology;
3. Optimization of proposed method and potential assessment of *Laminaria japonica* biomass into biofuel and value-added products.

2. Materials and Methods

2.1. Materials

Laminaria japonica biomass was purchased from the local market of Wando, Korea. Table 1 shows the compositions *Laminaria spp*. [17,30–33]. The biomass was washed manually using tap water to remove dirt. The *Laminaria japonica* was dried for 3 days at 80 °C in conventional oven [1] and milled to a size less then 2 mm by a grinder (HMF-600, Hanil Electric, Korea). The milled *Laminaria japonica* was stored in a desiccator at room temperature until use. Celluclast® 1.5L was provided by Novozymes Corporation, Copenhagen, Denmark.

Table 1. Chemical composition of dried *Laminaria spp.*

Seaweed	Species	Carbohydrate	Protein (% Dry Weight)	Lipid	Ash	Country	Ref.
Laminaria spp.	*L. japonica*	51.9	14.8	1.8	31.5	South Korea	[17]
	L. japonica	59.7	9.4	2.4	28.5	South Korea	[30]
	L. japonica	51.5	8.4	1.3	38.8	South Korea	[31]
	L. digitata (August)	64.2	3.1	1.0	11.9	Denmark	[32]
	L. digitata (July)	77.4	4.0	0.5	18.1	Iceland	[33]
	Mean ± SD	60.9 ± 10.7	7.9 ± 4.7	1.4 ± 0.7	25.8 ± 10.7		

2.2. Experimental Design and Statistical Analysis

Optimization of saccharification conditions for the enzymatic hydrolysis of the *Laminaria japonica* biomass was performed by taking the *RSy* as a response with the central composite design (CCD) of RSM. The design was determined according to four factors: enzyme dose, hydrolysis time, pH, and temperature. Coded variable levels and the corresponding independent variables are given in Table 2.

Table 2. Coded variable levels of independent variables used in the CCD *.

Factors	Unit	Levels				
Actual, Coded		$-\alpha$	−1	0	+1	$+\alpha$
Enzyme dose, A	%	7.5	8	8.5	9	9.5
Hydrolysis time, B	h	26	27	28	29	30
pH, C	-	3.7	3.9	4.1	4.3	4.5
Temp., D	°C	40	42	44	46	48

* CCD = central composite design.

The experimental points for *RSy* in the experimental design are shown in Figure 1.

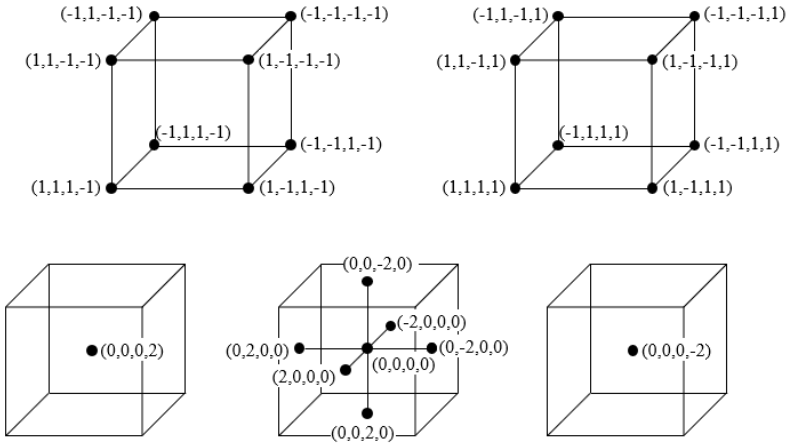

Figure 1. Experimental points in the experimental design, based on four independent factors.

A polynomial equation was fitted to evaluate the effect of the four independent factors on the dependent factor (Equation (1)):

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i x_i + \sum_{i=1}^{4} \beta_{ii} x_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} x_i x_j \quad (1)$$

where Y is the *RSy* (mg/g); β_0 is the intercept coefficient; β_i, $\beta_i i$, and β_{ij} are the coefficients of the linear, quadratic, and cubic, respectively. Statistical analysis was performed using Design-Expert statistical software (version 11, Stat-Ease, Inc., Minneapolis, MN, USA), and the remaining calculations were performed using Microsoft Excel (2019, Microsoft Corporation, Redmond, WA, USA).

2.3. Validation of the Analytical Methodology

Analysis of the *RSy* was performed as per the 3,5-dinitrosalicylic acid (DNS) analytical methods [34–37], in which the absorbance was measured by DNS and rochelle salt. The specimen was diluted with distilled water. Three milliliters of the DNS reagent was added to 1 mL of the diluted specimen, and the mixture was left to react at 90 °C for 5 min, after which it was diluted with 20 mL of distilled water. Using a UV–Vis Spectrophotometer (UV-1650, Shimadzu, Kyoto, Japan), the absorbance was measured at 550 nm. Accuracy is the proximity of measurement results to the true value [38]. The percentage of recovery was calculated by Equation (2).

$$\%R = \frac{(M_a - M_s)}{a} \quad (2)$$

where $\%R$ is the percentage of recovery, M_a is the initial concentration of algae extract with addition of standard, M_s is the initial concentration of algae extract without addition of standard, and a is the concentration of the standard solution added.

3. Results and Discussion

3.1. Experimental Design and Statistical Analysis

The relationship between the dependent factor and independent factors (enzyme dose (A), hydrolysis time (B), pH (C), and temperature (D)) was studied. In statistical modeling, regression analysis is a statistical method to fit the model with the experimental data [39]. The response was correlated with the four independent factors using the polynomial equation. The regression coefficients of the polynomial equation were determined and evaluated with the statistical software. The polynomial equation (quadratic model) in terms of coded factors was as follows Equation (3).

$$\begin{aligned} Y(mg/g) = &+ 137.57 - 2.35A - 12.99B - 7.66C - 18.74D + 11.74(A \times B) \\ &+ 8.15(A \times C) - 20.59(A \times D) - 5.05(B \times C) + 45.33(B \times D) \\ &+ 3.40(C \times D) - 29.08A^2 - 20.92B^2 - 75.89C^2 - 85.85D^2 \end{aligned} \quad (3)$$

The plus (+) and minus (–) signs in front of the terms indicate synergistic and antagonistic effects, respectively. ANOVA (analysis of variance) was performed to determine the statistical significance and the significant terms of the quadratic model. The results are listed in Table 3.

Table 3. ANOVA for the quadratic model.

Source	Sum of Squares	DF *	Mean Square	*F*-Value	*p*-Value	Remark
Model	27819.86	14	1987.13	8.76	<0.0001	significant
A	33.21	1	33.21	0.1463	0.7071	
B	1012.57	1	1012.57	4.46	0.0507	
C	352.28	1	352.28	1.55	0.2307	
D	2108.06	1	2108.06	9.29	0.0077	significant
AB	137.89	1	137.89	0.6076	0.4471	
AC	66.46	1	66.46	0.2929	0.5958	
AD	424.05	1	424.05	1.87	0.1905	
BC	25.48	1	25.48	0.1123	0.7419	
BD	2055.04	1	2055.04	9.06	0.0083	significant
CD	11.58	1	11.58	0.0510	0.8242	
A^2	1510.90	1	1510.90	6.66	0.0201	significant
B^2	782.22	1	782.22	3.45	0.0819	
C^2	10291.99	1	10291.99	45.35	<0.0001	significant
D^2	13172.48	1	13172.48	58.04	<0.0001	significant

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

The regression model for the *RSy* was significant by the *F*-test at the 5% confidence level. The *p*-value was less than 0.0001 for the *RSy* (Y), reflecting the significance of the model. Temperature (D) had the greatest effect on the *RSy*, showing the greatest *F*-value (9.29). The quadratic terms C^2 and D^2 were highly significant (>99.99%). The linear term D, the cubic term BD, and the quadratic term A^2 were also significant (>95%). Generally, the quadratic terms had greater effects on the *RSy* than the linear terms. The coefficient of variation (CV) was 15.60%, which indicated high degrees of accuracy and repeatability of the experimental values [40,41].

3.2. Validation of the Analytical Methodology

The influences of independent factors on the change in RS yield are shown by the perturbation plot in Figure 2. The yield of reducing sugars was most sensitive to the change in temperature (*D*) compared to the enzyme dose (*A*), hydrolysis time (*B*), and pH (*C*).

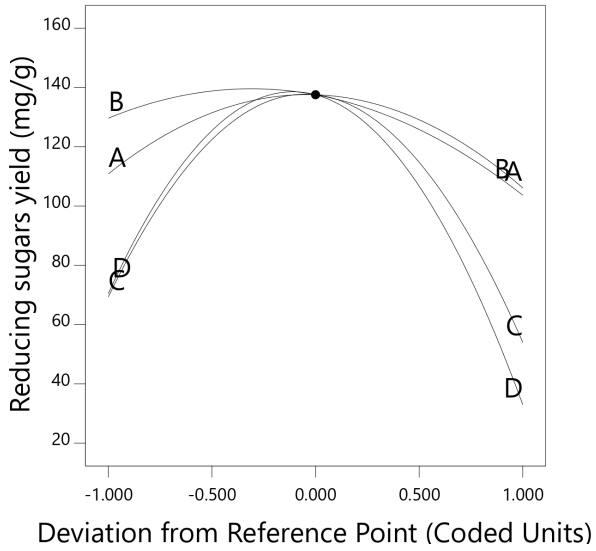

Figure 2. Perturbation plot with all factors.

A parity plot can be useful for the validation of the model and judging the standard error of the estimate. Figure 3 shows the parity plot for *RSy*, where each point represents one experimental run. Nearly 80% of the points were predicted with <10% error lines. As shown in Figure 3, the R^2 value for this response factor was greater than 0.80 (R^2 = 0.885) [42], which ensures a satisfactory fit of the regression model with the experimental data.

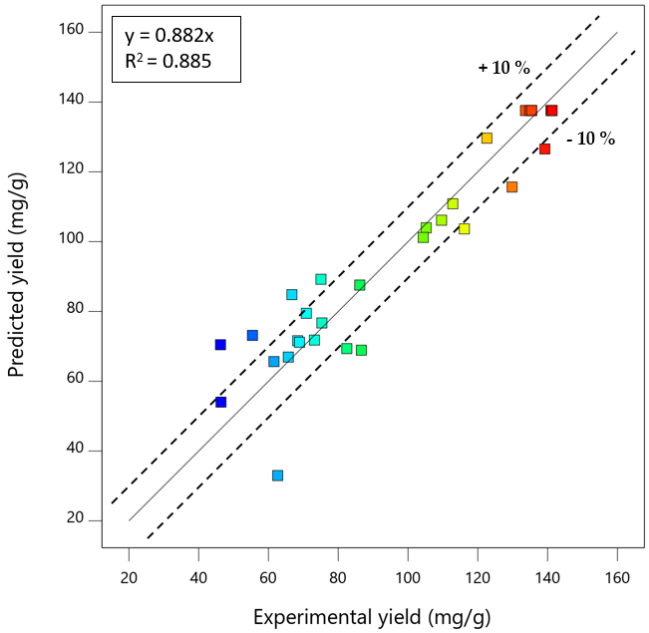

Figure 3. Parity plot for reducing sugars yield.

As mentioned above, the model *F*-value of 8.76 implied that the model was significant. Therefore, the proposed model was adequate to predict the *RSy* within the ranges investigated.

The results of the ANOVA showed that the interaction of two factors had a significant effect on the *RSy* ($p < 0.05$). Figure 4 presents the interaction effects of time and temperature.

The *RSy* was optimal with a temperature of 42 °C and a hydrolysis time of 26 h. A relatively low temperature and short hydrolysis time had a positive effect on the *RSy*, whereas a low temperature and a long hydrolysis time had a negative effect on the *RSy*.

Table 4 shows the results of the accuracy test. The mean recovery rate for the accuracy test resulted at 97.855%. According to A.O.A.C (Association of Official Analytical Chemists) [43], the range of the acceptable mean recovery was 90% to 107% at concentration over 100 ppm (mg/kg).

Table 4. Recovery rate and coefficient of variation for glucose sample (G) + *Laminaria japonica* extract (L).

Sample	Glucose g/L	G + L g/L	Average	Recovery (%)	Stand. Dev.	C.V. (%)
1	0.5	0.655 0.654 0.647	0.652	98.247	0.004	0.661
2	1.0	1.122 1.136 1.135	1.131	97.003	0.008	0.669
3	1.5	1.641 1.634 1.632	1.636	98.316	0.005	0.294
Laminaria japonica extract Recovery Average(%)	0.161			97.855		0.541

3.3. Optimization of Proposed Method

The saccharification of the *Laminaria japonica* seaweed biomass with the enzyme Celluclas® 1.5L was optimized with respect to the enzyme dose, hydrolysis time, pH, and temperature. A comparison of RS yields for different hydrolysis conditions reported for different brown seaweeds is given in Table 5. The *RSy* obtained in this study was comparatively greater than previously reported values. Therefore, the brown seaweed *Laminaria japonica* showed considerable potential as a renewable feedstock for the production of sustainable biofuel and value-added products.

Table 5. Comparison of hydrolysis conditions and their reducing sugar (RS) yields.

Brown Algae	Hydrolysis Condition	*RSy* (mg/g DW)	Ref.
Laminaria japonica	Celluclast® 1.5L (8.2 % v/w, 43 °C, pH 4.1, 26 h)	118	This study
Laminaria japonica	HCl (0.1N, 121 °C 15 min)	94	[17]
Laminaria japonica	H_2SO_4 (0.5 M, 121 °C, 15 min)	85	[44]
Hizikia fusiforme	Viscozyme L/Novozyme 188 = 9 : 1, (30% of substrate weight, 50 °C, 150 rpm, 24 h)	89	[45]
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)	80	[46]
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)	110	[47]

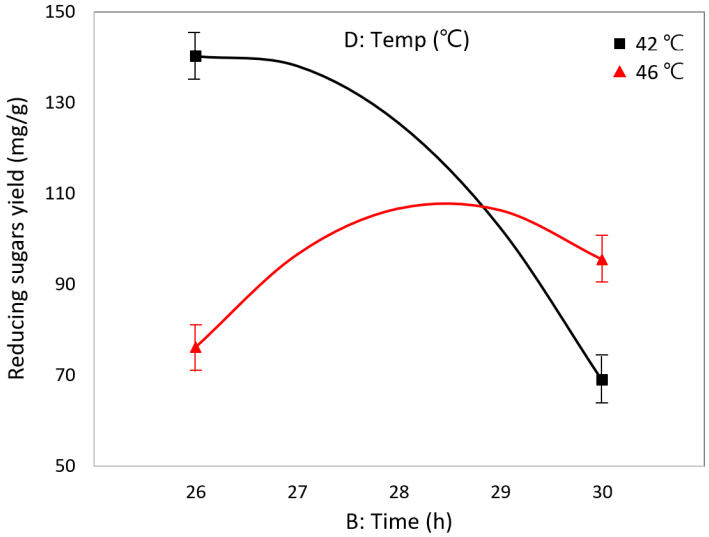

Figure 4. Interaction effects of time and temperature.

4. Conclusions

The enzymatic saccharification of the *Laminaria japonica* seaweed biomass was optimized by four independent factors (enzyme dose, hydrolysis time, pH, and temperature) using a CCD of a standard RSM. Analysis of variance confirmed that the model was highly significant, with p less than 0.0001. In the accuracy study, average recoveries were in the range of 97.00% to 98.32%. The optimum experimental conditions were an enzyme dose of 8.2%, a hydrolysis time of 26 h, a pH of 4.1, and a temperature of 43 °C. Temperature was the most important factor in the enzymatic saccharification. A relatively low temperature and short hydrolysis time were shown to improve the *RSy*.

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