Comparison of Ethanol Yield Coefficients Using \textit{Saccharomyces cerevisiae}, \textit{Candida lusitaniae}, and \textit{Kluyveromyces marxianus} Adapted to High Concentrations of Galactose with \textit{Gracilaria verrucosa} as Substrate

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The red seaweed \textit{Gracilaria verrucosa} has been used for the production of bioethanol. Pretreatment for monosaccharide production was carried out with 12\% (w/v) \textit{G. verrucosa} slurry and 500 mM HNO$_3$ at 121°C for 90 min. Enzymatic hydrolysis was performed with a mixture of commercial enzymes (Cellic C-Tec 2 and Celluclast 1.5 L; 16 U/ml) at 50°C and 150 rpm for 48 h. \textit{G. verrucosa} was composed of 66.9\% carbohydrates. In this study, 61.0 g/L monosaccharides were obtained from 120.0 g dw/l \textit{G. verrucosa}. The fermentation inhibitors such as hydroxymethylfurfural (HMF), levulinic acid, and formic acid were produced during pretreatment. Activated carbon was used to remove HMF. Wild-type and adaptively evolved \textit{Saccharomyces cerevisiae}, \textit{Candida lusitaniae}, and \textit{Kluyveromyces marxianus} were used for fermentation to evaluate ethanol production.

\textbf{Keywords:} Bioethanol, thermal acid hydrolysis, \textit{Gracilaria verrucosa}, enzymatic saccharification, adaptive evolution, fermentation

\textbf{Materials and Methods}

\textbf{Raw Material}

\textit{G. verrucosa} was obtained from Wando (Jeonnam, Korea). Dried seaweed samples were ground using a hammer mill. The seaweed powder was passed through a 150-mesh sieve before pretreatment [5]. The composition of \textit{G. verrucosa} was analyzed by the Feed and Foods Nutrition Research Center at Pukyong National University in Busan, Korea.

\textbf{Thermal Acid Hydrolysis}

Thermal acid hydrolysis conditions for \textit{G. verrucosa} were optimized by maintaining the temperature at 121°C and varying the slurry content (6.3–17.7\%), nitric acid (HNO$_3$) concentration (175.8–824.2 mM), and treatment...
time (17.58–102.42 min). The samples were transferred to a cold water bath and allowed to cool to room temperature [6]. The optimal HNO3 concentration was determined based on the acid hydrolysis efficiency (Ep) as defined in Eq. (1):

\[ E_p = \frac{\Delta S_{gal+gl}(g/l)}{TC(g/l)} \times 100 \% \]  

(1)

where \( E_p \) indicates the efficiency of thermal acid hydrolysis (%), \( \Delta S_{gal+gl} \) is the increase in galactose and glucose (g/l) concentrations during hydrolysis, and TC is the total carbohydrate content (g/l) of the biomass [6, 7, 9]. Sugars in \( G. \ verrucosa \) consist mainly of glucose and galactose. Therefore, \( \Delta S_{gal+gl} \) was considered as the total sugar obtained by pretreatment as shown in Eq. (2):

\[ Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \sum_{j=1}^{3} \beta_{ij} X_i X_j \]  

(2)

where \( Y \) is the response factor as sugar yield of \( E_p \), \( \beta_0 \) is the intercept term, \( \beta_i \) is the quadratic coefficient for factor \( i \), and \( \beta_{ij} \) is the linear model coefficient for the interaction coefficient between factors \( i \) and \( j \). The quality of fit for the polynomial model equation was expressed as the coefficient of determination (\( R^2 \)). Response surface methodology (RSM) was utilized to optimize the condition of pretreatment with HNO3 and to evaluate the effect of variables including pretreatment temperature (\( X_1 \)), HNO3 concentration (\( X_2 \)), and reaction time (\( X_3 \)) on sugar yield (\( Y \)). The slurry was then adjusted to pH 5.0 with NaOH to measure monosaccharide content by high-performance liquid chromatography (HPLC). All statistical calculations were performed with the response surface methodology (RSM) using SAS software (ver. 9.4; SAS Institute, Cary, NC, USA) as shown in Table 1 [10].

**Enzymatic Saccharification**

NaOH (10 N) was used to adjust the pH to 5.0 for enzyme activation [6, 7]. Enzymatic saccharification was conducted by adding 16 units/ml Celluclast 1.5L (854 EGU/ml; Novozymes, Bagsvaerd, Denmark) [6], 16 units/ml Cellic C-Tec2 (120 FPU/ml; Novozymes, Bagsvaerd, Denmark), and a mixture containing a 1:1 ratio of Celluclast 1.5 L and Cellic C-Tec2 (16 units/ml) [8]. Celluclast 1.5 L contains endoglucanase, and Cellic C-Tec2 is a complex of enzymes. Enzyme kinetics were determined using the Hanes-Woolf equation derived from the Michaelis-Menten equation as shown in Eq. (3):

\[ \frac{[S]}{V} = \frac{[S]}{V_{max}} \frac{K_m}{V_{max}} \]  

(3)

where \( [S] \) and \( V \) represent the substrate concentration (\( G. \ verrucosa \)) and the reaction rate, respectively. \( K_m \) is a Michaelis-Menten constant and indicates the maximum reaction rate at the substrate.

The amount of monosaccharides obtained from enzymatic saccharification was determined as shown in Eq. (4), and the efficiency was calculated:

\[ E_s(\%) = \frac{\Delta S_{gal+gl}(g/l)}{TC(g/l)} \times 100 \]  

(4)

**Table 1. RSM formula to determine optimal pretreatment conditions.**

| Design point | Slurry concentration, X₁ (w/v) | HNO₃ concentration, X₂ (mM) | Thermal hydrolysis time, X₃ (min) | Dependent variable Y Monosaccharides (g/l) |
|--------------|-------------------------------|-----------------------------|----------------------------------|------------------------------------------|
| 1            | 16                            | 700                         | 120                              | 50.08478                                 |
| 2            | 16                            | 300                         | 120                              | 35.71804                                 |
| 3            | 16                            | 700                         | 60                               | 42.11600                                 |
| 4            | 16                            | 700                         | 120                              | 50.08478                                 |
| 5            | 8                             | 300                         | 60                               | 17.39879                                 |
| 6            | 8                             | 300                         | 120                              | 22.63901                                 |
| 7            | 8                             | 700                         | 160                              | 27.88817                                 |
| 8            | 8                             | 700                         | 120                              | 30.63901                                 |
| 9            | 12                            | 500                         | 90                               | 57.45969                                 |
| 10           | 17.7                          | 500                         | 90                               | 53.34028                                 |
| 11           | 6.3                           | 500                         | 90                               | 15.33930                                 |
| 12           | 12                            | 824.2                       | 90                               | 54.27600                                 |
| 13           | 12                            | 175.8                       | 90                               | 50.94499                                 |
| 14           | 12                            | 500                         | 132.42                           | 56.18084                                 |
| 15           | 12                            | 500                         | 47.58                            | 49.33814                                 |
| 16           | 12                            | 500                         | 90                               | 57.45969                                 |
| 17           | 12                            | 500                         | 90                               | 57.45969                                 |
where $E_S$ is the efficiency of enzymatic saccharification, (%)$\Delta S_{\text{GLU}}$ is the increase in glucose concentration (g/L) during enzymatic saccharification, and $TC$ is the total carbohydrate content (g/l) of the biomass. Saccharification was carried out in 100 ml of 12% (w/v) seaweed slurry at 50°C with 150 rpm shaking for 48 h. Samples were collected for the determination of monosaccharide and hydroxymethylfurfural (HMF) concentrations by HPLC [12, 13].

**Removal of HMF**

HMF removal after enzyme saccharification was performed using activated carbon powder (Duksan Pure Chemical Co., Ltd., Korea). A shaking incubator was used to remove HMF produced during pretreatment and saccharification. The hydrolysate was treated with 2% (w/v) activated carbon (reaction temperature of 50°C, rotational speed of 150 rpm, and reaction time of 2 min). The adsorption surface area of the activated carbon powder was 1,400~1,600 m$^2$/g. The ethanol fermentation inhibitor was removed, and the samples were centrifuged at 8,000 × g for 20 min to remove activated carbon. The monosaccharide and residual HMF concentrations in the supernatant were evaluated by HPLC [5], and the HMF removal efficiency was calculated:

$$E_R = \frac{C_{\text{CHMF}} - R}{C_{\text{CHMF}}} \times 100$$

where $E_R$ is the efficiency of HMF removal (%) by activated carbon, $C_{\text{CHMF}}$ represents the monosaccharides before HMF removal by activated carbon, and $R$ represents the monosaccharides lost during HMF removal.

**Ethanol Fermentation**

Ethanol fermentation was performed with 100 mL of 12% (w/v) $G$. verrucosa hydrolysate in 250 mL Erlenmeyer flasks. Adaptive evolution to high concentrations of galactose was carried out for 48 h, and yeasts (1.0 g dcw/l) were inoculated into 100 mL of $G$. verrucosa hydrolysate [5].

Fermentation for ethanol production was performed at 30°C and 150 rpm using yeasts that were evolutionarily adapted to galactose and wild-type yeasts with $G$. verrucosa as the substrate. The ethanol yield coefficient ($Y_{\text{EtOH}}$, g/g) was defined as the maximum ethanol concentration (g/l) determined based on the total initial fermentable galactose and glucose concentration at the onset of fermentation (g/l) as shown in Eq. (6) [13]:

$$Y_{\text{EtOH}} (\text{g/g}) = \frac{[\text{EtOH}]_{\text{max}}}{[\text{Monosaccharide}]_{\text{ini}}}$$

where $[\text{EtOH}]_{\text{max}}$ is the maximum ethanol concentration, and $[\text{Monosaccharide}]_{\text{ini}}$ is the concentration of glucose and galactose (g/l) at the onset of fermentation.

**Analytical Methods**

The glucose, galactose, HMF, and ethanol concentrations in the samples were determined by HPLC (Agilent 1100 Series; Agilent Inc., USA) with a refractive index detector. An Aminex HPX-87H column (300 × 7.8 mm; Bio-Rad, USA) was used with filtered and degassed 5 mM sulfuric acid at an elution rate of 0.6 ml/min. Before analysis, aqueous samples were centrifuged at 8,000 × g for 12 min, and the supernatant was filtered through a 0.2 μm syringe filter.

**Statistical Analysis**

Optimal pretreatment conditions were determined with the RSM using SAS ver. 9.4 (SAS Institute, USA) [14, 15].

**Results**

**Thermal Acid Hydrolysis**

Seaweed samples were subjected to thermal acid hydrolysis. The reaction temperature and HNO$_3$ concentration with various thermal hydrolysis periods were plotted based on a three-dimensional response surface method. The monosaccharide concentration was increased with the acid concentration, reaction time, and slurry concentration. Variables including the hydrolysis temperature ($X_1$), HNO$_3$ concentration ($X_2$), and reaction time ($X_3$) were assessed using 12% (w/v) $G$. verrucosa slurry, and the results are summarized in Table 1.

The regression coefficients were calculated, and the predictive response model equation for $Y$ as the pretreatment efficiency ($E_p$) was expressed as Eq. (7):

$$Y = -128.853973 + 20.677701X_1 + 0.12802X_2 + 0.355691X_3 - 0.000881X_1X_2 + 0.000837X_1X_3 - 0.00026761X_2X_3 - 0.717684X_1X_1 - 0.000108X_2X_3 - 0.001863X_3X_3$$

Based on the high value of $R_2 = 0.9308$, the regression was statistically significant, indicating that thermal acid hydrolysis had a significant effect on monosaccharide release from $G$. verrucosa. The results obtained through this equation are shown in Fig. 1. The red-colored part indicates the optimal conditions for thermal acid hydrolysis to produce 57.4 g/l monosaccharides. Green and blue areas in Fig. 1. indicate lower pretreatment efficiency. When 500 mM acid, 90 min of treatment time, and 12% slurry were used, the maximum yield of monosaccharides was
obtained as shown in Fig. 1. The production of inhibitors such as formic acid, levulinic acid, and HMF occurred during acid hydrolysis. Therefore, the optimal pretreatment conditions for obtaining monosaccharides were 12% (w/v) *G. verrucosa* slurry and 500 mmol/l HNO₃ at 121°C for 90 min.

**Enzymatic Saccharification**

Enzymatic saccharification was performed to obtain glucose after thermal acid hydrolysis [16]. Cellulase is an effective enzyme for obtaining glucose from cellulose. As shown in Fig. 2, a synergistic effect was achieved with multiple enzymes (Cellic C-Tec 2 and Celluclast 1.5 L), and saccharification was the highest compared with that of single enzyme treatments using Cellic C-Tec 2 or Celluclast 1.5 L. Therefore, enzymatic saccharification was carried out using a mixture of Cellic C-Tec 2 and Celluclast 1.5 L for 72 h. When Cellic C-Tec 2 was used as the enzyme for hydrolysis, 60.2 g/l monosaccharides were obtained, and when Celluclast 1.5 L was used, 57.8 g/l monosaccharides were obtained. When a mixture of the enzymes (Cellic C-Tec 2 and Celluclast 1.5L) was used, 61.0 g/l monosaccharides were obtained.

**Removal of HMF**

HMF is formed by the dehydration of monosaccharides and is known as an inhibitor of ethanol production. HMF was removed using activated carbon [17]. HMF removal was performed using 2% activated carbon for 2 min at a reaction temperature of 50°C with a rotational speed of 150 rpm [5]. The reaction was carried out under optimal conditions. Therefore, there was no loss of monosaccharides, and the amount of HMF was decreased from 5.1 g/l to 0.8 g/l as shown in Fig. 3.

**Fermentation**

Three yeast strains, *S. cerevisiae*, *C. lusitaniae*, and *K. marxianus*, were used for fermentation [18]. Each fermentation was carried out with the addition of wild-type yeasts and yeasts adapted to high galactose.

![Fig. 1. Response surface curve showing the combined effect of HNO₃ concentration and slurry content on monosaccharide production.](image)

![Fig. 2. Enzymatic saccharification of *G. verrucosa* hydrolysate using a mixture of Celluclast 1.5L and Cellic C-Tec2 (1:1 ratio; 16 U/ml), Cellic C-Tec2, or Celluclast 1.5 L (16 units/ml).](image)
concentrations (adaptive evolution). Ethanol was produced over 96 h of fermentation as shown in Fig. 4-6. When wild-type *S. cerevisiae* was used, ethanol production reached 19.0 g/l as shown in Fig. 4A. However, *S. cerevisiae* adapted to high concentrations of galactose produced 23.5 g/l ethanol as shown in Fig. 4B. The ethanol yield coefficient ($Y_{\text{EtOH}}$) was 0.39 using adaptively evolved *S. cerevisiae* and 0.31 using wild-type *S. cerevisiae*. Therefore, the ethanol yield was higher using adaptively evolved *S. cerevisiae* instead of wild-type *S. cerevisiae*.

Ethanol production using wild-type *C. lusitaniae* was 26.0 g/l ($Y_{\text{EtOH}} = 0.43$) and using adaptively evolved *C. lusitaniae* was 26.7 g/l ($Y_{\text{EtOH}} = 0.44$) as shown in Figs. 5A and 5B, respectively. The use of *C. lusitaniae* adapted to high concentrations of galactose did not increase the ethanol yield coefficient significantly. This is because *C. lusitaniae* can consume galactose even if it is not adapted to high concentrations of galactose. However,
adaptive evolution could reduce the time required to consume galactose. The diauxic production of ethanol was observed with wild-type *C. lusitaniae*, which exhibited a distinct pattern in the shift from glucose to galactose consumption. After complete glucose exhaustion, galactose was consumed by *C. lusitaniae*. Fig. 5A shows the complete consumption of galactose in 72 h using wild-type *C. lusitaniae*, and Fig. 5B shows the complete consumption of galactose in 60 h using adaptively evolved *C. lusitaniae*.

The outcome following the use of *K. marxianus* was similar to that following the use of *S. cerevisiae*. Wild-type *K. marxianus* produced 24.5 g/l ethanol (YEtOH = 0.40), and adaptively evolved *K. marxianus* produced 28.4 g/l ethanol (YEtOH = 0.47), as shown in Figs. 6A and 6B, respectively. Among the yeasts used in the study, *K. marxianus* produced the highest concentration of ethanol with the highest ethanol yield coefficient (YEtOH = 0.47). In addition, *K. marxianus* adapted to high concentrations of galactose produced the maximum ethanol concentration in 36 h.

**Discussion**

The use of HNO₃ produced monosaccharides in a short reaction time and showed a high saccharification efficiency compared with the efficiency using other acids such as sulfuric acid or hydrochloric acid [18, 19]. In this study, pretreatment with HNO₃ resulted in a high monosaccharide production efficiency as shown in Fig. 1 [5]. The duration of heat treatment (60, 90, and 120 min) at 121°C was evaluated. Monosaccharide production for 90 and 120 min showed similar results. Therefore, 90 min was selected as the optimal treatment time, which was determined based on statistical evaluation using SAS software.

Monosaccharides could be obtained by thermal acid hydrolysis using HNO₃. The optimal conditions for thermal acid hydrolysis pretreatment were 12% slurry and 500 mmol/L HNO₃ for 90 min at 121°C. Pretreatment with *G. verrucosa* produced 57 g/l monosaccharides. The optimal conditions were used for obtaining a high concentration of monosaccharides.

A mixture (1:1 ratio) of Celluclast 1.5 L and Cellic C-Tec 2 was used in saccharification to efficiently produce glucose. The use of the two enzymes together had a synergistic effect on cellulose degradation [21]. Through the processes of thermal acid hydrolysis and enzymatic saccharification, 76% of monosaccharides were obtained from the carbohydrates of *G. verrucosa*.

A lag phase could be observed when the medium contains more than one sugar. This phenomenon, known as the diauxic production of ethanol, is caused by a shift in metabolic pathways with the change from glucose to galactose consumption as shown in Fig. 5A. After glucose exhaustion, the cells were adapted to utilize galactose. Glucose is more readily metabolized than galactose, and the presence of more readily available sugars such as glucose suppresses the synthesis of enzymes required for the metabolism of secondary sugars such as galactose. The red seaweed *G. verrucosa* contains glucose and galactose. Therefore, galactose was consumed as shown in Figs. 4B, 5B, and 6B.

For fermentation, three yeasts (*S. cerevisiae*, *C. lusitaniae*, and *K. marxianus*) were used to produce ethanol. Yeasts prefer glucose to galactose, and they have evolved to consume glucose rather than galactose. Wild-type yeasts consume minimal amounts of galactose. Therefore, the adaptive evolution of yeasts to high concentrations of galactose was performed, and the ethanol yield coefficient was improved using yeasts adapted to high concentrations of galactose. Ethanol production was increased using the adaptively evolved yeasts compared with the wild-type yeasts. Wild-type *S. cerevisiae* produced 19.0 g/l ethanol, which was increased to 23.5 g/l using *S. cerevisiae* adapted to high concentrations of galactose. Ethanol yield of 27% increase was obtained by the adaptive evolution with *S. cerevisiae*. *C. lusitaniae* adapted to high concentrations of galactose was used to increase ethanol production from 26.0 g/l to 26.7 g/l. Ethanol production by *C. lusitaniae* adapted to high concentration of galactose showed 2% increase comparing to that of wild-type strain. A significant increase in ethanol production was not observed for *C. lusitaniae* considering the active consumption of galactose by both wild-type *C. lusitaniae* and *C. lusitaniae* adapted to high concentrations of galactose. Wild-type *K. marxianus* produced 24.5 g/l ethanol, and ethanol production was increased to 28.4 g/l using *K. marxianus* adapted to high concentrations of galactose. When adaptive evolution-type *K. marxianus* was used, 16% more ethanol was produced than that of the wild-type.
K. marxianus. In contrast to C. lusitaniae, K. marxianus showed a higher ethanol production efficiency due to efficient galactose consumption following adaptive evolution. The ethanol yield coefficient was 0.40 using wild-type K. marxianus, whereas the ethanol yield coefficient was the highest at 0.47 using adaptive evolution-type K. marxianus. S. cerevisiae has been improved with the best adaptation effect. Adaptive evolution-type S. cerevisiae showed 27% higher ethanol production than wild-type S. cerevisiae. Among the three yeast strains, the highest ethanol yield coefficient was obtained from fermentation using galactose adaptive evolution-type K. marxianus.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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