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Optimization, Scale-Up, and Economic Analysis of the Ethanol Production Process Using *Sargassum horneri*

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Abstract: Recently, the extensive spread of some algae along coastlines has surged into unmanageable thick decomposition layers. This study aimed to demonstrate the use of *Sargassum horneri* as a biomass resource for ethanol production through the continuous hydrolysis, enzymatic saccharification, and fermentation process. Sugars from *S. horneri* were obtained using a combination of thermal acid hydrolysis and enzymatic saccharification. The optimal conditions for thermal acid hydrolysis involved a 10% (*w*/*v*) *S. horneri* slurry treated with 100 mM H_2SO_4 at 121 °C for 60 min; enzymatic saccharification using 16 U/mL Cellic CTec2 further boosted the monosaccharide concentration to 23.53 g/L. Fermentation experiments were conducted with mannitol-adapted *Saccharomyces cerevisiae* BY4741 using *S. horneri* hydrolysate. Enhanced ethanol production was observed in the hydrolysate, particularly with mannitol-adapted *S. cerevisiae* BY4741, which yielded 10.06 g/L ethanol. Non-adapted *S. cerevisiae* produced 8.12 g/L ethanol, as it primarily utilized glucose and not mannitol. Regarding ethanol fermentation using 5 L- and 500 L-scale fermenters, the ethanol concentrations reached 10.56 g/L and 7.88 g/L with yields of 0.51 and 0.45, respectively, at 48 h. This study confirmed the economic viability of ethanol production using waste seaweed with optimized pretreatment conditions and the adaptive evolution of *S. cerevisiae* to mannitol.

Keywords: *S. horneri*; ethanol production; optimization; adaptive evolution; continuous hydrolysis enzymatic saccharification and fermentation (CHEF); economical evaluation of bioenergy production

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1. Introduction

Sargassum algae are free-floating brown seaweeds belonging to the class Phaeophyceae. They are found in tropical and subtropical oceans worldwide and serve as shelter, refuge, and feed for many marine species, including sea turtles and shrimp [1]. However, in recent years, their widespread proliferation along coastlines has reached uncontrollable levels, forming dense mats of decaying weeds that slowly pollute beaches, leading to the accumulation of toxic solid waste along urban coastlines [2]. This phenomenon significantly depletes water oxygen, light, and underwater space when harmful seaweeds proliferate, causing serious problems ranging from disrupting coastal traffic for boats and swimmers to affecting fish survival [3,4]. Therefore, it is necessary to explore alternatives for utilizing algae.

Brown macroalgae show promise for the production of biofuels and bio-based chemicals. Moreover, algae have gained attention as a potential feedstock for biofuel production at various levels, from laboratory to commercial scale [5,6]. According to analyses of global ethanol production, most ethanol (97%) is produced through fermentation processes, whereas only 3% is produced through the catalytic hydration of petroleum-derived ethylene [7]. The production of synthetic ethanol is less economically attractive in the

United States than fermentation because of high ethylene prices and abundant agricultural feedstock such as corn for ethanol production [8]. Bioethanol production by fermentation involves biomass pretreatment, hydrolysis, fermentation, and ethanol recovery [9]. The long-term commercial prospects of ethanol production from algae depend significantly on the method selection, particularly in the hydrolysis and fermentation stages, owing to their substantial impact on ethanol yield. From an economic perspective, selecting methods for these critical stages of algal ethanol production is essential to ensure efficiency, cost-effectiveness, and sustainability, particularly in the context of commercial-scale production.

First, utilizing low-value by-products from nuisance algae for ethanol production could open the path to economically viable bio-refineries with zero-waste technologies [10]. Moreover, the efficiency of ethanol production is first constrained by the amount of fermentable sugars released during hydrolysis [11]. Subsequently, during the fermentation stage, ethanol is produced based on the efficiency of the available reducing sugars and fermentation micro-organisms. The development of robust yeast strains capable of using various sugars from pretreatment is a prerequisite for efficient fermentation [12]. Among the various strategies, adaptive laboratory evolution, which imitates natural selection, has been adopted as a simple and safe approach to enrich desired microbes, in contrast to rational genetic engineering [13]. Serial passages in defined environments significantly improve the robustness of engineered yeast strains against several unfavorable fermentable sugars and fermentation inhibitors present in the hydrolysates [14].

Furthermore, continuous processes are being researched for high productivity and cost savings. Separating the enzymatic hydrolysis and fermentation stages, known as separate hydrolysis and fermentation (SHF), follows traditional saccharification [15]. However, SHF has the drawback of reduced reaction rates due to the high glucose concentration that can cause substrate inhibition to yeast [16]. Alternatively, these hydrolysis and fermentation steps can be integrated into a single process known as simultaneous saccharification and fermentation (SSF) [17,18]. However, a limitation of SSF is that enzymes and yeast cannot operate optimally under the same conditions, such as temperature [19]. Therefore, there is a need for new processes to address these shortcomings.

This study aimed to demonstrate the cost-effectiveness of ethanol production using waste seaweed, optimization of hydrolysis, enzymatic saccharification, and fermentation using adaptive yeasts under the continuous hydrolysis, enzymatic saccharification, and fermentation (CHEF) process.

2. Materials and Methods

2.1. Biomass

Sargassum horneri was collected during the winter season in February 2022 along the shores of Jeju Island, Republic of Korea. *Sargassum* sp. was dried under sunlight and ground using a grinder (Shinil, Republic of Korea) and sieved through a 200-mesh sieve (Chunggye Merchandise Co., Seoul, Republic of Korea). The powdered biomass was stored at −20 °C until use.

A composition analysis for crude protein, crude lipid, crude ash, and fiber was performed at the Institute of Agricultural Science, Chungnam National University (Daejeon, Republic of Korea), according to the Association of Official Analytical Chemists (AOAC, Gaithersburg, MD, USA) methods [20].

2.2. Optimization of Thermal Acid Hydrolysis and Enzymatic Saccharification Conditions

Thermal acid hydrolysis and enzymatic saccharification were optimized to obtain sugars. First, acid catalysis was optimized with four different acid catalysts (H_2SO_4, HNO_3, HCl, and H_2O). One hundred mM of the four different acid catalysts was reacted with 10% (*w*/*v*) *S. horneri* at 121 °C for 60 min with a 50 mL working volume in a 100 mL flask. Before enzymatic saccharification, the pH of the acid hydrolysate was adjusted to 5 using 10 N NaOH. Sixteen units/mL of Cellic CTec2 (120 filter paper units/mL; Novozymes,

Copenhagen, Denmark) was added to the 50 mL working volume in the 100 mL flask at 50 °C on a shaking incubator under 150 rpm. The optimal thermal acid hydrolysis and enzymatic saccharification conditions were selected based on the monosaccharide concentrations and Fourier transform infrared (FT-IR) results.

After the selection of acid catalyst, biomass concentration (6–12% (*w*/*v*)), acid concentration (50–200 mM), hydrolysis time (30–120 min), and enzyme concentrations (8–24 units/mL) were optimized with one-factor-at-a-time method to obtain maximum monosaccharide from *S. horneri*.

The efficiency of thermal acid hydrolysis and enzymatic saccharification were calculated using the following equation:

$$E_{AE}(\%) = \Delta S_{AE}/TCF \times 100 \quad (1)$$

where E_{AE} is the efficiency of thermal acid hydrolysis and enzymatic saccharification (%), ΔS_{AE} is the increase in monosaccharide concentration (g/L) during acid hydrolysis and enzymatic saccharification, and TCF is the total carbohydrate and fiber concentration (g/L) in the initial biomass which determined by AOAC methods.

2.3. Adaptive Evolution of Saccharomyces cerevisiae BY4741 to Mannitol

Saccharomyces cerevisiae BY4741 was obtained from Soo-rin Kim's lab at Kyungpook National University. The strains were cultured in a YPD medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose). The seed was incubated with agitation at 150 rpm at 30 °C for 24 h under aerobic conditions.

Mannitol adaptation was performed using *S. cerevisiae* BY4741. Initially, the yeasts were cultivated in a liquid medium composed of yeast extract, peptone, and mannitol (YPM medium) at concentrations of 10 g/L yeast extract, 20 g/L peptone, and 20 g/L mannitol. Subsequently, a 5 mL inoculum of the yeast culture was transferred into a 50 mL medium with a higher mannitol concentration, referred to as YPHM medium (10 g/L yeast extract, 20 g/L peptone, and 60 g/L mannitol). This process involved 10 successive subcultures in the YPHM medium during the adaptation phase, with the yeast strains being incubated at 30 °C with agitation at 150 rpm for 24 h.

2.4. Ethanol Fermentation in an Erlenmeyer Flask and 5 L and 500 L Fermenters

Fermentation was performed using 25 mL, 2.5 L, and 250 L of *S. horneri* hydrolysate in a 50 mL flask and 5 L- and 500 L-scale fermenters, respectively. The *S. horneri* hydrolysates were subjected to separate hydrolysis and fermentation at 30 °C and 150 rpm. The fermentation samples were collected periodically and stored at −20 °C before the analysis of ethanol and sugars. The ethanol yield coefficient (Y_{EtOH}) was calculated using the following equation [21]:

$$Y_{EtOH} = [EtOH]_{max}/[Monosaccharide]_{ini} \quad (2)$$

where Y_{EtOH} is the ethanol yield (g/g), $[EtOH]_{max}$ is the maximum ethanol concentration achieved during fermentation (g/L), and $[Monosaccharide]_{ini}$ is the initial total fermentable sugar (glucose, mannitol, and xylose) concentration (g/L). The maximum Y_{EtOH} was 0.51 from glucose (max Y_{EtOH} = 0.51).

2.5. Aanlysis

2.5.1. Fourier Transform Infrared Spectroscopy

The interactions between chemical functional groups were investigated using FT-IR. The FT-IR spectra were recorded on a Bruker Cary 600 FT-IR spectrometer (Agilent Technologies, Santa Clara, CA, USA) from 4000 to 400 cm^{-1}. The samples were analyzed as freeze-dried hydrolysate pellets.

The FT-IR spectra of the samples were recorded at room temperature using a Spectrum 100 attenuated total reflection FT-IR spectrophotometer (PerkinElmer, Shelton, CT, USA). The FT-IR spectral analysis was conducted in the wavenumber range of 600–4000 cm^{-1}.

Sixty-four scans were run at a resolution of 1 cm^{-1} to collect each spectrum. The lateral order index and total crystallinity index, proposed by O'Connor [22] and Nelson and O'Connor [23], were estimated from the ratio between the absorption peaks at 1430 and 890 cm^{-1} bands and 1370 and 2900 cm^{-1} bands, respectively.

2.5.2. UV Spectrophotometer

Cell growth was assessed by measuring the optical density at 600 nm (OD_{600}) using a UV spectrophotometer (Amersham Biosciences Ultrospec 6300 Pro; Biochrom, Cambridge, UK). The OD_{600} values were then converted into dry cell weight (DCW) using a calibration curve correlating the DCW to the OD_{600} [21]. A pH meter (CH-8603; Mettler-Toledo AG, Schwerzenbach, Switzerland) was used to determine the pH levels.

2.5.3. High Performance Liquid Chromatography (HPLC)

The concentrations of glucose, xylose, 5-HMF, formic acid, levulinic acid, and ethanol were quantified using an HPLC (1100 series; Agilent Technologies, Santa Clara, CA, USA) equipped with a refractive index detector. An Aminex HPX-87H column (300 mm × 7.8 mm; Bio-Rad, Hercules, CA, USA) was used for chromatographic separation. The eluent used was 5 mmol/L H_2SO_4, which had been filtered and degassed, flowing at a rate of 0.6 mL/min, and the analysis was performed at a temperature of 65 °C [21].

2.6. Economic Analysis of Bioethanol Production

SuperPro Designer (Intelligen, Inc., Scotch Plains, NJ, USA) was used for the scale-up simulation and economic evaluation of ethanol production. The stepwise processes and the equipment to be included in the simulation with the optimized data obtained at the lab scale were used in the simulation and analysis. Then, specific parameters and operating conditions were input for each relevant piece of equipment into the software [24]. A capacity of 50 tons per batch was used to model the ethanol production process.

2.7. Statistical Analysis

Differences in monosaccharide concentrations were evaluated using a one-way analysis of variance and Duncan's multiple range test ($p < 0.05$). Statistical analyses were conducted using the SPSS software (ver. 23.0; SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Characterization of the Biomass

The composition of *S. horneri* was analyzed using the AOAC methods, and the results are presented in Table 1. It had 8.59% crude fiber and 43.9% carbohydrate content. Ethanol can be produced via carbohydrate fermentation [25]. The selection of high carbohydrate content has many advantages for improving the feasibility of ethanol production.

Table 1. Result analysis of composition of *S. horneri*.

Composition (%)	Moisture	Crude Protein	Crude Lipid	Crude Fiber	Crude Ash	Carbohydrate
	12.64	13.00	1.13	8.59	20.74	43.90

Environmental factors, such as salinity, water and atmospheric temperatures, pH, sunlight, and nutrients, influence the growth of seaweeds and stimulate or inhibit the biosynthesis of several compounds. The total carbohydrate content of different *Sargassum* species has been reported to range from 18% to 55.5%: *S. horneri* (55.5%), *S. polycystum* (33.5%), and *S. myriocystum* (18%) [26–28].

Comparing the year-round carbohydrate content of *Sargassum* sp., the seaweed showed the highest values in winter [29]. Therefore, in February, the large quantities of waste

S. horneri, which moved to the coast from China to Jeju Island, Korea, is a practical biomass for ethanol production.

3.2. Optimization of Thermal Acid Hydrolysis and Enzymatic Saccharification

Thermal acid hydrolysis was performed using H_2O (control) and 100 mM acids (H_2SO_4, HNO_3, and HCl) (Table 2). The maximum monosaccharide concentration obtained was 4.82 g/L following thermal acid hydrolysis using H_2SO_4. However, there were no significant differences between the control and acidic catalysts. Therefore, FT-IR analysis was employed to analyze the degradation status of *S. horneri*.

Table 2. Results of thermal acid hydrolysis and enzymatic saccharification.

		Glucose (g/L)	Galactose (g/L)	Mannitol (g/L)	Total Sugar (g/L)	EAE (%)
Thermal acid hydrolysis	H_2SO_4	1.35 ± 0.13	1.56 ± 0.26	1.91 ± 0.16	4.82	11
	HNO_3	0.84 ± 0.01	1.41 ± 0.07	1.33 ± 0.03	3.59	8
	HCl	0.76 ± 0.11	1.22 ± 0.06	1.97 ± 0.20	3.94	9
	H_2O	1.24 ± 0.01	1.23 ± 0.06	0.62 ± 0.02	3.10	7
Enzymatic saccharification	H_2SO_4	19.85 ± 0.03	1.56 ± 0.26	2.11 ± 0.07	23.53	54
	HNO_3	15.04 ± 0.07	1.47 ± 0.11	1.37 ± 0.14	17.88	41
	HCl	13.03 ± 0.06	1.35 ± 0.01	1.91 ± 0.06	16.29	37
	H_2O	10.03 ± 0.06	1.33 ± 0.01	0.94 ± 0.07	12.31	28

The FT-IR spectra of *S. horneri* powder treated with H_2O (control), HNO_3, H_2SO_4, and HCl are illustrated in Figure 1. The FT-IR spectra showed prominent peaks at 3400, 1640, 1350, and 1000–1100 cm^{-1}.

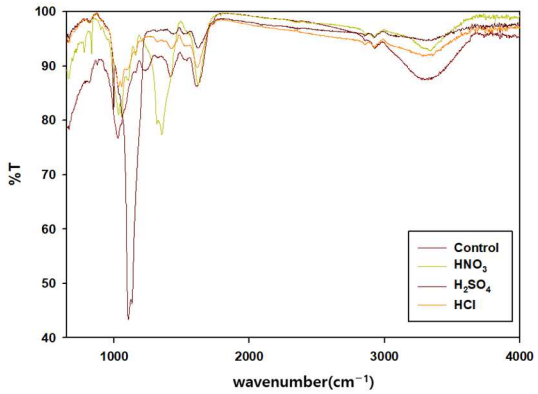

Figure 1. FT-IR spectra of pretreated *S. horneri* with H_2O and various acids.

The band located at 3400 cm^{-1} was assigned to O-H. The small peak at 2920 cm^{-1} indicated the polysaccharide substance. The band at 1640 and 1350 cm^{-1} indicated C=O and C-OH, respectively. The peak at 1350 indicated the S-O stretching vibration group. The strong absorption bands at 1000–1100 cm^{-1} were due to C-O-C [30,31].

All samples showed peaks at 3400 and 1640 cm^{-1}, which were due to O-H and C-O decrease, respectively. The control sample exhibited a decrease in both O-H and C-O following the addition of HCl, HNO_3, and H_2SO_4; this indicates that the degradation of O-H and C-O does not require a strong acid [32].

In the peak at 1350 cm^{-1}, S-O bond breaking was enhanced in the HNO_3-treated sample compared to other acids and H_2O. The peak at 1105 cm^{-1}, corresponding to the C-O-C asymmetric stretching of cellulose, strongly decreased in the H_2SO_4-treated sample, implying that during thermal acid hydrolysis with H_2SO_4, the cellulose content was highly exposed to H_2SO_4, which improved degradation. Based on the above results,

H_2SO_4 was selected as the optimal acid for thermal acid hydrolysis of *S. horneri* before enzymatic saccharification.

Enzymatic saccharification was conducted using Cellic Ctec2 (Table 2). After enzymatic saccharification, the maximum monosaccharide concentration was 23.52 g/L in the hydrolysate treated with H_2SO_4. In addition, the E_{AE}(%) of H_2SO_4 was 54%, which was 1.3–1.9 times higher than that of other acids and H_2O. A previous study showed that using H_2SO_4 not only increased the glucose yield but also significantly accelerated the enzymatic hydrolysis rate, especially during the initial stages of hydrolysis [33]. Therefore, H_2SO_4 effectively disrupts the cellulose structure during enzymatic saccharification.

To optimize other conditions, biomass concentration, H_2SO_4 concentration (50–200 mM), hydrolysis time (45–90 min), and enzyme loading (8–24 units/mL) were carried out to obtain maximum monosaccharide yield from *S. horneri* (Figure 2). The biomass concentration was evaluated with 6–12% (*w*/*v*) and 100 mM H_2SO_4 at 121 °C for 60 min followed by enzyme treatment by 16 units/mL of CTec2. Figure 2A illustrates a correlation between the increase in monosaccharide concentration and biomass concentration. Consequently, an analysis of the optimal biomass concentration was conducted using an E_{AE} approach. The EAE showed an upward trend until reaching a biomass concentration of 10% (*w*/*v*), after which it exhibited a decline. It is evident that a higher solid content in the biomass results in the production of more monosaccharides. However, it is worth noting that prior research has indicated that elevated solid content can lead to increased viscosity, making it challenging to handle the medium and diminishing the efficiency of pretreatment [34]. Moreover, the desirability of lower viscosity liquids becomes apparent as they can help prevent obstructions in bioreactors and facilitate the seamless transfer of desired liquid fractions in subsequent processes. Hence, 10% (*w*/*v*) was chosen as the optimal biomass concentration.

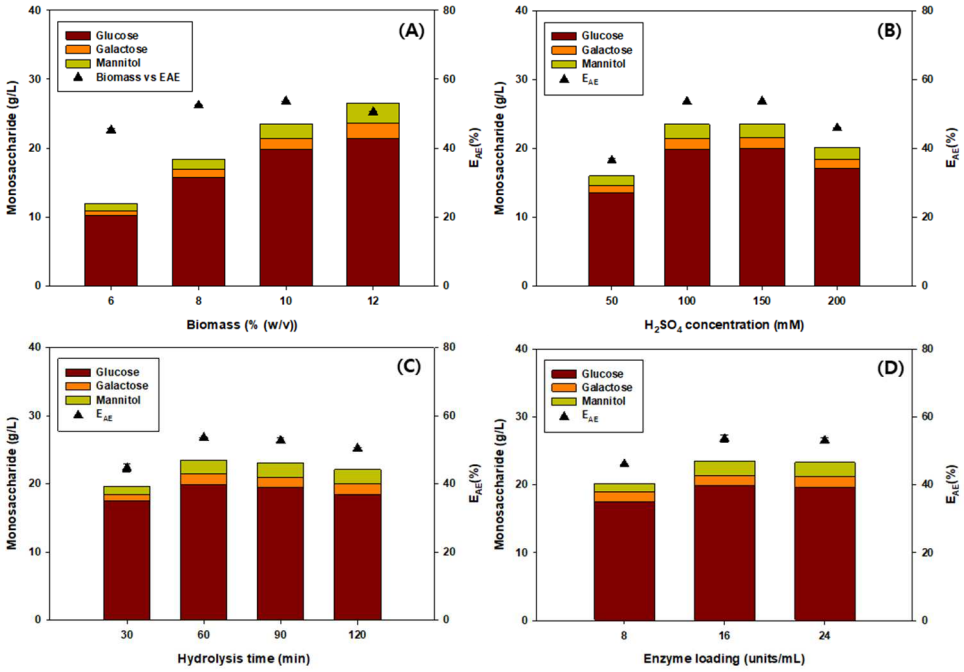

Figure 2. Optimization of *S. horneri* hydrolysate with (**A**) various biomass concentrations, (**B**) H_2SO_4 concentrations, (**C**) hydrolysis times, and (**D**) enzyme loadings.

H_2SO_4 concentration was assessed within the range of 50–300 mM using a solution of *S. horneri* at a concentration of 10% (*w*/*v*) under conditions of 121 °C for a duration of 60 min. Subsequently, enzyme treatment was administered with a concentration of 16 units/mL of CTec2. Figure 2B delineates the impact of varying H_2SO_4 concentrations. The highest monosaccharide yield, at 53.57%, was attained at an H_2SO_4 concentration of 100 mM,

closely followed by 53.37% at 200 mM. A decline in yield was observed beyond 150 mM. This reduction can likely be attributed to secondary reactions involving monosaccharides and other compounds, such as HMF or levulinic acid [35]. Consequently, the optimal H_2SO_4 concentration was determined to be 100 mM.

The treatment time spanned from 30 to 120 min, utilizing a 10% (*w*/*v*) *S. horneri* solution for 60 min, followed by enzyme treatment with a concentration of 16 units/mL of CTec2. Figure 2C illustrates that the maximum monosaccharide yield was achieved at 60 min. To obtain higher quantities of glucose, galactose, and mannitol, a longer hydrolysis time beyond 30 min was necessary. However, the E_{AE} did not exhibit an increase beyond 60 min. A similar rationale to the optimization of H_2SO_4 concentration applies here, as a longer pretreatment time results in a decrease in pretreatment yield due to glucose loss and the conversion of other compounds.

In various instances, cellulase mixtures have been employed for the enzymatic hydrolysis of brown seaweed [36,37]. Cellic CTec2 is comprised of two primary cellobiohydrolases, five distinct endo-1,4,-β-glucanases, β-glucosidase, β-xylosidase, and specific proprietary hydrolysis-boosting proteins. In this study, the pretreated *S. horneri* was treated with different Cellic CTec2 dosages ranging from 8 to 24 units/mL to assess their impact on monosaccharide release, as depicted in Figure 2D. The maximum monosaccharide yield was achieved at a dosage of 16 units/mL. Further increases in enzyme dosage did not result in higher monosaccharide yields. Therefore, a dosage of 16 units/mL proved sufficient to maximize monosaccharide release.

Pretreatment constitutes a crucial stage in the conversion of biomass into fuel or other chemicals, facilitating both saccharification and fermentation processes. Over time, numerous pretreatment methods have been developed, particularly in the context of seaweed-based biomass. In this study, maximum monosaccharides were obtained with 10% (*w*/*v*) *S. horneri* slurry treated with 100 mM H_2SO_4 at 121 °C for 60 min followed by enzymatic saccharification using 16 U/mL Cellic CTec2. However, further optimization needs to be performed under variable conditions that include the use of other pretreatment techniques, adjusting temperature, or using different enzymes.

3.3. Adaptive Evolution of S. cerevisiae BY4741 to Mannitol

Various organisms have been used to ferment sugars into ethanol. Furthermore, the adaptation of yeast to high sugar concentrations has been considered as a method to enhance ethanol yield in the fermentation of seaweed hydrolysates containing various types of sugars [13]. Glucose, galactose, and mannitol were detected in *S. horneri* hydrolysates. To ferment the hydrolysates, *S. cerevisiae* BY4741 was adapted to a mannitol-containing medium, rendering it capable of assimilating mannitol and using it for fermentation at 30 °C for 48 h (Figure 3).

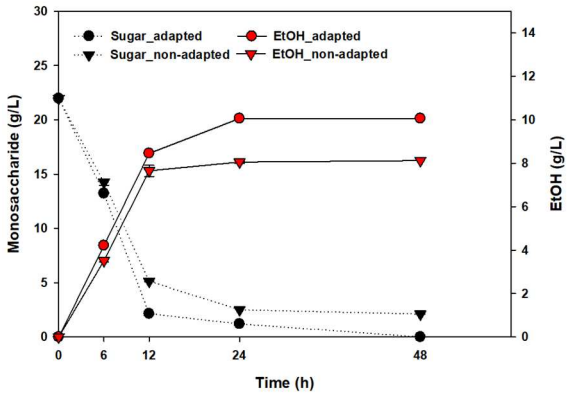

Figure 3. Ethanol fermentation with *S. cerevisiae* BY4741 and mannitol-adapted *S. cerevisiae* BY4741.

Typically, *S. cerevisiae* cannot consume mannitol as a carbon source. However, some *S. cerevisiae* strains cultured on mannitol for an extended period exhibit the ability to consume mannitol. Consequently, mannitol-adapted *S. cerevisiae* fermented *S. horneri* hydrolysates and produced 10.06 g/L of ethanol within 24 h. In contrast, non-adapted *S. cerevisiae*, which did not completely assimilate mannitol, produced 8.12 g/L of ethanol in 48 h. These observations suggest that BY4741 cells spontaneously acquired the ability to utilize mannitol. These cells adapted to mannitol-containing media, assimilated mannitol, and produced more ethanol than non-adapted cells.

According to previous studies, different *S. cerevisiae* strains (AH109, BY4742, DBY877, EBY100, SEY6210, T8-1D, and YPH500) form visible colonies on plates containing mannitol after extended cultivation periods [38]. These findings confirm that the acquisition of the mannitol assimilation capability is due to spontaneous mutations in genes encoding Tup1 or Cyc8, which constitute a general repressive complex that regulates various genes. Additionally, the evolved strains exhibited superior osmotolerance compared with other ethanol-producing micro-organisms [39]. These traits are expected to be highly valuable, especially for ethanol production from discarded marine biomass.

3.4. Continuous Hydrolysis, Enzymatic Saccharification, and Fermentation with Various Scales

The SHF process is commonly used primarily because of its flexibility in selecting hydrolysis methods. It also allows the utilization of optimal conditions for both hydrolysis and fermentation by micro-organisms [40]. Fermentation was conducted at various scales, ranging from 50 mL flasks to 5 L and 500 L fermenters, to optimize the pilot-scale design (Table 3).

Table 3. Summary of thermal acid hydrolysis and enzymatic saccharification and fermentation of *S. horneri* at 50 mL, 5 L-, and 500 L-scale fermenters.

Thermal Acid Hydrolysis and Enzymatic Saccharification	Glucose (g/L)	Galactose (g/L)	Mannitol (g/L)	Total Sugar (g/L)	EAE (%)
0.05 L	19.85 ± 0.03	1.56 ± 0.26	2.11 ± 0.07	23.52	53.58
5 L	13.32 ± 0.05	5.72 ± 0.05	1.83 ± 0.01	20.87	47.54
500 L	10.33 ± 0.04	5.18 ± 1.75	2.16 ± 0.14	17.67	40.24
0.05 L	0.00	0.00	0.83 ± 0.48	10.06	0.43
5 L	0.00	0.23 ± 0.01	0.62 ± 0.01	10.56	0.51
500 L	0.00	1.05 ± 0.40	0.07 ± 0.01	7.88	0.45

First, the 50 mL Erlenmeyer flask was used with a 25 mL working volume. *S. cerevisiae* BY4741 was used for ethanol production from the hydrolysate of *S. horneri*. The initial monosaccharide concentration comprised 19.85 g/L of glucose, 1.56 g/L of galactose, and 0.83 g/L of mannitol. Glucose and galactose were completely consumed at 48 h, but mannitol remained, producing 10.06 g/L of ethanol with an ethanol yield of 0.43 at 48 h.

Using the 5 L-scale fermenter, the initial concentrations of glucose, galactose, and mannitol were 13.32 g/L, 5.72 g/L, and 1.83 g/L, respectively. The maximum ethanol concentration was 10.56 g/L with an ethanol yield of 0.50 at 24 h. Glucose was completely consumed; however, galactose (0.23 g/L) and mannitol (0.62 g/L) were not. Although the pretreatment efficiency was lower, ethanol production showed a higher yield in L-scale fermentation than in the 50 mL flask, which may have been due to mechanical stirring. Previous studies also reported that mechanical stirring accelerates microbial growth and metabolic reactions during anaerobic fermentation [41,42].

For the mass-production of ethanol, a 500 L-scale fermenter was used with a 250 L working volume. The initial glucose concentration was 10.33 g/L, and it was totally consumed by *S. cerevisiae* BY4741 at 24 h. However, the initial concentrations of galactose and mannitol were 5.18 g/L and 2.16 g/L, respectively. The concentrations were 1.05 g/L

and 0.07 g/L, respectively, after fermentation. The maximum ethanol concentration was 7.88 g/L after 24 h.

3.5. Economic Analysis

The overall process flow for ethanol production formalized by the SuperPro Designer is presented in Figure 4. The first step in ethanol production was the pretreatment step. In this step, 250 kg of *S. horneri* was collected along the shores of Jeju Island, Republic of Korea. The biomass cost was set to zero because it was waste. Based on economic evaluations, the maximum dry seaweed price (MDSP) ranged from $28 to $91 [43,44]. In the context of sustainable biofuel production, the choice of feedstock plays a pivotal role. It is worth noting that, in the biofuel production process, the raw materials typically account for a significant portion, ranging from 50% to 70%, of the total operational expenses [24]. In our current investigation, we opted to utilize waste biomass for bioethanol production. This strategic decision effectively lowered both the overall expenditure on raw materials and the operational costs.

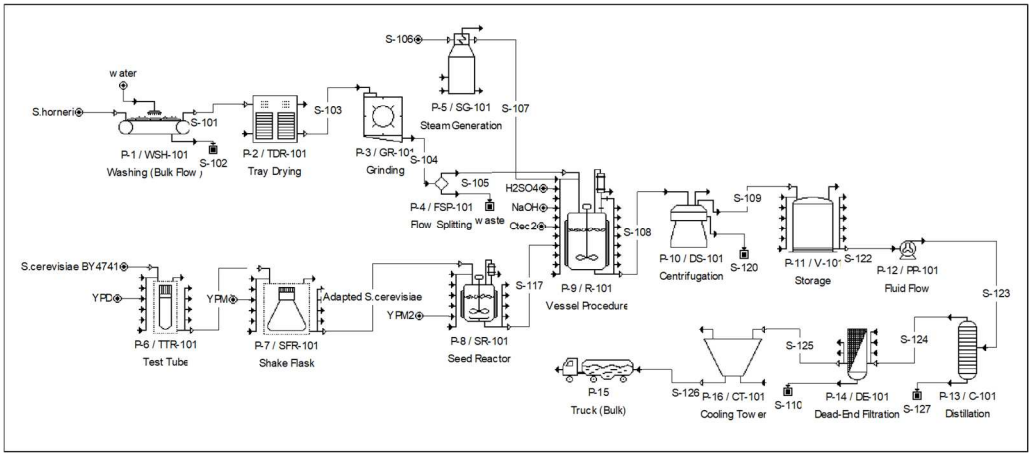

Figure 4. Process simulation for the production of ethanol from *S. horneri*.

Collected *S. horneri* underwent a cleaning process using a washing machine (P-1/WSH-101) and subsequent drying in a tray dryer (P-2/DDR-101). Following this, the biomass was subjected to grinding using a grinder (P-3/GR-101) and passed through a 200-mesh sieve before being transferred to the CHEF bioreactor (P9/R-101). Subsequently, ground *S. horneri* was transferred to the CHEF bioreactor, where pretreatment with H_2SO_4 was carried out at 121 °C for 60 min. The inlet stream is preheated to around 121 °C using a steam generator (P5/SG-101). The steam is provided by the water vapor generated during the expansion of the hydrolyzed *S. horneri*. The hydrolysate of *S. horneri* was neutralized with 10 N NaOH. Simultaneously, 16 units/mL of Cellic CTec2 was added to the same fermenter to facilitate saccharification. Various acid concentrations have been studied extensively for the hydrolysis of algal polysaccharides to optimize the process using diverse combinations of acid concentrations, reaction times, and temperatures [35]. It has been demonstrated that these parameters can more than double the yield of fermentable sugars obtained from algal biomass compared to the maximum achievable yield of sugars that can be obtained without optimizing these parameters [45].

The second section was responsible for medium preparation and fermentation. The sterilized YPD medium was inoculated with 10% (*v*/*v*) *S. cerevisiae* BY4741 in the test tube (P6/TTR-101) and moved to the shake flask (P-7/SFR-101) with YPM media for making mannitol-adapted *S. cerevisiae* BY4741. The yeast for seed culture was used to inoculate approximately 10% of the seed fermenter (P-8/SR-101), which was agitated at 150 rpm at 30 °C for 24 h. The adaptation of yeast could enhance the efficiency of industrial ethanol production through the effective fermentation of these sugars. *S. cerevisiae* BY4741, which adapted to and evolved in a mannitol medium, was inoculated into the CHEF bioreactor

containing *S. horneri* hydrolysate and efficiently fermented glucose, galactose, and mannitol. When considering algal biomass for ethanol production, only the cellulose fraction of the biomass (1.5–3.4% DW) is readily hydrolyzed and fermented among carbohydrates [46]. Converting the major sulfated polysaccharide portion (10–50%) of algal biomass into fermentable sugars remains challenging. Therefore, adapted and evolved yeast have been designed to overcome this challenge.

The final section of the process deals with the recovery of ethanol. The broth from the CHEF bioreactor is sent to the centrifuged (P-10/DS-101) and stored in a storage tank (P-11/V-101). Next, the mixture was transferred to the first distillation column(P-13/C-101) for 6 h to separate ethanol from the fermentation medium. Most of the water and fermentation broth were removed as bottom products. Simultaneously, the concentrated ethanol was transferred to secondary molecular sieve columns (P-14/C-101), which are used for ethanol dehydration in order to generate fuel-grade ethanol containing less than 0.1% of water. Subsequently, the product was cooled to 25 °C in a cooler (P-16/CT-101) then transferred to a truck and delivered (P-15).

All the equipment and raw materials costs are shown in Tables S1 and S2. The cost for the bioethanol production process was estimated at $17,493,597. Among the production steps, the distillation process incurred the highest cost, followed by the molecular sieve process and the CHEF bioreactor process (Figure S1a). In addition, the CHEF process, which uses only one fermenter, generally exhibits higher productivity than SHF and SSF, reducing downtime for cleaning and refilling vessel operations and enabling smaller factory sizes with the same annual production capacity. The initial continuous versions emerged in the 1970s but encountered various operational issues, such as high contamination levels, low productivity, low yields, and solid material flow problems. However, the continuous fermentation process in this study was optimized based on kinetic models to achieve high productivity, process flexibility, stability, and reduced chemical consumption, making it more cost-effective for ethanol production than separate batch processes.

When comparing raw material prices, it was observed that enzyme costs constituted a significantly higher expense, followed by medium costs, and then NaOH costs (Figure S1b). Previous research has also indicated that, similar to the production of feedstock, enzymatic saccharification is the second-most costly module, with enzyme costs alone potentially contributing up to 30% of the total operating expenditure [47]. To reduce the cost of enzymatic saccharification, various studies are being conducted, such as employing inexpensive enzyme production and mixing, partially substituting enzymes, or adjusting the enzymatic saccharification experimental conditions to facilitate enhanced hydrolysis [48].

Previous techno-economic studies have demonstrated that ethanol production from algae is economically viable and competitive with ethanol produced from energy crops and lignocellulosic biomass [43,44]. Roesijadi [43] and Fasahati [44] conducted techno-economic analyses and estimated the MDSP and minimum ethanol selling price for large-scale ethanol production from the giant kelp species *Laminaria*. These studies provided maximum annual plant capacities of 500,000 tons DW and 400,000 tons DW of *Laminaria* biomass, respectively. Factors such as feedstock prices, yield, seaweed moisture content, solids, and enzyme loading were considered. It was observed that with feedstock prices of $100/MT, the minimum ethanol selling price ranged from $3.6/gal to $8.5/gal, and a decrease in feedstock prices to $50/MT led to a further reduction. In addition, for the commercial-scale viability of ethanol production, approximately 4–5.0% ABV (alcohol by volume) of ethanol needs to be produced [34]. When yeast converts glucose to ethanol at a maximum rate of 0.51 g of ethanol per gram of glucose, economically producing ethanol from seaweeds would require a minimum of approximately 8% (80 g/L) of glucose in the hydrolysate. The process developed in this study has not yet been fully optimized and produce only 17.67–23.52 g/L monosaccharide; however, it achieved an ethanol yield of 1.0% ABV from the feedstock due to effective fermentation of monosaccharide using adaptive evolution and reduction of loss using the CHEF process. Still, with this ethanol yield, ethanol production alone (without additional bio-refining for high-value co-products) is not economically vi-

able and would not compete with ethanol produced from other sources [49]. Consequently, we need to find the optimal biomass price, yeast for fermenting, and a simple process. This study suggested that the low cost of discarded seaweeds makes them an advantageous raw material for ethanol production. From a technical perspective, optimizing the process for converting biomass into fermentable sugars and yeast development using adaptive evolution methods allows for more efficient and cost-effective ethanol production. In addition, a simple CHEF process could reduce ethanol production fees.

4. Conclusions

In conclusion, *S. horneri* is a valuable biomass resource for ethanol production. Acid hydrolysis and enzymatic saccharification were used to enhance monosaccharide production and improve cellulose digestibility by increasing enzyme accessibility. The optimal conditions for the pretreatment of *S. horneri* via acid hydrolysis involved using 100 mM H_2SO_4 with a 10% (*w*/*v*) slurry at 121 °C for 60 min. The ideal saccharification conditions included the use of 16 U/mL of Cellic CTec2 at 45 °C for 24 h. Mannitol-adapted *S. cerevisiae* showed a higher capacity for mannitol utilization than non-adapted *S. cerevisiae*, resulting in the highest ethanol yield. The highest ethanol concentration, achieved through thermal acid hydrolysis and enzymatic saccharification in conjunction with mannitol-adapted *S. cerevisiae*, was 10.6 g/L with a Y_{EtOH} of 0.43 using the 50 mL flask. As a result of the scale-up process, the 5 L- and 500 L-scale fermentations obtained a Y_{EtOH} of 0.51 and 0.45, respectively. To gain a more comprehensive understanding of the ethanol production process, a process simulation was conducted for ethanol production using *S. horneri*. Finally, an economic feasibility analysis was conducted on biomass and ethanol prices.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9121004/s1, Table S1: Equipment capacity and cost; Table S2: Input material cost; Figure S1: Comparison of cost (a) Equipment cost, (b) raw material cost.

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