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Hydrothermal Acid Pretreatment of *Chlamydomonas reinhardtii* Biomass for Ethanol Production

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Certain microalgae have been known to use light and various carbon sources to produce carbohydrates, mainly in the form of starch. This is one of the pertinent feedstocks replacing agricultural products for the production of bioethanol by yeast. This study focuses upon dilute acid hydrothermal pretreatments at low cost and high efficiency to compete with current methods, and employs *Chlamydomonas reinhardtii* UTEX 90 as the feedstock. With dry cells of 5% (w/v), the algal biomass was pretreated with sulfuric acid (1–5%) under temperatures from 100 to 120°C, from 15 to 120 min. As a result, the glucose release from the biomass was maximum at 58% (w/w) after pretreatment with 3% sulfuric acid at 110°C for 30 min. This method enabled not only starch, but also the hydrolysis of other oligosaccharides in the algal cell in high efficiency. Arrhenius-type of model equation enabled extrapolation of some yields of glucose beyond this range. The pretreated slurry was fermented by yeast, *Saccharomyces cerevisiae* S288C, resulting in an ethanol yield of 29.2% from algal biomass. This study suggests that the pretreated algal biomass is a suitable feedstock for ethanol production and can have a positive impact on large-scale applied systems.

Keywords: Acid pretreatment, ethanol, microalgae

Research to promote renewable energy has flourished in many countries as preventative maintenance for future lack of viable fuels. Bioethanol is one of the potential sources that can reduce gasoline consumption and environmental pollution. Most current bioethanols are derived from agricultural stock (starch, corn, rice, wheat, and cassava), or from raw materials that encroach on agriculture lands (sugar cane, sugar beet, and sweet sorghum) that depend on appropriate geographical conditions of each country [14, 16]. These are not prospective selections for bioethanol production as the human demand for food has yet to be met.

Thus, process technologies for bioconversion of other feedstocks into ethanol have been strategically developed [4, 6, 19, 20]. Green microalgae are photosynthetic microorganisms having the ability to fixate CO_2 and accumulate intracellular starch in high content [9]. In addition, this photosynthetic microalgae shows environmental-friendly metabolism through its CO_2 fixation and release of O_2. With a high growth rate, microalgae can be easily cultured at a high yield in a low-cost photobioreactor or an unused aquatic zone with unlimited energy, the sunlight. With such advantages, microalgae can be preferentially selected as a safe and clean feedstock for bioethanol production. According to the 2006 report of National Renewable Energy Laboratory (NREL), the commercial application of microalgae on industrial scales is still at low levels. *Chlamydomonas reinhardtii* is a microalgae that accumulates unusually high amounts of starch with a high growth rate. However, intracellular starch must be broken down into its constituent glucose molecules before the sugars can be fermented into ethanol. Intending to utilize this starch as feedstock, another obstacle is that the starch granules are bound within rigid cell walls [18], thus a pretreatment step should be requisitely introduced to release the fermentable sugars and other nutrients for yeast fermentation. Therefore, the conversion of algal cell mass into ethanol is composed of 2 stages: first, a pretreatment for the hydrolysis of the starch into simple sugars; and second, the fermentation of those sugars into ethanol. Since the fermentation process has been extensively investigated, the development of an effective pretreatment is the primary focus.

Traditionally, acid hydrolysis has been applied toward hardwoods and agricultural by-products where the carbohydrates are composed mainly of cellulose, xylan, and starch. Because mass transfer and kinetics in the acid hydrolysis are very

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complicated and heterogeneous, mathematical modeling has been carried out using simplified models and severity analysis [15]. The combined severity factor (CSF) has been used to predict the hydrolysis of polysaccharides in hydrothermal processes and to interpret data by including the effects of 3 major operational variables (reaction time, acid, and temperature) in a single parameter.

In this study, the hydrothermal pretreatment with dilute sulfuric acid was applied for the hydrolysis of high-density algal biomass, *Chlamydomonas reinhardtii* UTEX 90, in an attempt to determine the optimal conditions to generate monomeric sugars available for more efficient production of ethanol by yeast. A variety of pretreatment conditions (temperature, acid concentration, and residence time) were investigated for acidic digestibility. This work interpreted the acid hydrolysis mathematically by means of the CSF. We demonstrated that the hydrolysate from the microalgae has a potential usefulness as a fermentation feedstock by cultivating the ethanol-producing yeast, *Saccharomyces cerevisiae* S288C, with it.

Materials and Methods

Algal Cell Culture

A marine green alga, *Chlamydomonas reinhardtii* UTEX 90, was grown in Tris-acetate-phosphate (TAP) medium containing the following components (g/l): K_2HPO_4, 0.108; KH_2PO_4, 0.056; NH_4Cl, 0.4; $MgSO_4{\cdot}7H_2O$, 0.1; $CaCl_2{\cdot}2H_2O$, 0.05; $C_4H_{11}NO_3$, 2.422; $C_{10}H_{16}N_2O_8$, 0.05; BO_3H_3, 0.0114; $ZnSO_4{\cdot}7H_2O$, 0.022; $MnCl_2{\cdot}4H_2O$, 0.00506; $FeSO_4{\cdot}7H_2O$, 0.00499; $CoCl_2{\cdot}6H_2O$, 0.00161; $CuSO_4{\cdot}5H_2O$, 0.00167; $MO_7O_{24}(NH_4)_6{\cdot}4H_2O$, 0.0011; and 1.0 ml of glacial acetic acid [13]. Cell cultures were carried out at 23°C in a 2.5-ml photobioreactor containing 1.6 ml of autoclaved medium with 10% inoculation under continuous illumination (450 μE/m^2/s), provided by white fluorescent lamps, and rotary agitation (130 rpm). The pH of the medium was automatically adjusted to the range of 7.0 to 7.4 by the feeding of 1.0 M acetic acid. The growth of the algae cells was monitored by measuring the optical density (OD) of the appropriately diluted culture broth at 600 nm [20]. The cells were harvested by centrifugation at 5,000 ×*g* for 3 min. The harvested cells were characterized and used for pretreatment of the feedstock biomass (Fig. 1). The dry cell weight (DCW) was measured on filter paper (GC/F, England) dried at 80°C [12].

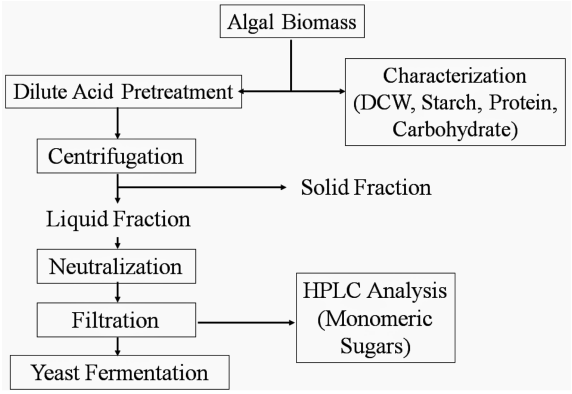

Fig. 1. Experimental procedure for characterization and pretreatment of algal biomass.

Analytic Methods

The cell pellets were broken down by vortexing with glass beads in a methanol solution with white pellets indicating the broken cell wall. After a methanol extraction, the total chlorophyll was measured by the Spreitzer method [8]. The cell lysates were put in the oven at 80°C for 6 h to vaporize the methanol. Starch was extracted at 0°C for 12 h by the addition of 72% perchloric acid. After filtering the extracted starch through a 0.2-μm filter, the contents of the starches in the filtrate were determined by an iodo-starch reaction method [16].

The anthrone reagent was used for colorimetric determinations of total cell carbohydrate. The algal cell pellets were mixed with 67% sulfuric acid for 30 min and then with anthrone reagent for 5 min at 100°C. The OD of the resulting solution was measured at 630 nm.

Total intracellular protein content was analyzed by the Bradford method [5]: After cell disruption by sonication in PBS buffer (pH 7.3), the supernatant was mixed with the Bradford reagent (Sigma, U.S.A.). The OD of the colored complex was measured at 595 nm. Light intensity in the photobioreactor was measured with a LI-250 Li-Cor quantum photometer (Lambda Instrument Corp., U.S.A.).

Hydrothermal Pretreatment of Algae with Dilute Sulfuric Acid

Cell pellets were washed once and slurried in water at a 5% solid to liquid ratio (w/v), mixed with sulfuric acid at a final concentration between 1% to 5% (v/v), and pretreated in an autoclave vessel at different temperatures (100, 110, and 120°C) from 15 to 120 min. The liquid containing the sugars hydrolyzed from the algal biomass was collected by centrifugation at 5,000 ×*g* for 3 min, and was then neutralized with $CaCO_3$ to adjust the pH to between 6.5 and 7. The formed solid precipitate was removed by centrifugation (4,000 ×*g*, 15 min). The liquid fraction was then filtered through a 0.2-μm membrane filter prior to analysis.

The concentration of the monomeric sugar, such as glucose, xylose, galactose, and arabinose, in the liquid fraction, was analyzed by high-pressure liquid chromatography (HPLC). The separation system consisted of a solvent delivery system (P2000 pump; Spectra-Physics, San Jose, CA, U.S.A.) equipped with an autosampler (N°. 60044, Spark, The Netherlands), a refractive index detector (Acme 9000, Younglin Instrument), and a computer software-based integration system (DS Chrom 99). Ion-moderated partition chromatography columns (Aminex HPX-87P, 300 mm×7.8 mm; Bio-Rad, Richmond, CA, U.S.A.) were used with a Carbo-P micro-guard cartridge. The Aminex HPX-87P column was maintained at 80.0°C, and the sugars were eluted with deionized water (Sigma, U.S.A.) as the mobile phase at a flow rate of 0.6 ml/min. Peaks detected by refractive index were identified and quantified by comparison with retention times of authentic standards.

Ethanol Fermentation by Yeast

Fermentability tests were performed on the pretreated algal biomass using ordinary compressed Baker's yeast, *Saccharomyces cerevisiae* S288C. The solid fraction of the pretreated algal biomass was removed by centrifugation (4,000 ×*g*, 15 min). The pH of the liquid

fraction was adjusted from 4.5 to 5.0 with $CaCO_3$, a pH range suitable for yeast for ethanol fermentation.

Yeast cells were precultured aerobically to the mid-logarithmic phase (approximately 15 g/l of dry weight) in a 250-l shake flask containing 100 ml of YEP medium (20 g/l yeast extract, 20 g/l sucrose, 10 g/l peptone) at 30°C for 24 h. The precultured yeast cells were added at 10% of inoculation size to the liquid fraction of the pretreated algal biomass, before the main culture was performed anaerobically at 30°C. Samples were withdrawn after 24 h and the cell growth was measured turbidometrically at 600 nm and converted to the cell dry weight by an appropriate calculation curve. The concentration of the produced ethanol was analyzed by HPLC under the same conditions as the analysis of monomeric sugars after pretreatment.

RESULTS AND DISCUSSION

Preparation of Algal Biomass by Photobioreactor

To obtain a large amount of algal biomass for usage in pretreatment, a photosynthetic microalgae, *C. reinhardtii* UTEX 90, was cultivated in a photobioreactor. Acetic acid (1.0 M) stock was added in the fed-batch mode to control the pH within a range of 7.0 to 7.4. The addition of acetic acid promoted the growth of the cells as well as the accumulation of starch [2, 13]. In the first 48 h, most of the nutrient was used as an energy source for growth of the algal cells, while little starch was produced (Fig. 2). The starch reached its highest level by the mid-exponential phase of cell growth but gradually decreased in the stationary phase. This result coincided with the previous study concerning the physiology of starch accumulation during the growth of *Chlamydomonas* [21]. After 50 h of culture time, the algae cells started to starve for other essential elements such as magnesium or potassium, and therefore, although the acetic acid was continuously provided as a carbon source under phototrophic conditions, cell growth was restricted. In the restricted culture conditions, large amounts of starch gradually accumulated, whereas the intracellular chlorophyll decreased. Finally, the cell mass was harvested in high density (2.4 g/l) with the highest starch content (35%, based on dry cell mass) after 96 h of culturing. The composition of the harvested algal biomass was characterized as 60.0% carbohydrate (35.0% starch) and 8.3% protein (Table 1), which are available sources of C and N, respectively, utilized as feedstock for ethanol fermentation.

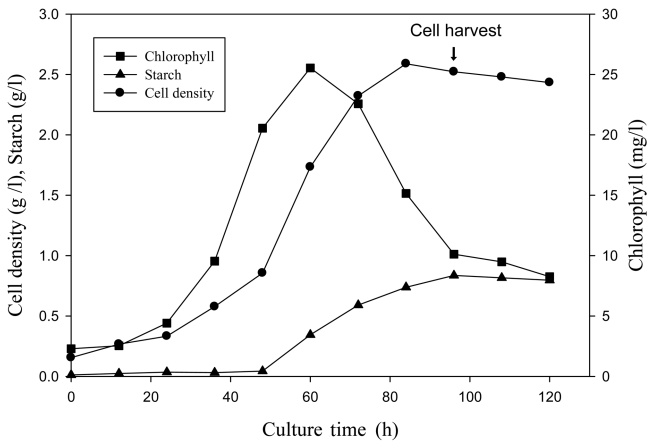

Fig. 2. Cell growth, chlorophyll content, and starch accumulation in the pH-stat fed-batch culture of *C. reinhardtii* UTEX 90 by feeding 1.0 M acetic acid under continuous illumination (450 μE/m²/s), using a 2.5-l photobioreactor.

Table 1. Characterization of cell mass composition for 2.4 g/l of *Chlamydomonas reinhardtii* UTEX 90 obtained from the pH-stat fed-batch culture by feeding 1.0 M acetic acid under continuous illumination (450 μE/m²/s).

Main components	Composition based on cell mass (%, w/w)
Protein	8.3
Carbohydrate (starch)	60.0 (35.0)
Others	31.7

Hydrothermal Hydrolysis of Algal Starch by Dilute Acid

Factors affecting dilute acid hydrolysis of algal biomass into fermentable sugars were examined. These included acid concentration, temperature, and residence time, as hydrothermal acid hydrolysis is generally regarded a chemical reaction having Arrhenius temperature-dependent rates [5]. The effect of acid dosage on the release of glucose was evaluated at several concentrations of sulfuric acid [1, 2, 3, 4, and 5% (v/v)]. At higher acid concentration, faster hydrolysis was observed, especially at 100°C (Fig. 3A), and allowed the shortening of residence time in the pretreatment process.

There should be a trade-off between acid concentration and temperature in acid hydrolysis because sulfuric acid is fairly expensive. Three temperature levels (100, 110, and 120°C) were tested to determine its effect on hydrolysis (Fig. 3). To achieve similar effects, the more dilute acid was used at higher temperatures, whereas the more concentrated acid at lower temperatures. Higher temperatures also shortened the residence time. To achieve 20% release of glucose (based on algal biomass) under hydrothermal hydrolysis using 1% acid, 60 min of residence time was required at 100°C, but a shorter residence time (15 min) was needed at 110°C. It was no wonder that extending the residence time would generate more released sugar, up to each saturation level according to acid dosage and temperature conditions. When the lower acid (1% and 2%) hydrolysis at 120°C was compared with that at 110°C, the release of glucose at 120°C was greater at an earlier residence time (15 min); however, at a later residence time (45 min), the maximum of glucose released at 120°C was lower than that at 110°C. This is likely due to the glucose

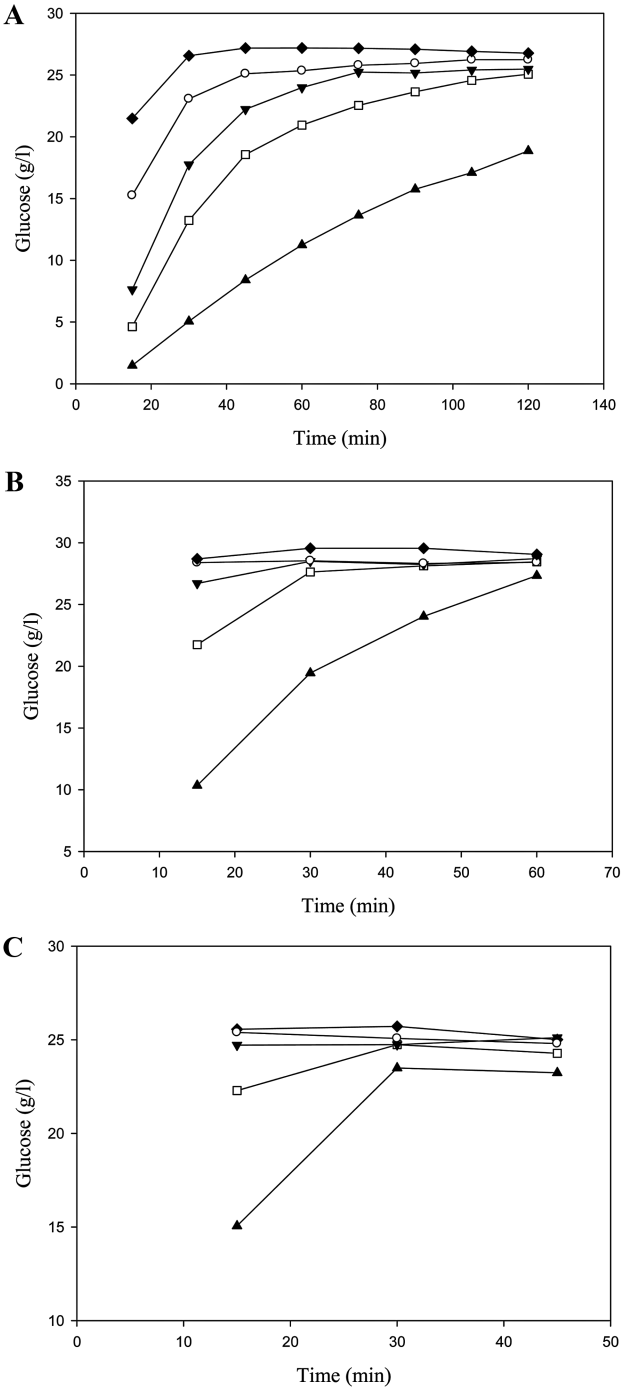

Fig. 3. Glucose released by hydrothermal dilute acid pretreatment under different conditions.
A. Dilute acid pretreatment at 100°C; **B**. Dilute acid pretreatment at 110°C; **C**. Dilute acid pretreatment at 120°C. ▲: 1% acid; □: 2% acid; ▼: 3% acid; ○: 4% acid; ◆: 5% acid.

released from starch decomposing into other compounds (by-products) under the treatment at higher temperatures [7]. Thus, the excessively high temperature pretreatment was excluded to minimize further decomposition of glucose. It was also reasonable that each acid pretreatment should be completed within the suitable residence time to minimize decomposition.

It was found by HPLC analysis that the liquid fraction of the pretreated algal biomass contained several kinds of monomeric sugars, 90% glucose, 6% galactose, 3% arabinose, and traces of xylose at 120°C, and at 110°C, 88% glucose, 7% galactose, 4% arabinose, and a trace of xylose. Starch is a polysaccharide carbohydrate consisting of a large number of glucose monosaccharide units. Therefore, the analysis showed that hydrothermal acid hydrolysis induced carbohydrates other than starch to be hydrolyzed [1]. Although glucose, which is a fermentable sugar by yeast, took the highest percent, it was a primary purpose to investigate which conditions of pretreatment gave the highest yield of glucose. The maximum release of glucose was 29.6 g/l with pretreatment with 5% sulfuric acid at 110°C (Fig. 3B), which corresponded to 59% (w/w) of the dry cell weight. Considering the original composition of the algal biomass (Table 1), the results suggested that almost all of the glucose contained within the cell was released from the carbohydrates by dilute acid hydrolysis [11]. Compared with another method such as enzymatic hydrolysis, this dilute acid hydrolysis had an advantage of nonspecific action towards carbohydrates in the feedstock, thus leading to the higher yield.

The maximum release of glucose was 28.5 g/l, which corresponded to 58% (w/w) of the dry cell weight, after 30 min of residence in pretreatment with 3% sulfuric acid at 110°C (Fig. 3B). However, it took 60 min to release 28.5 g/l of glucose with 2% acid concentration at the same temperature, too much time to operate the process in an efficient heating system. The dilute acid hydrolysis with 3 levels of acid concentration (3%, 4%, and 5%) at 110°C resulted in similar effects, averaging a release of 28.9 g/l glucose. Moreover, the use of higher concentrations of acid required greater amounts of the neutralizing agent in the following step and tended to corrode the experimental equipment. Therefore, it was desirable to perform the hydrothermal acid pretreatment at as low a concentration of acid as possible. Thus, the condition of 3% acid at 110°C at 30 min was chosen as the best for the dilute acid pretreatment in this study. Before pretreatment, the algal biomass did not hold as much intracellular glucose as the other feedstock (rice, corn, and cassava) (Table 1). Nevertheless, an amount of glucose could be efficiently extracted from it by the hydrothermal dilute acid pretreatment.

These results demonstrated the dilute acid steam pretreatment as a useful approach to the release of monomeric sugars from algal biomass containing 2.4 g/l of total solids. It is probable that this process can also be applied to other starch sources.

Estimation of the Optimal Conditions Based on Kinetics of Acid Hydrolysis

The experimental data obtained for this study were used to provide a reliable interpretation of data concerning starch

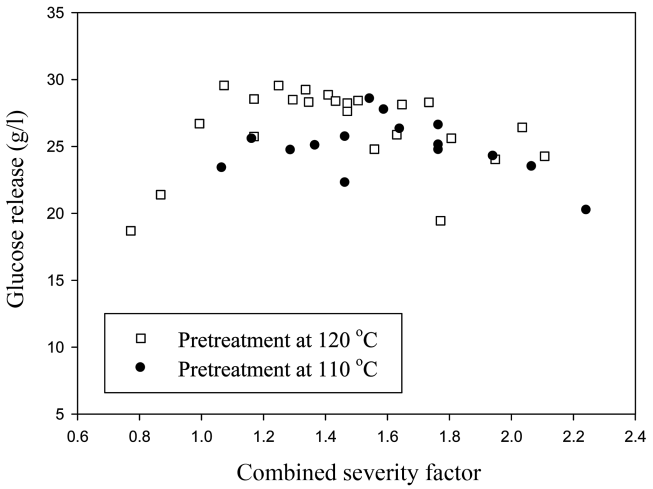

Fig. 4. Glucose release from pretreated biomass as a function of CSF.

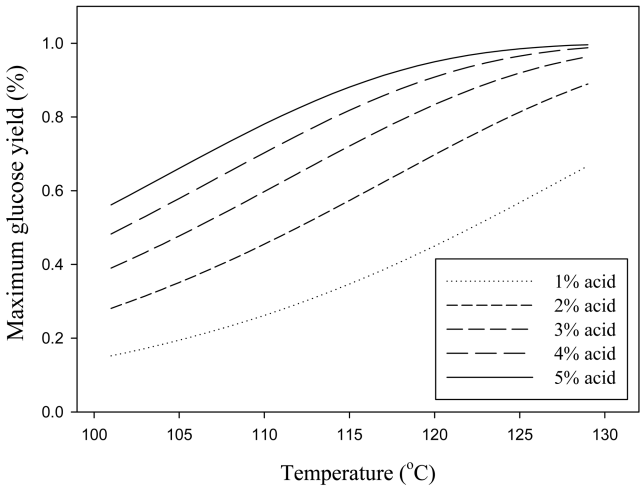

Fig. 5. Predicted maximum release of glucose as a function of temperature and pH.
The lines between temperatures 100 and 120°C show the range investigated in the experiments.

depolymerization under isothermal acid hydrolysis of algal biomass. If we assume that the kinetic equation for reagents (starch, S, and hydrogen, H^+) participates in a typical irreversible reaction of first order with an Arrhenius-type dependence on temperature, then

$$S+H^+ \rightarrow \text{Products}$$
$$-d[S]/dt=k_0\times\exp[-E_a/(R\times T)]\times[S]\times[H^+] \quad (1)$$

where [S] and [H^+] are the concentration of starch and acid respectively, t the reaction time, k_0 the rate parameter, E_a the activation energy, R the gas constant, and T the absolute temperature. By developing (1/T) as a 2-term Taylor series, Eq. (1) can be rewritten as

$$-d[S]/dt=k_0\times\exp[-E_a/(R\times T)] \times\exp[(T-T_r)/\omega]\times[S]\times[H^+] \quad (2)$$

where $\omega=R\times T_r^2/Ea$, and T_r is the reference temperature that is usually set to 100°C. After separation of the variables and integration ($S=S_0$ at t=0 and S=S at t=t), it follows that

$$[S]=[S_0]\times\exp(-k_r\times R_o\times[H^+]) \quad (3)$$

where k_r is the kinetic constant measured at T_r, and R_o is the severity factor, defined by $R_o=t\times\exp[(T-T_r)/\omega]$, where $\omega=R\times T_r^2/E_a$, which, in this and most other studies, is assigned the value of 14.75. The combined severity factor (CSF) is defined by $\log\{\ln[S]-\ln[S_0]\}=\log R_0-\log[H^+]$, which combines the effects of pretreatment temperature, residence time, and acid concentration into a single parameter for analysis of the results.

Fig. 4 presents the release of glucose as a function of the CSF, which showed a broad peak release of glucose at a CSF of 1.2 and 1.5 for pretreatments at 120°C and 110°C, respectively. Above a CSF of 1.7, the released glucose declined, likely because the coarse pretreatment conditions at these higher severities degraded more of the glucose into other products not included in the kinetic equation. Kinetic parameters were estimated using the following data values: E_a=58.4 (kcal/g mol) and $k_0=4.48\times10^{-29}$ ($min^{-1}\cdot M^{-1}$). Thus, the kinetic model was used to predict maximum yield of glucose as a function of temperature and pH (addition of 1%, 2%, 3%, 4%, and 5% acid) by adjusting residence time (Fig. 5). The maximum yields of glucose increased with increasing acid and increasing temperature. The graph enabled extrapolation of some yields of glucose beyond the range, within which the model parameters were estimated.

Yeast Fermentation

To utilize the pretreated biomass as a medium for yeast growth, the effective usefulness was estimated by checking yeast growth through separate hydrolysis and fermentation methods (SHF). Reference fermentation with a pure glucose

Table 2. Glucose, ethanol, and ethanol yields after 24 h fermentation of yeast, *Saccharomyces cerevisiae* S288C.

Growth medium	Glucose remaining (g/l)	Ethanol produced (g/l)	Ethanol yield[c] (g/g)
Reference[b]	11.6	5.7	20.1
Pretreated biomass[a]	0	14.6	51.2

[a]Yeast was cultured using the algal biomass pretreated with 3% acid at 110°C for 30 min.
[b]Growth medium contained the same amount of glucose as the pretreated algal biomass (28.5 g/l).
[c]Ethanol yield was calculated based on the amount of consumed glucose in the samples.

solution (28.5 g/l) instead of the pretreated algal biomass was also performed as a control. The reference medium lacked the additional components, such as nitrogen sources, whereas the pretreated algal biomass contained high contents of protein, an abundant source of nitrogen, and helped the yeast's growth and metabolism. Furthermore, it is not needed to add nitrate to the yeast growth medium separately. Thus, the rate of ethanol production with the reference medium was slower than with the pretreated algal biomass. In actuality, after 24 h of culture time using the pretreated algal biomass, the concentration of produced ethanol reached a maximum of 14.6 g/l (Table 2), whereas for the reference medium, only 13 h was required.

The biodegradability of the pretreated algal biomass was also tested in yeast fermentation. Nearly all the glucose contained in the pretreated algal biomass was consumed, whereas only a portion, 59.3%, contained in the reference medium was used. The remaining glucose is indicative of restriction of cell growth due to depletion of essential components for physiological functioning. As a result, the potential ethanol yield from pretreated algal biomass, 51.2 g/g glucose, was higher than that from the reference medium in the fermentation process, 20.1 g/g glucose. This result suggested that the pretreated algal biomass contained additional, major necessities for the yeast's physiology that served as feedstocks suitable for yeast fermentation to produce high yields of ethanol. Finally, 292 mg of ethanol was produced from 1.0 g of algal biomass through SHF. The main advantages of this SHF process include the low cost of chemicals, short residence time, and a simple equipment system, all of which would promote its large-scale application.

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