Improved Glucose Recovery from Sicyos angulatus by NaOH Pretreatment and Application to Bioethanol Production

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Date Submitted: 2022-10-25

Keywords: Sicyos angulatus, pretreatment, sodium hydroxide, bioethanol

Abstract:

As greenhouse gases and environmental pollution become serious, the demand for alternative energy such as bioethanol has rapidly increased, and a large supply of biomass is required for bioenergy production. Lignocellulosic biomass is the most abundant on the planet and a large part of it, the second-generation biomass, has the advantage of not being a food resource. In this study, Sicyos angulatus, known as an invasive plant (harmful) species, was used as a raw material for bioethanol production. In order to improve enzymatic hydrolysis, S. angulatus was pretreated with different NaOH concentration at 121 °C for 10 min. The optimal NaOH concentration for the pretreatment was determined to be 2% (w/w), and the glucan content (GC) and enzymatic digestibility (ED) were 46.7% and 55.3%, respectively. Through NaOH pretreatment, the GC and ED of S. angulatus were improved by 2.4-fold and 2.5-fold, respectively, compared to the control (untreated S. angulatus). The hydrolysates from S. angulatus were applied to a medium for bioethanol fermentation of Saccharomyces cerevisiae K35. Finally, the maximum ethanol production was found to be 41.3 g based on 1000 g S. angulatus, which was 2.4-fold improved than the control group.

Record Type: Published Article

Submitted To: LAPSE (Living Archive for Process Systems Engineering)

Citation (overall record, always the latest version):	LAPSE:2022.0096
Citation (this specific file, latest version):	LAPSE:2022.0096-1
Citation (this specific file, this version):	LAPSE:2022.0096-1v1

DOI of Published Version: https://doi.org/10.3390/pr9020245

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Improved Glucose Recovery from *Sicyos angulatus* by NaOH Pretreatment and Application to Bioethanol Production

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Keywords: *Sicyos angulatus*; pretreatment; sodium hydroxide; bioethanol

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Citation: An, H.-E.; Lee, K.H.; Jang, Y.W.; Kim, C.-B.; Yoo, H.Y. Improved Glucose Recovery from *Sicyos angulatus* by NaOH Pretreatment and Application to Bioethanol Production. *Processes* **2021**, *9*, 245. https://doi.org/10.3390/pr9020245

Received: 31 December 2020
Accepted: 27 January 2021
Published: 28 January 2021

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1. Introduction

After the Industrial Revolution, fossil fuels have been the world's primary energy source and now account for 88% of the world's energy consumption. However, with the rapid increase in the use of fossil fuels worldwide, greenhouse gases and environmental pollution have become serious, and the development of alternative energy such as biodiesel and bioethanol has become essential [1–4]. Bioethanol is the most widely used liquid biofuel in the world, with a global market amounting to $6.8 billion in 2019, which is estimated to grow to $7.2 billion in 2024 [5]. The technology of bioethanol production from sugar and starch-based edible crops such as sugar cane, corn, sugar beet and wheat are already at the commercial level [6]. However, the use of edible crops was faced with ethical problems due to competition for food resources, which has led to the utilization of non-edible crops as a sustainable feedstock for bioethanol production [7]. In particular, advanced biofuels, including bioethanol, biomethane, biodiesel, and aviation biofuels, have to be produced from sustainable feedstock according to the recent European regulation Renewable Energy Directive II [8].

Second-generation biomass is lignocellulosic biomass including industrial wastes (sawdust and bark), forestry wastes (hardwoods and softwoods), agricultural residues (straws and stover), and weedy plants [9]. Weeds are suitable as the feedstock for bioethanol production because they do not interfere with food supply and grow rapidly without effort to provide farmland, water, and energy [10]. *Sicyos angulatus* is known as an invasive plant species and is spread throughout Europe and Asia [11]. This species grows faster than crops such as corn, rice, ginseng, soybeans, and potatoes, and not only consumes agricultural

nutrients more quickly but also covers up the plants, disrupting photosynthesis, resulting in economic losses in terms of crop yield and management [12]. *S. angulatus* is listed as an invasive exotic plant by the Korean Ministry of Environment, and many efforts are being made to remove and prevent spread [13]. In general, invasive plants can be controlled by herbicide spraying, cutting, and burning, but these control methods incur treatment costs and lead to soil and air pollutions. [14,15]. Although there are no studies using *S. angulatus* as a raw material for biorefinery, there have been cases in which similar invasive plants such as *Acacia dealbata* and *Eichhornia crassipes* were used as raw materials to produce bioethanol and examine its potential usefulness [16,17]. The conversion of waste resources into energy or chemicals is considered strategically to address two issues simultaneously: reducing the amount of non-recyclable/non-reusable waste and a sustainable way to produce bio products [18,19]. Therefore, the strategy of using the removed invasive plants as a raw material for biorefinery without burning it can reduce environmental pollution and at the same time be converted into a sustainable bioproduct, thereby receiving higher valorization.

Lignocellulosic biomass is a complex compound composed of cellulose, hemicellulose, and lignin [20]. To convert lignocellulosic biomass into bioethanol, pretreatment and enzymatic hydrolysis are required to decompose the complex and recalcitrant structures and produce fermentable sugars [21]. Various pretreatment methods of lignocellulosic biomass such as chemical (acid and alkali), ionic liquid, proton and electron beam have been reported [22]. Chemical pretreatment is the most widely used method due to its high speed, high efficiency and low cost compared to other pretreatments [23]. Acid pretreatment with high temperature and pressure produces fermentation inhibitory compounds such as furfural, hydroxymethylfurfural, and acetic acid due to the decomposition of sugar [24]. Acids used for pretreatment are corrosive and toxic and should be recovered to design an economical process [25]. In contrast, alkali pretreatment is more effective to remove lignin under the mild reaction conditions than acid pretreatment and does not produce fermentation inhibitory compounds by minimizing the decomposition of sugar [26]. The intermolecular ester bonds between lignin, cellulose and hemicellulose are decomposed by saponification reactions with alkaline reagents, improving the enzyme accessibility [27]. NaOH, an inexpensive reagent, has been reported to be applied to alkaline pretreatment of various second-generation biomass. Enzymatic hydrolysis has the advantage of less generation of undesirable acidic waste, a high conversion rate to fermentation sugar, and no need for corrosion-resistant equipment [28]. Enzyme digestibility can be improved depending on reaction conditions such as NaOH concentration, treatment temperature, and time, and it can be seen that the optimum conditions are significantly affected depending on the type of biomass. In particular, the solid recovery decrease under severe reaction conditions, which can be a factor that significantly reduces the sugar recovery from biomass [29–32]. Therefore, it is required to derive appropriate pretreatment conditions according to the biomass to be used.

In this study, *S. angulatus* was used as the feedstock for bioethanol fermentation by *Saccharomyces cerevisiae* K35. NaOH pretreatment of *S. angulatus* was carried out at various NaOH concentrations, and the optimal concentration was determined based on the improved enzyme digestibility. The pretreated *S. angulatus* under optimal conditions were enzymatically hydrolyzed and the hydrolysates were used as a carbon source for bioethanol fermentation by *S. cerevisiae* K35. Finally, the overall process of biomass conversion to bioethanol was evaluated with a material balance based on 1000 g *S. angulatus*. Our research is expected to be useful in reducing soil and air pollution by converting invasive plants that cause environmental pollution into eco-friendly bioenergy.

2. Materials and Methods

2.1. Materials

Sicyos angulatus was collected from Yeongnam area of Korea. The biomass was milled to a particle size of 1–2 mm and dried in an oven at 105 °C for 72 h. The dried biomass was stored in plastic bags at room temperature. Celluclast® 1.5 L (cellulase), Cellic® CTeC2

(cellulosic enzyme) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium hydroxide (NaOH), sulfuric acid (H_2SO_4), calcium carbonate ($CaCO_3$) and citric acid monohydrate were purchased from Duksan Chemical (Ansan, Korea).

2.2. Alkaline Pretreatment

NaOH pretreatment was performed with 250 mL Erlenmeyer flask in an autoclave (VS-1221, Vision Scientific, Daejeon, Korea). 4 g dried biomass was reacted with 40 mL NaOH of various concentration (0%, 1%, 2%, 4%, 6% and 8%, *w/w*) at 121 °C for 10 min. 0% NaOH means that *S. angulatus* was pretreated using deionized water (DW). After NaOH pretreatment, the solid fraction was neutralized with DW until pH 7 using a 45 μm test sieve and dried in an oven at 105 °C for 72 h. All pretreatment was performed in triplicate to denote the standard deviation. Solid recovery (SR) of the sample was determined by following equation:

$$\text{Solid recovery, SR (\%)} = (\text{weight of dried sample after pretreatment}/\text{weight of initial dried sample}) \times 100 \quad (1)$$

2.3. Enzymatic Hydrolysis

Enzymatic hydrolysis was carried out according to the National Renewable Energy Laboratory (NREL) laboratory analytical procedure (TP-510-42629) [33]. 0.3 g sample was soaked in 10 mL 50 mM sodium citrate buffer (pH 4.8) and hydrolyzed with commercial enzyme at 50 °C for 72 h in shaking incubator at 180 rpm (SI-100R, Hanyang Science Lab, Seoul, Korea). One-unit filter paper (FPU) was defined as the amount of enzyme that released 1 μmol glucose per min under standard assay conditions. The enzyme loadings were 60 FPU/g-biomass Celluclast® 1.5L and 20 FPU/g-biomass Cellic® CTeC2. The activities of both Celluclast® 1.5L and Cellic® CTeC2 were about 120 FPU/mL. All enzymatic hydrolysis was performed in triplicate to denote the standard deviation. Enzymatic digestibility (ED) was determined by following equation:

$$\text{Enzymatic digestibility, ED (\%)} = (\text{weight of glucose released}/(\text{weight of glucan} \times 1.1)) \times 100 \quad (2)$$

where 1.1 is the conversion factor of glucan to glucose.

2.4. Bioethanol Production

Bioethanol production was performed by *Saccharomyces cerevisiae* K35 using *S. angulatus* hydrolysates. *S. cerevisiae* K35 was pre-cultured on YPD medium at 30 °C for 24 h in shaking incubator at 150 rpm. The composition of seed medium was as follows: 5 g/L yeast extract, 5 g/L peptone, 10 g/L glucose, 1 g/L $MgSO_4$ and 1 g/L K_2HPO_4. For bioethanol production, the main medium was inoculated with 10% (*v/v*) seed suspension. The composition of main medium was as follows: 5 g/L yeast extract, 5 g/L peptone, 10 g/L glucose (commercial glucose for control and glucose in *S. angulatus* hydrolysates for experimental group), 1 g/L $MgSO_4$ and 1 g/L K_2HPO_4. The inoculated main medium was incubated at 30 °C for 24 h in shaking incubator at 150 rpm. Cell growth was investigated with optical density (OD) at 600 nm using a UV-spectrophotometer (DU® 730, Beckman Coulter, Brea, CA, USA). All fermentation was performed in triplicate to denote the standard deviation. Ethanol yield from glucose was determined by following equation:

$$\text{Ethanol yield (\%)} = ((\text{weight of ethanol released} \times 2)/\text{weight of glucose consumed}) \times 100 \quad (3)$$

where, 2 is the conversion factor for glucose to ethanol.

2.5. Analytical Methods

The chemical composition of *S. angulatus* was analyzed according to the NREL laboratory analytical procedure (TP-510-42618) [34]. The sample weight of 0.3 g was soaked in 3 mL 72% (*w/w*) H_2SO_4 at 30 °C for 2 h. Then, the mixture was diluted to a concentration of 4% with DW and reacted at 121 °C for 1 h using an autoclave. The mixture was neutralized with $CaCO_3$ and the supernatant was filtered using a 0.2 μm syringe filter for high liquid performance chromatography (HPLC) analysis.

The concentration of glucose and ethanol was analyzed by HPLC equipped a Shodex SUGAR SH1011 H^+ ion exclusion column (300 mm × 8 mm, Shodex, Japan) and a refractive index detector (RID-10A, Shimadzu, Japan). The HPLC analysis conditions were as follows: mobile phase of 0.005 N H_2SO_4, flow rate of 0.8 mL/min, temperature of column at 50 °C and injection volume of 20 μL. Glucan content (GC) of the sample was determined by following equation:

$$\text{Glucan content, GC (\%)} = (\text{weight of glucan in dried sample/weight of dried sample}) \times 100 \quad (4)$$

3. Results and Discussion

3.1. Effect of NaOH Pretreatment on Carbohydrate Composition of Sicyos angulatus

The carbohydrate composition of *S. angulatus* was determined to be 16.4% glucan, 6.2% xylan, 11.8% lignin, 1.1% ash, and 64.5% others. *S. angulatus* showed a low lignin content compared to other lignocellulosic biomass such as rice straw (20.0%), corn stover (19.5%), *Imperata cylindrica* (19.0%), and canola straw (19.5%) [35]. In order to investigate the effect of NaOH pretreatment on biomass, *S. angulatus* was reacted with 0% (DW), 1%, 2%, 4%, 6% and 8% NaOH at 121 °C for 10 min. The biomass loading was selected as 10% (*w/w*) with a high sugar conversion since the high biomass loading (>15%, *w/w*) has disadvantages such as a high concentration of inhibitors and limitation of mass transfer [36]. In lignocellulosic biomass pretreatment process, alkaline concentration is a significant factor affecting degradation of the ester bonds between lignin-carbohydrate matrix [35]. Various study reported that the NaOH was suitable to remove lignin barrier, which increase the accessibility of enzyme to carbohydrate [37–39]. Carbohydrates of lignocellulosic biomass were comprised of cellulose and hemicellulose such as glucan, xyloglucan, xylan, arabinoxylan, mannan, gluco- or galactomannan [40]. Pentose including xylose and arabinose is not fermented by *S. cerevisiae*, which is commonly used for bioethanol production [41]. For this reason, the xylan content was not considered in this study.

Figure 1 is shown the carbohydrate composition and solid recovery (SR) of *S. angulatus* after NaOH pretreatment. Glucan content (GC) was determined to be 26.0%, 29.3%, 46.7%, 50.9%, 51.1% and 53.5% at NaOH concentration of 0% (DW), 1%, 2%, 4%, 6% and 8%, respectively. GC of *S. angulatus* pretreated with 2% NaOH was 2.8-fold higher than untreated *S. angulatus* and GC significantly increased with 2% or more NaOH concentrations. In addition, the lignin content of *S. angulatus* pretreated with 2% NaOH was found to be 8.8%, which was 3% lower than that of untreated *S. angulatus*. These results indicate that NaOH concentrations of 2% or more are suitable for *S. angulatus* pretreatment. SR following NaOH pretreatment was found to be 57.6%, 51.2%, 33.2%, 31.6%, 30.8% and 29.8% at NaOH concentration of 0% (DW), 1%, 2%, 4%, 6% and 8%, respectively. At NaOH concentrations of 2% or more, SR decreased below 40%. In the sugar conversion process from biomass, sugar recovery is hindered by low SR [42]. Severe pretreatment conditions such as high temperature and solvent concentration, and long reaction time lead to low SR [43]. These results are consistent with Yan et al. and Jiang et al., who reported that glucan content increased while the solid recovery decreased as NaOH concentration increased [44,45]. The aim of this study is to improve the enzymatic hydrolysis of *S. angulatus* by alkaline pretreatment for bioethanol production. Therefore, it is required to select the optimum NaOH concentration considering enzymatic digestibility (ED) of samples pretreated with different NaOH concentrations.

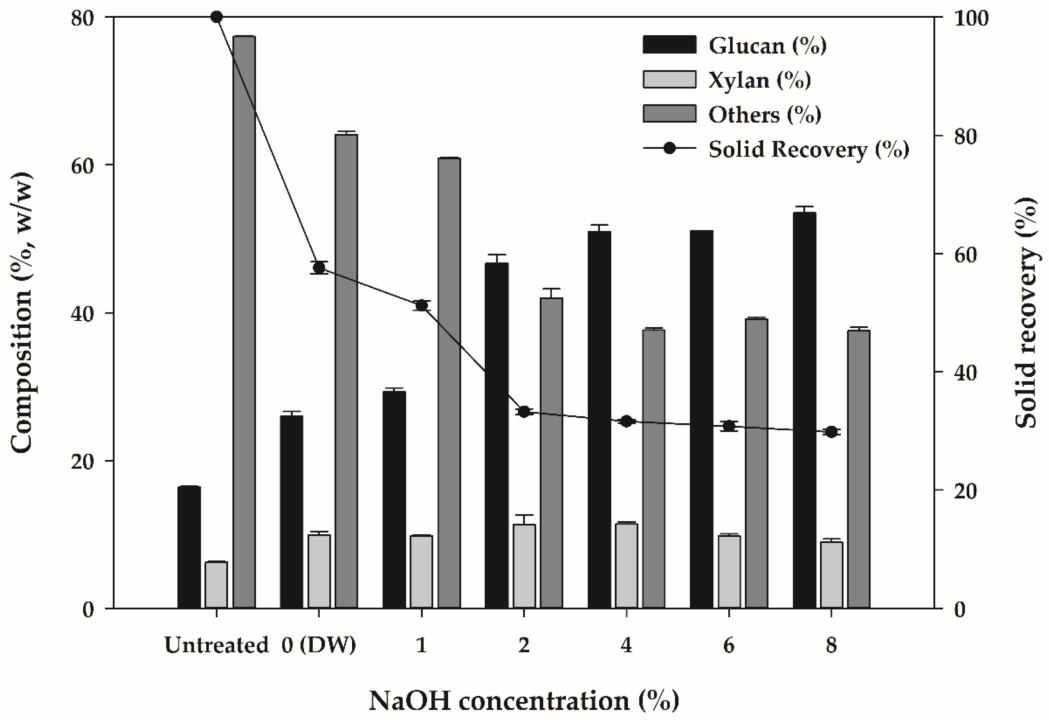

Figure 1. Chemical composition and solid recovery after pretreatment of *Sicyos angulatus* with different concentrations of NaOH at 121 °C for 10 min.

3.2. Effect of Alakaline Pretreatment on Enzymatic hydrolysis of Sicyos angulatus

The effect of NaOH pretreatment on ED is represented in Figure 2. ED of untreated *S. angulatus* was 22.1%. ED was found to be 47.5%, 50.1%, 55.3%, 48.2%, 47.6% and 40.5% at NaOH concentration of 0% (DW), 1%, 2%, 4%, 6% and 8%, respectively. It is indicated that ED was significantly improved by NaOH pretreatment of *S. angulatus*. The maximum ED was achieved with 55.3% at 2% NaOH, which is 2.5-fold higher than that of untreated *S. angulatus* (22.1%). The maximum ED of *S. angulatus* was higher than that of *Picea abies* (40.0%) and poplar (41.5%) and similar to that of sugarcane bagasse (55.1%) and *Typha angustifolia* (55.3%), compared to ED of lignocellulosic biomass pretreated with NaOH [29–32]. ED slightly decreased with increasing NaOH concentration above 2%. It is estimated that high concentrations (≥4%) of NaOH cause repolymerization or condensation of lignin, interfering with the accessibility of the enzyme to polysaccharides [46]. Therefore, 2% NaOH is determined as optimum concentration for the pretreatment of *S. angulatus*.

3.3. Bioethanol Production and Process Evalutaion

Figure 3 is shown the ethanol production profiling using *S. angulatus* hydrolysates (experimental group) by *S. cerevisiae* K35. In order to investigate the effect of fermentation inhibitory compounds that potentially exist in hydrolysates, glucose was used as the control group. The control and experimental group both showed the maximum ethanol production of 4.8 g/L at 9 h. Then, the ethanol yield of control and experimental group was 95.4% and 96.2%, respectively. The cell growth of the control and experimental group was similar, indicating that fermentation inhibitory compounds were negligibly produced during NaOH pretreatment. Kim et al. reported that the invasive plants, including *S. angulatus*, *Ambrosia artemisiifolia* and *Eupatorium rugosum*, have allelochemicals to survive in the ecosystem [47]. The allelochemicals such as flavonoids, terpenoids and organic acid have antibacterial activity [48–51]. In this study, the growth of *S. cerevisiae* K35 was not affected by the allelochemicals of *S. angulatus*. It is estimated that the allelochemicals contained in *S. angulatus* could be removed during pretreatment and washing stage. After 9 h of cultivation time, the initial 10 g/L glucose in both groups was almost consumed and

the ethanol concentration decreased, while the cell growth increased. Several studies were reported that *S. cerevisiae* can utilize maltose, galactose and ethanol, which are less preferred carbon sources than glucose when glucose is depleted [52,53]. In the overall fermentation process, the control and experimental group showed similar levels of ethanol production, ethanol yield and cell growth. These results indicate that hydrolysates of *S. angulatus* are suitable to utilize as a carbon source replacing glucose in the ethanol production process.

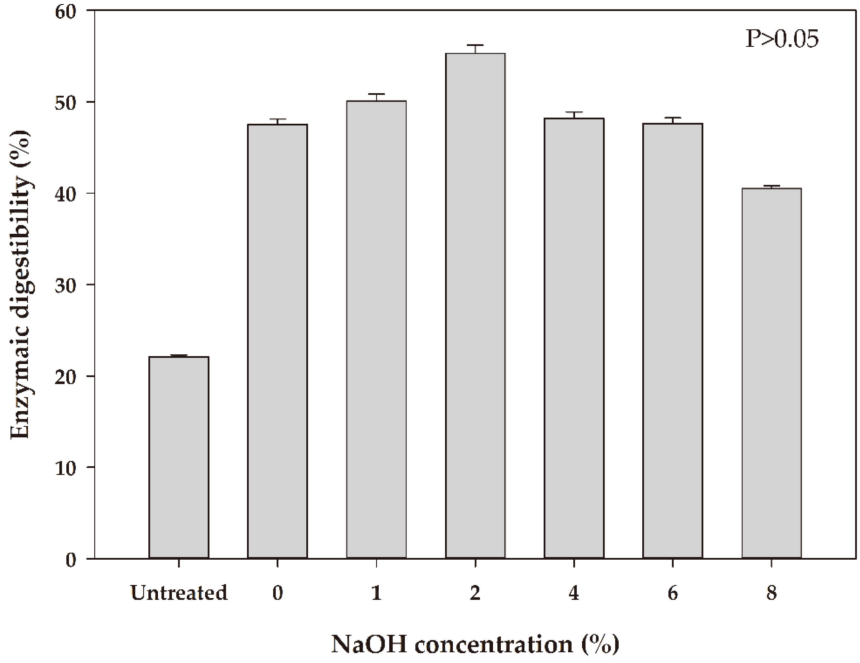

Figure 2. Enzymatic digestibility after pretreatment of *Sicyos angulatus* with different concentrations of NaOH at 121 °C for 10 min.

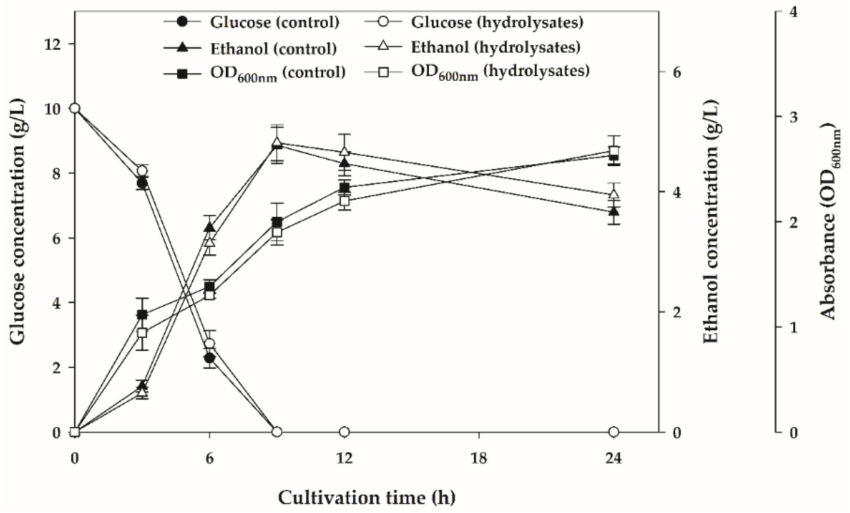

Figure 3. Profiles of ethanol fermentation by *Saccharomyces cerevisiae* K35 using glucose (filled symbol) and hydrolysates (hollow symbol) of *Sicyos angulatus* pretreated with 2% NaOH at 121 °C for 10 min as carbon sources.

Figure 4 shows a material balance of biomass into bioethanol to evaluate the overall bioethanol production process. *S. angulatus* consists of about 16.4% glucan and 6.2% xylan. When 1000 g *S. angulatus* was loaded in the process, 332 g solid fraction containing 155 g glucan and 37.5 g xylan was recovered after NaOH pretreatment (2% NaOH, 121 °C and 10 min). Enzymatic hydrolysis to convert *S. angulatus* into glucose was performed under

the following conditions: 2% (*w/w*) solid loading, 50 mM sodium citrate buffer pH 4.8, 60 FPU/g-biomass Celluclast® 1.5L, 20 FPU/g-biomass Cellic® CTeC2, 50 °C, 72 h, and 180 rpm. The enzymatic digestibility of untreated *S. angulatus* was determined to be 22.1%, and 36.2 g glucose was produced. On the other hand, the enzymatic digestibility and glucose production of the pretreated *S. angulatus* was determined to be 55.3% and 85.8 g, which were 2.4-fold and 2.5-fold higher than the untreated *S. angulatus*, respectively. Glucose produced by enzymatic hydrolysis of *S. angulatus* was used as a carbon source for *S. cerevisiae* K35 fermentation to produce bioethanol. The production of bioethanol was carried out at 30 °C for 9 h with 150 rpm, and the maximum ethanol yield was determined to be 96.2%. Finally, 1000 g *S. angulatus* was converted to 41.3 g bioethanol, which was 2.4-fold improved by NaOH pretreatment compared to the untreated *S. angulatus*. Both glucose and ethanol conversion from *S. angulatus* were improved by NaOH pretreatment, but the final produced glucose and ethanol were low concentration. Optimization of pretreatment and enzymatic hydrolysis was required to design the economical process.

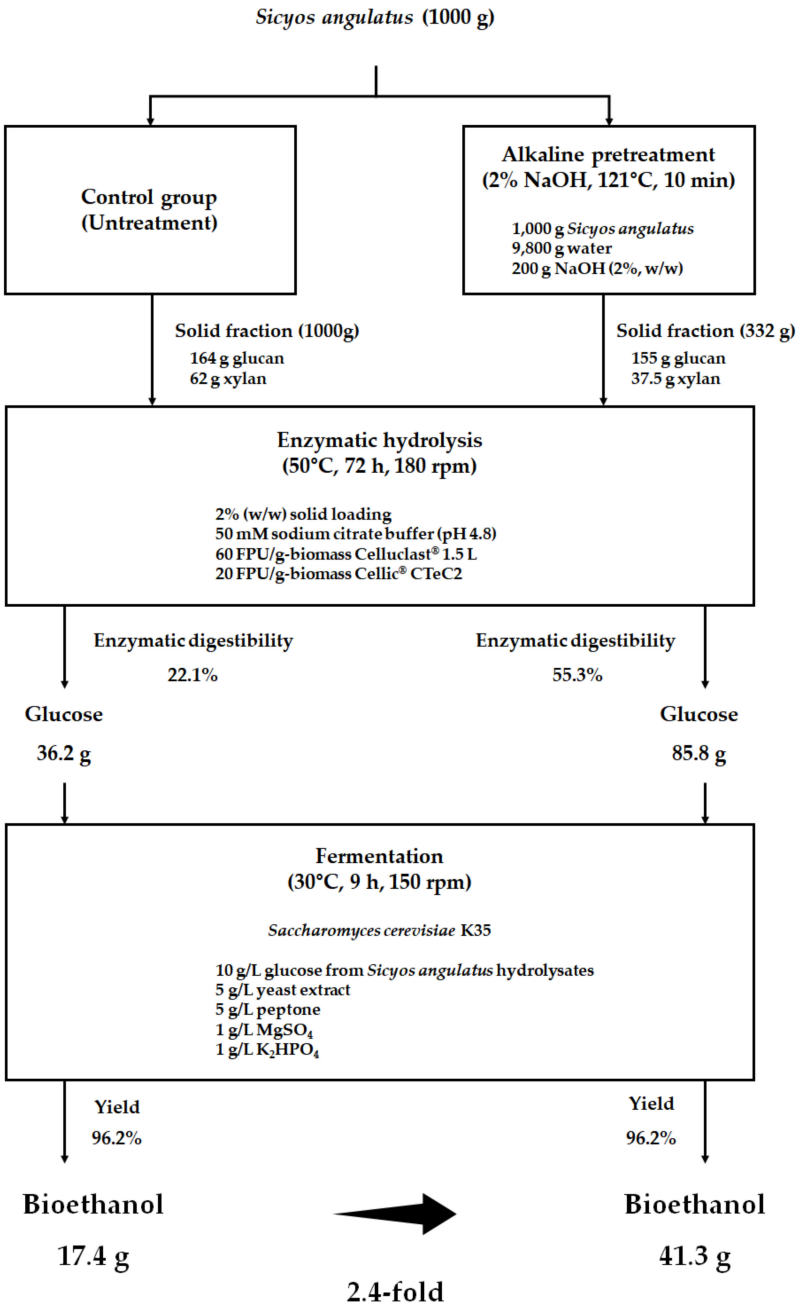

Figure 4. Material balance of bioethanol production from biomass based on 1000 g *Sicyos angulatus*.

4. Conclusions

In this study, *Sicyos angulatus* was selected as the feedstock for bioethanol production, and NaOH pretreatment was performed to improve enzymatic hydrolysis. The GC and ED of the untreated *S. angulatus* (control group) were found to be 16.4% and 22.1%, respectively. The optimum NaOH concentration for alkaline pretreatment of *S. angulatus* was determined to be 2%. The GC and ED of *S. angulatus* pretreated with 2% (*w*/*w*) NaOH at 121 °C for 10 min (experimental group) were found to be 46.7% and 55.3%, respectively. It was found that NaOH pretreatment is suitable for glucose recovery from *S. angulatus*, showing 2.8-fold and 2.5-fold improved GC and ED, respectively, compared to the control group. Finally, the hydrolysates from *S. angulatus* were applied to a medium for bioethanol fermentation by *Saccharomyces cerevisiae* K35, and a high ethanol yield of 96% or more was achieved. In particular, since the hydrolysate did not show an inhibitory effect during fermentation, it was confirmed that it can be used as a useful resource for biorefinery. Currently, bioethanol production using agricultural raw materials is causing food ethics issues, and it is required to discover biomass that can replace agricultural raw materials for bioenergy production. This study suggested the possibility of using invasive plant such as *S. angulatus* at the present time when a lot of non-edible biomass sources is required for bioenergy production, and it is expected to be useful information for bioprocesses design in the future.

Author Contributions: Conceptualization, H.-E.A. and H.Y.Y.; methodology, H.-E.A. and K.H.L.; validation, H.-E.A., K.H.L. and C.-B.K.; formal analysis, K.H.L. and Y.W.J.; writing—original draft preparation, H.-E.A. and K.H.L.; writing—review and editing, C.-B.K. and H.Y.Y.; visualization, H.-E.A. and Y.W.J.; supervision, C.-B.K. and H.Y.Y.; funding acquisition, H.Y.Y. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Ministry of Science and ICT (MSIT) NRF-2020R1C1C1005060.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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

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