

Enrichment of Second Generation Ethanol Concentration Obtained from Napier Grass Pretreated with Sulfuric Acid and Hydrothermal Pretreatment

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Abstract. Enhancing sugar concentration is crucial for improving ethanol yield in biorefinery processes, enabling more efficient downstream recovery. This study investigates the hydrothermal pretreatment of Napier grass with 2% sulfuric acid to boost sugar recovery and ethanol production by incorporating a concentration step. After pretreatment, the liquid fractions were concentrated two-fold and four-fold through rotary evaporation and freeze-drying, resulting in a significant increase in sugar levels, with a 3.5-fold rise in sugar concentration achieved through rotary evaporation compared to unconcentrated samples. However, ethanol production was limited by elevated levels of inhibitors, such as acetic acid and furfural. The maximum ethanol concentration reached was 2.43%, from a liquid fraction concentrated four-fold. These results highlight the necessity of concentration techniques to improve sugar recovery, while also emphasizing the importance of removing inhibitors to increase ethanol yields and enhance the overall efficiency of biorefining processes.

Keywords: Biofuel, Biorefinery, Circular economy, Lignocellulose biomass, Pretreatment

1 Introduction

The increasing demand for sustainable energy solutions has driven significant interest in biofuels, particularly second-generation (2G) ethanol, which is produced from non-food lignocellulosic biomass. Unlike first-generation biofuels, which rely on food crops such as corn or sugarcane, 2G ethanol uses agricultural residues, grasses, and other plant materials, offering a more sustainable alternative by reducing competition with food production [1]. This approach contributes to lowering greenhouse gas emissions, reducing agricultural waste, and promoting rural economic development [2].

One promising feedstock for 2G ethanol production is Napier grass (*Pennisetum purpureum*), a high-yielding tropical grass widely grown for fodder. Napier grass presents several advantages as a bioenergy crop. Its fast growth, high biomass yield, and ability to thrive on marginal lands make it an ideal candidate for biofuel production [3]. The grass consists of lignocellulosic components including cellulose, hemicellulose, and

lignin. Their carbohydrate compositions, cellulose and hemicellulose, can be hydrolyzed into fermentable sugars, which are then converted into ethanol and other value-added products [4], such as platform chemicals [5], polymers [4], foods and feeds [6], etc., through microbial fermentation.

In a lignocellulose biorefinery, the conversion of biomass into biofuels is achieved through several stages, including pretreatment, hydrolysis, fermentation and product recovery [7]. Pretreatment is a crucial step that enhances the accessibility of biomass by breaking down its recalcitrant structure to promote hydrolysis and conversion to final products. Lignocellulose has a recalcitrant structure where cellulose is tightly bound with hemicellulose and lignin, making it resistant to enzymatic degradation [8]. Pretreatment processes are designed to overcome this natural resistance, allowing for the efficient release of fermentable sugars from the cellulose and hemicellulose fractions, thereby increasing the production of biofuels and other products [9].

Various pretreatment methods, including physical, chemical, and biological approaches, have been

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developed. Currently, the operations of 2G ethanol production on a commercial scale use sulphuric acid as the main chemical for pretreatment due to its efficiency and cost [10]. Although there are many attempts to develop green chemicals and green processes to be applied for lignocellulose biorefinery, most are still in the lab-scale R&D stage [11]. Due to the similarity of 1G and 2G ethanol production during the fermentation and distillation process [12], the 2G process can use the existing facilities and technologies for industrial purposes. Therefore, the specificification of the process of 1G process should be applied to 2G to achieve an efficient process.

This study aimed to tackle one main problem of 2G ethanol production. In many studies, the low concentration of ethanol (less than 1%) obtained from fermentation was observed due to the low concentration of sugars released from lignocellulose biomass [13-15]. For instance, dilute acid pretreatment of sugarcane bagasse could produce only 0.50 % ethanol, even with increased sugar yield [15]. Similarly, dilute acid pretreatment of sugarcane bagasse and marine seaweed also reported 0.39 % ethanol concentration inspite of an increased sugar yield [13]. However, the ethanol concentration as an input stream for distillatory in 1G ethanol plant is 8-10% [12]. Therefore, this study applied two concentration methods, rotary evaporator (heat process) and freeze drying (cold process), and compared their efficiency in 2G ethanol production. There are few reported study on comparison of sugar concentration technique to enhance the ethanol yield from biomass to our knowledge. The inhibitor formation caused by sulphuric pretreatment was also analyzed to understand the mechanism for ethanol formation. This study could help to select the concentration method for 2G ethanol production to meet the requirement of industrial-scale facilities in the future.

2 Materials and methods

2.1 Biomass preparation and reagents

Napier grass was gathered from Kanchanaburi province, Thailand as the raw material for this study. The Napier grass was cut manually to 1-cm size and dried in a hot air oven until achieving a constant dried weight. The dried biomass was ground by using a household food processor and size-screening by using 20 mesh-size aluminium sieve to get consistency in the size of the sample. The chemicals and solvents used in this study were procured from Kemaus, India as analytical grades.

2.2 Biomass pretreatment and enzymatic saccharification

One hundred grams of ground Napier grass was mixed with 2%v/v sulphuric acid with a solid loading ratio of 10% w/v in a screw-capped bottle based on the preliminary study. The biomass hydrothermal pretreatment was conducted in an autoclave machine at 121 °C, 15 psi, for 45 min (Figure 1). The pretreated sample was cooled down to room temperature and was

fractionated by using filtration on a cheesecloth, yielding liquid (LF1) and solid fractions (SF1) (Figure 1). The pH of LF1 was adjusted to pH 4.8 by the addition of 3 M NaOH solution. The SF1 sample was washed with distilled water until pH 4.8 was achieved and proceeded to enzymatic saccharification. The reaction of enzymatic saccharification was set up by adding pretreated biomass at a solid loading ratio of 5% in a 50 mM citrate buffer (pH 4.8) containing CTec2 cellulase enzyme of 20 FPU/g [16-17]. The saccharification reaction was conducted at 50 °C, 150 rpm, for 72 h. After the hydrolysis process was completed, the samples were fractionated by centrifugation at 6,000 rpm for 10 min, yielding liquid (LF2) and solid fractions (SF2) (Figure 1). The volume of liquid fractions, LF1 and LF2, were measured and dried weights of solid fractions, SF1 and SF2, were recorded for mass balance and yield calculations. The concentrations of reducing sugars in liquid fractions were determined using the 3,5-dinitrosalicylic acid assay (DNS) [18]. The compositions (cellulose, hemicellulose and lignin) of SF1 and SF2 were analyzed by using the standard protocols from the National Renewable Energy Laboratory (NREL) [16-17]. Each experiment was conducted with three replicates. The inhibitors, including furfural, 5-HMF and acetic acid, in each liquid fraction were analyzed by using gas chromatography-mass spectrometry (GC-MS) as described in our previous studies [19-20].

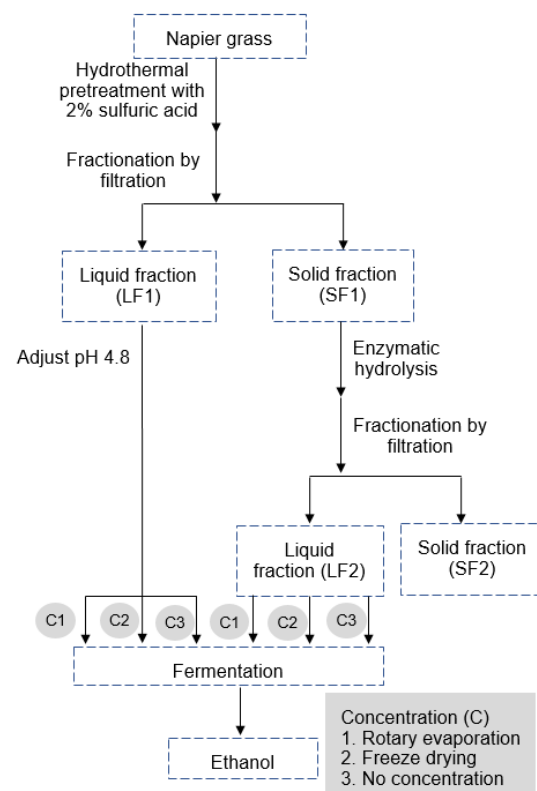


Fig. 1. Experimental flowchart to present pretreatment, fractionation, enzymatic saccharification and fermentation of this study.

2.3 Fermentation for ethanol production

To concentrate the liquid fractions (LF1 and LF2), a total of 650 ml of each liquid fraction was aliquot for concentration by using rotary evaporation (C1), freeze drying (C2), and no concentration (C3). For C1 and C2, 2-fold (2X) and 4-fold (4X) reduced volumes of samples were prepared. All liquid fractions were subjected to fermentation experiments (Figure 1). The fermentation was conducted by using Angel yeast, *Saccharomyces cerevisiae*, for the production of ethanol. Each fermentation sample contained 0.5% w/v glucose, 1% w/v yeast extract, 5% of yeast inoculum, and filled the volume to 50 ml [21] by liquid fractions (LF1 and LF2). The fermentation reactions were conducted in a rotary shaker at 30 °C for 48 h, 150 rpm. The sample was fractionated by centrifugation at 6000 rpm for 10 min. The supernatant fraction was analyzed for ethanol concentration by using spectrophotometry assay [22]. Each experiment was conducted with three replicates.

3 Results and discussion

To evaluate the potential of ethanol production from Napier grass, in this study, the pretreatment liquor (LF1) and liquid hydrolysate (LF2) were prepared and fractionated (Figure 1). Previously, most studies reported that the sugar concentrations of hydrolysate samples obtained from 2G ethanol production (~0.2-2%) are lower than that of 1G (~30-50%) [23-24]. When the sugar concentration in the sample is low, the ethanol concentration is even lower than the sugar concentration, due to the maximum theoretical yield is 51.15%. In conventional industrial processes, the minimum ethanol concentration required from a fermenter before entering a distillation column is typically around 8-10% (v/v) ethanol [12]. Therefore, it is important to enrich the sugar concentration of the pretreatment liquor (LF1) and liquid hydrolysate (LF2) to increase ethanol concentration for the downstream process during product recovery.

In this work, the rotary evaporation (C1) and freeze drying (C2) methods are selected as the representative for the heat and cold process to remove excess water content to increase sugar concentration in liquid fractions. After the concentration process, the reducing sugar concentrations were determined (Figure 2). The results showed that both pretreatment liquor (LF1) and liquid hydrolysate (LF2) had the same trend. That is the more reduction of volume by rotary evaporation (C1) and freeze drying (C2), the higher sugar concentration. For example, the sugar concentration in the unconcentrated sample of LF1 was 28.9 mg/ml, and the concentrations of 2X samples were 53.19 and 50.40 mg/ml obtained from rotary evaporation (C1) and freeze drying (C2), respectively. Likewise, the the sugar concentration in the unconcentrated sample of LF2 was 20.81 mg/ml, the concentrations of 2X samples were also increased about 2 fold too (32.08 and 36.34 mg/ml). The same trend of 4X concentrated samples was also observed from both methods, and the maximum sugar concentration of this study was obtained at 101.77 mg/ml (10.18%) from LF1 with rotary evaporation

(Figure 2). Simialr trend of increasing sugar concentration was reported by Chen et al., 2020, in an attempt to increase sugar concentration through reverse osmosis (RO) technique from corn straw synthetic hydrolysate [29]. The RO concentration experiment was carried out in batch recycling mode where the initial volume of 15L was concentrated to 10L and 5L to yield 1.4 fold and 2.9 fold more glucose and xylose respectively than in the initial volume [29].

After the measurement of sugar concentration, the ethanol concentration was analyzed by using the spectrophotometry method. The results showed that the ethanol concentration was increased to the maximum concentration at 2.43% in the 4X concentrated LF1 sample by rotary evaporation (C1), which was a 1.90-fold increase compared to the unconcentrated LF1 sample (Figure 3). A previous study on hydrothermal and dilute caid pretreatment of extruded Napier grass was only able to achieve 1.32% ethanol yield upon pretreatment with 3% H₂SO₄ for 190 and 30 min [30]. Another study on dilute sulfuric acid pretreatment of sugarcane bagasse could achieve only 0.50% ethanol yield due to the presence of inhibitors [15]. Another study on durian peel pretreated with dilute sulfuric acid has reported to attain a ethanol yield of 0.57% [31]. It could be noted that the higher ethanol concentration is obtained in this study up on concentration process. However, the trend of ethanol enrichment is unlike the trend of sugar (Figure 2). The concentration of ethanol was not doubly increased in the 2X concentrated sample, and the same in the 4X concentrated sample. This reduction from the expectation may be due to the presence of inhibitors released from lignocellulose biomass or formed during the pretreatment process [25-26].

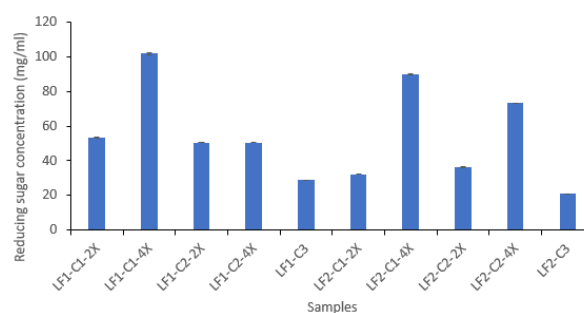


Fig. 2. Reducing sugar concentrations in liquid fractions (LF1 and LF2).

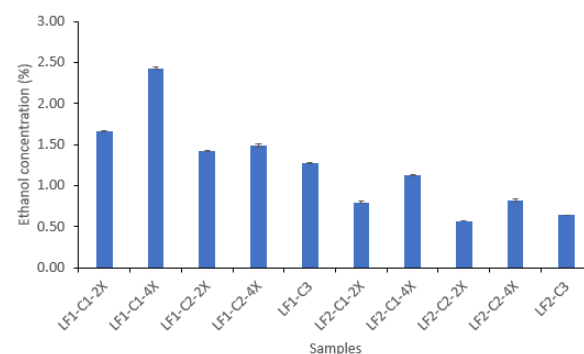


Fig. 3. Ethanol concentrations in liquid fractions (LF1 and LF2).

To understand the mechanism for the observed deviation of the ethanol concentration, composition analysis of solid fractions (SF1 and SF2) was conducted (Figure 4) and the inhibitors in liquid fractions (LF1 and LF2) were analyzed by using GC-MS (Table 1). After sulphuric acid pretreatment, it was observed that hemicellulose contents were completely removed in both SF1 and SF2 fractions when compared to the untreated sample (16.52%). Likewise, the lignin contents in both solid samples were decreased when compared to the untreated sample (22.4%). However, cellulose content was increased in the pretreated sample (SF1) by 19.71% but decreased in the hydrolysate solid (SF2) (Figure 4). This result suggested that the lignin and hemicellulose, which are molecules to function in hindering the interaction between cellulose and cellulose, were removed from samples and released into liquid fractions [27-28].

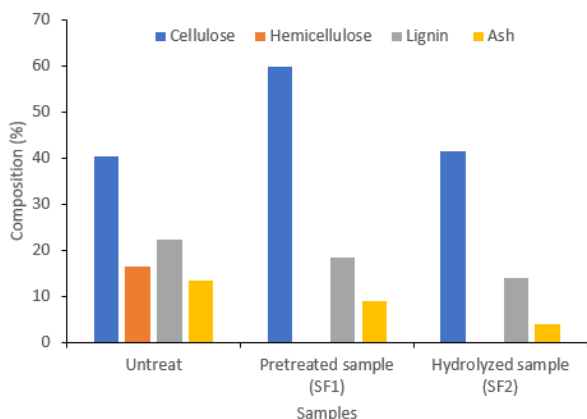


Fig. 4. Biomass compositions of solid fractions (SF1 and SF2).

Table 1. Concentrations of acetic acid and furfural in liquid fractions, LF1 and LF2.

Samples	Inhibitor concentration	
	Acetic acid (mg/L)	Furfural (mg/ml)
LF1-C1-2X	223.27±15.05	0.39±0.04
LF1-C1-4X	371.50±23.40	1.44±0.08
LF1-C2-2X	204.09±21.20	0.36±0.04
LF1-C2-4X	344.44±17.76	1.54±0.08
LF1-C3	61.92±7.36	N.D.*
LF2-C1-2X	61.67±4.49	N.D.
LF2-C1-4X	117.31±12.47	N.D.
LF2-C2-2X	68.78±4.34	N.D.
LF2-C2-4X	106.35±8.96	N.D.
LF2-C3	47.20±9.58	N.D.

* N.D. is not detected

Therefore, the inhibitor compounds, including furfural, 5-HMF and acetic acid, in liquid fractions were analyzed. It was observed that there was no 5-HMF detected in all samples, but furfural was presented in LF1 fractions (C1 and C2) (Table 1). Furfural was reported previously that it was hydrolyzed products of hemicellulose during pretreatment with harsh conditions [26]. This observed result was agreed and correlated to composition analysis that the removal of hemicellulose

in solid fraction caused the formation of furfural in liquid fraction. Furthermore, the more concentrated sample, 2X and 4X, the higher concentrations of furfurals were present. This trend was also observed in acetic concentration (Table 1). The increased concentration of inhibitors, furfural, and acetic acid was also noted in the corn starw synthetic hydrolysate concentrated with RO technique [29]. Acetic acid could be formed during hemicellulose degradation from the acetyl groups attached to the hemicellulose fraction [25]. The accumulations of acetic acid and furfural could be the reasons for a reduction in ethanol yields (Figure 2). The increased concentration of these inhibitors can impede the microbial growth and hinder the ethanol production [15].

4 Conclusion

This study investigated the hydrothermal pretreatment of Napier grass with 2% sulfuric acid to enhance sugar production by concentrating the liquid fractions, aiming to improve ethanol yields for biorefinery applications. The results demonstrated that the pretreatment significantly increased cellulose content, approximately 1.5 times higher than untreated biomass, while effectively removing hemicellulose and lignin. Concentration of the liquid fractions via rotary evaporation and freeze drying led to a substantial increase in sugar concentration, with rotary evaporation yielding up to a 3.5-fold improvement compared to unconcentrated samples. However, ethanol yields did not increase proportionally due to elevated levels of inhibitor, acetic acid, in the concentrated liquid fractions. The maximum ethanol concentration achieved was 2.43%, obtained from a liquid fraction concentrated 4x times. These findings underline the importance of utilizing concentration techniques to maximize biomass conversion efficiency. Additionally, they highlight the need for effective purification processes to remove inhibitors and improve both sugar and ethanol yields, enhancing the overall efficiency of ethanol production in biorefineries.

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