# การเพิ่มประสิทธิภาพการผลิตแก๊สมีเทนจากหญ้าเนเปียร์และหญ้าเนเปียร์หมักโดยการปรับสภาพด้วยน้ำร้อน

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**บทคัดย่อ**

หญ้าเนเปียร์เป็นหญ้าที่เจริญเติบโตได้รวดเร็วและมีองค์ประกอบของสารอินทรีย์ที่สามารถย่อยสลายได้ในปริมาณมาก โดยเฉพาะโครงสร้างคาร์โบไฮเดรต ด้วยสมบัติดังกล่าว หญ้าเนเปียร์และหญ้าเนเปียร์หมักจึงได้รับความสนใจในการนำมาเป็นวัตถุดิบสำหรับการผลิตแก๊สมีเทนในระบบการย่อยสลายแบบไร้อากาศ อย่างไรก็ตาม หญ้าเนเปียร์เป็นวัตถุดิบจำพวกลิกโนเซลลูโลสซึ่งย่อยสลายได้ยากด้วยเชื้อจุลินทรีย์ในระบบไร้อากาศ จึงจำเป็นต้องมีการปรับสภาพวัตถุดิบเพื่อเพิ่มความสามารถในการผลิตแก๊สชีวภาพก่อนนำเข้าสู่กระบวนการย่อยสลายแบบไร้อากาศ การปรับสภาพด้วยน้ำร้อนเป็นวิธีที่สามารถทำลายพันธะและละลายน้ำตาลโมเลกุลใหญ่ โดยเฉพาะเฮมิเซลลูโลสได้ ส่งผลต่อการเพิ่มปริมาณของน้ำตาลไซโลสและลดปริมาณของสารพิษ เช่น เฟอฟูรอลและไฮดรอกซีเมทิลเฟอฟูรอล จากผลการทดลองพบว่า ปริมาณน้ำตาลไซโลส เฟอฟูรอลและไฮดรอกซีเมทิลเฟอฟูรอล เพิ่มสูงขึ้นตามอุณหภูมิ (140–200 องศาเซลเซียส) และระยะเวลาในการทำปฏิกิริยา (0–30 นาที) โดยสภาวะที่เหมาะสมที่ให้ปริมาณน้ำตาลไซโลสสูงสุดและปริมาณเฟอฟูรอลและไฮดรอกซีเมทิลเฟอฟูรอลต่ำสุดคือการปรับสภาพด้วยน้ำร้อนที่อุณหภูมิ 200 องศาเซลเซียส เป็นเวลา 15 นาที สภาวะดังกล่าวส่งผลให้ปริมาณเฮมิเซลลูโลสในตัวอย่างถูกกำจัดออกไปมากกว่า 90% จากการศึกษาศักยภาพในการผลิตแก๊สมีเทนของหญ้าเนเปียร์และหญ้าเนเปียร์หมักที่ถูกปรับสภาพด้วยน้ำร้อนที่สภาวะดังกล่าว พบว่า มีปริมาณสูงกว่าตัวอย่างที่ไม่ปรับสภาพ 16% และ 23% ตามลำดับ

# Enhancement of Methane Production Potential from Napier Grass and Napier Grass Silage with Liquid Hot Water Pretreatment

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## Abstract

Napier grass is a fast-growing grass that contains high levels of organic compounds, particularly, structural carbohydrates, and exhibits potential to be used as a substrate for renewable energy production. In addition, napier grass can be converted into silage, which has gained attention as a feedstock for biogas production. However, both napier grass and napier silage are categorized as lignocellulosic materials that are recalcitrant to degradation by anaerobic digestion. To enhance their degradation and hence biogas production, liquid hot water (LHW) pretreatment was applied to break down and partially solubilize polysaccharides. Optimization of the process was performed to obtain a high amount of total xylose derived from hemicellulose component, along with lower concentrations of inhibitors, i.e., furfural (FF) and hydroxymethylfurfural (HMF). Our experimental results revealed that the amount of total xylose and concentrations of inhibitors increased with increasing LHW temperature (140–200 °C) and time (0–30 min). Optimum LHW pretreatment condition for napier grass and napier silage was noted at 200 °C for 15 min; such a condition resulted in the highest amount of total xylose, lower concentrations of FF and HMF, and more than 90% hemicellulose removal. Biochemical methane ($CH_4$) potential analysis of the untreated and LHW-pretreated napier grass and napier silage showed that the $CH_4$ yields from the LHW-pretreated napier grass and napier silage increased by 16% and 23%, respectively.

## 1. Introduction

In Thailand, the Alternative Energy Development Plan targets by 2036 include an installed biogas power capacity to produce 680 MW of electricity from energy crops [1]. Napier grass (*Pennisetum purpureum*) can be used as a raw material in agricultural biogas plants because of its high potential fresh yield, ease of cultivation, stress resistance (e.g. low soil quality without continuous irrigation, drought tolerance, and heavy metals endurance), and low nutrient requirement with a low environmental impact. Moreover, napier grass has a high organic content, and so should be a promising raw material for biogas production [2]. Grass can be used as silage by naturally fermenting the grass to supply a year-round availability of nutritious and palatable feed for livestock. Further, an anaerobic digestion (AD) facility may prefer the grass silage to using fresh grass [3]. Ensiling process increases the specific methane ($CH_4$) production by 25–42% because the easily digestible organic compounds like hemicellulose are fermented into organic acids [4, 5]. Both napier grass and napier silage are mainly composed of lignocellulosic material (LCM), as in cellulose, hemicellulose, and lignin. The LCM composition of the grass influences the biodegradability since lignin cannot be converted into biogas and only part of it can be depolymerized into soluble components. Cellulose can be hydrolyzed to glucose, while hydrolysis of hemicellulose yields xylose, arabinose, glucose, mannose, and galactose [6, 7]. These sugars can be used as carbon sources for $CH_4$ production. However, grasses have a high carbon content with a low nitrogen content. This high carbon to nitrogen (C:N) ratio is considered unsuitable for AD [8]. Therefore, pretreatment and co-digestion are frequently required for reducing AD limitations and improving biogas yields [9].

Different pretreatment methods affect different physical and chemical compositions or structures of lignocellulose, such as increasing the surface area of LCM, microorganism accessibility, substrate digestibility, and lignin and hemicellulose solubility [10]. Normally, the $CH_4$ yields from napier grass range from 134–249 $Nm^3/t\ VS_{added}$, depending on the harvesting age, fertility, farm management, and cultivar. Enhanced $CH_4$ production (175–630 $Nm^3/t\ VS_{added}$) can be achieved by pretreatment and co-digestion of napier grass and napier silage with animal manure and food waste to give a more balanced C:N ratio and reduced risk of ammonia inhibition and acidification [11-16].

Among various pretreatment methods, liquid hot water (LHW) pretreatment has several merits, including its relatively low cost in terms of capital investments, energy, and chemical inputs, as well as a minimal generation of inhibitory products and waste, among others. Overall, LHW pretreatment is a good alternative to increase the efficiency of biogas production since it provides a high sugar recovery from the hemicellulose with a low concentration of AD inhibitors, such as furfural (FF) and hydroxymethylfurfural (HMF) [17]. The LHW pretreatment is operated with water at a high temperature and pressure in a non-catalytic process. The water can penetrate the biomass cell structure at high temperatures (100–240 °C) under pressurized conditions for a short period ranging from a few minutes to hours, resulting in hydrolysis of the cellulose, solubilization of hemicellulose, and slight removal of lignin. However, the major challenge of this process is the optimization of the process to minimize the amount of water and to scale-up the reaction with economic feasibility [18].

Therefore, this study aimed to optimize the LHW pretreatment process of napier grass and napier silage that would result in a maximal sugar release from the

liquid and solid fractions (LF and SF, respectively) for maximal biogas production. The characteristics (i.e. inhibitory compounds, cellulose, hemicellulose, and lignin content, and sugar composition) of the SF and LF of the LHW-pretreated napier grass and napier silage were evaluated to determine their effects on the $CH_4$ yield in the subsequent AD process.

## 2. Materials and methods

### *2.1 Preparation and characterization of napier grass and napier silage*

Napier grass (Hybrid between *Pennisetum purpureum* and *Pennisetum Americanum*) and napier silage were collected from the Phetchaburi Animal Nutrition Research and Development Center, Sam Phraya, Cha-am, Phetchaburi province, Thailand after 60 d growth during the rainy season. The grasses were cut to a size of 4–5 cm long and dried in a hot air oven at 55 °C for 3 d until the moisture content was less than 10% to prevent degradation of the organic compounds by microorganisms [19]. The dried grasses were then ground to a particle size of 1–2 mm using a multi-function blender.

The moisture content, total solid (TS), volatile solid (VS), and ash content of the fresh grass were analyzed [20]. Crude protein and crude fat were respectively determined using the total Kjeldahl nitrogen and Soxhlet extraction methods [21, 22]. Starch content was analyzed using the iodine-starch complex method [23]. Cellulose, hemicellulose, and lignin contents were analyzed by the detergent fiber method developed by Van Soest and McQueen & Nicholson using Fibreterm® [24]. Elemental analysis, in terms of carbon (C), hydrogen (H), oxygen (O), nitrogen (N), and sulfur (S), of each sample was analyzed according to the AOAC protocol [25]. Structural carbohydrates of the grass samples were analyzed using the National Renewable Energy Laboratory protocol (NREL) [26]. Briefly, 300 mg of sample was hydrolyzed in 3 mL of 72% sulfuric acid ($H_2SO_4$) for 1 h at 30 °C. The solution was diluted with deionized water to 4% $H_2SO_4$ and placed in an autoclave at 121 °C for 1 h and then brought to a pH of 6–7 by the addition of calcium carbonate. An aliquot of the solution was used to determine the sugar concentration using high-performance liquid chromatography (HPLC) with a Biorad Aminex HPX-87P column at 85 °C. Deionized water was used as the mobile phase at a flow rate of 0.6 mL/min. Refractive index (RI) detector was employed for the detection.

### *2.2 Optimization of the LHW pretreatment*

The LHW reaction temperature and time were the main factors in the LHW pretreatment. A face centered central composite design (CCD) was used to design the reaction conditions for optimization, which are shown in Table 1. The reaction was conducted in a 100- to 600-mL mini bench top stirred reactor (Parr instrument company, Moline, USA). The grass sample and water were mixed to a final 5% (w/v) TS solution. Nitrogen ($N_2$) was used to control the pressure in the reactor, which was set to the saturated vapor pressure (SVP) at each specific reaction temperature. Reactor agitation was set at 500 rpm and the rate of heating was 10 °C/min. After reaching the desired reaction temperature and time, it was instantly cooled to terminate the heating process and rapidly decrease the solution temperature. The control (non-pretreated) condition was conducted at room temperature under atmospheric pressure. Each reaction was then separated into the SF and LF by filtration (Whatman No.1). The SF was dried at 55 °C to determine the residual weight of the sample.

The fiber composition of the SF was analyzed, while monosaccharides and total sugar were analyzed

in the LF using HPLC [27]. Direct injection of the LF was performed to measure the amount of monosaccharides. To determine the level of oligo- and di-saccharides, the sample was prior hydrolyzed by $H_2SO_4$ to convert all the oligo- and di-saccharides into monosaccharides before measurement and was then reported as the total sugar content. Moreover, the presence of FF and HMF were measured according to the LAP protocol using a Biorad Aminex HPX-87H column coupled with a photodiode array detector, in which the absorbance wavelength was set at 277 and 285 nm to detect the FF and HMF, respectively. The column temperature was set at 65 °C and the mobile phase was 5 mM $H_2SO_4$ at a flow rate of 0.6 mL/min. All samples were filtered through a 0.22 µm syringe filter before injection.

**Table 1** Face-centered CCD of the temperature and time in the LHW pretreatment optimization for napier grass and napier silage (Pressure is set to the SVP). Design 1: 140–180 °C for 0–30 min. Design 2: 160–200 °C for 0–30 min.

| Design | Run no. | Temperature (°C) | Time (min) | Pressure (bar) |
|---|---|---|---|---|
| | Control | 25 | 30 | 1.0 |
| Design 1 | 1 | 140 | 0 | 3.6 |
| | 2 | 140 | 15 | 3.6 |
| | 3 | 140 | 30 | 3.6 |
| | 4* | 160 | 0 | 6.2 |
| | 5 | 160 | 15 | 6.2 |
| | 6* | 160 | 15 | 6.2 |
| | 7 | 160 | 15 | 6.2 |
| | 8* | 160 | 30 | 6.2 |
| | 9* | 180 | 0 | 10.0 |
| | 10* | 180 | 15 | 10.0 |
| | 11* | 180 | 30 | 10.0 |
| Design 2 | 12 | 180 | 15 | 10.0 |
| | 13 | 180 | 15 | 10.0 |
| | 14 | 200 | 0 | 15.5 |
| | 15 | 200 | 15 | 15.5 |
| | 16 | 200 | 30 | 15.5 |

*Data was used in the Design 2 statistical analysis.

Validation of the optimum condition from statistical analysis was conducted in triplicate. An identical procedure of LHW pretreatment was performed. The SF was analyzed for residual solid and fiber contents, while the sugar composition and inhibitors were determined in the LF.

*2.3 Biochemical methane potential (BMP) analysis*

The pretreated sample at the optimum condition without separation was used to determine the BMP in comparison with the non-treated sample. All samples were performed in triplicate. Seed sludge was taken from a cassava starch wastewater treatment plant in Chonburi province, Thailand. The BMP assay was conducted in a 120 mL serum vial with a working volume of 80 mL using a feed and inoculum ratio of 1 g VS of sample: 3 g VSS of inoculum [28]. To create an anaerobic condition in the vial, 99.99% $N_2$ was flushed in the vial headspace prior to being sealed with a rubber stopper and an aluminum cap. All vials were kept at 37 °C. Biogas production and gas composition were determined using liquid replacement and gas chromatography coupled with a thermal conductivity detector [15]. Biogas and $CH_4$ accumulation data were used for curve fitting with the modified Gompertz model, as shown in Eq. (1) [29],

$$G = G_m \exp\{-\exp\left[\frac{R_m e}{G_m}(\lambda - t) + 1\right]\} \quad (1),$$

where $G$ is the cumulative $CH_4$ production yield (mL STP/g $VS_{added}$) at digestion time ($t$; d), $G_m$ is the maximum $CH_4$ production yield (mL STP/g $VS_{added}$), $R_m$ is the maximum $CH_4$ production rate (mL STP/g $VS_{added}$ *d), $e$ is the Euler's number (2.7183), $\lambda$ is the lag phase time (d), and $t$ is the digestion time (d).

The theoretical $CH_4$ yield ($Y_t$) of each sample was calculated using the chemical formula of the sample (C, H, O and N) as shown in Eqs. (2) and (3) [30]. The $CH_4$ yield obtained from the BMP test ($Y_{bmp}$) was compared with the theoretical $CH_4$ yield, to represent biodegradability, using Eq. (4).

$$C_aH_bO_cN_d + \left(\frac{4a-b-2c+3d}{4}\right) H_2O \rightarrow \left(\frac{4a+b-2c-3d}{8}\right) CH_4 + \left(\frac{4a-b+2c+3d}{8}\right) CO_2 + dNH_3 \quad (2),$$

$$Y_t = \frac{(4a+b-2c-3d)}{8\,(12a+b+16c+14d)} \times 22.4\ \frac{mL\ CH_4}{gVS} \quad (3),$$

$$\%\ Biodegradibility = \frac{Y_{bmp}}{Y_t} \times 100 \quad (4),$$

*2.4 Statistical analysis*

Minitab® 18.1 software was used to perform all statistical analyses. The optimization of reaction conditions for the LHW pretreatment was determined using analysis of variance (ANOVA), response surface plot (RSP), and contour plots of the optimum condition. The kinetic data from the BMP test was subject to one-way ANOVA and Tukey's test for multiple comparisons, accepting significance at the 95% ($\alpha$ = 0.05) level.

## 3. Results and discussion

*3.1 Chemical compositions and characteristics of napier grass and napier silage*

The napier grass and napier silage were considered as a potential alternative biomass feedstock for renewable energy production and categorized as LCMs. The moisture content, TS, and VS of the grass samples were 73–76%, 24–27%, and 90–91% dry weight basis, respectively. Since the VS of the grass samples was approximately 90%, they contained a

high level of organic compounds. The chemical composition of the respective grass samples is summarized in Table 2. The major composition of both napier grass and napier silage was lignocellulose, at approximately 80%. The hemicellulose content of napier silage (23%) was lower than that for napier grass (31%) because the ensiling process breaks down the easily hydrolyzed part, like hemicellulose. Thus, the cellulose and lignin content of napier silage was higher than that for napier grass. Starch, crude protein, and crude fat were found in small amounts in both samples.

**Table 2** Chemical composition of the napier grass and napier silage

| Component | % Dry basis | |
|---|---|---|
| | Napier grass | Napier silage |
| Moisture content* | 73.02 ± 0.13 | 75.69 ± 0.08 |
| Total solid (TS)* | 26.98 ± 0.13 | 24.31 ± 0.08 |
| Volatile solid (VS) | 90.97 ± 0.14 | 90.22 ± 0.28 |
| Ash | 9.03 ± 0.14 | 9.78 ± 0.28 |
| Starch | 0.04 | 0.09 |
| Cellulose | 41.78 ± 0.24 | 43.61 ± 0.47 |
| Hemicellulose | 31.42 ± 0.52 | 23.13 ± 0.06 |
| Lig nin | 4.48 ± 0.50 | 5.93 ± 0.11 |
| Crude protein | 4.66 | 2.03 |
| Crude fat | 1.29 | 0.82 |
| **Structural carbohydrate** | | |
| Glucose | 28.85 ± 3.15 | 32.82 ± 1.74 |
| Xylose | 17.95 ± 2.08 | 19.51 ± 1.15 |
| Galactose | 7.12 ± 1.04 | 6.82 ± 0.58 |
| L -arabinose | 0.08 ± 0.01 | 0.07 ± 0.00 |
| Mannose | ND | ND |
| **Ultimate analysis** | | |
| Carbon (C) | 40.65 | 38.29 |
| Hydrogen (H) | 6.18 | 6.07 |
| Oxygen (O) | 43.09 | 45.41 |
| Nitrogen (N) | 1.03 | 0.44 |
| Sulfur (S) | 0.10 | 0.09 |
| Chemical formula | $C_{46.04}H_{84}O_{36.61}N$ | $C_{101.53}H_{193.14}O_{90.30}N$ |

*; wet basis, ND; not detected. Data are shown as the mean ± SD, derived from three replicates.

More than 70% (on a dry weight basis) of the napier grass or napier silage samples was carbohydrate, especially cellulose and hemicellulose, so the determination of the carbohydrate monomer type was mandatory for the experimental analysis. From the result, glucose had the highest sugar composition in both the napier grass and napier silage, at 29 and 33%, respectively. Glucose could be obtained from several components, such as starch, cellulose, and hemicellulose. For the sugar derived from hemicellulose, the amount of xylose was higher than galactose and so xylan was potentially the main structural sugar.

The xylose content in napier silage (about 20%) was slightly higher than that in napier grass (18%). L-arabinose is the sugar-binding on the side chain of xylan, and was found in a small amount. The elemental analysis revealed that the principal content was CHO, related to its main carbohydrate structure. The N content, representing the protein content, was low but napier grass had a 2.3-fold higher N content than napier silage. A trace amount of sulfur (S) was found in both samples.

*3.2 The LHW pretreatment of napier grass and napier silage*

Both napier grass and napier silage were subjected to different reaction conditions for the LHW pretreatment (Table 1) and then the obtained SF and LF were analyzed, including the fiber composition and hemicellulose content in the SF and the monosaccharides (i.e. glucose, xylose, galactose, and L-arabinose), total sugar, FF, and HMF in the LF. The FF and HMF were measured to represent the level of inhibitors of biogas production in the subsequent AD, which were generated during the LHW pretreatment.

After the LHW pretreatment, the residual solid content (SF) is summarized in Figure 1. For the pretreated napier grass, the SF ranged from 49–81%, whereas it was slightly higher in the pretreated napier silage at 54–84%. Some parts of the solid were easily solubilized, as seen in the control condition, which was because grass contained soluble compounds [31, 32]. The TS compound in the pretreated napier grass (19%) was higher than in the pretreated napier silage (16%) due to the degradation of the compound during fermentation in the ensiling process [33]. Overall, the residual solid content after LHW pretreatment decreased with increasing reaction temperature and time, decreasing slightly at 140, 160, and 180 °C and dramatically at 200 °C at 15–30 min. For example, for napier grass at 200 °C, the solid content slightly decreased from 65% to 63% at 0 to 15 min but drastically decreased to 49% at 30 min of reaction time. For the pretreated napier silage, a substantial decrease in the residual solid content occurred from 0 min to 15 min of reaction time (81% to 56%), with a slight further decrease at 30 min of reaction time (54%).

The decrease in the TS content was mainly caused by the solubilization of grass components, particularly the fiber composition. The cellulose, hemicellulose, and lignin content in the SF after the LHW pretreatment of napier grass and napier silage was shown in Table 3. Both napier grass and napier silage showed a similar decrease in hemicellulose content with increasing LHW pretreatment temperature (140–200 °C) and time (0–30 min), while the cellulose and lignin remained in the SF. Between 140 and 160 °C, the hemicellulose content slightly decreased, whereas at temperatures higher than 180 °C, a significant decrease in the hemicellulose content was observed. The highest degree of hemicellulose removal (about 90%) occurred with an LHW pretreatment at 200 °C for 15–30 min.

A previous study on the hydrolysis kinetics of hemicellulose in LHW pretreatment revealed that a slow hydrolysis rate of hemicellulose was observed at temperatures lower than 180 °C, but a faster hydrolysis rate was observed at temperatures above 180 °C [34]. Saccharides derived from hemicellulose were solubilized into the LF. Although the cellulose and lignin content mostly remained in the solid residue at low temperatures, pretreatment at 200 °C for 30 min greatly reduced the cellulose content of napier grass and napier silage by 43% and 30%, respectively. The degradation of cellulose at this condition could occur on the less ordered regions, such as amorphous cellulose, while the crystalline region

required a higher temperature condition. A similar temperature profile for cellulose hydrolysis has been observed previously, where the amorphous region started to break down at ≥ 200 ℃ [35].

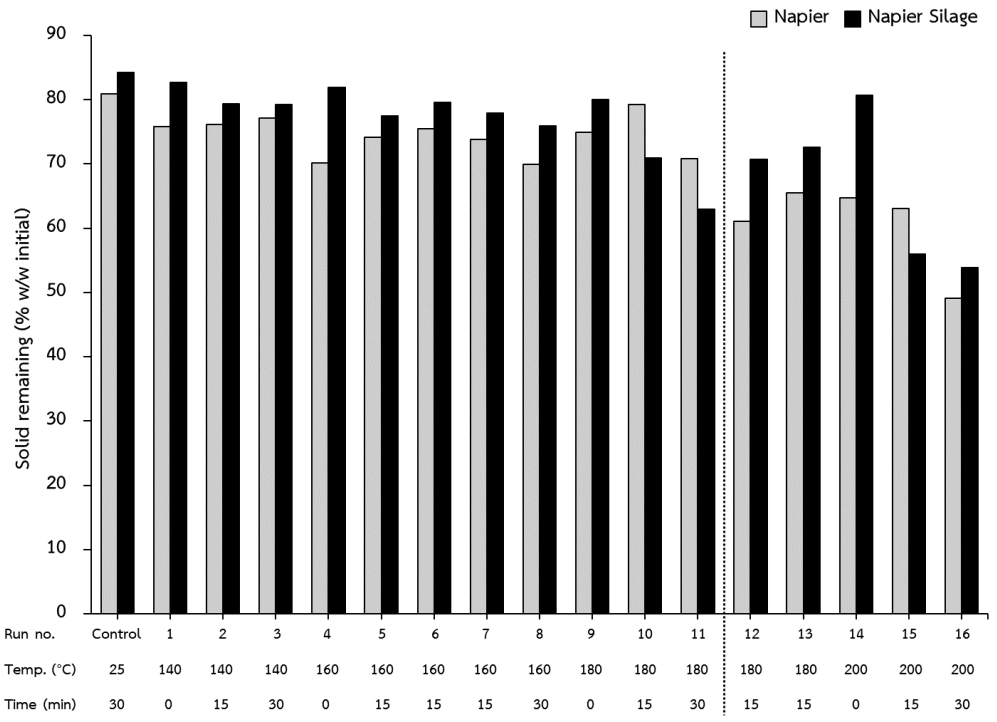


**Figure 1** Residual solid content in LHW pretreatment under different temperatures.

The sugar composition in the LF obtained after LHW pretreatment was determined as monosaccharides and total sugars (oligo- and mono-saccharides). The amount of monosaccharides after the LHW pretreatment is shown in Figure 2, where the pretreated napier grass sample contained higher monosaccharide and sugar content than the pretreated napier silage sample. Note that the control (untreated) sample of napier grass contained a high amount of glucose (about 5.5 mg/g-napier grass), whereas that for the untreated napier silage was only about 0.6 mg/g-napier silage. This is consistent with the reduction in the SF in the control condition, caused by solvation of the soluble compounds in the grass sample, which were higher in napier grass than in napier silage. The amount of glucose in the pretreated napier grass sample increased about two-fold as the LHW pretreatment temperature increased from 140 ℃ to 180 ℃ with a slight decrease when prolonging the reaction time from 15 to 30 min at any given temperature. At 200 ℃, the amount of glucose in the pretreated napier grass was greatly reduced from 6.6 mg/g-napier grass at 0 min to 2.9 mg/g-napier grass at 30 min of reaction time in the same manner as in the pretreated napier silage sample.

**Table 3** Fiber composition of the SF and hemicellulose removal in napier grass and napier silage after LHW pretreatment

| Run no. | Condition | | Napier grass [% (w/w) initial] | | | | Napier silage [% (w/w) initial] | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Temp. (°C) | Time (min) | Cellulose | Hemicellulose | Lignin | Hemicellulose removal* | Cellulose | Hemicellulose | Lignin | Hemicellulose removal* |
| Non-treated | | | 41.78 | 31.42 | 4.48 | | 43.61 | 23.13 | 5.93 | |
| Control | 25 | 30 | 35.33 | 25.57 | 4.94 | 18.62 | 40.17 | 24.02 | 5.70 | 0.00 |
| 1 | 140 | 0 | 35.51 | 24.88 | 3.97 | 20.82 | 40.45 | 23.61 | 5.83 | 0.00 |
| 2 | 140 | 15 | 37.17 | 22.32 | 4.62 | 28.98 | 40.71 | 21.06 | 6.07 | 8.95 |
| 3 | 140 | 30 | 36.70 | 23.17 | 4.94 | 26.26 | 41.79 | 22.30 | 5.37 | 3.60 |
| 4 | 160 | 0 | 33.32 | 22.25 | 4.16 | 29.18 | 39.53 | 24.29 | 5.82 | 0.00 |
| 5 | 160 | 15 | 39.20 | 21.63 | 3.38 | 31.17 | 39.54 | 20.29 | 5.72 | 12.29 |
| 6 | 160 | 15 | 36.42 | 21.35 | 4.37 | 32.06 | 40.74 | 22.40 | 5.22 | 3.15 |
| 7 | 160 | 15 | 35.61 | 21.01 | 4.35 | 33.12 | 40.66 | 21.46 | 5.32 | 7.21 |
| 8 | 160 | 30 | 35.19 | 18.86 | 3.65 | 39.97 | 40.24 | 17.62 | 5.58 | 23.84 |
| 9 | 180 | 0 | 36.24 | 23.13 | 4.02 | 26.38 | 39.93 | 22.85 | 5.33 | 1.23 |
| 10 | 180 | 15 | 37.66 | 23.88 | 4.58 | 23.99 | 39.06 | 13.42 | 5.01 | 42.00 |
| 11 | 180 | 30 | 35.98 | 17.97 | 4.16 | 42.82 | 37.25 | 7.21 | 4.57 | 68.81 |
| 12 | 180 | 15 | 33.18 | 10.75 | 3.99 | 65.78 | 38.60 | 13.77 | 5.13 | 40.48 |
| 13 | 180 | 15 | 35.09 | 11.92 | 3.69 | 62.06 | 40.44 | 16.11 | 4.27 | 30.35 |
| 14 | 200 | 0 | 34.65 | 13.23 | 3.42 | 57.91 | 42.56 | 14.46 | 4.29 | 37.50 |
| 15 | 200 | 15 | 37.37 | 2.01 | 4.01 | 93.61 | 32.69 | 3.80 | 5.20 | 83.55 |
| 16 | 200 | 30 | 23.61 | 2.61 | 2.91 | 91.70 | 30.37 | 3.21 | 5.65 | 86.13 |

* Amount of hemicellulose left in the SF divided by the amount of hemicellulose in the non-treated.

The other principal monosaccharides derived from hemicellulose, xylose and L-arabinose, showed a significant change with the different LHW reaction conditions, while galactose did not show a significant change in the LF. The amount of galactose obtained from the LHW-pretreated napier grass and napier silage ranged from 0.5 to 1.9 mg/g. For xylose, napier grass and napier silage had a similar amount after a LHW pretreatment at 140–180 °C at about 0.6–2.0 mg/g, but at 200 °C the amount of xylose in both samples rapidly increased. The xylose content in the pretreated napier grass increased about 14-fold from 0 to 15 min reaction time, and then slightly increased to 18.1 mg/g-napier grass at 30 min of reaction time. For the pretreated napier silage, the xylose level sample increased from 0.6 to 6.0 mg/g-napier silage between 15 and 30 min of reaction time, respectively. The increasing xylose level with increasing LHW temperature and time was observed at ≥ 200 °C and ≥ 15 min.

The highest amount of L-arabinose was obtained after LHW pretreatment at 180 °C at 15 min for the napier grass sample (8.8 mg/g-napier grass) and at 30 min for the napier silage sample (5.5 mg/g-napier silage). Increasing the LHW temperature and time did not increase the amount of L-arabinose. Like in the napier grass sample at 200 °C, the amount of L-arabinose decreased with increasing LHW time from 0 to 30 min. A similar phenomenon of increasing sugar content was also observed in the amount of total sugar in the LF of the LHW-pretreated napier grass and napier silage samples.

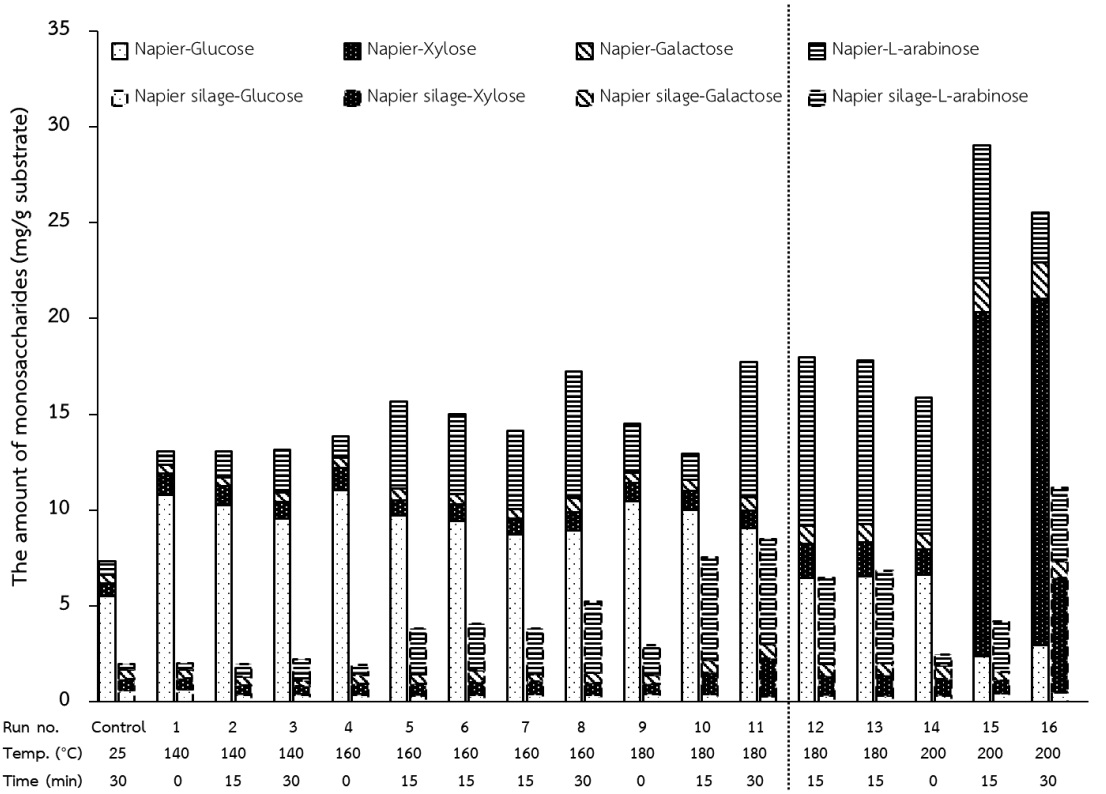


**Figure 2** Amount of monosaccharides in the LF obtained from napier grass and napier silage under different LHW pretreatment conditions.

Evaluation of the total sugar content in the LF was determined using the NREL protocol that hydrolyzed oligosaccharides into monosaccharides before measurement, with the results shown in Figure 3. The amount of total glucose, xylose, galactose, and Larabinose were higher than the monosaccharides, which indicated that most of the sugars in the LF were oligosaccharides. These oligosaccharides were obtained from the solubilization of polysaccharide components, such as starch, cellulose, and hemicellulose. Glucose could be obtained from several components, such as starch, cellulose, and hemicellulose, while xylose, galactose, and L-arabinose were mainly obtained from hemicellulose.

Among these sugars, xylose showed a significant change with different LHW pretreatment conditions. For the pretreatment of napier grass, the amount of total xylose started to increase when the temperature was greater than 160 °C. Prolonging the reaction time also enhanced the amount of total xylose. For example, at 160 °C it increased from 4.0 mg/g-napier grass at 0 min to 20.8 mg/g-napier grass at 30 min. This increase in the total xylose content in the LF of pretreated napier grass was related to the decreasing hemicellulose content in the corresponding SF. In contrast, at 200 °C the total xylose increased from 43.7 mg/g-napier grass at 0 min to 106.1 mg/g-napier grass at 15 min and then dramatically decreased to

36.6 mg/g-napier grass at 30 min, which conflicted with the residual hemicellulose content in the SF. Therefore, LHW pretreatment at a high temperature for longer reaction times could further degrade the sugars into other products. For the total xylose content of the pretreated napier silage sample, a significant increase was observed when the temperature was greater than 180 °C. At 180 °C the total amount of xylose increased from 5.8 mg/g-napier silage at 0 min to 30.6 mg/g-napier silage at 15 min and then a further two-fold increase to 61.9 mg/g-napier silage at 30 min. A similar phenomenon in decreasing hemicellulose content in the SF and increasing xylose content in the LF was observed, except no drastic decrease in the total xylose with prolonged reaction time was observed. During LHW pretreatment, it was inevitable that degradation of sugars into other products, including the AD inhibitors FF and HMF, would occur. Therefore, determination of the FF and HMF content was necessary because these inhibitors affect the activity of the microorganisms in the subsequent AD.

The concentrations of FF and HMF in the LF are shown in Figure 4. Note that FF is the degradation product from pentose sugars (C5), where hexose sugars (C6) can be degraded into HMF. The concentration of these compounds increased at higher LHW temperatures and longer times (Figure 4), with the highest concentration observed after LHW pretreatment at 200 °C for 30 min. For the pretreated napier grass, the generation of FF and HMF was observed at 180 °C and considerably increased at 200 °C. At 180 °C, the highest concentration of HMF and FF were 50 and 37 µg/mL, respectively. At 200 °C, they rapidly increased with increasing reaction times from 24 and 12 µg/mL for HMF and FF, respectively, at 0 min to 156 and 562 µg/mL, respectively, after 15 min and a further two-fold increase at 30 min. This was related to the decrease in the total sugar content observed in the napier grass treatment at 200 °C and 30 min.

Compared to the pretreated napier silage sample, the concentration of FF and HMF was lower than in the napier grass sample and these compounds were generated at a higher temperature (200 °C). This could have been caused by the initial existence of sugar, which was higher in treated napier grass than in napier silage. Thus, the sugar in the pretreated napier grass was easily degraded into FF and HMF during the LHW pretreatment. The HMF concentration in the napier grass sample was higher than that in the napier silage sample because the initial amount of soluble glucose in napier silage was lower than that in napier grass. Thus, samples with a high soluble sugar content were more likely to generate HMF during the LHW pretreatment. A similar observation for the FF concentration was found. To further optimize the utilization of these solutions after LHW pretreatment, the amount of total sugar and the concentration of these inhibitors should be considered, because simply optimizing for a high amount of total sugar could possibly result in a high concentration of inhibitors, which will negatively impact biogas production in the subsequent AD.

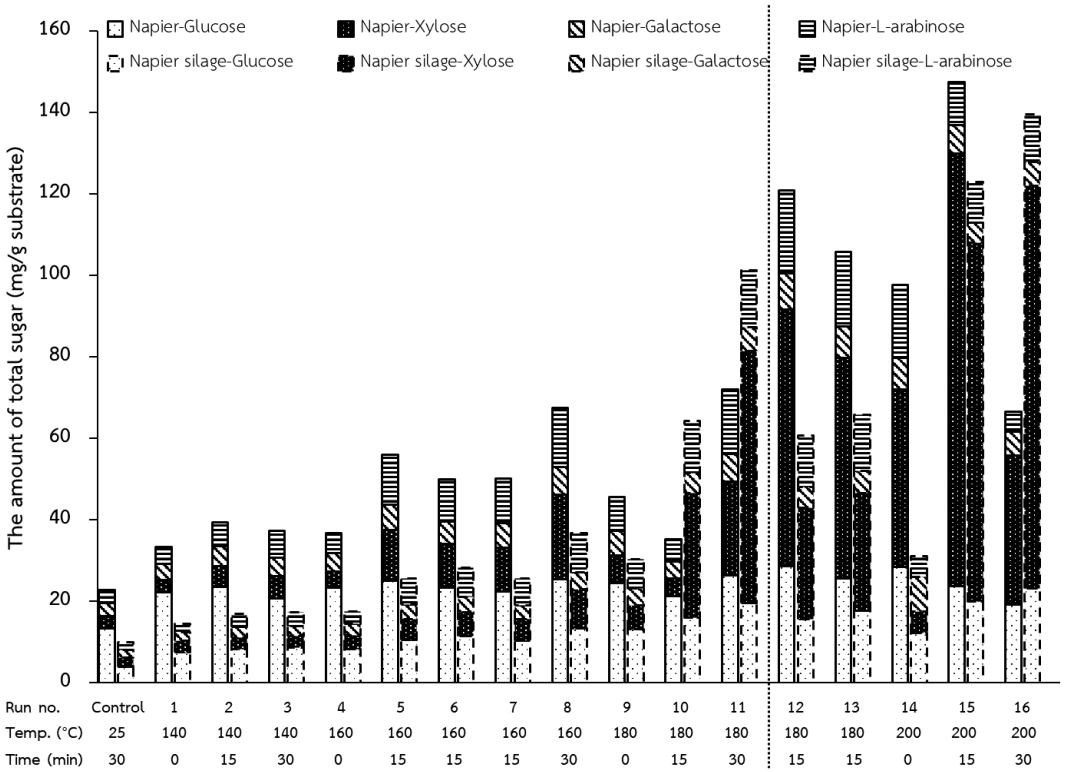


**Figure 3** Amount of total sugars in the LF obtained from LHW pretreatment of napier grass and napier silage under different conditions.

The solvation of solids in the napier grass and napier silage during LHW pretreatment, particularly hemicellulose, increased the amount of total sugars, and then the generation of inhibitors during the LHW pretreatment. Because of that, sugar derived from hemicellulose was found in the LF, especially xylose in an oligosaccharides form.

Increasing the temperature and reaction time of the LHW pretreatment resulted in the removal of hemicellulose from the SF to a high amount of oligosaccharides in the LF, some of which could further break down into monosaccharides. However, the amount of total sugar in the LF decreased with further increases in the LHW temperature and time to 200 °C and 30 min due to its degradation into other products, such as FF and HMF. Thus, to obtain a high amount of total sugar with a low concentration of inhibitors for use as a substrate in AD requires careful selection of the optimum reaction condition for the LHW pretreatment of napier grass and napier silage.

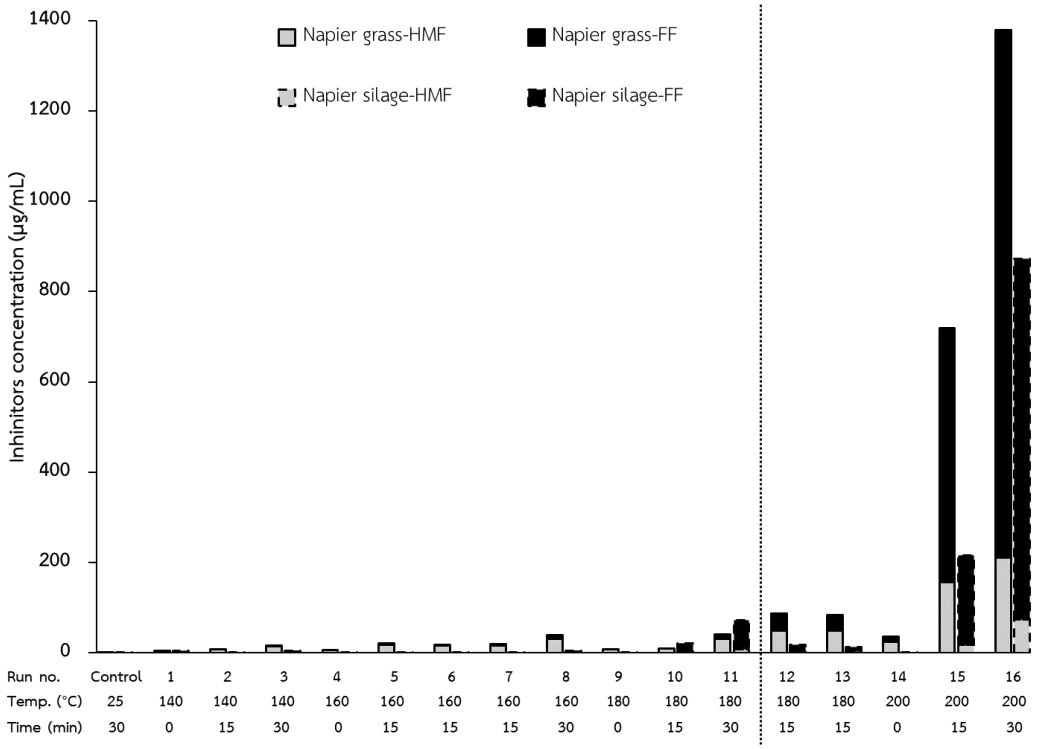


**Figure 4** The FF and HMF concentrations in the LF after LHW pretreatment of napier grass and napier silage under various conditions.

*3.2.1 Optimization of the LHW pretreatment of napier grass and napier silage*

The optimization of the LHW pretreatment of napier grass and napier silage to obtain a high total sugar content was performed to enhance or accelerate the $CH_4$ production in the subsequent AD. Since the reaction temperature (140–200 °C) and time (0–30 min) of the LHW pretreatment was focused on the solubilization of hemicellulose, xylose, as the main monomer of hemicellulose, was used as the response in the optimization. In Design 1, the temperature ranged from 140–180 °C over 0–30 min (11 experiment conditions). The Anderson-Darling test for normality revealed that the data for the total xylose content in the pretreated napier grass and napier silage samples were not parametric and so the data were transformed using Box-Cox transformation (Table S1), resulting in normally distributed data.

From the ANOVA, significant differences in xylose levels were found with the reaction time, where longer reaction times increased the amount of total xylose in the LF (Tables S2 and S3). On the other hand, only the pretreated napier silage sample showed a significant difference with the reaction temperature. The regression equation for the transformed total xylose level in the pretreated napier grass and napier silage in the uncoded equation is shown in Eqs. (5) and (6), where T is the temperature and t is the reaction time.

$$\text{Transformed napier-xylose} = 7.20 - 8.18\times10^{-2}\,T - 3.7\times10^{-3}\,t + 2.47\times10^{-4}\,T*T - 8\times10^{-6}\,t*t - 1.7\times10^{-5}\,T*t \quad (5),$$

$$\text{Transformed napier silage-xylose} = 2.29 - 2.01\times10^{-2}\,T + 1.29\times10^{-2}\,t + 4.6\times10^{-5}\,T*T + 1.02\times10^{-4}\,t*t - 1.24\times10^{-4}\,T*t \quad (6),$$

The coefficient of determination ($R^2$) and adjusted $R^2$ of the transformed data for napier-xylose was poor at 76% and 51%, respectively, meaning that data did not fit the equation. Thus, using this equation to predict the optimum condition was not reliable. In contrast, the transformed napier silage-xylose data had an $R^2$ of 96% and adjusted $R^2$ of 93%, meaning the data strongly fitted the equation. The reaction condition that gave the highest xylose amount was 180 °C and 30 min for both napier grass and napier silage, but the amount of xylose was different being 22.9 mg/g-napier grass and 61.9 mg/g-napier silage, respectively, which accounted for about 30% of the total xylose in the sample. In addition, the hemicellulose content also remained in the SF at about 55% and 23% of hemicellulose in the pretreated napier grass and napier silage, respectively. Increasing the reaction temperature to 200 °C increased the amount of total xylose in the LF by increasing the solubilization of hemicellulose.

Increasing the reaction temperature to 200 °C (five more experiment runs) was performed in Design 2 with a temperature range from 160–200 °C and reaction time of 0–30 min. The data for napier-xylose was parametric, whilst that for napier silage-xylose was not and so was subject to Box-Cox transformation with a 0 lambda value before statistical analysis. The transformed data of napier silage-xylose is shown in Table S4 and the ANOVA of these data is shown in Tables S5 and S6. The regression equation for napier-xylose and transformed napier silage-xylose are shown in Eqs. (7) and (8), respectively, with a poor $R^2$ and adjusted $R^2$ of napier-xylose of 64% and 27%, respectively. For the transformed napier silage-xylose, the $R^2$ and adjusted $R^2$ was 95% and 91%, respectively.

$$\text{Napier-xylose} = 901 - 11.5\,T - 7.74\,t + 3.62\times10^{-2}\,T*T - 1.29\times10^{-1}\,t*t - 1.99\times10^{-2}\,T*t \quad (7),$$

$$\text{Transformed napier silage-xylose} = -32.7 - 3.61\times10^{-1}\,T + 1.37\times10^{-1}\,t + 9.43\times10^{-4}\,T*T + 2.49\times10^{-3}\,t*t - 1.57\times10^{-3}\,T*t \quad (8),$$

In optimization of Design 2, the maximum amount of total xylose was set as the target. The optimum reaction condition for napier-xylose was 200 °C for 15 min, while for napier silage-xylose, it was 200 °C for 30 min. The contour plot and RSP of napier-xylose and transformed napier silage-xylose are shown in Figures 5 and 6, where the optimum condition with the highest amount of total xylose was observed at the peak at the top of the graph. From the amount of total sugar (Figure 3), the highest total xylose level was predicted to be 106.1 mg/g-napier grass and 98.7 mg/g-napier silage. However, consideration of the total sugar level only was not suggested due to the potential level of inhibitors (FF and HMF) that could have a toxic effect on the microorganisms in the subsequent AD. The optimum condition for the LHW pretreatment of napier silage was suggested at 200 °C for 15 min to give a slightly lower amount of total xylose at 88.0 mg/g-napier silage, since the HMF and FF concentrations were four-times lower at 200 °C for 30 min. The HMF and FF concentration at 200 °C for 15 min were only 17 and 199 µg/mL, respectively. Hence, the optimum LHW pretreatment condition for obtaining the highest amount of total

xylose yet a low concentration of inhibitors was at 200 ℃ for 15 min. High concentration of FF and HMF that occurred from LHW pretreatment could inhibit several metabolic pathways involving methanogens in AD system (e.g. growth rate and cell mass yield). The concentration of FF and HMF greater than 1 mg/mL could partially inhibit methanogenic activity, prolonging lag phase of fermentation. However, FF and HMF concentration over 2 mg/mL had fully inhibited the microorganism activity in AD process [36 - 37].

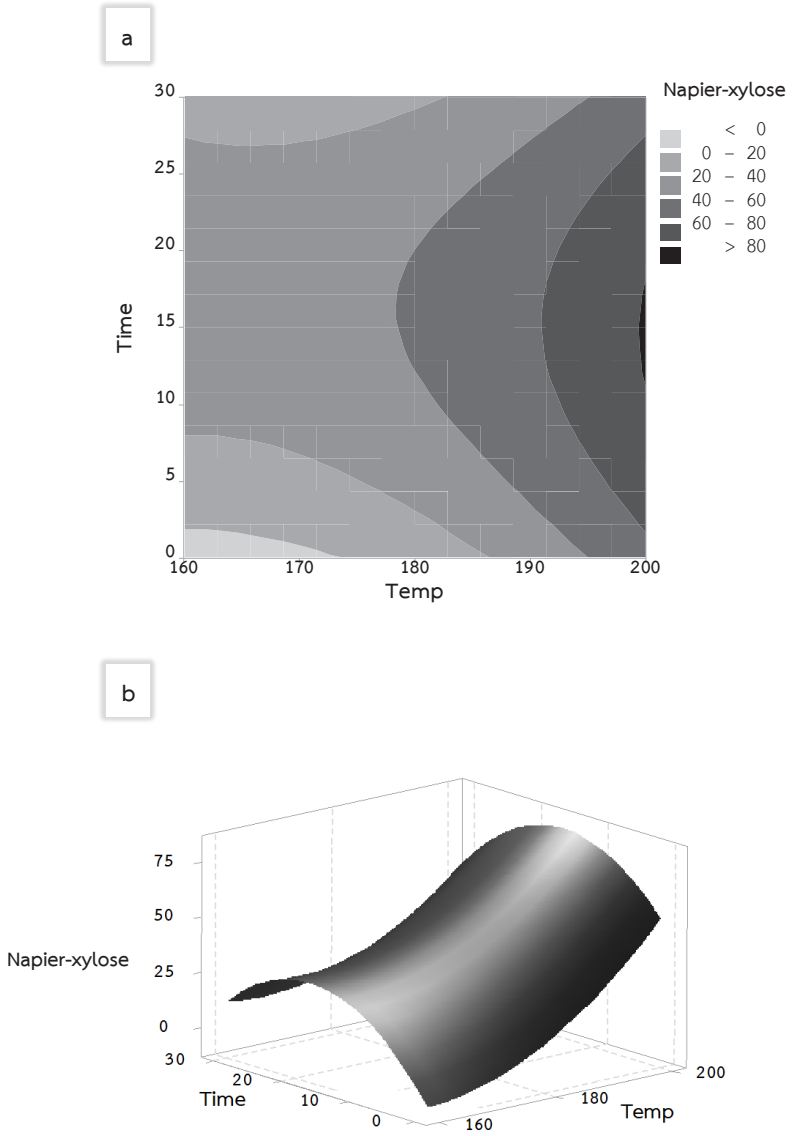


**Figure 5** (a) Contour plot and (b) RSP of napier grass-xylose from Design 2

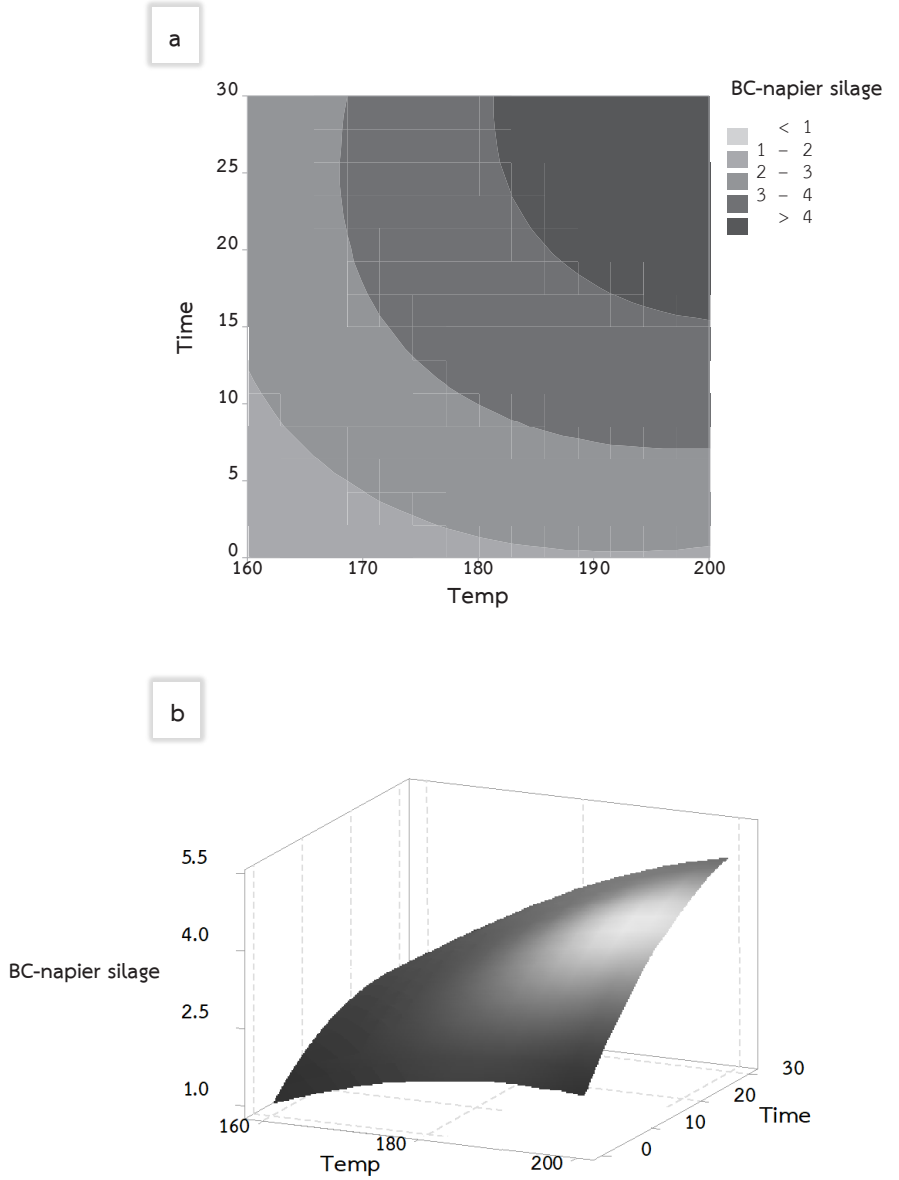


**Figure 6** a) Contour plot and (b) RSP of transformed napier silage-xylose from Design 2

*3.2.2 Validation of the optimum condition of napier grass and napier silage*

The LHW pretreatment of napier grass and napier silage at the derived optimum condition (200 °C, 15 min) was conducted in triplicate. The chemical composition of the obtained SF is shown in Table 4, where the residual solid content in napier grass and napier silage was 52% and 56%, respectively. Fiber composition analysis showed that cellulose and lignin mostly remained in the SF, while the hemicellulose content was largely (> 90%) removed from the sample, being hydrolyzed into oligo- and

monosaccharides in the LF. For the pretreated LF, the sugar composition and inhibitors concentration are shown in Table 5. In terms of monosaccharides, xylose had the highest concentration and was similar in the pretreated napier silage and grass samples at 20.5 mg/g-napier grass and 19.5 mg/g-napier silage, respectively. L-arabinose was the second most abundant sugar found in the LF in both samples. The presence of xylose and L-arabinose in the LF reflects hemicellulose removal, because xylose is the monomer of the main structural chain and L-arabinose is the sugar bound at the side chain of the hemicellulose structure. Glucose and galactose were observed in a small amount.

For total sugars, xylose still showed the highest amount in the LF and was at a higher level than the monosaccharide form, meaning that about 80% of the xylose was oligosaccharides with a total xylose content of 89.4 mg/g-napier grass and 96.2 mg/g-napier silage, of which only 50% of the total xylose from the initial sugar composition was obtained. Therefore, some of the total xylose was further degraded into other products. Determination of the FF concentration in the LF revealed signs of xylose degradation, with a high concentration of 674 and 620 µg/mL in the pretreated napier grass and napier silage, respectively.

**Table 4** Fiber compositions of the LHW-pretreated napier grass and napier silage at the optimum condition (200 °C and 15 min)

| Component | % (w/w) Initial | |
|---|---|---|
| | Napier grass | Napier silage |
| Solid remaining | 51.74 ± 1.09 | 56.32 ± 0.33 |
| Cellulose | 28.92 ± 0.35 | 34.00 ± 0.25 |
| Hemicellulose | 3.45 ± 0.43 | 2.69 ± 0.14 |
| Lignin | 4.53 ± 0.41 | 5.12 ± 0.03 |
| Hemicellulose removal* | 89.39 ± 1.33 | 91.35 ± 0.44 |

*Hemicellulose removal (%) was calculated from the remaining hemicellulose divided by the initial hemicellulose.
Data are shown as the mean ± SD, derived from three repeats. Means with a different letter are significantly different.

**Table 5** The amount of monosaccharides, total sugars, and concentration of inhibitors in the LF of LHW-pretreatment napier grass and napier silage at the optimum condition (200 °C and 15 min)

| Composition | Napier grass | Napier silage |
|---|---|---|
| **Monosaccharides (mg/g sample)** | | |
| Glucose | 1.42 ± 0.03 | 1.12 ± 0.10 |
| Xylose | 20.52 ± 0.54 | 19.47 ± 1.26 |
| Galactose | 1.78 ± 0.44 | 1.77 ± 0.08 |
| L-arabinose | 5.07 ± 0.26 | 5.03 ± 0.19 |
| **Total sugars (mg/g sample)** | | |
| Glucose | 33.09 ± 0.41 | 20.77 ± 0.83 |
| Xylose | 89.40 ± 5.75 | 96.18 ± 2.99 |
| Galactose | 5.98 ± 0.45 | 5.14 ± 0.13 |

**Table 5** The amount of monosaccharides, total sugars, and concentration of inhibitors in the LF of LHW-pretreatment napier grass and napier silage at the optimum condition (200 °C and 15 min) (Continue)

| Composition | Napier grass | Napier silage |
|---|---|---|
| L-arabinose | 5.07 ± 0.26 | 5.03 ± 0.19 |
| **Total sugars (mg/g sample)** | | |
| Glucose | 33.09 ± 0.41 | 20.77 ± 0.83 |
| Xylose | 89.40 ± 5.75 | 96.18 ± 2.99 |
| Galactose | 5.98 ± 0.45 | 5.14 ± 0.13 |
| L-arabinose | 8.65 ± 0.61 | 8.70 ± 0.24 |
| **Inhibitors (μg/mL)** | | |
| HMF | 104 ± 2 | 49 ± 4 |
| FF | 674 ± 81 | 620 ± 41 |

Data are shown as the mean ± SD, derived from three repeats. Means with a different letter are significantly different.

In addition, the total glucose level in the LHW pretreated napier grass and napier silage was 33.1 and 20.8 mg/g-sample, respectively, which was much higher than that in the monosaccharide form. The glucose could be derived from several components, such as starch, cellulose, and hemicellulose. Apart from the glucose obtained from hemicellulose, the cellulose content in the SF was slightly removed, which means some part of the cellulose was broken down and solubilized into the LF. Particularly at the optimum condition, the amorphous region of the cellulose structure is more easily broken down than the crystalline region [35]. The total galactose and L-arabinose levels were at small amounts because galactose is not the main chain of the polysaccharides and L-arabinose is attached as a side chain of the hemicellulose structure [38].

*3.3 The BMP of the untreated and LHW-pretreated napier grass and napier silage*

Evaluation of $CH_4$ production from the untreated and LHW-pretreated napier grass and napier silage was performed to determine the effect of LHW-pretreatment on the subsequent AD in terms of the BMP test. The untreated napier grass and napier silage, and the LHW-pretreated napier grass and napier silage were used in turn as the substrate and the $CH_4$ production yield from these individual substrates is shown in Figure 7.

During the first 30 d, a sharp increase in the cumulative $CH_4$ yield was observed in all four samples (napier grass, napier silage, LHW-pretreated napier grass, and LHW-pretreated napier silage), where 70–97% of the maximal $CH_4$ yield was produced. This was due to limiting substrates for the microbial hydrolysis in biogas production, such as high crystallinity of cellulose, high degree of polymerization, high moisture content, lower available surface area, and high lignin content [31, 39]. The $CH_4$ production from napier grass and napier silage was rapid at the beginning of the reaction (first 10 d) and then slightly increased afterwards. The $CH_4$ yield of napier grass (246 mL STP/g $VS_{added}$) was 17% higher than that from napier silage (211 mL STP/g $VS_{added}$), which could be due to the higher content of easily degradable compounds

in napier grass than in napier silage. During ensiling process, easily degradable compounds, such as soluble compounds, starch, and hemicellulose, are degraded and consumed by microorganisms, leaving the difficult-to-degrade compounds, like cellulose and lignin, in the napier silage sample. For the $CH_4$ production of the LHW-pretreated slurry, LHW-pretreated napier grass and LHW-pretreated napier silage showed a lag phase of 14 and 7 d, respectively, and thereafter $CH_4$ production of both LHW-pretreated samples rapidly increased until after 30 d when the $CH_4$ production only slightly increased. The $CH_4$ production from the LHW-pretreated samples was higher than that from the untreated samples. The lag phase of the LHW-pretreated samples was caused by the presence of inhibitors in sample, where the longer lag phase time was consistent with the higher inhibitor concentration in the LHW-pretreated napier grass sample, which had a two-fold higher HMF concentration than the LHW-pretreated napier silage (Table 5). The rapidly increasing $CH_4$ production yield in the LHW-pretreated samples could be caused by solubilization of sugar derived from hemicellulose compounds, which can easily be utilized by microorganisms in the AD. In addition, the $CH_4$ production from LHW-pretreated napier grass was about 12% higher than that from the LHW-pretreated napier silage sample, a similar result to that observed in the untreated sample. Comparing between the LHW-pretreated and untreated samples, the $CH_4$ yield of the LHW-pretreated napier grass was about 17% higher than that of the untreated napier grass, whereas the $CH_4$ yield of LHW-pretreated napier silage was 20% higher compared to that of the untreated napier silage.

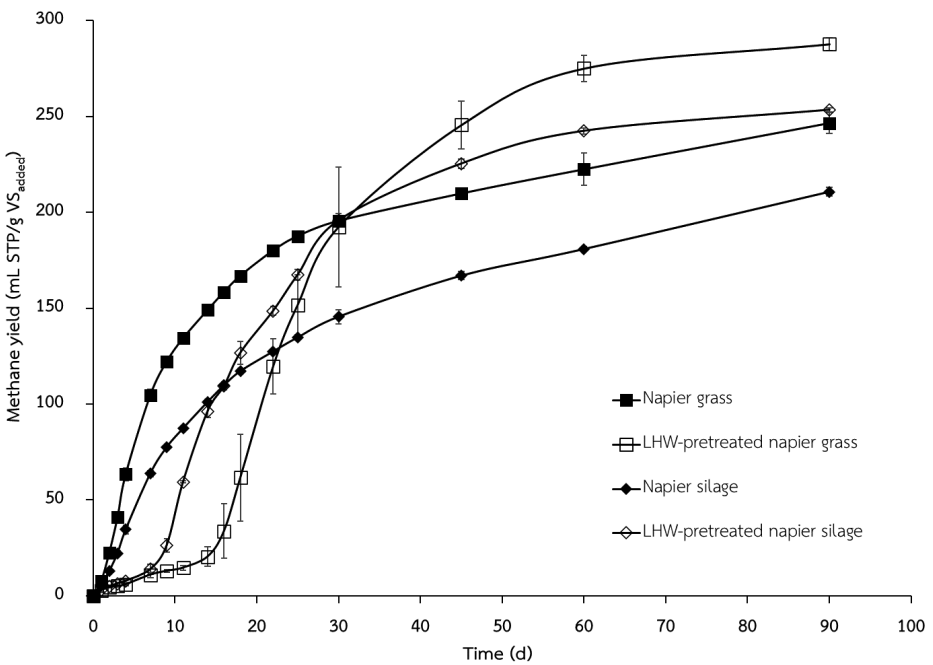


**Figure 7** The BMP of napier grass, napier silage, LHW-pretreated napier grass, and LHW-pretreated napier silage. Data are shown as the mean ± SD, derived from three replications.

The kinetic data from the BMP test for the four substrates (napier grass, napier silage, LHW-pretreated napier grass, and LHW-pretreated napier silage) are shown in Table 7 using the regression analysis of the experimental results and the modified Gompertz model. After curve fitting, all the data showed a satisfactory agreement with $R^2$ values higher than 0.96. The lag phase time showed a result corresponding to the $CH_4$ production, where LHW-pretreated napier grass had the longest lag phase time at 16 d followed by LHW-pretreated napier silage at 5 d and both of the untreated samples (napier grass and napier silage) did not show any lag phase. That the lag phase time was observed in the LHW-pretreated samples reflects the presence of inhibitors in the pretreated solution, particularly FF and HMF. Even though the LHW-pretreated samples contained easily degradable compounds, such as oligo- and mono-saccharides, other compounds were derived from the breakdown of complex carbohydrates by the LHW-pretreatment such as FF and HMF, which inhibit several metabolic pathways of microorganisms in the AD (e.g. growth rate and cell mass yield) [36 - 37]. Thus, the microorganisms in the AD take time to adapt and metabolize these inhibitors into less toxic compounds prior to utilization of the oligo- and mono-saccharides. The LHW-pretreated samples showed a significantly higher maximum $CH_4$ yield potential than the untreated samples, where the $CH_4$ yield of LHW-pretreated napier grass was 16% higher than napier grass and 23% higher in LHW-pretreated napier silage than LHW-pretreated napier grass. However, the $CH_4$ production rate was not significantly different between the pretreated and untreated samples, ranging from 7.7 to 15.0 mL STP/g $VS_{added}$*d. This is because all four samples contained a high cellulosic fraction with an unsuitable C:N ratio, resulting in a slower initial $CH_4$ production rate than feedstocks composed of easily degradable matter, such as sugar and starch. Therefore, solubilization of the carbohydrate structure, especially hemicellulose, using the LHW-pretreatment of napier grass and silage enhanced the $CH_4$ production yield but not the $CH_4$ production rate compared to the untreated sample.

**Table 7** The $CH_4$ accumulation data from napier grass, napier silage, LHW-pretreated napier grass, and LHW-pretreated napier silage obtained using the modified Gompertz model.

| Sample | $BMP_{30}$ (mL STP/g $VS_{added}$) | Cumulative $CH_4$ yield (mLSTP/g $VS_{added}$) | Lag phase (d) | $CH_4$ yield potential (mL STP/g $VS_{added}$) | $CH_4$ production rate (mL STP/g $VS_{added}$*d) | $R^2$ |
|---|---|---|---|---|---|---|
| Napier grass | 195 | 246 | $0^{c}$ | $235^{bc}$ | $13.2^{a}$ | 0.97 |
| Napier silage | 146 | 211 | $0^{c}$ | $205^{c}$ | $7.7^{b}$ | 0.96 |
| LHW-pretreated napier grass | 192 | 288 | $16^{a}$ | $274^{a}$ | $15.0^{a}$ | 0.99 |
| LHW-pretreated napier silage | 196 | 253 | $5^{b}$ | $253^{ab}$ | $9.5^{b}$ | 0.99 |

Data are shown as the mean, derived from three replicates.
Upper letter represented the multiple comparison of Tukey's test with significant level at 95% ($\alpha$ = 0.05).

In addition, calculation of the theoretical yield of $CH_4$ production using the elemental analysis of napier grass and silage was 435 and 405 mL STP/g $VS_{added}$, respectively. The higher theoretical $CH_4$ yield was caused by the characteristics of the samples, where napier grass contained a higher level of carbohydrate compounds than napier silage. The biodegradability of napier grass and napier silage was 54% and 51%, respectively, where the LHW pretreatment of both samples increased the biodegradability by about 10%. The low degradability and conversion of carbohydrate could be caused by the recalcitrant properties of lignocellulosic compounds in the SF, particular cellulose and lignin. The C:N ratio of napier grass and napier silage also affected the low biodegradability, where napier grass and napier silage had a 39.5 and 87.0 C:N ratio, respectively. Typically, LCMs containing low levels of nitrogen (high C:N ratio) have a low substrate pH, poor buffering capacity, and the possibility of high volatile fatty acid accumulation (potential inhibitor to subsequent AD) in the digestion process. The ideal C:N ratio range was reported to be between 20–35 in the co-digestion of LCM and manure [8, 11, 40 - 41]. Consequently, napier grass and LHW-pretreated napier grass had a higher $CH_4$ yield potential than napier silage and LHW-pretreated napier silage.

## 4. Conclusions

The LHW pretreatment significantly affected the $CH_4$ production yield from napier grass and napier silage, and the pretreatment was found to be efficient in the solubilization and partially breakdown of hemicellulose into oligo- and mono-saccharides, resulting in a larger amount of xylose becoming solubilized in the LF after the LHW pretreatment. The optimal LHW pretreatment condition of 200 °C for 15 min gave the highest amount of sugar with a low concentration of inhibitors (FF and HMF). However, the toxic effect of these inhibitors was potentially shown by the prolonged lag phase time during the BMP test. The $CH_4$ production yield of the LHW-pretreated napier grass and napier silage was 16% and 23% higher than the untreated samples.

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