Final report

Combined system of steam explosion and enzymatic pretreatment to improve anaerobic digestibility and recover value-added products from rubber wood sawdust

By

Boonya Charnnok

April 2018

สัญญาเลขที่ MRG5980051

Final report

Combined system of steam explosion and enzymatic pretreatment to improve anaerobic digestibility and recover value-added products from rubber wood sawdust

By

Boonya Charnnok

PSU Energy Systems Research Institute

Prince of Songkla University

สนับสนุนโดยสำนักงานคณะกรรมการอุดมศึกษา และสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกอ. และ สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

บทคัดย่อ

เศษไม้ยางพาราเป็นชีวมวลลิกโนเซลลูเลสที่พบได้มากในประเทศไทย ด้วยลักษณะความแข็งแรงตามธรรมชาติของชีวมวลลิกโนเซลลูโลสทำให้ยากต่อการผลิตพลังงานชีวภาพ การศึกษานี้ได้ปรับสภาพเศษไม้ยางด้วยการระเบิดไอน้ำที่ severity ในช่วง 2.27-4.35 จากการศึกษาพบว่า เศษไม้ยางพาราที่ผ่านการปรับสภาพแล้ว มีความสามารถในการย่อยสลายในสภาวะไร้อากาศสูงสุด (83.9 L CH4/kg-VS) ที่ severity เท่ากับ 4.35 เช่นเดียวกับความสามารถในการไฮโดรไลซ์ แสดงในรูปความสามารถย่อยสลายกลูแคน (45.20%) ด้วย cellulase Cellic®CTech2 ที่ความเข้มข้นเอนไซม์ 30 FPU/g ทั้งปริมาณผลผลิตมีเทน และน้ำตาลที่เกิดขึ้นสอดคล้องกับการเปลี่ยนแปลงความเป็นผลึกและลักษณะพื้นผิวชีวมวลที่ผ่านการปรับสภาพแล้ว นอกจากนี้ ยังได้ศึกษาการปรับปรุงประสิทธิภาพการย่อยสลายชีวมวลด้วยกระบวนการร่วมแบบต่อเนื่อง ประกอบด้วย การปรับสภาพด้วยการระเบิดไอน้ำ การย่อยสลายด้วยเอนไซม์ และการย่อยสลายในสภาวะไร้อากาศ กระบวนการดังกล่าวใช้การปรับสภาพด้วยการระเบิดไอน้ำที่ severity เท่ากับ 4.03 ให้ผลผลิตสองรูปแบบ คือ น้ำตาลและมีเทน เมื่อคิดเทียบเป็นค่าพลังงานแล้ว พบว่ามีค่าพลังงานรวมสูงกว่าการผลิตน้ำตาลหรือมีเทนเพียงอย่างเดียวที่การปรับสภาพ severity สูงกว่า (severity เท่ากับ 4.35) อย่างไรก็ตาม เศษเหลือชีวมวลจากกระบวนการร่วมดังกล่าว ยังคงมีส่วนเหลือพอลิแซคคาร์ไรด์ตกค้าง ซึ่งควรได้หาแนวทางใช้ประโยชน์ในรูปวัสดุชีวภาพต่อไป

คำสำคัญ: การระเบิดด้วยไอน้ำ การย่อยสลายด้วยเอนไซม์ การย่อยสลายสภาวะไร้อากาศ เศษไม้ยางพารา พลังงานชีวภาพ

Abstract

Rubber wood waste (RW) is an abundant biomass in Thailand, their lignocellulosic nature entails difficulties for bioconversion. Steam explosion with a severity of 2.27-4.35 was applied to pretreat RW. Pretreated RW with highest improvement in anaerobic digestibility achieved 83.9 L CH_4/kg-VS at severity of 4.35 together with the hydrolysability in terms of glucan conversion as high as 45.2% at a cellulase Cellic®CTech2 concentration of 30 FPU/g. These yields correlated to the changes in crystallinity and surface morphology of RW. Conversion efficiency was enhanced with an integrative process of SE followed by enzymatic hydrolysis and anaerobic digestion. In term of energy yield, this process with severity 4.03 could co-produce glucose and methane higher than only the production of glucose or methane alone or even higher SE severity at 4.35. The remainder RW is rich in polysaccharide and could be a good biomaterial for further utilization.

Keywords: Steam explosion; Enzymatic hydrolysis; Anaerobic digestion; Rubber wood waste; Bioenergy

Executive Summary

Rubber wood waste (RW) consists mainly of cellulose, hemicellulose and lignin that makes its structure rigid and recalcitrant to degradation, especially in hydrolysis step. Steam explosion (SE) is a thermo-mechano-chemical pretreatment which was chosen for pretreating RW to improve hydrolysability and anaerobic digestibility of RW because it has low energy intensive and environmental friendly due to no chemical addition.

This study aims to examine the effects of severity of SE pretreatment and enzyme loading on RW hydrolysis and anaerobic digestion due to changing in physical and chemical RW structures. In addition, an integrative process of SE pretreatment and enzymatic hydrolysis with various enzyme loading followed by anaerobic digestion was applied in order to improve biodegradability of RW and overall energy yield of co-product which was glucose and methane.

RW of size 2.4-4.7 mm was collected from a production process of a particle board factory, Panel Plus Co., Ltd. in Songkhla province, Thailand. It was subjected to steam explosion reactor at severity 2.70, 2.37, 2.73, 4.03 and 4.35. After SE pretreatment, the samples were washed with 800 mL hot water (70°C) for 10 min in order to remove the degradation products accumulated on the RW surface. The washing liquid was analyzed for degradation products while the steam exploded RW (SE-RW) was subjected to anaerobic digestion (AD) and enzymatic hydrolysis (EH) assays. Enzymatic hydrolysis optimization with Cellic®CTech2 enzyme loading of 3, 6, 12, 18, 24 and 30 FPU/g was performed. Moreover, integrative process including SE pretreatment, EH and AD was conducted in order to recover the remaining polysaccharide from enzymatic hydrolysis process (SE+EH-RW).

RW mainly consists of 44.87% glucan (cellulose), 14.51% xylan (hemicellulose) and 13.12% lignin. An advantage of SE pretreatment of RW is that glucan was preserved whereas degradation of xylan was enhanced with an increasing severity due to thermo-physical and

catalytic reaction of auto-hydrolysis by acetic acid, as a result, improvement of active area for accessible enzyme to cellulose.

Anaerobic digestibility test, methane production was significantly enhanced with an increasing SE severity above 3.73 in comparison to Untreated-RW (Un-RW). Methane yield was improved with an increasing severity and reached maximum value of 83.9 L CH_4/kg-VS at severity 4.35, interestingly with a 2-step pattern. During the first seven days of digestion, the methane production increased slowly then it rapidly evolved during days 7 to 13 of digestion by 3.8 folds (from 19.8 to 74.5 LCH_4/kg-VS). The sharp increase in methane yield might be a result of the weakening biomass cell wall due to intensive physical effect and auto-catalytic reaction particularly changing in C=O group in structure of SE-RW.

Enzymatic hydrolysis test, glucose yield and glucan conversion were reached maximum value of 38.44 and 45.20% at severity 4.35, respectively whereas maximum value of xylan conversion was achieved 92.44% lower severity of 4.03 at enzyme loading 30 FPU/g. Quadratic models of all enzymatic hydrolysis yields were performed in order to evaluate the influential factors. SE severity and enzyme loading (EL) were influential factors for production of glucose yield, glucan conversion and xylan conversion. All enzymatic hydrolysis yields could reach a maximum value with severity adjustment. However, reduction of solid recovery after SE pretreatment with an intensive severity applied should be taken in consideration for product recovery per mass of native feedstock. Contrast, applying intensive enzyme loading could not significantly enhance hydrolysability of both glucan and xylan in SE-RW. It might be due to inhibitory effects of cellubiose to enzymatic hydrolysis. Our study cellubiose formation potentially increased with an addition of enzyme loading.

The effect of enzymatic hydrolysis with enzyme loading between 3 to 30 FPU/g was studied in addition to the previous SE pretreatments at severity of 4.03 due to higher xylan conversion in enzymatic hydrolysis step compared to other SE conditions. This synergy effects

of steam explosion and enzymatic hydrolysis contributed the acceleration of initial anaerobic digestibility to improve initial methane potential yield. Not only the quick start, the overall methane yield of the SE+EH-RW combination accomplished a significantly higher methane than only steam explosion for a 28-day digestion by 81.6% at enzyme loading of 30 FPU/g. Although higher volume of methane from SE+EH-RW was observed, approximately 58.9% of holocellulose (at enzyme loading of 30 FPU/g) left after digestion was not able to convert into methane. Thus, the biomethanation of SE+EH-RW could be justified as the final recovery for biodegradable carbon.

Product and energy yields per ton-RW were evaluated at various options. Highest total energy of 3833 MJ/ton-RW was obtained from the integrated process including steam explosion (severity 4.03), enzymatic hydrolysis, and anaerobic digestion (SEA-process) caused by enlargement of SE+EH-RW biodegradability. This methane contributed 57.9% of total energy potential production while another was shared by glucose yield from enzymatic hydrolysis step. Compared to a single process of SE, SE+EH-RW with SE severity 4.03 corresponds to 45.6% and 42.5% higher energy yield than only from glucose or methane with SE pretreatment at severity of 4.35. In comparison to SE severity 4.35, lower energy supply to SE pretreatment at severity 4.03 with an integrative SEA-process showed an improvement of overall energy yield from rubber wood waste. Moreover, microbial inhibitors such as furfural and HMF were also minimized during SE pretreatment at severity 4.03 which could take into consideration for other purposes based on zero-waste strategy.

This research was concluded that severity of SE pretreatment played a vital role to improve the production of glucose and methane from RW by causing physical and chemical structural changes as well as reduction of hemicellulose xylan contents. Production of glucose thru enzymatic hydrolysis followed by anaerobic digestion of enzymatically hydrolyzed residue of the proposed SEA-process with lower severity of 4.03 of steam explosion

pretreatment has great potential to improve energy recovery compared to either glucose or methane production alone even at a higher severity of 4.35. While the SEA-process enhanced technical feasibility of bioenergy production from rubber wood waste, valuated end use of the purified processed rubber wood residue will be key for its economic feasibility which is subject to further research.

Table of contents

List of tables

List of figures

Chapter 1

Introduction

Rubber industry is one of the most important economic agro-industry sectors in over 20 countries including Thailand. While the main primary products are either dry or latex natural rubber, their co-product of rubber wood is an important material of great economic value for furnisher industry. With rubber planting area of over 19 million rais (7.6 milion acres) in 2016 (Center of Agricultural Information, 2016), this rubber wood not only could contribute to national export value but also a huge resource for lignocellulosic biomass for biochemicals and energy production. The economical lifespan of a rubber tree is approximately 25 years, after which rubber latex production is reduced and it will be cut down for replantation. The rubber biomass largely become a woody material for furnisher and toy industry. In rubber wood processing, a gigantic amount of rubber wood waste (RW) is generated from cutting and milling.

This lignocellulosic biomass consists mainly of cellulose, hemicellulose and lignin that makes its structure rigid and recalcitrant to degradation, especially in hydrolysis step. The carbohydrate in woody biomass are not readily accessible for hydrolytic enzymes. The pretreatment step is crucial in order to disrupt lignocellulosic biomass matrix and make available polysaccharides for enzymatic attack. Steam explosion (SE) is a thermo-mechano-chemical pretreatment by high pressure saturated steam with temperature between 160 and 260 °C (Rabemanolontsoa & Saka, 2016). The biomass is subjected to harsh environment of high pressure and temperature with saturated steam. The thermal expansion opens up the cell wall of the biomass prior to explosion and also induces auto-hydrolysis by acetic acid derived from acetyl residues in xylan hemicellulose. The pressure is then rapidly reduced to atmospheric condition where the explosion of the plant cell wall takes place. Not only this process is

relatively low energy intensive but also is environmental friendly due to no chemical addition (Jacquet et al., 2015; Singh et al., 2015). It is among the most efficient pretreatments to enhance enzymatic hydrolysis and digestion of woody feedstocks (Cotana et al., 2015; Li et al., 2016; Vivekanand et al., 2013). Removal of hemicellulose by SE is so effective that it increases exposure of cellulose surface to enzymes within the loosen cellulose microfibrils (Imman et al., 2014).

For each biomass, it is important to optimize SE condition by critical operating parameters. Those of particular interest include pressure, temperature and retention time, which impact structural transformation at different degrees (Asada et al., 2012; Ballesteros et al., 2011; Cotana et al., 2015; Lizasoain et al., 2017). Currently, RW is used as low value fuel in biomass power plant and industrial steam boiler. Its use as cellulosic biomass for the combined liquid biofuel and methane production had not been reported. Previous research had used residues from ethanol fermentation of steam exploded oat straw at 190°C for 10 min (Dererie et al., 2011) and steam exploded birch wood at 210 °C for 10 min (Kalyani et al., 2017) as substrate for producing biogas thru anaerobic digestion. Although the overall energy recovery was reportedly increased compared to only ethanol production, only one combination of ethanol-methane production from their biomasses was tested. Study on comparative yield of the different combinations in terms of energy and its by-products could give an insight for this biomass valorization in biorefinery perspective. Thus, the integrative strategy of steam explosion combined with enzymatic hydrolysis and anaerobic digestion is proposed that could potentially raise the value-chain of this lignocellulosic rubber wood waste.

This study aims to examine the effects of severity of steam explosion pretreatment and enzyme loading on RW hydrolysis and anaerobic digestion due to changing in physical and chemical RW structures. In addition, an integrative process of SE pretreatment and enzymatic hydrolysis with various enzyme loading followed by anaerobic digestion was applied in order

to improve biodegradability of RW and overall energy yield of co-product which was glucose and methane.

Chapter 2

Literature review

2.1 Rubber wood waste

Rubber industry is one of the most important economic agro-industry sectors in over 20 countries including Thailand. While the main primary products are either dry or latex natural rubber, their co-product of rubber wood is an important material of great economic value for furnisher industry. With rubber planting area of over 19 million rais (7.6 milion acres) in 2016 (Center of Agricultural Information, 2016), this rubber wood not only could contribute to national export value but also a huge resource for lignocellulosic biomass for biochemicals and energy production. The economical lifespan of a rubber tree is approximately 25 years, after which rubber latex production is reduced and it will be cut down for replantation. The rubber biomass largely become a woody material for furnisher and toy industry. In rubber wood processing, a gigantic amount of rubber wood waste (RW) is generated from cutting and milling.

The rubber wood waste has been used as solid fuel to generate heat energy. However, it is widely considered low efficiency of energy conversion. Moreover, in combustion system, polycyclic aromatic hydrocarbons (PAHs) are formed from the incomplete combustion of organic matter. PAHs could be created by secondary aromatization reactions in char in the pyrolysis phase of incomplete biomass combustion at temperatures higher than 400 °C (Fisher et al., 2002). Anthracene, fluoranthene, pyrene, benzo[a]anthracene and chrysene are predominant PAHs emitted from combustion of biomass fuels (Singh et al., 2013). PAHs are considered as carcinogenic compounds, environmental and health concern in biomass power plant. Hence, utilization of rubber wood waste is still being a problem.

2.2 Lignocellulosic biomass

Typically, wood is a lignocellulose that mainly composed of cellulose (40–45%), lignin (25–30%), hemicelluloses (20–30%) and extractives (1–5%). Cellulose is a polysaccharide having the generic formula $(C_6H_{10}O_6)_n$. The cellulose can be broken down to glucose on hydrolysis process. Glucose is a six-carbon (C6) sugar called hexoses. Hemicellulose is a macromolecular polysaccharide forming a mixture of straight and highly branched chains of pentoses (xylose, arabinose) called C5, hexoses (mannose, glucose, galactose) and acetylated sugars (Agbor et al., 2011). Lower molecular weight of hemicellulose compared to cellulose and branches with short lateral chains, hemicelluloses are easily hydrolyzed (Saha, 2003). Lignin consists of complex phenolic polymers which has benzene ring as dominant monomeric units. The benzene rings bear methoxyl, hydroxyl, and propyl groups that can be attached to other units. It binds the different components of lignocellulosic biomass together, resulting lignin is able to protect microbial digestion of structural carbohydrates.

The complex structure of lignocellulose contains fermentable sugars which can be utilized for the production of biofuel such as ethanol, hydrogen, methane etc. In order to develop conversion processes for lignocellulose, it is important to understand its chemical composition. The composition of difference lignocelluloses is shown in Table 2-1. For biochemical conversion processes, the proportions of cellulose and lignin in lignocellulose are significant. It is one of important factors when selecting types of lignocellulosic biomass for biochemical processing. Lignocellulosic biomass with a high proportion of cellulose/hemicellulose content is required to provide a high biofuel yield. The biodegradability of cellulose is greater compared to lignin. Thus, the overall conversion of lignocellulose with a higher proportion of cellulose is greater than for lignocellulose with a higher proportion of lignin. Rubber wood has higher potential degradability compared to Acacia wood chip and Eucalypt wood chip because of higher cellulose/lignin.

Lignocellulosic biomass consists mainly of cellulose, hemicellulose and lignin that makes its structure rigid and recalcitrant to degradation, especially in hydrolysis step. The carbohydrate in woody biomass are not readily accessible for hydrolytic enzymes. The pretreatment step is crucial in order to disrupt lignocellulosic biomass matrix and make available polysaccharides for enzymatic attack to promote enzymatic hydrolysis and anaerobic digestibility for co-production of sugar and biogas production.

Table 2-1 Chemical composition (%w/w) for different lignocellulosic biomass

Biomass	Cellulose	Hemicellulose	Lignin	Cellulose/lignin	References
Aspen wood	47.0	22.7	26.8	1.7	(Fougere et al., 2015)
Acacia wood chip	26.60	66.80	41.80	0.6	(Ko et al., 2012)
Eucalypt wood chip	22.90	69.90	47.20	0.5	(Ko et al., 2012)
Rubber wood (trunk)	42.6c	n/a	27.0	1.6	(Jahan et al., 2011)
Rubber wood (branch)	40.9c	n/a	29.3	1.4	(Jahan et al., 2011)
Olive tree wood	34.4	20.3	20.4	1.7	(Cara et al., 2006)
Softwood	35-40	25-30	27.30	1.3	(McKendry, 2002)
Hardwood	45-50	20-25	20-25	2.2-2.0	(McKendry, 2002)
Wheat straw	33-40	20-25	15-20	2.2-20.0	(McKendry, 2002)
Sugarcane straw	39.8	28.6	22.5	1.3	(Oliveira et al., 2013)
Switch grass	30-50	10-40	5-20	3.0-2.5	(McKendry, 2002)
Palm empty fruit bunch	37.6	38.8	23.6	1.6	(Saritpongteeraka et al., 2015)
Apple pomace	16.91	20.00	24.11	0.7	(Wang et al., 2010)

2.3 Steam explosion pretreatment

Steam explosion (SE) is a thermo-mechano-chemical pretreatment by high pressure saturated steam with temperature between 160 and 260 °C (Rabemanolontsoa & Saka, 2016) and pressure 7-48 bar (0.7-4.8 MPa) (Agbor et al., 2011; Qiu & Chen, 2012) ranging from seconds to several minutes. The biomass is subjected to harsh environment of high pressure and temperature with saturated steam. The thermal expansion opens up the cell wall of the biomass prior to explosion and also induces auto-hydrolysis by acetic acid derived from acetyl

residues in xylan hemicellulose. The pressure is then rapidly reduced to atmospheric condition where the explosion of the plant cell wall takes place.

The steam explosion pretreatment combines mechanical forces and chemical effects. The mechanical effects are the action of explosive with the rapid depressurization. This rapid expansion vaporize the saturated water within the fibrils, break down the molecular linkages resulting fiber are opened up. The chemical effects are presented due to auto-hydrolysis of acetyl groups in hemicellulose. Breaking off of these acetyl groups into acetic acid as well as the acidic nature of water at high temperatures will promote further hydrolysis of the hemicellulose.

Key factors for the steam explosion process are reaction time, temperature, particle size and moisture. The combined effect of both temperature (T) and reaction time (t) is described by the severity index (R_0). High severity promotes an increased removal of hemicellulose from the solid fraction. Hemicelluloses are thermo-chemical sensitive that are partially hydrolyzed and solubilized into the liquid phase during pretreatment. A linear correlation between the solubilization of hemicellulose and pretreatment severity has been found by (Oliveira et al., 2013). Hemicellulose solubilization was increased from 65% to 90% as the severity increased from 3.6 to 4.1 (Oliveira et al., 2013).

Not only this process is relatively low energy intensive but also is environmental friendly due to no chemical addition (Jacquet et al., 2015; Singh et al., 2015). It is among the most efficient pretreatments to enhance enzymatic hydrolysis and anaerobic digestion of woody feedstock (Cotana et al., 2015; Li et al., 2016; Vivekanand et al., 2013). Removal of hemicellulose by SE is so effective that it increases exposure of cellulose surface to enzymes within the loosen cellulose microfibrils (Imman et al., 2014).

2.4 Enzymatic hydrolysis

Enzymatic hydrolysis is a promising technology to convert cellulose into sugar. It is carried out at mild conditions. For example, cellulase has been reported working at pH 4.8-5.0 and temperature 45–50 °C that is desirable for biochemical process such as anaerobic digestion in downstream process. Various enzymes have been used for fractioning lignocellulose with specific region in lignocellulose. Laccase is capable of selectively degrading lignin without cellulose intact (Qiu & Chen, 2012) while cellulases have been used for converting cellulose into glucose (Qiu & Chen, 2012; Sun & Cheng, 2002). The cellulases involved in the hydrolysis are divided into three major groups (1) endoglucanase (EG, endo-1,4-D-glucanohydrolase, or EC 3.2.1.4.), attacks regions of low crystallinity in the cellulose, creating free chain-ends; (2) exoglucanase or cellobiohydrolase (CBH, 1,4-b-D-glucan cellobiohydrolase, or EC 3.2.1.91.), removing cellobiose units from the free chain-ends; and (3) ß-glucosidase (EC 3.2.1.21), hydrolyzes cellobiose for glucose production (Sun & Cheng, 2002). The products of the hydrolysis are usually reducing sugars including glucose which is intermediate product for biofuel and value-added product production.

Bacteria and fungi are able to produce cellulases for enzymatic hydrolysis of lignocellulose with flexible condition, aerobic or anaerobic, mesophilic or thermophilic. Bacteria, *Cellulomonas fimi* and *Thermomonospora fusca* have been extensively studied for cellulase production. *Clostridium thermocellum* and *Bacteroides cellulosolvens* are cellulolytic anaerobes, available in digester which could produce cellulases with high specific activity. However, the anaerobes have a very low growth rate, therefore, most research for commercial cellulase production has focused on fungi. Fungal pretreatment has been previously studied for enzymatic hydrolysis of lignocellulosic biomass to produce biofuel. White, soft rots and brown fungi have been used to degrade lignin and hemicellulose. White and soft rots attack cellulose and lignin, while brown rots mainly disrupt cellulose.

Several commercial enzymes are available in market for conversion of glucan into sugar as shown in Table 2-2.

Table 2-2 Available hydrolysis enzyme in market

Enzyme	Company	Substrate	References
Celluclast 1.5 L and Novozyme 188	SigmaeAldrich (St. Louis, MO)	Wood chip	(Fougere et al., 2015)
CTec2 (cellulase enzyme mixture)	Novozymes (Bagsvaerd, Denmark)	Wood chip	(Fougere et al., 2015)
Cellulase	Challenge Bioproducts Co., Ltd. (Touliu, Taiwan)	News paper	(Wu et al., 2014)
Celluclast®	Novozymes	Corn strove	(Diaz et al., 2015)
Novoayme 188	Novozymes (Bagsvaerd, Denmark)	Sugarcane straw	(Oliveira et al., 2013)
Cellubrix (mixture of cellulase and ß-glocosidase)	Novo Nordisk Ferment AL, Dittingen, Swizerland	Olives stones	(Fernández-Bolaños et al., 2001)

Several factors including substrate, cellulase dosage and reaction conditions have been considered affect the efficiency of yield and rate of enzymatic hydrolysis (Sun & Cheng, 2002). In order to improve the capability of enzymatic hydrolysis, these factors should be optimized for a specific substrate.

2.5 Anaerobic digestion

Anaerobic digestion process is a complex-biochemical process in which biomass is metabolized in an environment with free of dissolved oxygen. The transformation of complex macromolecules in biomass into end products, methane and carbon dioxide, is accomplished through four individual phases by different groups of microorganisms.

Hydrolysis stage is first step in anaerobic digestion process. Complex organic matters such as proteins, carbohydrates and lipids are hydrolyzed into simple soluble products such as amino acids, sugars and long-chain fatty acids, by the action of extracellular enzymes produced by the fermentative bacteria. There are facultative and obligatorily anaerobic bacteria (Deublein & Steinhauser, 2008). Hydrolysis can be a rate-limiting step in the anaerobic

digestion processes for lignocellulosic biomass (Khanal, 2008). Lignocellulosic biomass and lignin are degraded only slowly and incompletely. Rubber wood waste has a complex and rigid structure, consisting of cellulose, hemicellulose and lignin. Lignin is the most unmanageable component to biodegradation and hindering cellulose biodegradability. In addition, hydrolysis may be constrained by high cellulose crystallization, resulting in low biogas production. Hence, pretreatment process are considered to overcome this constrain and enhance hydrolysis by rendering the substrate matrix more accessible to enzyme attack through various activities in order to improve anaerobic digestibility.

Acidogenesis stage involves a group of fermentative microorganisms converting the simple soluble products to a mixture of intermediary products such as volatile fatty acids, alcohols, hydrogen, carbon dioxide and other simpler organic compounds. Volatile fatty acids (VFAs) (Carbon > 2), such as propionic, butyric acid, etc. are produced in this step. Consequently, these VFAs along with ethanol are converted to acetic acid, hydrogen and carbon dioxide by hydrogen-producing acetogenic bacteria**.** This process is called **acetogenesis**.

Methanogenesis stage, two groups of microorganisms are able to produce methane, the acetoclastic methanogens can convert acetic acid into methane and carbon dioxide while the hydrogen utilizing methanogens consume hydrogen and carbon dioxide.

Biogas is a byproduct of anaerobic digestion of organic matter. It consists of methane, carbon dioxide and other trace gases. The most advantage of anaerobic digestion is the production of a methane rich gas, 40-60% containing in biogas, which can be used to produce energy. Biogas has been acceptable to be renewable energy and widely used for heat and electricity production.

Chapter 3

Methodology

3.1 Preparation of substrate

Rubber wood waste (RW) was collected from a production process of a particle board factory, Panel Plus Co., Ltd. in Songkhla province, Thailand. It was firstly air-dried for one week and sieved. RW of size 2.4-4.7 mm was chosen for this study. It was further dried in a hot air oven at 60 °C until constant weight and stored in plastic bag at room temperature. Characteristics of the RW are shown in Table 3-1.

3.2 Steam explosion pretreatment

Steam explosion (SE) of RW was carried out at the Kasetsart University, Bangkok, Thailand. SE reactor Model FD-97137, CHINO corporation, Japan was used throughout this study. A 200-g RW on dry basis was added into the 2-L pressure vessel connected to a reception chamber and a steam generator. High-pressure steam provided by the electric steam generator was fed into the reactor until the pressure reached the designated values and stayed at such condition for different retention times. The biomass was rapidly exploded by a sudden pressure release by opening the valve, through which it passed into the reception chamber. The samples were steamed at different temperatures from 191 to 214 °C during 1 to 10 min in triplicate. The severity factor which is used to represent the combined effect of reaction temperature and retention time was calculated according to Eq. 3-1 (Iroba et al., 2014; Overend et al., 1987). Severity conditions used in this study were 2.70, 2.37, 2.73, 4.03 and 4.35.

$$logR_o = logt\ exp\left[\frac{(T-100)}{14.75}\right] \quad (3\text{-}1)$$

where $logR_o$ is severity factor; t is retention time (min); T is reaction temperature (ºC).

After SE, the samples were washed with 800 mL hot water (70°C) for 10 min in order to remove the degradation products accumulated on the RW surface. The washing liquid was immediately separated from the steam exploded RW (SE-RW). The washing liquid was analyzed for degradation products including glucose, xylose, hydroxylmethylfurfural (HMF), furfural and acetic acid. SE-RW samples were stored at 4 °C before being dried in a hot air oven at 60 °C until constant weight, then kept in plastic bag at room temperature until use. The solid recovery of different pretreatments was determined based on Eq. 3-2.

$$\text{Solid recovery}(\%) = \frac{\text{mass of pretreated RW (g)}}{\text{mass of initial RW (g)}} \times 100\% \quad (3\text{-}2)$$

3.3 Enzymatic hydrolysis assay

Un-RW (untreated RW) and SE-RW were used as substrate for enzymatic hydrolysis (EH) optimization with enzyme loading of 3, 6, 12, 18, 24 and 30 FPU/g. The assays were performed in triplicate using 8 % (w/v) of substrates in 50-mL reaction vessels. The tests were put into 0.05 M, pH 4.8 citric acid monohydrate buffer solution and 0.8 % (v/v) of sodium azide (20 mg/mL). Commercial enzyme Cellic®CTec2 (Novozyme) with filter paper activity of 150 FPU/mL was used in this experiment. Its activity was measured according to Adney and Baker (2008). All tests were incubated at 50 °C for 72 h in an incubator shaker operated at 150 rpm. The mix liquors were filtered by vacuum system to separate the enzymatic residue (EH residue) and enzymatic hydrolyzate. The hydrolysis yields as glucose, cellobiose and xylose were analyzed using high performance liquid chromatography (HPLC). All experiments were carried out in triplicate and average results were reported. Glucose yield, glucan conversion (summation of glucose and cellobiose) and xylan conversion were determined relative to their theoretical yields according to Eq. 3-3, 3-4 and 3-5, respectively (Pengilly et al., 2016).

$$\text{Glucose yield (\%)} = \frac{\text{Glucose (g)}}{f \times (\text{pretreated RW}) \times 1.111} \times 100 \quad (3\text{-}3)$$

$$\text{Glucan conversion (\%)} = \frac{\text{Glucose (g)} + (1.053 * \text{Cellobiose (g)})}{f \times (\text{pretreated RW}) \times 1.111} \times 100 \quad (3\text{-}4)$$

$$\text{Xylan conversion (\%)} = \frac{\text{Xylose (g)}}{f \times (\text{pretreated RW}) \times 1.136} \times 100 \quad (3\text{-}5)$$

where glucose/cellobiose/xylose are the amount of glucose/cellobiose/xylose in the enzymatic hydrolysate (g); 1.053 is a conversion factor for cellobiose to equivalent glucose; 1.111 is a conversion factor for cellulose to equivalent glucose; 1.136 is a conversion factor for xylan to equivalent xylose; *f* is the glucan/xylan fraction by dry mass; pretreated RW is the amount of substrate loading on dry basis (g).

3.4 Anaerobic digestibility test

Anaerobic digestibility tests of Un-RW, SE-RW and the residue from enzymatic hydrolysis of stream exploded RW (SE+EH-RW) were carried out in triplicate in sealed batch reaction vessels. The method used was described by Dechrugsa et al. (2013). Briefly, the total reaction volume was 60 mL containing 0.5 g of substrate. The ratio of inoculum to substrate was 3 on dry mass basis. The inoculum was a mesophilic sludge taken from a full scale anaerobic digester treating wastewater from a concentrated rubber latex factory in Songkhla province, Thailand. Prior to the experiments the inoculum was incubated at room temperature (28-30°C) for 4 weeks to reduce its endogenous biogas production. The reaction vessels were added with 10 % (v/v) of buffer solution (50 g/L $NaHCO_3$), 1 % (v/v) of trace elements and nutrient solution (Rincón et al., 2010). The final volume was made up with distilled water to 60 mL and small quantity of 10 % HCL to adjust pH to 7.0±0.2. The headspace of reaction

vessels was purged for 2 min with pure nitrogen gas and sealed immediately. The tests were incubated at 35 ± 0.5 °C and shaken at 150 rpm for 28 days. The blanks containing the same amount of inoculum without substrate were conducted. Daily biogas generation was measured using glass syringe and its composition was determined by gas chromatography. Methane yield was calculated based on volatile solid of the pretreated RW according to Eq. 3-6.

$$CH_4\ yield\ \left(\frac{L}{kg-VS}\right) = \frac{Vol.\ of\ CH_4\ substrate - Vol.of\ CH_4\ blank}{VS_{substrate}} \quad (3\text{-}6)$$

where Vol. of CH_4 substrate and blank is the amount of methane (L) releasing from reaction vessels and $VS_{substrate}$ is the amount of substrate loading on VS basis (kg).

The biodegradability index (BI) is defined as the ratio of methane yield to theoretical yield based on holocellulose content (cellulose plus hemicellulose). This value describes the biodegradability efficiency of the feedstock in the batch digestion. The determination of BI value was conducted according to Eq. 3-7.

$$Biodegradability\ index\ (\%) = \frac{Methane\ yield}{Theoretical\ methane\ yield} \times 100 \quad (3\text{-}7)$$

Theoretical methane yield was calculated using the Buswell's equation (Buswell & Mueller, 1952). The calculated methane yield of cellulose as glucan ($C_6H_{10}O_5$) is 415 $mLCH_4$/g cellulose and of hemicellulose as xylan ($C_5H_8O_4$) is 424 $mLCH_4$/g hemicellulose at STP on dry basis, respectively.

3.5 Analytical methods for aqueous and gaseous samples

Total solid, volatile solid and ash contents were characterized using gravimetric method following National Renewable Energy Laboratory's (NREL) (Sluiter et al., 2008a). The polysaccharide and lignin of RW were analyzed according to Sluiter et al. (2008b) and Goering and Van Soest (1970), respectively.

The concentration of sugar species and by-products (furfural, HMF, and acetic acid) in the washing liquid was measured with Water e2695 HPLC, equipped with a refractive index detector (RID) using 300 mm × 7.8 mm Aminex HPX-87H Ion Exclusion Column (Bio-Rad, USA) operating at 40 °C with 0.005 M H_2SO_4 as mobile phase at a flow rate of 0.6 mL/min. The glucose in the enzymatic hydrolyzate and sugar species in carbohydrate content of RW were measured using HPLC (Agilent 1100) equipped with a refractive index detector (RID) using 300 mm × 7.8 mm Aminex HPX-87H Ion Exclusion Column (Bio-Rad, USA) at 55 °C with 0.4 mL/min eluent of 0.005 M H_2SO_4.

Biogas composition from anaerobic digestibility test was analyzed by gas chromatography (GC 7820A Agilent Technologies) equipped with a thermal conductivity detector (TCD) and a stainless steel packed column SS Hayesep Q80/100 (6 m x 1/8 in.) using helium as carrier gas.

3.6 Analytical methods for physical properties of solid samples

Microstructure of Un-RW and SE-RW were analyzed by scanning electron microscopy (SEM), model Quanta 400, FEI, Czech Republic. The total surface area and average adsorption pore diameter of the samples were determined according to the method of Brunauer, Emmett, and Teller (BET) using the surface area and porosity analyzer, ASAP2460, Micromeritics, USA equipped with a thermal conductivity detector. Fourier Transform Infrared Spectrometer, Vertex 70, Bruker, Germany with Pellet KBr technique was used to identify functional groups

of the samples. Crystallinity of the sample was measured by X-ray diffractometer (XRD), model X'Pert MPD, PHILIP: XRD (The Netherland) using CuKα (λ = 1.54 A°) generated at 40 kV, 30 mA. The samples were scanned from 5 to 90 degree 2θ in a step size of 0.05 degree. Crystallinity was determined according to Eq. 3-8 (Segal et al., 1959).

$$\text{CrI}\ (\%) = \left[\frac{I_{002} - I_{am}}{I_{002}}\right] \times 100 \qquad (3\text{-}8)$$

where CrI is the crystallinity (%), I_{002} represents the maximum intensity for the crystalline portion of biomass at 2θ around 22.4°, and I_{am} is the peak for the amorphous portion at 2θ around 18.4°.

3.7 Statistical analysis

The Minitab statistical software 16 was used for statistical analysis including analysis of variance *(*ANOVA*)*. Multiple means were compared with one-way ANOVA followed by Fisher's Least Significant Difference method for post hoc–comparison. The level of significance was set at $p < 0.05$.

Chapter 4

Results and Discussion

4.1 Chemical composition of exploded RW

Rubber wood waste was consists of 44.87% glucan (cellulose), 14.51% xylan (hemicellulose) and 13.12% lignin (Table 4-1). With its high polysaccharide content as holocellulose of 59.38% and lower lignin content made RW a potential feedstock for various bioenergy conversions. SE pretreatment clearly improved degradation of xylan by 3.50% while glucan and lignin content increased by 0.80 and 1.34 %, respectively at severity of 3.37 compared to Un-RW (Table 4-1). One advantage of SE pretreatment of RW is that glucan as a main fraction of bioenergy feedstock was preserved whereas degradation of xylan could improve active area for accessible enzyme (Table 4-2) at severity of 4.03 and 4.35. Reduction of xylan content was caused by the degradation of hemicellulose which took place at temperature above 150 °C (Li et al., 2016) as our SE pretreatment was performed between 191 and 214 °C. Compared to glucan, the amorphous short chain structure of hemicellulose is easier degrade because of its lack of crystallinity (Li & Khanal, 2017) as indicated by the higher xylose concentration in the washing liquid (Table 4-3). Without external chemical addition, hydrolysability of biomass during pretreatment could be accelerated by auto-hydrolysis reaction that occurred as a result of acetic acid formation from the degradation of acetyl groups in hemicellulose. With an increasing severity to 4.03 and 4.35, the large amount of acetic acid (Table 4-3) released acted as a catalyst. This acid catalyzed the hydrolytic reaction of hemicellulose into monomeric sugar (Imman et al., 2015) and subsequently degraded to furfural (Chen et al., 2014) resulted in a dramatic reduction of xylan content in pretreated RW to 4.23 and 3.51%, respectively. SE pretreatment could thus contribute to the combined effects of thermal and acetic acid-catalytic reactions to disrupt RW amorphous structure in

particularly. The efficiency of SE pretreatment will be further discussed in the enzymatic and anaerobic digestibility assays performed.

Table 4-1 Chemical compositions of untreated and steam exploded RW

Steam explosion conditions		Solid recovery (%)[a]	TS (%)[b]	VS (%)[a]	Glucan (%)[a]	Xylan (%)[a]	Lignin (%)[a]
Pressure/RT	Severity						
Un-RW	-	100±0	95.2±0.55	98.2±0.20	44.87±1.58	14.51±0.20	13.12±0.09
12 bar-1 min	2.70	99.1±0.45	97.4±0.15	98.7±0.06	43.72±4.59	11.28±0.71	12.75±0.14
12 bar-5 min	3.37	97.9±0.10	95.3±0.14	98.9±0.08	45.67±7.89	11.01±1.58	14.46±0.28
16 bar-5 min	3.73	93.2±0.65	97.8±1.03	99.0±0.07	47.77±0.79	9.29±0.53	16.86±0.13
16 bar-10 min	4.03	89.6±0.40	96.3±0.09	98.7±0.08	49.48±0.39	4.23±0.20	16.39±0.19
20 bar-10 min	4.35	82.2±0.57	96.9±0.02	98.4±0.15	51.84±0.54	3.51±0.56	17.30±0.10

[a] Mass percentage

[b] Solid content-as wet weight

Table 4-2 Structural analysis of Un-RW and SE-RW

Steam explosion severity	Crystallinity (%)	Surface area (m^2/g)	Adsorption average pore diameter (A°)
Un-RW	69.6	0.5617	115.93
4.03	77.2	0.6646	121.05
4.35	84.0	0.9795	143.47

Table 4-3 Yields of soluble sugar and degradation products of washing liquid

Steam explosion severity	Concentration (g/L)				
	Glucose	Xylose	Acetic acid	Furfural	HMF
2.70	0.12 (0.04-0.19)	0.26 (0.13-0.46)	0.54 (0.50-0.57)	n.d.	n.d.
3.37	0.14 (0.04-0.20)	0.44 (0.33-0.52)	0.97 (0.83-1.09)	n.d.	n.d.
3.73	0.36 (0.31-0.40)	1.01 (0.96-1.06)	2.05 (1.74-2.29)	n.d.	n.d.
4.03	0.82 (0.57-1.11)	2.43 (1.87-3.02)	4.75 (4.42-5.08)	0.95 (0.80-1.06)	0.51 (0.37-0.63)
4.35	1.50 (1.29-1.63)	2.72 (2.38-2.92)	7.83 (7.69-8.12)	3.40 (3.15-3.73)	2.48 (2.44-2.57)

Note n.d.= not detected, the value in the parenthesis represents the range of data.

4.2 Chemical composition of washing liquid from exploded RW

The water-soluble products accumulated on surface of pretreated RW were removed by deionized water. The composition of this washing liquid which makes up of monomeric sugars and degradation products is shown in Table 4-3. The pretreatment intensity had a strong influence on the formation of soluble products particularly furfural and HMF which were initially derived from xylose and glucose, respectively, at high severity of 4.03. These dehydration products are considered damaging to the cellular membrane and inhibitive to metabolism of microorganisms in anaerobic digestion. Both furfural and HMF completely inhibited the specific methanogenic activity at 2.0 g/L (Ghasimi et al., 2016). The inhibitory levels of furfural and HMF (0.12 and 0.02 g/L, respectively) have been reported for reducing glucan conversion in enzymatic hydrolysis (Pengilly et al., 2016). Thus, washing of SE-RW was necessary to eliminate inhibitory effects and helped preserve the degree of enzymatic hydrolysability and anaerobic digestibility of the pretreated RW solid.

4.3 Methane production of steam exploded RW

Methane production of 10.9 L CH_4/kg-VS was obtained from Un-RW solid fraction after 28-day anaerobic digestion assay (Figure 4-1). According to methane generation, SE pretreatment could be divided into 3 groups; low (2.70-3.37), medium (3.73-4.03) and high (4.35) severities. Compared to Un-RW, methane production was significantly enhanced (*p*-value <0.05) with an increasing SE severity from medium to high.

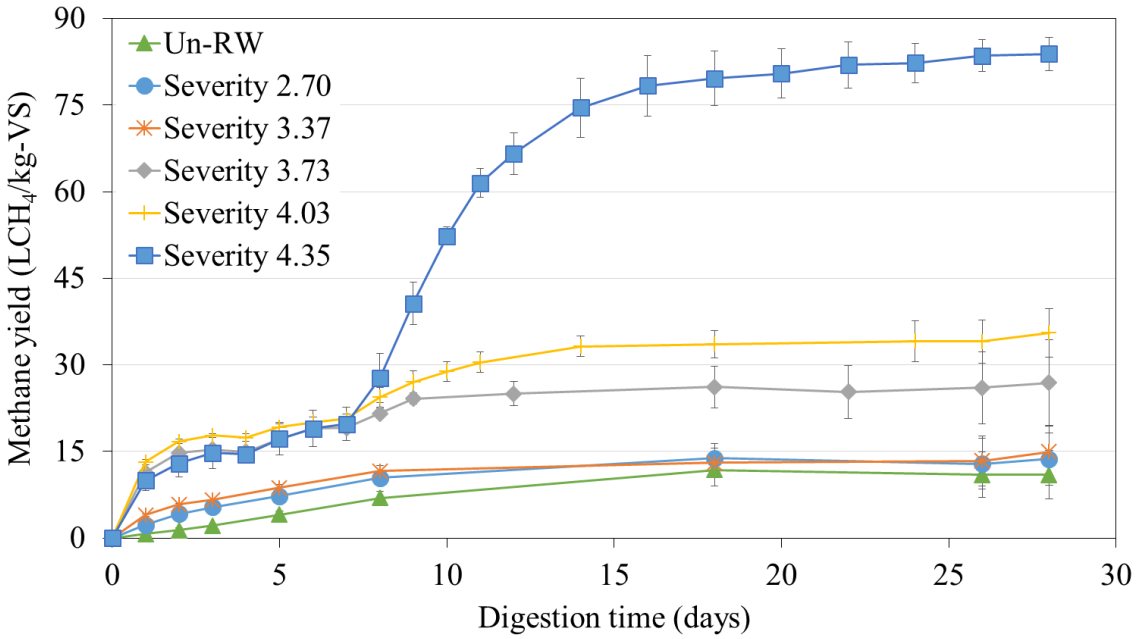
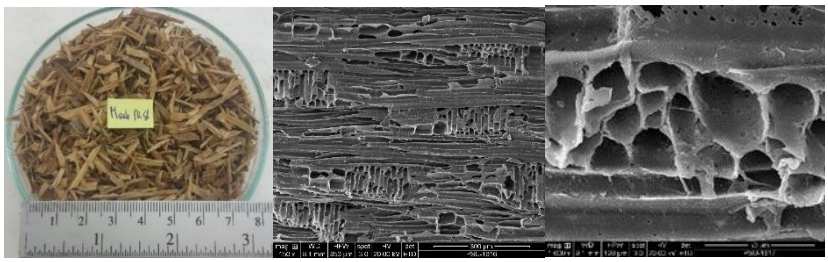


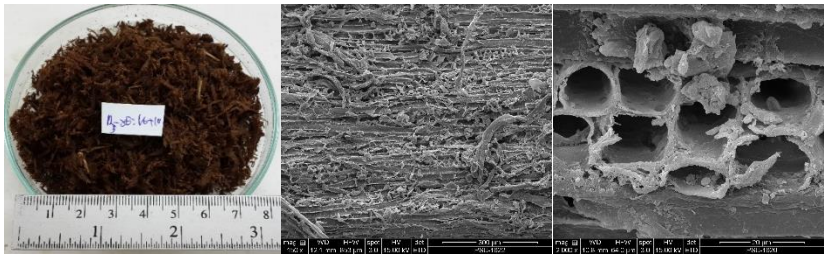
Figure 4-1 Cumulative methane yield in anaerobic digestion assays of Un-RW and SE-RW with difference degree of severities

Methane yield reached maximum value of 83.9 L CH_4/kg-VS at severity 4.35, interestingly with a 2-step pattern. During the first seven days of digestion, SE-RW was gradually degraded, as a result, the methane production increased slowly up to 19.8 LCH_4/kg-VS. It rapidly evolved during days 7 to 13 of digestion by 3.8 folds (from 19.8 to 74.5 LCH_4/kg-VS). The sharp increase in methane yield might be a result of the weakening biomass cell wall to the point that was sufficient for anaerobic digestion to destroy its structure as observed by SEM images (Figure 4-2c). The SE pretreatment at severity 4.35 showed intensive physical defects that the sudden thermal explosion had deconstructed the RW cell, also evidenced by the drastic shift to smaller size distribution compared to SE-$RW_{4.03}$ and Un-RW (Figure 4-2 b and a, respectively).

(a) Un-RW



(b) SE-RW at severity of 4.03



(c) SE-RW at severity of 4.35

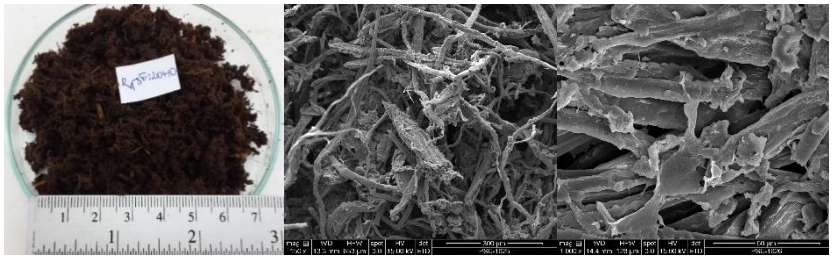


Figure 4-2 SEM photos of the morphological surface of (a) Un-RW and SE-RW at severity of 4.03 (b) and 4.35 (c), at magnification of 150X and 1000X

In comparison to Un-RW, an intensive auto-catalytic reaction with acetic acid generated as high as 31.31 g/kg-RW took place as a result of changes in functional group in structure of SE-RW. Figure 3 shows similar behavior by FTIR spectra but different transmission intensities between Un-RW and SE-$RW_{4.35}$ particularly the wavenumber around 1734 cm^{-1} which corresponds to the C=O functional group. This carbonyl group is a characteristic peak of ester-linked between hemicelluloses and lignin (Phitsuwan et al., 2015; Siddhu et al., 2016). Reduction of this peak intensity indicated that SE pretreatment was

effective to improve anaerobic digestibility of SE-RW by breaking down ester bond in hemicelluloses.

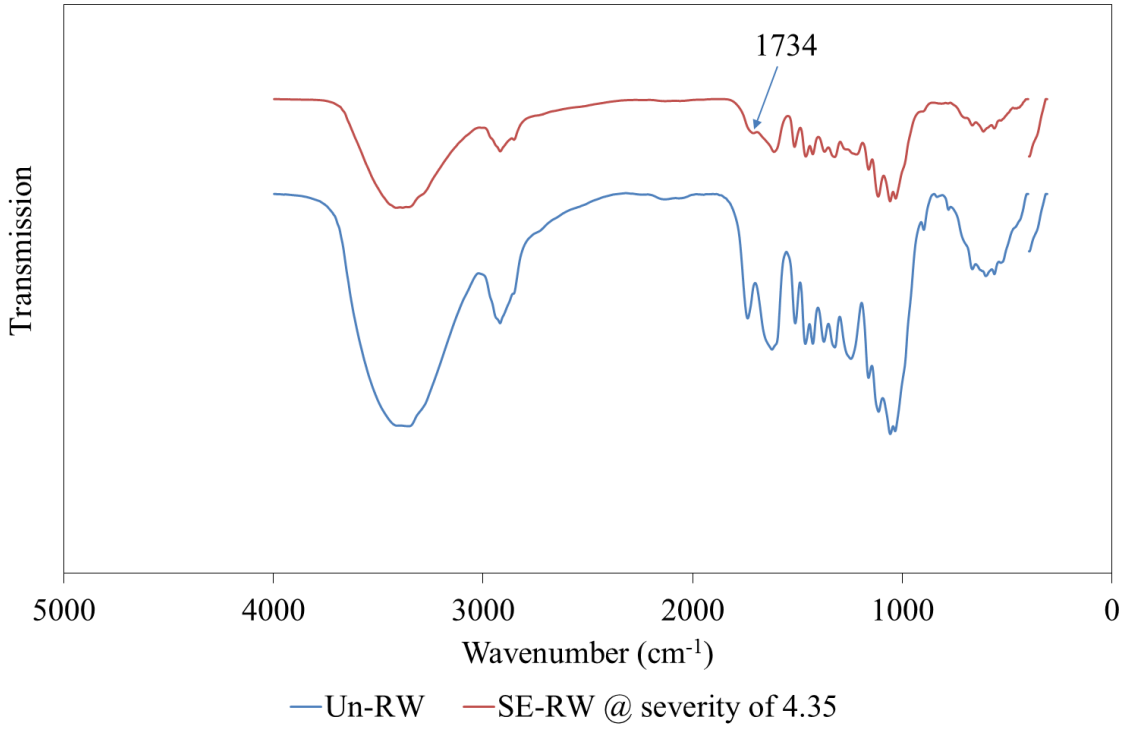


Figure 4-3 FTIR spectra of Un-RW and SE-RW at severity 4.35

4.4 Enzymatic hydrolysis of SE-RW

In addition to the direct anaerobic digestion of the SE-RW, the other objective is to evaluate the enzymatic hydrolysibility of the SE pretreated RW. In this study, cellulase Cellic®CTec2 formulated to target cellulose hydrolysis with aggressive β-glucosidase activity was used. Without SE pretreatment, hydrolysability of Un-RW generated glucose yield in range of 2.91±0.11 to 7.37±0.24% with an enzyme loading from 3 to 30 FPU/g. In contrast, the glucose yield of SE-RW was successfully improved with both increasing severity of SE pretreatment and enzyme loading which was shown in Figure 4-4 (a). Moreover, cellobiose, a disaccharide, was also one of enzymatic hydrolysis yields which was derived from glucan. In order to estimate glucan conversion in enzymatic hydrolysis step, summation of glucose and cellubiose yields was determined as shown in Figure 4 (b). The glucan conversion (GC) showed

similar behavior to glucose yield. The highest glucan conversion of 45.20±1.59 was achieved at severity 4.35 and enzyme loading 30 FPU/g. The hydrolysability behaviors of the cellulose in RW correlated with intensity of severity applied. The glucan conversion at different enzyme loadings fell closely on the lower and medium severities whereas ones with high severity fell widely. This hydrolysability at high severity of 4.35 could be attributed to large values of both active area and adsorption pore average diameter of SE-RW (Table 2), which associated with the reduction in hemicellulose providing improved accessibility to cellulose in SE-RW. Additionally, an increased CrI value (Table 2) indicates the exposure of the reactive cellulose portion in the pretreated biomass to enzyme adsorption (Phitsuwan et al., 2016). The active area and chemical changes in SE-RW structure in turn enhance the enzymatic hydrolysis of cellulose with addition of enzyme loading.

Cellic®CTec2 contains not only cellulase activities but also an endo-xylanase activities which has been proven to promote xylan degradation on Triticale straw (Pengilly et al., 2016). Figure 4-4 c shows the enzymatic hydrolysis profiles of xylan conversion. The efficiency of xylan conversion obtained was higher than glucan conversion. SE-RW contained a relatively low initial xylan content with an increasing severity (Table 4-1), and it is possible that the xylan fraction was present more in hydrolysable forms. Highest xylan conversion was achieved at severity of 4.03 while lower value was found with an increasing severity to 4.35. This could be that there was competition for adsorbing cellulose by cellulases and xylaneses since much reduction of hemicellulose led to the increase in pore size diameter (Table 4-2) of the cell walls and unwinding of the cellulose bundles (Figure 4-2). This phenomenon could certainly improve accessibility to cellulose of pretreated RW.

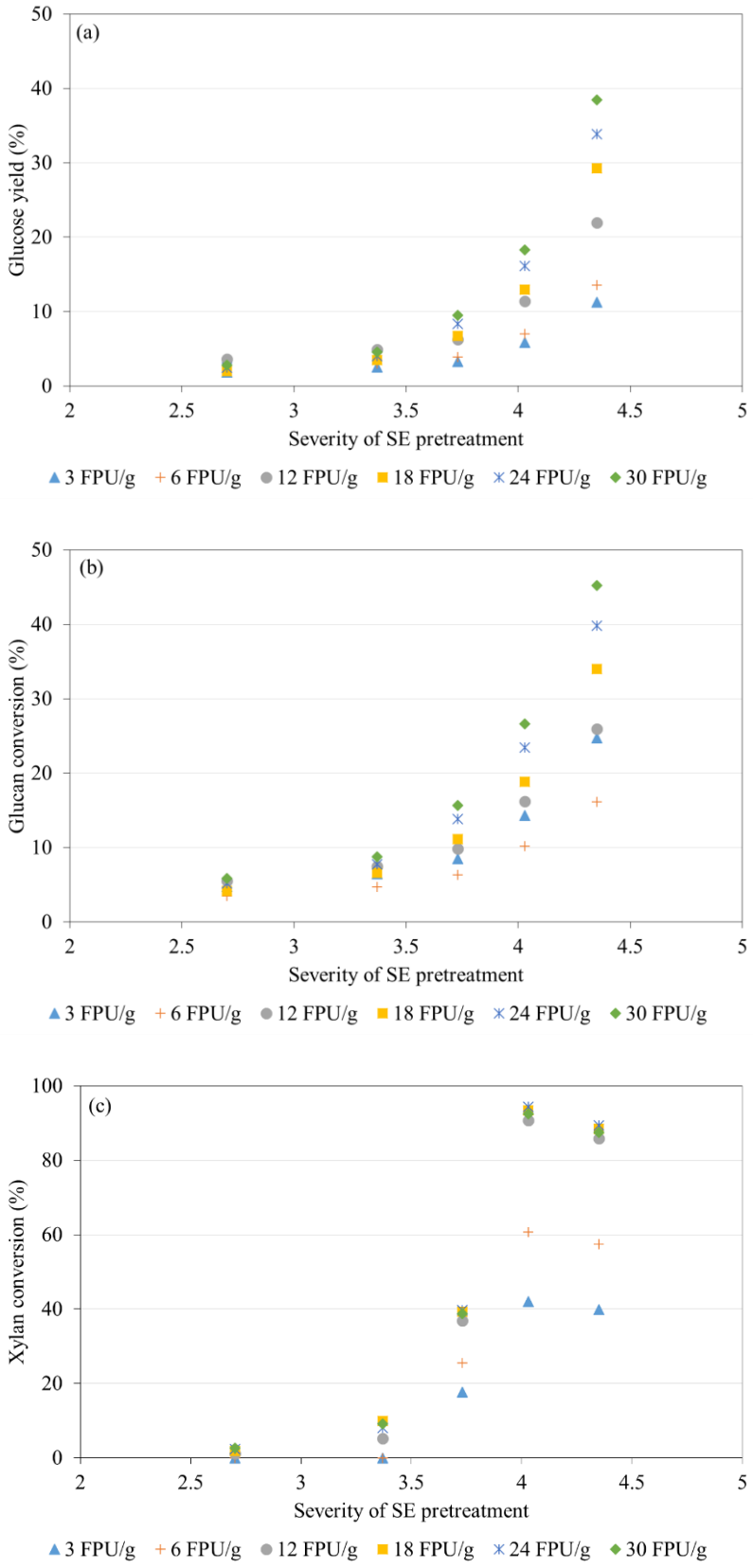


Figure 4-4 Enzyematic hydrolysis yields of SE-RW (a) glucose yield, (b) glucan conversion and (c) xylan yield

The influence of SE severity and enzyme loading (EL) of enzymatic hydrolysis on glucose yield, glucan conversion, and xylan conversion was further analyzed by fitting the data (Figure 4-4) to quadratic model using MINITAB 16 software. The model coefficients are presented in Table 4-4. The *p*-value for all three models: (1) glucose yield, (2) glucan conversion and (3) xylan conversion, indicated that these models were statistically significant (*p*-value <0.05). According to the coefficients, both severity and enzyme loading were influential factor for production of all enzymatic hydrolysis yields. Furthermore, the significant quadratic interaction of severity showed that all enzymatic hydrolysis yields could reach a maximum value with severity adjustment. Reduction of solid recovery after SE pretreatment with an intensive severity applied should be taken in consideration for product recovery per mass of native feedstock.

Table 4-4 Analysis of variance (ANOVA) of the quadratic model for the enzymatic hydrolysis yield of SE-RW

Terms	Glucose yield (%)		Glucan conversion (%)		Xylan conversion (%)	
	Coefficient estimate	P-value	Coefficient estimate	P-value	Coefficient estimate	P-value
Model		0.000		0.000		0.000
b_0	159.807		160.884		194.245	
b_1*EL	-1.525	0.000	-1.758	0.000	-0.522	0.0136
b_2*Severity	-95.491	0.000	-96.213	0.000	-147.681	0.000
b_3*EL*Severity	0.567	0.000	0.529	0.000	1.0717	0.0416
b_4*EL^2	-0.00530	**0.420**	0.00707	**0.2991**	0.0753	**0.057**
b_5*Severity^2	14.080	0.000	14.650	0.000	26.141	0.0095
R^2		0.9433		0.9532		0.8609
adjusted R^2		0.9315		0.9434		0.8320
Predicted R^2		0.8945		0.9182		0.7814
CV (%)		25.80		18.22		38.32

Note: Significant under 95% level of confidence

Moreover, insignificant quadratic function of enzyme loading means that applying intensive enzyme loading could not significantly enhance hydrolysability of both glucan and xylan in SE-RW. It might be due to inhibitory effects of cellubiose to enzymatic hydrolysis

which is in agreement with (Olsen et al., 2016). Our study cellubiose formation potentially increased with an addition of enzyme loading (data not show).

4.5 Methane production of SE-RW and SE+EH-RW

The effect of enzymatic hydrolysis with enzyme loading between 3 to 30 FPU/g was studied in addition to the previous SE pretreatments at severity of 4.03 due to higher xylan conversion in enzymatic hydrolysis step compared to other SE conditions.

In order to estimate chemical composition of $SE\text{-}RW_{4.03}$ which was enzymatically hydrolyzed by Cellic®CTec2 (SE+EH-RW), subtracting glucan and xylan content in SE-RW with glucan and xylan degraded in enzymatic hydrolysis experiment was performed. Figure 4-5 shows polysaccharide composition in SE+EH-RW obtained.

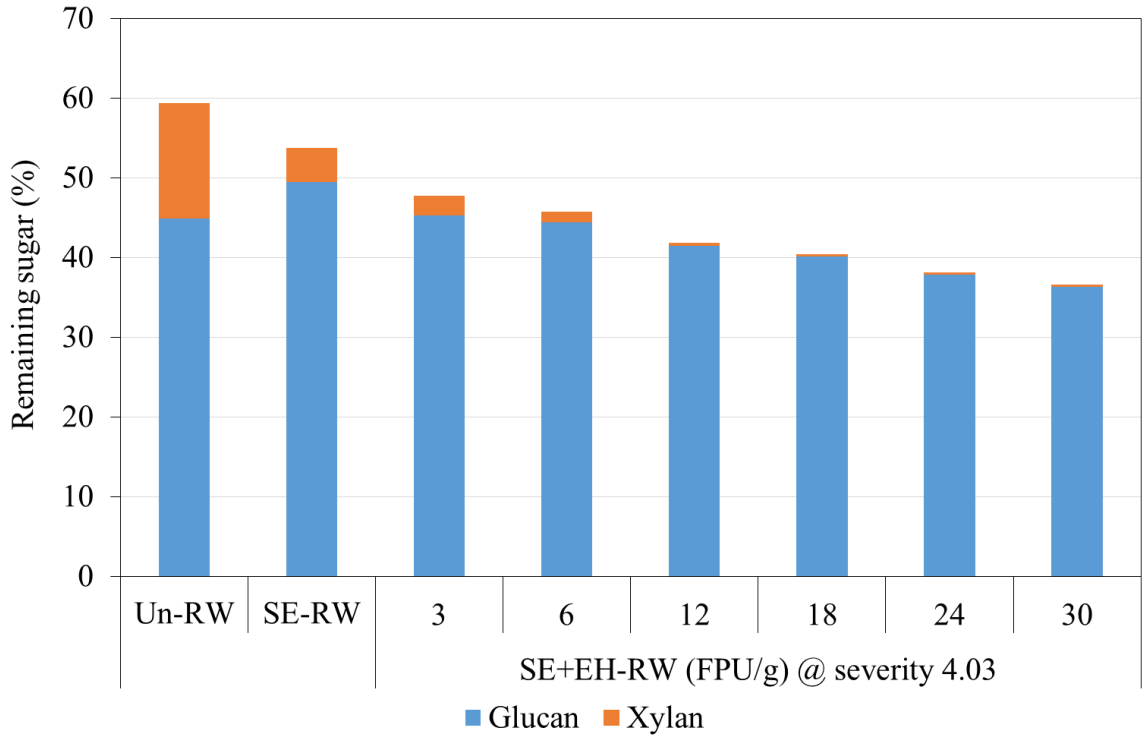


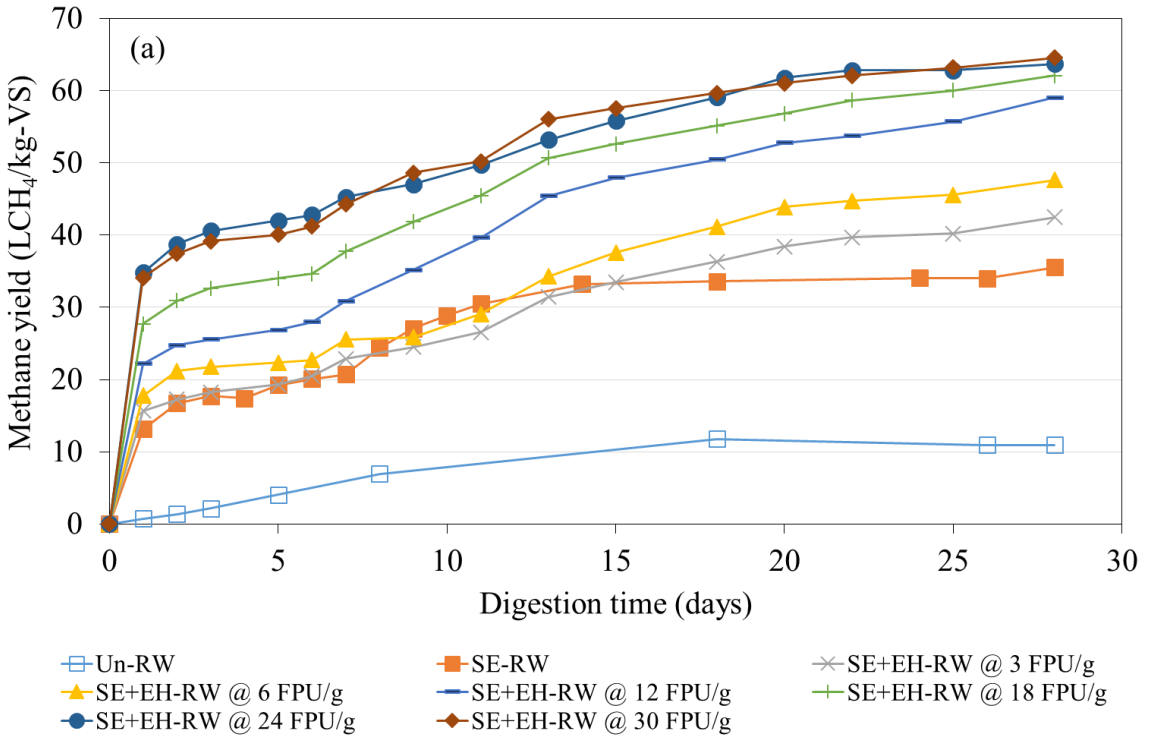
Figure 4-5 Remaining sugar content in Un-RW, SE-RW and SE+EH-RW at enzyme loading between 3 and 30 FPU/g

All SE+EH-RW samples were subjected in to anaerobic digestibility assays. Cumulated methane production profiles are presented in Figure 4-6 (a). In a combination of steam explosion and enzymatic hydrolysis, the initial methane production rate was obviously significantly faster during the first 2 days of digestion. It is noticeable that EH had clear effect on the SE-RW as methane yield increased from 8.61±0.91 to 38.77±1.24 LCH_4/kg-VS/day with an increasing enzyme loading from 3 to 30 FPU/g, while SE pretreatment gave only 5.98±0.13 LCH_4/kg-VS/day. This synergy effects of steam explosion and enzymatic hydrolysis contributed the acceleration of initial anaerobic digestibility to improve initial methane potential yield. Not only the quick start, the overall methane yield of the SE+EH-RW combination accomplished a significantly higher methane than only steam explosion for a 28-day digestion by 81.6% at enzyme loading of 30 FPU/g (Figure 6b).

Biodegradability index (BI) which is the ratio of methane yield to theoretical yield based on holocellulose content would be preferable for revealing degree of anaerobic bioconversion of substrate into methane. The SE+EH-RW with higher enzyme loading was successful in improving the anaerobic degradability of RW. As a consequence, higher BI was obtained (Figure 4-6 b). The highest BI of 41.1% was observed at enzyme loading of 30 FPU/g, while the lower was 15.7% obtained with SE severity 4.03. Polysaccharide fractions as glucan and xyan are considered the main carbon sources for biomethanation. In fact, disruption of biomass in enzymatic hydrolysis by cellulase enzyme caused depolymerization of both glucan and xylan. The cleavage of some β-glucan molecules into glucose could unwind the remaining cellulose bundles. Moreover, higher xylan conversions with an increasing enzyme loading are correlated with increased xylan hydrolysis. Reduction of hemicellulose as xylan (Figure 4-5) led to additional reactive area in the SE+EH-RW micro-structure (Imman et al., 2014) which in turn improves the accessibility of cellulose and remaining xylan for microbial enzyme. The

change in cellulose and hemicellulose properties could be the source of the increased anaerobic digestibility of SE+EH-RW.

Although higher volume of methane from SE+EH-RW was observed, approximately 58.9% of holocellulose (at enzyme loading of 30 FPU/g) left after digestion was not able to convert into methane. Thus, the biomethanation of SE+EH-RW could be justified as the final recovery for biodegradable carbon.



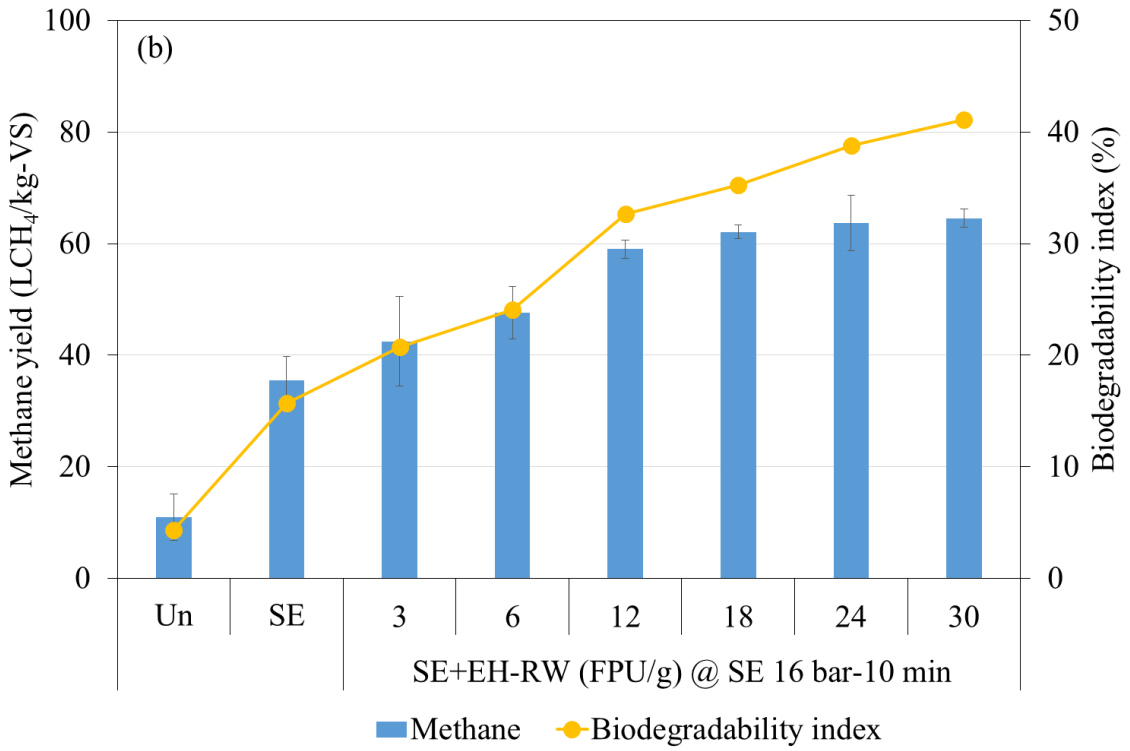


Figure 4-6 Comparison of (a) methane yield profile and (b) methane yield production and biodegradability index, between Un-RW, SE-RW and SE+EH-RW at enzymatic hydrolysis loading of 3 to 30 FPU/g

4.6 Product and energy yields

Our study showed that both efficiency of glucan conversion and biodegradability index could be enhanced by SE pretreatment resulted in improvement of glucose and methane yield, respectively (Table 4-5). Glucose is being a substrate in fermentation process for industrial ethanol production. Thus, in order to evaluate the possible energy recovery of glucose obtained from enzymatic hydrolysis of SE-RW, stoichiometry glucose to theoretical ethanol conversion was applied (Li & Khanal, 2017). The energy production per ton-RW was then evaluated at various options. Energy values of expected ethanol and methane were determined based on the higher heating value of 26.8 and 55.7 MJ/kg, respectively.

Highest total energy of 3833 MJ/ton-RW was obtained from the integrated process including steam explosion, enzymatic hydrolysis, and anaerobic digestion (SEA-process)

(option 4) caused by enlargement of SE+EH-RW biodegradability. This methane contributed 57.9% of total energy potential production. Compared to a single process of SE, SE+EH-RW with SE severity 4.03 corresponds to 45.6% and 42.5% higher energy yield than only from glucose or methane with SE pretreatment at severity of 4.35 (option 2). In thermal pretreatment, energy cost is considered as one of the main factors that influences overall project appraisal and investment. In comparison to SE severity 4.35, lower energy supply to SE pretreatment at severity 4.03 with an integrative SEA-process showed an improvement of overall energy yield from rubber wood waste. Moreover, microbial inhibitors such as furfural and HMF were also minimized at severity 4.03 (Table 3) which could take into consideration for other purposes based on zero-waste strategy.

Table 4-5 Conversion efficiency, product yields and energy yields

Options	Process	Conversion efficiency (%)	Product yields (kg-glucose/ton-RW or m^3CH_4/ton-RW)	Energy yields (MJ/ton-RW)
1. Un-RW	EH	GC = 11.03	16.42	224
	AD	BI = 4.34	7.82	310
2. SE-$RW_{4.35\text{-}30FPU/g}$	EH	GC = 45.20	192.62	2633
	AD	BI = 35.78	67.66	2690
3. SE-$RW_{4.03\text{-}30FPU/g}$	EH	GC = 26.62	118.02	1613
	AD	BI = 15.72	31.44	1250
4. SE-$RW_{4.03\text{-}30FPU/g}$ + SE+EH-$RW_{4.03\text{-}30FPU/g\text{-}AD}$	EH+AD	GC = 26.62	118.02	1613
		BI = 41.10	55.84	2220
				Total = 3833

Note: [a]EH = enzymatic hydrolysis and [b]AD = anaerobic digestion

These results suggest that enzymatic hydrolysis of SE-RW followed by anaerobic digestion is a good alternative for bioenergy production in the forms of glucose and methane. Additionally, higher lignin residue after SEA process, due to no degradation of lignin during enzymatic hydrolysis as well as in anaerobic digestion, are of interest. This lignin-rich and purer solid from residue of the SEA-process has higher heating value and less impurities that can be an excellent fuel, or could be used as material to produce higher value products. Thus,

the valuation of RW thru bioenergy and biomaterial production will increase if co-production of sugar, biogas and lignin-rich biomass are realized.

Chapter 5

Conclusions

Severity of steam explosion played a vital role to improve the production of glucose and methane from rubber wood waste by causing physical and chemical structural changes as well as reduction of hemicellulose xylan contents. Production of glucose thru enzymatic hydrolysis followed by anaerobic digestion of enzymatically hydrolyzed residue of the proposed SEA-process with lower severity of 4.03 of steam explosion pretreatment has great potential to improve energy recovery compared to either glucose or methane production alone even at a higher severity of 4.35. While the SEA-process enhanced technical feasibility of bioenergy production from rubber wood waste, valuated end use of the purified processed rubber wood residue will be key for its economic feasibility which is subject to further research.

References

Adney, B., Baker, J. 2008. Measurement of Cellulase Activities Laboratory Analytical Procedure (LAP) (Technical Report NREL/TP-510-42628 January 2008). National Renewable Energy Laboratory (NREL).

Agbor, V.B., Cicek, N., Sparling, R., Berlin, A., Levin, D.B. 2011. Biomass pretreatment: Fundamentals toward application. *Biotechnology Advances*, **29**(6), 675-685.

Asada, C., Sasaki, C., Uto, Y., Sakafuji, J., Nakamura, Y. 2012. Effect of steam explosion pretreatment with ultra-high temperature and pressure on effective utilization of softwood biomass. *Biochemical Engineering Journal*, **60**, 25-29.

Ballesteros, I., Ballesteros, M., Cara, C., Sáez, F., Castro, E., Manzanares, P., Negro, M.J., Oliva, J.M. 2011. Effect of water extraction on sugars recovery from steam exploded olive tree pruning. *Bioresource Technology*, **102**(11), 6611-6616.

Buswell, A.M., Mueller, H.F. 1952. Mechanism of methane fermentation. *Ind. Eng. Chem.*, **44**(3), 550-552.

Cara, C., Ruiz, E., Ballesteros, I., Negro, M.J., Castro, E. 2006. Enhanced enzymatic hydrolysis of olive tree wood by steam explosion and alkaline peroxide delignification. *Process Biochemistry*, **41**(2), 423-429.

Center of Agricultural Information. 2016. Thailand rubber planting area. Thaniland Office of Agricultural Economics.

Chen, J., Zhang, W., Zhang, H., Zhang, Q., Huang, H. 2014. Screw extrude steam explosion: A promising pretreatment of corn stover to enhance enzymatic hydrolysis. *Bioresource Technology*, **161**(Supplement C), 230-235.

Cotana, F., Buratti, C., Barbanera, M., Lascaro, E. 2015. Optimization of the steam explosion and enzymatic hydrolysis for sugars production from oak woods. *Bioresource Technology*, **198**, 470-477.

Dechrugsa, S., Kantachote, D., Chaiprapat, S. 2013. Effects of inoculum to substrate ratio, substrate mix ratio and inoculum source on batch co-digestion of grass and pig manure. *Bioresource Technology*, **146**, 101-108.

Dererie, D.Y., Trobro, S., Momeni, M.H., Hansson, H., Blomqvist, J., Passoth, V., Schnürer, A., Sandgren, M., Ståhlberg, J. 2011. Improved bio-energy yields via sequential ethanol fermentation and biogas digestion of steam exploded oat straw. *Bioresource Technology*, **102**(6), 4449-4455.

Deublein, D., Steinhauser, A. 2008. *Biogas from waste and renewable resources. An introduction*. Wiley-VCH.

Diaz, A.B., Moretti, M.M., Bezerra-Bussoli, C., Carreira Nunes Cda, C., Blandino, A., da Silva, R., Gomes, E. 2015. Evaluation of microwave-assisted pretreatment of lignocellulosic biomass immersed in alkaline glycerol for fermentable sugars production. *Bioresour Technol*, **185**, 316-23.

Fernández-Bolaños, J., Felizón, B., Heredia, A., Rodríguez, R., Guillén, R., Jiménez, A. 2001. Steam-explosion of olive stones: hemicellulose solubilization and enhancement of enzymatic hydrolysis of cellulose. *Bioresource Technology*, **79**(1), 53-61.

Fisher, T., Hajaligol, M., Waymack, B., Kellogg, D. 2002. Pyrolysis behavior and kinetics of biomass derived materials. *Journal of Analytical and Applied Pyrolysis*, **62**(2), 331-349.

Fougere, D., Clarke, K., Zhao, Y., Li, K. 2015. Chemical–mechanical pretreatment of wood: Reducing downsizing energy and increasing enzymatic digestibility. *Biomass and Bioenergy*, **80**, 17-29.

Ghasimi, D.S.M., Aboudi, K., de Kreuk, M., Zandvoort, M.H., van Lier, J.B. 2016. Impact of lignocellulosic-waste intermediates on hydrolysis and methanogenesis under thermophilic and mesophilic conditions. *Chemical Engineering Journal*, **295**, 181-191.

Goering, H., Van Soest, P.J. 1970. Forage Fiber Analysis (Apparatus, Reagents, Procedures and Some Application), in: Agricultural Handbook. Agricutural Research Service, U.S. Department of Agriculture.

Imman, S., Arnthong, J., Burapatana, V., Champreda, V., Laosiripojana, N. 2014. Effects of acid and alkali promoters on compressed liquid hot water pretreatment of rice straw. *Bioresource Technology*, **171**, 29-36.

Imman, S., Arnthong, J., Burapatana, V., Champreda, V., Laosiripojana, N. 2015. Influence of alkaline catalyst addition on compressed liquid hot water pretreatment of rice straw. *Chemical Engineering Journal*, **278**, 85-91.

Iroba, K.L., Tabil, L.G., Sokhansanj, S., Dumonceaux, T. 2014. Pretreatment and fractionation of barley straw using steam explosion at low severity factor. *Biomass and Bioenergy*, **66**, 286-300.

Jacquet, N., Maniet, G., Vanderghem, C., Delvigne, F., Richel, A. 2015. Application of Steam Explosion as Pretreatment on Lignocellulosic Material: A Review. *Industrial & Engineering Chemistry Research*, **54**(10), 2593-2598.

Jahan, M.S., Haider, M.M., Rahman, M., Biswas, S., Misbahuddin, M., Mondal, G.K. 2011. Evaluation of rubber wood (*Hevea brasiliensis*) as a raw material for kraft pulping. *Nordic Pulp and Paper Research Journal*, **26**(3), 258-262.

Kalyani, D.C., Zamanzadeh, M., Müller, G., Horn, S.J. 2017. Biofuel production from birch wood by combining high solid loading simultaneous saccharification and fermentation and anaerobic digestion. *Applied Energy*, **193**, 210-219.

Khanal, S.K. 2008. *Anaerobic Biotechnology for Bioenergy Production: Principles and Applications*. Wiley-Blackwell, Singapore.

Ko, C.-H., Wang, Y.-N., Chang, F.-C., Chen, J.-J., Chen, W.-H., Hwang, W.-S. 2012. Potentials of lignocellulosic bioethanols produced from hardwood in Taiwan. *Energy*, **44**(1), 329-334.

Li, C., Liu, G., Nges, I.A., Liu, J. 2016. Enhanced biomethane production from Miscanthus lutarioriparius using steam explosion pretreatment. *Fuel*, **179**(Supplement C), 267-273.

Li, Y., Khanal, S.K. 2017. *Bioenergy:Principles and Applications*. Wiley Blackwell, United States of America.

Lizasoain, J., Trulea, A., Gittinger, J., Kral, I., Piringer, G., Schedl, A., Nilsen, P.J., Potthast, A., Gronauer, A., Bauer, A. 2017. Corn stover for biogas production: Effect of steam explosion pretreatment on the gas yields and on the biodegradation kinetics of the primary structural compounds. *Bioresource Technology*, **244**(Part 1), 949-956.

McKendry, P. 2002. Energy production from biomass (part 1): overview of biomass. *Bioresource Technology*, **83**(1), 37-46.

Oliveira, F.M.V., Pinheiro, I.O., Souto-Maior, A.M., Martin, C., Gonçalves, A.R., Rocha, G.J.M. 2013. Industrial-scale steam explosion pretreatment of sugarcane straw for enzymatic hydrolysis of cellulose for production of second generation ethanol and value-added products. *Bioresource Technology*, **130**, 168-173.

Olsen, J.P., Alasepp, K., Kari, J., Cruys-Bagger, N., Borch, K., Westh, P. 2016. Mechanism of product inhibition for cellobiohydrolase Cel7A during hydrolysis of insoluble cellulose. *Biotechnology and Bioengineering*, **113**(6), 1178-1186.

Overend, R.P., Chornet, E., Gascoigne, J.A. 1987. Fractionation of Lignocellulosics by Steam-Aqueous Pretreatments [and Discussion]. *Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences*, **321**(1561), 523-536.

Pengilly, C., Garcia-Aparicio, M.P., Diedericks, D., Gorgens, J.F. 2016. Optimization of Enzymatic Hydrolysis of Steam Pretreated Triticale Straw. *Bioenergy Research*, **9**(3), 851-863.

Phitsuwan, P., Charupongrat, S., Klednark, R., Ratanakhanokchai, K. 2015. Structural features and enzymatic digestibility of Napier grass fibre treated with aqueous ammonia. *Journal of Industrial and Engineering Chemistry*, **32**, 360-364.

Phitsuwan, P., Sakka, K., Ratanakhanokchai, K. 2016. Structural changes and enzymatic response of Napier grass (Pennisetum purpureum) stem induced by alkaline pretreatment. *Bioresource Technology*, **218**, 247-256.

Qiu, W., Chen, H. 2012. Enhanced the enzymatic hydrolysis efficiency of wheat straw after combined steam explosion and laccase pretreatment. *Bioresource Technology*, **118**, 8-12.

Rabemanolontsoa, H., Saka, S. 2016. Various pretreatments of lignocellulosics. *Bioresource Technology*, **199**, 83-91.

Rincón, B., Banks, C.J., Heaven, S. 2010. Biochemical methane potential of winter wheat (Triticum aestivum L.): Influence of growth stage and storage practice. *Bioresource Technology*, **101**(21), 8179-8184.

Saha, B.C. 2003. Hemicellulose bioconversion. *J Ind Microbiol Biotechnol*, **30**(5), 279-91.

Saritpongteeraka, K., Chaiprapat, S., Boonsawang, P., Sung, S. 2015. Solid state co-fermentation as pretreatment of lignocellulosic palm empty fruit bunch for organic acid recovery and fiber property improvement. *International Biodeterioration & Biodegradation*, **100**, 172-180.

Segal, L., Creely, J.J., Martin, A.E., Jr., Conrad, C.M. 1959. An Empirical Method for Estimating the Degree of Crystallinity of Native Cellulose Using the X-Ray Diffractometer. *Textile Research Journal*, **29**(10), 786-794.

Siddhu, M.A.H., Li, J., Zhang, J., Huang, Y., Wang, W., Chen, C., Liu, G. 2016. Improve the Anaerobic Biodegradability by Copretreatment of Thermal Alkali and Steam Explosion of Lignocellulosic Waste. *BioMed Research International*, **2016**, 10.

Singh, D.P., Gadi, R., Mandal, T.K., Saud, T., Saxena, M., Sharma, S.K. 2013. Emissions estimates of PAH from biomass fuels used in rural sector of Indo-Gangetic Plains of India. *Atmospheric Environment*, **68**, 120-126.

Singh, J., Suhag, M., Dhaka, A. 2015. Augmented digestion of lignocellulose by steam explosion, acid and alkaline pretreatment methods: A review. *Carbohydrate Polymers*, **117**, 624-631.

Sluiter, A., Hames, B., Hyman, D., Payne, C., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Wolfe, J. 2008a. Determination of Total Solids in Biomass and Total Dissolved Solids in Liquid Process Samples (Ed.) N.R.E.L. (NREL).

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D. 2008b. Determination of Structural Carbohydrates and Lignin in Biomass. National Renewable Energy Laboratory (NREL) Laboratory Analytical Procedures (LAP).

Sun, Y., Cheng, J. 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresource Technology*, **83**(1), 1-11.

Vivekanand, V., Olsen, E.F., Eijsink, V.G.H., Horn, S.J. 2013. Effect of different steam explosion conditions on methane potential and enzymatic saccharification of birch. *Bioresource Technology*, **127**, 343-349.

Wang, H., Wang, J., Fang, Z., Wang, X., Bu, H. 2010. Enhanced bio-hydrogen production by anaerobic fermentation of apple pomace with enzyme hydrolysis. *International Journal of Hydrogen Energy*, **35**(15), 8303-8309.

Wu, F.-C., Huang, S.-S., Shih, I.-L. 2014. Sequential hydrolysis of waste newspaper and bioethanol production from the hydrolysate. *Bioresource Technology*, **167**, 159-168.

Output

- Boonya Charnnok, Sumate Chaiprapat, Tokla Eom (2018) “Steam explosion and enzymatic pretreatment to improve anaerobic digestibility of rubber wood waste” ในงาน “นักวิจัยรุ่นใหม่พบเมธีวิจัยอาวุโส สกว.” ครั้งที่ 17 ระหว่างวันพุธที่ 10-วันศุกร์ที่ 12 มกราคม พ.ศ. 2561 ณ โรงแรมเดอะรีเจ้นท์ ชะอำ บีช รีสอร์ท จังหวัดเพชรบุรี (Oral presentation)
- Tokla Eom, Sumate Chaiprapat, Boonya Charnnok (2017) “Effect of steam explosion on enzymatic hydrolysis of rubber wood waste” The 7th International Conference on Fermentation Technology for Value Added Agricultural Products. July 25-28, 2017 Pullman Khon Kaen Raja Orchid, Khon Kaen, Thailand.

Appendix

Proceeding

FerVAAP 2017
The 7th International Conference on Fermentation Technology for Value Added Agricultural Products

Effect of steam explosion on enzymatic hydrolysis of rubber wood waste

Tokla Eom[1], Sumate Chaiprapat[1,2] and Boonya Charnnok[2,*]

[1] Department of Civil Engineering, Faculty of Engineering, Prince of Songkla University, Hat Yai Campus, Songkhla, 90110, Thailand
[2] PSU Energy System Research Institute (PERIN), Prince of Songkla University, Hat Yai Campus, Songkhla, 90110, Thailand
** Corresponding author: boonya.c@psu.ac.th*

Abstract

Improvement of enzymatic hydrolysis and anaerobic digestion of rubber wood waste (RW) using steam explosion (SE) pretreatment was investigated. Total reducing sugar (TRS) and methane production were improved with an increased retention time of SE. The highest yield of TRS and methane of steam exploded RW reached the maximum of 192.9 g/kgDM and 35.1 LCH_4/kgDM, respectively, at 16 bars for 10 min. TRS improved by 85.6% when enzyme dose increased from 10 to 30 FPU/gDM. The dose above 30 FPU/gDM was less effective giving only 27.5% increase between 30 to 50 FPU/gDM. Hence, TRS yield at 30 FPU/gDM was selected to determine theoretical energy in case of subsequent conversion to ethanol. The highest energy yield of 2.14 MJ/kg DM feedstock was achieved from TRS. Anaerobic digestion of the remaining residue was also conducted to evaluate possible energy yield of RW feedstock.

Keywords: Steam explosion, Enzymatic hydrolysis, Total reducing sugar, Anaerobic digestion, Rubber wood waste

1. Introduction

In Thailand, rubber wood waste (RW) is one of the most abundant lignocellulosic residues. It is high potential raw material for energy and bio-based chemical productions. Lignocellulosic biomass, a structure formed mainly by cellulose, hemicellulose, and lignin, is rigid and recalcitrant to bioconversion, especially in hydrolysis phase. Currently, pretreatment methods have been developed, including biological pretreatment, microwave radiation, wet oxidation, liquid hot water, and steam explosion (SE) (1).

Among them, SE pretreatment is considered a highly efficient pretreatment for lignocellulosic biomass. During SE pretreatment the biomass is rapidly pressurized at saturated steam. The rapid thermal expansion opens up the cell wall of the biomass prior to explosion and partial hydrolysis. Then, the pressure is rapidly reduced to atmospheric condition and only solid fraction was obtained. Steam exploded biomass can be enzymatically hydrolyzed by cellulase to glucose, which is an intermediate product for biofuel and biochemical production (2), as well as in anaerobic digestion for producing methane (3). SE has been reported on pretreatment of numerous lignocellulosic biomass such as Oak woods (4), corn stover (5), and etc. for improvement of methane yield. Several factors such as physicochemical component of biomass, pretreatment condition, and enzyme dose (for enzymatic hydrolysis) can affect the efficiency of enzymatic hydrolysis and anaerobic digestibility. Hence, in order to improve their conversion, these factors should be optimized for a specific biomass. Strategy of RW utilization to efficiently produce biofuel and biogas is still needed. Therefore, this study aims to investigate the effect of retention time in SE pretreatment on enzymatic and anaerobic digestibility of RW.

2. Material and Methods

2.1. Steam explosion pretreatment

Rubber wood waste (size 2.36-4.75 mm) was collected from a particle board factory, Panel Plus Co., Ltd. in Songkhla province, Thailand. It was dried in a hot air oven at 60 °C until constant weight. Steam explosion (SE) pretreatment was performed using steam explosion reactor FD-97137, CHINO Corporation, Japan. A 200-g RW on dried basis was put into the 2 L pressure vessel at 16 bar (203 °C) with retention times 1, 5 and 10 min. The steam exploded RWs were washed with 800 mL hot water (70°C) for 10 min and dried in a hot air oven at 60 °C until constant weight. The experiment was performed in triplicate.

2.2. Enzymatic hydrolysis

Commercial enzyme Celli CTec2 (Novozyme) with filter paper activity of 254 FPU/mL was used in enzymatic hydrolysis steam exploded RW. A-8 % (w/v) substrates was mixed with citric acid monohydrate buffer solution (0.05 M, pH = 4.8) in a glass bottle with reaction volume 50 mL and different enzymatic loading (0 to 60 FPU/g DM). A-0.8 % (v/v) sodium azide (20 mg/mL) was added to prevent microbial growth during incubation. All samples were incubated at 50 °C in incubator shaker at 150 rpm for 72 h. The enzymatic hydrolyzate was centrifuged at 10000 rpm for 5 min. The supernatant taken was analyzed for the total reducing sugar (TRS) according to 3,5-dinitrosalicylic acid (DNS) method (6) and calculated according to the following equation:

$$TRS = \frac{V \times 0.9 \times TRScon.}{Substrate} \quad (1)$$

Where TRS is total reducing sugar yield (g/kg DM), V is reaction volume (mL), TRScon. is concentration of TRS (g/L) and substrate is mass in gDM

2.3. Anaerobic digestion

Anaerobic digestion of steam exploded RW and solid residue from enzymatic hydrolysis was carried out in a sealed batch glass bottle using method described by (7). The inoculum used was sludge from the anaerobic digester from a concentrated latex factory (Chalong Latex Rubber Co., Ltd. Songkhla, Thailand). The feedstock (0.5 g) was mixed with inoculum with initial inoculum to substrate ratio on total solid basis of 3. Buffer solution and nutrient (8) were added 10% (v/v) and 1% (v/v), respectively. The final reaction volume was adjusted to 60 mL by adding deionized water and small amount of 10 % HCL to adjust pH to 7.0±0.5. Anaerobic conditions were provided by purging the reactors with a nitrogen gas for 2 min at the beginning of the digestion process. The samples were incubated at 35 ± 0.5 °C in an incubator shaker at 150 rpm for 28 days. Three bottles of blank containing the same amount of inoculum without substrate were used as corrections. Daily biogas yield and its composition were measured and analyzed by using a glass syringe and gas chromatography (GC), respectively.

2.4. Analytical methods

Cellulose, hemicellulose, and lignin contents were analyzed on dry basis (9),(10), and (11). Biogas composition was analyzed by gas chromatography (GC 7820A Agilent Technologies) equipment with thermal conductivity detector (TCD) using helium as carrier gas. TRS was determined using UV spectrophotometer (Spectroquant® Pharo 100 M) at absorbance 540 nm.

3. Results and discussion

3.1 Chemical composition

Steam explosion (SE) pretreatment was used to alter the structure of RW in order to improve the enzymatic hydrolysis and anaerobic digestibility. Rubber wood waste (RW) used in this study is composed of

cellulose, hemicellulose and lignin were 66.72, 12.72 and 13.12%, respectively (Table 1). Cellulose, a glucose polymer, is a good source of carbon for various bioenergy productions. The cellulose content of RW was higher than other biomasses such as corn stover 40.2% (12) and Eucalyptus (*Eucalyptus grandis*) 45.2% (13). This higher cellulose content made the RW biomass an interesting renewable feedstock. After SE pretreatment, the RW was broken into smaller pieces compared to untreated RW (Figure 1). Solid recovery was determined on dry solid basis as the percentage of recovered pretreated RW of the feedstock weight. The decrease of solid recovery was found with an increased retention time due to degradation of its chemical composition. Hemicellulose content mostly was degraded during SE pretreatment. The degree of hemicellulose degradation was found with increasing retention time. Results (Table 1) showed that hemicellulose remaining in pretreated RW was lower than the untreated RW while hemicellulose decreased by 4.4% when RT increased from 1 to 5 min, and reached the minimum remain at 10 min (4.82%DM). On the other hand, lignin content of steam exploded RW was increased in all retention times compared to untreated RW. It can be described by the formation of pseudo-lignin (14). During SE pretreatment, cellulose and hemicellulose were degraded into hexose and pentose, which subsequently dehydrolyzed to form hydroxymethylfurfural (HMF) and furfural, respectively. The HMF and furfural were found 0.506 and 0.951 g/L, respectively at SE pretreatment for 10 min. The result of rearragament of furfural and HMF formation can be depolymerized to form pseudo-lignin (detected as lignin) (14). Similar study also reported the decrease of hemicellulose with increasing lignin of steam exploded corn stover at 200 °C (1.55 MPa) for 12 min, lignin increased from 16.00% to 33.60% (5), and steam exploded hay at 220 °C for 15 min, lignin increased from 5.80% to 24.00% (15) compared to the native feedstocks.

Table 1 Chemical composition of steam exploded RW

RT	Solid recovery	CL	HCL	Lignin
U	100	66.72	12.72	13.12
1	93.98	59.32	9.97	16.53
5	93.16	55.20	5.57	17.90
10	89.55	54.50	4.82	16.39

Note: Unit = % on dry basis, CL = cellulose, HCL = hemicellulose, RT = retention time (min), U = untreated

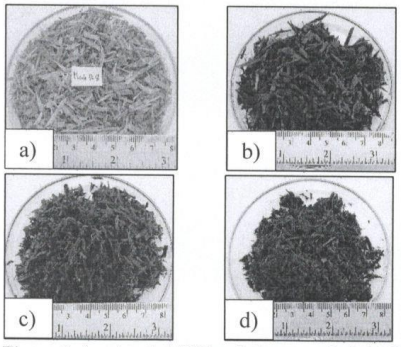


Figure 1 a) untreated RW and steam exploded RW at 16 bar for b) 1 min c) 5 min and d) 10 min

3.2 Enzymatic hydrolysis of steam exploded RW

Steam exploded RW as well as the untreated RW were subjected to enzymatic hydrolysis to produce total reducing sugar (TRS). The hydrolysis yield are presented in Figure 2, TRS yield of untreated RW was 27.92 and 74.14 g/kgDM after 72 h hydrolysis with enzyme dose of 5 and 60 FPU/gDM. TRS production could be improved by pretreatment of RW using steam explosion at 16 bar for 1-10 min. Although, the increase of hydrolysis yield was found with longer retention time, it is not significant improvement at RT 1 min at all enzyme doses compared to the untreated RW ($p \geq 0.05$). Hence, in order to increase effectiveness of TRS production, RT higher than 1 min which equivalent to severity factor of 3.03 (temperature and retention time) is

recommended. The SE pretreatment increased the TRS yield by 8.6% when retention time increased from 5 to 10 min at 5 FPU/gDM, which relate to the decrease of hemicellulose content by 13.5%.

The steam exploded RW at different pretreatment conditions were also investigated with enzyme doses ranging from 5 to 60 FPU/gDM. The increase of TRS yield was found with rising enzyme dose for both untreated and pretreated RW. The highest biomass digestibility was achieved at 10 min SE pretreatment and enzyme dose 60 FPU/gDM, which led to TRS yield of 192.89 g/kgDM. With the solid recovery of 89.55%, this was equivalent to 17.3% TRS recovery from the native feedstock. Our results are in accordance to Cotana et al. (4), who reported the TRS yield of steam exploded holm, Turkey oak, and downey were ranged from 21.80-38.38, 12.29-32.04, and 14.79%, respectively at severity of 4.31. However, the TRS improved by 85.6% when enzymatic dose increased from 10 to 30 FPU/gDM. The dose above 30 FPU/gDM was less effective returning at only 27.5% increment between 30 to 50 FPU/gDM. Hence, TRS yield at 30 FPU/gDM was selected to determine theoretical energy assuming that ethanol is to be produced.

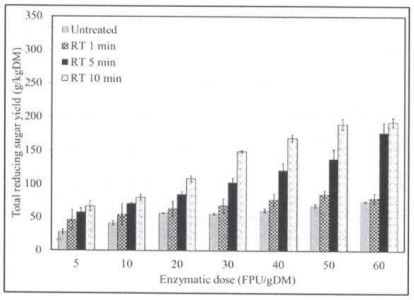


Figure 2 Total reducing sugar in enzymatic hydrolysis of steam exploded RW

3.3 Anaerobic digestion of steam exploded RW

The methane production of 10.75 LCH_4/kg DM was obtained from untreated RW after 28 days anaerobic digestion (Figure 3). SE pretreatment showed a high potential for improving methane production with a significant increase ($p<0.05$) compared to untreated RW. The methane yield of 23.59 LCH_4/kg DM for 1 min is around 2 times higher than that of the untreated RW. The increase of methane production is related to the harshness of the SE pretreatment conditions, as higher auto-hydrolysis occurred. The highest methane productions was found for the RW pretreated for 10 min (35.09 L CH_4/kgDM or 35.5 L CH_4/kgVS or 31.42 L CH_4/kg feedstock), whereas the sample pretreated for 1 and 5 min had lower yields by 32.8 and 25.6%, respectively. This is probably due to the lower hemicellulose content (Table 1) and increase the contacting area of the pretreated RW. The removal of hemicellulose could improve the internal surface area of biomass (16) leading to an increased enzyme accessibility to the cellulose, thus resulted in higher enzymatic susceptibility of the pretreated RW. It subsequently led to greater anaerobic and enzymatic digestibility. However, the methane production level reported in this study (severity = 4.03 for retention time 10 min) is lower than steam exploded *Salix* wood (240 LCH_4/kgVS) but was pretreated at higher severity as 4.5 (17). Hence, in order to improve the anaerobic digestibility of the rubber wood, higher harshness of the SE pretreatment conditions are required.

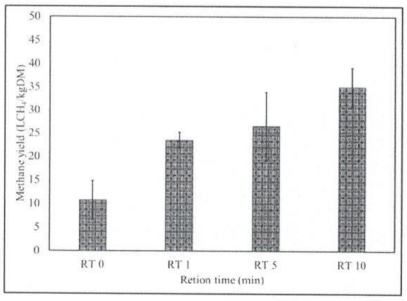


Figure 3 methane yield of steam exploded RW

3.4 Methane recovery from the enzymatic hydrolysis residue

After separation of TRS in enzymatic hydrolysis process, the remaining residue (EH-residue) from enzymatic hydrolysis at 30 FPU/g DM was used as substrate in anaerobic digestion in order to evaluate possible energy yield of RW feedstock. Table 2 shows the methane production of the EH-residue for 28-day anaerobic digestion. The combination of SE pretreatment and enzymatic hydrolysis affected the methane production of EH-residue compared to untreated EH-residue by 43.2 and 79.8% for 1 and 5 min. Increase in SE retention time seemed to have a positive effect for methane recovery. However, methane recovery slightly decreased with the harshness of the pretreatment condition up to 10 min, whereas enzymatic and anaerobic digestibility reached the highest value (Figure 2 and 3). It can be explained that at SE higher retention time the structure of the rubber wood was highly degraded, resulted in high conversion of carbohydrate into TRS during enzymatic hydrolysis. The remaining residue for10 min had lower potential carbohydrate content and thus produce lower methane yield.

Table 2 Methane yield of EH-residue at different conditions of SE pretreatments

Retention time (min)	Methane yield (LCH_4/kgDM)
Untreated + EH	12.80±8.81
1 min	18.33±1.76
5 min	23.02±6.26
10 min	21.95±2.39

Note Enzymatic hydrolysis at 30 FPU/gDM

3.5 Evaluation of energy recovery

The composition of glucose and xylose containing in TRS from enzymatic hydrolysis was used to evaluate the theoretical ethanol yield. A 100 g glucose or xylose can theoretically yield up to 51.1 g ethanol. Energy values of ethanol and methane were determined based on the higher heating value of 26.8 MJ/kg and 55.7 MJ/kg, respectively. The integrated process including steam explosion (16 bar 10 min), enzymatic hydrolysis (30 FPU/gDM), and anaerobic digestion of EH-residue could yield the energy 2.96 MJ fuel/kgDM of feedstock. This energy yield is higher than only production of ethanol (2.14) or biogas (1.24) MJ fuel/kg DM of feedstock alone. Similarly, Kalyani et al. (18) reported combined production of ethanol and biogas from birch wood (using steam explosion pretreatment at 210 °C for 10 min) yielding 9.1 MJ fuel/kg DM of feedstock that 1.4-1.7 fold higher energy yield than only production of ethanol or biogas.

4. Conclusion

Both SE pretreatment and enzymatic dose played an important role to improve the TRS yield from rubber wood waste. Production of TRS followed by methane recovery from enzymatic hydrolysis residue has potential to improve energy production compared to only production of ethanol. Further attempts to raise in retention time of SE for co-production of TRS and methane recovery should be undertaken to maximize energy recovery from rubber wood waste.

5. Acknowledgements

This research was financially supported by Thailand Research Fund (TRF) contract no. MRG5980051 as well as by the Thailand's Education Hub for Southern Region of ASEAN Countries (TEH-AC) and Graduate School of Prince of Songkla University. The authors would like to thank Panel Plus Co. Ltd. for rubber wood waste and Chalong Latex Industry Co., Ltd. Songkhla, Thailand for anaerobic inoculum. The authors would like also to recognize the support from the Biogas & Biorefinergy Laboratory, Prince of Songkla University.

6. References

(1) Bhutto AW, Qureshi K, Harijan K, Abro R, Abbas T, Bazmi AA, et al. Insight into progress in pre-treatment of lignocellulosic biomass. Energy. 2017 Mar 1;122:724–45.

(2) Wang W, Ling H, Zhao H. Steam explosion pretreatment of corn straw on xylose recovery and xylitol production using hydrolysate without detoxification. Process Biochem. 2015 Oct;50(10):1623–8. \

(3) Li C, Liu G, Nges IA, Liu J. Enhanced biomethane production from Miscanthus lutarioriparius using steam explosion pretreatment. Fuel. 2016 Sep 1;179:267–73.

(4) Cotana F, Buratti C, Barbanera M, Lascaro E. Optimization of the steam explosion and enzymatic hydrolysis for sugars production from oak woods. Bioresour Technol. 2015 Dec;198:470–7.

(5) Liu Z-H, Chen H-Z. Xylose production from corn stover biomass by steam explosion combined with enzymatic digestibility. Bioresour Technol. 2015 Oct;193:345–56.

(6) Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem. 1959;31(3):426–8.

(7) Dechrugsa S, Kantachote D, Chaiprapat S. Effects of inoculum to substrate ratio, substrate mix ratio and inoculum source on batch co-digestion of grass and pig manure. Bioresour Technol. 2013 Oct;146:101–8.

(8) Raposo F, de la Rubia MA, Borja R, Alaiz M. Assessment of a modified and optimised method for determining chemical oxygen demand of solid substrates and solutions with high suspended solid content. Talanta. 2008 Jul 15;76(2):448–53.

(9) Goering H., Van Soest PJ. Forage Fiber Analysis (Apparatus, Reagents, Procedures and Some Application). In: Agricultural Handbook. Agricutural Research Service, U.S. Department of Agriculture; 1970.

(10) Horwitz W. Official methods of analysis of AOAC International. Gaithersburg, Md.: AOAC International; 2000.

(11) Van Soest PJ, Robertson JB, Lewis BA. Methods for Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition. J Dairy Sci. 1991 Oct 1;74(10):3583–97.

(12) Chen J, Zhang W, Zhang H, Zhang Q, Huang H. Screw extrude steam explosion: A promising pretreatment of corn stover to enhance enzymatic hydrolysis. Bioresour Technol. 2014 Jun;161:230–5.

(13) Klamrassamee T, Champreda V, Reunglek V, Laosiripojana N. Comparison of homogeneous and heterogeneous acid promoters in single-step aqueous-organosolv fractionation of eucalyptus wood chips. Bioresour Technol. 2013 Nov 1;147:276–84.

(14) Vivekanand V, Olsen EF, Eijsink VGH, Horn SJ. Effect of different steam explosion conditions on methane potential and enzymatic saccharification of birch. Bioresour Technol. 2013 Jan;127:343–9.

(15) Bauer A, Lizasoain J, Theuretzbacher F, Agger JW, Rincón M, Menardo S, et al. Steam explosion pretreatment for enhancing biogas production of late harvested hay. Bioresour Technol. 2014 Aug;166:403–10.

(16) Imman S, Arnthong J, Burapatana V, Champreda V, Laosiripojana N. Effects of acid and alkali promoters on compressed liquid hot water pretreatment of rice straw. Bioresour Technol. 2014 Nov;171:29–36.

(17) Horn SJ, Estevez MM, Nielsen HK, Linjordet R, Eijsink VGH. Biogas production and saccharification of Salix pretreated at different steam explosion conditions. Bioresour Technol. 2011 Sep;102(17):7932–6.

(18) Kalyani DC, Zamanzadeh M, Müller G, Horn SJ. Biofuel production from birch wood by combining high solid loading simultaneous saccharification and fermentation and anaerobic digestion. Appl Energy. 2017 May 1;193:210–9.

Manuscript

Enhanced enzymatic hydrolysis and methane production from rubber wood waste using steam explosion

Tokla Eom[a], Sumate Chaiprapat[a,b], Boonya Charnnok[b*]

[a]Department of Civil Engineering, Faculty of Engineering, Prince of Songkla University, Hat Yai campus, Hat Yai, Songkhla, 90110, Thailand

[b]PSU Energy Systems Research Institute (PERIN), Prince of Songkla University, Hat Yai campus, Hat Yai, Songkhla, 90110, Thailand

*Corresponding author address: PSU Energy Systems Research Institute (PERIN), Prince of Songkla University, Hat Yai campus, Hat Yai, Songkhla, 90110, Thailand. Tel.: +74282000 ext. 749957. Email address: boonya.c@psu.ac.th

Abstract

Rubber wood waste (RW) is an abundant biomass in Thailand, their lignocellulosic nature entails difficulties for bioconversion. Steam explosion with a severity of 2.27-4.35 was applied to pretreat RW. Pretreated RW with highest improvement in anaerobic digestibility achieved 83.9 L CH_4/kg-VS at severity of 4.35 together with the hydrolysability in terms of glucan conversion as high as 45.2% at a cellulase Cellic®CTech2 concentration of 30 FPU/g. These yields correlated to the changes in crystallinity and surface morphology of RW. Conversion efficiency was enhanced with an integrative process of SE followed by enzymatic hydrolysis and anaerobic digestion. In term of energy, this process with severity 4.03 could co-produce glucose and methane higher than only the production of glucose or methane alone or even higher SE severity at 4.35. The remainder RW is rich in polysaccharide and could be a good biomaterial for further utilization.

Keywords: Steam explosion; Enzymatic hydrolysis; Anaerobic digestion; Rubber wood waste; Bioenergy

1. Introduction

Rubber industry is one of the most important economic agro-industry sectors in over 20 countries including Thailand. While the main primary products are either dry or latex natural rubber, their co-product of rubber wood is an important material of great economic value for furnisher industry. With rubber planting area of over 19 million rais (7.6 milion acres) in 2016 (Center of Agricultural Information, 2016), this rubber wood not only could contribute to national export value but also a huge resource for lignocellulosic biomass for biochemicals and energy production. The economical lifespan of a rubber tree is approximately 25 years, after which rubber latex production is reduced and it will be cut down for replantation. The rubber biomass largely become a woody material for furnisher and toy industry. In rubber wood processing, a gigantic amount of rubber wood waste (RW) is generated from cutting and milling.

This lignocellulosic biomass consists mainly of cellulose, hemicellulose and lignin that makes its structure rigid and recalcitrant to degradation, especially in hydrolysis step. The carbohydrate in woody biomass are not readily accessible for hydrolytic enzymes. The pretreatment step is crucial in order to disrupt lignocellulosic biomass matrix and make available polysaccharides for enzymatic attack. Steam explosion (SE) is a thermo-mechano-chemical pretreatment by high pressure saturated steam with temperature between 160 and 260 °C (Rabemanolontsoa & Saka, 2016). The biomass is subjected to harsh environment of high pressure and temperature with saturated steam. The thermal expansion opens up the cell wall of the biomass prior to explosion and also induces auto-hydrolysis by acetic acid derived from acetyl residues in xylan hemicellulose. The pressure is then rapidly reduced to atmospheric

condition where the explosion of the plant cell wall takes place. Not only this process is relatively low energy intensive but also is environmental friendly due to no chemical addition (Jacquet et al., 2015; Singh et al., 2015). It is among the most efficient pretreatments to enhance enzymatic hydrolysis and digestion of woody feedstocks (Cotana et al., 2015; Li et al., 2016; Vivekanand et al., 2013). Removal of hemicellulose by SE is so effective that it increases exposure of cellulose surface to enzymes within the loosen cellulose microfibrils (Imman et al., 2014).

For each biomass, it is important to optimize SE condition by critical operating parameters. Those of particular interest include pressure, temperature and retention time, which impact structural transformation at different degrees (Asada et al., 2012; Ballesteros et al., 2011; Cotana et al., 2015; Lizasoain et al., 2017). Currently, RW is used as low value fuel in biomass power plant and industrial steam boiler. Its use as cellulosic biomass for the combined liquid biofuel and methane production had not been reported. Previous research had used residues from ethanol fermentation of steam exploded oat straw at 190°C for 10 min (Dererie et al., 2011) and steam exploded birch wood at 210 °C for 10 min (Kalyani et al., 2017) as substrate for producing biogas thru anaerobic digestion. Although the overall energy recovery was reportedly increased compared to only ethanol production, only one combination of ethanol-methane production from their biomasses was tested. Study on comparative yield of the different combinations in terms of energy and its by-products could give an insight for this biomass valorization in biorefinery perspective. Thus, the integrative strategy of steam explosion combined with enzymatic hydrolysis and anaerobic digestion is proposed that could potentially raise the value-chain of this lignocellulosic rubber wood waste.

This study aims to examine the effects of severity of steam explosion pretreatment and enzyme loading on RW hydrolysis and anaerobic digestion due to changing in physical and chemical RW structures. In addition, an integrative process of SE pretreatment and enzymatic

hydrolysis with various enzyme loading followed by anaerobic digestion was applied in order to improve biodegradability of RW and overall energy yield of co-product which was glucose and methane.

2. Materials and Methods

2.1 Preparation of substrate

Rubber wood waste (RW) was collected from a production process of a particle board factory, Panel Plus Co., Ltd. in Songkhla province, Thailand. It was firstly air-dried for one week and sieved. RW of size 2.4-4.7 mm was chosen for this study. It was further dried in a hot air oven at 60 °C until constant weight and stored in plastic bag at room temperature. Characteristics of the RW are shown in Table 1.

2.2 Steam explosion pretreatment

Steam explosion (SE) of RW was carried out at the Kasetsart University, Bangkok, Thailand. SE reactor Model FD-97137, CHINO corporation, Japan was used throughout this study. A 200-g RW on dry basis was added into the 2-L pressure vessel connected to a reception chamber and a steam generator. High-pressure steam provided by the electric steam generator was fed into the reactor until the pressure reached the designated values and stayed at such condition for different retention times. The biomass was rapidly exploded by a sudden pressure release by opening the valve, through which it passed into the reception chamber. The samples were steamed at different temperatures from 191 to 214 °C during 1 to 10 min in triplicate. The severity factor which is used to represent the combined effect of reaction temperature and retention time was calculated according to Eq. 1 (Iroba et al., 2014; Overend et al., 1987). Severity conditions used in this study were 2.70, 2.37, 2.73, 4.03 and 4.35.

$$logR_o = logt\ exp\left[\frac{(T-100)}{14.75}\right] \quad (1)$$

Where $logR_o$ is severity factor; t is retention time (min); T is reaction temperature (ºC).

After SE, the samples were washed with 800 mL hot water (70°C) for 10 min in order to remove the degradation products accumulated on the RW surface. The washing liquid was immediately separated from the steam exploded RW (SE-RW). The washing liquid was analyzed for degradation products including glucose, xylose, hydroxylmethylfurfural (HMF), furfural and acetic acid. SE-RW samples were stored at 4 °C before being dried in a hot air oven at 60 °C until constant weight, then kept in plastic bag at room temperature until use. The solid recovery of different pretreatments was determined based on Eq. 2.

$$\text{Solid recovery}(\%) = \frac{\text{mass of pretreated RW (g)}}{\text{mass of initial RW (g)}} \times 100\% \quad (2)$$

2.3 Enzymatic hydrolysis assay

Un-RW (untreated RW) and SE-RW were used as substrate for enzymatic hydrolysis (EH) optimization with enzyme loading of 3, 6, 12, 18, 24 and 30 FPU/g. The assays were performed in triplicate using 8 % (w/v) of substrates in 50-mL reaction vessels. The tests were put into 0.05 M, pH 4.8 citric acid monohydrate buffer solution and 0.8 % (v/v) of sodium azide (20 mg/mL). Commercial enzyme Cellic®CTec2 (Novozyme) with filter paper activity of 150 FPU/mL was used in this experiment. Its activity was measured according to Adney and Baker (2008). All tests were incubated at 50 °C for 72 h in an incubator shaker operated at 150 rpm. The mix liquors were filtered by vacuum system to separate the enzymatic residue (EH residue) and enzymatic hydrolyzate. The hydrolysis yields as glucose, cellobiose and xylose

were analyzed using high performance liquid chromatography (HPLC). All experiments were carried out in triplicate and average results were reported. Glucose yield, glucan conversion (summation of glucose and cellobiose) and xylan conversion were determined relative to their theoretical yields according to Eq. 3, 4 and 5, respectively (Pengilly et al., 2016).

$$\text{Glucose yield (\%)} = \frac{\text{Glucose (g)}}{f \times (\text{pretreated RW}) \times 1.111} \times 100 \quad (3)$$

$$\text{Glucan conversion (\%)} = \frac{\text{Glucose (g)} + (1.053 * \text{Cellobiose (g)})}{f \times (\text{pretreated RW}) \times 1.111} \times 100 \quad (4)$$

$$\text{Xylan conversion (\%)} = \frac{\text{Xylose (g)}}{f \times (\text{pretreated RW}) \times 1.136} \times 100 \quad (5)$$

where glucose/cellobiose/xylose are the amount of glucose/cellobiose/xylose in the enzymatic hydrolysate (g); 1.053 is a conversion factor for cellobiose to equivalent glucose; 1.111 is a conversion factor for cellulose to equivalent glucose; 1.136 is a conversion factor for xylan to equivalent xylose; *f* is the glucan/xylan fraction by dry mass; pretreated RW is the amount of substrate loading on dry basis (g).

2.4 Anaerobic digestibility test

Anaerobic digestibility tests of Un-RW, SE-RW and the residue from enzymatic hydrolysis of stream exploded RW (SE+EH-RW) were carried out in triplicate in sealed batch reaction vessels. The method used was described by Dechrugsa et al. (2013). Briefly, the total reaction volume was 60 mL containing 0.5 g of substrate. The ratio of inoculum to substrate was 3 on dry mass basis. The inoculum was a mesophilic sludge taken from a full scale anaerobic digester treating wastewater from a concentrated rubber latex factory in Songkhla

province, Thailand. Prior to the experiments the inoculum was incubated at room temperature (28-30°C) for 4 weeks to reduce its endogenous biogas production. The reaction vessels were added with 10 % (v/v) of buffer solution (50 g/L $NaHCO_3$), 1 % (v/v) of trace elements and nutrient solution (Rincón et al., 2010). The final volume was made up with distilled water to 60 mL and small quantity of 10 % HCL to adjust pH to 7.0±0.2. The headspace of reaction vessels was purged for 2 min with pure nitrogen gas and sealed immediately. The tests were incubated at 35 ± 0.5 °C and shaken at 150 rpm for 28 days. The blanks containing the same amount of inoculum without substrate were conducted. Daily biogas generation was measured using glass syringe and its composition was determined by gas chromatography. Methane yield was calculated based on volatile solid of the pretreated RW according to Eq. 6.

$$CH_4\ yield\ \left(\frac{L}{kg-VS}\right) = \frac{Vol.\ of\ CH_4\ substrate - Vol.of\ CH_4\ blank}{VS_{substrate}} \quad \text{(Eq. 6)}$$

where Vol. of CH_4 substrate and blank is the amount of methane (L) releasing from reaction vessels and $VS_{substrate}$ is the amount of substrate loading on VS basis (kg)

The biodegradability index (BI) is defined as the ratio of methane yield to theoretical yield based on holocellulose content (cellulose plus hemicellulose). This value describes the biodegradability efficiency of the feedstock in the batch digestion. The determination of BI value was conducted according to Eq. 7.

$$Biodegradability\ index\ (\%) = \frac{Methane\ yield}{Theoretical\ methane\ yield} \times 100 \quad \text{(Eq.7)}$$

Theoretical methane yield was calculated using the Buswell's equation (Buswell & Mueller, 1952). The calculated methane yield of cellulose as glucan ($C_6H_{10}O_5$) is 415 mLCH_4/g

cellulose and of hemicellulose as xylan ($C_5H_8O_4$) is 424 mLCH_4/g hemicellulose at STP on dry basis, respectively.

2.5 Analytical methods for aqueous and gaseous samples

Total solid, volatile solid and ash contents were characterized using gravimetric method following National Renewable Energy Laboratory's (NREL) (Sluiter et al., 2008a). The polysaccharide and lignin of RW were analyzed according to Sluiter et al. (2008b) and Goering and Van Soest (1970), respectively.

The concentration of sugar species and by-products (furfural, HMF, and acetic acid) in the washing liquid was measured with Water e2695 HPLC, equipped with a refractive index detector (RID) using 300 mm × 7.8 mm Aminex HPX-87H Ion Exclusion Column (Bio-Rad, USA) operating at 40 °C with 0.005 M H_2SO_4 as mobile phase at a flow rate of 0.6 mL/min. The glucose in the enzymatic hydrolyzate and sugar species in carbohydrate content of RW were measured using HPLC (Agilent 1100) equipped with a refractive index detector (RID) using 300 mm × 7.8 mm Aminex HPX-87H Ion Exclusion Column (Bio-Rad, USA) at 55 °C with 0.4 mL/min eluent of 0.005 M H_2SO_4.

Biogas composition from anaerobic digestibility test was analyzed by gas chromatography (GC 7820A Agilent Technologies) equipped with a thermal conductivity detector (TCD) and a stainless steel packed column SS Hayesep Q80/100 (6 m x 1/8 in.) using helium as carrier gas.

2.6 Analytical methods for physical properties of solid samples

Microstructure of Un-RW and SE-RW were analyzed by scanning electron microscopy (SEM), model Quanta 400, FEI, Czech Republic. The total surface area and average adsorption pore diameter of the samples were determined according to the method of Brunauer, Emmett,

and Teller (BET) using the surface area and porosity analyzer, ASAP2460, Micromeritics, USA equipped with a thermal conductivity detector. Fourier Transform Infrared Spectrometer, Vertex 70, Bruker, Germany with Pellet KBr technique was used to identify functional groups of the samples. Crystallinity of the sample was measured by X-ray diffractometer (XRD), model X'Pert MPD, PHILIP: XRD (The Netherland) using CuKα (λ = 1.54 A°) generated at 40 kV, 30 mA. The samples were scanned from 5 to 90 degree 2θ in a step size of 0.05 degree. Crystallinity was determined according to Eq. 8 (Segal et al., 1959).

$$\text{CrI}\ (\%) = \left[\frac{I_{002} - I_{am}}{I_{002}}\right] \times 100 \qquad (8)$$

where CrI is the crystallinity (%), I_{002} represents the maximum intensity for the crystalline portion of biomass at 2θ around 22.4°, and I_{am} is the peak for the amorphous portion at 2θ around 18.4°.

2.7 Statistical analysis

The Minitab statistical software 16 was used for statistical analysis including analysis of variance *(*ANOVA*)*. Multiple means were compared with one-way ANOVA followed by Fisher's Least Significant Difference method for post hoc–comparison. The level of significance was set at $p < 0.05$.

3. Results and Discussion

3.1 Chemical composition of exploded RW

Rubber wood waste was consists of 44.87% glucan (cellulose), 14.51% xylan (hemicellulose) and 13.12% lignin (Table 1). With its high polysaccharide content as holocellulose of 59.38% and lower lignin content made RW a potential feedstock for various

bioenergy conversions. SE pretreatment clearly improved degradation of xylan by 3.50% while glucan and lignin content increased by 0.80 and 1.34 %, respectively at severity of 3.37 compared to Un-RW (Table 1). One advantage of SE pretreatment of RW is that glucan as a main fraction of bioenergy feedstock was preserved whereas degradation of xylan could improve active area for accessible enzyme (Table 2) at severity of 4.03 and 4.35. Reduction of xylan content was caused by the degradation of hemicellulose which took place at temperature above 150 °C (Li et al., 2016) as our SE pretreatment was performed between 191 and 214 °C. Compared to glucan, the amorphous short chain structure of hemicellulose is easier degrade because of its lack of crystallinity (Li & Khanal, 2017) as indicated by the higher xylose concentration in the washing liquid (Table 3). Without external chemical addition, hydrolysability of biomass during pretreatment could be accelerated by auto-hydrolysis reaction that occurred as a result of acetic acid formation from the degradation of acetyl groups in hemicellulose. With an increasing severity to 4.03 and 4.35, the large amount of acetic acid (Table 3) released acted as a catalyst. This acid catalyzed the hydrolytic reaction of hemicellulose into monomeric sugar (Imman et al., 2015) and subsequently degraded to furfural (Chen et al., 2014) resulted in a dramatic reduction of xylan content in pretreated RW to 4.23 and 3.51%, respectively. SE pretreatment could thus contribute to the combined effects of thermal and acetic acid-catalytic reactions to disrupt RW amorphous structure in particularly. The efficiency of SE pretreatment will be further discussed in the enzymatic and anaerobic digestibility assays performed.

Table 1 Chemical compositions of untreated and steam exploded RW

Steam explosion conditions		Solid recovery (%)[a]	TS (%)[b]	VS (%)[a]	Glucan (%)[a]	Xylan (%)[a]	Lignin (%)[a]
Pressure/RT	Severity						
Un-RW	-	100±0	95.2±0.55	98.2±0.20	44.87±1.58	14.51±0.20	13.12±0.09
12 bar-1 min	2.70	99.1±0.45	97.4±0.15	98.7±0.06	43.72±4.59	11.28±0.71	12.75±0.14
12 bar-5 min	3.37	97.9±0.10	95.3±0.14	98.9±0.08	45.67±7.89	11.01±1.58	14.46±0.28
16 bar-5 min	3.73	93.2±0.65	97.8±1.03	99.0±0.07	47.77±0.79	9.29±0.53	16.86±0.13
16 bar-10 min	4.03	89.6±0.40	96.3±0.09	98.7±0.08	49.48±0.39	4.23±0.20	16.39±0.19
20 bar-10 min	4.35	82.2±0.57	96.9±0.02	98.4±0.15	51.84±0.54	3.51±0.56	17.30±0.10

[a] Mass percentage

[b] Solid content-as wet weight

Table 2 Structural analysis of Un-RW and SE-RW

Steam explosion severity	Crystallinity (%)	Surface area (m^2/g)	Adsorption average pore diameter (A°)
Un-RW	69.6	0.5617	115.93
4.03	77.2	0.6646	121.05
4.35	84.0	0.9795	143.47

Table 3 Yields of soluble sugar and degradation products of washing liquid

Steam explosion severity	Concentration (g/L)				
	Glucose	Xylose	Acetic acid	Furfural	HMF
2.70	0.12 (0.04-0.19)	0.26 (0.13-0.46)	0.54 (0.50-0.57)	n.d.	n.d.
3.37	0.14 (0.04-0.20)	0.44 (0.33-0.52)	0.97 (0.83-1.09)	n.d.	n.d.
3.73	0.36 (0.31-0.40)	1.01 (0.96-1.06)	2.05 (1.74-2.29)	n.d.	n.d.
4.03	0.82 (0.57-1.11)	2.43 (1.87-3.02)	4.75 (4.42-5.08)	0.95 (0.80-1.06)	0.51 (0.37-0.63)
4.35	1.50 (1.29-1.63)	2.72 (2.38-2.92)	7.83 (7.69-8.12)	3.40 (3.15-3.73)	2.48 (2.44-2.57)

Note n.d.= not detected, the value in the parenthesis represents the range of data.

3.2 Chemical composition of washing liquid from exploded RW

The water-soluble products accumulated on surface of pretreated RW were removed by deionized water. The composition of this washing liquid which makes up of monomeric sugars and degradation products is shown in Table 3. The pretreatment intensity had a strong influence on the formation of soluble products particularly furfural and HMF which were initially derived from xylose and glucose, respectively, at high severity of 4.03. These dehydration products are considered damaging to the cellular membrane and inhibitive to metabolism of microorganisms in anaerobic digestion. Both furfural and HMF completely inhibited the specific methanogenic activity at 2.0 g/L (Ghasimi et al., 2016). The inhibitory levels of furfural and HMF (0.12 and 0.02 g/L, respectively) have been reported for reducing glucan conversion in enzymatic hydrolysis (Pengilly et al., 2016). Thus, washing of SE-RW was necessary to eliminate inhibitory effects and helped preserve the degree of enzymatic hydrolysability and anaerobic digestibility of the pretreated RW solid.

3.3 Methane production of steam exploded RW

Methane production of 10.9 L CH_4/kg-VS was obtained from Un-RW solid fraction after 28-day anaerobic digestion assay (Figure 1). According to methane generation, SE pretreatment could be divided into 3 groups; low (2.70-3.37), medium (3.73-4.03) and high (4.35) severities. Compared to Un-RW, methane production was significantly enhanced (*p*-value <0.05) with an increasing SE severity from medium to high.

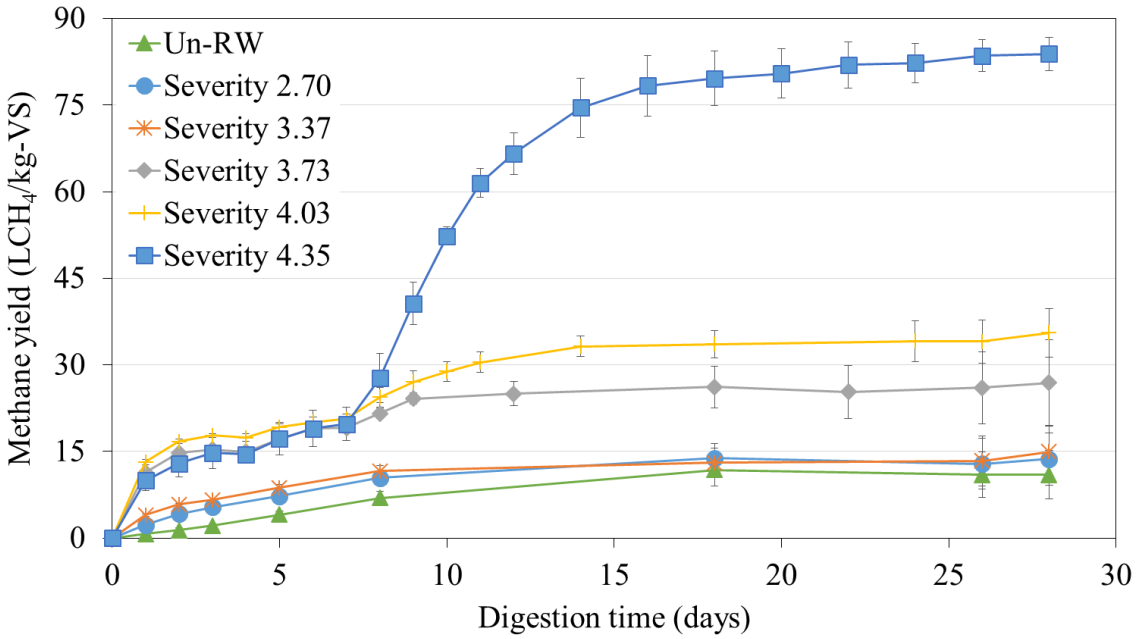
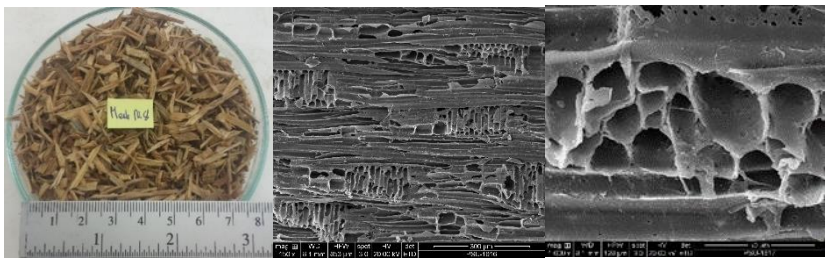


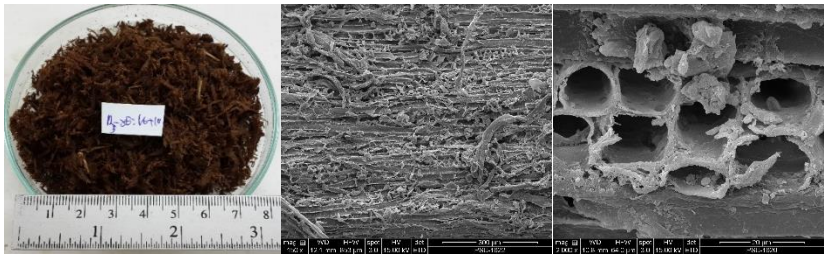
Figure 1 Cumulative methane yield in anaerobic digestion assays of Un-RW and SE-RW with difference degree of severities

Methane yield reached maximum value of 83.9 L CH_4/kg-VS at severity 4.35, interestingly with a 2-step pattern. During the first seven days of digestion, SE-RW was gradually degraded, as a result, the methane production increased slowly up to 19.8 LCH_4/kg-VS. It rapidly evolved during days 7 to 13 of digestion by 3.8 folds (from 19.8 to 74.5 LCH_4/kg-VS). The sharp increase in methane yield might be a result of the weakening biomass cell wall to the point that was sufficient for anaerobic digestion to destroy its structure as observed by SEM images (Figure 2c). The SE pretreatment at severity 4.35 showed intensive physical defects that the sudden thermal explosion had deconstructed the RW cell, also evidenced by the drastic shift to smaller size distribution compared to SE-$RW_{4.03}$ and Un-RW (Figure 2 b and a, respectively).

(a) Un-RW



(b) SE-RW at severity of 4.03



(c) SE-RW at severity of 4.35

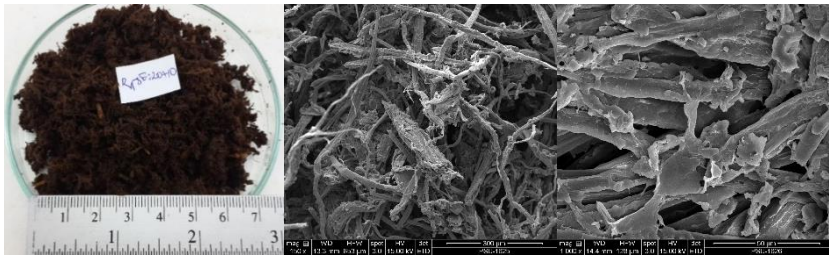


Figure 2 SEM photos of the morphological surface of (a) Un-RW and SE-RW at severity of 4.03 (b) and 4.35 (c), at magnification of 150X and 1000X

In comparison to Un-RW, an intensive auto-catalytic reaction with acetic acid generated as high as 31.31 g/kg-RW took place as a result of changes in functional group in structure of SE-RW. Figure 3 shows similar behavior by FTIR spectra but different transmission intensities between Un-RW and SE-$RW_{4.35}$ particularly the wavenumber around 1734 cm^{-1} which corresponds to the C=O functional group. This carbonyl group is a characteristic peak of ester-linked between hemicelluloses and lignin (Phitsuwan et al., 2015; Siddhu et al., 2016). Reduction of this peak intensity indicated that SE pretreatment was

effective to improve anaerobic digestibility of SE-RW by breaking down ester bond in hemicelluloses.

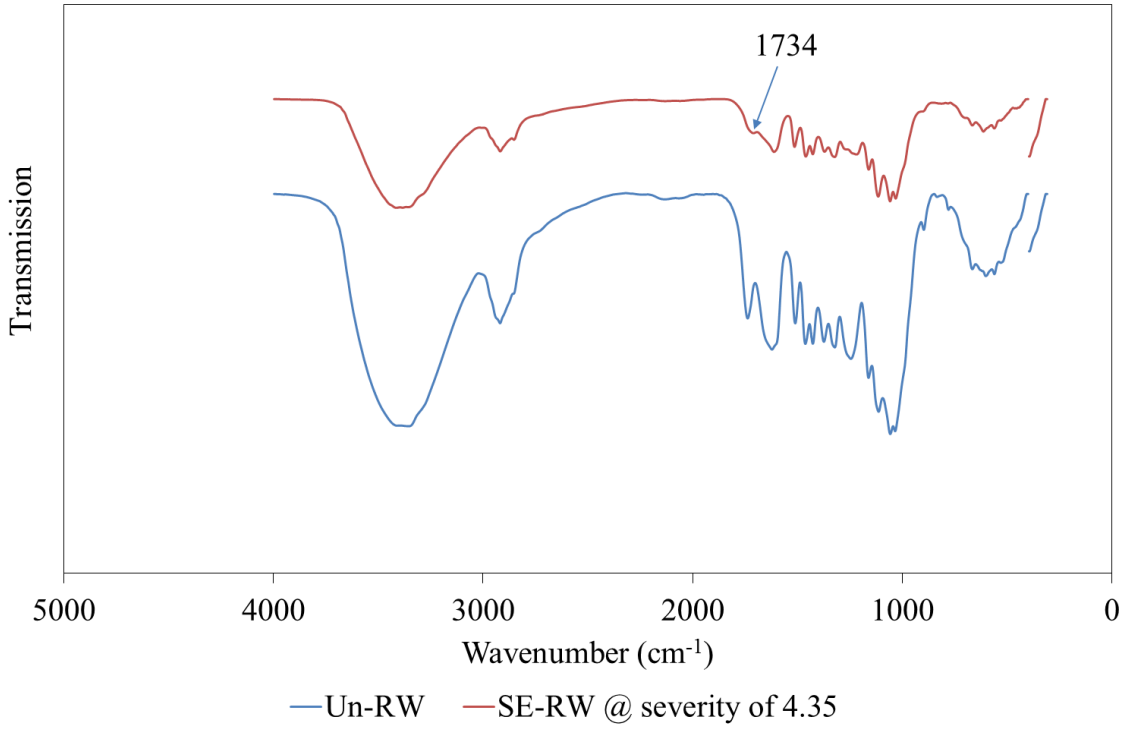


Figure 3 FTIR spectra of Un-RW and SE-RW at severity 4.35

3.4 Enzymatic hydrolysis of SE-RW

In addition to the direct anaerobic digestion of the SE-RW, the other objective is to evaluate the enzymatic hydrolysibility of the SE pretreated RW. In this study, cellulase Cellic®CTec2 formulated to target cellulose hydrolysis with aggressive β-glucosidase activity was used. Without SE pretreatment, hydrolysability of Un-RW generated glucose yield in range of 2.91±0.11 to 7.37±0.24% with an enzyme loading from 3 to 30 FPU/g. In contrast, the glucose yield of SE-RW was successfully improved with both increasing severity of SE pretreatment and enzyme loading which was shown in Figure 4 (a). Moreover, cellobiose, a disaccharide, was also one of enzymatic hydrolysis yields which was derived from glucan. In order to estimate glucan conversion in enzymatic hydrolysis step, summation of glucose and cellubiose yields was determined as shown in Figure 4 (b). The glucan conversion (GC) showed

similar behavior to glucose yield. The highest glucan conversion of 45.20±1.59 was achieved at severity 4.35 and enzyme loading 30 FPU/g. The hydrolysability behaviors of the cellulose in RW correlated with intensity of severity applied. The glucan conversion at different enzyme loadings fell closely on the lower and medium severities whereas ones with high severity fell widely. This hydrolysability at high severity of 4.35 could be attributed to large values of both active area and adsorption pore average diameter of SE-RW (Table 2), which associated with the reduction in hemicellulose providing improved accessibility to cellulose in SE-RW. Additionally, an increased CrI value (Table 2) indicates the exposure of the reactive cellulose portion in the pretreated biomass to enzyme adsorption (Phitsuwan et al., 2016). The active area and chemical changes in SE-RW structure in turn enhance the enzymatic hydrolysis of cellulose with addition of enzyme loading.

Cellic®CTec2 contains not only cellulase activities but also an endo-xylanase activities which has been proven to promote xylan degradation on Triticale straw (Pengilly et al., 2016). Figure 4 c shows the enzymatic hydrolysis profiles of xylan conversion. The efficiency of xylan conversion obtained was higher than glucan conversion. SE-RW contained a relatively low initial xylan content with an increasing severity (Table 1), and it is possible that the xylan fraction was present more in hydrolysable forms. Highest xylan conversion was achieved at severity of 4.03 while lower value was found with an increasing severity to 4.35. This could be that there was competition for adsorbing cellulose by cellulases and xylaneses since much reduction of hemicellulose led to the increase in pore size diameter (Table 2) of the cell walls and unwinding of the cellulose bundles (Figure 2). This phenomenon could certainly improve accessibility to cellulose of pretreated RW.

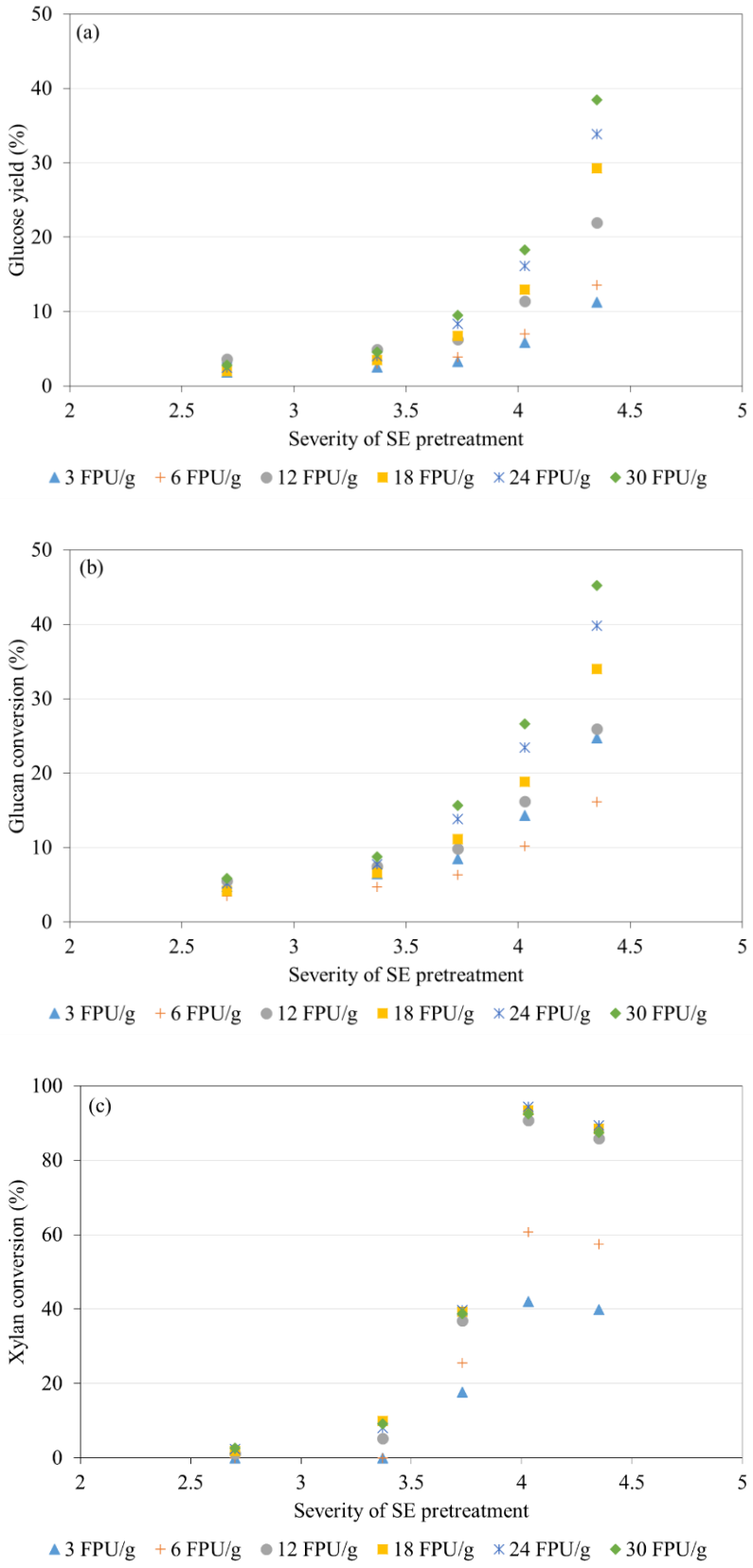


Figure 4 Enzymatic hydrolysis yields of SE-RW (a) glucose yield, (b) glucan conversion and (c) xylan yield

The influence of SE severity and enzyme loading (EL) of enzymatic hydrolysis on glucose yield, glucan conversion, and xylan conversion was further analyzed by fitting the data (Figure 4) to quadratic model using MINITAB 16 software. The model coefficients are presented in Table 4. The *p*-value for all three models: (1) glucose yield, (2) glucan conversion and (3) xylan conversion, indicated that these models were statistically significant (*p*-value <0.05). According to the coefficients, both severity and enzyme loading were influential factor for production of all enzymatic hydrolysis yields. Furthermore, the significant quadratic interaction of severity showed that all enzymatic hydrolysis yields could reach a maximum value with severity adjustment. Reduction of solid recovery after SE pretreatment with an intensive severity applied should be taken in consideration for product recovery per mass of native feedstock.

Moreover, insignificant quadratic function of enzyme loading means that applying intensive enzyme loading could not significantly enhance hydrolysability of both glucan and xylan in SE-RW. It might be due to inhibitory effects of cellubiose to enzymatic hydrolysis which is in agreement with (Olsen et al., 2016). Our study cellubiose formation potentially increased with an addition of enzyme loading (data not show).

Table 4 Analysis of variance (ANOVA) of the quadratic model for the enzymatic hydrolysis yield of SE-RW

Terms	Glucose yield (%)		Total glucan conversion (%)		Xylan conversion (%)	
	Coefficient estimate	P-value	Coefficient estimate	P-value	Coefficient estimate	P-value
Model		0.000		0.000		0.000
b_0	159.807		160.884		194.245	
b_1*EL	-1.525	0.000	-1.758	0.000	-0.522	0.0136
b_2*Severity	-95.491	0.000	-96.213	0.000	-147.681	0.000
b_3*EL*Severity	0.567	0.000	0.529	0.000	1.0717	0.0416
b_4*EL^2	-0.00530	**0.420**	0.00707	**0.2991**	0.0753	**0.057**
b_5*Severity^2	14.080	0.000	14.650	0.000	26.141	0.0095
R^2		0.9433		0.9532		0.8609
Adjusted R^2		0.9315		0.9434		0.8320

Predicted R^2	0.8945	0.9182	0.7814
CV (%)	25.80	18.22	38.32

Note: Significant under 95% level of confidence

3.5 Methane production of SE-RW and SE+EH-RW

The effect of enzymatic hydrolysis with enzyme loading between 3 to 30 FPU/g was studied in addition to the previous SE pretreatments at severity of 4.03 due to higher xylan conversion in enzymatic hydrolysis step compared to other SE conditions.

In order to estimate chemical composition of $SE\text{-}RW_{4.03}$ which was enzymatically hydrolyzed by Cellic®CTec2 (SE+EH-RW), subtracting glucan and xylan content in SE-RW with glucan and xylan degraded in enzymatic hydrolysis experiment was performed. Figure 5 shows polysaccharide composition in SE+EH-RW obtained.

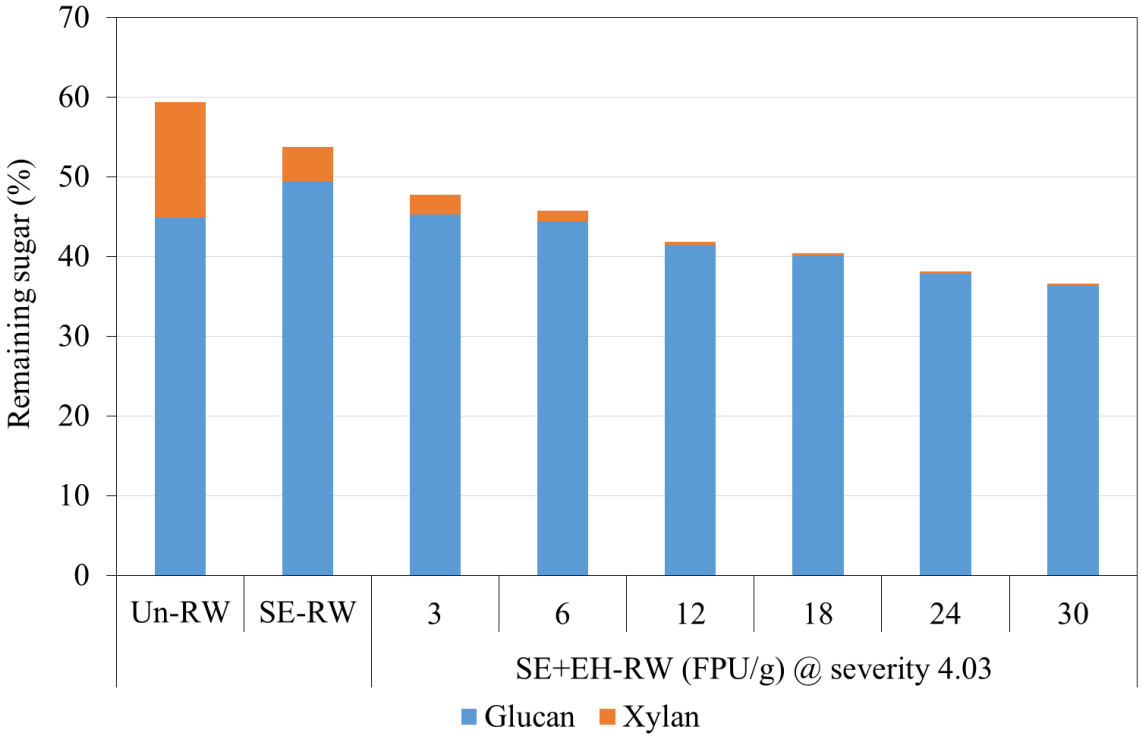


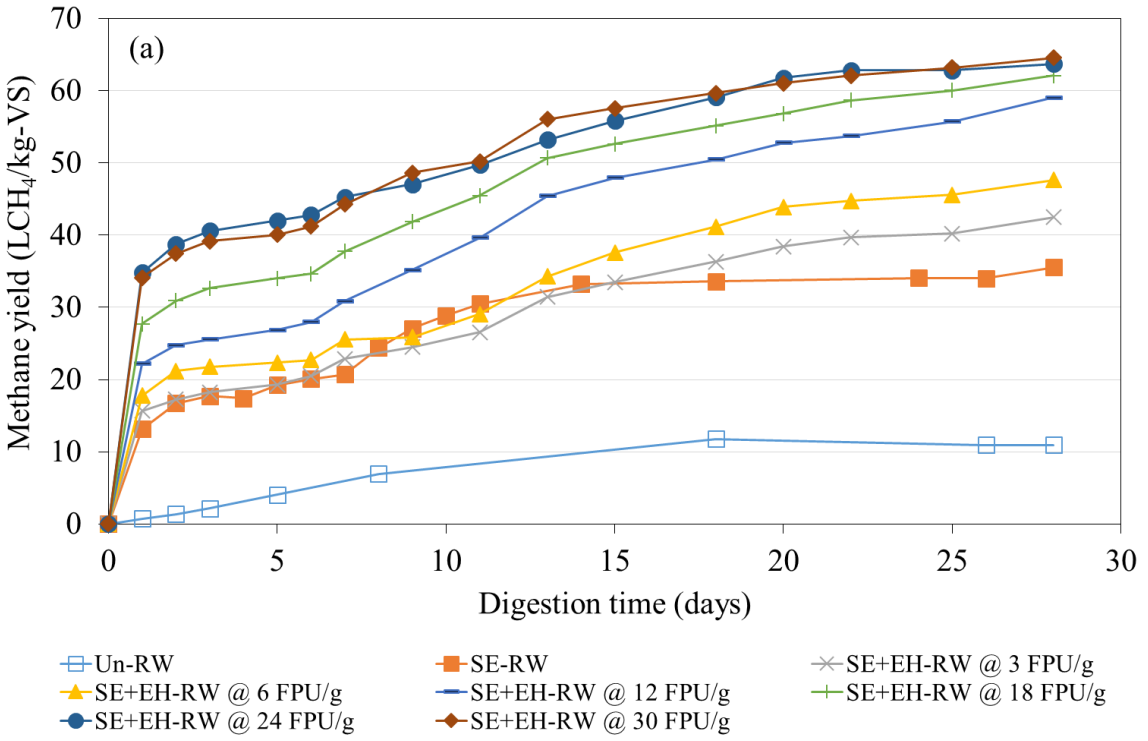
Figure 5 Remaining sugar content in Un-RW, SE-RW and SE+EH-RW at enzyme loading between 3 and 30 FPU/g

All SE+EH-RW samples were subjected in to anaerobic digestibility assays. Cumulated methane production profiles are presented in Figure 6 (a). In a combination of steam explosion and enzymatic hydrolysis, the initial methane production rate was obviously significantly faster during the first 2 days of digestion. It is noticeable that EH had clear effect on the SE-RW as methane yield increased from 8.61±0.91 to 38.77±1.24 LCH_4/kg-VS/day with an increasing enzyme loading from 3 to 30 FPU/g, while SE pretreatment gave only 5.98±0.13 LCH_4/kg-VS/day. This synergy effects of steam explosion and enzymatic hydrolysis contributed the acceleration of initial anaerobic digestibility to improve initial methane potential yield. Not only the quick start, the overall methane yield of the SE+EH-RW combination accomplished a significantly higher methane than only steam explosion for a 28-day digestion by 81.6% at enzyme loading of 30 FPU/g (Figure 6b).

Biodegradability index (BI) which is the ratio of methane yield to theoretical yield based on holocellulose content would be preferable for revealing degree of anaerobic bioconversion of substrate into methane. The SE+EH-RW with higher enzyme loading was successful in improving the anaerobic degradability of RW. As a consequence, higher BI was obtained (Figure 6 b). The highest BI of 41.1% was observed at enzyme loading of 30 FPU/g, while the lower was 15.7% obtained with SE severity 4.03. Polysaccharide fractions as glucan and xyan are considered the main carbon sources for biomethanation. In fact, disruption of biomass in enzymatic hydrolysis by cellulase enzyme caused depolymerization of both glucan and xylan. The cleavage of some β-glucan molecules into glucose could unwind the remaining cellulose bundles. Moreover, higher xylan conversions with an increasing enzyme loading are correlated with increased xylan hydrolysis. Reduction of hemicellulose as xylan (Figure 5) led to additional reactive area in the SE+EH-RW micro-structure (Imman et al., 2014) which in turn improves the accessibility of cellulose and remaining xylan for microbial enzyme. The

change in cellulose and hemicellulose properties could be the source of the increased anaerobic digestibility of SE+EH-RW.

Although higher volume of methane from SE+EH-RW was observed, approximately 58.9% of holocellulose (at enzyme loading of 30 FPU/g) left after digestion was not able to convert into methane. Thus, the biomethanation of SE+EH-RW could be justified as the final recovery for biodegradable carbon.



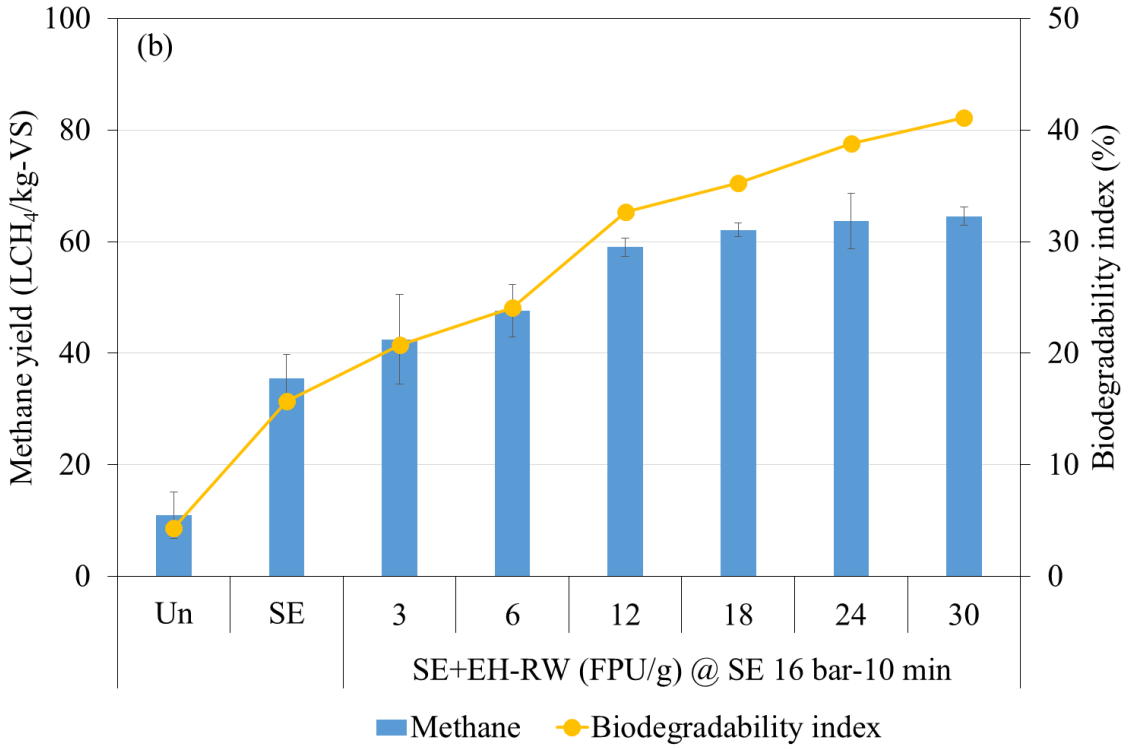


Figure 6 Comparison of (a) methane yield profile and (b) methane yield production and biodegradability index, between Un-RW, SE-RW and SE+EH-RW at enzymatic hydrolysis loading of 3 to 30 FPU/g

3.6 Product and energy yields

Our study showed that both efficiency of glucan conversion and biodegradability index could be enhanced by SE pretreatment resulted in improvement of glucose and methane yield, respectively (Table 5). Glucose is being a substrate in fermentation process for industrial ethanol production. Thus, in order to evaluate the possible energy recovery of glucose obtained from enzymatic hydrolysis of SE-RW, stoichiometry glucose to theoretical ethanol conversion was applied (Li & Khanal, 2017). The energy production per ton-RW was then evaluated at various options. Energy values of expected ethanol and methane were determined based on the higher heating value of 26.8 and 55.7 MJ/kg, respectively.

Highest total energy of 3833 MJ/ton-RW was obtained from the integrated process including steam explosion, enzymatic hydrolysis, and anaerobic digestion (SEA-process) (option 4) caused by enlargement of SE+EH-RW biodegradability. This methane contributed

57.9% of total energy potential production. Compared to a single process of SE, SE+EH-RW with SE severity 4.03 corresponds to 45.6% and 42.5% higher energy yield than only from glucose or methane with SE pretreatment at severity of 4.35 (option 2). In thermal pretreatment, energy cost is considered as one of the main factors that influences overall project appraisal and investment. In comparison to SE severity 4.35, lower energy supply to SE pretreatment at severity 4.03 with an integrative SEA-process showed an improvement of overall energy yield from rubber wood waste. Moreover, microbial inhibitors such as furfural and HMF were also minimized at severity 4.03 (Table 3) which could take into consideration for other purposes based on zero-waste strategy.

Table 5 Conversion efficiency, product yields and energy yields

Options	Process	Conversion efficiency (%)	Product yields (kg-glucose/ton-RW or m^3CH_4/ton-RW)	Energy yields (MJ/ton-RW)
1. Un-RW	EH	GC = 11.03	16.42	224
	AD	BI = 4.34	7.82	310
2. SE-$RW_{4.35\text{-}30FPU/g}$	EH	GC = 45.20	192.62	2633
	AD	BI = 35.78	67.66	2690
3. SE-$RW_{4.03\text{-}30FPU/g}$	EH	GC = 26.62	118.02	1613
	AD	BI = 15.72	31.44	1250
4. SE-$RW_{4.03\text{-}30FPU/g}$ + SE+EH-$RW_{4.03\text{-}30FPU/g\text{-}AD}$	EH+AD	GC = 26.62	118.02	1613
		BI = 41.10	55.84	2220
				Total = 3833

Note: [a]EH = enzymatic hydrolysis and [b]AD = anaerobic digestion

These results suggest that enzymatic hydrolysis of SE-RW followed by anaerobic digestion is a good alternative for bioenergy production in the forms of glucose and methane. Additionally, higher lignin residue after SEA process, due to no degradation of lignin during enzymatic hydrolysis as well as in anaerobic digestion, are of interest. This lignin-rich and purer solid from residue of the SEA-process has higher heating value and less impurities that can be an excellent fuel, or could be used as material to produce higher value products. Thus,

the valuation of RW thru bioenergy and biomaterial production will increase if co-production of sugar, biogas and lignin-rich biomass are realized.

4. Conclusions

Severity of steam explosion played a vital role to improve the production of glucose and methane from rubber wood waste by causing physical and chemical structural changes as well as reduction of hemicellulose xylan contents. Production of glucose thru enzymatic hydrolysis followed by anaerobic digestion of enzymatically hydrolyzed residue of the proposed SEA-process with lower severity of 4.03 of steam explosion pretreatment has great potential to improve energy recovery compared to either glucose or methane production alone even at a higher severity of 4.35. While the SEA-process enhanced technical feasibility of bioenergy production from rubber wood waste, valuated end use of the purified processed rubber wood residue will be key for its economic feasibility which is subject to further research.

5. Acknowledgements

This research was financially supported by Thailand Research Fund (TRF) contract no. MRG5980051 as well as by the Thailand's Education Hub for Southern Region of ASEAN Countries (TEH-AC) and Graduate School of Prince of Songkla University. The authors would like to thank Panel Plus Co. Ltd. for rubber wood waste, Chalong Latex Industry Co., Ltd. Songkhla, Thailand for anaerobic inoculum and Department of Mathematics and Statistics, Faculty of Science, Prince of Songkla University for statistical analysis. The authors would also like to recognize the support from the Biogas & Biorefinergy Laboratory, Prince of Songkla University.

6. References

Adney, B., Baker, J. 2008. Measurement of Cellulase Activities Laboratory Analytical Procedure (LAP) (Technical Report NREL/TP-510-42628 January 2008). National Renewable Energy Laboratory (NREL).

Asada, C., Sasaki, C., Uto, Y., Sakafuji, J., Nakamura, Y. 2012. Effect of steam explosion pretreatment with ultra-high temperature and pressure on effective utilization of softwood biomass. *Biochemical Engineering Journal*, **60**, 25-29.

Ballesteros, I., Ballesteros, M., Cara, C., Sáez, F., Castro, E., Manzanares, P., Negro, M.J., Oliva, J.M. 2011. Effect of water extraction on sugars recovery from steam exploded olive tree pruning. *Bioresource Technology*, **102**(11), 6611-6616.

Buswell, A.M., Mueller, H.F. 1952. Mechanism of methane fermentation. *Ind. Eng. Chem.*, **44**(3), 550-552.

Center of Agricultural Information. 2016. Thailand rubber planting area. Thaniland Office of Agricultural Economics.

Chen, J., Zhang, W., Zhang, H., Zhang, Q., Huang, H. 2014. Screw extrude steam explosion: A promising pretreatment of corn stover to enhance enzymatic hydrolysis. *Bioresource Technology*, **161**(Supplement C), 230-235.

Cotana, F., Buratti, C., Barbanera, M., Lascaro, E. 2015. Optimization of the steam explosion and enzymatic hydrolysis for sugars production from oak woods. *Bioresource Technology*, **198**, 470-477.

Dechrugsa, S., Kantachote, D., Chaiprapat, S. 2013. Effects of inoculum to substrate ratio, substrate mix ratio and inoculum source on batch co-digestion of grass and pig manure. *Bioresource Technology*, **146**, 101-108.

Dererie, D.Y., Trobro, S., Momeni, M.H., Hansson, H., Blomqvist, J., Passoth, V., Schnürer, A., Sandgren, M., Ståhlberg, J. 2011. Improved bio-energy yields via sequential ethanol fermentation and biogas digestion of steam exploded oat straw. *Bioresource Technology*, **102**(6), 4449-4455.

Ghasimi, D.S.M., Aboudi, K., de Kreuk, M., Zandvoort, M.H., van Lier, J.B. 2016. Impact of lignocellulosic-waste intermediates on hydrolysis and methanogenesis under thermophilic and mesophilic conditions. *Chemical Engineering Journal*, **295**, 181-191.

Goering, H., Van Soest, P.J. 1970. Forage Fiber Analysis (Apparatus, Reagents, Procedures and Some Application), in: Agricultural Handbook. Agricutural Research Service, U.S. Department of Agriculture.

Imman, S., Arnthong, J., Burapatana, V., Champreda, V., Laosiripojana, N. 2014. Effects of acid and alkali promoters on compressed liquid hot water pretreatment of rice straw. *Bioresource Technology*, **171**, 29-36.

Imman, S., Arnthong, J., Burapatana, V., Champreda, V., Laosiripojana, N. 2015. Influence of alkaline catalyst addition on compressed liquid hot water pretreatment of rice straw. *Chemical Engineering Journal*, **278**, 85-91.

Iroba, K.L., Tabil, L.G., Sokhansanj, S., Dumonceaux, T. 2014. Pretreatment and fractionation of barley straw using steam explosion at low severity factor. *Biomass and Bioenergy*, **66**, 286-300.

Jacquet, N., Maniet, G., Vanderghem, C., Delvigne, F., Richel, A. 2015. Application of Steam Explosion as Pretreatment on Lignocellulosic Material: A Review. *Industrial & Engineering Chemistry Research*, **54**(10), 2593-2598.

Kalyani, D.C., Zamanzadeh, M., Müller, G., Horn, S.J. 2017. Biofuel production from birch wood by combining high solid loading simultaneous saccharification and fermentation and anaerobic digestion. *Applied Energy*, **193**, 210-219.

Li, C., Liu, G., Nges, I.A., Liu, J. 2016. Enhanced biomethane production from Miscanthus lutarioriparius using steam explosion pretreatment. *Fuel*, **179**(Supplement C), 267-273.

Li, Y., Khanal, S.K. 2017. *Bioenergy:Principles and Applications*. Wiley Blackwell, United States of America.

Lizasoain, J., Trulea, A., Gittinger, J., Kral, I., Piringer, G., Schedl, A., Nilsen, P.J., Potthast, A., Gronauer, A., Bauer, A. 2017. Corn stover for biogas production: Effect of steam explosion pretreatment on the gas yields and on the biodegradation kinetics of the primary structural compounds. *Bioresource Technology*, **244**(Part 1), 949-956.

Olsen, J.P., Alasepp, K., Kari, J., Cruys-Bagger, N., Borch, K., Westh, P. 2016. Mechanism of product inhibition for cellobiohydrolase Cel7A during hydrolysis of insoluble cellulose. *Biotechnology and Bioengineering*, **113**(6), 1178-1186.

Overend, R.P., Chornet, E., Gascoigne, J.A. 1987. Fractionation of Lignocellulosics by Steam-Aqueous Pretreatments [and Discussion]. *Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences*, **321**(1561), 523-536.

Pengilly, C., Garcia-Aparicio, M.P., Diedericks, D., Gorgens, J.F. 2016. Optimization of Enzymatic Hydrolysis of Steam Pretreated Triticale Straw. *Bioenergy Research*, **9**(3), 851-863.

Phitsuwan, P., Charupongrat, S., Klednark, R., Ratanakhanokchai, K. 2015. Structural features and enzymatic digestibility of Napier grass fibre treated with aqueous ammonia. *Journal of Industrial and Engineering Chemistry*, **32**, 360-364.

Phitsuwan, P., Sakka, K., Ratanakhanokchai, K. 2016. Structural changes and enzymatic response of Napier grass (Pennisetum purpureum) stem induced by alkaline pretreatment. *Bioresource Technology*, **218**, 247-256.

Rabemanolontsoa, H., Saka, S. 2016. Various pretreatments of lignocellulosics. *Bioresource Technology*, **199**, 83-91.

Rincón, B., Banks, C.J., Heaven, S. 2010. Biochemical methane potential of winter wheat (Triticum aestivum L.): Influence of growth stage and storage practice. *Bioresource Technology*, **101**(21), 8179-8184.

Segal, L., Creely, J.J., Martin, A.E., Jr., Conrad, C.M. 1959. An Empirical Method for Estimating the Degree of Crystallinity of Native Cellulose Using the X-Ray Diffractometer. *Textile Research Journal*, **29**(10), 786-794.

Siddhu, M.A.H., Li, J., Zhang, J., Huang, Y., Wang, W., Chen, C., Liu, G. 2016. Improve the Anaerobic Biodegradability by Copretreatment of Thermal Alkali and Steam Explosion of Lignocellulosic Waste. *BioMed Research International*, **2016**, 10.

Singh, J., Suhag, M., Dhaka, A. 2015. Augmented digestion of lignocellulose by steam explosion, acid and alkaline pretreatment methods: A review. *Carbohydrate Polymers*, **117**, 624-631.

Sluiter, A., Hames, B., Hyman, D., Payne, C., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Wolfe, J. 2008a. Determination of Total Solids in Biomass and Total Dissolved Solids in Liquid Process Samples (Ed.) N.R.E.L. (NREL).

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D. 2008b. Determination of Structural Carbohydrates and Lignin in Biomass. National Renewable Energy Laboratory (NREL) Laboratory Analytical Procedures (LAP).

Vivekanand, V., Olsen, E.F., Eijsink, V.G.H., Horn, S.J. 2013. Effect of different steam explosion conditions on methane potential and enzymatic saccharification of birch. *Bioresource Technology*, **127**, 343-349.