Final Report

Project Title: Exploring cost effectiveness of bioethanol production from palm empty fruit bunch using waste glycerol as a solvent for pretreatment

By Dr. Supawadee Polprasert

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Project Granted by the Thailand Research Fund

Abstract

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Project Period: 2 years

1. Abstract:

This research aimed to enhance the production of fermentable sugar and bioethanol from palm empty fruit bunch (EFB). To accomplish the objectives of this study three parts were involved. The first part attempted to evaluate the effect of the type of solvent, pH, substrate loading, and reaction time on the chemical components of EFB. Steam pretreatment was set up at 121°C temperature and 1.18 bar pressure, using an autoclave (LS-2D, REXALL) with substrate loading of 5, 10, 15 and 20% (w/v) at reaction times of 15 and 60 min. Distilled water, waste glycerol, alkaline glycerol and acidic glycerol were used as solvents during steam pretreatment. The results showed that with distilled water, better pretreatment was achieved at 5% and 10% loading for 60 min. During the pretreatment with waste glycerol at 5% loading an increase on the reaction time from 15 to 60 min reaction resulted in a remarkable increase in reducing sugar in the liquid phase. Overall, the best condition of steam pretreatment was observed using alkaline glycerol at 5% w/v and 15 min reaction time, resulting in a holocellulose (cellulose plus hemicellulose) increase to 87.98% and a lignin decrease to 9.17%. The results suggest that waste glycerol during steam pretreatment of EFB can be utilized effectively at short reaction times and at an increased pH to achieve a high output of cellulose and hemicellulose for sugar conversion in the bioethanol fermentation process.

The second part targeted to optimize fermentable sugar production from mixed enzymes, Celluclast: Cellic CTec2: Celli HTec2 (1:1:1). Glucose and xylose were produced from pretreated EFB, detected by HPLC (Alliance-e2695, U.S.A). The highest glucose concentration was achieved at 96 hours of hydrolysis with 10% w/v of pretreated EFB with

distilled water for 60 minutes (146.69 g/L) while the highest of total sugar was obtained from 15-min steam pretreatment with alkaline glycerol (152.36 g/L).

In the fermentation experiment (third part), ethanol concentration was determined by a GC-FID (Agilent-6890N, U.S.A) and carried out in a 150 rpm shaker at room temperature. The highest concentration of 10.81 and 10.27 g/L were obtained from applying 5% *Saccharomyces cerevisiae* at 6 hours hydrolysis time, using EFB pretreated either distilled water for 60 minutes or alkaline glycerol for 15 minutes, respectively. It can be concluded that alkaline glycerol helps reduce the time required for pretreatment.

Keywords: Palm empty fruit bunch/ Steam pretreatment/ Waste glycerol/ Fermentable sugar/ bioethanol

บทคัดย่อ

งานวิจัยนี้มีจุดประสงค์หลักเพื่อศึกษาวิธีการเพิ่มปริมาณน้ำตาลและเอทานอลที่สกัดได้จากทะลายปาล์มเปล่าเพื่อใช้เป็นสารตั้งต้นในการหมักให้ได้เชื้อเพลิงชีวภาพหรือเอทานอล งานวิจัยถูกแบ่งเป็น 3 ส่วน โดยในส่วนแรกเป็นการศึกษาผลของชนิดสารละลาย ความเป็นกรด-ด่างของสารละลาย ปริมาณสารตั้งต้น และเวลาที่ใช้ในการให้ความร้อนและความดันด้วย autoclave (LS-2D, REXALL) ที่อุณหภูมิ 121 องศาเซลเซียล ความดัน 1.18 บาร์ ที่มีต่อส่วนประกอบของโครงสร้างทะลายปาล์มเปล่าที่มีความเข้มข้น 5 10 15 และ 20% (น้ำหนักต่อปริมาตร) ผลจากการศึกษาพบว่า การใช้น้ำกลั่นเป็นสารละลายนั้นมีผลดีต่อโครงสร้างเมื่อใช้ปริมาณสารตั้งต้นที่ 5 และ 10% ที่ระยะเวลา 60นาที ส่วนการใช้กลีเซอรอลที่ระยะเวลา 15 และ 60 นาทีนั้น พบว่ามีปริมาณน้ำตาลรีดิวซ์ในของเหลวหลังการบำบัดเพิ่มมากขึ้น ในภาพรวมทั้งหมดของงานวิจัยส่วนแรกพบว่าสภาวะที่ดีที่สุดจากการใช้สารตั้งต้น 5% กับกลีเซอรอลที่ถูกปรับให้เป็นด่าง โดยให้ความร้อนและความดันตามที่กำหนดเป็นเวลา 15 นาที ทำให้เปอร์เซ็นต์โฮโลเซลลูโลส (ผลรวมของเซลลูโลสและเฮมิเซลลูโลส)ที่เป็นโพลิเมอร์ของน้ำตาลมีค่าเพิ่มขึ้นไปถึง 87.98% และมีเปอร์เซ็นต์ลิกนินลดลงไปที่ 9.17%.จึงมีความเป็นไปได้ว่า กลีเซอรอลที่ถูกปรับให้มีความเป็นด่างนั้นมีประสิทธิภาพในการปรับโครงสร้างของทะลายปาล์มเปล่าให้มีโครงสร้างที่เหมาะแก่การย่อยให้ได้น้ำตาล

ตัวอย่างทะลายปาล์มเปล่าที่ผ่านการบำบัดข้างต้นทั้งหมด ถูกนำมาผ่านกระบวนการย่อยโดยการใช้เอนไซม์ 3ชนิด ได้แก่ Celluclast: Cellic CTec2: Celli HTec2 (1: 1: 1) ซึ่งพบว่าน้ำตาลที่สามารถผลิตได้มี 2 ชนิดคือ กลูโคสและไซโลสโดยตรวจวัดจากเครื่อง HPLC (Alliance-e2695, U.S.A) กลูโคสที่มีปริมาณมากที่สุดถูกพบจากการย่อยตัวอย่าง 96 ชั่วโมงที่ผ่านการบำบัดโดยใช้ตัวอย่าง10%w/v ต่อน้ำกลั่น 60 นาที (148.57g/L) แต่อย่างไรก็ตาม ผลรวมของกลูโคสและไซโลสที่มากที่สุดคือ 152.36 g/L จากตัวอย่าง 10%ที่ใช้กลีเซอรอลที่ถูกปรับให้เป็นด่างเมื่อย่อยเป็นเวลา 72 ชั่วโมง

เมื่อทำการหมักน้ำตาลเพื่อให้ได้เอทานอลด้วยยีสต์และตรวจวัดเอทานอลด้วยเครื่อง GC-FID (Agilent-6890N, U.S.A) ณ สภาวะการแกว่งผสม 150 รอบต่อนาที ที่สภาวะอุณหภูมิห้อง ผลปรากฏว่าการหมักด้วย *Saccharomyces cerevisiae* เพียงชนิดเดียวเป็นเวลา 6ชั่วโมงให้ปริมาณเอทานอลสูงสุดคือ 10.81 และ 10.27 g/Lจากตัวอย่าง 10%ที่ผ่านการบำบัดด้วยคามร้อนและความดันโดยใช้น้ำกลั่นเป็นเวลา 60นาทีและกลีเซอรอลที่ถูกปรับให้เป็นด่างเป็นเวลา 15นาทีตามลำดับ ดังนั้นสามารถสรุปได้ว่าการบำบัดทะลายปาล์มเปล่าด้วยความร้อนและความดันโดยใช้สารละลายกลีเซอรอลที่ปรับให้เป็นด่างสามารถลดเวลาในการบำบัดได้สั้นกว่าการใช้น้ำกลั่น ซึ่งให้ผลเอทานอลใกล้เคียงกัน

2. Executive summary

2.1 Introduction to Research

Nowadays, bioethanol production from lignocellulosic materials such as agricultural residues, food and agricultural processing wastes is of great interest. The use of lignocellulosic waste presents a sustainable and renewable source because the starch-based product such as corn, sugarcane and potato are used as human food and animal feed, thereby resulting in limited supply and being too costly to produce bioethanol. In Thailand, palm empty fruit bunch (EFB) is one of the most abundant lignocellulosic waste (3.21 million ton per year) generated from crude palm oil mill (DEDE, 2014). Its cost is minimal but it has a high content of cellulose and hemicellulose, which can serve as raw material for bioethanol production (Shamsudin et al., 2012; Park et al., 2013). Because of the recalcitrance of the lignocellulosic structure, pretreatment of the raw material is necessary to enhance the accessibility of enzymes to the substrate (Njoku et al., 2013). The obstacles in the existing pretreatment processes include insufficient separation of cellulose and lignin, and high consumption of chemicals and/or energy. Moreover, pretreatment has been recognized as one of the most expensive processing steps in bioethanol production. Pretreatment technologies for lignocellulosic biomass include biological, physical, chemical methods and various combinations thereof. The pretreatment process depends on the objective of the biomass pretreatment and its economic assessment and environmental impact (Harmsen et al., 2010). The energy and chemical substances used in pretreatment should be cost effective. Therefore, pretreatment with steam generated during crude palm oil processing and waste glycerol produced from biodiesel production are chosen to be used. Moreover, waste glycerol can be used as co-substrate for bioethanol fermentation (Suzuki et al., 2014; Bensah and Mensah, 2013).

Steam pretreatment of EFB for ethanol production could be the most economical option to be implemented in a palm oil mill. During oil palm processing, steam is continuously used for electricity generation and for sterilizing the palm fresh fruit bunches. The steam is an inexpensive resource which is readily available in the mill (Shamsudin et al., 2012). Glycerol is a by-product of biodiesel production which accounts for about 10% (w/w) (Yang et al., 2012). The expansion of the biodiesel industry has thus created a surplus of waste glycerol. Waste glycerol, the liquid content after methanol recovery, is a non-toxic, high-boiling point organic solvent and has a pH around 10 (high alkaline pH). Thus, it is attractive to be used as a solvent for fractionating biomass in order to improve the economics of cellulosic ethanol (Bensah and Mensah, 2013). Previous research has

shown that glycerol has been used as a solvent for pretreatment of lignocellulosic biomass (Diaz et al.,2015; Zhang et al., 2015; Martin et al., 2011), or in combination with alkaline catalysts such as Na_2SO_3 and NaOH for delignification (Fu and Mazza, 2011; Novo et al., 2011). Up to now, glycerol pretreatment of lignocellulosic material has been investigated only for a few crop residues. Moreover, the combination pretreatment of palm EFB between steam and waste glycerol has never been studied.

Following pretreatment, mixed enzymes can be used to enhance cellulose and hemicellulose hydrolysis (Chen et al., 2012; Hamzah et al., 2011). Complete hydrolysis of cellulose results in glucose whereas the hemicellulose results in several pentoses and hexoses (Millati et al., 2011). Previous research has revealed that the low ethanol yields were obtained from hydrolysate of palm EFB because the native *Saccharomyces cerevisiae* could not assimilate all reducing sugar. Other pentose-consuming microorganisms must be evaluated with the aim to completely utilize the sugar released after pretreatment and hydrolysis processes (Piarpuzan et al., 2011). *Pichia stipitis* is one of the robust xylose-fermenting yeasts that has been investigated in many laboratories around the world because of its capacity for using pentose sugars beside hexoses with high ethanol yield (Njoku et al., 2013; Singh et al., 2014). Therefore, *Saccharomyces cerevisiae* and *Pichia stipilis* are selected to be used for improving the bioethanol fermentation.

In this study, the effects of steam and waste glycerol on enzymatic hydrolysis of palm EFB to produce bioethanol are investigated at a laboratory scale. Moreover, the co-cultures of two yeasts species *(Saccharomyces cerevisiae* and *Pichia stipitis)* are evaluated to enhance the bioethanol fermentation from hydrolysate of palm EFB.

2.2 Literature review

2.2.1 By-products from Crude Palm Oil Mills and Biodiesel Productions

1) Palm Empty fruit Bunch

Solid wastes and by-products generated in crude palm oil extraction processes are empty fruit bunches (EFB), fibers, shell, decanter cake, and ash from the boiler. Based on 1 ton FFB, the amounts of wastes and by-products are shown in Figure 1. Palm EFB is an abundant and low-cost residual by-product of crude palm oil mill. Moreover, EFB is a rich source of sugar, with a high content (75-80%) of cellulose and hemicellulose. Therefore, EFB may be a good source of bioethanol and other biofuel (Park et al., 2013). The components of EFB are shown in Table 1. In addition, other lignocellulosic biomass sources commonly found in Thailand are compared with palm EFB as presented in Table 2.

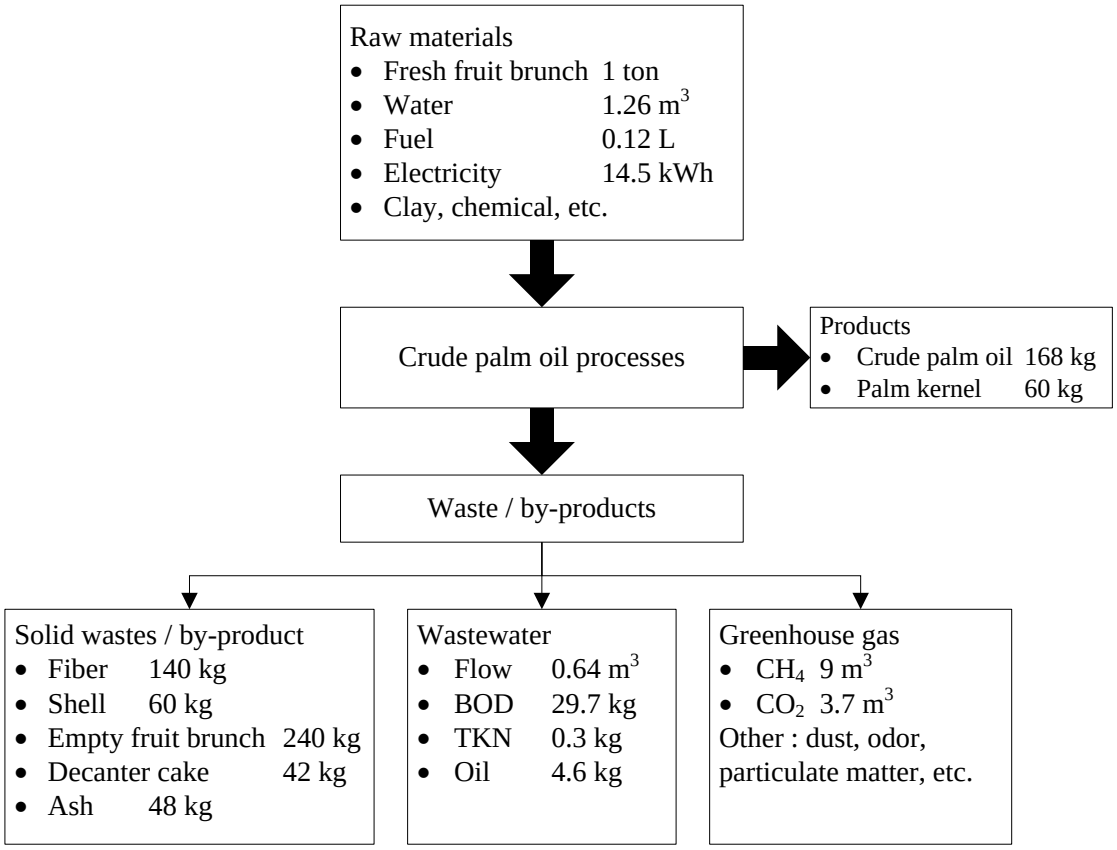

Figure 1 Average value of waste generation rate (per ton FFB) from five selected crude palm oil mills (Chavaprarit, 2006)

Table 1 The components of palm empty fruit bunch

Components	**Content (wt.%)**				
	Triwahyuni et al. (2015)	Sudiyani et al. (2013)	Park et al. (2013)	Hamzah et al. (2011)	Piarpuzan et al. (2011)
Cellulose	36.59	37.26	39.8±0.5	43.8±0.02	13.75±0.44
Hemicellulose	24.97	14.62	17.3±0.7	35.0±0.59	12.79±0.40
Lignin	26.53	31.68	28.8±0.6	16.4±0.23	7.79±0.08
Ash	1.79	6.69	3.8±0.5	-	0.63±0.04
Moisture	10.12	-	-	-	65.04±1.80
extractives		-	10.3±0.3	4.8±1.03	-

Table 2 Different lignocellulosic biomass sources (%dry weight)

Lignocellulosic biomass	Cellulose	Hemicellulose	Lignin	References
Palm empty fruit bunch	36.59-43.8	14.62-36.58	16.4-31.68	From Table 1
Palm press fiber	30.18	23.16	22.85	Ponthien and Cheirsilp, 2011
Corncob	33.7	31.9	6.1	
Corn stover	38.3	25.8	17.4	
Rice straw	31.1	22.3	13.3	Adapted from Mood et al., 2013
Soybean straw	34.5	24.8	9.8	
Sunflower stalks	42.1	29.7	13.4	
Sweet sorghum bagasses	27.3	14.5	14.3	
Sugarcane bagasses	37.6	23.9	39	Zhang et al., 2015

2) Waste Glycerol

Glycerol (or glycerine or 1,2,3-propanetriol) is a colourless, odourless, viscous and non toxic alcohol to both humans and the environment. The chemical formula of glycerol is $C_3H_5(OH)_3$. While boiling point, melting point and flash point of glycerol are 290°C, 18°C and 177°C, respectively. Glycerol can be obtained from biodiesel production which generated about 10% (w/w) glycerol. In other words, every 100 liters of biodiesel produced generates approximately 10 kilograms of glycerol (Yang et al., 2012). Although crude glycerol can be further upgraded to pharmaceutical, cosmetic and food industry applications, the extensive biodiesel production has already started to flood the market and drive the price down (Martin et al., 2011). The expansion of the biodiesel industry has thus created a surplus of glycerol, resulting in an inevitable abundance of waste glycerol now considered as a waste stream with associated disposal cost. The flow chart of biodiesel production is illustrated in Figure 2.

The chemical composition of crude glycerol mainly varies with the type of catalyst used to produce biodiesel, the transesterification efficiency, recovery efficiency of the biodiesel, other impurities in the feedstock, and whether the methanol and catalysts were recovered. The characteristics of waste glycerol from palm oil biodiesel production are shown in Table 3.

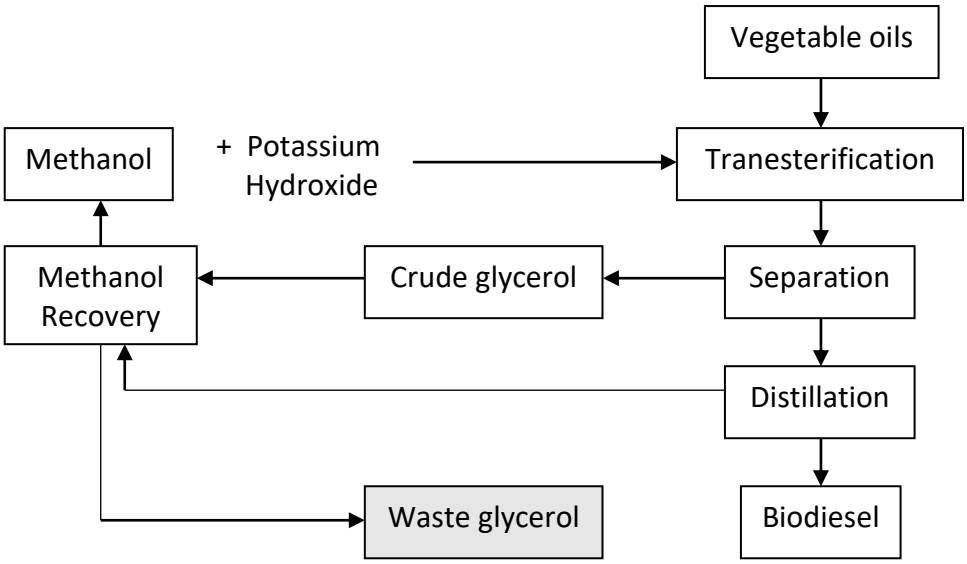

Figure 2 Flow diagram of biodiesel production and waste glycerol (adapted from Li et al., 2013)

Table 3 Characteristics of waste glycerol from palm oil biodiesel production (Xie et al., 2011)

Parameters	unit	Concentration
pH		9.7-10.4
COD	10^6 mg/L	1.7-1.9
BOD5	10^6 mg/L	0.9-1.2
TSS	10^6 mg/L	21.3-38.7
Total glycerol	g/L	413-477
Soap	10^6ppm	2.1-2.7
Methanol	g/L	112-203
Water	% by weight	9.3-11.9

2.2.2 Pretreatment and Enzymatic Hydrolysis of Lignocellulosic Material

Lignocellulose, the most abundant renewable biomass on earth, is composed mainly of cellulose, hemicellulose and lignin. Both the cellulose and hemicellulose fractions are polymers of sugars and thereby a potential source of fermentable sugars. Lignin can be used for the production of chemicals, combined heat and power or other purposes (Harmsen et al., 2010). The goals of the pretreatment are to decompose the polymeric components of the lignocellulose and form monomer sugars thus enhance enzymatic

conversion of the cellulose fraction that increases the digestibility of the material for microbial and enzymatic bioconversion and obtain a higher ethanol yield as shown in Figure 3.

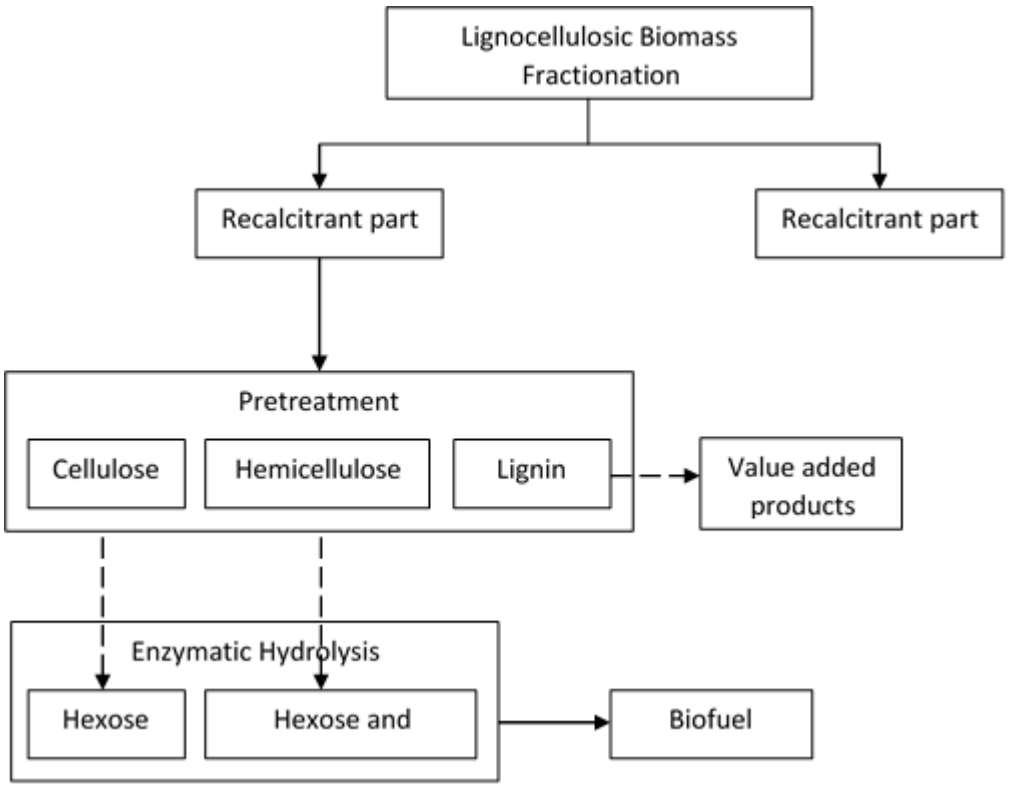

Figure 3 Lignocellulosic Biomass Fractionation (adapted from Singh et al., 2014)

Pretreatment is one of the most expensive steps in biological conversion of cellulosic biomass. An effective pretreatment should produce significant percent of cellulose supports, the lesser production of inhibitors and be cost effective (Singh et al., 2014). The review of pretreatment methods and sugar productions of palm EFB are summarized in Table 4.

Table 4 Pretreatment methods and sugar productions of palm EFB

Raw material	Pretreatment	Hydrolysis	Sugar production	References
EFB	Dilute acid, 161.5°C, 9.44 min and 1.51% acid loading	Commercial cellulose cocktail	85.5% glucose yield	Chiesa and Gnansounou, 2014
EFB	Water, Acid and Alkaline pretreatment	Celluclast with activity 70 FPU/ml	Total glucose yields were increase from 2.6% to 34.9% (water), 34.2% (acid) and 27.9% (alkaline) pretreatment	Ying et al., 2014
EFB	Steam pretreatment (140°C. 0.28Mpa, 15 min)	Celluclast with activity 117.6 FPU/ml	The sugar yield was 0.21 g/g EFB	Shamsudin et al., 2012
EFB	Soaked with NaOH solution and autoclaved for 15 min at 121°C	Cellulase and β 1-4 glucosidase with the ratio of 5:1	The maximum glucose concentration of 2.4 g/L	Hamzah et al., 2011
EFB	Soaked with 2% NaOH 4 h at 30°C and autoclaved for 6 min at 121°C and 117 kPa	Cellulase cocktial	The reducing sugar yield was 0.59 g/g EFB	Piarpuzan et al., 2011

From this table, the effective pretreatment methods generally are stream, acid, and alkaline pretreatments. After initial biomass processing, the production of fermentable sugars from biomass is usually acchived in two steps:

1. A pretreatment process in which the cellulose polymers are made accessible for further conversion. In this step hydrolysis of hemicellulose may occur, as well as separation of the lignin fraction, depending on the process applied. The example of pretreatment techniques is showed as follow.

a. Steam pretreatment

Biomass is treated with high pressure saturated steam at temperature about 160-240°C and pressure between 0.7-4.8 MPa. Different studies have reported that steam

pretreatment can efficiently hydrolyze the hemicelluloses (partially), modify the lignin, increase the surface area and decrease the cellulose crystallinity and degree of polymerization (Singh et al., 2014). The mass balance for steam pretreated under pressure is illustrated in Figure 4.

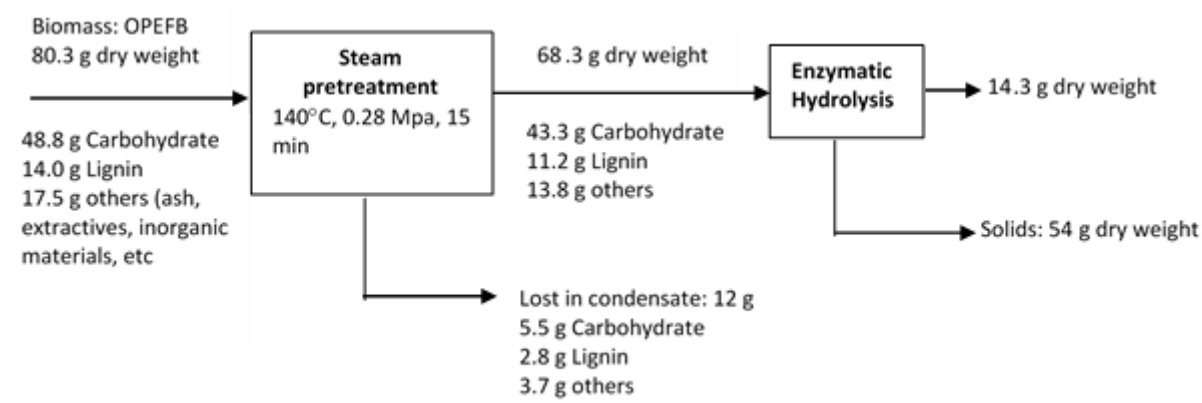

Figure 4 Mass balance for steam pretreatment of EFB after 15 min (Shamsudin et al., 2012).

b. Glycerol pretreatment

Pretreatment generally leads to significant delignification and/or improved fibre porosity, thereby improving cellulose accessibility to cellulase and it is commonly refer to as “organosolv” pretreatment (Zhang et al., 2015). Organic solvents i.e. methanol, ethanol, acetone, glycerol, ethylene glycol and tetrahydrofurfuryl alcohol, with or without the addition of a catalyst agent could be used in organosolv process (Mood et al., 2013). The catalysts used include organic or inorganic acid (Hydrochloric and sulfuric acid), and bases (sodium hydroxide, ammonia and lime). Glycerol is a non-toxic, high boiling-point (290 $^{\circ}$C) organic solvent generated in large quantities as a by-product in biodiesel factories. The increasing production of biodiesel has significantly reduced the price of industrial-grade glycerol in recent years. Glycerol has been used as a standalone solvent, or in combination with alkaline catalysts such as Na_2CO_3 and NaOH, for delignification and pretreatment of lignocellulosic biomass (Zhang et al., 2015). The glycerol organosolv pretreatment (GOP) can effective disrupt the complex, recalcitrant structure of lignocellulosic substrates (Sun et al., 2015). In addition, Guragain et al. (2011) investigated the best conditions in the use of crude glycerol (water : glycerol = 1 : 1) in pretreating wheat straw at 230°C for 4h.Enzymatic hydrolysis of pretreated wheat straw produced reducing sugar yields (mg/g of sample) of 423 and 487 for crude and pure glycerol, respectively, compared to a low yield

of dilute acid. Moreover, Sun and Chen (2008) reported that the aqueous glycerol pretreatment can enhance enzymatic hydrolysis of wheat straw. With a liquid-solid ratio of 20g/g at 220^{O}C for 3h, 70% hemicelluloses and 65% lignin were removed, however 98% cellulose was retained. The pretreated fiber was achieved with 90% of enzymatic hydrolysis yield after 48h. Nevertheless, enzymatic hydrolysis was inhibited slightly with 2.0 wt% glycerol (Zhang et al., 2015).

2. Enzymatic cellulose hydrolysis to fermentable sugars using cellulase enzyme cocktails produced on location or acquired from enzyme manufacturers (Chen et al., 2012; Harmsen et al., 2010). Hydrolysis process separates long chain of carbohydrate (from cellulose or starch) with addition of water molecule and is usually catalyzed by enzyme or acid (Aditiya et al., 2016). Hydrolysis of cellulose produces mostly the glucose (6-carbon) whereas hemicellulose produces mostly pentose sugars (Palamae et al., 2017). Hydrolysis and fermentation process can be achieved by several process strategies. They include separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), prehydrolysis and simultaneous saccharification and fermentation (PSSF) (Triwahyuni et al., 2015). The efficacy of enzyme hydrolysis is highly dependent upon the type of substrate. The pretreatment process which dictates the reactivity of the cellulose ease of solubilization and hydrolysis of hemicellulose (Saville et al., 2016). Moreover the pretreatment, the type of enzyme is mainly effect to concentration of sugars from hydrolysis process. Different preparations of cellulose, hemicellulose and β-glucosidase enzyme was studied on enzymatic hydrolysis of the palm date fibers. The best results for hydrolysis yields of xylose, galactose, arabinose and mannose from the hemicellulose were obtained when using the mixture of cellulase, β-glucosidase and hemicellulase enzymes while using only hemicellulase had no effect on glucan or other polysaccharides using (Shafiei et al., 2010). For lignocellulosic biomass, the general type of enzyme is commercial enzyme that specific for hydrolyzation. Hassan et al. (2013) found that the optimum sugar recovery from EFB pretreated with steam and 5% acetic acid (xylose and glucose) was achieved after 35.08h of enzymatic hydrolysis using a cellulolytic enzyme mixture (0.90Celluclast: 0.03Viscozyme: 0.07Novozyme). Muryanto et al. (2015) reported that the maximum glucose concentration after 72hours of saccharification process was carried out using two kinds of enzyme, CTec2 and HTec2, with ratio 5:1.

2.2.3 Bioethanol Production from Palm Empty Fruit Bunch

EFBs have a great potency as basic raw materials used for the fermentative production because they contain 37.3 – 46.5 % cellulose, 25.3 – 33.8 % hemicelluloses. Being abundant and outside the human food chain makes these cellulosic materials relatively inexpensive feedstocks for ethanol production and no conflict with the food supply (Sudiyani et al., 2013). There are many studies on bioethanol production from palm EFB, on different pretreatment methods are also applied to enhance the enzymatic hydrolysis and bioethanol yield as summarized in Table 5. *Saccharomyces cerevisiae* is usually used for bioethanol fermentation of palm EFB.

Table 5 Comparison of ethanol production results from palm EFB

Raw Material	Strain	Pretreatment	Ethanol Production	Productivity (g/L/h)	Ref.
EFB	*S. cerevisiae* L2524a	Alkaline (1M NaOH at 121^{O}C, stirred for 15 min)	64.2 g/L	2.4	Park et al., 2013
EFB	*S. cerevisiae* Mk	NaOH solution10% w/w at 140-145^{O}C and 4-7 kg/cm^3 of pressure for 30 min	50 g/L	1.38	Sudiyani et al., 2013
EFB	native *S. cerevisiae*	Soaked with NaOH 2% at 30^{O}C for 4 hours and heated the soaked fiber in an autoclave at 121^{O}C and 117 kPa for 6 min	66.5 L /t EFB		Piarpuzan et al., 2011

2.3 Objective

General objective:

To enhance the bioethanol production from palm empty fruit bunch

Specific objectives:

1) To Study the combinations of steam and waste glycerol pretreatments on enzymatic hydrolysis
2) To evaluate the efficiency of mixed enzymes for cellulose and hemicellulose hydrolysis
3) To investigate the bioethanol production from hydrolysate using co-cultures of *Saccharomyces cerevisiae* and *Pichia stipites*

2.4 Research Methodology

2.4.1 Materials

1) Palm empty fruit bunch (Raw material)

Palm empty fruit bunch (EFB), used as raw material to produce fermentable sugar will be collected from Suksomboon's oil palm mill factory located in Chonburi province, Thailand. To prepare the experimental samples, PEFB will be washed to remove soil and other particles and dried at 100-105°C for 5 hours. Subsequently, it will be chopped and sieved to about 1-3 mm size and stored in sealed plastic bags at room temperature prior proceed to next experiment to avoid moisture and biological degradation as shown in Figure 5.

Figure 5 The collected and stored EFB for experiment

2) Waste glycerol

Waste glycerol will be collected from biodiesel's plant of Suksomboon's oil palm mill factory located in Chonburi province, Thailand (Figure 6). The characteristic of the waste glycerol is weak alkaline in pH 8.7.

Figure 6 The collection of waste glycerol from biodiesel's plant

3) Enzymes

Three types of enzymes as Celluclast (100FBG/g), Cellic CTec2 (1000BHU2-/g) and Cellic HTec2 (2500 FXU-S/g) were supported from Brenntag Ingredients (Thailand) Public Co.,Ltd. Celluclast or Beta-glucanase (endo-1,3(4)-) contains activity of Cellulase, Hemicellulase and Xylanase. The declared enzymes of Cellic CTec2 and Cellic HTec2 are Cellulase and Xylanase (endo-1,4-), respectively.

4) Microorganisms

Saccharomyces cerevisiae (2.10×10^7 CFU/ml) and *Pichia stipites* (6.30×10^7 CFU/ml) were obtained from Thailand Institute of Scientific and Technology Research (TISTR), Thailand. They were maintained at 4°C on yeast-malt agar (YMA). The cells of *Saccharomyces cerevisiae* and *Pichia stipitis* were first grown in YM broth, shaking 150 rpm for 24 h, at 30°C.

2.4.2 Experimental methods

This research was divided into three phases, (1) steam pretreatment, (2) enzymatic hydrolysis and (3) fermentation. The flow diagram of experiment methods for bioethanol production was explained in Figure 7.

Phase I: Pretreatment

The efficiency of steam pretreatment was investigated employing laboratory Erlenmeyer flasks (125ml volume) as batch reactors. The pretreatment was carried out by using steam in an autoclave (LS-2D, REXALL) at a temperature and pressure of 121°C and 1.18 bars, respectively. The four different solvents used in pretreatment were distilled water (DW), waste glycerol (WG) at pH=8.7, alkaline waste glycerol at pH=11.0 and acidic waste glycerol at pH=3.8. The alkaline waste glycerol was prepared with the addition of 1M NaOH while the acidic waste glycerol was prepared using concentrated H_2SO_4. Each liquid was added to EFB to formulate substrate loading of 5, 10, 15 and 20%, respectively. Two reaction times of 15 and 60 minutes were tested for each substrate loading. After the completion of pretreatment, the samples were filtrated through a 0.45 μm GF/C filter paper. A portion of the solid samples was washed with distilled water and dried at 100-105°C for 2 hours. Subsequently, it was analyzed to determine the percentages of cellulose, hemicellulose and lignin following the forage fiber analytical methods. Briefly stated, this method involves three analytical procedures: the neutral-detergent fiber (NDF), the acid-detergent fiber (ADF) and the acid-detergent lignin (ADL). The percentages of the three organic components mentioned above can be calculated as follows:

%Cellulose = %ADF - %ADL

%Hemicellulose = %NDF - %ADF

%Lignin = %ADL

The liquid phase from the pretreatment was analyzed for soluble chemical oxygen demand (SCOD) using the closed reflux titrimetric method as outlined in 3.3.2. In addition, reducing sugar (RS) was determined by the 3,5-dinistrosalicylic acid (DNS) method.

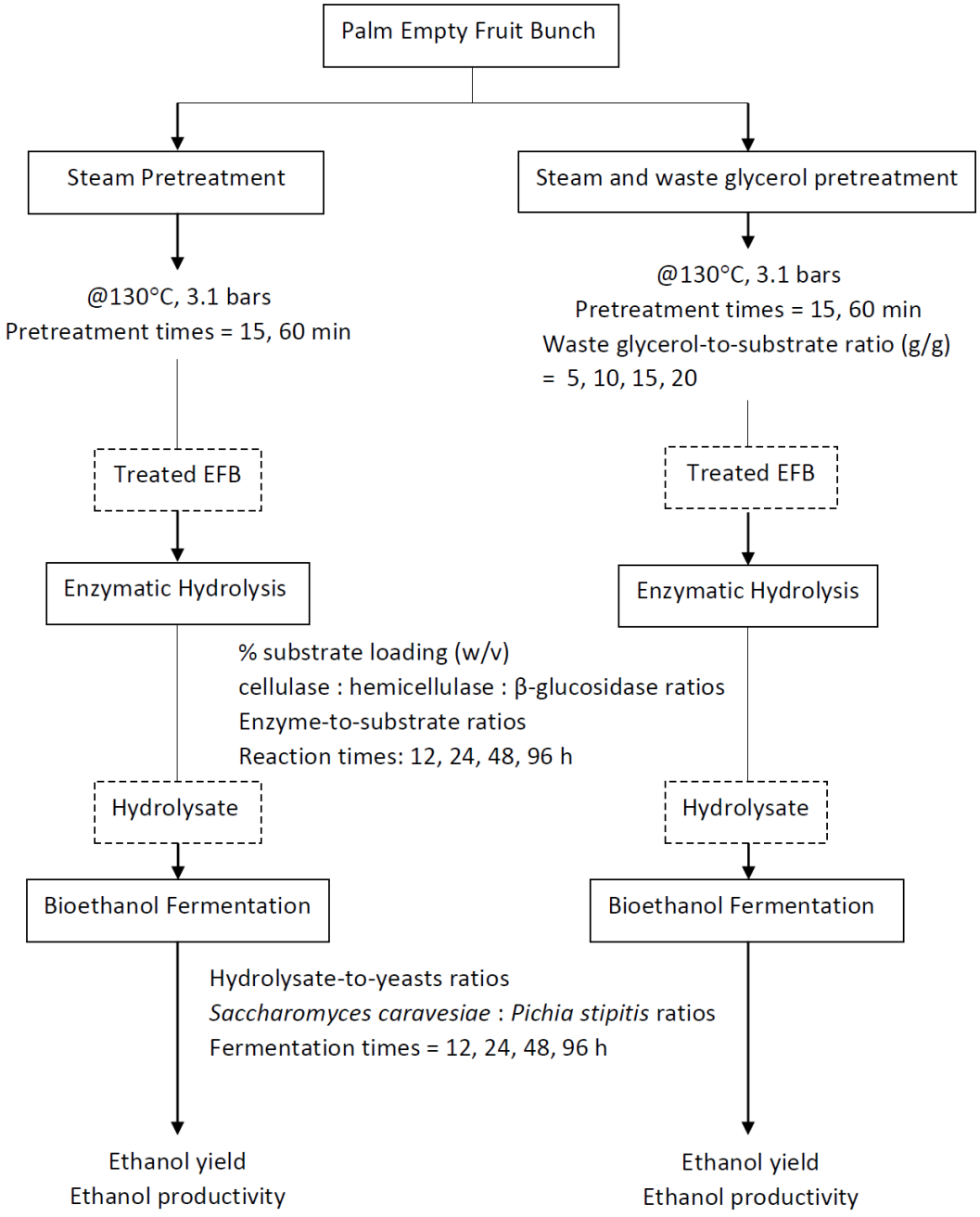

Figure 7 The experimental design

Phase II: Enzymatic hydrolysis

The pretreated EFB loading of 5% were digested to produce fermentable sugars using 2ml of three mixed enzymes, Celluclast (100 FBG/g), Cellic CTec2 (1000 BHU2-/g) and Cellic HTec2 (2500 FXU-S/g) in 2 ratios as shown in Table 6. Enzymatic hydrolysis process was held in 50ml Erlenmeyer flask with 10 ml of 50 mmol citrate buffer (pH 4.8) at 50±1°C in water bath for contact times 0, 2, 4, 6, 12, 24, 48, 72 and 96 hours. The sample was then put into boiling water for 10 minutes as to stop enzyme activity and, after that, was filtrated. Glucose and Xylose in liquid phase were monitored using High performance Liquid Chromatography (HPLC, Alliance-e2695, U.S.A).

Table 6 Ratios of 3 enzymes in hydrolysis phase

Celluclast	Cellic CTec2	Cellic HTec2
1	1	1
1	0	0

Phase III: Fermentation

After completion of enzymatic hydrolysis, 30 ml of the liquid phase was fermented by two cultures of yeasts, *Saccharomyces cerevisiae* (2.10×10^7 CFU/ml) and *Pichia stipites* (6.30×10^7 CFU/ml) in 3 conditions following Table 7. The fermentation was carried out at shaking condition with 150 rpm at room temperature. The sample was collected in 0, 6, 12, 24, 48 and 72 hours and bioethanol was analyzed using gas chromatography (GC-FID, Agilent-6890N, U.S.A.).

Table 7 Conditions of bioethanol fermentation

Condition	Ratio of yeast *Saccharomyces cerevisiae*: *Pichia stipitis*	Yeast loading (%v/v)
1	1:1	10
2	1:1	5
3	1:0	5

2.4.3 Analytical methods

1) Analysis of lignocellulosic contents

The contents of cellulose, hemicellulose and lignin were evaluated following Goering (1970). Sample of 0.7 g air-dried weigh (W_0) ground to pass 1-3 mm screen into refluxing container. One-hundred ml of preheated solution (30 g of Sodium lauryl sulfate, 18.61 g of Disodium ethylene diaminetetraacetate (EDTA), 6.81 g Sodium borate decahydrate (Borax), 4.56 g Disodium hydrogen phosphate and 10 ml 2-ethoxyethanol are dissolved in 1 L distilled water) was added to the sample. Heated to boiling for 1hour and filter the sample with weighed GF/C filter paper (W_1). Rinsed sample with hot water (90-100°C) and washed twice with acetone. Dry at 105±3°C in an oven for 3 hours, removed from oven, allowed to cool in a desiccator and weigh (W_2). The neutral detergent fiber (NDF) content was calculated using the following equation:

$$\%NDF = (W_2 - W_1) \times 100 / W0$$

Acid detergent fiber (ADF) content was estimated by the same procedure of NDF but using a different solution. Acid detergent solution (20 g Cetyl trimethylammonium bromide (CTAB) was dissolved in distilled water and 27.56 ml of conc. Sulfuric acid was added then adjusted to 1 L with distilled water). The ADF content was calculated using the following equation:

$$\%ADF = (W_2 - W_1) \times 100 / W_0$$

For acid detergent lignin (ADL) procedure, the ADF residue was placed in a 50 ml beaker, covered with 72% H_2SO_4 and stirred at hourly with glass rod for a minimum of 3 hours. After 3hours, the sample was filtrated though a filter paper and washed with hot water. The filtrate was dried at 105±3°C for 3hours and weighed (W_3). Then ignited the filtrate in 550°C furnace for 2 hours, cooled and weighed (W_4). The ADL content was calculated using the following equation:

$$\%ADL = (W_3 - W_4) \times 100 / W_0$$

The percentages of cellulose, hemicellulose and lignin were computed as follows:

$$\%Cellulose = \%ADF - \%ADL$$

$$\%Hemicellulose = \%NDF - \%ADF$$

$$\%Lignin = \%ADL$$

2) Analysis of Soluble Chemical Oxygen Demand (SCOD) in liquid phase

Part of liquid from filtration of pretreated sample was determined the concentration of soluble chemical oxygen demand (SCOD) with closed reflux, titrimetric method following

standard methods for the examination of water and wastewater (APHA, 2012). The SCOD was calculated as follows:

SCOD as mgO_2/L = ((A-B) × M × 8000) / (ml sample)

Where:

A = ml FAS used for blank

B = ml FAS used for sample

M = Molarity of FAS and

8000 = milliequivalent weight of oxygen × 1000ml/L

3) Analysis of reducing sugar in liquid phase

Reducing sugar in liquid sample was analyzed applying the Dinitrosalicylic acid (DNS) method (Miller, 1959). Sample of 0.5 ml was used and 2 ml of DNS (10 g DNS, 300 g Potassium sodium tartrate and 800 ml 0.5 N NaOH) was added into test tube. Heated with boiled water for 5 minute and suddenly soaked with cooled water for 5 minute to stop reaction. After that added 2.5 ml distilled water and analyzed reducing sugar with spectrophotometer at 540 nm.

4) Determination of fermentable sugar

The polysaccharides present in a biomass sample could be hydrolyzed to their component sugar monomers by enzyme and could be quantified by ion-moderated partition HPLC. This procedure had been adopted by ASTM as the Standard Test Method for Determination of Carbohydrates in Biomass by High Performance Liquid Chromatography. Prepared a series of sugar calibration standards in deionized water at concentrations appropriate for creating a calibration curve for each sugar of interest. Analyzed the calibration standards and the samples by HPLC using a Waters carbohydrate column (3.9×300mm.) for glucose and xylose. The following instrumental conditions were used for water carbohydrate column:

Sample volume: 10 µL

Mobile phase: 80% Acetone nitrile

Flow rate: 1.0 mL/min

Column temperature: 35°C

Detector: refractive index (RI)

Created a calibration curve by linear regression analysis for each sugar to be quantified. From these curve, determined the concentration in g/L of the sugar present in each solution analyzed by HPLC.

5) Determination of ethanol content

Ethanol content was analyzed by gas chromatography (GC). The gas chromatograph system equipped with flame ionization detector (FID), HP-INNOWAX polyethylene glycol column (30m×0.25mm×0.25μm).

Sample volume: 1 μL

Carrier gas: Helium

Flow rate: 1.3 ml/min

Temperature limit: 40 to 260°C

Detector: Flame ionization detector (FID)

3 Result

3.1 Characteristics of raw material

3.1.1 Palm empty fruit bunch (EFB)

The palm empty fruit bunch (EFB) used in this study was obtained from Suksomboon's oil palm mill factory located in Chonburi province, Thailand. It was shredded into fibrous material as shown in Figure 8 before being used in the experiment. The main composition of EFB consisting of cellulose, hemicellulose and lignin was analyzed. The characteristics of EFB used in this study compared with those from previous research on EFB as shown in Table 8. The organic content of EFB, measured as volatile solids, was about 90% with cellulose comprising approximately half of the organic matter, while the remainder is attributed to moisture and ash

Figure 8 Palm empty fruit bunch (EFB) and shredded EFB.

Table 8 Characteristics of raw EFB

Characteristics	Value (%weigh)				
	Sudiyani et al., 2013	**Mahammed et al., 2012**	**Abdullah et al., 2011**	**Khor et al., 2009**	**This study**
Cellulose	37.26	22.24	59.70	38.52	48.19±1.30
Hemicellulose	14.62	20.58	22.10	33.52	29.44±3.48
Holocellulose[a]	51.88	42.82	81.80	72.04	77.63±3.56
Lignin	31.68	30.45	18.10	20.36	13.75±0.74
Volatile solids	(N/A)[b]	82.58	83.86	75.09	89.57±0.27
Moisture	(N/A)[b]	5.18	7.95	8.65	6.57±0.06
Ash	6.69	3.45	5.36	3.92	3.85±0.24

Notes: [a] — Summation of cellulose and hemicellulose

[b] — Not available

3.1.2 Waste glycerol (WG)

The waste glycerol (WG) generated from the biodiesel production unit in the Suksomboon factory was used as solvent in the steam pretreatment. It contains 0.97±0.09 g/L of reducing sugar and 2090 g/L of soluble chemical oxygen demand (SCOD). After pretreatment with autoclave (121°C, 1.18bars) for 15 and 60 minutes, the concentration of reducing sugar was increased with increasing steaming time, whereas, SCOD was decreased as shown in Table 9. Jahn et al (2014) reported that increasing temperature for aqueous glycerol gave a smaller density. Also, the total number of hydrogen-bonds between molecules decreased with increasing temperature. This may indicated that reducing sugar was also released from waste glycerol.

Table 9 Concentration of reducing sugar and SCOD in waste glycerol

Sample	Reducing sugar (g/L)	SCOD (g/L)
Untreated waste glycerol	0.97±0.09	2090±0.61
Waste glycerol with autoclave 15 mins	0.98±0.04	1830±0.60
Waste glycerol with autoclave 60 mins	1.41±0.06	1380±0.46

3.2 Effect of pretreatment

3.2.1 Effect of pretreatment on the components of EFB

The percentage of cellulose was increased whereas hemicellulose was decreased when using distilled water (DW) as solvent for steam pretreatment at 15 minutes as shown in Figure 9 (experiment A). As a consequence, no significant changed in the percentage of holocellulose was found. While the percentage of lignin, the obstacle of enzymatic accessibility, was not significantly different from that of untreated EFB with 95% confidence level, regardless of the substrate loading applied. Similarly as shown in Figure 9, the results from 15 minutes steam pretreatment with waste glycerol (WG) at pH 8.7 (experiments C) show that the percentage of cellulose increased while that of hemicellulose decreased when 5 and 10 % of substrate loadings were applied. The percentage of holocellulose was not significantly different from the untreated EFB (1C and 2C experiments). However, 15 and 20% resulted in the decrease of both cellulose and hemicellulose, 3C and 4C experiments. The concentration of holocellulose produced from these experiments were found to be lower than those of EFB pretreated with distilled water and glycerol in autoclave for 15minutes. Although the percentage of holocellulose was decreased but 20% of substrate loading with waste glycerol pretreatment (4C) showed the significant decrease of lignin from 13.75% to 11.32%. These are consistent related with previous studies as percentage of holocellulose of 3 and 5% of dried EFB in aqueous glycerol were found increased, indicating complete a delignification, while 10% of dried EFB applied gave poor result (Ibrahim et al., 2012). Therefore it can be concluded that waste glycerol performed well at low substrate loading.

As shown in Figure 10, steam pretreatment for 60 minutes with distilled water, holocellulose mostly remained at the average of 80.89% excepted those from 3D and 4D experiments which dropped to 58.59% with 95% confidence level. These indicated that higher degradation of EFB was observed when increasing substrate loading, thereby resulting in lower percentage of holocellulose as these compounds have already been converted to reducing sugar in the liquid phase. Overall, results indicated that longer time of steam pretreatment with waste glycerol can convert the components of EFB to reducing sugar. Moreover, waste glycerol used as a solvent appeared to be more effective in the conversion of EFB into reducing sugar when compared with distilled water pretreatment.

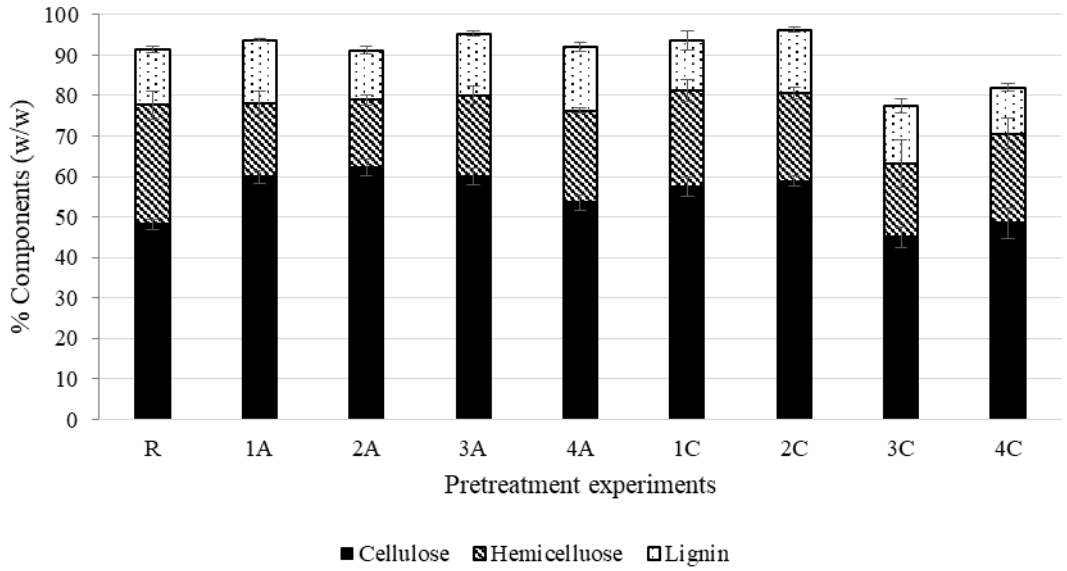

Figure 9 Percentage of cellulose, hemicellulose and lignin of EFB (w/w): Raw material (R); DW 15minutes (A) and WG 15minutes (C) with substrate loading: 5% (1); 10% (2); 15% (3) and 20% (4).

Following the completion of steam pretreatment with alkaline glycerol and acidic glycerol (experiments E and F), alkaline glycerol proved to be more effective at the lowest substrate loading of 5% thereby resulting in the highest percentage of holocellulose (87.98%, condition 1E) for all conditions investigated. Moreover, the use of acidic glycerol did not seem to improve the percentage of holocellulose content while the percentage of lignin was significantly lower than the untreated EFB for both alkaline and acidic conditions as shown in Figure 11. These results indicated that alkaline environment appears to enhance the performance of steam pretreatment of EFB, resulting in higher output of holocellulose. Similarly, Choi et al., (2013) reported that NaOH-catalyzed steam pretreatment of EFB can remove lignin efficiently and requires only a short reaction time. Another study has also pointed out that glycerol used as delignification solvent was more efficient in the presence of acidic or alkaline compounds (Ibrahim et al., 2012).

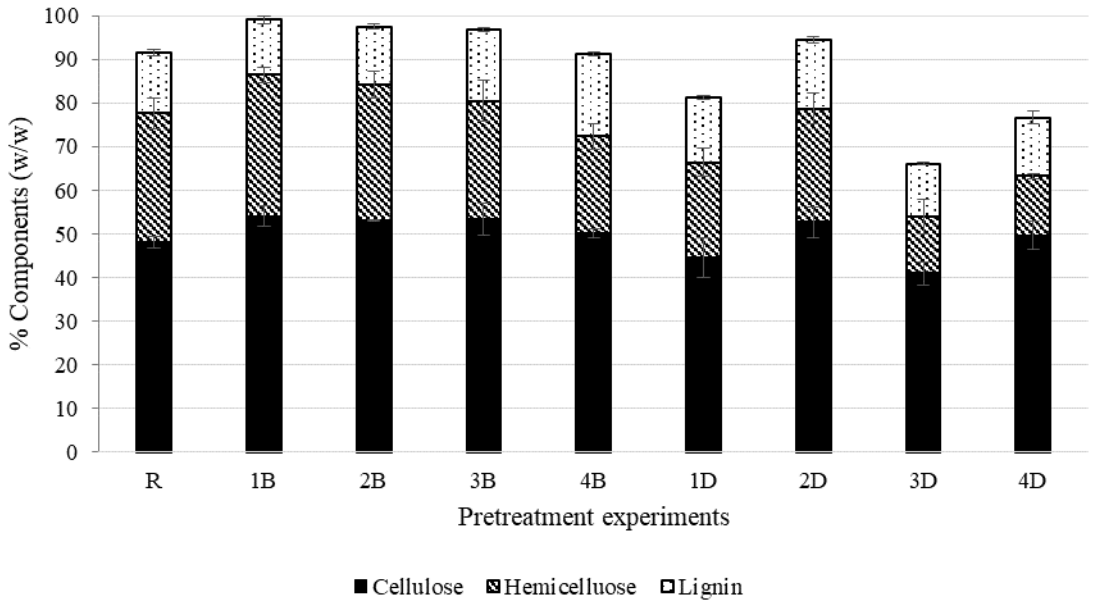

Figure 10 Percentage of cellulose, hemicellulose and lignin of EFB (w/w): Raw material (R); DW 60minutes (B) and WG 60minutes (D) with substrate loading: 5% (1); 10% (2); 15% (3) and 20% (4).

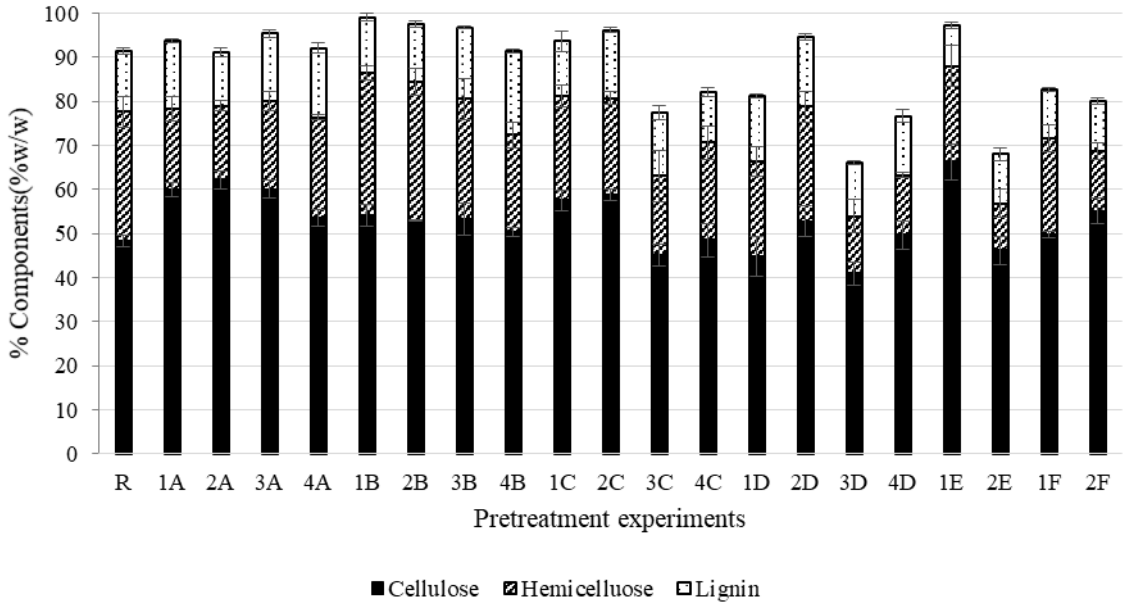

Figure 11 Percentage of cellulose, hemicellulose and lignin of EFB (w/w): Raw material (R); DW 60minutes (B); WG 60minutes (D); Alkaline WG (E) and Acidic WG (F) with substrate loading: 5%(1); 10%(2); 15%(3) and 20%(4).

3.2.2 Reducing sugar in liquid phase

After pretreatment, the treated samples were filtrated and the filtrate was analyzed for reducing sugar (R.S.) concentration. In general, as the EFB loading increased, the reducing sugar concentration also increased as shown in Figure 12. The highest concentration was observed during pretreatment with waste glycerol at 60 minutes (experiment D), regardless of the loading variation, suggesting that glycerol can release more reducing sugar at a longer reaction time. Regarding the effects of pretreatment, pretreatment time of 60 minutes with waste glycerol was found that both the percentage of cellulose and hemicellulose decreased and were lower than the corresponding values obtained using distilled water. Such a decrease in cellulose and hemicellulose may indicate that these compounds were already degraded to sugars in the liquid phase. Whereas at 15 minutes, both alkaline and acidic conditions encourage a higher reducing sugar generation from glycerol than that observed with waste glycerol without pH adjustment. The results of alkaline waste glycerol pretreatment relate to the composition of EFB, that is, a low initial percentage of cellulose and hemicellulose may increase the reducing sugar content. Overall, it is apparent that steam pretreatment can convert the components of EFB to reducing sugar.

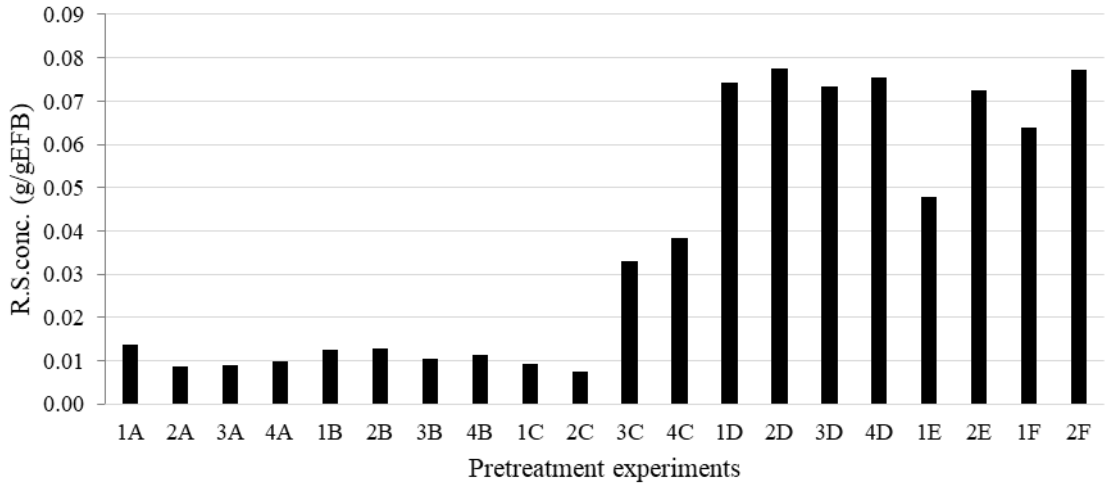

Figure 12 Concentration of reducing sugar in liquid phase (mg/L): Reducing sugar (R.S.); DW 15minutes (A); DW 60minutes (B); WG 15minutes (C); WG 60minutes (D); Alkaline WG 15minutes (E) and Acidic WG 15minutes (F) with substrate loading: 5% (1), 10% (2),15% (3) and 20% (4). (S.D.<0.0025g/L).

3.3 Fermentable sugars from pretreated EFB

To determine the effect of enzymatic hydrolysis on fermentable sugar production, the three enzymes including Celluclast, Cellic CTec2 and Cellic HTec2 were used at two activity ratio of 1:1:1 and 1:0:0. The results indicated that the fermentable sugars produced under different pretreatment conditions as described above were depended on the pretreatment method. Results of fermentable sugar concentration obtained from different pretreatment methods are described below.

3.3.1 Hydrolysis with three types of enzymes

1) Sugar concentration from hydrolysis of EFB in the pretreatment with distilled water

After the complete pretreatment, the pretreated EFB was hydrolyzed by three type of enzymes, Celluclast, Cellic Ctec2 and Cellic HTec2. The samples were taken at different sampling times (0, 2, 4, 6, 12, 24, 48, 72 and 96 hours) to determine the effect of hydrolysis time on different pretreated EFB. For untreated EFB, concentration of glucose was increased from 91.91±0.14 g/L at 0 hour to the highest concentration of 113.05±0.73 g/L at 96 hours when increasing reaction times. Similar with concentration of xylose, the highest concentration of 1.30±0.00 g/L was obtained at 96 hours. Enzymatic hydrolysis of untreated EFB yielded only low glucose and xylose level of about 0.1 ± 0.021 g/g EFB and 0.02 ± 0.005 g/g EFB, respectively. However, concentration of xylose was significantly lower about 10 fold than glucose. Moreover, the conversion of hemicellulose to xylose at lower hydrolysis time (<6 hours) was not effective as shown in Figure 13.

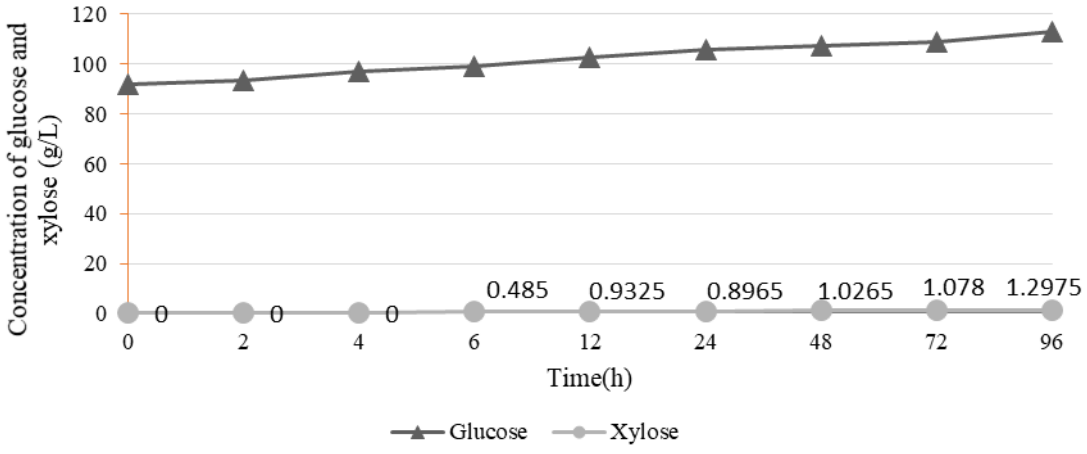

Figure 13 Concentration of glucose and xylose (g/L) from hydrolysis the untreated EFB.

As shown in Figure 14, the pretreated EFB with distilled water for 15 minutes released higher total sugar than untreated. For 15 minutes of pretreatment, the highest glucose and xylose concentrations were found at 20% of substrate loading for 96 hours of digestion that were 133.31±1.43 and 13.16±0.07 g/L, respectively. However, for other substrate loading, total sugar concentration increased when increasing substrate loading. According to statistical analysis, it was found that the total sugar concentrations obtained from pretreated EFB were significantly higher than those of untreated EFB. It should be noted that the lowest substrate loading (5%) of pretreatment with distilled water for 15 minutes needed 4 hours for the highest sugars production but it was decreased after 6 hours. Moreover, the lowest sugar concentration was produced from this condition.

The pretreated EFB with distilled water at 5 and 15% of substrate loading for 60 minutes released the highest glucose concentration of 129.47±0.05 and 145.27±0.05g/L for 72 hours. While 10 and 20% of substrate loading needed 96 hours to produced 146.69±0.96 and 143.85±0.05 g/L of glucose as shown in Figure 15 a. The glucose concentrations obtained from all condition of EFB pretreatment were higher than those of untreated EFB. Whereas, glucose achieved from 10% pretreated EFB with distilled water for 60 minutes in autoclave was the highest concentration in this study (significance at 95% confidence). In contrast, the xylose concentrations at 5, 10, 15 and 20% of substrate loading with the same condition (Figure 15 b) were lower than those of DW pretreatment for 15 minutes. However, hydrolysis of steam pretreated EFB with distilled water can produce more glucose and total sugar than those of untreated EFB. The results indicated that increasing the steam pretreatment time with distilled water resulted in a higher efficiency of enzyme hydrolysis from EFB.

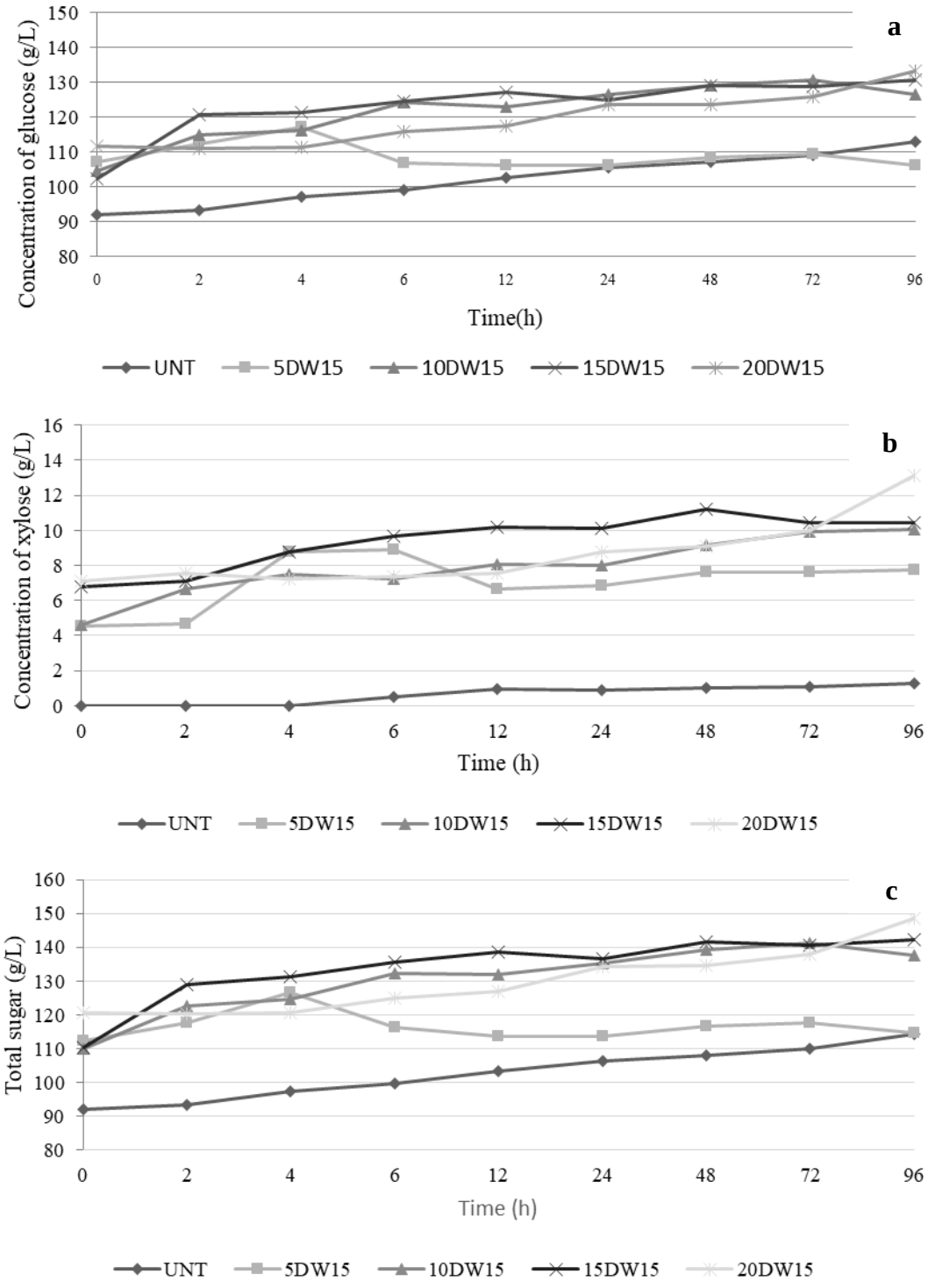

Figure 14 Concentration of glucose (a), xylose (b) and total sugar (c) (g/L) from hydrolysis the untreated (UNT) and pretreated EFB with distilled water for15 minutes (DW15). 5% (5); 10% (10); 15% (15); 20% (20).

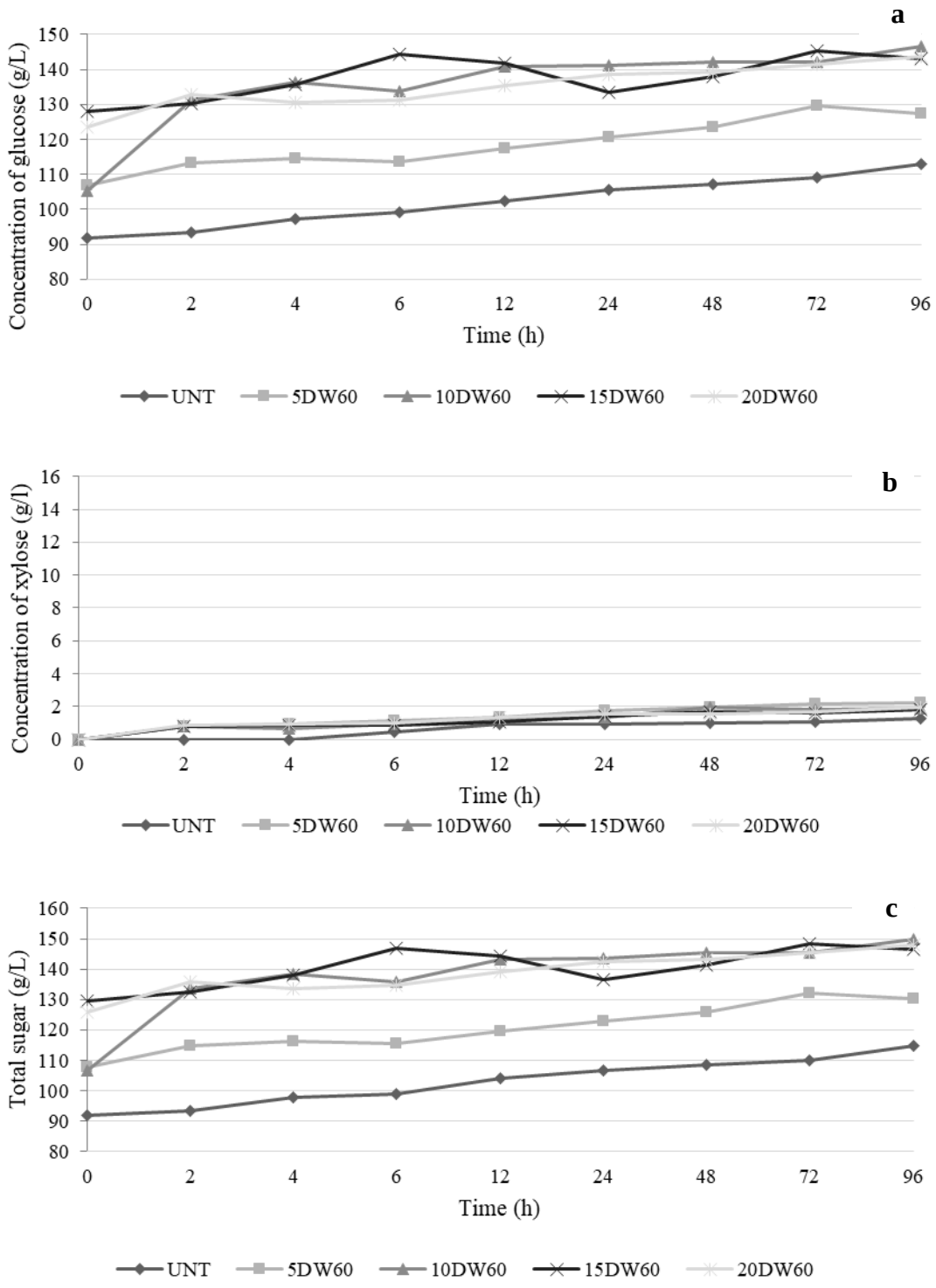

Figure 15 Concentration of glucose (a), xylose (b) and total sugar (c) (g/L) from hydrolysis the untreated (UNT) and pretreated EFB with distilled water for 60 minutes (DW60). 5% (5); 10% (10); 15% (15); 20% (20).

2) Sugar concentration from hydrolysis of EFB in the pretreatment with glycerol

Figure 16 shows the result of the 15-min pretreatment with waste glycerol as a solvent. The concentrations of glucose and xylose obtained were found to be similar with those pretreated with distilled water. The results indicated that more than 48 hours of hydrolysis are needed to produce the highest concentration of glucose. From all experiments, the average glucose concentration of 128.46 g/L was produced which is higher than those from untreated EFB. When considered the concentration of xylose, it was in the range of 2 to 8 g/L which is higher than that of untreated EFB (1.30±0.01 g/L). As shown in Figure 17, the concentrations of glucose and xylose obtained from pretreated EFB with waste glycerol for 60 minutes in all substrate loading were not significantly different from untreated EFB. The result showed that waste glycerol was found to accelerate EFB degradation, especially in longer reaction time as described in section 3.2.1. It is also obvious that the fiber consisted in EFB has already been converted to the reducing sugar in liquid phase, which may also result in low concentrations of cellulose and hemicellulose were observed as shown in Figure 17 c.

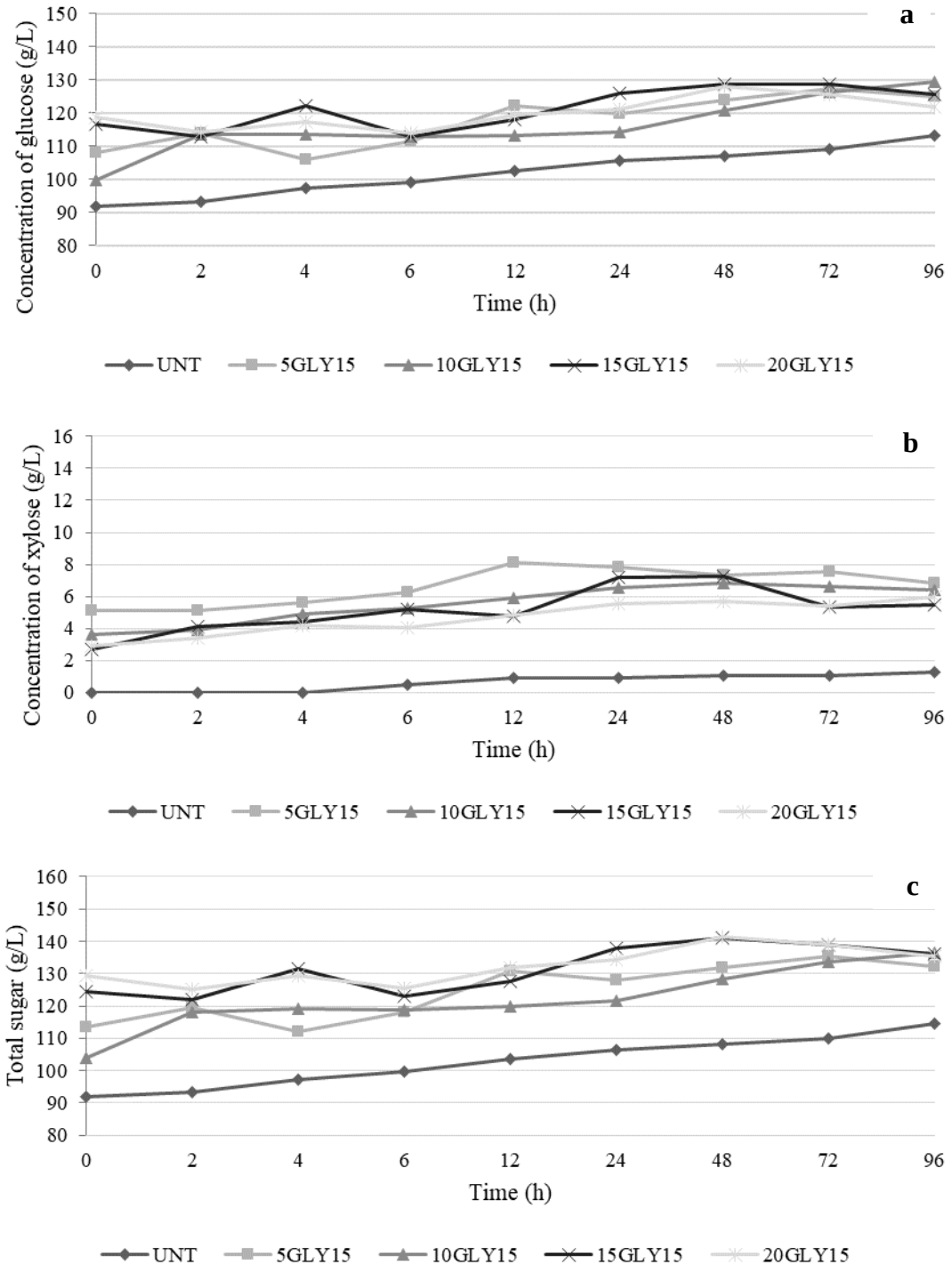

Figure 16 Concentration of glucose (a), xylose (b) and total sugar (c) (g/L) from hydrolysis the untreated (UNT) and pretreated EFB with glycerol for15 minutes (GLY15). 5% (5); 10% (10); 15% (15); 20% (20).

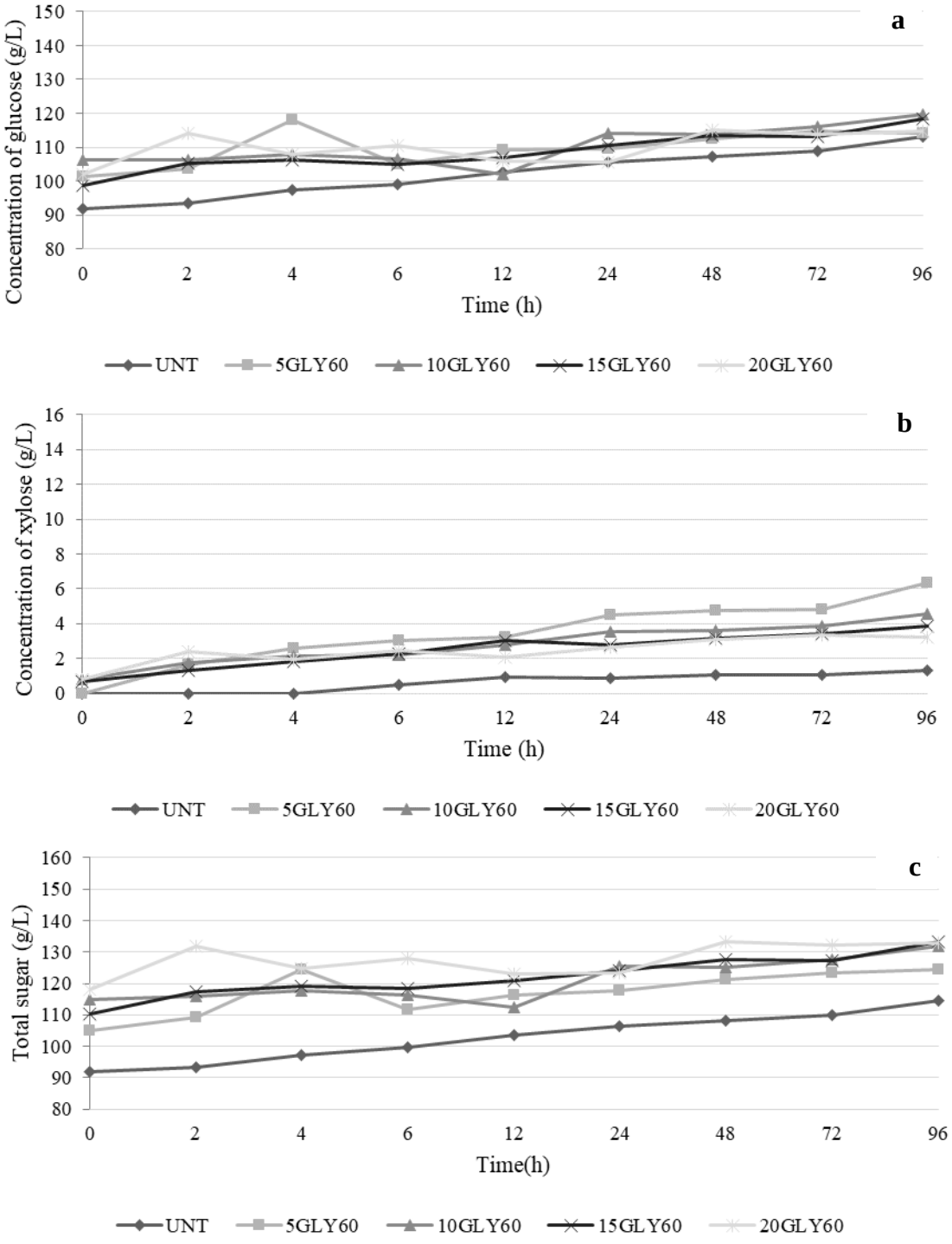

Figure 17 Concentration of glucose (a), xylose (b) and total sugar (c) (g/L) from hydrolysis the untreated (UNT) and pretreated EFB with waste glycerol for 60 minutes (GLY60). 5% (5); 10% (10); 15% (15); 20% (20).

3) Sugar concentration from hydrolysis of EFB in the pretreatment with alkaline and acidic glycerol

It is interesting to note that the highest total sugar concentration (glucose and xylose) was appeared after hydrolysis the pretreated EFB with alkaline glycerol (pH 11.0). Results showed that glucose is the major sugar received from pretreated EFB. Total sugar concentration about 150 g/L was obtained after 24 hours of hydrolysis. It should be noted that high concentrations of glucose were observed after 24 hours of hydrolysis time but at lower time showed a similar glucose concentration to that of untreated EFB. However concentration of glucose was decreased after 72 hours (Figure 18). While 10% of substrate loading of pretreatment with alkaline glycerol produced total sugar in range 140 to 152 g/L after 24 hours but promptly reduced after 72 hours of reaction time.

On the other hand, pretreated EFB with acidic glycerol at 10% substrate loading cannot produced glucose after hydrolysis. While 5% of substrate loading can produce small amount of glucose but it was not significantly different with untreated EFB as shown in Figure 19. After 24 hours, glucose concentration was decreased lower than untreated EFB. From previous study, hardwood and softwood pretreated using H_2SO_4 of 0.55 to 0.82% with steam explosion for 15 minutes can improved sugar concentration after hydrolysis. Whereas increasing H_2SO_4 to 1.23% resulted in much poorer than those in the above-mentioned study because excess concentration of acidic solution had already formed sugars to be destroyed. It was indicated that pH adjustment to 3.8 in this study might result in excess acidity to degrade biomass.

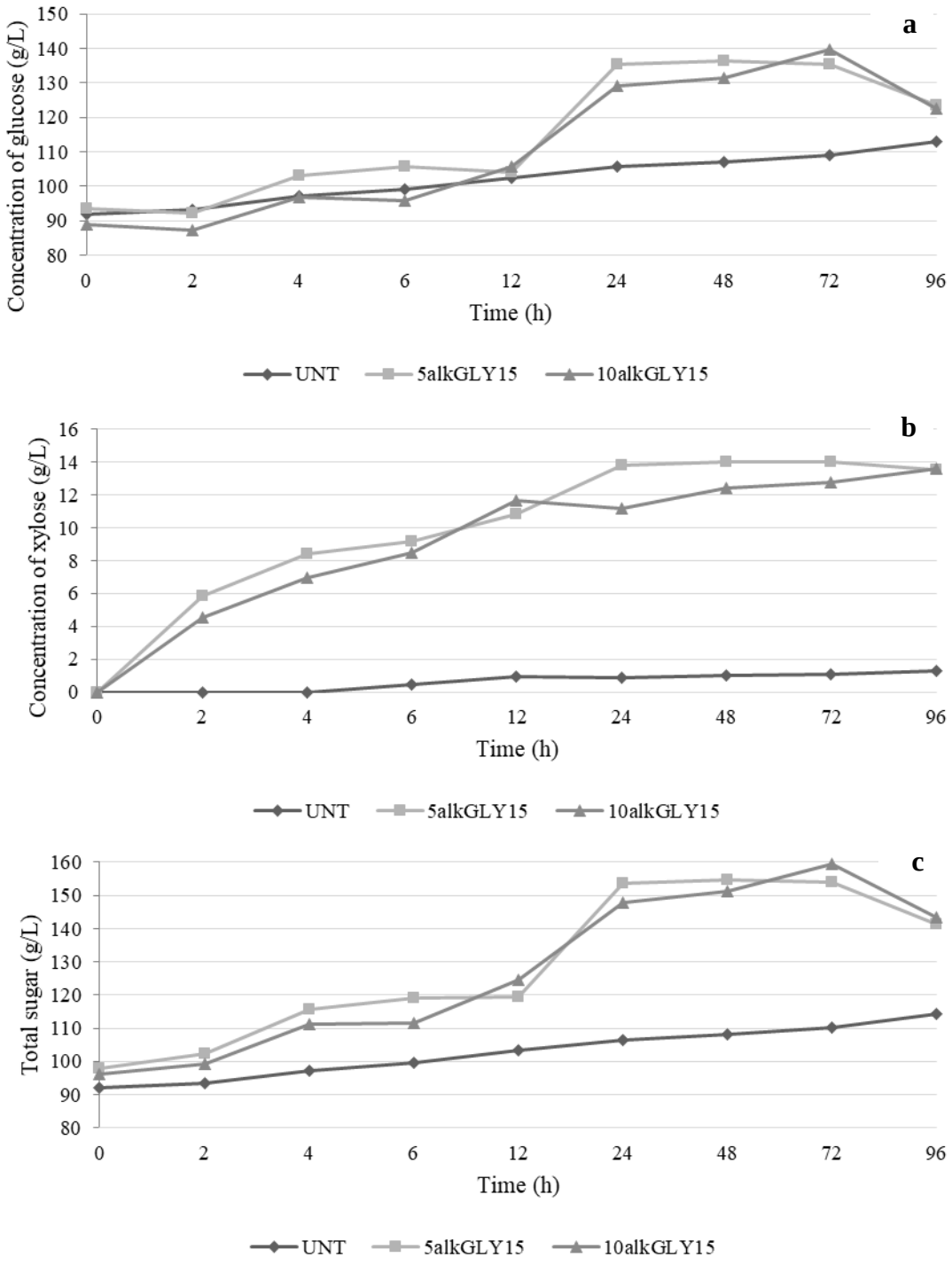

Figure 18 Concentration of glucose (a), xylose (b) and total sugar (c) (g/L) from hydrolysis the untreated (UNT) and pretreated EFB with alkaline glycerol for 15 minutes (alkGLY15) 5% (5); 10% (10).

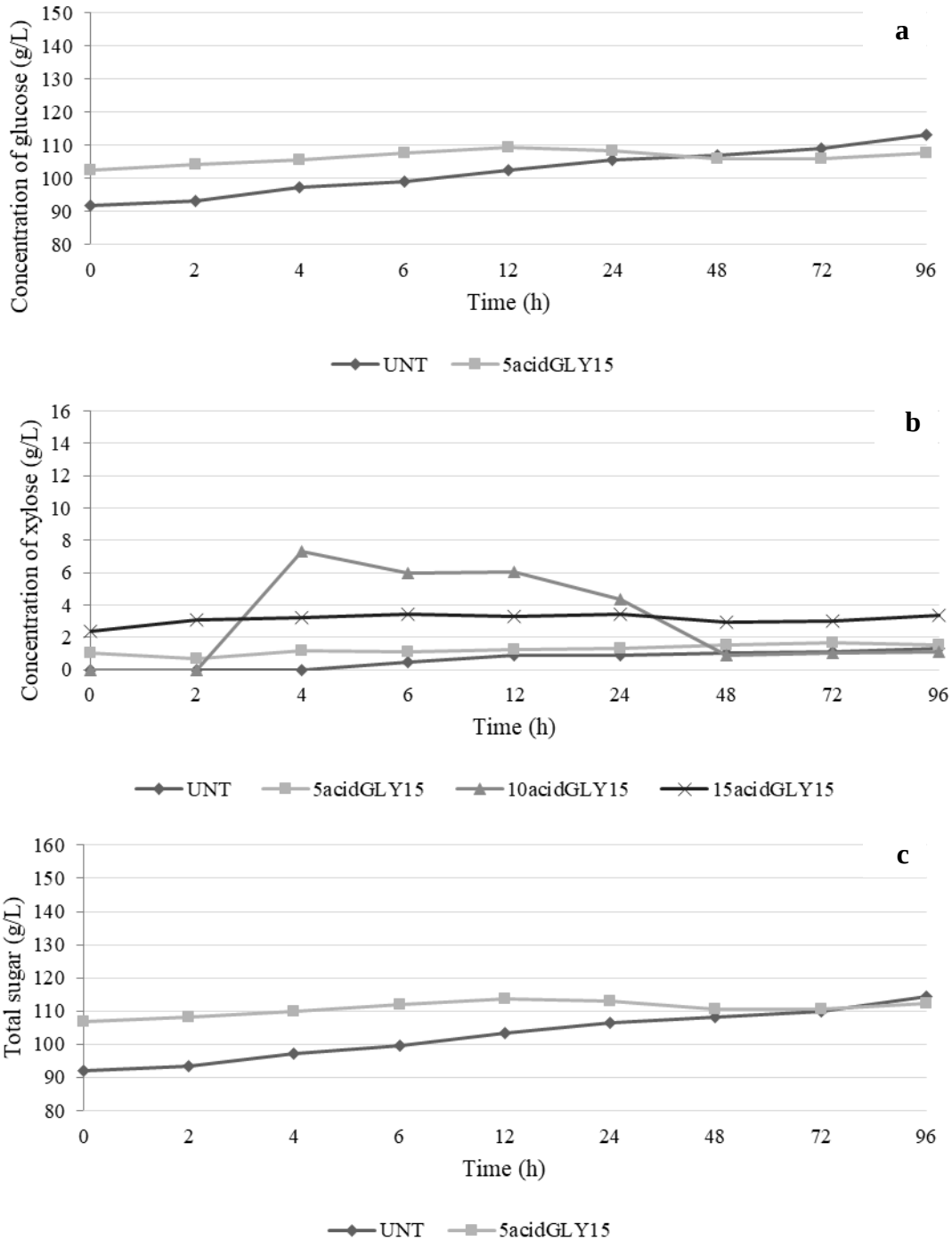

Figure 19 Concentration of glucose (a), xylose (b) and total sugar (c) (g/L) from hydrolysis the untreated (UNT) and pretreated EFB with acidic glycerol for15 minutes (acidGLY15). 5% (5); 10% (10).

3.3.2 Hydrolysis with one type of enzyme

Using only Celluclast as an enzyme for hydrolysis, very low concentrations of glucose and xylose were produced for all of the pretreated EFB experiments. Regarding three types of enzyme namely Celluclast, Celli CTec2 and Cellic HTec2 were applied, the total sugar was found to be produced significantly higher than that using only one type of enzyme Celluclast. As shown in Figure 20, the hydrolysis with one type of enzyme produced total sugar ranging of 8 to 38 g/L and the highest concentration was obtained from 10% EFB for 15 minutes pretreatment with alkaline glycerol. It was indicated that mixed enzyme can enhance the efficiency of hydrolysis. Relating to the properties of enzyme, Celluclast is a mixture of Cellulase, Hemicellulase and Xylanase while Cellic CTec2 and HTec2 is Cellualse and Xylanase, respectively, which function to support the main enzyme (Novozyme A/S, 2010). Most of previous studies reported similar results, successful hydrolysis and removal of palm fibers (up to 84.3% of cellulose and 31% of hemicellulose) resulted in using cellulase and hemicellulase enzymes together while only hemicellulase had no effect on glucan or ther polysaccharides in the fibers (Shafiei et al., 2010). Hassan et al. (2013) studied the optimization of enzyme combination for EFB. Glucose and xylose that obtained from hydrolysis with mixed enzymes of Celluclast, Viscozyme and Novozym were higher than those from using only one of them in same volume.

From the above results, the high sugar concentration was fermented by yeast in the phase III of experiment. The interesting results were 10% EFB at 60-min pretreated with distilled water and 15-min pretreated with alkaline glycerol produced 143.66±2.56 and 147.68±1.97 g/L, respectively, after 24 hours of hydrolysis with mixed enzyme.

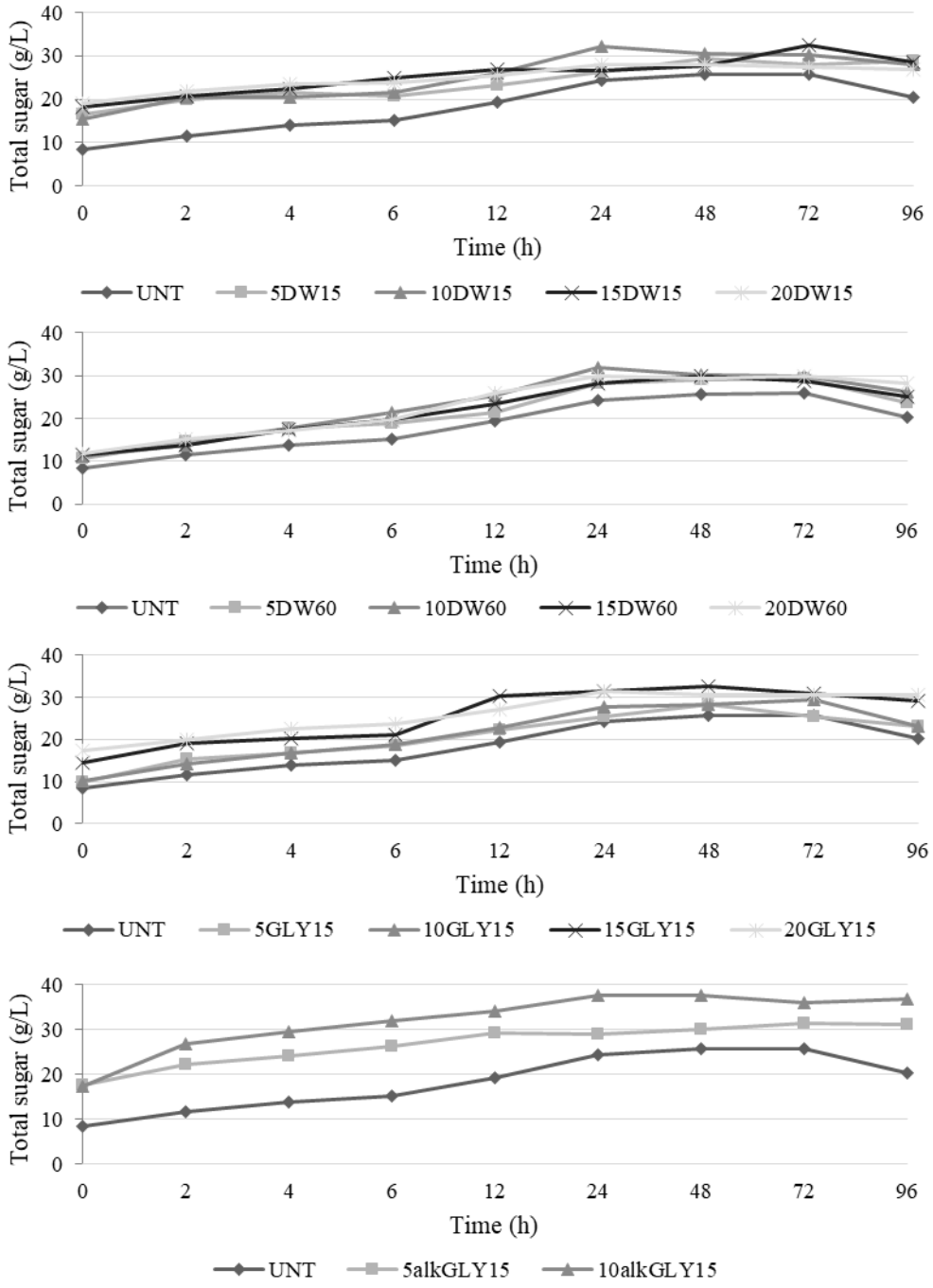

Figure 20 Total sugar (g/L) from hydrolysis the untreated (UNT) and pretreated EFB by Celluclast: 5% (5); 10% (10); 15% (15); 20% (20): Distilled water (DW); Waste glycerol (WG); Alkaline glycerol (alkgly): 15-min (15); 60-min (60).

3.4 Ethanol production

Two strains of yeasts (*Saccharomyces cerevisiae* and *Pichia stipites*) were used in the fermentation process. Three types of feed including untreated EFB, 10% pretreated EFB with DW for 60 minutes, and 10% pretreated EFB with alkaline glycerol for 15 minutes were fed into the fermenter. With the ratio of *Saccharomyces cerevisiae* to *Pichia stipites*at 1:1, the highest ethanol concentration of 9.28±0.33 and 8.50±0.18g/L were produced from the 10% and 5% yeasts loadings, respectively. The maximum concentrations mentioned above occurred at the elapsed time of 6 hours as shown in Figure 21 a and b. when using only *Saccharomyces cerevisiae* in the fermenter, the maximum concentration of ethanol of 10.81±0.28 and 10.27±0.11 g/L were obtained from pretreated EFB with DW for 60 minutes and alkaline glycerol for 15 minutes at 6 hour fermentation time, respectively as shown in Figure 21 c. These apparently indicate that alkaline glycerol assistances to reduce the time required for pretreatment thereby saving cost and energy consumed in the process. Moreover, ethanol concentrations after 6 hours were found to decrease whereas fermentable sugar was not consumed by yeast. This may indicate that carbon metabolism of yeast switched from a fermentative metabolism producing ethanol to a respiratory metabolism of Kreb's cycle generating acetaldehyde and acetate. This metabolic switch occurs at low concentrations of pyruvate (a key intermediate compound in glucose biodegradation) (Dickinson et al., 2004). These results are consistent with Azhar et al. (2017) for the toxicity of microbial growth in the long fermentation time. Meanwhile alkaline treatment was found to reduce inhibition in fermentation of pretreated biomass (Piarpuzan et al., 2011).

3.5 Yield of fermentable sugars and ethanol

In general, during enzymatic hydrolysis, cellulose is degraded by cellulose and hemicellulose is degraded by hemicellulose to reducing sugar that can be fermented by yeast to ethanol. Figure 22 shows the flow diagram of ethanol fermentation process employed in this study, starting from enzymatic hydrolysis of pretreated EFB until ethanol fermentation using difference loading of yeast strain (*Saccharomyces cerevisiae: Pichia stipites*). The untreated EFB was hardly to be hydrolyzed, in which ethanol yielded only 0.048 g ethanol/g EFB. This results was similar to those of Ying et al. (2014). Besides, Medina et al. (2016) reported that the enzymatic hydrolysis of the steam explosion pretreated EFB produced twice glucose concentration comparing with untreated EFB.

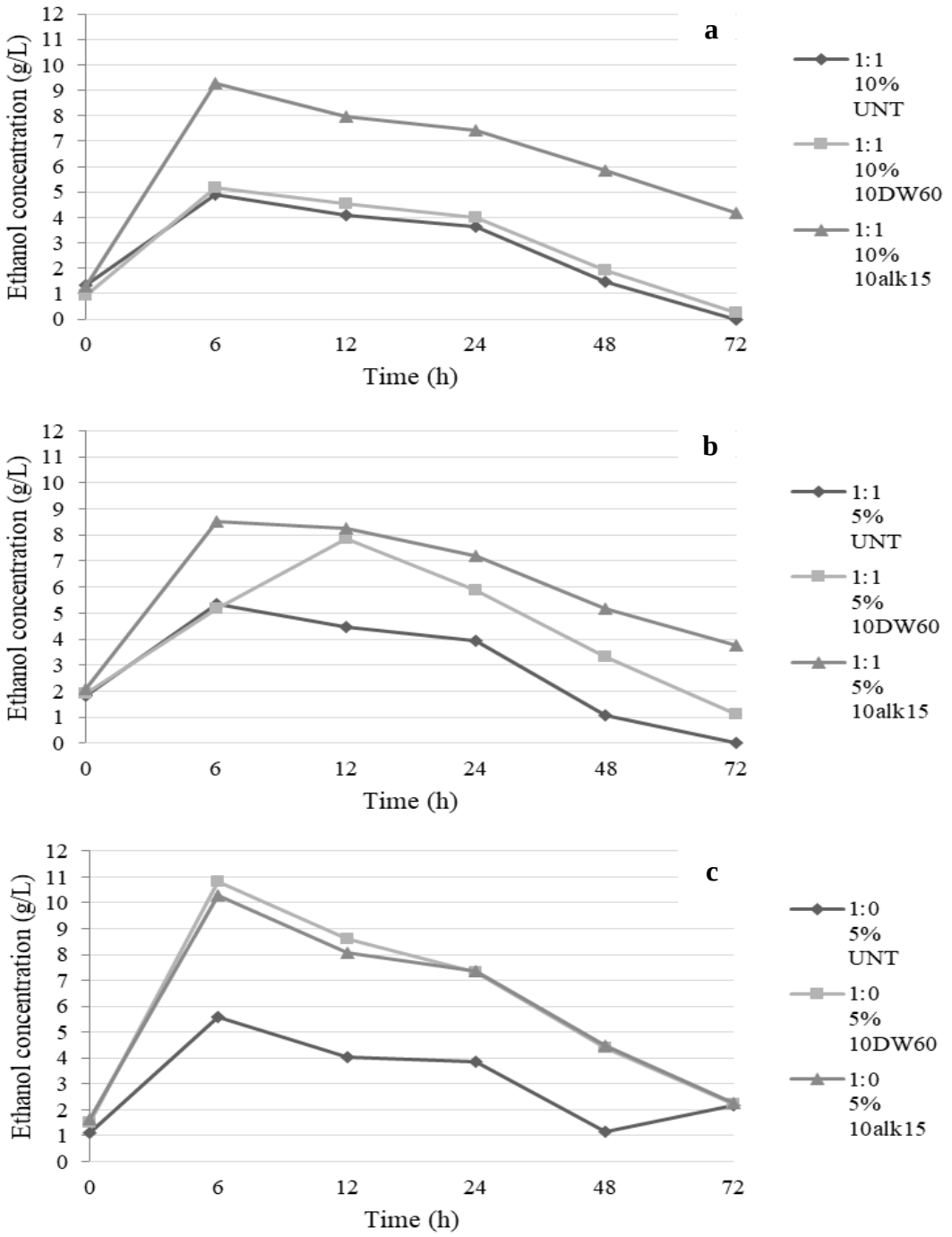

Figure 21 Ethanol production from hydrolyzed EFB (g/L); untreated (UNT), 10% pretreated with distilled water for 60 minutes (10 DW60) and 10% pretreated with alkaline glycerol for 15 minutes (10 alk15) by 3 condition of yeasts, ratio of *S. cerevisiae*: *P. stipites*, 1:1 with 10% concentration (a), 1:1 with 5% concentration (b) and 1:0 with 5% concentration (c).

In 60-min steam pretreatment with distilled water as shown in Figure 23, glucose yield (0.73±001 g/g EFB) was higher than that obtained from untreated EFB (0.10±0.21 g/g EFB). Moreover, ethanol yield achieved from this pretreatment condition was higher than those of untreated EFB produced. These may be because the chemical composition analysis of pretreated EFB indicated that the increase of sugar production was highly related to the removal of lignin in the fibers (Ying et al., 2014). In addition, the flow diagram of ethanol production from pretreated EFB with alkaline glycerol for 15 minutes (Figure 24) showed that the highest xylose of 0.16±0.01 g/g EFB was produced in competition with glucose production of 0.41±0.01 g/g EFB at a shorter time of pretreatment. Moreover, ethanol yield that obtained from pretreated EFB with alkaline glycerol for 15 minutes was about 0.18-0.23 g ethanol/g EFB which is similar to that of steam pretreatment with distilled water for 60 min. The results obviously indicated that using alkaline glycerol as a solvent for EFB pretreatment can enhance the enzymatic hydrolysis in shorten steam autoclaving time. Furthermore, the maximize both glucose and xylose concentrations are suitable substrate for both strain of yeast (*Saccharomyces cerevisiae* and *Pichia stipites*) used in this study.

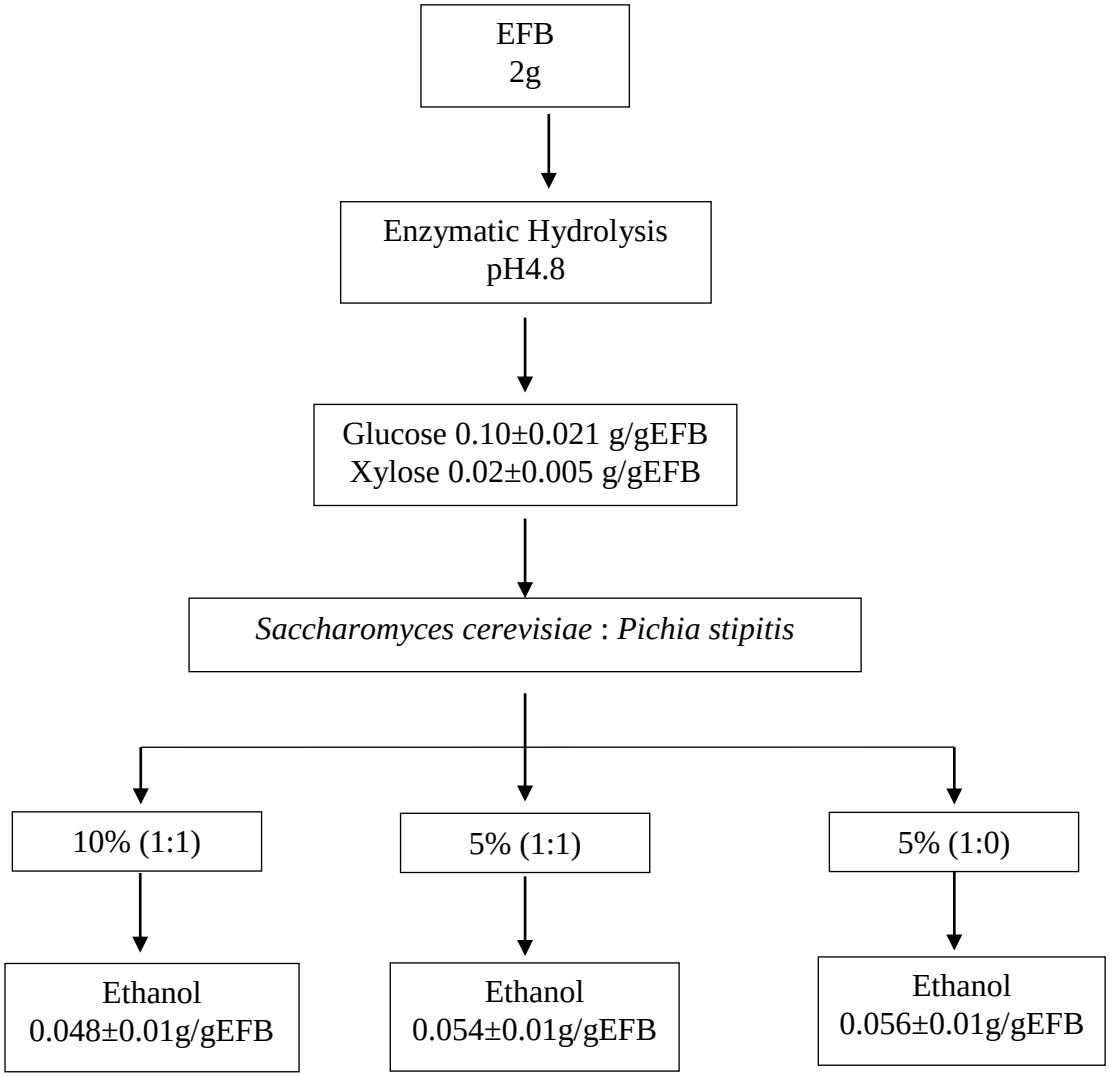

Figure 22 Fermentable sugar and ethanol yields from untreated EFB

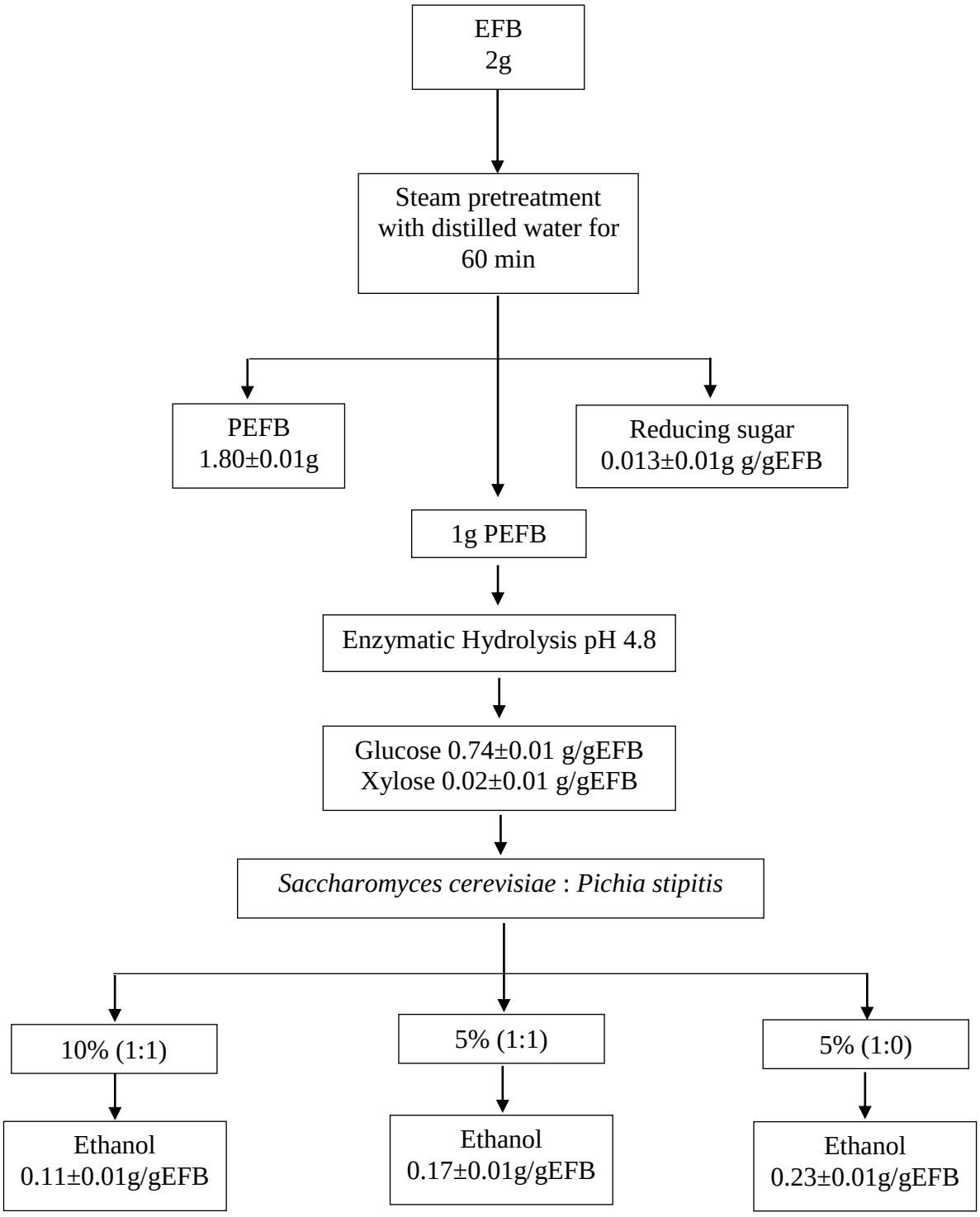

Figure 23 Fermentable sugar and ethanol yields from pretreated EFB with distilled water for 60 minutes

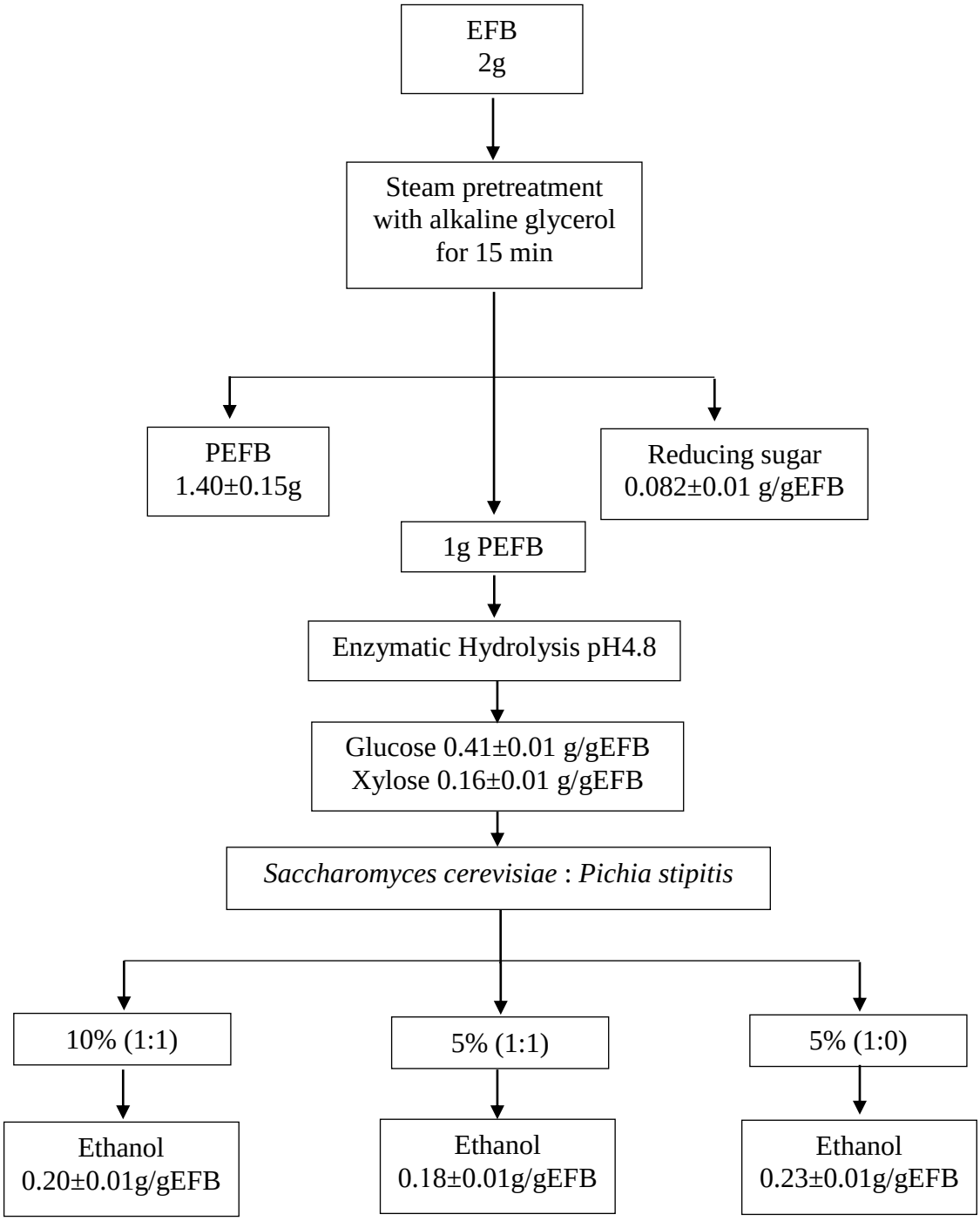

Figure 24 Fermentable sugar and ethanol yields from pretreated EFB with alkaline glycerol for 15 minutes

4 Conclusion

The effect of pretreated EFB with and without waste glycerol was determined under difference conditions to optimize the suitable condition for sugar production. Moreover, the co-cultures of two yeasts species *(Saccharomyces cerevisiae* and *Pichia stipitis)* were evaluated to enhance the bioethanol fermentation from hydrolysate of palm EFB. Results of this study can be concluded as follows;

4.1 Effects of pretreatment on components of EFB

The chemical compositions of treated EFB during steam pretreatment were changed depending on the types of solvent used, the pH of the liquid content and reaction time. The 60 minutes steam pretreatment with distilled water generated a higher content of holocellulose than that for 15 minutes. However, it produced the same holocellulose concentration of about 0.87 g holocellulose/ g EFB as did the 15-min pretreatment with waste glycerol. This clearly indicates that EFB pretreatment with alkaline glycerol results in better performance to produce intermediate holocellulose for further ethanol fermentation.

4.2 Production of fermentable sugars and ethanol

For enzymatic hydrolysis experiments, the mixed enzymes show highest glucose concentration produced from 10% EFB at the 60 minutes steam pretreatment with distilled water and the highest total sugar concentration was obtained from the 15-min pretreatment with alkaline glycerol. This indicated that EFB can be used as substrate to produce higher value fermentable sugar. However, pretreatment or enzyme loading may not be the only factors that enhance lignocellulose degradation. The composition of the lignocellulose material should be determined in order to select the appropriate pretreatment processes.

In the fermentation experiments, high ethanol concentrations of 10.81 and 10.27 g/L were achieved at the reaction time of 6 hours for both EFB pretreated with distilled water for 60 minutes and alkaline glycerol for 15 minutes, respectively. Overall from the results mention above, it can be concluded that alkaline glycerol can enhance the production of fermentable sugars to produce higher concentration at a short period of time, thereby saving cost and energy consumed in the pretreatment process.

5 Appendix

- Choopakar O., Polprasert C., Elefsiniotis T., Polprasert S. (2018). The Effect of Steam and Glycerol Pretreatment on Chemical Contents of Oil Palm Empty Fruit Bunch (EFB). Applied Environmental Research, 40(2): 61-67. (attached document 1)

6 Output (Acknowledge the Thailand Research Fund)

6.1 International Journal Publication

- In process

6.2 Research Utilization and Application

- Create new researcher (Ornjira Choopakar, Master student on Environmental Technology)

6.3 Others e.g. national journal publication, proceeding, international conference, book chapter, patent

- Choopakar O., Polprasert C., Elefsiniotis T., **Polprasert S.** (2018). The Effect of Steam and Glycerol Pretreatment on Chemical Contents of Oil Palm Empty Fruit Bunch (EFB). *Applied Environmental Research*, 40(2): 61-67.
- Choopakar O., Polprasert C., Elefsiniotis P., and **Polprasert S.** (2017). The effect of Steam and Glycerol Pretreatment on Chemical Contents of Oil Palm Empty Fruit Bunch (EFB), Proceeding of The 4th EnvironmentAsia International Conference, 21-23 June 2017, Bangkok, Thailand.

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